Characterization and Isolation of Fecal Indicator Bacteria, *Staphylococcus aureus*, and Methicillin-resistant *Staphylococcus aureus* from Pacific Northwest Marine Beach Samples

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Abstract

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The aims of this study were to quantify fecal indicator bacteria (FIB) and *Staphylococcus* spp. in algal wrack and freshwater streams at twelve Washington State marine beaches, and to isolate *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant Enterococci (VRE) from algal wrack (n=93), freshwater streams traversing marine beaches (n=13), and seabird feces (n=63) found on public marine recreational beaches. Samples were collected from May through November 2011. Algal wrack and freshwater stream samples were processed using IDEXX^{TM} Most Probable Number (MPN) method to quantify *Enterococcus* spp., coliforms, *E. coli*, and a modified method for *Staphylococcus* spp. Algae wrack, freshwater, and bird feces were enriched and isolated for *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*. Isolates were biochemically verified as MRSA and typed by SCC*mec* typing. Of the 92 *Staphylococcus*-enrichment wrack samples, 15 [16.3%] were methicillin-susceptible *S. aureus* [MSSA], and 1 [1.1%] sample was MRSA positive. From 13 freshwater samples enriched for *Staphylococcu*s, 4 [31%] were MSSA, and 1 [7.7%] sample was MRSA positive. Of the 64 bird feces samples, 2 [3.1%] samples were positive for MSSA, and 3 [4.7%] were MRSA positive. Of the 5 MRSA characterized, 1 (20%) was SCC*mec* type IV, one (20%) type II, and three (60%) non-typeable. No VRE was isolated. FIB was found at all beaches sampled for wrack and freshwater. This study extends our knowledge of the types of microbes distributed throughout recreational beach environments. Further quantitative microbial risk assessment is needed to determine potential effects on human health.

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1. Introduction

Beaches, both freshwater and marine, are popular recreational destinations throughout the United States. While swimming areas are closely monitored by state and federal agencies, few people visiting the beach would suspect that other substrates, such as aquatic plants, could contain any possible dangers. Yet algal deposits in Lake Michigan have been shown to contain fecal indicator bacteria at higher levels than the water surrounding the vegetation (Olapade et al., 2006, Ishii et al., 2006). In recreational waters, fecal indicator bacteria (FIB) are used as an indicator of water quality--they function as a means of estimating the number of more fastidious human pathogens that are typically present when the FIB concentrations reach specific numbers. A 2006 study by Olapade et al. found *Escherichia coli* (*E. coli*) levels in *Cladaphora* spp., a filamentous algal species, ranged from 2,700 CFU/100 gm to 7,500 CFU/100 gm on average, while the surrounding waters had less than 235 CFU/100 ml of the bacteria. *E. coli* are closely associated with human pathogens, making them favored indicator bacteria for swimming-associated gastroenteritis in marine and fresh water (Myers & Sylvester, 1997, Piérard et al., 2012).

Another study done at Lake Michigan found the algal deposits could also harbor Shiga toxin-producing *E. coli*, a human pathogen, found in 25% of the samples gathered from the lake (Ishii et al., 2006). When California's Cowell Beach closed for 67 days in 2008, and over 170 days in 2009, the Santa Cruz Environmental Health Services suspected large piles of rotting kelp could be contributing to the bacteria counts, in a manner similar to the Lake Michigan *Cladaphora* spp. (Gaura, 2010; http://santacruzwire.com/index.php/la-vida-local/21-la-vida-local/345-rotting-kelp-breeds-bacteria-at-cowell-beach.html). Authorities ruled out human sources of the bacteria, and in 2011, Stanford University began a two year study to determine whether the algal wrack or

animal contamination from birds and sea lions was the cause of unsafe bacteria levels in the water (Gaura, 2011; http://www.santacruzwire.com/index.php/la-vida-local/21-la-vida-local/430-stanford-study-aims-to-clear-the-water-at-santa-cruzs-cowell-beach.html). Algal wrack is aquatic vegetation which has washed upon the shore or is floating on the surface of water. A 2011 study by Imamura et al. of Cowell Beach concluded that marine algal wrack could act as a reservoir for fecal indicator bacteria, but did not address whether human pathogens could persist in marine vegetation in a manner similar to the Lake Michigan studies. No work has been done to assess marine algal wrack as a reservoir for indicator bacteria or pathogens in the colder climate of the Pacific Northwest.

Washington State possesses over 3,200 miles of coastline, attracting millions of visitors each year (State of Washington Water Research Center, 2012; http://www.swwrc.wsu.edu/). Human pathogens, including antibiotic-resistant strains, have recently been isolated from Pacific Northwest beaches (Soge et al., 2009, Levin-Edens et al., 2011c). In a 2009 study, vancomycin-resistant enterococci [VRE] were isolated from marine water and sand samples originating from four of six Washington State marine beach sites (Soge et al., 2009). VRE are *Enterococcus* spp. which have acquired resistance to high levels of vancomycin due to carriage of the *vanA* or *vanB* genes, and vancomycin-resistant *Enterococcus faecalis* or *E. faecium,* are important clinical pathogens (Cetinkaya et al., 2000). The study concluded it was unlikely this contamination was unique to the geographic area tested, and suggested United States coastal beaches may act as reservoirs for the pathogen (Soge et al., 2009). The Soge et al. (2009) study did not attempt to identify sources of VRE deposition at the beaches tested.

A 2011 study by Levin-Edens et al. of marine and freshwater recreational beaches in the Seattle area (WA), found *Staphylococcus aureus* and methicillin-resistant *S. aureus* [MRSA],

opportunistic human pathogens, in marine water, freshwater, and sand (Levin-Edens et al., 2011c). MRSA is resistant to traditional Gram-positive β-lactam antibiotics, such as methicillin and penicillin (Yamamoto et al., 2009). In the past decade MRSA has emerged as a major health threat in nonclinical settings in the general population without known risk factors (Yamamoto et al., 2009). Percentages of MRSA positive samples found at freshwater recreational beaches were 19.4% in the study, while the percentage of marine beach MRSA positive samples ranged from 7.9% to 11.4% (Levin-Edens et al., 2011c). The majority of MRSA isolates (9.7%) in this study came from freshwater drainages and creeks traversing marine beaches, but the study no attempt was made to identify the sources of the contamination (Levin-Edens et al., 2011c).

Washington State marine algal wrack has not been studied to determine if it can act as a reservoir for fecal indicator bacteria or pathogens such as VRE or MRSA. The Soge et al. (2009) study on marine environmental VRE contamination only tested marine water and sand samples, and no other environmental samples at Washington State beaches have been assessed for VRE contamination. Only two marine beaches were assessed for *S. aureus* and MRSA in the Levin-Edens et al. (2011c), and the study did not quantify indicator bacteria or relate S. aureus/MRSA presence to weather conditions at time of sampling.

1.2 Study Background

Bacteria in Marine Wrack

In a study of fecal coliform (FC) contamination of a marine embayment in Massachusetts, researchers found marine shoreline algal wrack elution contributed 6% of the yearly terrestrial input of fecal coliforms to the bay (Weiskel et al., 1996). Fecal coliforms are bacteria that are part of the intestinal microbiome of man and animals, and used as indicators of potential human pathogens (APHA, 1995). The decaying wrack deposited fecal coliforms in the bay at a rate of

~59,000 to ~530,000 colonies per year, and the bacterial deposition was possibly influenced by tidal flooding and major rain events causing elution of bacteria from the vegetation (Weiskel et al., 1996).

It has been suggested by Imamura et al. (2011) that marine algal wrack may promote bacterial survival by acting as a nutrient substrate, offering UV protection, as well as offering protection from predation and desiccation. Imamura et al. (2011) designed a study to assess the relationship between beach algal wrack, FIB, and surrounding water and sediment using environmental samples from Cowell Beach (Santa Cruz, CA). The study showed a statistically significant difference given in orders of magnitude on a $\log_{10}$ scale between each wrack type. Dry wrack harbored enterococci at a rate two orders of magnitude (100x) higher than concentrations found in wet wrack, and wet wrack contained one order of magnitude (10x) higher levels of enterococci than suspended surf wrack. The *E. coli* bacteria levels were one order of magnitude higher in dry wrack than wet, and three logs higher (1000x) in wet wrack than surf wrack. The findings suggest that wrack may increase the amount of FIB in sands and beach water by acting as a protective nutrient source (Imamura et al., 2011). The study did not address whether algal wrack could act as a reservoir for other types of bacteria, and potentially harbor human pathogens such as VRE and MRSA.

A study on *S. aureus* and MRSA survival in marine waters at typical Pacific Northwest temperatures (13 ºC) compared to the warmer (20 ºC) temperatures associated with other study sites found increased survival at colder temps, which could affect the persistence of *S. aureus*/MSRA in algal wrack in the Northwest (Levin-Edens et al., 2011b). MRSA has been isolated and characterized in marine waters and sands in Washington State, Florida, and California (Levin-Edens et al., 2011b, Soge et al., 2009, Abdelzaher et al., 2010, Yamahara et al.,

2012). A Californian study by Yamahara et al. found sand at marine beaches to contain *S. aureus* (14%) and MRSA (3%) (2012). These findings indicate *S. aureus* and MRSA may be present in other beach materials, such as wrack.

In 2012, researchers quantified the risk of illness caused by exposure to *S. aureus* in marine beach sand from Florida. Using a risk analysis model set to find an illness equivalency to the risk of illness set by the EPA for marine water exposure (1.9×10^{-2} cfu/ml) *S. aureus* levels need to be between 10^5 to 10^6 cfu/g of sand to cause a probabilistic skin infection (Shibata & Solo-Gabriele, 2012). No standard has been set yet as to the amount of MRSA considered acceptable at public beaches. The risk model designed by Shibata and Solo-Gabriele could be applied to other marine beach samples, such as wrack, if bacteria levels were quantified. There are no studies from the Pacific Northwest quantifying FIB in wrack, and no published studies attempting to isolate *S. aureus*/MRSA or VRE from marine wrack.

Bacteria in Freshwater Streams

Marine recreational waters have been associated with a four-fold increase in risk of *S. aureus* infection in Hawaii (Charoenca & Fujioka, 1995), indicating a potential for acquiring disease at public contaminated beaches. Another study characterizing the *S. aureus* and MRSA in marine water in Florida showed the bacteria were of the same genetic strains as bacteria cultured from nasal swabs of individuals frequenting the beach, indicating that human *S. aureus* shedding may inoculate marine waters with bacteria (Plano et al., 2011).

A study by Viau et al. (Hawaii, 2011) aimed to find a relationship between *S. aureus* concentrations and microbial indicator concentrations, finding a marginal positive association ($P = <0.1$) between *Enterococcus* spp. and *S. aureus*. The study found *E. coli* and *Enterococcus* spp. in all 22 streams tested, and *S. aureus* was isolated at 19 of 22 streams. One isolate (4.5%) was

MRSA positive. Their results implicated streams as a potential source of pathogens to coastal waters, and also indicated that recreational activities in coastal streams may have the potential to cause illness through direct contact.

In 2011, Levin-Edens et al. (2011c) tested for MRSA in Washington State freshwater streams at two marine beaches. The study found 9.7% of samples from freshwater streams traversing marine beaches were positive for MRSA, indicating that marine freshwater streams might serve as a transport system and source of bacteria at marine beaches. No nonpathogenic bacteria were enumerated or isolated in the Levin-Edens et al. study, and weather conditions at time of sampling were not recorded in relationship to *S. aureus* presence. Further assessment could identify the factors which contribute to MRSA presence in Washington State streams.

Bacteria and Seabirds

The capability of birds to travel large distances gives them a unique ability to spread disease, bringing emerging zoonotic diseases to new areas (Reed et al., 2003). Zoonotic diseases may be transmitted from animals to humans. In 2005 researchers tested seabirds at wildlife rehabilitation centers along the Pacific coast of Washington State and California for carriage of zoonotic bacteria, bacteria which is transmissible from animals to humans (Steele et al., 2005). The study tested common murres, gulls, and other seabirds; findings included multiple strains of *E. coli.* Steele et al. (2005) found antibiotic resistance in 13 of 19 total isolates tested, including resistant *E. coli* strains. The study further suggested that wild birds were able to contract drug-resistant enteropathogens from farms and agricultural waste (Steele et al., 2005). Their results concluded that zoonotic transmission of pathogens or antibiotic resistant bacteria could potentially pose a threat to workers and seabirds at rehabilitation centers.

Seagulls are known for carriage of various enteropathogens as a result of scavenging food at sites where raw sewage is released (Tizard, 2004). A 2010 study by Radhouani et al. in Portugal assessed seagull feces for *vanA*-containing enterococcal strains, finding 10.5% of 57 seagull fecal samples were positive for vancomycin-resistance. European studies have isolated VRE from a greater number of environmental samples than the United States (Cetinkaya et al., 2000), and results from Europe cannot be applied directly to Washington State seabirds. Environmental avian fecal samples in Washington State could potentially be a source of VRE, deserving further study in light of VRE isolation from Washington State sands and water (Soge et al., 2009).

Seabirds are also capable of carrying *Staphylococcus* spp. including *S. aureus* (Saleki, 2002). Domestic fowl have been known to harbor *S. aureus* and MRSA (Hermans et al., 2000), with the potential to spread the disease to humans (AVMA, 2012; http://www.avma.org/animal_health/mrsa_faq.asp). The contribution of seabirds to the bacterial loads of *S. aureus* and MRSA at recreational beaches is not understood, and merits further study.

1.3 Marine Beach Safety Standards

The Environmental Protection Agency (EPA) has worked throughout the US to create comprehensive testing guidelines and monitoring methods for pathogenic bacteria in both marine and freshwater bodies (EPA BEACHES, 2006). The criteria designed by the EPA requires a geometric mean count of bacteria concentration over the sampling period, which allows for assessment of longer term impacts and is used to measure the water quality based effluent levels (WQBELS), as well as single sample maximums (SSM) used for beach monitoring (Nappier, 2012). The SSM is a maximum allowed value for a single environmental sample, and values exceeding the SSM are assumed with a given amount of confidence that the geometric mean standard would be violated if a greater number of samples were taken (Ambient Water Quality

Criteria for Bacteria [AWQCB], 2012). The geometric mean value (geometric mean is created using running total of arithmetic means for at least 5 sampling dates over a period of 30 days) is most closely linked to illness rates, while SSM is used for beach closures and advisories, but both the geometric mean and SSM must be collected for all primary contact recreation waters.

The geometric mean and SSM may be applied to other beach samples in Washington State, and may be used as an assessment tool for algal wrack samples. The EPA freshwater water criteria are set at a geometric mean of 33 enterococci/100 ml water, or 126 *E. coli*/100 ml. The EPA is drafting a Revised Water Quality Criteria (RWQC), which will cause a shift from the SSM to the statistical threshold value (STV), which would allow 25% of samples to exceed the 75^{th} percentile value of individual values about the mean, while the geometric mean will be maintained (Nappier, 2012). States may have different standards than those recommended by the EPA, but they must be as stringent as those set forth by the federal government.

The current water quality criteria for marine beach waters in Washington State for primary contact recreational marine waters (waters where swimming or high water exposure activities would take place) are that fecal coliform organism levels must not exceed a mean of 14 colonies/100 ml (no more than 10% of all samples within 30 days exceeding 43 colonies/100 ml). Secondary contact recreational waters (activities with low risk of water exposure to eyes, ears, respiratory system, digestive system, and urogenital area, such as wading) are not to exceed an enterococci geometric mean value of 70 colonies/100 ml (no more than 10% of all samples within 30 days exceeding 208 colonies/100ml) (Marine Water Quality Criteria, 2012; http://www.ecy.wa.gov/progs/wq/swqs/criteria-marine/wac173201a_210-bacteria.html). In Washington State, beach safety includes water and sediment monitoring for bacteria, temperature, nutrients, and salinity with preferably five sample dates during each season (Marine

Water Quality Criteria, 2012; http://www.ecy.wa.gov/progs/wq/swqs/criteria-marine/wac173201a_210-bacteria.html).

Washington State freshwater standards for primary contact recreational waters are that fecal coliform organism levels must not exceed a geometric mean of 100 colonies/100 ml, with not more than 10% of all samples collected in a season exceeding 200 colonies/100 ml, and secondary contact recreational waters are not to exceed a fecal coliform geometric mean value of 200 colonies/100 ml, with not more than 10% of all samples exceeding 400 colonies/100 ml (Freshwater Quality Criteria, 2012; http://www.ecy.wa.gov/progs/wq/swqs/criteria-freshwater/wac173201a_200-bacteria.html). These standards may provide a background as other marine environmental samples are assessed for bacteria carriage.

1.4 Study Objectives

Environmental samples were taken to test the hypothesis that Pacific Northwest beach algal wrack and freshwater streams harbor FIB, *E. coli*, and *Enterococcus* spp., as well as to test whether wrack, freshwater, and bird feces contain VRE, and *S. aureus* /MRSA. The objectives of this pilot study were: 1) to determine if algal wrack may act as a bacterial reservoir for total coliforms, *Enterococcus* spp., *E. coli*, and *Staphylococcu*s spp., VRE, *S. aureus*, and MRSA; 2) to quantify total coliforms, *E. coli*, *Enterococcus* spp., and *Staphylococcu*s spp., and isolate VRE, *S. aureus*, and MRSA from freshwater streams draining into marine beaches; 3) to determine if avian feces from Washington State marine beaches contains *S. aureus,* and MRSA, and VRE. Secondary objectives were to find associations between ambient weather conditions (precipitation and temperature) and bacterial loads of algal wrack and freshwater samples.

2. Materials and Methods:

2.1 Sample collection procedures

Twelve Western Washington State marine beaches, including Alki Beach Park (King County), Carkeek Park (King County), Discovery Park (King County), Golden Gardens Park (King County), Grandma Cove (San Juan County), Tulalip Bay (Snohomish County), Kalaloch Beach (Jefferson County), Kayak Point Park (Snohomish County), Leadbetter Point State Park (Pacific County), Penrose Park (King County), Pickering Passage (Mason County), and Saltwater State Park (King County) were sampled between May 2011 and November 2011 (Figure 1). Alki Beach Park and Golden Gardens Park were sampled three times; Carkeek Park was sampled on seven occasions, while the remaining nine beaches were sampled once. All samples were transported and stored at 4º C and processed within 48 h of collection.

Ninety-three algal wrack samples were collected with ~100 gm total placed into sterile 1L plastic Nalgene bottles. The wrack was collected at the high and low tide lines and composed of convenience grab samples of different algal species available at the beach. The types of wrack in the composite included; *Callophyllis* spp., *Desmarestia ligulata*, *Fucus distichus*, *Fucus vesiculosus,* M*astocarpus* spp*., Nereocystis luetkeana, Osmundea spectabilis, Palmaria mollis, Ulva intestinalis, Ulva lactuca, Ulva linza,* and *Zostera marina.* Algal wrack was identified prior to processing using the guide *Common Intertidal Seagrasses of the Salish Sea, Including Puget Sound, Georgia Basin, and the Strait of Juan de Fuca* (Periwinkle Press, 2011).

Fresh water (n=13) grab samples [~500 ml] were directly collected into sterile 1L Nalgene bottles from freshwater drainages and creeks traversing through marine recreational beaches. Freshwater was collected midstream at a depth of 5.0 cm, from running stream water. No marine water was collected in this study.

Sixty-four bird feces grab samples were collected including; 43 collected directly from the beach surface as a feces/sand mixture, and 20 samples scraped off of rocks and logs into 50 ml sterile Falcon tubes. Feces samples were collected from surfaces as available, thus avian species of origin and age of sample could not be determined. Feces was collected from Alki Beach Park, Carkeek Park, Golden Gardens Park, Tulalip Bay, Kalaloch Beach, Kayak Point Park, Leadbetter Point State Park, as well as two freshwater beaches sampled only for feces, Seward Park (King County), and Lake Union (King County).

2.2 IDEXX Most Probable Number [MPN]/100 ml for *Enterococcus* spp., Total Coliforms, *E. coli*, and *Staphylococcus* spp.

Water Samples

To enumerate *Enterococcus* spp., a packet of IDEXX Enterolert™ (IDEXX Laboratories, Westbrook, ME) media was added to 110 ml of freshwater sample, sealed into a Quanti-Tray 2000® and incubated at 41°C for 24 h. The volume of sample used in this study is greater than the validated protocol 100 ml samples, but 110 ml of freshwater ensured all 0.1 ml and 1.0 ml Quanti-Tray wells were filled. Wells which fluoresced under UV light were considered positive for *Enterococcus* spp. To enumerate total coliforms (group of rod-shaped Gram-negative bacterial indicators) and *E. coli*, a packet of IDEXX Colilert™ (IDEXX Laboratories) media was added to 110 ml of freshwater sample, sealed into a Quanti-Tray 2000® and incubated at 36.5° C for 24 h. Yellow wells were considered positive for total coliforms, and wells which fluoresced under UV light were considered positive for *E. coli*. Any sample with at least one positive IDEXX well of bacteria was considered positive. The MPN/110 ml for each was calculated using the MPN software provided by IDEXX, as described in the manufacturer's instructions and then

this value was adjusted to find the MPN/100 ml. An example calculation for this procedure for either *Enterococcus* spp. or total coliforms/*E. coli* is as follows:

$$\frac{MPN}{110ml} x \frac{100}{110} = \frac{MPN}{100ml}$$

Presumptive *Staphylococcus* spp. were enumerated with 55 ml freshwater sample 1:1 with 1.5 x Bacto *Staphylococcus* Medium 110 broth (Difco Laboratories, Becton Dickinson & Co., Sparks, MD), supplemented with a final concentration of 75 μg/ml polymyxin B (Sigma Chemical Co., St. Louis, MO) and 0.01% potassium tellurite (Sigma). The final volume was sealed into a Quanti-Tray 2000® and incubated at 36.5° C with 5.0% CO_2 as previously described (Levin-Edens et al. 2011a). Any sample with at least one positive IDEXX well was considered positive. From the incubated trays, *S. aureus* and MRSA were enriched and selected for as described below in section 2.4. The formula for adjusting for the dilution of freshwater to calculate the MPN/100 ml of sample water is as follows:

$$\frac{MPN}{55ml} x \frac{100}{55} = \frac{MPN}{100ml}$$

Algal Wrack Samples

Seventy-five gm consisting of a mixed sample of wrack types were placed in a sterile bottle, 175 ml sterile dH_20 added, and manually shaken vigorously for 2 min, as modified from Imamura et al. (2011). The algal extract was used to find the Most Probable Number (MPN)/gm sample for *Enterococcus* spp., total coliforms, *E. coli*, and *Staphylococcus* spp. A 1:50 dilution of the extract [2.2 ml of sample extract and 107.8 ml of sterile dH_2O], was mixed with a packet of Enterolert ™ enrichment media (IDEXX Laboratories) and processed as described above for the freshwater *Enterococcus* samples. For total coliforms and *E. coli*, a 1:10 dilution of the algal extract [11 ml of sample extract and 99 ml of sterile dH_2O] was mixed with one packet of

Colilert™ (IDEXX Laboratories) and processed as described above for total coliforms and *E. coli* in water. The sample MPN/ 2.2 ml or MPN/11 ml (enterococci or total coliforms/*E.coli*) was calculated using IDEXX software provided by the manufacturer, then adjusted for dilution and sample mass to MPN/gm wrack. Adjustments followed the formula below, where X is the amount of wrack extract diluted above for each specific bacteria type:

$$\frac{MPN}{X\ ml} x \frac{175}{X} x \frac{1}{75} = \frac{MPN}{gm}$$

For enumeration of presumptive *Staphylococcus* spp., a 1:1 dilution of 55 ml of algal sample extract was added to 55 ml of 1.5x Bacto *Staphylococcus* Medium 110 broth (Difco Laboratories, Div. Becton Dickinson & Co., Sparks, MD, USA) supplemented with a final concentration of 75 μg ml^{-1} polymyxin and 0.01% potassium tellurite (Sigma) and incubated at 36.5 °C with 5.0% CO_2 until samples were turbid with black precipitate as adapted from Levin-Edens et al. (2011a).

2.4 Identification of *S. aureus*, methicillin-resistant *S. aureus* [MRSA] and vancomycin-resistant *Enterococcus* spp. [VRE].

The Quanti-Trays wells positive for *Enterococcus* spp. and *Staphylococcus* spp. were further tested for the presence of *S. aureus* and MRSA as previously described (Levin-Edens et al., 2011b quantitation). The method was modified for VRE as follows: the backs of the Quanti-Trays were sprayed with 70% ethanol, incised with a sterile scalpel in the positive wells (limited to 20 positive 1.0 ml wells) and 10 μl of broth extracted and plated on Difco™ m-*Enterococcus* [mE] (Difco Laboratories) supplemented with 6 μg/ml vancomycin [mEv6] (Difco; Sigma) and incubated at 41° C for 24 h. Colonies that grew after incubation were streaked onto Brain Heart

Infusion (Difco) agar supplemented with 18 μg/ml vancomycin [BHIv18] then incubated at 41° C for 24 h. No colonies grew on BHIv18 in this study, and no further analysis was performed .

To isolate *S. aureus* and MRSA, 10 μl of broth was extracted from the Quanti-Trays and plated on Vogel and Johnson agar (Difco Laboratories) supplemented with 75 μg/ml polymyxin B (Sigma) and 0.01% potassium tellurite (Sigma) and incubated at 36.5°C with 5.0% CO_2 for 24 h. Positive growth was plated onto Brucella agar (Difco) supplemented with 5% sterile sheep blood and incubated at 36.5 C for 24 h. The β-hemolytic colonies were tested for coagulase activity using Remel Staphaurex® rapid latex kit (Thermo Fisher Scientific Remel Products, Lenexa, KS). Samples that had β-hemolytic coagulase-positive Gram-positive bacteria were labeled *S. aureus*. The *S. aureus* colonies were plated on Brain Heart Infusion (Difco Laboratories) agar supplemented with 10 μg/ml of methicillin (USP, Rockville, MD) [BHI Meth10]. Growth on BHI Meth10 was presumptive MRSA, and tested for the presence of the *mecA* gene by PCR as previously described (Levin-Edens et al., 2011a).

2.5 Bird feces enrichment and testing for *Staphylococcus aureus*

The bird feces/sand (n= 44) samples were homogenized in a 50 ml conical vial by stirring with a sterile swab. With the same swab, sample feces/sand mixture was streaked onto Difco™ m-Enterococcus [mE] agar (Difco Laboratories, Becton Dickinson & Co., Sparks, MD) supplemented with 6 μg/ml vancomycin [mEv6] (Difco Laboratories; Sigma Chemical Co., St. Louis, MO) and incubated for 24 h at 41° C. The samples of directly scraped feces (n=20) were streaked for isolation on mEv6 agar and incubated at 41° C for 24 h. Colonies that grew after incubation were plated onto Brain Heart Infusion (Difco) agar supplemented with 18 μg/ml vancomycin [BHIv18] (Sigma) incubated at 37° C for 24 h.

For *S. aureus* and MRSA enrichment, 20 gm of feces/sand was weighed into a sterile bottle and 20 ml of dH_2O added. In cases where feces was removed off of logs or stones, the entire feces sample was weighed, then mixed with 20 ml of dH_2O. The slurry was shaken for 2 min and 10 ml added to 10 ml of 1.5x Bacto *Staphylococcus* 110 broth (Difco Laboratories), supplemented with a final concentration of 75 μg/ml polymyxin (Sigma) and 0.01% potassium tellurite (Sigma) and incubated at 36.5° C with 5% CO_2. Samples which had growth with black precipitate were plated onto Brucella agar (Difco) supplemented with 5% sterile sheep blood and incubated at 36.5 C for 24 h, as in the extraction of *S. aureus* described above for IDEXX wells (Levin-Edens et al. 2011a). The β-hemolytic colonies were coagulase tested using Staphaurex® rapid latex kit (Thermo Fisher Scientific Remel Products). The samples that had β-hemolytic coagulase-positive Gram-positive bacteria were *S. aureus* positive. *S. aureus* colonies were plated on BHI Meth10. Those colonies that grew were presumptive MRSA, and verified for the presence of the *mecA* gene by PCR (Levin-Edens et al., 2011a).

2.6 PCR Assay for *mecA* and SCC*mec* Typing

PCR assay for the *mecA* gene used Type IV (USA 300) as a positive control for the assay, and sterile PCR dH_20 was used as the negative control. SCC*mec* typing was done by multiplex PCR assay using positive controls of Types I-V (MS361, MS1053, 4-29, USA 300, and 2-36), and negative control was sterile PCR dH_20 as previously described (Zhang et al., 2005). Isolates that were negative were categorized as non-typeable [NT].

2.7 Temperature and precipitation data

Weather data was obtained by the National Weather Service (NWS) (http://www.nws.noaa.gov/climate/index.php?wfo=sew). Archival data for 2011 was available for four weather stations in Washington State; Seattle-Sand Point, Seattle-Tacoma Airport,

Olympia, and Quillayute Airport. Each beach sampling site was matched to the NWS climate station geographically closest for temperature and precipitation data for each sampling date. Temperature data used for analysis was daily high temperature, and precipitation was daily cumulative centimeters for day of sampling and the two days prior to sample collection.

2.8 Analytic techniques

For algal wrack and water, the significance of association between bacterial type, ambient temperature, and precipitation using the geometric mean MPN/100 ml bacterial counts was analyzed by Spearman Rank Order Correlation using Statistica v9.1 (StatSoft, Inc., Tulsa, OK). $P < 0.05$ was considered statistically significant. Nonparametric tests were used as it was assumed environmental microbiological data was not normally distributed. Confidence intervals around the geometric mean were calculated using the Excel 2010® (Microsoft, Seattle, WA) function "Confidence" set at $\alpha = 0.05$ and the geometric standard deviation calculated using the array formula:

$$Confidence = Exp(St.Dev(ln(individual\ sample\ values\ for\ each\ sampling\ date\ /location)))$$

(Wyatt, 2012; http://excelribbon.tips.net/T011208_Calculating_a_Geometric_Standard_Deviation.html).

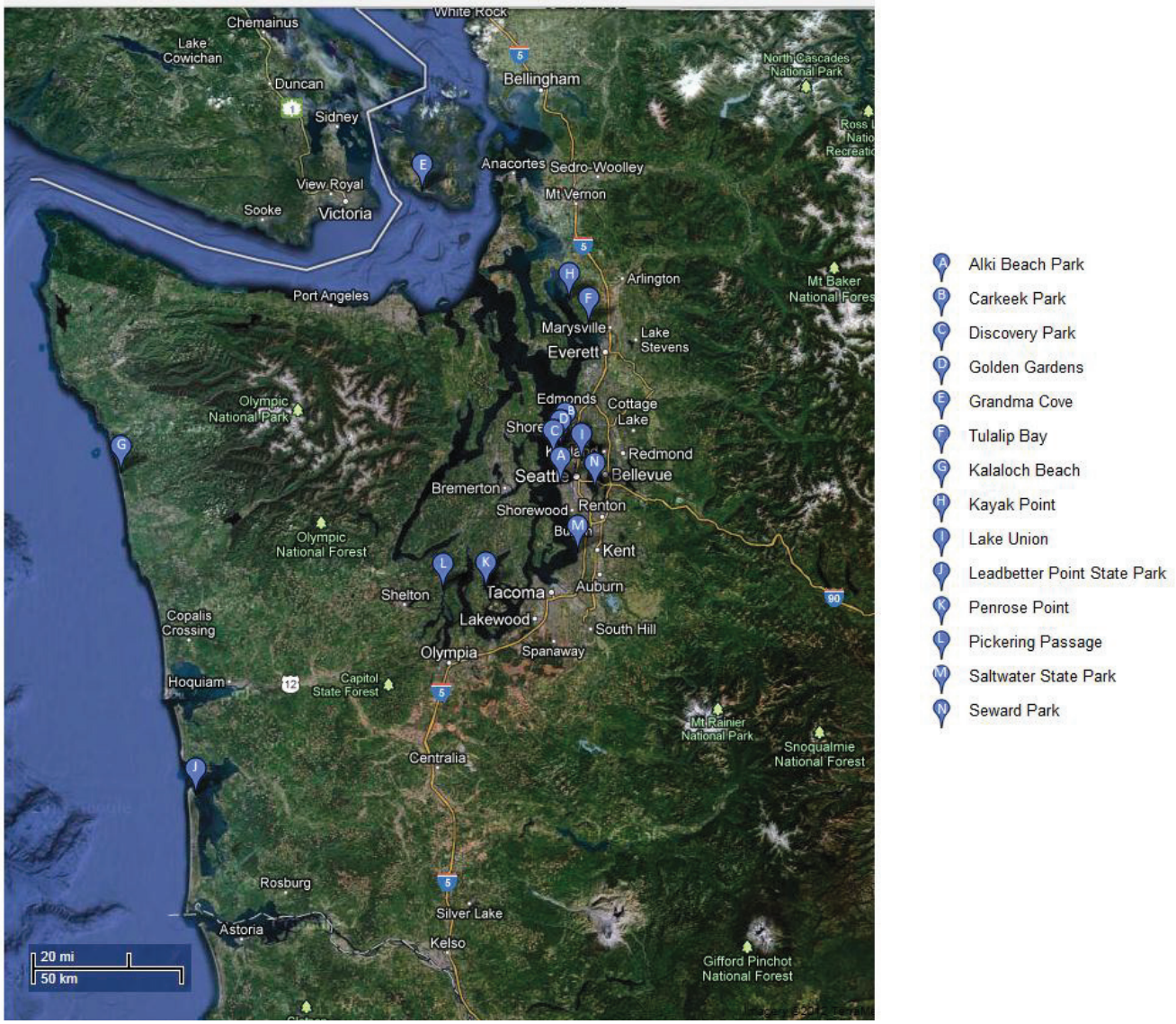

Figure 1: Map of Washington State locations sampled for wrack, freshwater, and feces in this study.

3. Results:

3.1 Bacterial Distributions

Wrack

Enterococcus spp., total coliforms, *E. coli*, and *Staphylococcus* spp. were compared to one another for all wrack samples using Spearman Rank Order Correlation tests. Weather data for precipitation (cm) on day of sampling was assessed for a relationship with bacterial type geometric mean bacterial MPN/gm. Ambient air temperature (ºC) on day of sampling was compared to geometric mean bacterial MPN/gm for all four bacterial types enumerated from wrack. Relationships were considered significant if $P < 0.05$, and R values below 0.5 were considered weakly correlated.

Ninety of ninety-two samples [97.8%] were positive for *Enterococcus* spp. The MPN/gm algal wrack for *Enterococcus* spp. individual samples (total samples taken during the study and tested for *Enterococcus* spp.) ranged from <1 to 2,541, with a geometric mean from all samples combined of 18 MPN/gm (95% CI = 15 to 21). Wrack samples had no isolates capable of growth on BHIvan18 (no VRE was isolated in this study). Wrack *Enterococcus* spp. geometric mean MPN/gm values ranged from 2 MPN/gm (Discovery Park, 95% CI = 0 to 4) to 1,655 MPN/gm (Kalaloch Beach, 95% CI = 1,652 to 1,657). The Kalaloch Beach geometric mean MPN of 1,655 MPN/gm wrack was over two orders of magnitude (10^2) higher than the average for all samples, and ~9x than the second highest value geometric mean of 181 MPN/gm wrack from Leadbetter State Park (Figure 2). Confidence intervals around the geometric mean per sample location were large, for example, Saltwater State Park had a geometric mean of 12 MPN/gm, but the 95% CI ranged from 0 to 154 (Table 1). *Enterococcus* spp. were not significantly associated with any other bacteria when geometric means were used, but *Enterococcus* spp. and total coliforms were

weakly correlated [R=0.259] at the P <0.05 level when comparing total individual samples from the study, as were *Enterococcus* spp. and *E. coli* [R=0.237]. No relationships were found for precipitation or ambient temperature and *Enterococcus* spp. at the P <0.05 level.

Eighty-seven of ninety [96.7%] wrack samples were positive for total coliforms with a geometric mean MPN/gm of 17 (95% CI = 13 to 20). Individual MPN values for all wrack samples taken during the course of the study ranged from <1 to 2,541 bacteria (Table 1). Total coliforms had no significant associations with any other bacteria when using geometric mean MPN/gm, but total coliforms and *E. coli* were correlated at the P <0.05 level [R=0.537] when using sample by sample (all samples taken individually from each beach during collection) MPN/gm values. No significant relationships were found between ambient temperature or precipitation and total coliforms at the P <0.05 level.

Seventy of ninety [77.8%] wrack samples were positive for *E. coli*, with a combined sample geometric mean MPN/gm wrack of 3 (95% CI = 1 to 5). The individual sample MPN/gm ranged from <1 to 508 bacteria for all locations and sampling dates. *E. coli* MPN/gm geometric means varied from site to site, ranging from <1 MPN/gm (Grandma Cove, 95% CI = 0 to 3) to 27 MPN/gm (Leadbetter State Park, 95% CI = 12 to 42) (Table 1)(Figure 2). *E. coli* was not correlated to other bacteria based upon the geometric mean MPN/gm, but *E. coli* and *Staphylococcus* spp. were weakly related at the P <0.05 [R=0.315] when using individual total sample data. Spearman Rank Order tests found a weak relationship [R = 0.216] between precipitation on sampling date and *E. coli* MPN/gm wrack using individual sample data significant at the P <0.05 level. No significant relationships were found for ambient temperature and *E. coli* geometric mean MPN/gm at the P <0.05 level.

Eighty-four of ninety-two [91.3%] wrack samples were positive for *Staphylococcus* spp. growth. The geometric mean for *Staphylococcus* spp. MPN/gm from all wrack samples combined was 2 (95% CI = <1 to 4), while individual sampling MPNs ranged from <1 to 466 MPN/gm. *Staphylococcus* spp. geometric mean MPN/gm wrack values ranged from <1 to 44 MPN/gm, though the trend was for lower numbers between <1 and 12 bacteria per gm (Table 1) (Figure 2). *Staphylococcus* spp. were weakly correlated with precipitation levels two days prior to sampling [R= 0.221]. No relationships were found for ambient temperature and *Staphylococcus* spp. at the P <0.05 level. A total of 15 [16.3%] of the wrack samples were positive for *S. aureus,* and were isolated from Golden Gardens, Carkeek Park, Saltwater State Park, Grandma Cove, and Kayak Point. One of the *S. aureus* samples [1.1% of samples, 6.7% of total *S. aureus* samples isolated from wrack] was MRSA positive, isolated from Carkeek Park (Table 2).

Repeated Wrack Samples

Carkeek Park was sampled on seven occasions for algal wrack from May 8 to Nov 27, 2011. Both *E. coli* and *Enterococcus* spp. geometric mean MPN/gm values remained consistent over time within one standard deviation difference between sampling dates (Figure 3). *Staphylococcus* spp. average MPN/gm wrack over time showed an increasing trend from May to November, with initial values ranging from 1 to 4 MPN/gm May through August, and increasing to 43-44 MPN/gm in November (Figure 3), a change greater than one standard deviation from earlier sampling dates. Spearman Rank Order tests on Carkeek Park individual sampling data found total coliforms to be correlated with *E. coli* and *Enterococcus* spp. at the P <0.05 level [R = 0.635, R = 0.593]. *E. coli* was significantly correlated with *Enterococcus* spp. and *Staphylococcus* spp. [R = 0.586, R = 0.45]. *E. coli* was also correlated significantly with precipitation one day prior to sampling, precipitation on day of sampling, and ambient

temperature [R = 0.526, R = 0.443, R = 0.597]. *Enterococcus* spp. was also weakly correlated at the P <0.05 level with ambient temperature [R = 0.394], and *Staphylococcus* spp. was correlated with precipitation on day of sampling [R = 0.428]. No other correlations were found for bacteria with other bacteria, precipitation, or temperature.

Freshwater

Freshwater *Enterococcus* spp., total coliforms, *E. coli*, and *Staphylococcus* spp. were compared to one another for all samples using Spearman Rank Order Correlation tests. Weather data for precipitation on day of sampling was assessed for a relationship with bacterial type geometric mean MPN/100 ml using Spearman Rank Order tests. Ambient air temperature was compared to bacterial geometric mean MPN/100 ml for all four bacterial types enumerated from freshwater by correlation using Spearman Rank Order tests.

All thirteen freshwater samples were positive for *Enterococcus* spp. [100%]. The freshwater geometric mean MPN/100 ml for *Enterococcus* spp. for all samples combined was 1,022 (95% CI = 1,009 to 1,035) (Table 3). Individual samples taken in this study ranged from 19 MPN/100 ml to 110,092 MPN/100 ml enterococci. *Enterococcus* spp. varied from beach to beach--Kayak Point and Golden Gardens exceeded the combined geometric mean of 1,022 MPN/100 ml by approximately two $\log_{10}$ orders of magnitude (78,847 MPN/100 ml and 110,092 MPN/100 ml, respectively), and Saltwater State Park exceeded the combined geometric mean by approximately one $\log_{10}$ order of magnitude (17,622 MPN/100 ml)(Figure 4) (Table 3). *Enterococcus* spp. and total coliform geometric mean MPN/100 ml were correlated [R = 0.859] at the P <0.05 level, and were also correlated for individual samples [R = 0.914]. *Enterococcus* spp. and *E. coli* showed a correlation at the P <0.05 level for geometric means [R = 0.75] and between individual sample values [R = 0.818]. This correlation represents the association

between the presence of one bacteria to another being present, and there was a very strong relationship for presence of *Enterococcus* spp. Indicating presence of other bacteria. No VRE was isolated from freshwater.

The geometric mean total coliform count from 13 positive freshwater samples [100%] was 2,816 (95% CI = 2,813 to 2,818) (MPN/100 ml of freshwater). Individual sample MPN/100 ml ranged from 265 to 22,018 bacteria (Table 2). *E. coli* was found in all 13 water samples, with a geometric mean of 71 (95% CI = 63 to 78) (MPN/100 ml). Sample MPN/100 ml ranged from <1 to 3,325 bacteria. Total coliforms and *E. coli* were correlated for individual sample values at the $P < 0.05$ level [$R = 0.79$]. The *E. coli* MPN/100 ml single sample mean for Kayak Point was approximately one $\log_{10}$ order of magnitude larger (170 MPN/100 ml) than the combined geometric mean for all *E. coli* water samples. The largest *E. coli* MPN/100 ml from Golden Gardens (July 11, 788 MPN/100 ml) was 100x larger than the combined geometric mean for all locations, suggesting variation in *E. coli* numbers at various freshwater stream locations (Table 3) (Figure 4).

The combined geometric mean *Staphylococcus* spp. count from 12 [92.3%] positive freshwater samples was 51 (95% CI = 46 to 56) (MPN/100 ml). Individual sample MPN/100 ml ranged from <1 to 4,404 bacteria. The 12 positive *Staphylococcus* spp. samples were isolated at six of seven total beaches, all those sampled for freshwater except Saltwater State Park. The Kayak Point sample geometric mean of 4,404 MPN/100 ml was approximately 86x larger than the combined geometric mean MPN/100 ml of all the beaches tested, and 45x larger than the next largest single sample value (99 MPN/100 ml) isolated from Golden Gardens (Table 3) (Figure 4). From freshwater samples enriched for *Staphylococcus* spp. 4 [30.8%] were *S. aureus* and of those, 1 [7.7% of total freshwater samples, 25% of total freshwater *S. aureus* isolates]

sample was MRSA positive. *S. aureus* was isolated at Carkeek Park, Golden Gardens, and Kayak Point. The MRSA-positive sample was isolated from Carkeek Park.

There were no significant correlations between precipitation and bacterial MPN/100 ml for any bacterial type at the $P < 0.05$ level. There were some slight positive trends that were not significant at $P < 0.05$. *Enterococcus* spp., total coliforms, *E. coli*, or *Staphylococcus* spp. and ambient temperature had no relationships of significance at the $P < 0.05$ level for geometric mean or individual sample MPN/100 ml.

3.2 Detection of *S. aureus*/MRSA in Bird Feces

Bird feces samples had 5 [7.8%] isolates positive for *S. aureus*, and of the total *S. aureus* samples, 3 [4.7%] were MRSA positive samples. Bird feces samples were divided into 44 sand/feces composite samples and 20 directly scraped feces samples. From the sand/feces composites, 4 [9.1%] samples were positive for *S. aureus*, and 2 of those samples [4.5% of total sand/feces samples] MRSA-positive. From the 20 samples directly scraped off of surfaces, 1 [5%] MRSA-positive sample was isolated (Nov 27, Carkeek Park). *S. aureus* positive samples were isolated from Carkeek Park (sand/feces) and Golden Gardens (sand/feces). The MRSA positive *S. aureus* samples were isolated at Alki Beach Park (sand/feces) and Carkeek Park (directly removed from surface) (Table 4). Eight [12.5%] of the 64 samples were collected at freshwater beaches, and 0 of these samples were positive for *S. aureus* or MRSA. . The number of positive samples for *S. aureus* and MRSA were too low for statistical analysis.

3.3 MRSA Molecular Characteristics

There were five isolates from all sample types which tested positive for MRSA. SCC*mec* types were one [20%] SCC*mec* II isolate from Carkeek Park algal wrack, one [20%] SCC*mec* IV isolate from Carkeek Park freshwater, and three isolates [60%] were non-typeable [NT] from

bird feces (Alki Park and Carkeek Park) (Table 5). NT samples did not any of SCC*mec* types I-V.

Table 1: Geometric mean bacterial MPN/gm for algal wrack

Location	Date	N	Coliform [95% CI]	*E. coli* [95% CI]	*Enterococcus* spp. [95% CI]	*Staphylococcus* spp. [95% CI]
Alki	May 1	4	2 [0-13]	1 [0-9]	106 [103-110]	3 [1-5]
	Oct 1	5	4 [0-11]	1 [0-8]	11 [2-21]	<1 [0-1]
	Oct 23	2	35 [30-40]	10 [8-12]	54 [43-65]	6 [0-17]
Carkeek	May 8	4	2 [0-3]	1 [0-9]	12 [9-15]	3 [1-5]
	May 15	3	508 [507-509]	8 [4-12]	43 [27-60]	4 [2-6]*
	Jul 1	6	15 [0-34]	1 [0-5]	3 [0-41]	1 [0-5]
	Jul 31	6	1 [0-24]	<1 [0-4]	3 [0-10]	1 [0-13]
	Aug 21	4	72 [24-121]	11 [0-25]	11 [0-36]	2 [1-4]
	Nov 13	2	<1 [0-2]	3 [2-5]	1 [0-8]	44 [6-83]
	Nov 27	2	<1 [0-33]	25 [18-31]	11 [2-20]	43 [30-56]
Golden Gardens	Jun 21	4	72 [60-83]	17 [14-20]	8 [5-11]	12 [5-19]
	Jul 11	5	24 [21-27]	9 [6-13]	57 [49-64]	4 [1-7]
	Jul 17	8	91 [88-95]	27 [24-30]	10 [4-16]	1 [0-7]
Discovery Park	Jul 4	4	65 [53-78]	<1 [0-4]	2 [0-4]	1 [0-5]
Grandma Cove	Oct 1	5	65 [45-85]	<1 [0-3]	709 [694-724]	<1 [0-6]
Kalaloch Beach	Aug 8	3	38 [11- 65]	21 [0-88]	1655 [1652-1657]	<1 [0-14]
Kayak Point	Oct 11	5	46 [44-48]	1 [0-5]	2 [<1-4]	3 [0-14]
Leadbetter Pt.	Aug 26	4	83 [76-89]	27 [12-42]	181 [175-187]	27 [24-30]
Penrose	May 29	7	2 [0-9]	1 [0-6]	166 [162-170]	12 [9-15]
Pickering Passage	Jun 18	5	15 [8-21]	5 [0-12]	3 [<1-6]	2 [0-9]
Saltwater State Park	Sept 5	3	12 [7-16]	3 [0-14]	12 [0-154]	3 [0-26]
Tulalip Bay	Oct 30	2	159 [151-161]	<1 [0-6]	3 [1-5]	1 [0-5]
Total		93	17 [13-20]	3 [1-5]	18 [15-21]	2 [<1-4]

*Indicates sample which contained a methicillin-resistant *S. aureus* [MRSA] positive sample.

Table 2: Algal Wrack Sampling *Staphylococcus aureus* and MRSA distributions

Beach	Date	Sample Type	No. samples	No. *S. aureus* *(MRSA incl.)*	% *S. aureus*	No. MRSA	% MRSA
Alki Beach Park	May 1	Wrack	4	0	0	0	0
	Oct 1	Wrack	5	0	0	0	0
	Oct 23	Wrack	2	0	0	0	0
Carkeek Park	May 8	Wrack	4	0	0	0	0
	May 15	Wrack	3	0	0	0	0
	Jul 1	Wrack	6	1	16.7	0	0
	Jul 31	Wrack	6	3	50	1	15
	Aug 21	Wrack	4	0	0	0	0
	Nov 13	Wrack	2	0	0	0	0
	Nov 27	Wrack	2	0	0	0	0
Golden Gardens Park	Jun 21	Wrack	3	2	66	0	0
	Jul 11	Wrack	5	3	60	0	0
	Jul 17	Wrack	8	3	37.5	0	0
Discovery Park	Jul 4	Wrack	4	0	0	0	0
Grandma Cove	Oct 1	Wrack	5	1	20	0 0	0 0
Kalaloch Beach	Aug 8	Wrack	3	0	0	0	0
Kayak Point	Oct 11	Wrack	5	1	20	0	0
Leadbetter Point State Park	Aug 26	Wrack	4	0	0	0	0
Penrose Park	May 29	Wrack	7	0	0	0	0
Pickering Passage	Jun 18	Wrack	5	0	0	0	0
Saltwater State Park	Sept 5	Wrack	3	1	25	0	0
Tulalip Bay	Oct 30	Wrack	2	0	0	0	0
		Total	92	15	16.3	1	1.1

MRSA: methicillin-resistant *Staphylococcus aureus*. Total *S. aureus* counts include MRSA.

Table 3: Freshwater Stream from Marine Beach Geometric Mean Bacterial MPN/100 ml

Location	Date	Coliform MPN/100 ml [95% CI]		*E. coli* MPN/100 ml [95% CI]		*Enterococcus* spp. MPN/100 ml [95% CI]		*Staphylococcus* spp. MPN/100 ml [95% CI]	
Alki	May 1	590	[NA]	78	[NA]	226	[NA]	267	[NA]
Carkeek	May 8	1,948	[1945-1952]	103	[101-104]	49	[34-63]	83	[76-91]
Carkeek	Nov 13	4,980	[NA]	131	[NA]	5,026	[NA]	3	[NA]
Carkeek	Nov 27	1,577	[NA]	169	[NA]	590	[NA]	40	[NA]*
Golden Gardens	Jun 21	1,448	[1443-1453]	262	[258-266]	184	[119-248]	55	[48-62]
Golden Gardens	Jul 11	11,827	[NA]	788	[NA]	110,092	[NA]	99	[NA]
Kayak Point	Oct 11	22,018	[NA]	170	[NA]	78,847	[NA]	4,404	[NA]
Penrose	May 29	265	[NA]	63	[NA]	19	[NA]	45	[NA]
Pickering Passage	Jun 18	1,577	[NA]	<1	[NA]	71	[NA]	45	[NA]
Saltwater State Park	Sep 5	22,018	[NA]	170	[NA]	17,622	[NA]	<1	[NA]
Total		2,816	[2813-2818]	71	[63-78]	1,022	[1009-1035]	51	[46-56]

NA (not applicable); applies to data sets where only one sample was taken at a location and time point, no confidence interval could be calculated. *Indicates that methicillin-resistant *S. aureus* [MRSA] was isolated from the sample.

Table 4: Avian feces *Staphylococcus aureus* and MRSA distributions

Beach	Date	Sample Type	No. samples	No. *S. aureus* (MRSA incl.)	% *S. aureus*	No. MRSA	% MRSA
Alki Beach Park	Oct 1	Feces	3	2	0	2	66.7
	Oct 23	Feces	5	0	0	0	0
Carkeek Park	Jul 1	Feces	2	1	50	0	0
	Jul 31	Feces	6	0	0	0	0
	Aug 21	Feces	4	0	0	0	0
	Nov 13	Feces	5	0	0	0	0
	Nov 27	Feces	5	1	0	1	20
Golden Gardens Park	Jul 11	Feces	3	1	33	0	0
	Jul 17	Feces	2	0	0	0	0
Tulalip Bay	Oct 30	Feces	5	0	0	0	0
Kalaloch Beach	Aug 8	Feces	4	0	0	0	0
Kayak Point	Oct 11	Feces	3	0	0	0	0
Lake Union*	Oct 20	Feces	5	0	0	0	0
Leadbetter Point State Park	Aug 26	Feces	8	0	0	0	0
Seward Park*	Jul 24	Feces	4	0	0	0	0
		Total	64	5	7.1	3	4.7

*Indicates freshwater beach, all others are marine beaches. MRSA: methicillin-resistant *Staphylococcus aureus*. *S. aureus* total numbers include MRSA as a subset.

Table 5: Molecular characteristics of MRSA-positive isolates

Isolate number	Collection Date	Sample Type	Source	SCC*mec* Type
30	May 31	Algal Wrack	Carkeek Park	II
1	Oct 1	Feces	Alki Park	NT
3	Oct 2	Feces	Alki Park	NT
10	Nov 27	Feces	Carkeek Park	NT
121	Nov 27	Freshwater	Carkeek Park	IV

SCC*mec* type 1-V, NT [non-typeable]. NT isolâtes do not match known SCC*mec* types I-V.

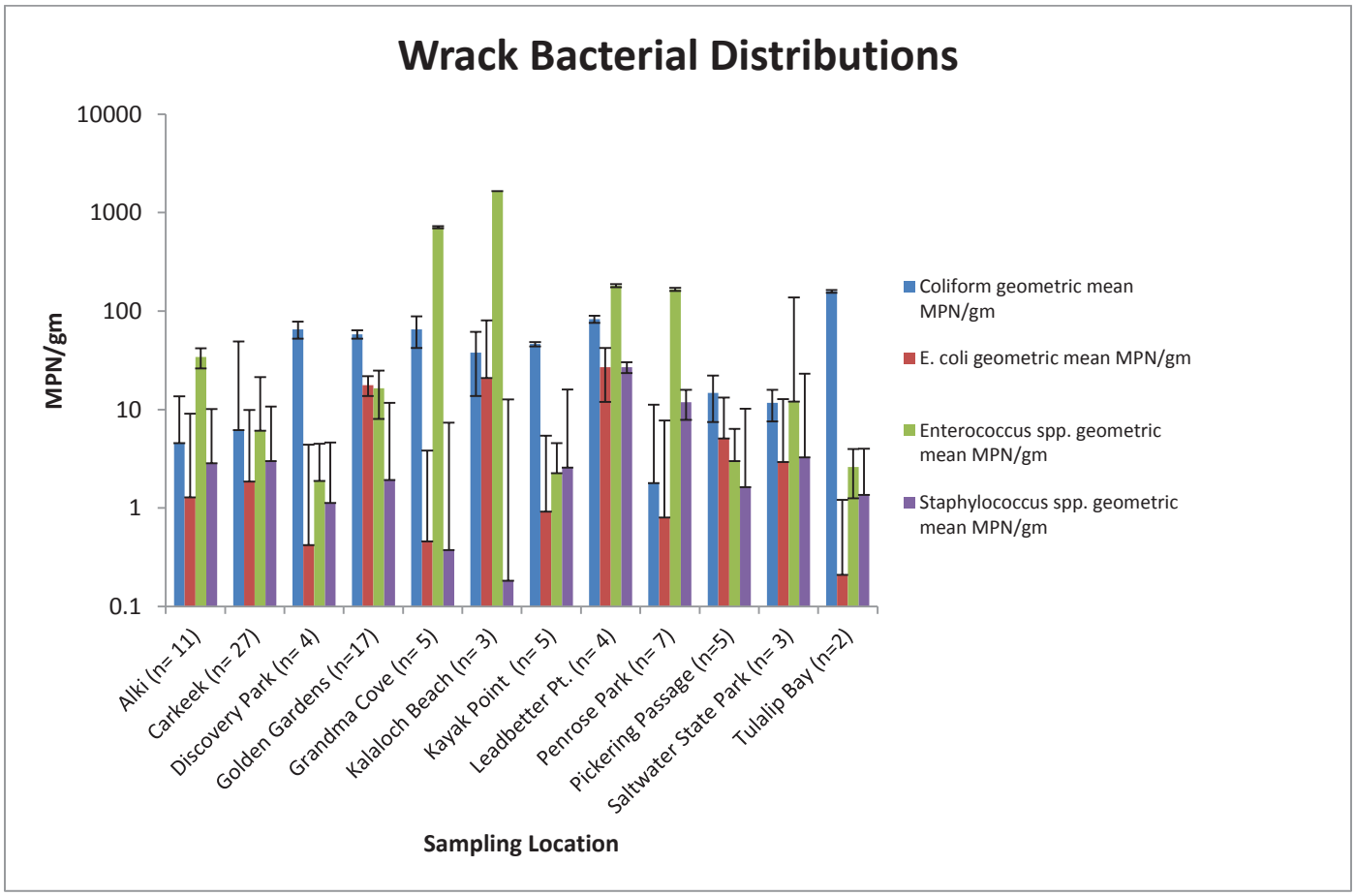

Figure 2: Algal wrack geometric mean MPN/gm wrack was calculated for all sampling locations in this study. Error bars represent the geometric standard deviation of all samples for each location. N is equal to the total number of samples taken at each site over the course of the study. Wrack was collected between May 1, 2011 and November 27, 2011.

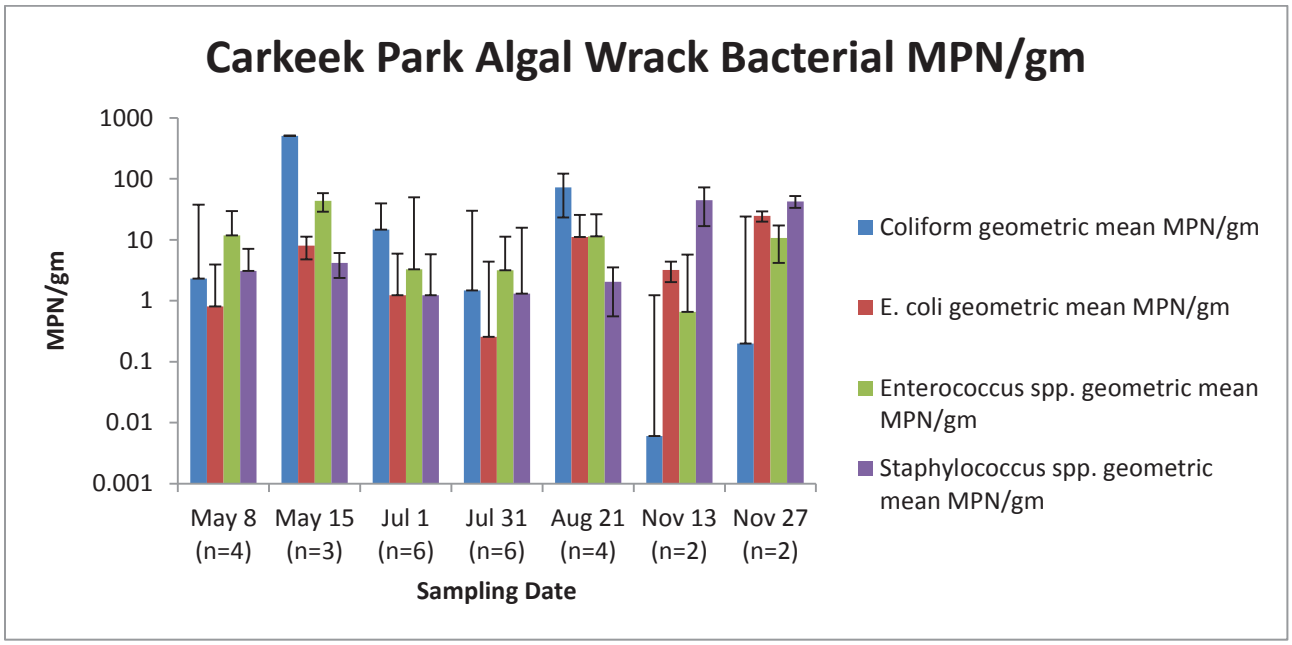

Figure 3: Algal wrack arithmetic mean MPN/gm wrack was calculated for all sampling dates for Carkeek Park, Seattle, WA. Error bars represent the standard deviation of all samples for each date. N is equal to the total number of samples taken on each sampling date.

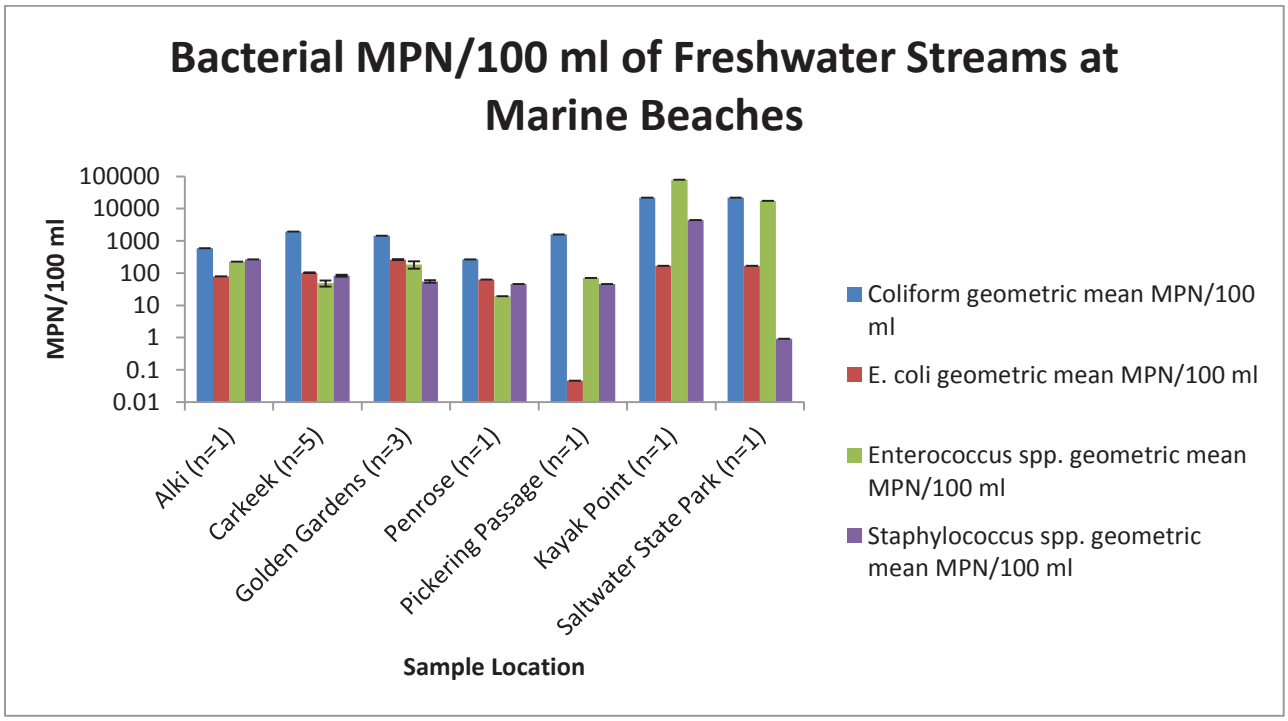

Figure 4: Freshwater geometric mean MPN/100 ml was calculated for all sampling locations in this study. Error bars represent the geometric standard deviation of all samples for each location, samples with n=1 do not have calculated error for a single sample. N is equal to the number of samples taken at each site over the course of the study. Freshwater was collected between May 1, 2011 and November 27, 2011.

4. Discussion

In the current study, marine algal wrack was assessed as a potential reservoir for fecal indicator bacteria and *Staphylococcus* spp. at various Washington State beaches. *Enterococcus* spp., *E. coli*, and *Staphylococcus* spp. were isolated from wrack at all twelve beach sites, though not all individual samples were positive for each sampling event. Total coliform data was collected to give a background rate of bacteria in Washington State marine algal wrack. Wrack *E. coli* were positively associated with precipitation when individual sample MPN/gm values were used for data analysis. This is contrary to the relationship seen in Weiskel et al. (1996), where rain and tidal events were associated with elution of bacteria from shore wrack. In the current study, it is conceivable rain could transport FIB along the shore where it may be absorbed by algal wrack.

No relationships of significance were found for freshwater MPN and precipitation, though in general there were positive trends at the $P > 0.05$ level, which could suggest that rain events flush bacteria into coastal streams. Correlations between bacteria geometric mean MPN and temperature were not statistically significant for either wrack or freshwater.

This study assessed wrack bacteria through late spring and mid-autumn, but low numbers of repeat samples make it difficult to assess bacterial levels over time for all sites. Carkeek Park was sampled most often, showing potential bacterial associations over time. The relationship between *Enterococcus* spp. and ambient temperature could suggest *Enterococcus* spp. were more able to persist in the environment when associated with algal wrack than other bacterial types. In a study by Goodwin et al. (2012), enterococci persistence was positively correlated with the presence of *S. aureus*, including MRSA *E. coli* and *Staphylococcus* spp. were associated with precipitation on the day of sampling, possibly suggesting wrack absorbs staphylococci from the

environment during rain events. Given the associations found with repeat samples over seven months from Carkeek Park, further studies should assess other Northwest beaches to see if these trends continue at other geographic locations. More research should be done to test for seasonality of beach wrack bacterial levels to assess major changes in bacterial MPN/gm throughout the calendar year. Expansion of repeated samples over time at a single site might elucidate if there are potential relationships between peak use recreation periods and wrack bacterial loads.

All sites tested in this study for freshwater had *Enterococcus* spp. MPN/100 ml geometric means above the recommended EPA surface water standard geometric mean 33 /100 ml, with the single exception of Penrose Park. Comparison of *E. coli* MPN/100 ml values from the current study to the EPA freshwater standard of geometric mean 126 *E. coli*/100 ml found Carkeek Park, Golden Gardens, Kayak Point, and Saltwater State Park coastal streams and drainages exceeded the recommended standard. In the current study, freshwater streams were tested once or twice, while both Washington State and EPA require multiple single date arithmetic mean samples for calculation of geometric mean (a minimum of 5 samples per 30 days). Future comparisons of indicator bacteria from freshwater streams at marine beaches would be enhanced by meeting the 5 sample per 30 day minimum standard. Total coliform geometric mean MPN/100 ml ranged from 73 to 22,018, but no direct comparisons to current Washington State freshwater surface water quality fecal coliform criteria may be made because the samples in this study were tested for total coliforms only. In general, the number of total coliforms is much greater than fecal coliforms in a sample. In future studies, IDEXX QuantiTrays with Colilert^{TM} media may be incubated at 44.5 ºC, rather than the 36.5 ºC used in this study, as a means of enumerating fecal coliforms in order to make comparisons to state and EPA standards (Yakub & Castric, 2002).

This temperature allows for use of IDEXX total coliform media (Colilert^{TM}) to isolate fecal coliforms given by IDEXX^{TM}.

Freshwater *Enterococcus* spp. and total coliforms were correlated using both geometric mean MPN/100 ml values and individual sample MPN/100 ml values for data analysis. Further study could assess if there is a predictive relationship between these MPN/100 ml values. No other freshwater bacterial types were significantly associated in this study.

This study was the first to assess marine algal wrack for the presence of *S. aureus* and MRSA. Isolation found 16.3% of samples were positive for *S. aureus* and 1.1% were positive for MRSA. The level of risk to recreational beach users at these levels for algal wrack is not currently known. A risk analysis model similar to Shibata and Solo-Gabriele (2012) could be developed in the future for algal wrack, quantifying the amount of wrack needed to contain a bacterial dose-equivalent to the EPA or Washington State standards for marine waters. The findings of this study concur with the hypothesis that wrack may serve as a reservoir for FIB and other microorganisms (Weiskel et al., 1996, Imamura et al., 2011). Since wrack serves as an important aspect of the beach ecosystem, removal of the shore algal wrack should be avoided except in cases of extreme contamination (Imamura et al., 2011).

MRSA findings were consistent with expectations for freshwater streams traversing marine beaches [7.7%] based upon previous research results of 7.9% to 11.4% by Levin-Edens et al. (2011c). The findings were also within range of the 4.5% found in Viau et al. (2011) given the small sample size. One of 13 samples contained MRSA, similar to the results of the Hawaiian beach study by Viau et al. (2011) where 1 of 22 distinct stream samples contained MRSA. The freshwater MRSA isolated in this study was SCC*mec* IV, in agreement with the findings of Levin-Edens et al. (2011c).

S. aureus and MRSA were isolated from avian feces samples taken from marine beaches (7.1% and 4.7% of samples, respectively). Bird feces at marine beach sites have not been studied in this manner, so there are no studies to compare rates of *S. aureus* and MRSA isolation. These results suggest avian feces could potentially act as a source of *S. aureus* and MRSA at public beaches. MRSA has been isolated from sand at marine beaches (Soge et al., 2009, Levin-Edens et al., 2011, Yamahara et al., 2012), so using sand/feces mixtures for future testing is disadvantageous for determining the origin of the bacteria. The amount of sand gathered in this study was small, and the effect of sand on fecal *S. aureus*/MRSA presence is predicted to be negligible. Ongoing studies in the Northwest by Dr. Marilyn C. Roberts' laboratory include trapping birds and using cloacal swabs taken for enrichment and isolation of *S. aureus* and MRSA to ensure the origin of the bacteria from avian species and not transference from sand or recreational surfaces.

MRSA isolates should be further typed by multilocus sequencing typing (MLST) and pulse-field gel electrophoresis (PFGE) analysis. Three of five MRSA isolates in this study were non-typeable, which is contradictory results from Levin-Edens et al. (2011), where the majority (67.7%) of environmental beach samples were SCC*mec* IV. All non-typeable isolates were extracted from avian feces, which have not been studied previously. This may account for environmental SCC*mec* typing differences from the previous study. The majority of freshwater samples (12 of 20) were SCC*mec* IV in Levin-Edens et al. (2011c), which is consistent with the findings in this study.

No VRE was isolated in this study from wrack, water, or feces, which is contrary to expectations from prior study of Washington State and California marine sand and water samples (Soge et al., 2009).

5. Conclusions

The primary objectives of this research was to survey marine algal wrack and freshwater streams for *Enterococcus* spp., *E. coli*, and *Staphylococcu*s spp., and to determine if wrack, freshwater, and seabird feces harbor VRE, *S. aureus*, and MRSA. Secondary aims were assessment of any associations between ambient weather conditions and bacterial loads of algal wrack and freshwater samples.

This study found *Enterococcus* spp., total coliforms, *E. coli*, and *Staphylococcus* spp. present throughout Washington State marine beaches in both stranded algal wrack and freshwater from drainages and creeks that traverse and drain into marine beaches. This is the first study in the Pacific Northwest to quantify fecal indicator bacteria (bacteria used to monitor for the potential presence of human pathogens) and *Staphylococcus* spp. from marine beach wrack, and the results are in agreement with studies indicating that algal wrack can act as a potential reservoir of fecal indicator bacteria at marine beaches (Imamura et al., 2011, Weiskel et al., 1996). This study is the first for the Pacific Northwest to search for relationships between bacterial wrack loads and ambient temperature or precipitation at time of sampling.

S. aureus and MRSA were isolated from all three substrates tested in this study; wrack, freshwater, feces. *S. aureus* and MRSA have not been previously isolated from marine algal wrack. The results of this study are in agreement with previous work that freshwater drainages and creeks traversing marine beaches are significant sources of *S. aureus* and MRSA contamination (Viau et al., 2011, Levin-Edens et al., 2011). This is the premier study of Northwestern marine birds to isolate *S. aureus* and MRSA from avian feces, indicating a greater need for study of marine wild bird ability to carry and transmit *S. aureus* and MRSA to recreational beach users.

The current study has shown that marine recreational samples are consistently contaminated by fecal indicator bacteria, as well as capable of harboring *S. aureus* and MRSA, proposing a need for further study of recreational environments to determine sources and extent of contamination.

References:

1. **Ambient Water Quality Criteria for Bacteria (AWQCB).** 2011. USEPA.
2. **American Public Health Association (APHA)**. 1995. *Standard Methods for the Examination of Water and Wastewater* (19th ed.). APHA, Washington, DC.
3. **American Veterinary Medical Association (AVMA)**. 2012. Animal Health: Frequently asked questions about Methicillin-resistant *Staphylococcus aureus.* Available at : http://www.avma.org/animal_health/mrsa_faq.asp.
4. **Abdelzaher AM, Wright ME, Ortega C, Solo-Gabriele HM, Miller G, Elmir S, Newman X, Shih P, Bonilla JA, Bonilla TD, Palmer CJ, Scott T, Lukasik J, Harwood VJ, McQuaig S, Sinigalliano C, Gidley M, Plano LR, Zhu X, Wang JD, Fleming LE**. 2010. Presence of pathogens and indicator microbes at a non-point source subtropical recreational marine beach. Appl. Environ. Microbiol. **76**:724-732.
5. **Cetinkaya Y, Falk P, Mayhall CG**. 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. **31**:686-707.
6. **Charoenca N, Fujioka RS**. 1995. Assessment of *Staphylococcus* bacteria in Hawaii recreational waters. Water Sci. Technol. **31**:11-17.
7. *Common Intertidal Seagrasses of the Salish Sea, Including Puget Sound, Georgia Basin, and the Strait of Juan de Fuca.* 2011. Periwinkle Press, 3178 Weida View Ln, Oak Harbor WA 98277.
8. **Englebert ET, McDermott C, Kleinheinz GT**. 2008. Effects of the nuisance algae, *Cladophora*, on *Escherichia coli* at recreational beaches in Wisconsin. Sci. Total Environ. **404**:10-17.

9. EPA BEACHES (Beaches Environmental Assessment and Coastal Health Act). Report to Congress. 2006. Available: http://water.epa.gov/type/oceb/beaches/report_index.cfm

10. EPA Surface Water Standards and Guidance. 2012. Available: http://water.epa.gov/scitech/swguidance/

11. EPA Water Quality Standards for Surface Waters. 2012. Available: http://water.epa.gov/scitech/swguidance/standards/

12. **Guara M**. 2010. Unsafe for Swimming - Rotting Kelp Trumps Swimmers at Cowell Beach. Santa Cruz Wire. Available: http://santacruzwire.com/index.php/la-vida-local/21-la-vida-local/345-rotting-kelp-breeds-bacteria-at-cowell-beach.html.

13. **Guara M**. 2011. Stanford Study Aims to Clear the Water at Santa Cruz's Cowell Beach. Available: http://www.santacruzwire.com/index.php/la-vida-local/21-la-vida-local/430-stanford-study-aims-to-clear-the-water-at-santa-cruzs-cowell-beach.html.

14. **Goodwin KD, McNay M, Cao Y, Ebentier D, Madison M, Griffith JF**. 2012. A multi-beach study of *Staphylococcus aureus*, MRSA, and enterococci in seawater and beach sand. Water Research **46**:4195-4207.

15. **Hermans K, Devriese LA, De Herdt P, Godard C, Haesebrouck F.** 2000. *Staphylococcus aureus* infections in psittacine birds. Avian Pathology. **29**:411-415.

16. **Imamura GJ, Thompson RS, Boehm AB, Jay JA**. 2011. Wrack promotes the persistence of fecal indicator bacteria in marine sands and seawater. FEMS Microbiol. Ecol. **77**:40-49.

17. **Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL, Sadowsky MJ**. 2006. *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. Applied and Environmental Microbiology. **72**:4545-4553.

18. **Levin-Edens E, Meschke JS, Roberts MC (a)**. 2011. Quantification of methicillin-resistant *Staphylococcus aureus* strains in marine and freshwater samples by the most-probable-number method. Appl. Environ. Microbiol. **10**:3541-3.

19. **Levin-Edens E, Bonilla N, Meschke JS, Roberts MC (b)**. 2011. Survival of environmental and clinical strains of methicillin-resistant *Staphylococcus aureus* [MRSA] in marine and fresh waters. Water Res. **45**:5681-5686.

20. **Levin-Edens E, Soge OO, No D, Stiffarm A, Meschke JS, Roberts MC (c)**. 2011. Methicillin-resistant *Staphylococcus aureus* from Northwest marine and freshwater recreational beaches. Fems. Microbiol. Ecol. **79**:412-420.

21. **Nappier S**. 2012. 2012 Draft Recreational Water Quality Criteria. USEPA.

22. National Weather Service Forecast Office (NWS). 2012. Seattle, WA, Observed Weather Reports. Available: http://www.nws.noaa.gov/climate/index.php?wfo=sew

23. **Olapade OA, Depas MM, Jensen ET, McLellan SL**. 2006. Microbial communities and fecal indicator bacteria associated with *Cladophora* mats on beach sites along Lake Michigan shores. Applied and Environmental Microbiology. **72**:1932-1938.

24. **Piérard D, De Greve H, Haesebrouck F, Mainil J.** 2012. O157:H7 and O104:H4 Vero/Shiga toxin-producing *Escherichia coli* outbreaks: respective role of cattle and humans. Veterinary Research. 43:13 doi:10.1186/1297-9716-43-13.

25. **Plano LRW, Garza AC, Shibata T, Elmir SM, Kish J, Sinigalliano CD, Gidley ML, Miller G, Withum K, Fleming LE, Solo-Gabriele HM**. 2011. Shedding of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* from adult and pediatric bathers in marine waters. BMC Microbiology 11:5 doi:10.1186/1471-2180-11-5.

26. **Radhouani H, Poeta P, Pinto L, Miranda J, Coelho C, Carvalho C, Rodrigues J, López M, Torres C, Vitorino R, Domingues P, Igrejas G**. 2010. Proteomic characterization of *vanA*-containing *Enterococcus* recovered from Seagulls at the Berlengas Natural Reserve, W Portugal. Proteome Sci. **8**:48.

27. **Reed KD, Meece JK, Henkel JS, Shukla SK**. 2003. Birds, migration, and emerging zoonoses: West Nile Virus, Lyme disease, Influenza A and Enteropathogens. Clin. Med. Res. Available: http://www.clinmedres.org/content/1/1/5.full

28. **Roberts MC, Soge OO, No D, Beck NK, Meschke JS**. 2011. Isolation and characterization of methicillin-resistant *Staphylococcus aureus* from fire stations in two northwest fire districts. American Journal of Infection Control. doi: 10.1016/j/ajoc/2010.09.008.

29. **Roberts MC, Soge OO, No D, Helgeson SE, Meschke JS**. 2011. Characterization of methicillin-resistant *Staphylococcus aureus* isolated from public surfaces on a university campus, student homes and local community. Journal of Applied Microbiology. doi: 10.1111/j.1365-2672.2011.05017.x.

30. **Saleki K**. 2002. *C*haracterisation of avian isolates of *Staphylococcus aureus.* PhD Thesis. University of Glasgow. Available at :http://theses.gla.ac.uk/2802/01/2002salekiphd.pdf

31. **Soge OO, Roberts MC, Giardino MA, Mazengia E, Ma G, Meschke JS**. 2009. Vancomycin-resistant *Enterococcus* spp. in marine environments from the West Coast of the USA. J. Appl. Microbiol. **107**:300-7.

32. State of Washington Water Research Center. 2012. Available: http://www.swwrc.wsu.edu/.

33. **Steele CM, Brown RN, Botzler RG**. 2005. Prevalences of zoonotic bacteria among seabirds in rehabilitation centers along the Pacific coast of California and Washington, USA. Jwildlifedis. **42**:735-744.

34. **Stewart JR, Gast RJ, Fujioka RS, Solo-Gabriele HM, Meschke JS, Amaral-Zettler LA, Castillo E, Polz MF, Collier TK, Strom MS, Sinigalliano CD, Moeller PDR, Holland AF**. 2008. The coastal environment and human health: microbial indicators, pathogens, sentinels and reservoirs. Environ. Health. **7**:S3.

35. **Tizard I**. 2004. Salmonellosis in wild birds. Texas A&M University Veterinary College. Available: http://vetmed.tamu.edu/common/docs/public/schubot/SalmonellosisinWildBirds.pdf

36. **Viau EJ, Goodwin KD, Yamahara KM, Layton BA, Sassoubre LM, Burns SL, Tong H, Wong SHC, Lu Y, Boehm AB**. 2010. Bacterial pathogens in Hawaiian coastal streams – Associations with fecal indicators, land cover, and water quality. Water Research. **45**:3279 – 3290.

37. WA Department of Ecology(a). 2012. Table 200(2)(b) Water Contact Recreation Bacteria Criteria in Fresh Water. Available: http://www.ecy.wa.gov/progs/wq/swqs/criteria-freshwater/wac173201a_200-bacteria.html

38. WA Department of Ecology(b). 2012. Table 210 (3)(b) Water Contact Recreation Bacteria Criteria in Marine Water. http://www.ecy.wa.gov/progs/wq/swqs/criteria-marine/wac173201a_210-bacteria.html

39. **Weiskel PK, Howes BL, Heufelder GR**. 1996. Coliform contamination of a coastal embayment: sources and transport pathways. Environ. Sci. Technol. **30**:1872-1881.

40. **Wyatt A**. 2012. Calculating a Geometric Standard Deviation. ExcelTips. Avaiable: http://excelribbon.tips.net/T011208_Calculating_a_Geometric_Standard_Deviation.html.

41. **Yakub G, Castric D, et al.** 2004. Evaluation of Colilert and Enterolert Defined Substrate Methodology for Wastewater Applications. Water Environment Research 74. Available: http://www.idexx.com/pubwebresources/pdf/en_us/water/64194006K.pdf

42. **Yamahara KM, Sassoubre LM, Goodwin KD, Boehm AB**. 2012. Occurrence and persistence of bacterial pathogens and indicator organisms in beach sand along the California coast. Appl. Environ. Microbiol. **78**:1733-1745.

43. **Yamamoto T, Nishiyama A, Takano T, Yabe S, Higuchi W, Razvina O, Shi D**. 2010. Community-acquired methicillin-resistant *Staphylococcus aureus*: community transmission, pathogenesis, and drug resistance. Journal of Infection and Chemotherapy **16**:225-254.

44. **Zhang K, McClure JA, Elsayed S, Louie T, Conly JM**. 2005. N Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **43**:5026-33.