

The introduction and dissemination of pathogenic bacteria in the coastal waters of southern California and Mexico

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Non-technical summary

I investigated the introduction and dispersion of potentially disease-causing bacteria in the coastal waters of southern California and Mexico between March 16th and March 27th, 2012. These bacteria can be transported into coastal waters via a number of pollution sources, and studying their presence and abundance can be useful for assessing the safety of water for recreational and fisheries uses. I found *E. coli* present in coastal waters near San Diego and Cabo San Lucas, Mexico, and *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) present near Manzanillo, Mexico. The presence of *S. aureus* and MRSA could pose health risks to recreational swimmers, especially individuals who are immunosuppressed or individuals who have open cuts or abrasions.

Abstract

Foreign and potentially disease-causing bacteria can be introduced to marine coastal waters through a number of pollution sources. Studying the biogeography of these bacteria in seawater is useful for assessing the safety of waters for recreational and fisheries uses. The extent of pathogen contamination can also be used as a proxy for understanding the relative impact of anthropogenic contaminants on the marine environment. I investigated the dispersion of human pathogenic and fecal indicator bacteria into the coastal waters of southern California and Mexico. Samples were collected aboard the R/V *Thomas G. Thompson* between March 16th and March 27th, 2012. Total DNA was purified from all samples and PCR assayed for the presence of *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, the *Staphylococci* *mecA* gene that codes for methicillin resistance, and *Vibrio cholerae* serotype O1. *S. aureus* was determined to be dispersed over a large geographic area around Manzanillo, Mexico, and the *mecA* gene was found in two of six samples from the area. *E. coli* was found in two of four samples taken near Cabo San Lucas, Mexico and in two of three samples taken near San Diego, California. Pathogens were found mostly in samples taken closest to the coast, implicating coastal cities as the source of these human pathogens to the marine environment.

Contamination of seawater with human pathogens is increasingly being recognized as a significant environmental threat to human health. Pathogens in seawater can cause infections via recreational water use and contaminated seafood. Worldwide, recreational use of coastal waters alone has been implicated in 120 million gastrointestinal infections and 50 million acute respiratory infections each year (Viau et al. 2011). Microbial contamination of seawater has also been connected to harmful infections of marine mammals (Gulland and Hall 2007). Thus if microbes are introduced to marine waters, and then infect marine mammals in order to persist and proliferate in the environment, these pathogens could later be reintroduced to human populations and contamination could represent a significant potential driver of the emergence of new zoonotic diseases (Stewart et al. 2008). There are some significant point source contamination pathways of coastal waters, such as sewage intrusion and ballast water discharge, but also a number of non-point sources, such as freshwater runoff from land and recreational swimmers. For example, a study in 2011 by Plano et al. found that over the course of 15 minutes, recreational swimmers release approximately 10^5 colony-forming units (CFU) of *Staphylococcus*

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aureus, 20% of which they confirmed to be methicillin-resistant *Staphylococcus aureus* (MRSA), into seawater. Although these non-point sources of contamination can be diffuse over large geographical scales, they are still quantifiable and ecologically significant.

Temporal and spatial distributions of pathogenic bacteria in seawater reveal contamination sources and transmission pathways of human-associated microbes. Pathogens not native to marine environments provide useful tracers of contamination. Only by examining their dispersion can we understand the dominant regional sources of pollution and the various disease threats that recreational and commercial uses of coastal waters could represent. For example, *E. coli* and *Enterococcus faecalis* naturally inhabit the gastrointestinal tracts of animals and have therefore been determined by the U.S. Environmental Protection Agency to be useful fecal indicator bacteria (FIB) in seawater (Griffin et al. 1999, Stewart et al. 2008). In seawater, the cell density of the opportunistic pathogen *S. aureus*, and the *mecA* gene that codes for resistance to methicillin in *Staphylococcus* species (Borjesson et al. 2009), has been shown to be strongly correlated with the number of recreational swimmers in a region (Plano et al. 2011). *S. aureus* also has the potential to remain viable in seawater for 3-4 days (Gabutti et al. 2000), suggesting that recreational water use could be an effective transmission pathway for this particular microbe (Plano et al. 2011).

For microbes that can thrive in marine environments, introduction into coastal waters may offer an ideal opportunity by which they might be able to transfer from one host to another and spread disease over large distances and time scales. *Vibrio cholerae* is one example of a pathogen that can thrive in both the human body and seawater, and the toxigenic strains of this organism, subgroups O1 and O139, can cause the particularly harmful disease cholera. Because this organism can survive naturally in seawater, coastal waters in areas of the world that are

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regularly subjected to outbreaks of cholera in humans have been theorized to be environmental reservoirs for *V. cholerae*. This means that areas like Peruvian coastal waters, which were previously reported to contain high numbers of toxigenic and non-toxigenic *V. cholerae* (Lipp et al. 2003), may provide safe haven for this organism between epidemics. One particular danger of these environmental reservoirs is that ships can pick up and transport organisms over large geographical distances when they take on and exchange ballast water. For example, ship ballast water was implicated in transporting and introducing toxigenic *V. cholerae* into near shore waters and causing a disease epidemic that spread throughout Peru and Latin America in 1991 (McCarthy and Khambaty 1994). This same strain of *V. cholerae* later infected shellfish in Mobile Bay, Alabama, with ship ballast water again being suspected as the major transportation vector (McCarthy and Khambaty 1994). Thus understanding the geographical locations and extents of these reservoirs is a necessary component of reducing the danger they represent to human health.

Along the Pacific coast, examining the gradients in pathogen presence and concentration will be necessary to understanding the dominant sources of contamination and the relative threat that each unique pathogen transmission and infection pathway represents. The impacts of coastal habitation and anthropogenic activities on near shore waters have been well documented. Human wastewater intrusion and other pollution sources have been implicated in the loading of a number of pathogens into shallow beach waters, including *Enterococcus faecalis*, hepatitis viruses, *S. aureus*, and *Vibrio vulnificus*, just to name a few (Griffin et al. 1999, Viau et al., Mote et al. 2012). However, little is known about the extent to which these microbes are transported throughout the marine environment, including into deeper off shore waters. This project was therefore designed with four goals: (1) to examine horizontal gradients in surface seawater

pathogen presence as well as total bacteria concentrations near three major cities, (2) to determine the variety of pathogenic and anthropogenically-sourced bacteria that are loaded into the coastal waters of southern California and Mexico, (3) to examine the potential for dispersion and the extent of environmental transport of foreign microbes that contaminate seawater, and (4) to assess the safety of Pacific coastal water in these regions for near shore recreational activities and for commercial activities that can span both near shore and open ocean settings.

Materials and methods

Sample Collection

Seawater was collected aboard the *R/V Thomas G. Thompson* near San Diego (SD), Cabo San Lucas (Cabo), and Manzanillo (MZ) (Fig. 1). In each of the three geographic regions, one sample was collected as near shore as the ship could navigate and the remaining samples were collected at progressively more offshore locations. Seawater was collected between 2 and 50 meters depending on the depth of the chlorophyll-maximum. At all sampling locations, 500 ml of seawater was filtered through a 0.2 μm -pore-size filter. At stations SD 1, SD 2, SD 3, Cabo 1, Cabo 2, Cabo 4, MZ 1, MZ 2, and MZ 4, an additional 2 liters of seawater was filtered through a 0.2 μm -pore-size sterivex filter. All filters were stored at -80°C .

In addition to coastal seawater collection, samples were also collected from a sunbathing pool that was placed on the fantail of our ship. The first sample was collected as the pool was being filled with surface seawater, and a second sample was collected a few days later after numerous students had used the pool.

Bacterial cell counts

At each sampling location, 1 ml of seawater was fixed with a 2% final concentration of

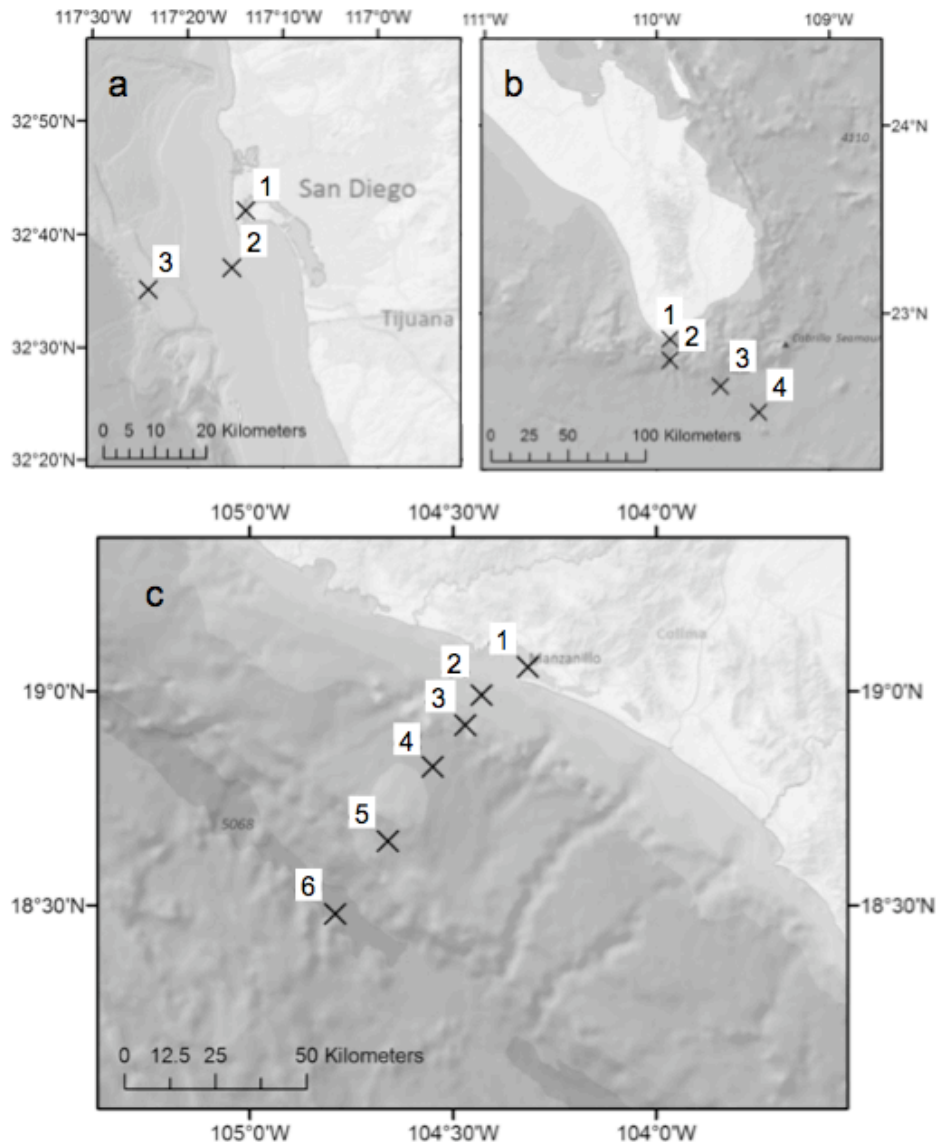


Fig. 1. Map of sampling stations near San Diego (a), Cabo (b), and Manzanillo (c). San Diego stations are abbreviated “SD” and Manzanillo stations are abbreviated “MZ”.

paraformaldehyde and filtered through a 0.2 μm -pore-size filter. 10 μL of DAPI-citifluor, a fluorescent nucleic acid stain, was applied to each filter, and the filters were mounted on microscope slides. Bacterial cells were visualized in the UV spectrum using an epifluorescent microscope and counted in ten distinct fields of view.

Nucleic Acid Purification

Total DNA was extracted from sterivex filters using a phenol-chloroform extraction protocol modified from Anderson (personal communication) and Thurber et al. (2009). DNA Extraction Buffer (0.1 M Tris-HCl, 0.1 M Na-EDTA, 0.1 M NaH₂PO₄, 1.5 M NaCl, and 1% cetyltrimethylammonium bromide), 50 mg/ml Lysozyme, Proteinase K, and 20% SDS were added to the filters to lyse microbial cells. Chloroform and phenol/chloroform/isoamyl alcohol were used to purify nucleic acids. DNA was precipitated with isopropanol, washed with 70% Ethanol, and resuspended in 150 µl of MilliQ water.

Total DNA from all flat 47 mm 0.2 µm-pore-size filters was purified according to the phenol-chloroform extraction methods described in Thurber et al. (2009). All DNA was

Table 1. Primer sequences and PCR conditions for the amplification of *E. faecalis*, *E. coli*, *S. aureus*, *mecA*, and *V. cholerae* O1.

Target	Primer Sequences	Amplicon Length (bp)	Annealing Temp (°C)	Source
<i>E. faecalis</i>	F-primer: CGCTTCTTTCCCTCCCGAGT R-primer: GCCATGCGGCATAAACTG	125	61	Santo Domingo et al. 2002
<i>E. coli</i>	F-primer: GCAGTCTTACTTCCATGATTTCTTTA R-primer: TAATGCGAGGTACGGTAGG	522	57	Srinivasan et al. 2011
<i>S. aureus</i>	F-primer: GCAAAAATCCAGCACAAACAGGAAACGA R-primer: CTTGATCTCCAGCCATAATTGGTGG	638	55	Viau et al. 2011
<i>mecA</i>	F-primer: CATTGATCGCAACGTTCAATTT R-primer: CGGTTTTAAAGTGGAACGAAGGT	329	54	Soge et al. 2009
<i>V. cholerae</i> O1	F-primer: CAACAGAATAGACTCAAGAA R-primer: TATCTTCTGATACTTTTCTAC	647	50	Lipp et al. 2003

resuspended in 100 μl of MilliQ water, quantitated with a Nanodrop spectrophotometer, diluted and normalized to 2 ng/ μl , and stored at -20°C .

PCR

PCR reactions were carried out in 20 μl volumes containing 1X PCR buffer, 3.75 mM MgCl_2 , 0.4 mM deoxynucleoside triphosphates, 0.8 units Taq polymerase, and 0.25 μM of both forward and reverse primers. Between 5 and 10 ng total DNA template was added to each reaction and the reactions were run in a MJ Research PTC225 Peltier thermal cycler. Primer sequences and reaction conditions are listed in Table 1.

For primer sets that were designed to amplify fragments of 300 base pairs or more, PCR products were run on 1.5% agarose gels. PCR products of expected amplicon sizes below 200 base pairs were run on 3% agarose gels. Gels were stained with ethidium bromide and visualized with a UV transilluminator.

Results

Bacterial concentrations

The bacterial abundances observed at the times of our sampling did not correlate with any environmental parameters, including sea surface temperature, salinity, chlorophyll, oxygen, and transmissivity. The highest cell concentrations were generally observed at near shore locations and then decreased with distance from shore. Near San Diego, a maximum cell concentration of 1.95×10^6 cells ml^{-1} was observed 0.9 km from shore at SD 1, and lower concentrations were observed offshore. Cell concentrations near Cabo San Lucas, Mexico varied little with depth or distance from shore, ranging between 3.2×10^5 and 4×10^5 cells ml^{-1} (Fig. 2).

MZ 1, where the R/V *Thompson* was docked in Manzanillo's industrial port, had the highest surface seawater cell concentration observed during the entire cruise, 2.8×10^6 cells ml⁻¹ of seawater. Cell concentration decreased to 1.07×10^6 cells ml⁻¹ at MZ 3, located 20 km from shore, and then progressively decreased again to 4.55×10^5 cells ml⁻¹ at station MZ 6, located 80 km from shore (Fig. 2).

PCR

S. aureus fragments amplified in samples from MZ 1, MZ 3, and MZ 5 near Manzanillo, Mexico. In two of those three, MZ 1 and 2, fragments of the *mecA* gene also amplified (Table 2). *S. aureus* tested negative at all sampling locations near San Diego and Cabo. *E. coli* tested positive at SD 1 and 2 near San Diego,

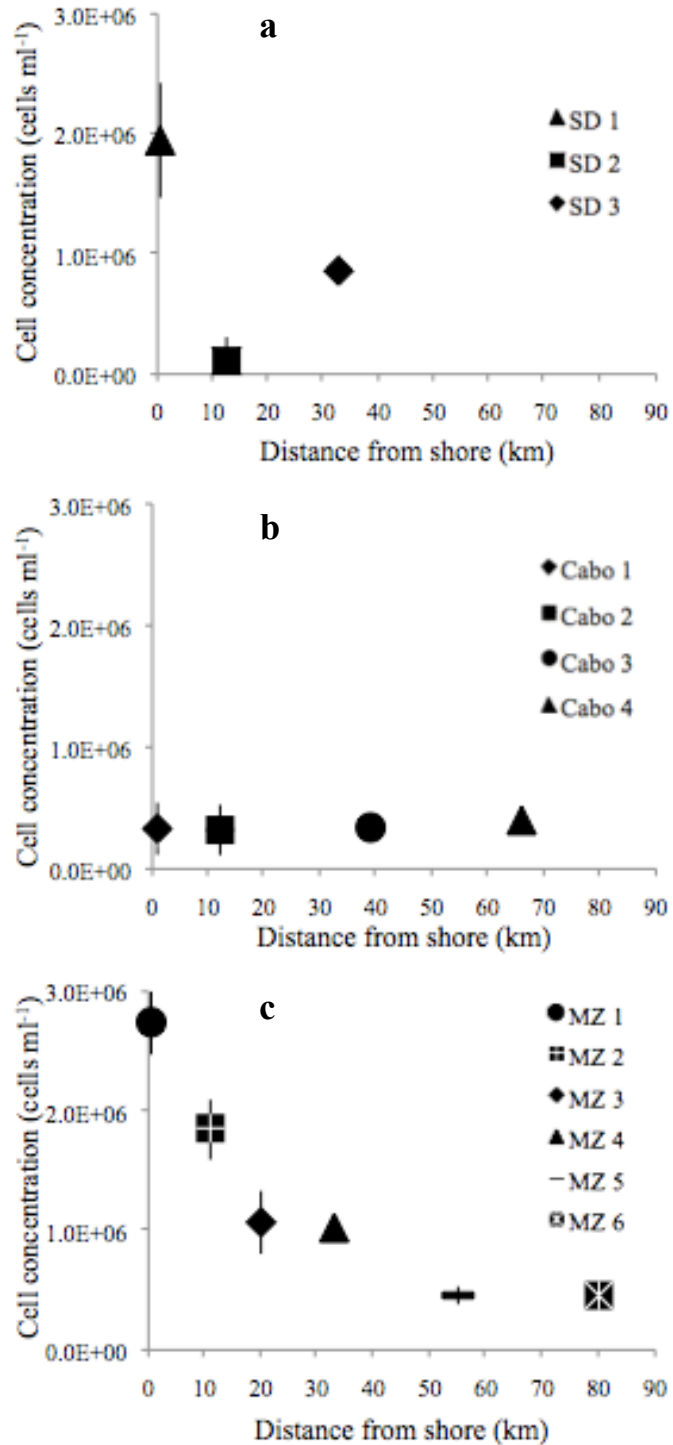


Fig. 2. Cell concentration data for sampling stations near (a) San Diego, (b) Cabo, and (c) Manzanillo. Error bars represent the standard deviation.

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Table 2. Occurrence and distribution of *E. coli*, *E. faecalis*, *S. aureus*, *mecA*, and *V. cholerae* O1 in Pacific coastal waters and the pool.

Station	<i>E. coli</i>	<i>Enterococcus faecalis</i>	<i>S. aureus</i>	<i>mecA</i>	<i>Vibrio cholerae</i> O1
SD 1	+	?	-	-	-
SD 2	+	?	-	-	-
SD 3	-	?	-	-	-
Cabo 1	+	?	-	-	-
Cabo 2	-	?	-	-	-
Cabo 3	+	?	-	-	-
Cabo 4	-	?	-	-	-
MZ 1	-	?	+	+	-
MZ 2	-	?	-	-	-
MZ 3	-	?	+	+	-
MZ 4	-	?	-	-	-
MZ 5	-	?	+	-	-
MZ 6	-	?	-	-	-
Pool #1	-	?	-	-	-
Pool #2	-	?	+	+	-

+ positive result, - negative result, ? inconclusive result

and Cabo 1 and Cabo 3 near Cabo San Lucas. PCRs for species-level detection of *Enterococcus faecalis* were unsuccessful as there was non-specific amplification of multiple DNA bands in every sample assayed (data not shown). *Vibrio cholerae* O1 was not detected in any sample (Table 2).

The first sample taken from the pool as it was first constructed and filled on the ship's fantail did not reveal the presence of any of the target pathogens. A second pool sample was taken three days later after many students had bathed in the pool. The second sample tested positive for *S. aureus* and the *mecA* gene (Table 2).

Discussion

Bacterial concentrations

The bacterial cell abundances observed in the coastal waters near San Diego and Manzanillo follow trends that may be indicative of contamination (Fig. 2). It is not uncommon in the oceans to observe high abundances of bacteria at the surface of the water column close to shore, and lower concentrations offshore in oligotrophic conditions. Such horizontal gradients can often be attributed to greater nutrient availability, increased total biological production, and greater amounts of suspended particles in the water column for attachment. However, in the coastal waters near Manzanillo in particular, the only parameter that cell densities seemed to correlate with was proximity to shore. Thus, being unable to correlate abundances with environmental parameters other than proximity to coastal cities, anthropogenic enhancement of bacterial abundance remains a likely possibility.

Pathogen detection

The pathogenic signatures and horizontal gradients detected in Pacific coastal waters in this project are indicators of anthropogenic and terrestrial enhancement of bacterial abundance. In particular, the detection of *S. aureus* and the *mecA* gene in surface waters near Manzanillo, Mexico is likely not a natural phenomenon. *S. aureus* was detected at MZ 1 and 3, and at MZ 5, approximately 55 km from shore. The *Staphylococci* *mecA* gene was detected at MZ 1 and 3, the two stations of that subgroup that are closer to shore (Fig. 3, Table 2). *S. aureus* normally colonizes the nasal cavity, skin, and gastrointestinal tract of humans and animals (Karst 2005), and its presence in seawater near Manzanillo can most likely be attributed to shedding from recreational swimmers, as was shown in Plano et al. (2011). In addition, our own pool

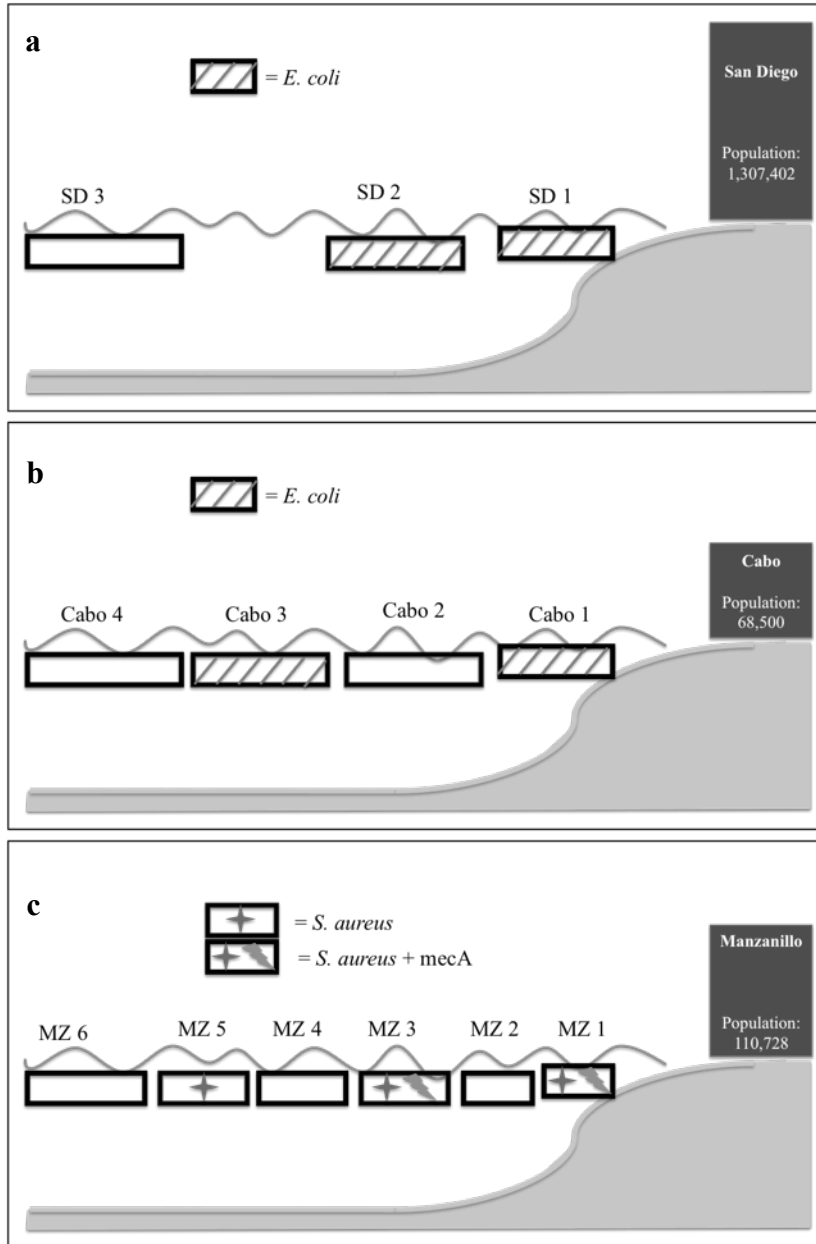


Fig. 3. Presence/absence of *E. coli*, *S. aureus*, and *mecA* in coastal waters near (a) San Diego, (b) Cabo, and (c) Manzanillo. Boxes with dashed lines indicate the presence of *E. coli*. Boxes with a star indicate the presence of *S. aureus*. Boxes with a star and a lightning bolt indicate the presence of both *S. aureus* and the *mecA* gene.

experiment verified that bather shedding can increase both cell density and pathogen concentrations in water, significantly enough to be detected by PCR (Table 2).

The smaller geographical range of *mecA* dispersion could be explained by the naturally smaller abundance of *Staphylococci* that carry this resistance gene. The *mecA* gene detected could be from any species of *Staphylococci*, and is commonly found in *S. aureus*, as methicillin-resistant *S. aureus* (MRSA). However,

only about 20% of human carriers of *S. aureus* are carriers of MRSA (Plano et al. 2011). Therefore, it is understandable that if *S. aureus*

and MRSA were being shed into seawater in this location, that MRSA would occur in lower

concentrations at the source of contamination and would be more quickly diluted beyond PCR detection limits.

The significance of the detection of the indicator bacteria *E. coli* in the coastal waters near San Diego and Cabo San Lucas, Mexico is less clear than detection of a definitively foreign pathogen such as *S. aureus* near Manzanillo, Mexico. Measurements of indicator cell densities in water, such as *E. coli*, are used by the United States E.P.A. as a proxy for evaluating the density of pathogenic bacteria that are introduced into the environment via sewage contamination. However, *E. coli* may be able to reside in the environment and even proliferate if conditions are suitable (Stewart et al. 2008). Because *E. coli* was the only indicator or pathogen detected at SD 1, SD 2, Cabo 1, and Cabo 3 (Table 2), it is especially unfortunate that the primers used to target *Enterococcus faecalis* were ineffective. *E. faecalis* and *E. coli* are both found in the gastrointestinal tract of animals, and therefore environmental presence of either is not solely indicative of sewage contamination. However, assessing the presence of *E. faecalis* would be particularly helpful in assessing the safety of coastal waters for human use because *E. faecalis* densities in seawater have been shown to correlate strongly with gastrointestinal illness in recreational swimmers (Dufour and Ballentine 1986).

A pattern of patchy positive results for *S. aureus* and *mecA* near Manzanillo and *E. coli* near Cabo is apparent in the PCR data (Table 2, Fig. 3), and can be addressed with a number of possibilities. The fact that MZ 1, MZ 3, and MZ 5 tested positive for *S. aureus*, and MZ 2 and MZ 4 did not seems counter-intuitive given that the *S. aureus* cells being detected were mostly likely terrestrially sourced (Fig. 3). However, it should be noted that a negative result in a PCR reaction does not conclusively determine that a target organism or gene is not present in a sample. It instead indicates either that DNA extractions failed to adequately purify nucleic acids for

amplification, that the organism was not present at an adequate concentration for PCR amplification of a target gene, or that the PCR primers were inadequate for targeting the organism at its given concentration in a sample. There is the possibility that the organisms were actually not present in the waters in that area, and that the dominant regional currents had transported any *S. aureus* either north or south of MZ 2 and MZ 4 at the time of our sampling. Patchy distributions of *E. coli* (Fig. 3b) might also indicate complicated regional transport mechanisms, or that shore based pollution is not the only source of *E. coli* near Cabo. Marine mammals or ship ballast water and waste discharge could be contributing to the observed biogeography of this organism.

Overall, it is difficult to assess the safety of San Diego and Cabo coastal waters with the given data. Although *E. coli* was found in both locales, the concentration of this organism is unknown because the results were not quantitative. No other target organism was detected in San Diego or Cabo, but there are a number of pathogenic bacteria not targeted in this study that can contaminate coastal waters and pose health risks for swimmers. The safety and quality of Manzanillo's near shore waters is almost certainly poor, and probably not ideal for recreational swimmers. Although *S. aureus* can colonize and thrive on human skin and surfaces quite harmlessly, the relationship can quickly turn dangerous when *S. aureus* is permitted entrance to the blood through a break in the skin or it finds itself in contact with an immunosuppressed individual (Karst 2005). Recreational use of *S. aureus*-contaminated water has also been reported to cause a four-fold increase in risk in children of developing a *S. aureus* infection (Charoenca and Fujioka 1995), and Manzanillo had both *S. aureus* and *mecA* in its beach waters.

The complete absence of toxigenic *V. cholerae* in all samples collected for this project indicates that Pacific coastal waters may be safe for commercial fishery uses. The environmental

reservoir of this organism in Peruvian coastal waters was implicated as the source of an international cholera pandemic in 1991 (Lipp et al. 2003), and its absence near San Diego and the Mexican coast might at least imply that this area will not be a source of this organism for future pandemics. That being said, my project's coastal water quality assessments are limited by the narrow time scale I had available to sample. Lipp et al. (2011) identified seasonality in the peaks of *V. cholerae* O1 abundances in Peruvian coastal waters, with the highest O1 detection occurring between January and March (Lipp et al. 2011). Similar trends in abundances of O1, or any pathogen for that matter, could occur in Mexico's coastal waters. Year-round sampling would be necessary to determine seasonal trends and to truly determine if these waters may be environmental reservoirs for pathogens.

The use of PCR for this project was useful for constructing a baseline understanding of the presence and distribution of pathogenic bacteria near San Diego, Cabo, and Manzanillo. That being said, being a non-quantitative evaluative technique, PCR was not fully adequate for assessing the safety of water for human use as it was unable to quantify the concentration of pathogens in the water. In addition, the detection limit of the primers I used was never determined. In the future I would like to compare the detection limits of my PCR primers to detection limits of other techniques in order to determine the most quantitative and accurate method to assess seawater safety.

Conclusions

- (I) Concentrations of bacteria were higher close to shore near San Diego and Manzanillo, and changed little with distance from shore near Cabo (Fig. 2). Pathogens, when detected, were detected at sampling locations closest to coastal cities. These coastal

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- cities were generally suspected as the source of the pathogens to the marine environment (Table 2, Fig. 3).
- (II) *E. coli* was found in surface seawater near San Diego and Cabo. *S. aureus* and the *mecA* gene was detected at 3 stations near the Mexican coastal city of Manzanillo (Table 2, Fig. 3). The density of this species was not determined.
 - (III) *S. aureus* was detected directly adjacent to shore in Manzanillo at MZ 1, and a maximum of 55 km away from shore at MZ 5. This confirms that this organism is indeed highly persistent in seawater, as shown in Gabutti et al. (2000). *S. aureus* coastal contamination could therefore pose a human health threat over a large geographic area around contamination sources.
 - (IV) The coastal and beach waters of Manzanillo appear to be highly impacted by anthropogenic activities and urban development in the region, and are probably not safe for all beach-goers. Children have been reported to be at highest risk of infection by *S. aureus* (Chaorenca et al. 1995), and other at-risk individuals could be harmed by this opportunistic pathogen.

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