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Creating a microcosm to examine salinity tolerance of *Escherichia coli* in beach sand

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ABSTRACT

Escherichia coli (*E. coli*) is a gram-negative bacteria species that thrives in a variety of environments around the world. Due to its widespread prevalence, it is commonly used as an indicator for fecal pollution and other pathogens. One place where it is not often looked for is oceanic beaches because *E. coli* is inhibited by salt. However, recent research has shown that *E. coli* often thrives in sand at many oceanic beaches. To determine how it persists in sand, we created a microcosm simulating the intertidal zone of an oceanic beach. Using this microcosm, we examined how varying levels of salinity (0-6‰) affect the persistence of *E. coli* in these sandy environments. We found that there was a negative correlation between increasing salinity and the most probable number of *E. coli* colony forming units, which suggests that *E. coli* is being inhibited by salinity to a degree. However, we still found that *E. coli* was able to persist at all salt concentrations including those that exceed normal oceanic salinity. Collectively, our findings suggest that *E. coli* may be able to persist on sandy beaches despite the stress of salinity and may be a useful tool in the future for assessing these ecosystems for fecal contamination levels.

INTRODUCTION

Escherichia coli (*E. coli*) are gram-negative bacteria that are typically found in the intestinal tract of endotherms where they can thrive in warm, moist conditions. *E. coli* has also been shown to persist outside of the host in varying environments (Boehm et al. 2009a) and is commonly considered to be a fecal indicator bacteria (FIB) because it can be used to measure the presence of fecal matter and indicate potential signs of pollution (Halliday et al. 2014). The presence of *E. coli* can also be used to signal potential pathogenic bacteria (Halliday et al. 2014). Due to *E. coli*'s association with pathogenic bacteria and fecal material, *E. coli* can be used as an important tool to assess ecological quality and sanitation (Brady et al. 2014).

One environment in which *E. coli* are commonly found includes oceanic beaches. Because the *E. coli* no longer persist in the constantly warm and moist environment of the intestinal tract, they face many stressors that can threaten their survival. Several of these stressors include temperature variation due to seasonal and daily changes, microbial competition, and

ultraviolet radiation from the sun (Halliday et al. 2014; Whitman et al. 2014). While temperature and sunlight influence *E. coli* growth, salinity has shown to be a major factor for the inhibition of *E. coli* growth (How et al. 2012; Ortega et al. 2009). Salinity, an important stressor at oceanic beaches, can inhibit the growth and survival of *E. coli* (Ortega et al. 2009). Due to the inhibition of *E. coli* by salt, the growth of *E. coli* at oceanic beaches where ocean water has a 3‰ salinity was thought to be hindered. However, our research and previous research has shown that *E. coli* can tolerate salinity and persist in oceanic environments where the salinity levels exceed typical oceanic salt concentrations (Ortega et al. 2009; How et al. 2012; Lewis et al. in prep). In previous studies, *E. coli* strains have been shown to be able to persist in high NaCl concentrations after multiple generations and that suggests that *E. coli* may be able to become increasingly halophilic over time (Lee et al. 2012; How et al. 2013).

One potential issue that arises from *E. coli*'s perceived lack of salinity tolerance is that the Environmental Protection Agency (EPA) does not test for *E. coli* on oceanic beaches

(EPA 2014). While the persistence of *E. coli* in freshwater sand is documented, limited research on the salinity tolerance of *E. coli* in beach sand exists (but see Yamahara et al. 2012). Testing of *E. coli* on oceanic beaches may provide greater insight into health and safety protocols for marine and beach recreation.

In our study, we created a microcosm of the intertidal beach zone to simulate the natural environment where *E. coli* can thrive as well as assess how *E. coli* can persist in a sandy environment with varying levels of salinity. Our goal is to better understand how salinity affects growth and persistence of *E. coli* in beach sand.

METHODS

An intertidal microcosm was created in order to simulate oceanic conditions and test the salinity tolerance of *E. coli* in intertidal sand at varying levels of salinity (0%-6% NaCl). Oceanic salinity levels range from around 3-4% NaCl, and the salinity level at Folly Beach, SC, is normally 3.8% (Heard, personal observation).

Microcosm Experimental Set-up

We set up an intertidal microcosm with sterilized materials using 96.25g of play grade sand that was placed in 600mL beakers. The play grade sand was sterilized using high heat from a drying oven. Following the addition of sterilized sand, nutrient broth with varying salt concentrations (0-6%) and a standard amount of *E. coli* was added. To obtain standardized levels of *E. coli*, we created a McFarland Standard (1.5 X 10⁸CFU/mL) (ThermoScientific Remel™, KS, USA 2015) of K-12 *E. coli*, a commonly used lab strain. We diluted the McFarland standard and added 1μL to 25mL of nutrient broth, which was mixed with varying concentrations of salt (NaCl; 0%-6%) and replicated in triplicate. The resulting mixture of nutrient broth inoculated with K-12 *E. coli* was placed onto the sterile sand. The sand and broth was mixed by swirling the beaker until the sand was saturated to simulate the intertidal beach region.

Bacteria Extraction & Incubation

E. coli was extracted from sand after 0 hours and 6 hours of bacteria deposition to determine the change in *E. coli* growth in response to salinity. *E. coli* was extracted by removing 7.0g of sand via a sterilized spatula and placing it into 35mL of water. The mixture was then shaken for two minutes and allowed to settle for 30 seconds (Boehm et al. 2009b; Velonakiset al. 2014). For each mixture, 300μL was micropipetted into each well of a 96-well Coliplate in triplicate for each salinity concentration. Coliplates were incubated for 48 hours at 37°C.

E. coli Enumeration

After incubation for 48 hours at 37°C, the blue wells of the Coliplate were counted (Bluewater Biosciences Inc., Mississauga, ON, Canada 2015). Subsequently, the fluorescent wells were recorded using an ultraviolet light. The fluorescent well amount for each Coliplate was correlated to the most probable number (MPN) of *E. coli* colony forming units per 100mL.

Analysis

We examined whether salinity inhibited the growth of *E. coli* using linear regression analysis. We analyzed changes in the abundance of *E. coli* over the six hours using ANOVA and Pairwise t-tests.

RESULTS

Salinity had a significant effect on the growth of *E. coli*. As the salinity concentration increased in the intertidal microcosm, the change in the MPN of *E. coli* colony forming units decreased (Graph 1). We observed a significant decrease in the amount of *E. coli* with increasing concentrations of salinity between 0 and 6 hours. Our pairwise t-tests also indicate that as salinity increases, the change in the MPN of *E. coli* colony forming units decreases; our results indicate that there are three distinct regions of *E. coli* levels between 0%-6% salinity between 0%-1%, 2%-3%, and 3%-6% (Graph 2).

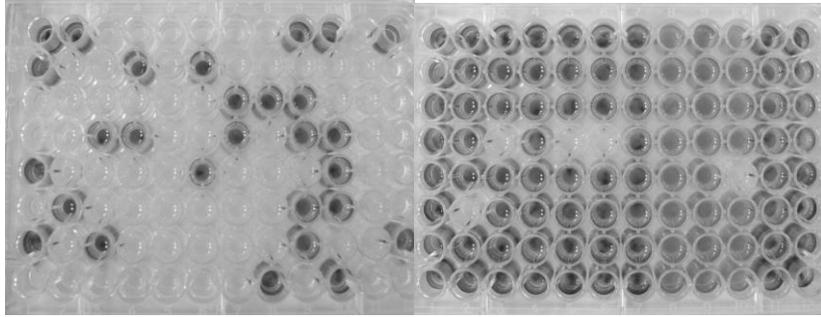


Figure 4. (A) contains 1% salinity at 0hr. (B) contains 1% salinity at 6 hours. As shown, there is an increase in blue wells and MPN (most probable number) of *E. coli* at this low concentration of salinity.

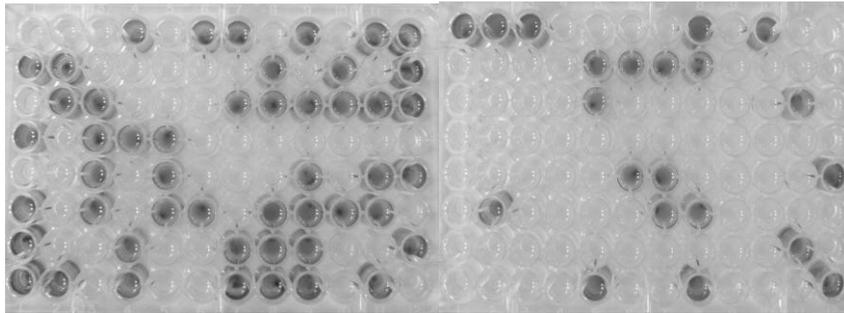
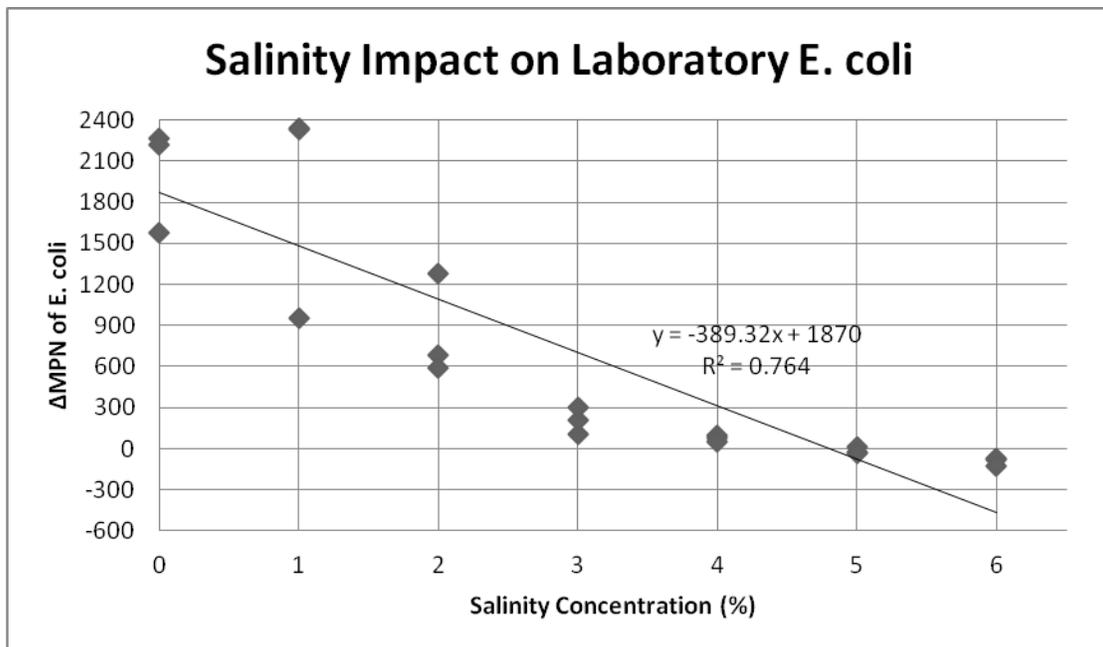
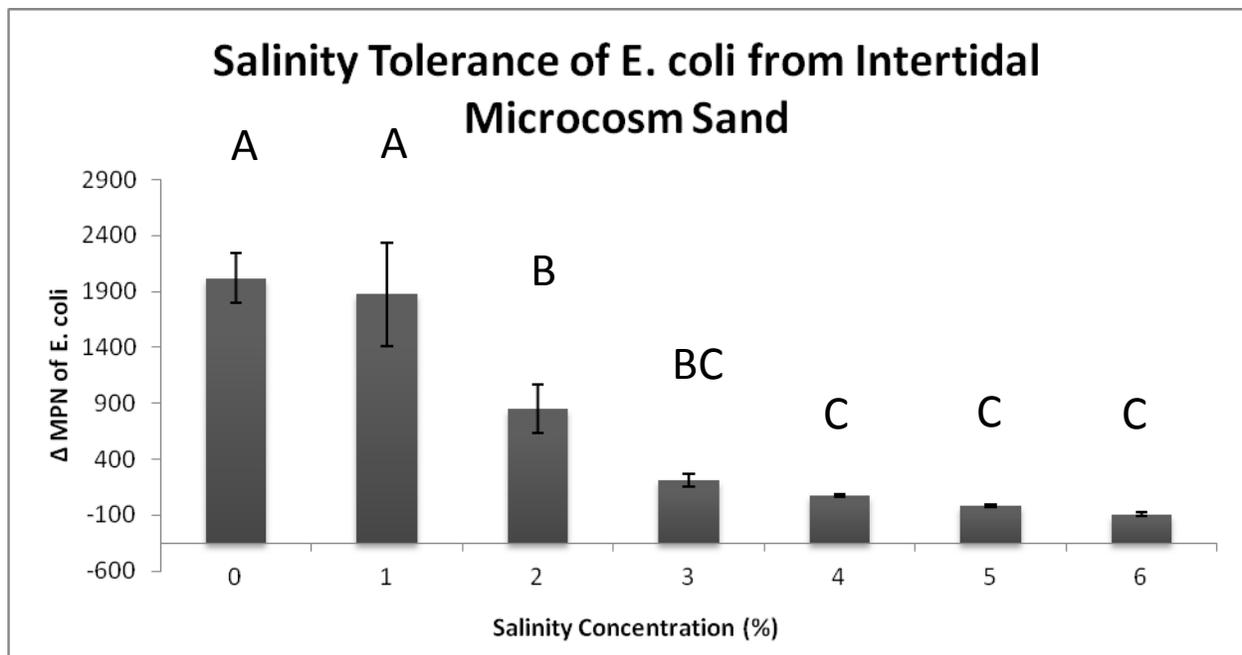


Figure 5. (A) contains 6% salinity at 0hr. (B) contains 6% salinity at 6 hours. There is a decrease in blue wells and MPN of *E. coli* at this higher concentration of *E. coli*.



Graph 1. We observed a significant effect of salinity concentration on the growth of *E. coli* ($R^2=0.764$; $p<0.0001$). Our linear regression analysis showed that the inhibition of *E. coli* growth increased as salinity concentration increased; a negative correlation was observed.



Graph 2. We observed a significant effect of salinity concentration on the growth of *E. coli* (F6,14=18.2553; $p < 0.0001$). Our Pairwise t-tests showed that the higher oceanic salinity concentrations (4-6%) were significantly different than the lower salinity levels (0-2%).

DISCUSSION

We found that *E. coli* is capable of surviving at salinity levels greater than present (approximately 3-4% NaCl) in most oceanic ecosystems. Although *E. coli* growth was inhibited by increased salinity as suggested by the decrease in the change in the number of MPN of *E. coli* forming units, *E. coli* persisted at salinity levels greater than 4%, which is the salinity of the ocean. Our microcosm findings imply that *E. coli* is capable of persisting on oceanic beaches in beach sand. As suggested by previous research, *E. coli* may undergo halophilization in which after several generations of exposure to saline conditions, bacteria can develop higher salinity tolerance levels (How et al. 2013). Because oceanic *E. coli* are continuously exposed to salinity, salinity tolerance may have resulted, allowing the persistence of *E. coli* growth and reduced inhibition.

Our findings also suggest that when health agencies like the EPA test for fecal contamination on oceanic beaches, they may want to consider including tests for *E. coli*. Rather than solely testing water for *E. coli*

contamination, levels of *E. coli* in sand should also be evaluated. Previous studies testing marine beaches suggest that the bacterial composition of marine water and sediment host different bacteria (Halliday et al. 2014). Not only are *E. coli* present in beach sand, but the sand may act as a bacteria reservoir and provide protection from UV radiation in marine environments (Beverdorf and Bornstein-Forst 2006). Because oceanic marine sand serves as a reservoir for *E. coli* and other associated enteric bacteria, *E. coli* testing is also important due to *E. coli*'s potential for pathogenicity. While the majority of *E. coli* bacteria are harmless, some *E. coli* can cause disease. An example is pathogenic *E. coli* O157:H7, which produces an enterotoxin that can cause diarrhea and gastrointestinal upset (Whitman et al. 2014; Yamahara et al. 2012).

In future studies, environmental isolates of *E. coli* could be used in our intertidal microcosm to assess their levels of salinity tolerance. Because environmental isolates of *E. coli* are constantly exposed to salinity levels, the bacteria may have a higher tolerance for salt

conditions than the K-12 lab *E. coli*. Further research may include microcosm testing of other beach zones, environmental *E. coli* strains, and temperature. Other beach zones that may be tested in a microcosm include the dune and subtidal zones. The combination between moisture and salinity of sand may play a role in *E. coli* survival. Previous research suggests that the intertidal region exhibits more *E. coli* growth than the subtidal sand region (Abdelzaher et. al 2009). While moisture or salinity may not account for this difference, the salinity levels amongst these regions may differ as the water evaporates from the sand, possibly influencing the salt concentration.

This work represents an important step in assessing *E. coli*'s ability to persist in oceanic beach environments. Furthermore, it suggests that testing sand in addition to water for the presence of *E. coli* is essential for identifying beaches that may be impacted by pollution and other pathogens.

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