Pilot- and bench-scale testing of fecal indicator bacteria survival in marine beach sand near point sources

Kathryn B. Mika[1], Greg Imamura[1], Crystal Chang[1], Victoria Conway[2], Genevieve Fernandez[3], John F. Griffith, Rita A. Kampalath[1], Christine M. Lee[1], Chu-Ching Lin[1], Raylene Moreno[1], Shawn Thompson[2], Richard L. Whitman[4] and Jennifer A. Jay[1]

Abstract

The microbiological quality of water at public bathing beaches is regularly monitored using fecal indicator bacteria (FIB) as a surrogate for the presence of human sewage and pathogens. Exposure to FIB and associated pathogens may also occur through contact with contaminated beach sand, but no standards limiting levels of microbes in sand or required monitoring program has been established. As a result, the factors affecting FIB and pathogen survival/persistence in sand remain largely unstudied. This goal of this study was to examine biological and physical factors that affect die-off of FIB in beach sand following sewage spills. Solar disinfection with mechanical mixing was pilot-tested as a disinfection procedure after a large sewage spill in the Los Angeles area. Effects of solar exposure, mechanical mixing, predation and/or competition, season, and moisture were tested at bench scale. First order decay constants for *Escherichia coli* ranged between -0.23 and -1.02 per day, and for enterococci between -0.5 and -1.0 per day. Desiccation was a dominant factor for *E. coli* but not enterococci. Initial microbial community and sand temperature were also important factors. Mechanical mixing, common in beach grooming, did not consistently reduce bacterial levels. Chlorination was an effective disinfection treatment in sand microcosms inoculated with raw sewage influent.

Introduction

Beach sand in both freshwater (LaLiberte and Grimes 1982; Bolton *et al*. 1987; Burton *et al*. 1987; Davies *et al*. 1995; Fujioka *et al*. 1999; Solo-Gabriele *et al*. 2000; An *et al*. 2002; Desmarais *et al*. 2002; Alm *et al*. 2003; Whitman and Nevers 2003; Whitman *et al*. 2003; Alm *et al*. 2006; Byappanahalli *et al*. 2006; Ishii *et al*. 2006a,b; Whitman *et al*. 2006; Ishii *et al*. 2007) and marine (Gerba and McLeod 1976, Martinez-Manzanares *et al*. 1992, Davies *et al*. 1995, Oshiro and Fujioka 1995, Craig *et al*. 2002, Desmarais *et al*. 2002, Craig *et al*. 2004, Boehm and Weisberg 2005, Ferguson *et al*. 2005, Jeong *et al*. 2005, Boehm 2006, Lee *et al*. 2006, Yamahara *et al*. 2007) environments is increasingly recognized as an important reservoir for *Escherichia coli* and enterococci, which are referred to as fecal indicator bacteria (FIB). The abundance of FIB in sand can have two distinct implications for public health. First, persistence and regrowth of non-pathogenic FIB in sediment (Byappanahalli and Fujioka 2004, Byappanahalli *et al*. 2006) may weaken the relationship between FIB and the pathogens they are meant to proxy. Second, sand may provide a favorable environment for pathogens and may be an understudied route of human exposure.

FIB survival in the water column depends on a variety of factors including salinity and exposure to solar radiation (Boehm *et al*. 2004, 2005, 2006;

[1] *University of California, Department of Civil and Environmental Engineering, Los Angeles, CA*
[2] *Sanitation Districts of Los Angeles County, Whittier, CA*
[3] *Port of Los Angeles, San Pedro, CA*
[4] *United States Geological Survey, Great Lakes Science Center, Ann Arbor, MI*

Boehm and Weisberg 2005; Grant *et al*. 2005; Pallud and Van Cappellen 2006; Santoro *et al*. 2006), while FIB fate in sediments is less well understood. In general, sediment may be more conducive to FIB survival relative to the water column due to reduced sunlight inactivation (Sinton *et al*. 1999), protection from predators (Brettar and Holfe 1992, Davies and Bavor 2000), nutrient and organic carbon availability (Gerba and McLeod 1976, LaLiberte and Grimes 1982, Blumenroth and Wagner-Dobler 1998, Craig *et al*. 2004), and the presence of a surface for the formation of biofilms (Brettar and Holfe 1992, Davies *et al*. 1995, Decho 2000). However, influences of these factors on the prevalence and persistence of FIB in sediment are not understood. In addition, there is ample evidence that sediments provide as favorable an environment for pathogens as they do for FIB. For example, decay rates for two species of *Salmonella* were shown to be higher in overlying water than in sand, suggesting that sand can act as a reservoir for these pathogens (Craig *et al*. 2003). Similarly, viruses adhere strongly to sands and show increased persistence when adsorbed (Gerba and Schaiberger 1975, Ferguson *et al*. 1996, Gantzer *et al*. 1998, Meschke and Sobsey 1998, Green and Lewis 1999).

This field and laboratory study investigates the dynamics of FIB in sand through both controlled experiments testing environmental effects on decay rates and a pilot-scale treatment study in Southern California. Specific goals are as follows: 1) measure first-order decay constants for FIB in plots of sand exposed to sunlight and raking following a sewage spill onto Manhattan Beach, CA; 2) investigate the influence of factors such as raking, light, and moisture on decay constants for FIB from sewage applied to sand in laboratory microcosms; and 3) investigate the differences between methods for measuring FIB in sand.

Methods

Manhattan Beach Pilot Test

Experimental Sand Plots

Plywood drying beds were constructed on the beach in the sewage-impacted area that consisted of a plywood base with a sand bag perimeter to contain the sand, as well as a plastic liner to prevent contamination from adjacent sand (Figure 1). In preparation for the start of the drying test, the surface of the sand within the sewage-impacted study area was raked to a 15 cm depth bihourly throughout the day. Sand from two depth intervals (0 to 31 cm and 31 to 61 cm) was excavated from a 10 x 10 cm plot of sand. This sand was spread 15 cm deep on a 10 x 20 cm plywood drying bed. Sand from a third depth interval, 41 to 92 cm, was excavated from the test plot, homogenized, and divided in two. Half of the sand from the third depth interval was placed on a 10 x 10 cm plywood drying bed. The other half of this sand was spread on the previously raked sand surface to an approximate depth of 15 cm (Figure 2). Triplicate Day-0 samples were taken from each of the four test drying beds at a depth of 8 cm. Throughout the experiment, the four test plots were raked with a leaf rake to turn over the top 15 cm of sand bihourly from 800 to 1600 PST.

Figure 1. Representative test beds raked and analyzed during Manhattan Beach experiment.

Sample Collection

Samples were collected from three locations within each sand plot every morning for eight days, as well as evenings for Day 3 and Day 6. Data from these three sample locations within the plot were averaged to obtain a value for the plot that would take some of the variation within the plot into account. All samples were collected with gloved hands in sterile containers, and kept on ice until analysis. Approximately 100 g of sand were collected per sample. Personnel at the Sanitation Districts of Los Angeles County (LACSD) conducted all field sampling and delivered samples curbside to the University of California, Los Angeles (UCLA). As a control, sand was also collected from three depths (31, 61, and 92 cm) along a core in a nearby unraked control area. This sand area was neither excavated nor treated.

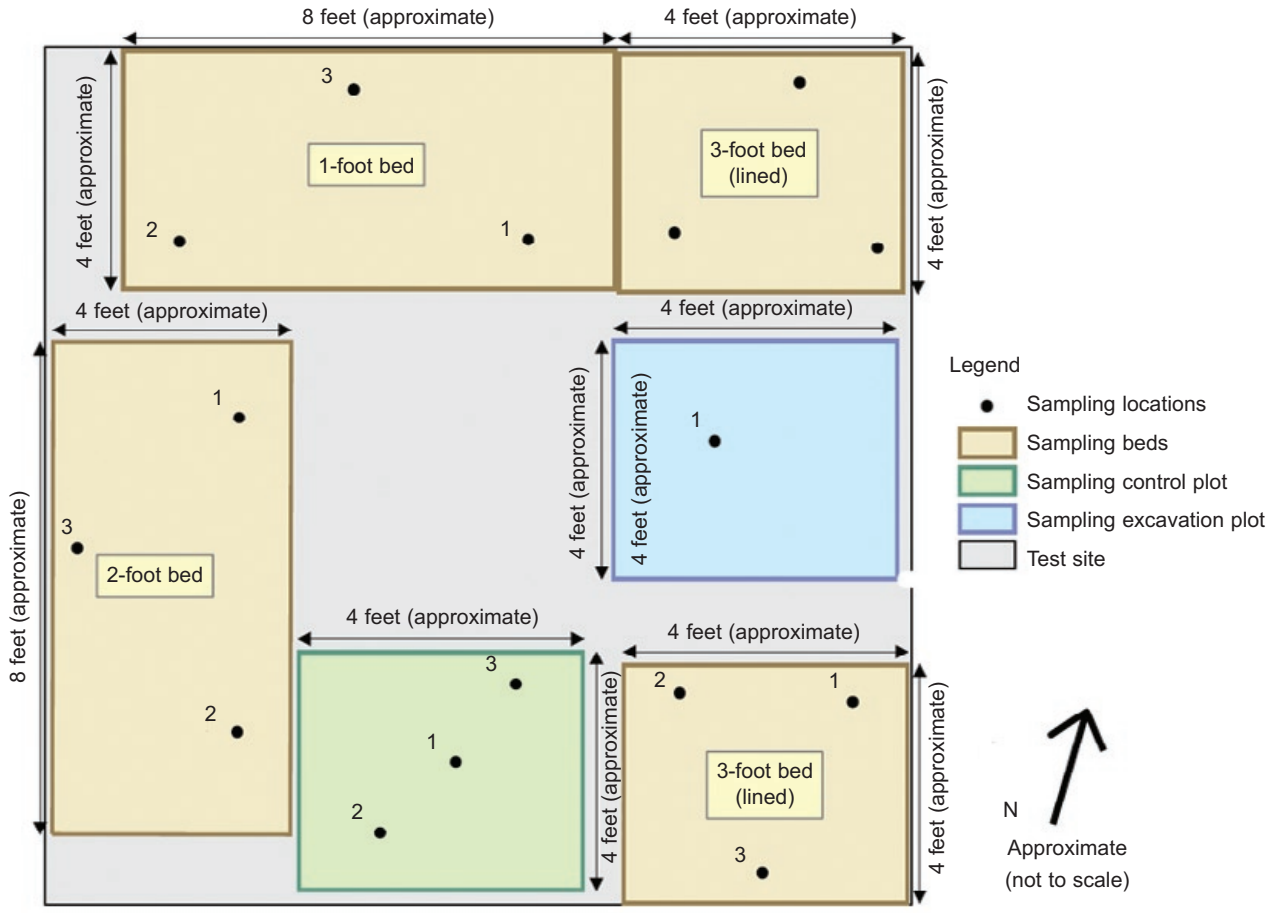

Figure 2. Manhattan Beach plot area. The 1-ft bed contained sand from 0 to 31 cm; the 2-ftb ed contained sand from 31 to 61 cm; the 3-ft bed (lined) contained sand from 61 to 92 cm with a plywood base; and the 3-ft bed (unlined) contained sand from 61 to 92 cm with no plywood. Samples were taken from three different locations within each plot.

Sand Analysis

Each sand sample was homogenized by stirring with a sterile metal spatula. Approximately 15 g were weighed into a 50-ml centrifuge tube, and 35 to 40 ml phosphate-buffered saline (PBS) with pH of 7.0 ±0.2 were added to the tube. Bacteria were detached from sand by vigorously shaking for 60 seconds, sonicating for 10 minutes, and shaking for another 60 seconds before decanting the buffer and repeating. The two volumes of buffer were pooled before enumeration of FIB with a defined substrate technology, IDEXX (Westbrook, ME).

Microcosm Experiments - Rooftop FIB Inactivation in Beach Sand

Sample Collection

Beach sand was collected from Santa Monica Beach, CA and autoclaved at 121ºC for 15 minutes in glass beakers for most microcosm experiments. For the competition microcosm, sand was collected from Manhattan Beach, CA and used within 24 hours. Autoclaved beach sand was inoculated with pure cultures of *Enterococci faecalis* (ATCC #11420) in October 2006 to compare the efficiency of solar inactivation in the presence and absence of raking over the course of several days.

To determine the effectiveness of iodine as a FIB disinfection agent, 250 ml of 80 parts per million (ppm) elemental iodine in solution were added to 100 g of sand in a perforated plastic beaker. The excess iodine was given 5 minutes to drain through the perforated bottom, and 45 g of sand were sampled. This process was repeated six times, per the manufacturer's instructions. Similarly, one 250-ml wash of 300 ppm aqueous chlorine was applied to inoculated sand to test the efficiency of chlorine as a disinfection agent. This sand was inoculated with either a pure culture of enterococci or raw influent. One chlorine-rinsed and

one iodine-rinsed beaker were sampled on Day 1 of all experiments. On the final day, one chlorine-rinsed and one iodine-rinsed beaker were sampled to test for regrowth. In a separate experiment, enterococci levels were measured in filtrate from water, chlorine, and iodine-rinsed microcosms to test the concentration of enterococci rinsed out of the sand. The iodine and chlorine were reconstituted in tap water, which would more closely resemble sewage spill clean-up dilutions than reconstitution in milli-Q water.

Sample Preparation

For pure culture experiments, enterococci cultures were transferred into fresh medium and allowed to grow overnight. Autoclaved sand was then saturated with a solution consisting of PBS and enterococci. For experiments simulating a sewage spill, sand was saturated with raw influent. In both experimental types, sand was homogenized by stirring with a sterile metal spatula.

Using ethanol-rinsed slotted spoons, approximately 150 g of sand were transferred into autoclaved, perforated plastic beakers. A sample was taken from the top, middle, and bottom layers of sand in the bucket to analyze for consistency in FIB levels throughout the inoculated sand. The bottom of each beaker was lined with sterile gauze to minimize sand loss through the perforations as the sand dried. All sand was placed in perforated microcosms for the rooftop experiments to allow direct comparison of bacterial counts in chlorine or iodine-rinsed beakers with counts in beakers with no external disinfection agent. All inoculated samples were transported to the flat roof of Boelter Hall, an eight-story building on the UCLA campus. Samples were placed on a low wall on the rooftop to maximize sunlight exposure.

Raking*:* The top 2.5 cm of sand was turned over to expose the sand to sun using an ethanol-rinsed spatula bihourly during daylight hours.

Moisture: The microcosm sand was moistened every bihourly during daylight. Five to 15 ml of 0.1X PBS were pipetted over the sand surface until the sand just reached saturation. While some salt residue could have been left in the microcosm sand by evaporation of the 0.1X PBS, this would have been a small amount relative to the high concentration of organic matter in the raw influent.

Predation/Competition: In April 2008, 16 beakers containing autoclaved sand and 16 beakers containing un-autoclaved sand were inoculated with raw influent. Beakers containing unautoclaved sand would still have the resident population of microorganisms, including predators. Beakers with autoclaved sand would have no potential predators, and could thus serve as a control for predation, if any, in the unautoclaved sand.

Solar Inactivation: The effectiveness of solar inactivation was studied alongside the competition microcosm experiment in April. Eight of the beakers containing autoclaved sand and eight of the beakers containing unautoclaved sand were placed on the roof beneath cardboard boxes to prevent sunlight from reaching the sand. The remaining eight beakers from each treatment type were left exposed to sunlight to test the effect of solar inactivation. Temperature in the sand in both microcosm types was measured with a sterilized kerosene-filled thermometer at 1000, 1200, and 1400 PST.

Seasonality/Temperature: Experiments with the same treatments were conducted in February, April, June, and December to look for effects of seasonality and ambient temperature on FIB survival.

Sand Analysis

One beaker per treatment type was sampled daily during the course of the microcosm experiments and discarded. The top 2.5 cm was homogenized by stirring with a sterile spatula before weighing out the sand aliquot for analysis.

Between 30 and 45 g of sand were weighed into a sterile plastic Nalgene bottle. Bacteria were detached from sand aliquots by vigorous shaking for 120 seconds. Samples were not sonicated in follow-up microcosm experiments because a previous experiment on a sand sample showed approximately 80% percent of bacteria are dislodged from sand without sonication. In addition, after the Manhattan experiment, hand shaking was determined to be the method most easily replicated across labs to dislodge bacteria as it requires no specific equipment. After allowing the bacteria to settle for 120 seconds, the buffer was decanted into a second sterile plastic bottle. The process was repeated, and the two volumes of buffer were pooled before enumeration with membrane filtration. Sterilized PBS was added to the washes for the first four days of the experiment to dilute the high concentration of FIB present in raw influent.

In accordance with EPA method 1600, three different volumes of the pooled buffer (0.3, 3, and 30 ml) were filtered onto 47-mm membranes (Fisher Brand) with 0.45-µm pores and placed onto 15 x 60 mm plastic Petri dishes containing membrane-Enterococcus Indoxyl-ß-D-Glucoside Agar (mEI). The plates were incubated overnight at 41ºC for 24 hours, and any colonies with visible blue halos were counted as enterococci. Similarly, according to EPA method 1603, *E. coli* were plated on modified membrane Thermotolerant *E. coli* (mTEC) agar. Plates were incubated at 35ºC for 2 hours, followed by incubation at 45ºC for 22 hours. Colonies with magenta halos were counted as *E. coli*. Colonies were counted by hand on the plates.

Method Comparison for FIB in Sand Samples

Samples were collected in conjunction with Southern California Coastal Water Research Project during the summer of 2007. From July through September, sand samples were collected at Avalon Beach on Catalina Island at 800, 1200, and 1600 PST. Both rooftop sand samples and field samples from Avalon Beach were analyzed for enterococci and *E. coli* using both membrane filtration and IDEXX. Samples were analyzed using the same pooled sediment wash.

Statistics

Regression plots were generated in STATA using the natural log of the concentration of bacteria over time. Slope differences between different treatments (i.e., raked vs. unraked) during the same experiment and between the same treatments (i.e., unraked in February and June) were then compared using an F test. Concentration differences between membrane filtration and IDEXX on the same sand sample were analyzed in STATA using a Wilcoxon signed-rank test. Standard errors were calculated using Excel to plot error bars (Figures 3, 6, 7). Half of the detection limit was used for samples that were non-detect in both methods. For samples above detection limit in IDEXX, the largest exceeded value was used as the bacterial count. For membrane filtration, 150 colonies, the maximum consistently countable, was set as the upper detection limit. The 150 colonies on the smallest wash filtered were used for the maximum detection limit after all dilutions had been maximized.

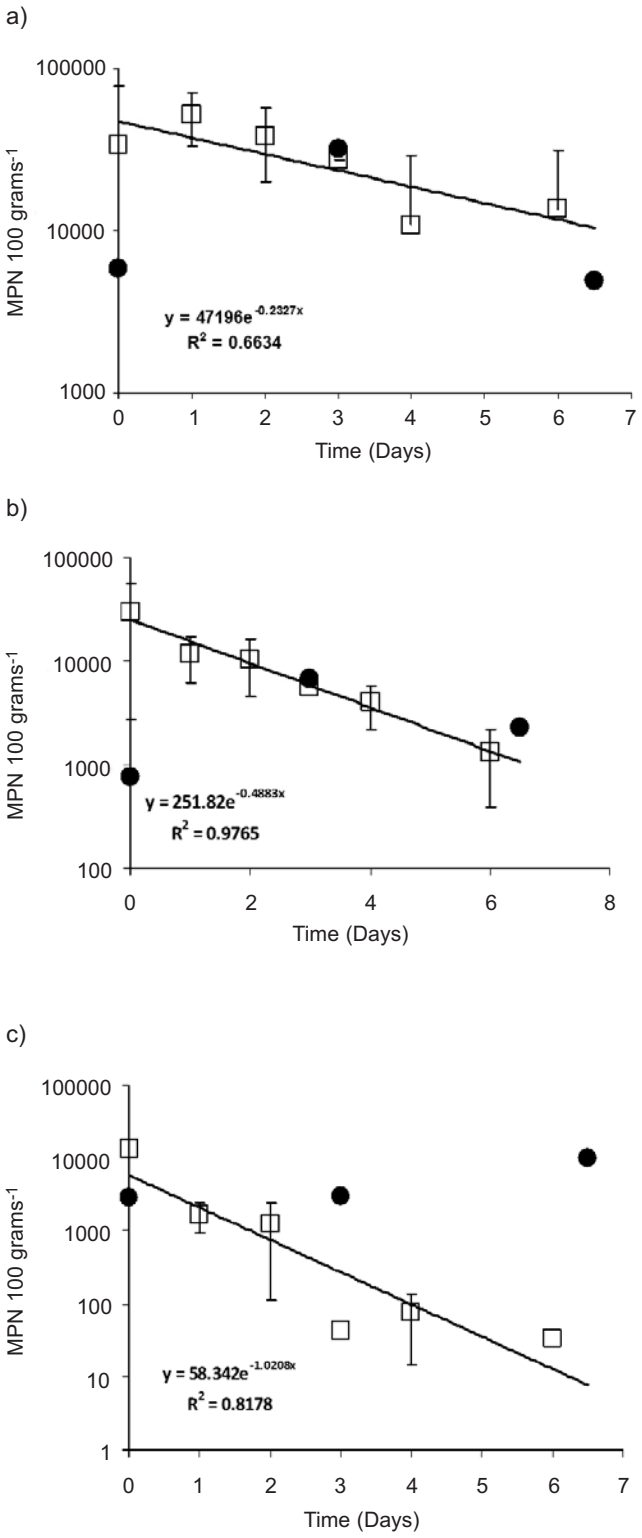

Figure 3. Manhattan Beach K-value graphs. Diamond denotes raked sample time points, while square denotes control values taken from sediment core. Plot 1contained sand from 0 to 31 cm depth (a); Plot 2 contained sand from 31 to 61 cm depth (b); and Plot 3 contained sand from 61 to 92 cm depth (c). Sample values in raked plots are denoted by ❐; control plots are denoted by ●.

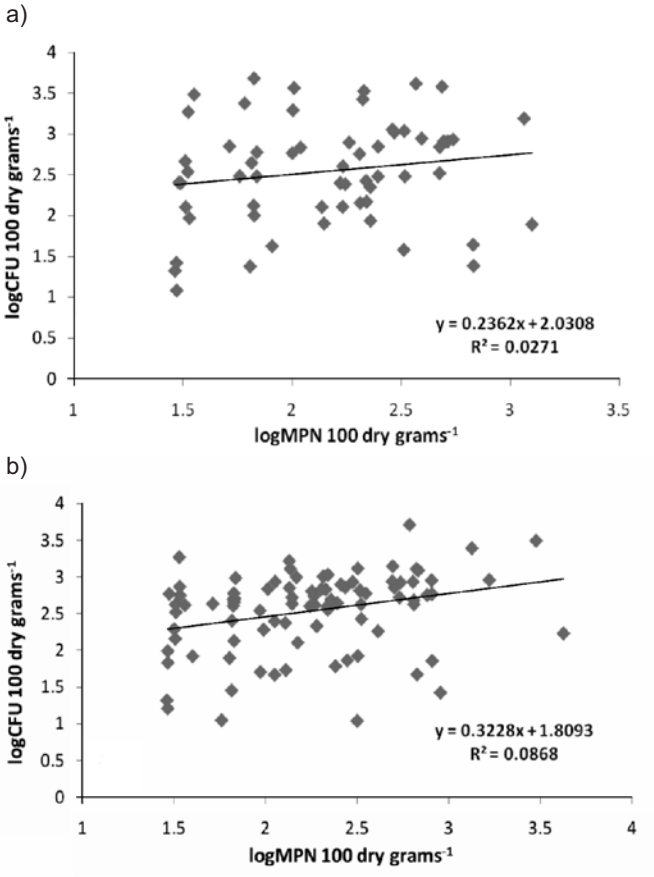

Figure 4. *E. coli* levels in sand at Avalon Beach measured by IDEXX and MF (a). Enterococci levels in Avalon Beach sand measured by IDEXX and MF (b). In environmental samples, there is very little correlation between the two methods for either FIB.

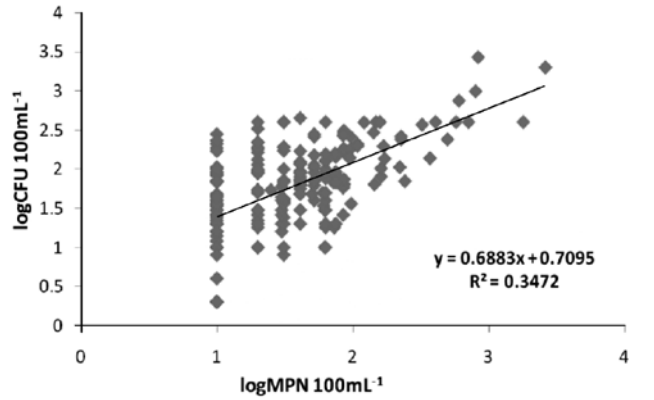

Figure 5. Enterococci levels as measured in Avalon ocean water had a better correlation between membrane filtration and IDEXX than in sand.

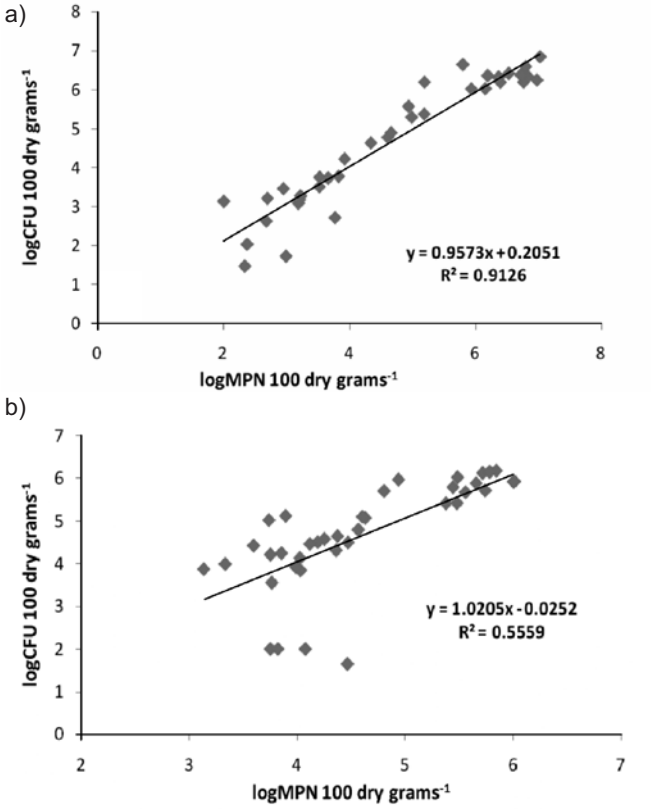

Figure 6. *E. coli* levels measured by MF and IDEXX in sand inoculated with raw influent (a). Enterococci levels measured by MF and IDEXX in sand inoculated with raw influent (b). In raw influent there is a high correlation between the two; when membrane filtration detects high levels of FIB, so does IDEXX.

Results

Pilot Test of Mechanical Mixing with Raking

To test the effectiveness of raking and exposure to sun on sewage-contaminated sand following a sewage spill at Manhattan Beach, CA, *E. coli* levels were measured in triplicate sand samples taken from each of four experimental plots each morning (Figure 3). Data were fit with a first-order decay regression line, with decay constants ranging from -0.23/day ($F_{1,4}$ = 7.9, P = 0.05), -0.48/day ($F_{1,4}$ = 166, P <0.01) and -1.02/day ($F_{1,4}$ = 18, P = 0.01) for the 0 to 31, 31 to 61, and 61 to 92 cm depth intervals, respectively. Sand from the deepest interval, treated on an unlined plot, had a decay constant of -0.44/day ($F_{1,4}$ = 5.9, P = 0.09). The heterogeneity in the unexcavated sand and limited number of plots constrained the extent of the experiment with respect to replicates and control over environmental factors.

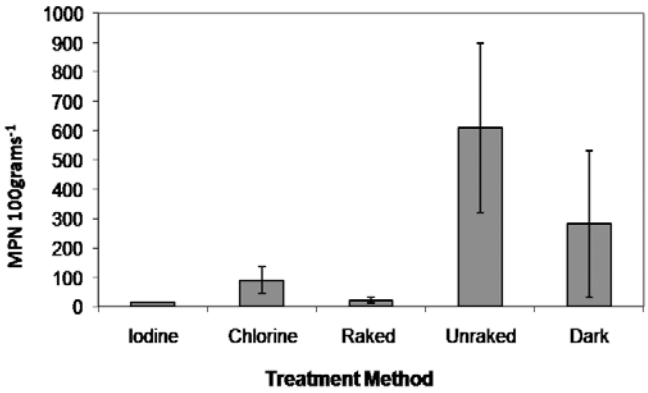

Figure 7. Final timepoint for October enterococci; in pure culture experiments raking effectively increased die-off by the end of the experiment.

Method Comparison

Membrane filtration (MF) experiments on the rooftop detected levels of enterococci and *E. coli* up to 20 times higher than those detected by IDEXX in sand. The geometric mean for enterococci using MF was 63,000 ±16 MPN/100 g compared to 53,000 ±8 MPN/100 g in IDEXX; for *E. coli* the geometric means were 84,000 ±40 MPN/100 g using MF compared to 83,000 ±40 MPN/100 g using IDEXX. Membrane filtration also detected slightly higher numbers on average at Avalon Beach. The geometric mean for enterococci as measured by IDEXX was 180 ±3 MPN/100 g compared to 343 ±4 CFU/100 g as measured by MF (sample values ranged from 3.5 to 4,000 MPN/100 g IDEXX and 3.5 to 5,100 CFU/100 g). Similarly, values for *E. coli* were 142 ±3 MPN/100 g and 346 ±4 CFU/100 g (sample values ranged from 3.5 to 700 MPN/100 g and 3.5 to 1,000 CFU/100 g). The MF and IDEXX measurements were significantly different in rooftop microcosm samples and environmental samples (Wilcoxon Test, $Z = -5$, $P < 0.01$). However, the correlation between MF and IDEXX results varied greatly in field and microcosm experiments. In field measurements, MF and IDEXX values in sand had little correlation ($R^2 = 0.03$ for *E. coli* and $R^2 = 0.09$ for enterococci; Figure 4). Environmental water samples showed better correlation between methods than was observed in sand samples ($R^2 = 0.35$; Figure 5). In microcosm experiments using raw influent; however, MF and IDEXX results showed much better correlation ($R^2 = 0.91$ for *E. coli* and $R^2 = 0.55$ for enterococci; Figure 6). Microcosm experiments had an N of 28; Avalon *E. coli* in sand had an N of 107; Avalon enterococci in sand had an N of 113; and Avalon enterococci in water had an N of 155.

Rooftop Microcosms

Pure Culture Microcosms/Disinfection Treatments

The first experiment was conducted in October 2007, with autoclaved sand inoculated with pure culture enterococci in sand and raked every two hours. Variables being considered were the effects of solar exposure and raking. First order decay constants were seen in both raked ($k = -0.75$/day, $F_{1,5} = 0.77$, $P = 0.43$) and unraked (-0.63/day, $F_{1,5} = 1.9$, $P = 0.24$) treatments exposed to the sun. Raking and iodine had a greater effect on FIB die-off than chlorine after five days exposure to sunlight on the roof (Figure 7).

A follow up experiment was done to measure bacteria levels in filtrate from chlorine and iodine-rinsed microcosms. Microcosms rinsed with water had enterococci present in filtrate after the sixth and final wash (135 MPN/100 ml). In microcosms rinsed with iodine, low levels of enterococci remained in the wash even after the sixth wash (26 MPN/100 ml). In microcosms rinsed with chlorine, enterococci dropped below detectable levels from the first wash to the sixth wash (2.7 MPN/100 ml). Iodine and chlorine achieved a reduction of one or two logs, respectively, of enterococci concentrations (Initial concentration were 2400 MPN/100 ml).

Sunlight Exposure, Temperature, and Seasonal Effects on Microcosms

Weather varied from 10ºC to 33ºC over the course of the rooftop microcosm experiments. In February and December 2007, temperatures ranged from 10ºC to 14ºC, with overcast skies. Although still overcast in June 2007, the weather was warmer, with temperatures ranging from 18ºC to 22ºC. In April 2008, temperatures ranged from 21ºC to 33ºC, with clear sunny skies. Sand temperatures in the microcosms exposed to sun in April climbed above 50ºC in the microcosms. Two days of intense heat at the beginning of the experiment caused very rapid FIB die-off. Seasonality had a greater effect on the inactivation rates of *E. coli* under the same treatment (i.e., raked) from month to month, with four out of five comparisons of the same treatments between months having P values less than or equal to 0.01. By contrast, statistically significant seasonal differences were only seen in enterococci in two out of five seasonal comparisons.

In April, half of the microcosms were left on the roof under a cardboard box, sheltered from sunlight.

Sand temperatures in microcosms kept in the dark never climbed above 41ºC and no rapid die-off was observed. In enterococci, a significant difference between light and dark microcosms was only observed between light and dark in unautoclaved sand ($F_{1,35}$ = 3.6, P = 0.04). In *E. coli*, however, a significant difference ($F_{1,32}$ >4.9, P ≤0.01) was observed between all light and dark microcosms, regardless of sand type. Despite the rapid die-off observed between Day 2 samples and Day 3 samples in the microcosms exposed to sunlight, the decay rates over the course of the experiments for light and dark microcosms were similar (*E. coli*: k = -1.2 to -1.5/day, $F_{1,8}$ >17, P <0.01; Enterococci: k= -0.50 to -0.64/day, $F_{1,8}$ >13, P <0.01).

Moistened Microcosms

Treatments in June 2007 were moistened by pipetting. Five to 10 ml of 0.1X PBS were applied to the microcosms to determine how desiccation affects FIB decay rates. The average percentage of moisture in moistened sand samples was 14 ±5%. In non-moistened microcosms, the moisture content of the sand had dropped below 1% by the third day of the experiment. Interestingly, decay constants for enterococci were very similar between moistened and non-moistened microcosms (Tables 1, 2). Microcosm experiments showed no statistical difference in enterococci survival as measured by moisture content (MF: $F_{1,23}$ <0.6, P >0.46 in all microcosms, IDEXX: $F_{1,37}$ <0.1, P >0.80) (Figure 8). However, a significant difference was found between all moistened and non-moistened treatments in *E. coli* (membrane filtration: $F_{1,23}$ >3.9, P <0.03, IDEXX: $F_{1,35}$ >7.0, P <0.01; Table 3). The addition of moisture significantly decreased the decay rate of *E. coli* as measured by both MF and IDEXX to below the decay rates of enterococci (Tables 1, 2).

Raked Microcosms

Mechanical mixing (raking) did not significantly affect decay rates when a microcosm was inoculated with sewage in either July or February microcosms. Although the difference between microcosms was not significant, raking appeared to modestly increase the rate of *E. coli* decay in February (-0.73/day, $F_{1,8}$ = 32, P <0.01 for raked compared to -0.62/day, $F_{1,8}$ = 9.3, P = 0.03 for unraked). In July, however, raked microcosms for *E. coli* had a slightly lower decay rate than unraked (Table 3).

Predation/Competition

No significant difference was seen between microcosms using autoclaved and unautoclaved sand in the April experiment with enterococci ($F_{1,35}$ <3.6, P >0.07). In *E. coli*, no significant difference was seen between autoclaved sand and unautoclaved sand in either the dark microcosms ($F_{1,32}$ = 0.04, P = 0.85) or the light microcosms ($F_{1,32}$ = 0.11, P = 0.74).

Table 1. P values for regression of each treatment. Bacterial concentrations were measured by IDEXX. CI = confidence interval; AD = autoclaved dark treatment; UD = unautoclaved dark treatment; AL = autoclaved light treatment; UL = unautoclaved light treatment; UR = unraked; R = raked; MR = moist raked; and MU = moist unraked.

Month, Treatment	*E. coli*		Enterococci	
	P value	K/day (CI)	P value	K/day (CI)
April AD, UR	<0.01	-1.4 (-2.2, -0.62)	<0.01	-0.64 (-0.95, -0.33)
April AL, UR	<0.01	-1.5 (-2.2, -0.74)	<0.01	-0.57 (-0.9, -0.24)
April UD, UR	<0.01	-1.2 (-1.6, -0.82)	<0.01	-0.51(-0.81, -0.21)
April UL, UR	<0.01	-1.37 (-2.0, -0.76)	<0.01	-0.5(-0.82, -0.18)
December UR	0.25	-0.31 (-0.94, -0.31)	0.02	0.68 (0.19, 1.2)
July R	<0.01	-1.35 (-2.0,-0.75)	<0.01	-0.68 (-1.1, -.26)
July UR	<0.01	-1.7 (-2.4, -0.97)	<0.01	-0.75 (-1.1, -0.35)
July MR	0.04	-0.55 (-1.1, -0.03)	0.02	-0.9 (-1.6, -0.18)
July MU	0.06	-0.44 (-0.90, -0.02)	0.01	-0.73 (-1.2, -0.25)

Table 2. P values for regression of each treatment. Bacterial concentrations were measured by membrane filtration. CI = confidence interval; UR = unraked microcosms; R = raked microcosms; MR = moistened raked microcosms; and MU = moist unraked microcosm.

Month, Treatment	*E. coli*		Enterococci	
	P value	K/day (CI)	P value	K/day (CI)
February UR	0.03	-0.62 (-1.2, -0.1)	0.34	-0.2 (-0.64, 0.27)
February R	<0.01	-0.73 (-1.1, -0.4)	0.44	-1 (-1.5, 0.27)
July R	<0.01	-1.4 (-2.1, -0.65)	<0.01	-0.77 (-1.1, -0.48)
July UR	<0.01	-1.5 (-2.2, -0.8)	<0.01	-0.83 (-1.2, -0.50)
July MR	0.05	-0.63 (-1.2, -0.01)	<0.01	-0.94 (-1.4, -0.50)
July MU	0.05	-0.68 (-1.35, 0)	0.02	-0.81 (-1.4, -0.25)

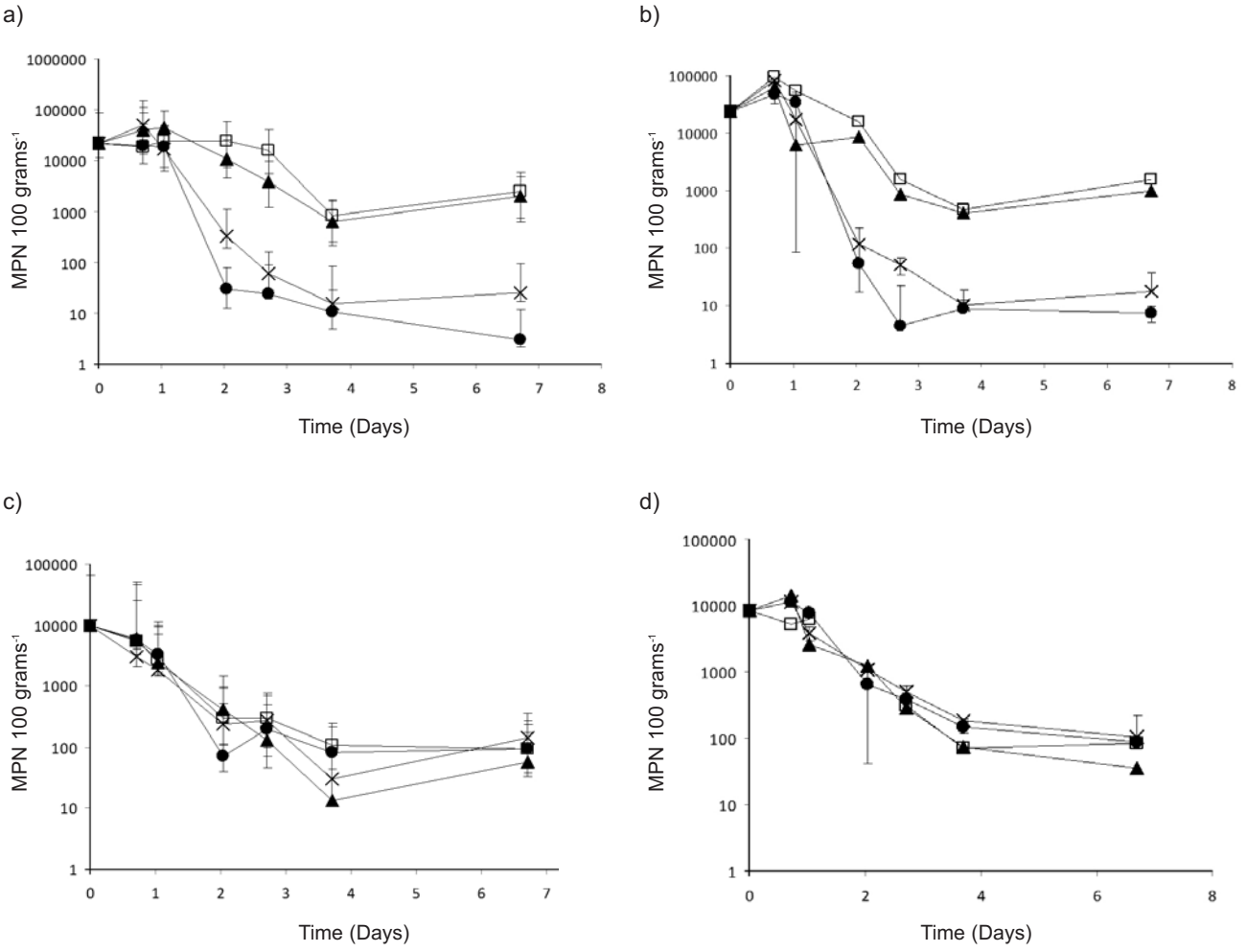

Figure 8. *E. coli* concentrations per 100 grams dry sand inoculated with sewage measured by IDEXX (a). *E. coli* concentrations per 100 grams dry sand measured by MF (b). Enterococci concentrations per 100 grams dry sand-dmeasured by IDEXX (c). Enterococci concentrations per 100 grams dry sand measured by MF (D). Treatments: Moist Unraked (❐), Moist Raked (▲), Unraked (●), and Raked (X).

Table 3. P values for slope comparison of the different treatments within the same experiment. AD = autoclaved dark treatment; UD = unautoclaved dark treatment; AL = autoclaved light treatment; UL = unautoclaved light treatment; UR = unraked; R = raked; MR = moist raked; and MU = moist unraked.

Month	Method	Treatments	F-test	P value	F-test	P value
July	IDEXX	MR, MU	F(1,37)=0.00	0.95	F(1,35)=0.1	0.75
July	IDEXX	MR, R	F(1,37)=0.00	0.95	F(1,35)=8.9	<0.01
July	IDEXX	MR, U	F(1,37)=0.07	0.8	F(1,35)=8.4	<0.01
July	IDEXX	MU, R	F(2,37)=0.01	0.99	F(2,35)=7.6	<0.01
July	IDEXX	MU, U	F(2,37)=0.04	0.96	F(2,35)=7.0	<0.01
April	IDEXX	AD, UD	F(1,35)=0.57	0.46	F(1,32)=.04	0.85
April	IDEXX	AD, AL	F(1,35)=1.96	0.17	F(1,32)=8.4	<0.01
April	IDEXX	AD, UL	F(1,35)=3.44	0.07	F(1,32)=7.0	<0.01
April	IDEXX	UD, AL	F(1,35)=2.39	0.11	F(1,32)=5.7	<0.01
April	IDEXX	UD, UL	F(1,35)=3.61	0.04	F(1,32)=4.9	0.01
April	IDEXX	AL, UL	F(1,35)=1.87	0.17	F(1,32)=0.11	0.74
July	MF	MR, MU	F(1,23)=0.13	0.72	F(1,23)=0.04	0.85
July	MF	MR, R	F(1,23)=0.00	0.98	F(1,23)=9.9	<0.01
July	MF	MR, U	F(1,23)=0.57	0.46	F(1,23)=6.3	0.02
July	MF	MU, R	F(1,23)=0.08	0.92	F(1,23)=6.2	<0.01
July	MF	MU, U	F(1,23)=0.28	0.76	F(1,23)=3.9	0.03
February	MF	R, U	F(1,11)=0.04	0.85	F(1,11)=0.19	0.67

Discussion

Membrane Filtration and IDEXX Comparison

Membrane filtration had higher counts on average for both enterococci and *E. coli* in sand in both microcosm and field experiments. It is possible that the overnight incubation required by the IDEXX method provides more time for environmental factors in the sediment wash, such as metals, to interfere with the production of the fluorescent byproduct used to determine concentration. Environmental factors do not have the same potential for interference in membrane filtration since the bacteria are removed from the wash during the filtration process prior to incubation. Similar patterns were seen in river water samples with high concentrations of FIB. While Colilert and membrane filtration results were the same at levels below 1,200 *E. coli* organism per 100 ml, Colilert values were significantly lower than membrane filtration results at levels above 10,000 *E. coli* per 100 ml (Colilert 12,400 ±2,600 vs. membrane filtration 16,700 ±2,300; Solo-Gabriele *et al*. 2000). Membrane filtration has been shown to have an 11 to 26% false positive rate in seawater samples (Ferguson *et al*. 2005), which may be another contributing factor to higher detected values of enterococci using this method. However, other work has shown the opposite effect in soil.

Effects of Environmental Factors on FIB Survival

Moisture

Sustained moisture greatly affected *E. coli* survival in microcosm experiments in this study, but did not appreciably change the survival of enterococci. Additional moisture decreased the decay rates of *E. coli* to less than one half of non-moistened decay rates, and caused *E. coli* to decay slower than enterococci. Enterococci may be more tolerant to desiccation in a sandy matrix than E.coli, and less tolerant to increased moisture. Previous studies have found a negative correlation between enterococci levels and moisture in a survey of sand along the California coast (Yamahara *et al*. 2007). Levels of *E. coli* in soil have been shown to decrease dramatically with distance from water (and decreasing water content), while enterococci levels remained relatively constant along this gradient (Desmarais *et al*. 2002). Microcosm and field studies at a river showed that drying and wetting cycles help foster *E. coli* regrowth and enhance certain sub-populations (Solo-Gabriele *et al*. 2000) in sand. In the same study, microcosm results showed that, while desiccation would initially decrease *E. coli* levels, the subsequent addition of moisture would result in a resurgence of *E. coli* levels. These results indicate that although *E. coli* can be inactivated through desiccation, some cells can recover and regrow upon the addition of new moisture. Increased persistence of

E. coli was observed in raked wet sand that was then compacted, presumably due to slower desiccation (Kinzelman *et al*. 2004). Beversdorf *et al*. showed *E. coli* levels in sand were highest with soil moisture at 15 to 19%, with lower levels at 0 to 14% and 20 to 24% (Beversdorf *et al*. 2006). Whitman *et al*. (2006) showed that fully saturated sands decreased in *E. coli* depth but remained homogeneous in the upper fringe zone.

Sunlight Exposure, Temperature, and Seasonal Effect

Looking at various seasonal factors including sunlight intensity, temperature, and seasonal effect, the most important factor appeared to be the sand temperature itself. Rapid die-off in both enterococci and *E. coli* was observed when sand temperatures climbed above 50ºC in April. Similarly, Beversdorf *et al*. (2006) recovered no cells in sand microcosm experiments maintained at 50 or 55ºC for several days. *E. coli* survival has been shown to be inversely related to temperature in both water and sediment. *E. coli* can survive for greater than 28 days at 10ºC (Craig *et al*. 2004). Despite the rapid initial die-off of FIB in sunlit microcosms, FIB levels in both microcosms exposed to sunlight and those kept in the dark were at similar levels (9 to 26 MPN/100 g) after eight days exposure to sunlight. The overall decay rates were very similar as well, only varying by 0.1/day between the light and dark treatments. This is consistent with other research, which had found that solar inactivation alone is relatively ineffective in sand relative to water. Where controlled exposure to UV light achieved 94 to 99% reduction in *E. coli* levels in pure culture, the same UV light application had no significant effect on *E. coli* levels in sand microcosms. While seasonality may be a significant factor in FIB decay rates, the significant difference may also reflect changes across experiments based on effects of solar exposure and temperature, different initial bacterial concentrations, or different species compositions across experiments.

Previous Exposure to Sunlight

The Manhattan Beach pilot test results exhibited inactivation rates for *E. coli* ranging from -0.22 to -1.02/day, which bracket the rates observed in rooftop experiments. While the heterogeneity within each depth interval could not be determined, as just one treatment existed for each depth interval, the results suggest that the starting population may be important (as all treatments were exposed to identical insolation, raking, and temperature). FIB in the top foot of sand, which had previously been exposed to sunlight, were the most resistant, while those in the deepest layer were more susceptible to inactivation. These results are consistent with previous research. Fecal coliforms (FC) surviving WSP treatment had higher sunlight resistance than cells in raw sewage (Sinton *et al*. 1999, 2002). Using optical filters, studies (Sinton *et al*. 2002) showed that FC and somatic coliphage were mainly inactivated by shorter wavelengths, and work by Davies-Colley *et al*. (1997, 1999) observed that *E. coli* inactivation occurred mostly from exposure to shorter wavelengths. Shorter wavelength (or uvB) radiation causes photobiological damage, or direct damage to DNA. While it can be bactericidal, photobiological damage is also largely reparable, through processes such as dark repair, photoreactivation, and post-replicative repair (part of the "SOS" response.) Thus, surviving cells from this type of damage have repair systems activated. In contrast, enterococci were inactivated by a wider range of wavelengths, and the resulting photooxidative damage lead to greater susceptibility to further damage. Thus, the greater resistance seen here for previously exposed *E. coli* cells would not be expected to be observed for enterococci, and may be due to physical differences between *E. coli* and enterococci.

Both sediment and water (salt or fresh) microcosms inoculated with different sources of FIB (dog feces, contaminated sediment, and sewage) showed very different decay rates under the same environmental conditions (Anderson *et al*. 2005). FIB persistence was observed to be greatest in contaminated sediments, followed by wastewater and then dog feces. This trend supports the idea that the persistence of FIB may be positively correlated with the time of exposure to selective pressure in the environment. The lack of correlation between concentration of bacteria detected by MF and IDEXX in Avalon sand samples also supports the idea of a changing FIB population with time spent in the environment, as these methods detect different subsets. However, in other work with environmental water samples, temperature and solar radiation were shown to impact inactivation rates of FIB (Noble *et al*. 2004), without much rate difference from differing sources of pollution.

Raking

The influence of raking on decay rate was

examined in microcosms that included unraked controls. For the various sets of microcosm experiments, the effect of raking was relatively weak compared to the effect of moisture, for example, and not consistent. Raking can have a negative or positive effect on the survival of FIB in sand. In February, raking slightly increased the decay rate for *E. coli*; in July, raking slightly decreased the decay rate for *E. coli*. Raking can potentially increase the efficiency of solar inactivation by exposing more bacteria to sunlight. However, raking can also increase access to nutrients and oxygen by the mixing of the sand, which could help the bacteria persist. Plots of sand groomed with a tractor had higher levels of *E. coli* than plots that were raked manually or left undisturbed. In contrast, sand plots with daily raking, twice weekly raking, and no raking had statistically indistinguishable levels of *E. coli*. (Kinzelman *et al*. 2003) A follow-up study showed that raking followed by leveling, which results in compaction after aeration, resulted in a favorable environment for *E. coli* survival (Kinzelman *et al*. 2004). Exposure to solar inactivation through raking was not an effective method of increasing FIB decay rates. Raked and unraked microcosm decay rates were not significantly different in both July and February experiments.

Predation/Competition

Microcosms comparing inactivation rates in autoclaved and unautoclaved dry sand from Manhattan Beach, taken from the location of the sewage spill in 2006, were used to investigate the influence of native biota, if any. There are other factors that may be influenced by autoclaving, such as nutrient release; thus, it is difficult to isolate the effects of predation and/or competition. The effect of autoclaving was weak in both microcosms exposed to sunlight and those kept in the dark. It has been shown that predation effects on bacteria are the same in microcosms kept consistently moist or subjected to drying periods, even though the effectiveness of soil protozoa are reduced in dry conditions (Kuikman *et al*. 1989). While some studies have shown predation by various protozoan communities in some environments to be a significant impact on bacterial populations (Recorbet *et al*. 1992, Ronn *et al*. 2002, Bomo *et al*. 2004), a significant predation effect on bacterial decay was not observed in this sand sample.

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