WATER RESEARCH XXX (2012) 1–13

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A multi-beach study of *Staphylococcus aureus*, MRSA, and enterococci in seawater and beach sand

Kelly D. Goodwin [a,*,1], *Melody McNay* [b,c], *Yiping Cao* [b], *Darcy Ebentier* [b], *Melissa Madison* [b], *John F. Griffith* [b]

[a] *National Oceanic and Atmospheric Administration (NOAA), AOML, 4301 Rickenbacker Cswy, Miami, FL 33149, USA*
[b] *Southern California Coastal Water Research Project, Costa Mesa, CA 92626, USA*
[c] *Cooperative Institute of Marine and Atmospheric Studies, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Florida 33149, USA*

ARTICLE INFO

Article history:
Received 26 November 2011
Received in revised form
1 April 2012
Accepted 2 April 2012
Available online xxx

Keywords:
Staphylococcus aureus
MRSA
Recreational water quality
Enterococci
Seawater
Beach sand

ABSTRACT

Incidences of *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA) have risen worldwide prompting a need to better understand routes of human exposure and whether standard bacterial water quality monitoring practices adequately account for this potential threat. Beach water and sand samples were analyzed during summer months for *S. aureus*, enterococci, and MRSA at three southern California beaches (Avalon, Doheny, Malibu Surfrider). *S. aureus* frequently was detected in samples of seawater (59%, $n = 328$) and beach sand (53%, $n = 358$). MRSA sometimes was detected in seawater (1.6%, $n = 366$) and sand (2.7%, $n = 366$) at relatively low concentrations. Site specific differences were observed, with Avalon Beach presenting the highest concentrations of *S. aureus* and Malibu Surfrider the lowest in both seawater and sand. *S. aureus* concentrations in seawater and sand were correlated to each other and to a variety of other parameters. Multiple linear regression on the combined beach data indicated that significant explanatory variables for *S. aureus* in seawater were *S. aureus* in sand, water temperature, enterococci in seawater, and the number of swimmers. In sand, *S. aureus* concentrations were related to *S. aureus* in seawater, water temperature, enterococci in seawater, and inversely to surf height classification. Only the correlation to water temperature held for individually analyzed beaches and for *S. aureus* concentrations in both seawater and sand. To provide context for these results, the prevalence of *S. aureus* in sand was compared to published fomite studies, and results suggested that beach prevalence was similar to that in homes.

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1. Introduction

Microbial contamination of marine waters worldwide is estimated to cause millions of gastrointestinal and acute respiratory infections (ARIs) (Shuval, 2003) and numerous skin infections (Yau et al., 2009) every year. Marine-borne pathogens in the US cost over $900 million per year, with $300 million from gastrointestinal illness from beach recreation (Ralston et al., 2011). Marine-related food-borne illness from *Staphylococcus aureus* was estimated at less than $500,000 per

Abbreviations: CHROMagar™ Staph aureus (SCA), fecal indicator bacteria (FIB).

* *Corresponding author*. Tel.: +1 858 546 7142; fax: +1 858 546 7003.
E-mail address: kelly.goodwin@noaa.gov (K.D. Goodwin).
[1] Stationed at SWFSC, La Jolla, CA 92037, USA.

0043-1354/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.watres.2012.04.001

year; unfortunately, data were not available to consider costs due to skin infections.

S. aureus is an opportunistic pathogen carried by 20–40% of people (Al-Zu'Bi et al., 2004 Kluytmans and Wertheim, 2005; Kuehnert et al., 2006) with an estimated ~0.8% of the US population (~2.3 million people) colonized by MRSA (Kuehnert et al., 2006). Incidence of infection from hospital- and community-onset *S. aureus* and MRSA is on the rise worldwide (Chambers, 2001; Zetola et al., 2005). Unlike typical hospital-associated strains, some strains of community-associated *S. aureus* can cause infections in healthy people with no traditional risk factors for infection (Chambers, 2001; Baba, 2002; Eguia and Chambers, 2004; Mulvey et al., 2005; Gorwitz, 2008). Even though most patients are treated as outpatients, hospitalization rates remain substantial (Kuehnert et al., 2005; Jarvis et al., 2007). The proportion of skin and soft tissue infections caused by MRSA has risen substantially (Moran et al., 2005), and drug resistant infections place an additional $5 billion burden on the United States health care system annually (Zhang et al., 2011).

Beaches have been suggested as a potential source of community-acquired *S. aureus* infection (Charoenca and Fujioka, 1995; Soge et al., 2009). Support for this suggestion derives from concentrations of *S. aureus* and total staphylococci being correlated to GI illness and to skin, eye and ear infections among bathers (Seyfried et al., 1985; Calderon et al., 1991; Gabutti et al., 2000). *S. aureus* and MRSA are shed by swimmers (Robinton and Mood, 1966; Hanes and Fossa, 1970; Smith and Dufour, 1993; Elmir et al., 2007; Plano et al., 2011), and both are found in beach seawater and sand (Goodwin and Pobuda, 2009; Soge et al., 2009; Sinigalliano et al., 2010; Shah et al., 2011; Enns et al., 2012). *S. aureus* concentrations have been correlated to bather density and attributed to human activity (Calderon et al., 1991; Charoenca and Fujioka, 1995; Papadakis et al., 1997; World Health Organization, 2003).

It has been suggested that human activity is the source of *S. aureus* at the beach (El-Shenawy, 2005). However, *S. aureus* also is found in stormwater (Selvakumar and Borst, 2006) and in coastal streams that drain to the coast (Viau et al., 2011), and wastewater may be another source of *S. aureus* to the environment. Although some studies have not found viable cells or genetic signatures in treated municipal wastewater (Volkmann et al., 2004; Shannon et al., 2007), other studies have found viable *S. aureus* and MRSA in raw (Ahtiainen et al., 1991; Rusin et al., 2003; Börjesson et al., 2009, 2010; Goldstein, 2010) and secondary treated wastewater (Goldstein, 2010). In addition to human inputs, domestic pets can be reservoirs for *S. aureus* and MRSA (Malik et al., 2006; Weese et al., 2006; Nuttall et al., 2008; Baptiste et al., 2009), and dogs can be significant contributors of fecal indicator bacteria (FIB), to the beach (Wright et al., 2009; Zhu et al., 2011).

In an effort to reduce human exposure to microbial contaminants, recreational waters are monitored for FIB, such as enterococci (USEPA, 2004; Dorman and Stoner, 2007). In turn, beach closures can be costly; for example, a 4-month closure of a Southern California beach resulted in millions of dollars of lost revenue, and almost 2 million dollars was spent in closure investigation fees (Dwight et al., 2005). Despite the investment in FIB monitoring, there are concerns that it may overlook pathogens that are not primarily associated with feces (such as *S. aureus*), and that a complementary indicator may be warranted (Cheung et al., 1990). Staphylococci have been suggested as an alternative or complementary indicator for marine water quality (Seyfried et al., 1985; Cheung et al., 1990; Gabutti et al., 2000).

An additional concern is that current FIB guidelines do not monitor the concentrations of bacteria in beach sands, and concern is growing with regard to this exposure route (Heaney et al., 2009; Yamahara et al., 2009; Hartz et al., 2010; Halliday and Gast, 2011; Phillips et al., 2011; Shibata and Solo-Gabriele, 2012). There also is concern that even dead and injured cells deposited to the environment from treated wastewater may pose a threat because antibiotic resistance genes can be taken up by live bacteria (Ahtiainen et al., 1991; Martinez, 2009; Börjesson et al., 2009, 2010). The transfer of *mec*A genes, which confers resistance to methicillin and other beta-lactam antibiotics, is thought to be relatively rare; however, as the abundance of *mec*A DNA has increased in the environment, the chance of transfer has increased (Chambers, 2001).

In this study, the prevalence and concentration of *S. aureus* and MRSA were studied in both seawater and beach sand. Results were compared to enterococci seawater concentrations and environmental parameters. Correlation, regression and multivariate statistical analyses were performed to identify parameters descriptive of the measured bacterial concentrations and to explore whether FIB monitoring might reflect concentrations of *S. aureus* (a non-fecal pathogen) and whether FIB and pathogens might share conditions suggestive of environmental persistence. The study represents a large collection of samples analyzed for *S. aureus* and MRSA from beach water (>320) and sand (>350) taken on 89 different sampling days.

2. Materials and methods

2.1. *Sample collection, processing, and bacterial identification*

Samples of sand and seawater were collected from beaches at Avalon, Doheny, and Malibu Surfrider beaches in California. Bulk water samples were collected as part of the Pacific Coast Water Study and processed as described previously in Converse et al. (2012). Processing of samples for *S. aureus* analysis was as described in Goodwin and Pobuda (2009) for Avalon and Doheny beaches. In addition, samples of seawater (40–300 ml) or sand (~120 g) were collected from Malibu Surfrider beach from May to September in 2009. A total of 12 beach sites, excluding sites in the lagoons at Malibu and Doheny, were tested on 89 different sampling days during the summers of 2007–2009. At Avalon (Fig. 1) samples were collected on 46 different days; sites A, B, and C were tested in both 2007 and 2008, whereas site D (Descanso Beach; separate watershed with less commercial development) was tested only in 2008. For Malibu Beach (Fig. 1), samples were collected on 38 different days. Malibu sites A and B were near the lagoon outlet, and Site A also was near a housing development on septic system. Only a small number of samples were successfully processed for *S. aureus* from Doheny Beach (5

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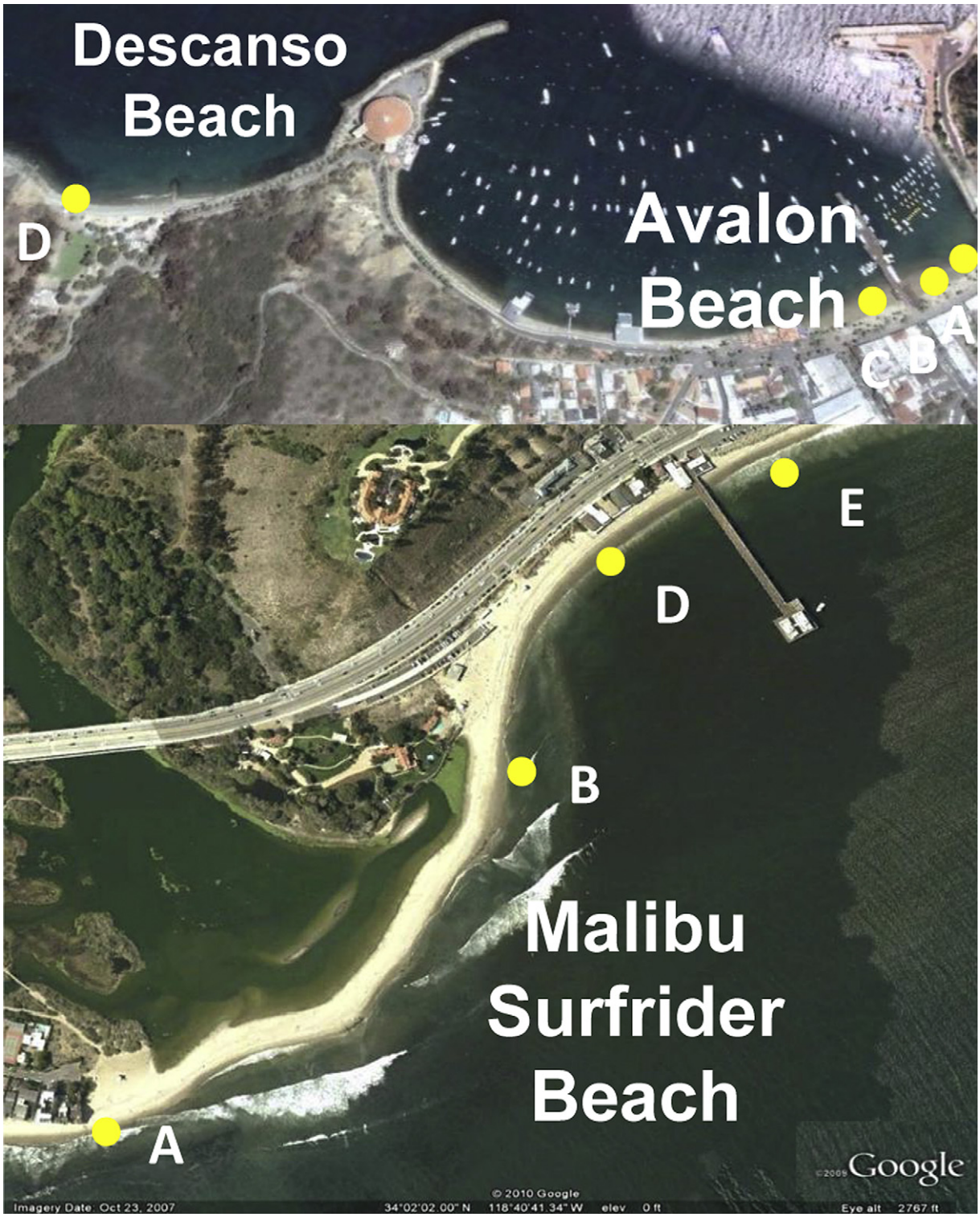

Fig. 1 – Aerial photos of Avalon and Malibu beaches (available from google.com) with sample sites depicted. Doheny beach is not shown because data from only one site is presented herein.

sampling days) and from lagoon sites (e.g., between Malibu Sites A and B; Fig. 1) due to overgrowth of non-target colonies; data from lagoon sites were not included in the analysis here.

Filtered samples of water were incubated either on CHROMagar™ Staph aureus (SCA) or CHROMagar™ MRSA (BD Biosciences, San Jose, CA, USA) at 37 °C for 24 h for SCA and 48 h for CHROMagar™ MRSA. Plates were refrigerated overnight prior to counting to allow for better color development. No enrichment or recovery step was used in this study. Putative *S. aureus* colonies were counted, and typically all or

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sometimes a representative number of colonies (~50%) was picked, streaked for isolation, and incubated as described above to confirm/adjust the initial count. Isolates were identified as *S. aureus* or MRSA through a combination of morphology and PCR as previously described (Goodwin and Pobuda, 2009). Briefly, *S. aureus* was identified on SCA or CHROMagar™ MRSA plates as a mauve colony with matte halo. For the study here, 3360 colonies were streaked for isolation to determine morphology and 846 isolates were checked by PCR. Previous work (Goodwin and Pobuda, 2009) showed that combined filter and isolate appearance with these seawater and sand samples provided a good balance between sensitivity and specificity, with a positive % agreement (sensitivity), a negative % agreement (specificity), and % positive predictive accuracy of 84%, 95%, and 99%, respectively (Goodwin and Pobuda, 2009).

PCR confirmation of isolate identity utilized primers to amplify the *clf*A gene (ClfA-F, 5′-GCAAAATCCAGCACAACAGGAAACGA-3′; ClfA-R, 5′-CTTGATCTCCAGCCATAAT TGGTGG-3′) (Mason et al., 2001) (Fig. 2A) and some samples also were tested for the presence of the staphylococcal 16S rRNA gene (Mason et al., 2001). For colonies isolated from CHROMagar™ MRSA and that were positive for *S. aureus* by PCR, MRSA confirmation utilized primers to amplify the *mec*A gene (mecA-F, 5′-TCCAGGAATGCAGAAAGACCAAAGC-3′; mecA-R, 5′-GACACGATAGCCATCTTCATGTTGG-3′) (Mason et al., 2001; Goodwin and Pobuda, 2009) (Fig. 2B). Amplification reactions were carried out using 1X Phusion™ HF Buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTPs (BioRad), 0.5 μM of each primer, 0.3 mg/ml bovine serum albumin (BSA), 0.5 μl (1 U) Finnzymes Phusion Hot Start High Fidelity DNA Polymerase (NEB, Ipswich, MA), 1 μl of cell colony lysate, and nuclease-free water for a final volume of 50 μl. Amplification conditions were as follows: 98 °C for 30 s; 35 cycles of 98 °C for 5 s, 60 °C for 10 s, 72 °C for 15 s; a final 8 min extension at 72 °C. Inhibition controls consisted of a sample reaction spiked with positive control DNA (0.5 μl of 10 pg/μl stock). The presence of an inhibition control band but not a sample band was used to verify negative reactions (Fig. 2). Inhibited samples were diluted 1:5 and re-run to verify the negative reaction. Positive controls consisted of a DNA control and crude lysate control as described in Goodwin and Pobuda (2009).

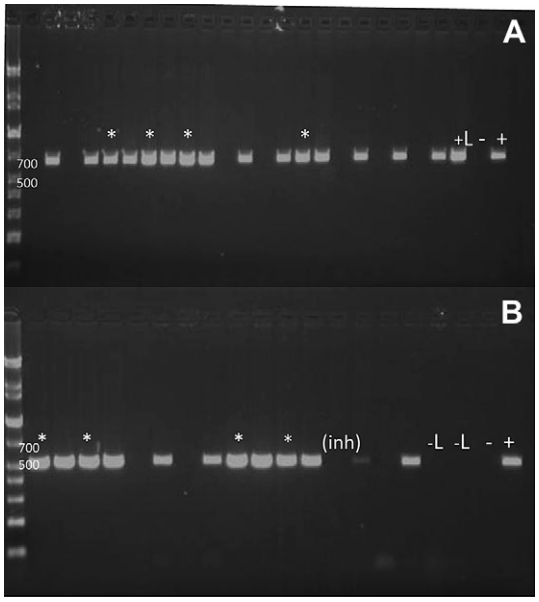

Fig. 2 – Agarose gel electrophoresis photos for bacteria isolated from Avalon beach and amplified with A) *clf*A primers or B) *mec*A primers. Pairs of wells contain sample amplicon and the corresponding inhibition control (sample plus spike of positive control DNA). * represents a positive sample, (inh) represents a partially inhibited sample, − represents a no template control, + represents a positive DNA control, +L represents a crude lysate from a *S. aureus* colony, and −L represents *S. aureus* crude lysate, however the culture was not MRSA, therefore, no *mec*A amplicon was expected. The first lane of each gel contains a DNA ladder with the 500 and 700 bp bands identified.

2.2. Data analysis

Two main types of data analysis were conducted: i) descriptive statistics to explore general frequency and trend results at the study sites and ii) correlation and regression analyses to explore relationships between *S. aureus*, enterococci, and other non-microbial parameters measured in the study. To best deal with samples below the limit of detection ("nondetects") and varying detection limits, statistical methods specially designed to handle censored data were used (Helsel, 2005). This method is regarded as superior to value substitution for nondetects in data analysis (Helsel, 2010). For comparison with the censored data approach, data were analyzed with standard linear regression using traditional value substitution for nondetects (referred as non-censored data hereon).

2.2.1. Descriptive statistics

Percent frequency of detection described the percentage of samples positive for *S. aureus*, enterococci, or MRSA; calculated as the number samples with one or more target colonies out of the total number of samples in which countable data was obtained for that target. The mean, standard error, standard deviation, median, confidence intervals, and percentiles of *S. aureus* and enterococci concentrations were computed using the nonparametric Kaplan–Meier method with Efron bias correction via the macros %KMSTATS and % BootKM for Minitab®16 software (available at www.practicalstats.com). These macros adapted the Kaplan–Meier method to deal with nondetects (left-censored data) by flipping the data to right-censored before calculation (Helsel, 2005). Descriptive statistics also were calculated by ROS estimation with the macro %bootros; however, results were equivalent so only the Kaplan–Meier statistics were presented here.

The median detection limit for *S. aureus* was 0.67 CFU/100 ml (range 0.33–4 CFU/100 ml), and the median detection limit for enterococci was 1 CFU/100 ml (range 1–100 CFU/100 ml). Nondetects with a detection limit greater than 2 CFU/100 ml for *S. aureus* and 4 CFU/100 ml for enterococci were removed for calculation and comparison of descriptive statistics. This removed 8 data points for *S. aureus* (6 from Doheny, 2

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from Avalon 2008) and 5 for enterococci (3 from Doheny, 2 from Avalon 2007). The actual detection limit for each sample was otherwise utilized. No values were removed from the sand data (the typical detection limit was 3.03 CFU/100 dry g). For MRSA data, the number of nondetects was greater than 50%, rendering the descriptive statistics of limited value due to high uncertainty. Therefore, MRSA data were described in terms of the % of samples above threshold and the 95% upper confidence limit (UCL) for the mean (Helsel, 2005).

Median concentrations for *S. aureus* and enterococci for three or more groups of censored data were compared using a Kruskal–Wallis multiple comparison testing using the %censKW macro with an alpha 0.05 subcommand. Comparisons between two groups of censored data used a Mann Whitney-U test by executing the %censMW macro. Censored boxplots were generated using the %cbox macro.

2.2.2. Correlation and regression analyses

Relationships between *S. aureus*, enterococci, and other non-microbial parameters were explored via simple correlation/regression and multiple linear regression analyses. In general, a bacterial concentration (*S. aureus* in seawater, *S. aureus* in sand, or enterococci in seawater) was compared to the set of remaining variables. The following continuous variables were utilized: 1) *S. aureus* seawater concentration (ln CFU/L); 2) *S. aureus* sand concentration (ln CFU/100 g dry sand); 3) enterococci seawater concentration (ln CFU/L); 4) water temperature (°C); 5) number of swimmers counted during sampling; 6) number of persons with water contact determined via questionnaire; 7) number of dogs; 8) number of birds; 9) tide (m). The following ordinal variables were utilized: 10) surf height classification (0 = none; 0.76 = low, 0.3–0.9 m; 1.5 = moderate, 1.2–1.8 m; high = 2.1 + m); 11) wind classification (0 = none; 1 = light; 2 = moderate; 3 = strong); and 12) turbidity classification (0 = clear; 1 = calm; 2 = slightly turbid; 3 = choppy; 4 = turbid). Data were analyzed per individual beach and for the combined data. In addition, analysis of Malibu Surfrider beach included berm flow (0 = open; 1 = closed).

First, simple correlation was used to test association between two variables: a bacterial concentration of interest and another variable (see above). The nonparametric Kendall's tau correlation coefficient and test of significance was calculated using the macro %Ckend for Minitab®16 software (Helsel, 2005). The Kendall's tau analysis used a rank-based measure of association and allowed one or both variables to include nondetects, multiple detection limits, and required no assumption about the distribution of the data. The macro returned values for both tau a and tau-b (which ignores ties) in a range of −1 to 1, with a zero value supporting the null hypothesis of no correlation between the variables. One advantage of a Kendall tau test was that it generated a *p*-value, which tested whether the value was significantly different from zero. For comparison, the Kendall tau value runs about 0.2 lower than a Pearson's *r* value for an equivalent strength of correlation (D. Helsel, personal communication). The nonparametric regression line associated with the Kendall's tau, the Akrita-Theil-Sen (ATS) line, was calculated using the %ATS macro command (Helsel, 2005).

Next, multiple linear regression was conducted between one dependent and multiple independent variables (described above) to obtain a best-fit model describing bacterial concentrations. The goal of the regression analysis was descriptive so interaction and nested terms were not evaluated. Regression with censored data was performed using the Minitab®16 option for regression with life data (arbitrary censored option) and maximum likelihood estimation (MLE). Model selection was based on *p*-values for the coefficients (typically $\alpha = 0.01$), with insignificant terms sequentially removed. Models with significant terms were further evaluated using the likelihood-ratio test which compared the model to the null using the equation $-2 \times$ (log-likelihood of the null model − log-likelihood of model); test statistics with *p*-values less than 0.01 were considered significant. Test statistic *p*-values were calculated via the chi-square distribution with the degrees of freedom equal to the number of estimated coefficients in the model subtracted by the number of estimated coefficients in the null model, as per Minitab® instructions.

In addition, typical regression analysis with non-censored data (i.e., value substitution) also was performed. This analysis returned information regarding model fit including residual plots, Anderson-Darling statistics, and variance inflation factors to identify multi-co-linearity. Furthermore, principal component analysis, stepwise regression, and best subset analysis was performed to help understand the data structure and to inform and confirm results from the multiple linear regression analysis. Additionally, the distributions of *S. aureus* in sand and seawater and of enterococci in seawater were analyzed using both censored and non-censored data, with output including probability plots and goodness-of-fit tests.

3. Results

3.1. Microbe detection frequency and concentrations at the study sites

3.1.1. S. aureus in seawater

S. aureus was frequently detected in seawater. The average % detection frequency for seawater was 59% ($n = 328$) for the combined beaches (Table 1). For Avalon, the average % detection frequency was 76% for the two summers studied. For Malibu Surfrider, the average % detection frequency was 46%. Only eight Doheny seawater samples were successfully enumerated for *S. aureus* due to overgrowth of plates, and all were positive for *S. aureus* (100%). The mean concentration of *S. aureus* in the seawater samples estimated by Kaplan–Meier statistics was 10 CFU/100 ml and the median concentration was 0.83 CFU/100 ml, with data for individual beaches given in Table 1.

Kruskal–Wallis multiple comparisons tests ($\alpha = 0.05$) showed that the median seawater concentration of *S. aureus* was significantly lower at Malibu Surfrider compared to the other beaches (Fig. 3). Within Malibu, the four beach sites (Fig. 1) had similar seawater concentrations (although site B was significantly lower than site D). Site A, with presumed septic influence, was not significantly different from any of the other sites. Within Avalon, the four beach sites (Fig. 1) also were similar to one another (although Site B in 2008 was significantly higher than Site C in 2007). Overall, *S. aureus* at Avalon was significantly higher in 2008 than 2007 (7.5 versus

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Table 1 – Detection frequency and concentration of *S. aureus* and MRSA in seawater and sand. NC = not calculated.

Site	*S. aureus*	Enterococci	MRSA
Detection frequency seawater			
All	59%	79%	1.6%
Concentration seawater (CFU/100 ml)			
	Mean (95% CI); median (*n*)	Mean (95% CI); median (*n*)	95% UCL of mean; range observed (*n*)
All	10 (7.3–15); 0.83 ($n = 328$)	42 (28–59); 6 ($n = 331$)	0.65; 0.33–2.5 ($n = 366$)
Avalon	23 (15–33); 5.0 ($n = 132$)	68 (45–105); 30 ($n = 132$)	NC
Doheny	5.3 (2.7–7.9); 4.0 ($n = 8$)	148 (15–388); 20 ($n = 11$)	NC
Malibu	1.8 (1.3–2.5); 0.69 ($n = 188$)	17 (8.8–27); 2.0 ($n = 188$)	NC
Detection frequency sand			
All	53%	71%	2.7%
Concentration sand (CFU/100 dry g)			
	Mean (95% CI); median (*n*)	Mean (95% CI); median (*n*)	95% UCL of mean; range observed (*n*)
All	187 (98–390); 7.7 ($n = 358$)	5086 (3331–10,755); 13 ($n = 238$)	2.5; 5–78 ($n = 366$)
Avalon	402 (192–830); 111 ($n = 159$)	24,009 (16,197–47,930); 3198 ($n = 49$)	NC
Doheny	58 (37–117); 25 ($n = 11$)	8646 (1146–16222); 3976 ($n = 5$)	NC
Malibu	21 (12–40); 4.9 ($n = 188$)	54 (39–75); 12 ($n = 184$)	NC

3.0 CFU/100 ml median concentration, $\alpha = 0.05$). Except for Site B in 2008, Site D was not lower than the other sites, even though Site D was envisioned as a control site because it was located in a different watershed with less commercial development adjacent to the beach (Fig. 1). There was only one beach site sampled at Doheny beach, thus no site comparisons were possible.

3.1.2. *Enterococci in seawater*

Enterococci concentrations are presented only for samples in which *S. aureus* analysis also was conducted. Enterococci were frequently detected in seawater, with an average % detection frequency of 79% ($n = 331$) for the beaches combined (Table 1). For Avalon, the average % detection frequency for seawater was 88% for both summers. At Malibu Surfrider, the average % detection frequency was 72%. Only eleven Doheny seawater samples had corresponding *S. aureus* data; all of them were positive for enterococci (100%). The mean concentration of enterococci in all the seawater samples estimated by Kaplan–Meier statistics with Efron bias correction was 42 CFU/100 ml and the median concentration was 6 CFU/100 ml, with data for individual beaches given in Table 1.

Kruskal–Wallis multiple comparisons tests ($\alpha = 0.05$) showed that the median seawater concentration of enterococci was significantly lower at Malibu compared to the other beaches (Fig. 3). At Malibu, the median enterococci concentration at site A (with presumed septic influence) was significantly lower than sites B and E, but did not differ significantly from Site D. At Avalon, median seawater concentrations of enterococci were not statistically different between 2008 and 2007, in contrast to *S. aureus*. At Avalon, the median enterococci concentration at Site A in 2008 was significantly lower than sites B and C in both 2007 and 2008, despite the fact that the sites were located close to one another (Fig. 1).

3.1.3. *S. aureus in sand*

S. aureus was frequently detected in beach sand, similar to that observed for seawater. The average % detection frequency was 53% ($n = 358$) for the combined beach data (Table 1). For Avalon, the average % detection frequency was 62% for both years combined. For Malibu Surfrider, the average % detection frequency was 46% for sand. Although only a few samples were successfully analyzed for Doheny beach ($n = 11$), the detection frequency was similar to that observed at the other beaches (45%). The mean concentration of *S. aureus* in the sand samples estimated by Kaplan–Meier statistics with Efron bias correction was 187 CFU/100 dry g and the median concentration was 7.7 CFU/100 dry g, with data for individual beaches given in Table 1.

Kruskal–Wallis multiple comparisons tests ($\alpha = 0.05$) showed that the median concentration of *S. aureus* in sand was lower at Malibu than at Avalon (Fig. 3). Concentrations of *S. aureus* in sand were higher at Avalon in 2008 than in 2007, as was found for the seawater concentrations. No significant differences among individual beach sites were observed for Avalon or Malibu.

3.1.4. *Enterococci in sand*

Enterococci concentrations are presented only for those samples which also had collection for *S. aureus* analysis. The

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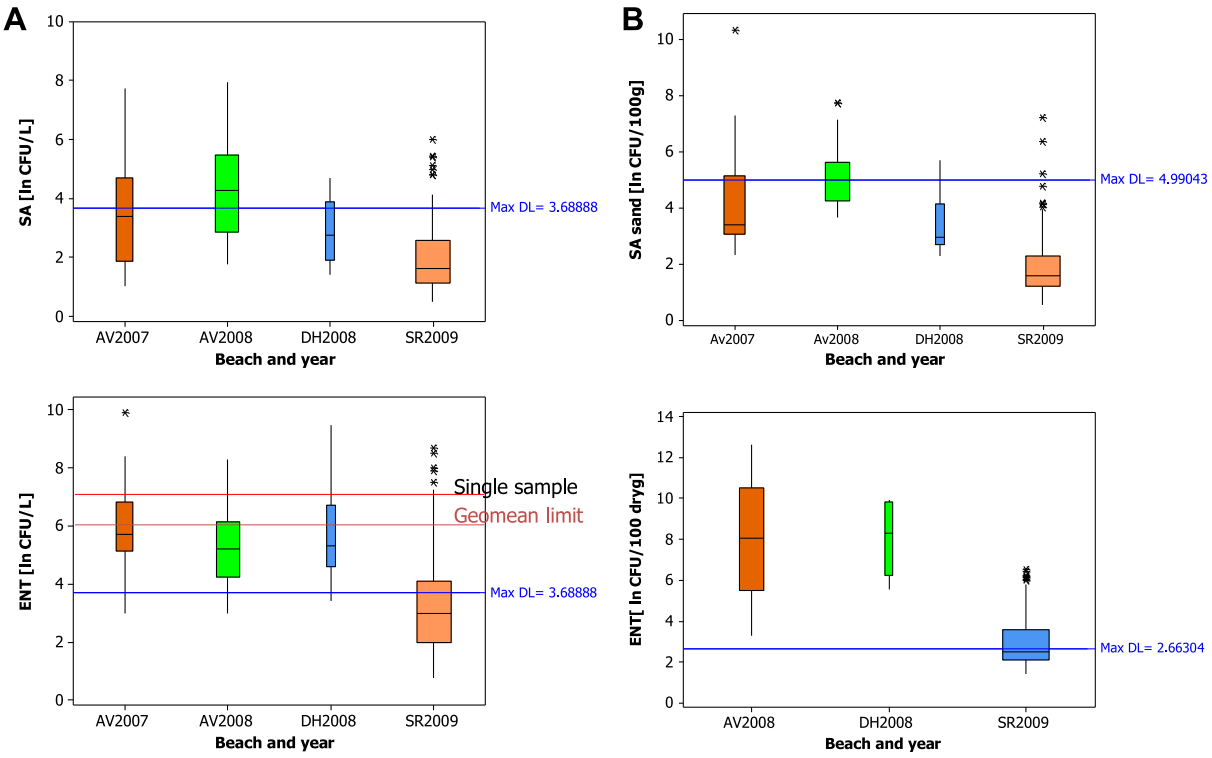

Fig. 3 – Box plots of *S. aureus* (SA) and enterococci (ENT) concentrations in A) seawater and B) sand generated by Kruskal–Wallis analysis ($\alpha = 0.05$) for samples collected from beach sites in Avalon during 2007 (AV2007) and 2008 (AV2008), Doheny in 2008 (DH2008), and Malibu Surfrider in 2009 (SR2009). Box width is proportional to sample size. Horizontal lines depict the maximum detection limit (Max DL) for the data. For enterococci in seawater, the EPA recreational water quality guidelines for a single sample and the geometric mean (Geomean limit) also are shown.

average % detection frequency was 71% ($n = 238$) for the combined beaches (Table 1). For Avalon (2008), the average % detection frequency was 94% ($n = 49$). Enterococci concentrations in terms of CFU/dry g sand were not available for Avalon in 2007. For Malibu Surfrider, the average % detection frequency was 63%. Only five Doheny sand samples had corresponding *S. aureus* data; all of them were positive for enterococci (100%). The mean concentration of enterococci in sand samples estimated by Kaplan–Meier statistics with Efron bias correction was 5086 CFU/100 dry g and the median concentration was 13 CFU/100 dry g, with data for individual beaches given in Table 1.

Kruskal–Wallis multiple comparisons tests ($\alpha = 0.05$) showed that the median concentration of enterococci in sand at Malibu was significantly lower than at the other beaches tested (Fig. 3). No significant differences across beach sites were observed except that the median enterococci concentration in sand at Malibu (Fig. 1) was significantly lower at Site E than Site B.

3.1.5. *MRSA in seawater and sand*

MRSA was found on occasion in seawater and sand at relatively low concentrations. Of the 12 beach sites tested, the overall % detection frequency was 1.6% (6/366) for seawater and 2.7% (10/366) for sand (Table 1). The % detection frequency varied by beach with Avalon having the highest frequency of detection for seawater (3.8%, 5/131) and sand (5.9%, 8/136). In contrast, the % detection frequency at Malibu was 0.4% (1/224) for seawater and 0.5% (1/221) for sand. No seawater samples were positive for MRSA at Doheny ($n = 14$) but 14% (1/7) were positive in the sand. Greater than 50% of the samples were nondetects; therefore, the concentration data are presented in terms of the 95% upper confidence limit (UCL) (Helsel, 2005) along with the range of concentrations observed (Table 1). The 95% UCL indicates that there was only a 5% chance of encountering a concentration higher than that given in Table 1.

3.1.6. *Enterococci concentrations relative to* S. aureus

Enterococci concentrations were higher than *S. aureus* in both seawater and sand. For seawater, the ratio of enterococci to *S. aureus* was fairly consistent across beaches and generally less than an order of magnitude. The median seawater concentration of enterococci was 7× higher than *S. aureus* for the combined beaches, ranging from 3–6× higher for the individual beaches. For sand, the ratio of enterococci to *S. aureus* was more variable. The median sand concentration of enterococci was 2× higher than *S. aureus* for the beaches combined, but the range across beaches was 2–159×.

3.2. *Relationships between* S. aureus, *enterococci, and non-microbial parameters measured in the study*

3.2.1. *Simple correlations and simple linear regression*

Kendall tau analysis and simple linear regression with life data were comparable and showed that *S. aureus* concentrations in seawater and in sand were correlated to a number of

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the analyzed variables (Table 2). *S. aureus* concentrations in seawater and sand were strongly correlated to each other (Table 2). Both seawater and sand concentrations were correlated to water temperature, the seawater concentration of enterococci, and surf height classification (inversely correlated). In addition, *S. aureus* in seawater (but not in sand) was significantly correlated with the number of swimmers as determined by observation at the time of collection. *S. aureus* concentrations were not correlated to the number of people having water contact as determined by questionnaire, although this was the parameter used in subsequent epidemiology analysis (Colford et al., 2012). *S. aureus* in sand (but not seawater) was inversely correlated to wind strength classification. Wind, tide height, and turbidity all became significant at $\alpha = 0.05$ (Table 2). Only water temperature and the number of swimmers were significantly correlated to the *S. aureus* concentration in seawater for both the combined beach data and for Avalon and Malibu beaches analyzed separately (Table 2). Doheny beach was not analyzed separately due to the small sample size. Other significant correlations for individual beaches are given in Table 2.

Table 2 – Results of analyses by Kendall tau correlation and simple linear regression with life data, each calculated for one dependent (*S. aureus* concentration in seawater or sand) and one independent variable for the combined beach data. Coefficients are given from the Akritas-Theil-Sen (ATS) line of the Kendall tau analysis and from MLE (in parenthesis) for significant *p*-values ($\alpha = 0.01$). NS = not significant.

	Dependent variable	
	Seawater *S. aureus* (ln CFU/L)	Sand *S. aureus* (ln CFU/100 dry g)
Independent variable	Coefficient from ATS line (from MLE)	
S. aureus sand (ln CFU/100 dry g)	0.67 (0.63)[b]	–
S. aureus seawater (ln CFU/L)	–	0.81 (0.72)
Water temperature (°C)	0.66 (0.62)[a]	0.68 (0.64)
Enterococci (ln CFU/L)	0.49 (0.44)	0.64 (0.59)
Surf height classification (m)	−1.4 (−1.4)	−0.94 (−2.0)
#Swimmers (counted)	0.046 (0.028)[a]	NS
# Swimmers (reported)	NS	NS
Wind classification	NS[c]	−1.18 (−0.92)
Tide height (m)	NS[c]	NS[c]
Turbidity classification	NS[c]	NS[c]
Birds (#)	NS	NS
Dogs (#)	NS	NS

a Also correlated for Avalon (2007, 2008 combined) and Malibu beaches analyzed separately.
b Also correlated for Malibu beach analyzed separately.
c Significant at $\alpha = 0.05$.

Kendall tau analysis was useful for comparing concentrations of bacteria because it could account for nondetects in both variables and made no assumptions about distribution shape. For the combined beach data, the relationship between the *S. aureus* concentration in seawater and sand was described by the following ATS line: *S. aureus* (ln CFU/L) = 0.18 + 0.67 × *S. aureus* (ln CFU/100 dry g) (tau-b = 0.29, $p < 0.001$). With the concentration in sand as the dependent variable, the relationship was described by the following ATS line: *S. aureus* (ln CFU/100 dry g sand) = 0.053 + 0. 81 × *S. aureus* (ln CFU/L) (tau-b = 0.29, $p < 0.001$). The relationship between the seawater concentrations of *S. aureus* and enterococci was described by the ATS line *S. aureus* (ln CFU/L) = 9.30 + 0.49 × enterococci (ln CFU/L seawater) (tau-b = 0.26, $p < 0.001$). The relationship between the sand concentrations of *S. aureus* and enterococci in seawater was described by the ATS line *S. aureus* (ln CFU/100 dry g sand) = −0.0061 + 0.64 × enterococci (ln CFU/L seawater) (tau-b = 0.27, $p < 0.001$).

For Malibu Surfrider beach, the seawater concentration of *S. aureus* was weakly correlated to whether or not the outlet to the lagoon (Fig. 1, Malibu Site C) was open (tau-b = 0.18, $p = 0.01$; open was coded as 0, therefore the relationship was inverse). There was no significant relationship between seawater enterococci concentration and lagoon flow, and there was no significant relationship between sand *S. aureus* concentration and lagoon flow. Attempts to quantify *S. aureus* in the lagoon water were unsuccessful due to overgrowth by non-target bacteria.

Although the focus of this study was *S. aureus*, a limited number of correlation analyses were performed with enterococci treated as the dependent variable. In addition to the correlation between enterococci and *S. aureus* described above, the seawater concentration of enterococci was weakly correlated to water temperature for the combined beach data (tau-b = 0.18, $p < 0.001$). However, water temperature was not significantly correlated to enterococci for the individual beaches, in contrast to the findings for *S. aureus*. The concentration of enterococci was not correlated to the number of swimmers for any of the beaches. It is interesting to note that water temperature was significantly correlated to the number of swimmers for the combined beaches (tau-b = 0.25, $p < 0.001$) and for Avalon (tau-b = 0.35, $p < 0.001$) and Malibu (tau-b = 0.19, $p < 0.001$) analyzed separately. There was no significant difference in the number of swimmers between beaches (data not shown).

3.2.2. *Multivariate and multiple linear regression analysis results*

Multivariate examination was performed on the combined beach data using principal component analysis. Principal component analysis was consistent with the results obtained by stepwise and best subset regression, and the best description by principal component analysis was obtained with the parameters *S. aureus* seawater concentration, *S. aureus* sand concentration, and water temperature (data not shown). Results were consistent with the analyses by Kendall tau and simple linear regression with life data (MLE with one dependent and one independent variable) (Table 2).

The best-fit for MLE analysis for *S. aureus* in sand was obtained using multiple linear regression with life data assuming a lognormal distribution (Table 3; all coefficient *p*-values significant at $\alpha = 0.01$). For *S. aureus* in seawater, the

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Table 3 – Best-fit multiple linear regression models (MLE with censored data) for *S. aureus* in beach seawater and sand.

Dependent variable	MLE model[a]
S. aureus seawater (ln CFU/L)	$=-4.6 + 0.58 \times$ *S. aureus* (ln CFU/100 dry g sand) $+ 0.22 \times$ water temperature (°C) $+ 0.22 \times$ enterococci (ln CFU/L seawater) $+ 0.018 \times$ swimmers (# counted at time of sample collection)
S. aureus sand (ln CFU/100 dry g)	$=-6.9 + 0.45 \times$ *S. aureus* (ln CFU/L seawater) $+ 0.37 \times$ water temperature (°C) $+ 0.24 \times$ enterococci (ln CFU/L seawater) $+ -0.77 \times$ surf height classification (m)

a Assumed distribution for *S. aureus*: lognormal in sand, Weibull in seawater (result for shape parameter, $\beta = 0.56$).

best-fit was obtained using multiple linear regression analysis with life data assuming a Weibull distribution (Table 3; all coefficient *p*-values significant at $\alpha = 0.01$, except # of swimmers at $\alpha = 0.03$). Variable inflation factors (VIF) revealed moderate co-linearity between *S. aureus* concentrations in seawater, enterococci in seawater, *S. aureus* in sand, and water temperature.

4. Discussion

Of the many relationships identified by simple correlation (Table 1), some parameters repeatedly emerged as significant descriptive variables for *S. aureus* in seawater regardless of analysis methods or approach for nondetects (censored vs. non-censored). These parameters were *S. aureus* sand concentration, water temperature, the number of swimmers, and the enterococci seawater concentration. Of these parameters, water temperature appeared to be particularly salient because it was correlated to *S. aureus* in both seawater and sand for both the combined data and for beaches analyzed individually (Avalon and Malibu). The only other correlation that held for both combined and individual beach data was the number of swimmers to *S. aureus* in seawater. In addition, the correlation between *S. aureus* concentrations in sand and in seawater held for both the combined data and for Malibu beach analyzed separately.

The correlation between the *S. aureus* concentration in sand to the concentrations of *S. aureus* and enterococci in seawater supports other studies that suggested beach sands can be a source of both fecal indicator bacteria and pathogens to adjacent waters (Lee et al., 2006; Bonilla et al., 2007; Yamahara et al., 2007, 2009; Goodwin et al., 2009; Halliday and Gast, 2011; Sabinro et al., 2011; Zhu et al., 2011; Shah et al., 2011). Furthermore, studies have implicated sand itself as an important vehicle for human exposure (Whitman et al., 2009) and epidemiology studies indicated that sand can pose a health risk (Bonilla et al., 2007; Heaney et al., 2009, 2012). In contrast to correlations seen between pathogens and indicators in some studies, other studies have found *S. aureus* to not be correlated to a variety of other indicators (El-Shenawy, 2005), including enterococci (Calderon et al., 1991; Selvakumar and Borst, 2006; Yamahara et al., 2012; Enns et al., 2012). The study here comprised a relatively large data set and days of sampling which may have allowed more patterns to emerge (Table 1). A common sand reservoir for both *S. aureus* and enterococci perhaps could explain the correlations seen here; however, it should be noted that the correlation existed only for the data combined across beaches but not for the beaches analyzed individually.

The positive correlation of water temperature to *S. aureus* concentrations in sand and seawater supports the concern that pathogens may grow and/or persist in the environment with sand as a source to adjacent waters, analogous to the case for FIB (Beversdorf et al., 2007; Shah et al., 2011; Phillips et al., 2011). In support of this suggestion, other studies have shown that *S. aureus* and MRSA can survive for days in river and seawater, with better survival in seawater because of a preference for higher salinity (Gabutti et al., 2000; Tolba et al., 2008; Levin-Edens et al., 2011). Both methicillin sensitive *S. aureus* (MSSA) and MRSA can survive on environmental surfaces and are resistant to desiccation (Cimolai, 2008). In addition, the ability for *S. aureus* to be resuscitated from a viable but non-culturable (VBNC) state has been demonstrated (Masmoudi et al., 2010); therefore, survival in sand and seawater appears plausible. Potential to transfer virulence and antibiotic resistance genes also is a concern, particularly if genes may be obtained from wounded or dead organisms contained in treated wastewater (Börjesson et al., 2009, 2010; Goldstein, 2010).

Compared to this study (Table 1), similar detection frequencies for *S. aureus* and MRSA in seawater (37% and 1%, respectively; Sinigalliano et al., 2010) and similar median concentrations (Shah et al., 2011) were reported for a subtropical beach using a substantially smaller data set. During a ten-day intensive study of this same beach, the detection frequency for *S. aureus* in knee-deep seawater was 19% and the detection frequency in sand ranged from 0.42 to 10%, depending on the sampling location (Enns et al., 2012). Compared to this study, similar MRSA but lower *S. aureus* prevalence in sand was observed for a survey of 37 beaches along the entire California coast (prevalence by beach: 13.5% *S. aureus*, 3% MRSA; Yamahara et al., 2012; prevalence by sample: 8.5% *S. aureus*, 1.7% MRSA; Goodwin unpublished results). Both the California beach survey and this study used the same methodology, but the beach survey investigated only dry sand and included beaches with few visitors (Yamahara et al., 2012); whereas the collections here were all moist sand from beaches with high visitor numbers. *S. aureus* prevalence in freshwater coastal streams in O'ahu, Hawaii (86%, 19/22; Viau et al., 2011) was higher than that observed here, with similar concentrations (below detection – 5.2 CFU/100 ml for the December sampling; Goodwin unpublished results).

It should be noted that the concentrations here are expected to be biased low despite the positive performance of SCA and C-MRSA with beach water and sand samples (Goodwin and Pobuda, 2009) because the performance only applies to countable plates. In this study, it was often the case that no data were obtained from samples of the poorest water quality because plates were overgrown by non-target

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microbes. Furthermore, it appeared that *S. aureus* could be outcompeted on the plates by non-target organisms because sometimes it could be identified on less crowded filters but not on more crowded filters that had received a higher volume of water. Therefore, the concentration estimates reported here should be considered conservative.

It should be helpful to view the potential risk from *S. aureus* and MRSA via beach exposure in context with other exposure routes, such as fomites or food products (Lindenmayer et al., 1998; Scott et al., 1982; Scott et al. 2008; Miller et al., 2009; Scott et al., 2009; Waters et al., 2011). To enable comparison, the concentrations given here can be converted to CFU per area using the specific surface area of coarse sand (~0.01 m^2/g; Yerima and Ranst, 2005), resulting in equivalent concentrations for units in CFU/dry g and CFU/m^2. Given that the typical detection limit for *S. aureus* in sand was 3.03 CFU/100 dry g, the frequency of detection for 25 cm^2 samples would drop from 53% to 42%, which is comparable to that reported in homes by Scott et al. (1982) (to express Table 1 sand concentrations in units of CFU/25 cm^2, divide values by 400).

Ultimately, the significance of *S. aureus* and MRSA prevalence and concentration in the environment depends on the infectious dose (Shibata and Solo-Gabriele, 2012) for both ingestion and, importantly, wound inoculation, particularly under real-world conditions (e.g., multiple organisms and particles contaminating the wound). One study of *S. aureus* surface inoculation of intact human skin found an ED_{50} of 10^3/cm^2, with an inoculation of 40/cm^2 causing infection in 20% of subjects; growth of the organisms after inoculation was necessary to cause infection (Singh et al., 1971; Rose and Haas, 1999). The ability for *S. aureus* to infect intact skin appeared dependent on a variety of parameters, including initial dose, maintained moisture of the skin surface, and competition with indigenous flora. An inoculation dose lower than 40/cm^2 was not tested; however, this dose was almost 1000 times higher than the highest concentration in sand observed here (using the conversion discussed above). However, the infectious dose of *S. aureus* is substantially reduced by factors such as foreign bodies or synergy with other contaminating microbes (Elek, 1956; Schaad, 1983; Brook et al., 1984; Rissing et al., 1987; Hendricks et al., 2001). Both of these modifying conditions could be expected in beach settings, particularly for contamination of a wound with sand. Therefore, obtaining a more accurate assessment of infectious dose under a variety of real-world conditions is an important area for future research. In any case, based on human studies (Singh et al., 1971), rinsing and drying the skin should help provide protection, particularly for intact skin.

5. Conclusions

- The frequent detection (>50%) of *S. aureus* seawater and beach sand samples and the correlation with water temperature supports the concern that bacterial pathogens exist and may persist in the environment, including at beaches.
- Although the correlation between *S. aureus* and the number of swimmers was weak and apparent only for *S. aureus* in seawater and not sand, the correlation held for data analyzed by individual beach and combined across beaches. These data support the possibility that beach goers are one source of this organism but suggests that other sources not identified in this study are important, as well.
- Although the prevalence of MRSA was much lower (<3% of samples) than for *S. aureus*, the data indicate the potential for virulent and antibiotic resistant strains to be encountered in this environment.
- *S. aureus* was correlated to enterococci, even though *S. aureus* is not considered a typical fecal organism. Perhaps the finding that *S. aureus* can sometimes be found in wastewater and in companion animal feces explains this observation. However, the relationship held only for the combined beach data, suggesting the need for further study; particularly to ensure whether current FIB guidelines are adequately protective against this pathogen.

Acknowledgements

We thank the many volunteers and workers of the Pacific Coast Water Study and the laboratory of Dr. Jenny Jay, UCLA, for analysis of enterococci in sand. This research was carried out in part under the auspices of the Cooperative Institute for Marine and Atmospheric Studies (CIMAS), a joint institute of the University of Miami and the National Oceanic and Atmospheric Administration, cooperative agreement #NA17RJ1226.

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Please cite this article in press as: Goodwin, K.D., et al., A multi-beach study of *Staphylococcus aureus*, MRSA, and enterococci in seawater and beach sand, Water Research (2012), doi:10.1016/j.watres.2012.04.001

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Please cite this article in press as: Goodwin, K.D., et al., A multi-beach study of *Staphylococcus aureus*, MRSA, and enterococci in seawater and beach sand, Water Research (2012), doi:10.1016/j.watres.2012.04.001