Photoinactivation of uncultured, indigenous enterococci

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
...
60

Photoinactivation of uncultured, indigenous enterococci

Jill S. McClary[1][#], Nathaniel A. Ramos[1], Alexandria B. Boehm[1]*

[1]Civil & Environmental Engineering, Stanford University, Stanford, CA, USA

*Correspondence: Alexandria B. Boehm, aboehm@stanford.edu

[#]Present address: School of Freshwater Sciences, University of Wisconsin-Milwaukee,

Milwaukee, WI, USA

Abstract

Enterococci are used to monitor recreational water quality worldwide so understanding their fate

and transport in the environment is essential to the protection of human health. As such,

researchers have documented enterococci inactivation under various exposure conditions and in

diverse water matrices. However, the majority of studies have been performed using lab-cultured

bacteria, which are distinct from indigenous, un-cultured bacteria found in the environment. Here

we investigate the photoinactivation of indigenous, un-cultured enterococci from a range of

sources, including wastewater treatment plants (WWTPs), marine beaches, urban streams, and a

wastewater-influenced pond. We concentrated indigenous enterococci from their sources using

filtration and centrifugation, placed them in a clear, buffer solution, and then exposed them to

simulated sunlight to measure their photoinactivation rates. Indigenous, uncultured enterococci

first order decay rate constants (k) spanned an order of magnitude, from 0.3 to 2.3 $m^2kJ_{UVB}^{-1}$. k of

indigenous enterococci from WWTPs tended to be larger than those from surface waters. k of

lab-cultured *Enterococcus faecalis* was larger than those of indigenous, un-cultured enterococci

from most sources. Positive associations between the fraction of pigmented enterococci and

sunlight susceptibility were observed. This work suggests that caution should be taken when

extending results on bacterial photoinactivation obtained using lab-cultured bacteria to

environmental bacteria, and that enterococci pigmentation may be a useful metric for estimating

photoinactivation rate constants.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Environmental Significance Statement

Enterococci are known to decay under sunlight exposure through a process called photoinactivation. However, it remains a challenge to predict their photoinactivation decay rates in natural water systems, which limits the development of accurate and dependable water quality models. In this study we document photoinactivation rates of indigenous populations of enterococci from diverse natural and wastewater sources. We find that photoinactivation differs among indigenous enterococci from diverse sources and that the pigmentation of enterococci communities is associated with photoinactivation rate constants.

1. Introduction

Enterococci are used to monitor recreational water quality, and their presence in recreational waters is associated with swimmer and surfer gastrointestinal illness [1–5]. In the United States, microbial pollution at recreational beaches is estimated to cause 90 million illnesses each year, resulting in an economic burden of over $2 billion [6]. Microbial water quality in the natural environment varies significantly on both spatial and temporal scales [7–10]. Modeling efforts that consider enterococci fate and transport aim to understand and predict enterococci concentration variability in natural systems [11–13].

Sunlight is one of the most important factors affecting pathogen and pathogen indicator concentrations in surface waters [14–19]. Previous work to understand rates and mechanisms of bacterial pathogen and indicator photoinactivation has relied mostly on lab-cultured bacteria, including reference strains purchased from culture collections and strains isolated from the environment [20–27]. Fewer studies have used indigenous or wastewater bacterial pathogens or indicators to study photoinactivation rates in mesocosms or in situ in surface waters.[15,16,28–30] Lab-cultured, environmental strains have been observed to decay differently under sunlight exposure than lab-cultured, reference strains [21,27,31,32]. For example, Nguyen et al. observed that a lab-cultured, environmental isolate of enterococci from wastewater photoinactivated significantly more slowly than lab-cultured, reference strain *Enterococcus faecalis* [31]. These differences may be due to phenotypic or genomic differences between strains including their degree of pigmentation [32,33].

Indigenous bacteria in environmental reservoirs differ from lab-cultured bacteria in several ways. First, the lab-culturing procedure alters bacterial physiology by exposing

indigenous bacteria to nutrient-rich growth conditions. This change in physiology likely has an impact on measured photoinactivation rates [34], possibly by altering intracellular concentrations of photosensitizers [21]. Second, bacteria in lab cultures are in active growth states and are therefore phenotypically different from bacteria in environmental waters, which are more likely to be expressing non-growing phenotypes due to nutrient limitations. Bacteria in non-growing states have been shown to be more resistant to a range of environmental stresses [35,36]. Third, bacteria in lab cultures have likely not been previously exposed to the range of stresses present in environmental waters. Sublethal exposure to stresses can increase resistance to those stresses [37]. Despite these biases that may be introduced during lab-culturing, few studies have investigated photoinactivation of indigenous enterococci in the laboratory without first lab-culturing isolates [31,38–42], and in almost all cases, indigenous enterococci in those studies were sourced from a single wastewater or fecal source [31,38,39,42]. Field studies of indigenous enterococci photoinactivation almost exclusively examine photoinactivation of wastewater enterococci.[15,28,30]

The goal of this research is to investigate the photoinactivation kinetics of indigenous, uncultured enterococci from a broad range of aqueous environments including WWTPs and surface waters. Enterococci were concentrated from waters using filtration and centrifugation; they were not sub-cultured or exposed to exogenous nutrient sources, in order to maintain their environmental physiological profile as much as possible. We then measured enterococci photoinactivation in a lab-controlled system. Finally, as enterococci pigmentation has been previously associated with decreased photoinactivation rate constants [31,32], we measured the fraction of pigmented enterococci in each sample and investigated the impact of pigmentation on photoinactivation.

2. Methods

2.1 Sample collection. Samples were collected from WWTP and surface waters between September 2017 and February 2018 (Figure 1, Table S1). All surface water samples were collected within one hour of sunrise. Two liter samples of primary treated effluent were collected at WWTPs, and 20-40 L samples were collected from surface waters. Surface waters included three marine beaches, three urban streams, and one wastewater-influenced pond with a large population of ducks, and are subsequently referred to as beach, stream, and pond samples, respectively. Locations were chosen based on expected high concentrations of enterococci.[18,43,44] All samples were collected in autoclaved plastic containers and placed on blue ice for transport to the laboratory. Upon arrival at the laboratory, approximately 500 mL of each sample was used to measure enterococci concentrations in the raw water using EPA Method 1600 [45] and other physicochemical parameters (see ESI). The remaining sample volumes were then processed to concentrate bacteria using tangential filtration and centrifugation.

2.2 Wastewater and surface water bacteria concentration. Bacteria were concentrated from wastewater and surface water using a combination of filtration and centrifugation. These methods were chosen because they are relatively simple and do not require exposing the bacteria to additional nutrient sources that may change the physiological profile of bacteria in the samples. As the separation methods rely on size and density, solids of similar size and density were co-concentrated with the enterococci.

Surface water samples were first concentrated using a tangential filtration membrane system (OMEGA suspended screen cassette; 30 kDa pore size; Pall Corporation, Port Washington, NY) until approximately 5 L of retentate remained. The wastewater was not concentrated first with

6

tangential filtration due to its relatively high bacterial concentrations. Bacteria in wastewater samples and the surface water retentate were then concentrated using three rounds of centrifugation (10 minutes at 9600 x g (retaining pellet), 5 minutes at 500 x g (retaining supernatant), 10 minutes at 9600 x g (retaining pellet)). The final pellet was resuspended in phosphate buffered saline (PBS, pH 6.77; Fisher Scientific, Fair Lawn, NJ) for a final volume of 10-30 mL for each sample. Concentrated bacteria were stored in the dark at 4 °C until photoinactivation experiments were performed (within 48 h of initial water sample collection) while we determined the enterococci concentration in the concentrate (which informed the volume needed to seed the experimental reactors, as described below). See ESI for further details.

2.3 Photoinactivation experiments. Concentrated bacteria were diluted into PBS to a final volume of 50 mL in three sterile 100-mL beakers wrapped in black electrical tape. The volume of concentrated bacteria added to the PBS varied among experiments in order to achieve initial enterococci concentrations in the reactors that ranged between 10^0 and 10^5 CFU/mL. Two beakers were designated experimental reactors (i.e. biological duplicates) and were exposed to full spectrum simulated sunlight in an Atlas Suntest XLS+ solar simulator (Chicago, IL) with a 1.1 kW xenon arc lamp set to an intensity of 250 W/m^2 and equipped with a glass filter to remove wavelengths below 290 nm (see Figure S1 for spectrum which approximates the spectrum of late morning sun during the summer in Palo Alto, CA, USA). The third reactor (dark control) was covered with foil to prevent exposure to simulated sunlight and placed next to the experimental reactors in the solar simulator. The solar simulator was equipped with a recirculating water bath to maintain reactors at 15°C. Photoinactivation experiments were run for 40-160 minutes, depending on expected inactivation kinetics. A time course of samples was

taken from both experimental and control reactors for enterococci quantification following EPA Method 1600[45] (see ESI). The number of samples collected and their volumes varied between experiments and depended on the starting enterococci concentration; our goal was to not remove more than 10% of the volume of the reactors, yet still be able to measure enterococci. 3-7 samples were taken from each experimental reactor, and 3 samples were taken from each control reactor.

2.4 Enterococci pigmentation tests. The fraction of enterococci in the raw water samples that were pigmented was quantified using a previously described method [46], with slight variation. Between 1 and 3 mEI plates that were used to measure enterococci concentrations in the raw water (prior to concentration) were chosen for each water sample, sealed with parafilm, and stored at 4 °C until pigmentation analysis (within 6 months). Individual enterococci colonies from stored mEI agar plates were sub-cultured in tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO) and then streaked onto tryptic soy agar (TSA; BD Difco, Sparks, MD). Each colony was identified as pigmented or nonpigmented by picking a small amount of biomass from the streak on a sterile cotton swab and visually examining the swab for pigmentation (see ESI). The total number of isolates examined for each water sample ranged from 20 to 89 (Table 1).

2.5 Photoinactivation of lab-cultured *Enterococcus faecalis*. *Enterococcus faecalis* (ATCC 19433) batch cultures were grown in Brain Heart Infusion broth (Fluka Analytical, Steinheim, Germany) at 37 °C for ~20 hours. 1 mL of the batch culture was washed by centrifuging at 9600 x g for 10 minutes and resuspending the pellet in 1 mL PBS. The bacteria were washed in PBS two more times, centrifuging for 5 minutes between each wash, and then diluted into 50 mL PBS for photoinactivation experiments as described above.

8

2.6 Data analysis. Upon visual examination of concentration time series (C) from the various experiments, we observed that some inactivation curves had an apparent shoulder while others did not. Therefore, data were fit using a least-squares method to log-linear (Equation 1) and shoulder log-linear (Equation 2) models [47]:

$$\ln\left(\frac{C}{C_0}\right) = -kF \qquad\qquad \text{Equation 1}$$

$$\ln\left(\frac{C}{C_0}\right) = \ln\left(e^{-kF}\frac{e^{kS}}{1 + (e^{kS} - 1)e^{-kF}}\right) \qquad\qquad \text{Equation 2}$$

where $ln(C/C_0)$ is the ln-transformed relative concentration, C_o is the concentration measured at $F = 0$, k is the photoinactivation rate constant with units m^2/kJ_{UVB}, F is fluence in kJ_{UVB}/m^2 in the UVB range (280-320 nm), and S is the shoulder constant with units of kJ_{UVB}/m^2 and was constrained to values >0 during the curve fitting. Fluence was corrected for light screening using the absorbance of reactor solutions in each experiment (see ESI & Figure S2). Data points from biological replicates were combined prior to fitting equations 1 and 2. The fit of the two model types, as measured by the residual standard error (RSE), was compared, and the model with the lowest RSE was chosen as the final model. $F_{90\%}$ (UVB fluence required to achieve 90% inactivation) was calculated by solving Equation 1 or 2 for F using the fitted k and S parameters (if applicable) and setting $(C/C_0) = 0.1$ using Microsoft Excel Solver. k values from either Equation 1 or 2 for bacteria sourced from WWTP, beach, or stream, were averaged, respectively, to generate k_{WWTP}, k_{beach}, and $k_{stream.}$ Pair-wise comparisons of k averaged for each sample-type were performed using a z-test at a confidence level of 95% [48]. Photoinactivation rate constants with respect to fluence in the UVA & UVB range (280-400 nm) are also provided in the ESI.

The fraction of pigmented isolates was calculated as the number of pigmented isolates divided by the total number of isolates for each sample or source type, and the standard error was calculated as

$$SE_f = \sqrt{\frac{f(1-f)}{n}}$$
                                                                                                Equation 3

where f is the fraction of pigmented isolates and n is the total number of isolates. Pearson correlations between the fraction of pigmented isolates and k or $F_{90\%}$ were calculated.

To compare the photoinactivation rate constants in this study to those in previous studies, we systematically reviewed the literature to identify studies that measured sunlight inactivation of enterococci, as well as studies that measured enterococci pigmentation and sunlight inactivation. Details of the systematic reviews are provided in the ESI. We performed unit conversions and accounted for light screening where necessary to conform rate constants to the same units used in this study. One of the identified studies also includes a model using biological weighting functions to predict photoinactivation rates of wastewater-sourced enterococci;[24] we therefore also used this model to compare to the wastewater photoinactivation rate constants observed in this study. All details for unit conversions, light screening calculations, and applying the biological weighting function model are described in the ESI.

All data analyses described herein were performed in Microsoft Excel and R v3.4.0.

3. Results

At the time of sampling, water sources had diverse temperatures (range = 8.4 to 26.4 °C), salinities (0.1 to 32.7 ppt), dissolved oxygen concentrations (0.30 to 12.08 mg/L), and turbidities (1.37 to 232.5 NTU). Enterococci concentrations in WWTP and surface water samples ranged

from 2×10^3 to 3×10^4 CFU/mL (WWTP) and 0.5 to 8.8 CFU/mL (surface water), respectively

(Table S2). The fraction of pigmented enterococci isolates ranged from 0 to 42% across the

samples (Table 1). The absorbance spectra of the environmental waters are provided in Figure

S3.

Enterococci inactivation in the sunlit (Figure 1) reactors were modeled using log-linear or

shoulder log-linear equations. All inactivation was modeled with respect to fluence in the UVB

range, taking into account light attenuation due to absorbance in the water column (described

further in the ESI). This was necessary as the absorbance of the solutions placed in the reactors

varied among experiments (Figure S4). The method for calculating fluence requires an

assumption that light attenuation due to absorbance is more important than that due to scattering.

This assumption was valid for all samples except beach2. Sample beach2 likely violated this

assumption due to the particularly low absorbance of the reactor solution for this sample (Figure

S4).

Decay rate constants for dark controls (k_d, Figure S5) for all but one experiment were not

statistically different from zero ($p > 0.05$, data not shown), suggesting that inactivation observed

in sunlit reactors was due to sunlight exposure. Statistically significant decay occurred in the

dark control reactor containing enterococci from a stream (stream3), but the effect size was small

($k_d \pm SE = 0.04 \pm 0.003$ m^{2}kJ$_{UVB}^{-1}$, $p = 0.04$, compared to $k \pm SE = 0.8 \pm 0.1$ m^{2}kJ$_{UVB}^{-1}$ in sunlit

reactor), so no correction for dark decay was made.

Inactivation curves of uncultivated, indigenous enterococci from eleven of the thirteen

waters were best fit using the shoulder log-linear model. The shoulder lengths (S) from these

models ranged from 0.6 to 3.9 kJ$_{UVB}$/m^2. The remaining two waters (beach1 and beach3) were

best fit using the log-linear model. k values from the inactivation curves considering k from both log-linear and shoulder log linear models together, varied over an order of magnitude, from 0.3 to 2.3 $m^2kJ_{UVB}^{-1}$.

The inactivation of a laboratory-cultured, reference strain of *E. faecalis* was best fit with a shoulder log-linear model with a k value was 3.1 $m^2kJ_{UVB}^{-1}$ (SE = 0.6), significantly larger than the k values measured for the indigenous, uncultured bacteria ($p < 0.05$, Z test) except for those from WWTP1 ($k \pm SE = 2.5 \pm 0.2$ $m^2kJ_{UVB}^{-1}$) and pond1 ($k \pm SE = 2.2 \pm 0.7$ $m^2kJ_{UVB}^{-1}$).

We tested the hypotheses that photoinactivation varied (1) among enterococci from diverse source types and (2) as a function of pigmentation. We excluded results from beach2 due to the relative importance of light scattering that yielded potentially inaccurate estimates of UVB fluence (see ESI). We excluded pond1 from our testing of the first hypothesis because $n = 1$ for this source type. Average k_{WWTP}, k_{beach}, and k_{stream} were statistically different from each other (Z test, $p < 0.05$, Figure 1) with $k_{WWTP} > k_{stream} > k_{beach}$. To test hypothesis (2), we evaluated the correlation between pigmentation fraction and k or $F_{90\%}$. There was an association between the fraction of pigmented enterococci in the raw water and sample type (Chi-square test, $p < 0.05$, excluding pond sample type because $n_{pond} = 1$). Including data from pond1, we also evaluated correlations between pigmentation and photoinactivation. There was a significant, positive correlation between the fraction of pigmented enterococci in the water at the time it was collected and $F_{90\%}$ for the indigenous, uncultured enterococci (Pearson correlation, $R = 0.61$, $p < 0.05$, n=12) and a negative correlation between the fraction of pigmented enterococci and k ($R = 0.51$, $p < 0.1$, n=12, curve fit: k = -2.5*f+1.6) (Figure 2). Overall, 35%, 24%, and 11% of enterococci isolates were pigmented from beach, stream, and WWTP sources (Figure 2).

We performed all data analyses described above after calculating rate constants that accounted for light screening of both UVA and UVB wavelengths (280-400 nm; see Table S3 for rate constants). Inclusion of the UVA wavelengths in the light screening calculation did not significantly alter any of the relationships presented above. As UVB photons are the most energetic UV photons incident on the Earth's surface, they are expected to exert the most influence on photoinactivation of bacteria when inactivation is dominated by endogenous processes. As such, we feel justified to choose the energy in the UVB as the basis for our rate constant calculations.[31,42,49] Therefore, additional discussion of results will be centered on inactivation after adjusting for screening of UVB.

As experiments were carried out in the laboratory by adding indigenous bacteria to buffer (PBS), we did not explore associations between photoinactivation rates and raw water properties like temperature and salinity.

4. Discussion

4.1 Indigenous, uncultured enterococci inactivate at different rates than laboratory-cultured *E. faecalis*. In our evaluation of indigenous, uncultured enterococci photoinactivation from thirteen different sources, indigenous uncultured enterococci from eleven of the sources had smaller k values than a common lab-cultured reference strain *E. faecalis*. Thus, caution should be taken in applying photoinactivation rate constants obtained for a single lab-culture strain to indigenous, environmental enterococci.

One procedural difference in the experiments that used indigenous, environmental enterococci and lab-cultured enterococci is that the indigenous uncultured enterococci were stored prior to commencement of the photoinactivation experiments up to 48 hours while the lab-

13

cultured *E. faecalis* were used immediately after overnight culturing. While we did not specifically test the effect of storage on photoinactivation of the decay of *E. faecalis*; previous work has shown that growth rate and growth phase of *E. faecalis* minimally affects its photoinactivation kinetics under full spectrum simulated sunlight.[50]

Previous studies have calculated photoinactivation rate constants of lab-cultured *E. faecalis* that range between 0.4-2.0 $m^2kJ_{UVB}^{-1}$ [24,31,42,51], which are smaller than the value presented herein ($k= 3.1$ $m^2kJ_{UVB}^{-1}$) but are also larger than photoinactivation rate constants from the majority of indigenous uncultured enterococci presented herein.

Waterborne indigenous enterococci likely represent a mixture of various *Enterococcus* species with potentially different decay characteristics. There are more than 30 species of *Enterococcus* [52]. To our knowledge, the photoinactivation of each of these species has not been documented with the exception of lab-cultured *E. casseliflavus* ($k = 0.2$-0.6 $m^2kJ_{UVB}^{-1}$) which is a pigmented species [31,32]. The photoinactivation rate constants of the indigenous, uncultured enterococci photoinactivation rate constants in our study are similar to those reported previously for lab-cultured *E. casseliflavus*. This led us to suspect that pigmentation may be an important factor controlling photoinactivation kinetics of the un-cultured indigenous enterococci.

The analysis presented herein assumes that correcting for light screening in the UVB portion of the spectrum adequately controls for differences between the experimental solutions, so that *k* values can be compared among the various experiments. Figure S4 clearly shows that absorbance varies among the solutions, likely due to the co-concentration of colored material along with the indigenous bacteria. Inherent in this assumption is the exogenous indirect photoinactivation, or inactivation caused by reactive species generated through the interactions

of photons and colored molecules or particles in the reactors, plays an insignificant role compared to endogenous photoinactivation. This assumption is probably valid given that evidence suggests that it is unusual for exogenous indirect photoinactivation to play an important role in the photoinactivation of enterococci.[23] However, some work using treated wastewater effluent as a matrix has highlighted that the exogenous, indirect mechanism of photoinactivation can sometimes be important for enterococci.[31] Interestingly, the pond1 sample was obtained from a pond filled with treated wastewater, and its k value was among the highest reported even though the community was highly pigmented, which may suggest that exogenous indirect mechanisms were at work and enhancing photoinactivation during the experiments.

4.2 The fraction of pigmented enterococci affects photoinactivation. Previous studies suggest enterococcal pigments can influence photoinactivation [31,32,46,53,54]. We observed a positive association between pigmentation and the environment from which the enterococci were isolated (WWTP, streams and beaches). This could be due to differences in enterococci speciation or previous sunlight exposures between environments, as pigmentation is associated with sunlight exposure and certain enterococci species are more likely to exhibit pigmentation than others [32,55]. Furthermore, we observed a negative association between pigmentation fraction and k providing support for a causal relationship between the two. The association suggested a linear relationship between k and f.

We investigated whether the linear relationship between k and f derived through or laboratory studies could predict k from other studies where both k and f were measured concurrently. There is only one study in the literature that reports both k and f for indigenous enterococci at a field site [16,32]. Boehm et al. determined *in situ* enterococcal photoinactivation at a beach in southern California using an mechanistic model [16] and found $k = 0.8$ m^{2}kJ$_{UVB}^{-1}$ (see

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ESI). In another study at the same location and time, Maraccini et al. [32] report that f varies as a function of time of day with average f=0.22. Assuming k = -2.5*f+1.6, k is predicted to be 1.1 $m^2kJ_{UVB}^{-1}$, similar to the k previously reported for this beach using *in situ* measurements and empirical modeling [16]. Further work to optimize enterococci decay modeling based on easily measurable community characteristics, such as pigmentation, is warranted.

A few previous studies have evaluated photoinactivation of indigenous, uncultured enterococci from wastewater and reported k as ranging from 0.08-0.6 $m^2kJ_{UVB}^{-1}$.[24,31] These k values are smaller than those calculated for indigenous wastewater enterococci published herein (1-2.5 $m^2kJ_{UVB}^{-1}$). We applied a previously developed model using biological weighting functions to determine photoinactivation rates of wastewater-source enterococci, these models predict k_{WWTP} =0.09-0.1 $m^2kJ_{UVB}^{-1}$,[24] smaller than the k values we report. We hypothesize that differences in the fraction of pigmented enterococci in the wastewater-sourced enterococcal communities in our study and those published previously may explain some of the differences in reported and modeled decay rate constants.

4.3 A shoulder log-linear model was needed to describe inactivation of most indigenous enterococci. The simplest and most common method used for modeling decay rates of pathogen indicators like enterococci is a log-linear model. However, studies observe deviations from log-linear kinetics.[23,26,56] The shoulder log-linear model includes a shouldering behavior that is attributed to some protective component in the bacteria or water matrix that must be destroyed before inactivation is observed.[47,57] For eleven of the thirteen indigenous enterococci communities evaluated, a shoulder log-linear model provided the best fit for the photoinactivation data, suggesting that such a model may be needed for modeling enterococci decay in sunlit waters. Shoulder log-linear models have been used in previous work on the decay

of indicator bacteria and bacterial pathogens.[21,27,58] Incorporating shoulder log-linear kinetics into predictive or mechanistic water quality models presents a challenge because it implies bacterial decay is dependent on previous exposure duration, and often this is not a reasonable parameter to estimate since contamination sources are usually unknown.[59] However, in specific instances where there are known sources of pollution, using a shoulder log-linear model may be feasible.

4.4 Practical Implications. The use of water quality models to aid in pollution source identification or health risk assessment is becoming increasingly common.[13,60,61] Such models require knowledge on bacterial inactivation and common practice is to use lab-cultured strains to parameterize this process. The variability of enterococci photoinactivation rates observed in this study and the influence of pigmentation point toward the importance of physiological characteristics and community heterogeneity on inactivation in environmental systems. Furthermore, the results call into question the appropriateness of using laboratory-cultured bacteria for estimating inactivation rates of indigenous, uncultured enterococci. A recent study on viral disinfection identified variability between environmental and laboratory strains as a key factor in determining a virus's susceptibility to inactivation.[62]

While homogenous laboratory cultures are ideal for improving experimental reproducibility and evaluating specific decay processes, we recommend that future work should attempt to better address the differences between bacterial communities generated in laboratory culture versus those occurring in environmental reservoirs. Additionally, our work demonstrates that easy-to-measure characteristics like pigmentation significantly influence enterococci decay and that a shoulder log-linear model provided the best fit in most cases. These parameters should

be included in future water quality modeling efforts in order to improve prediction of microbial

pollutant decay.

5. Conclusions

- Photoinactivation rate constants of un-cultured enterococci from wastewater and surface

  waters are different from lab-cultured *E. faecalis*.

- Enterococcal pigmentation is associated with decreasing photoinactivation rate constants.

- Photoinactivation of indigenous un-cultured enterococci often displays a shouldering

  behavior before decay.

Acknowledgements

Electronic Supplementary Information

Contains methods for calculation of light scattering and depth-averaged light intensity;

explanation for exclusion of sample pond1 from pigmentation analyses; and additional results

including complete physicochemical and microbial measurements of samples, irradiation spectra,

absorbance spectra of reactor solutions, dark control data, and absorbance spectra of raw water

samples.

References

(1)   Cabelli, V. J.; Dufour, A. P.; McCabe, L. J.; Levin, M. A. Swimming-Associated Gastroenteritis and Water Quality. *Am. J. Epidemiol.* **1982**, *115* (4), 606–616.

(2)   Arnold, B. F.; Schiff, K. C.; Ercumen, A.; Benjamin-Chung, J.; Steele, J. A.; Griffith, J. F.; Steinberg, S. J.; Smith, P.; McGee, C. D.; Wilson, R.; et al. Acute Illness among Surfers after Exposure to Seawater in Dry-and Wet-Weather Conditions. *Am. J. Epidemiol.* **2017**, *186* (7), 866–875.

(3)   Pruss, A. Review of Epidemiological Studies on Health Effects from Exposure to Recreational Water. *Int. J. Epidemiol.* **1998**, *27* (1), 1–9.

(4)   Cabelli, V. J. *Health Effects Criteria for Marine Recreational Waters*; U.S. Environmental Protection Agency: Research Triangle Park, 1983.

(5)   Wade, T. J.; Pai, N.; Eisenberg, J. N. S.; Colford, J. M. Do U.S. Environmental Protection Agency Water Quality Guidelines for Recreational Waters Prevent Gastrointestinal Illness? A Systematic Review and Meta-Analysis. *Environ. Health Perspect.* **2003**, *111* (8), 1102–1109.

(6)   DeFlorio-Barker, S.; Wing, C.; Jones, R. M.; Dorevitch, S. Estimate of Incidence and Cost of Recreational Waterborne Illness on United States Surface Waters. *Environ. Heal. A Glob. Access Sci. Source* **2018**, *17* (1), 1–10.

(7)   Brendel, C.; Soupir, M. Relating Watershed Characteristics to Elevated Stream Escherichia Coli Levels in Agriculturally Dominated Landscapes: An Iowa Case Study. *Water* **2017**, *9* (3), 154.

(8)   Aranda, D.; Lopez, J. V.; Solo-Gabriele, H. M.; Fleisher, J. M. Using Probabilities of

        Enterococci Exceedance and Logistic Regression to Evaluate Long Term Weekly Beach

        Monitoring Data. *J. Water Health* **2016**, *14* (1), 81–89.

(9)   Goodwin, K. D.; Schriewer, A.; Jirik, A.; Curtis, K.; Crumpacker, A. Consideration of

        Natural Sources in a Bacteria TMDL - Lines of Evidence, Including Beach Microbial

        Source Tracking. *Environ. Sci. Technol.* **2017**, acs.est.6b05886.

(10)   Boehm, A. B.; Ashbolt, N. J.; Colford, J. M.; Dunbar, L. E.; Fleming, L. E.; Gold, M. A.;

        Hansel, J. A.; Hunter, P. R.; Ichida, A. M.; McGee, C. D.; et al. A Sea Change Ahead for

        Recreational Water Quality Criteria. *J. Water Health* **2009**, *7* (1), 9–20.

(11)   Cho, K. H.; Cha, S. M.; Kang, J. H.; Lee, S. W.; Park, Y.; Kim, J. W.; Kim, J. H.

        Meteorological Effects on the Levels of Fecal Indicator Bacteria in an Urban Stream: A

        Modeling Approach. *Water Res.* **2010**, *44* (7), 2189–2202.

(12)   Liu, L.; Phanikumar, M. S.; Molloy, S. L.; Whitman, R. L.; Shively, D. A.; Nevers, M. B.;

        Schwab, D. J.; Rose, J. B. Modeling the Transport and Inactivation of E. Coli and

        Enterococci in the near-Shore Region of Lake Michigan. *Environ. Sci. Technol.* **2006**, *40*

        (16), 5022–5028.

(13)   Hou, D.; Rabinovici, S. J. M.; Boehm, A. B. Enterococci Predictions from Partial Least

        Squares Regression Models in Conjunction with a Single-Sample Standard Improve the

        Efficacy of Beach Management Advisories. *Environ. Sci. Technol.* **2006**, *40* (6), 1737–

        1743.

(14)   Viau, E. J.; Goodwin, K. D.; Yamahara, K. M.; Layton, B. A.; Sassoubre, L. M.; Burns, S.

L.; Tong, H. I.; Wong, S. H. C.; Lu, Y.; Boehm, A. B. Bacterial Pathogens in Hawaiian

Coastal Streams-Associations with Fecal Indicators, Land Cover, and Water Quality.

Water Res. **2011**, *45* (11), 3279–3290.

(15)   Davies-Colley, R. J.; Donnison, A. M.; Speed, D. J.; Ross, C. M.; Nagels, J. W.

Inactivation of Faecal Indicator Micro-Organisms in Waste Stabilisation Ponds:

Interactions of Environmental Factors with Sunlight. *Water Res.* **1999**, *33* (5), 1220–1230.

(16)   Boehm, A. B.; Yamahara, K. M.; Love, D. C.; Peterson, B. M.; Mcneill, K.; Nelson, K. L.

Covariation and Photoinactivation of Traditional and Novel Indicator Organisms and

Human Viruses at a Sewage-Impacted Marine Beach. *Environ. Sci. Technol.* **2009**, *43*

(21), 8046–8052.

(17)   Corsi, S. R.; Borchardt, M. a.; Carvin, R. B.; Burch, T. R.; Spencer, S. K.; Lutz, M. a.;

McDermott, C. M.; Busse, K. M.; Kleinheinz, G. T.; Feng, X.; et al. Human and Bovine

Viruses and Bacteria at Three Great Lakes Beaches: Environmental Variable Associations

and Health Risk. *Environ. Sci. Technol.* **2015**, acs.est.5b04372.

(18)   Russell, T. L.; Sassoubre, L. M.; Wang, D.; Boehm, A. B. A Coupled Modeling and

Molecular Biology Approach to Microbial Source Tracking at Cowell Beach, Santa Cruz,

CA, United States. *Environ. Sci. Technol.* **2013**, *47* (18), 10231–10239.

(19)   Boehm, A. B.; Graham, K. E.; Jennings, W. Can We Swim yet? Systematic Review,

Meta-Analysis, and Risk Assessment of Aging Sewage in Surface Waters. *Environ. Sci.*

Technol. **2018**, acs.est.8b01948.

(20)   Berney, M.; Weilenmann, H.-U.; Simonetti, A.; Egli, T. Efficacy of Solar Disinfection of

Escherichia Coli, Shigella Flexneri, Salmonella Typhimurium and Vibrio Cholerae. *J. Appl. Microbiol.* **2006**, *101* (4), 828–836.

(21)   Boehm, A. B.; Soetjipto, C.; Wang, D. Solar Inactivation of Four Salmonella Serovars in Fresh and Marine Waters. *J. Water Health* **2012**, *10* (4), 504–510.

(22)   Sassoubre, L. M.; Nelson, K. L.; Boehm, A. B. Mechanisms for Photoinactivation of Enterococcus Faecalis in Seawater. *Appl. Environ. Microbiol.* **2012**, *78* (21), 7776–7785.

(23)   Maraccini, P. A.; Wenk, J.; Boehm, A. B. Photoinactivation of Eight Health-Relevant Bacterial Species: Determining the Importance of the Exogenous Indirect Mechanism. *Environ. Sci. Technol.* **2016**, *50* (10), 5050–5059.

(24)   Silverman, A. I.; Nelson, K. L. Modeling the Endogenous Sunlight Inactivation Rates of Laboratory Strain and Wastewater E. Coli and Enterococci Using Biological Weighting Functions. *Environ. Sci. Technol.* **2016**, acs.est.6b03721.

(25)   Boyle, M.; Sichel, C.; Fernández-Ibáñez, P.; Arias-Quiroz, G. B.; Iriarte-Puña, M.; Mercado, A.; Ubomba-Jaswa, E.; McGuigan, K. G. Bactericidal Effect of Solar Water Disinfection under Real Sunlight Conditions. *Appl. Environ. Microbiol.* **2008**, *74* (10), 2997–3001.

(26)   McClary, J. S.; Sassoubre, L. M.; Boehm, A. B. Staphylococcus Aureus Strain Newman Photoinactivation and Cellular Response to Sunlight Exposure. *Appl. Environ. Microbiol.* **2017**, *83* (17), 1–14.

(27)   Al-Jassim, N.; Mantilla-Calderon, D.; Wang, T.; Hong, P.-Y. Inactivation of a Virulent Wastewater Escherichia Coli and Non-Virulent Commensal Escherichia Coli DSM1103

Strains and Their Gene Expression upon Solar Irradiation. *Environ. Sci. Technol.* **2017**, *51* (7), acs.est.6b05377.

(28)　Sinton, L. W.; Hall, C. H.; Lynch, P. A.; Davies-Colley, R. J. Sunlight Inactivation of Fecal Indicator Bacteria and Bacteriophages from Waste Stabilization Pond Effluent in Fresh and Saline Waters. *Appl. Environ. Microbiol.* **2002**, *68* (3), 1122–1131.

(29)　Whitman, R. L.; Nevers, M. B.; Korinek, G. C.; Byappanahalli, M. N. Solar and Temporal Effects on Escherichia Coli Concentration at a Lake Michigan Swimming Beach. *Appl. Environ. Microbiol.* **2004**, *70* (7), 4276–4285.

(30)　Fujioka, R. S.; Hashimoto, H. H.; Siwak, E. B.; Young, R. H. F. Effect of Sunlight on Survival of Indicator Bacteria in Seawater. *Appl. Environ. Microbiol.* **1981**, *41* (3), 690–696.

(31)　Nguyen, M. T.; Jasper, J. T.; Boehm, A. B.; Nelson, K. L. Sunlight Inactivation of Fecal Indicator Bacteria in Open-Water Unit Process Treatment Wetlands: Modeling Endogenous and Exogenous Inactivation Rates. *Water Res.* **2015**, *83*, 282–292.

(32)　Maraccini, P. A.; Ferguson, D. M.; Boehm, A. B. Diurnal Variation in Enterococcus Species Composition in Polluted Ocean Water and a Potential Role for the Enterococcal Carotenoid in Protection against Photoinactivation. *Appl. Environ. Microbiol.* **2012**, *78* (2), 305–310.

(33)　Hagi, T.; Kobayashi, M.; Nomura, M. Aerobic Condition Increases Carotenoid Production Associated with Oxidative Stress Tolerance in Enterococcus Gilvus. *FEMS Microbiol. Lett.* **2014**, *350* (2), 223–230.

(34) Curtis, T. P.; Mara, D. D.; Silva, S. A. Influence of PH, Oxygen, and Humic Substances on Ability of Sunlight to Damage Fecal Coliforms in Waste Stabilization Pond Water. *Appl. Environ. Microbiol.* **1992**, *58* (4), 1335–1343.

(35) Metselaar, K. I.; Abee, T.; Zwietering, M. H.; Besten, M. W. Den. Modeling and Validation of the Ecological Behavior of Wild-Type Listeria Monocytogenes and Stress-Resistant Variants. **2016**, *82* (17), 5389–5401.

(36) Sousa, A. M.; Machado, I.; Pereira, M. O. Phenotypic Switching: An Opportunity to Bacteria Thrive. *Sci. against Microb. Pathog. Commun. Curr. Res. Technol. Adv.* **2011**, 252–262.

(37) Probst-Rud, S.; McNeill, K.; Ackermann, M. Thiouridine Residues in TRNAs Are Responsible for a Synergistic Effect of UVA and UVB Light in Photoinactivation of Escherichia Coli. *Environ. Microbiol.* **2016**, *19* (2), 434–442.

(38) Fisher, M. B.; Iriarte, M.; Nelson, K. L. Solar Water Disinfection (SODIS) of Escherichia Coli, Enterococcus Spp., and MS2 Coliphage: Effects of Additives and Alternative Container Materials. *Water Res.* **2012**, *46* (6), 1745–1754.

(39) Brown, K. I.; Boehm, A. B. Comparative Decay of Catellicoccus Marimmalium and Enterococci in Beach Sand and Seawater. *Water Res.* **2015**, *83*, 377–384.

(40) Sinton, L. W.; Davies-Colley, R. J.; Bell, R. G. Inactivation of Enterococci and Fecal Coliforms from Sewage and Meatworks Effluents in Seawater Chambers. *Appl. Environ. Microbiol.* **1994**, *60* (6), 2040–2048.

(41) Anderson, K. L.; Whitlock, J. E.; Valerie, J.; Harwood, V. J. Persistence and Differential

Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. *Appl. Environ. Microbiol.* **2005**, *71* (6), 3041–3048.

(42)   Maraccini, P. A.; Mattioli, M. C. M.; Sassoubre, L. M.; Cao, Y.; Griffith, J. F.; Ervin, J. S.; Van De Werfhorst, L. C.; Boehm, A. B. Solar Inactivation of Enterococci and Escherichia Coli in Natural Waters: Effect of Water Absorbance and Depth. *Environ. Sci. Technol.* **2016**, *50* (10), 5068–5076.

(43)   Wuertz, S.; Wang, D.; Zamani, K.; Bombardelli, F. *An Analysis of Water Circulation in Pillar Point Harbor, Half Moon Bay , California, Based on the Dye Distribution Study of September 27, 2008*; 2011.

(44)   Sassoubre, L. M.; Walters, S. P.; Russell, T. L.; Boehm, A. B. Sources and Fate of Salmonella and Fecal Indicator Bacteria in an Urban Creek. *J. Environ. Monit.* **2011**, *13* (8), 2206–2212.

(45)   United States Environmental Protection Agency. *Method 1600: Enterococci in Water by Membrane Filtration Using Membrane-Enterococcus Indoxyl- $ -D-Glucoside Agar (MEI)*; 2002.

(46)   Facklam, R. R.; Collins, M. D. Identification of Enterococcus Species Isolated from Human Infections by a Conventional Test Scheme. *J. Clin. Microbiol.* **1989**, *27* (4), 731–734.

(47)   Geeraerd, A. H.; Valdramidis, V. P.; Van Impe, J. F. GInaFiT, a Freeware Tool to Assess Non-Log-Linear Microbial Survivor Curves. *Int. J. Food Microbiol.* **2005**, *102* (1), 95–105.

(48)   Brooks, L. E.; Field, K. G. Bayesian Meta-Analysis to Synthesize Decay Rate Constant

       Estimates for Common Fecal Indicator Bacteria. *Water Res.* **2016**, *104*, 262–271.

(49)   Nelson, K. L.; Boehm, A. B.; Davies-Colley, R. J.; Dodd, M. C.; Kohn, T.; Linden, K. G.;

       Liu, Y.; Maraccini, P. A.; McNeill, K.; Mitch, W. A.; et al. Sunlight-Mediated

       Inactivation of Health-Relevant Microorganisms in Water: A Review of Mechanisms and

       Modeling Approaches. *Environ. Sci. Process. Impacts* **2018**, *20* (8), 1089–1122.

(50)   Maraccini, P. A.; Wang, D.; McClary, J. S.; Boehm, A. B. Growth-Dependent

       Photoinactivation Kinetics of Enterococcus Faecalis. *J. Appl. Microbiol.* **2015**, *118* (5),

       1226–1237.

(51)   Maraccini, P. A.; Wenk, J.; Boehm, A. B. *Photoinactivation of Eight Health-Relevant

       Bacterial Species: Determining the Importance of the Exogenous Indirect Mechanism*; In

       Review; 2016.

(52)   Köhler, W. The Present State of Species within the Genera Streptococcus and

       Enterococcus. *Int. J. Med. Microbiol.* **2007**, *297* (3), 133–150.

(53)   Ferguson, D. M.; Moore, D. F.; Getrich, M. A.; Zhowandai, M. H. Enumeration and

       Speciation of Enterococci Found in Marine and Intertidal Sediments and Coastal Water in

       Southern California. *J. Appl. Microbiol.* **2005**, *99* (3), 598–608.

(54)   Halstead, F. D.; Thwaite, J. E.; Burt, R.; Laws, T. R.; Raguse, M.; Moeller, R.; Webber,

       M. A.; Oppenheim, B. A. The Antibacterial Activity of Blue Light against Nosocomial

       Wound Pathogens Growing Planktonically and as Mature Biofilms. *Appl. Environ.

       Microbiol.* **2016**, *82* (April), AEM.00756-16.

(55)   Mundt, J. O. Occurrence of Enterococci on Plants in a Wild Environment. *Appl. Microbiol.* **1963**, *11* (2), 141–144.

(56)   Brouwer, A. F.; Eisenberg, M. C.; Remais, J. V.; Collender, P. A.; Meza, R.; Eisenberg, J. N. S. Modeling Biphasic Environmental Decay of Pathogens and Implications for Risk Analysis. *Environ. Sci. Technol.* **2016**, No. 3.

(57)   Mossel, D. A. A.; Corry, J. E. L.; Struijk, C. B.; Baird, R. M. *Essentials of Microbiology of Foods*; John Wiley & Sons, 1995.

(58)   Sinton, L. W.; Finlay, R. K.; Lynch, P. A. Sunlight Inactivation of Fecal Bacteriophages and Bacteria in Sewage- Polluted Seawater. *Appl. Environ. Microbiol.* **1999**, *65* (8), 3605–3613.

(59)   Nevers, M. B.; Boehm, A. B. Modeling Fate and Transport of Fecal Bacteria in Surface Water. In *Fecal Bacteria*; Sadowsky, M. J., Whitman, R. L., Eds.; ASM Press, 2010; pp 165–188.

(60)   Boehm, A. B.; Ismail, N. S.; Sassoubre, L. M.; Andruszkiewicz, E. a. Oceans in Peril: Grand Challenges in Applied Water Quality Research for the 21st Century. *Environ. Eng. Sci.* **2015**, *00* (00), ees.2015.0252.

(61)   Safaie, A.; Wendzel, A.; Ge, Z.; Nevers, M. B.; Whitman, R. L.; Corsi, S. R.; Phanikumar, M. S. Comparative Evaluation of Statistical and Mechanistic Models of Escherichia Coli at Beaches in Southern Lake Michigan. *Environ. Sci. Technol.* **2016**, acs.est.5b05378.

(62)   Meister, S.; Verbyla, M. E.; Klinger, M.; Kohn, T. Variability in Disinfection Resistance between Currently Circulating Enterovirus B Serotypes and Strains. *Environ. Sci. Technol.*

2018, *52* (6), 3696–3705.

Tables

Table 1 Results of photoinactivation modeling and pigmentation tests. Values in parentheses represent standard error. * Note that light scattering may influence k and S values for this result and was not corrected.

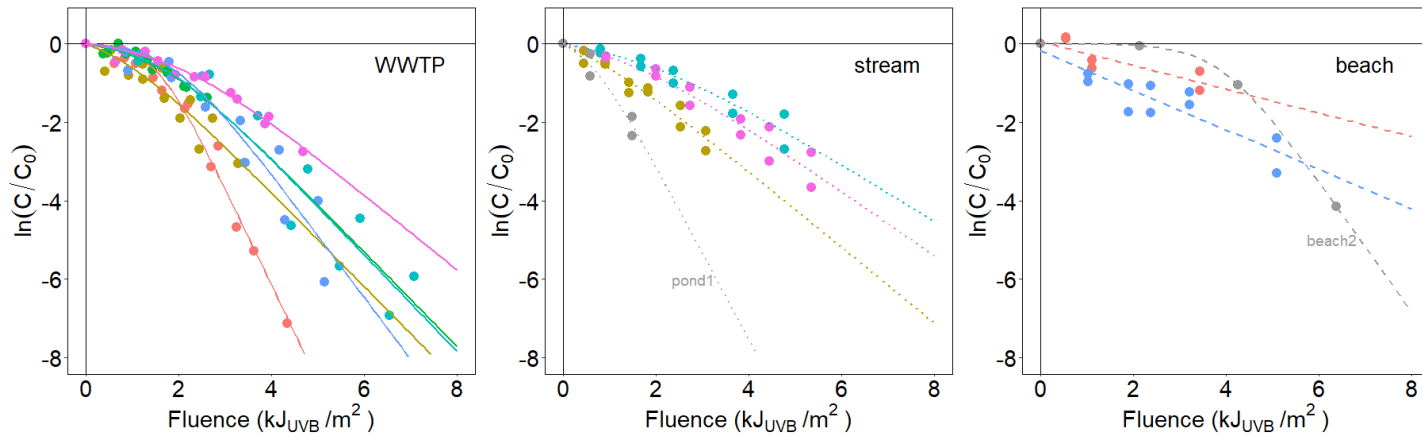| Sample Name	k $(m^2kJ_{UVB}^{-1})$	S (kJ_{UVB}/m^2)	Total Isolates Tested	Isolates Pigmented	Fraction Pigmented
WWTP1	2.5 (0.2)	1.5 (0.1)	20	1	5.0% (4.9%)
WWTP2	1.2 (0.3)	0.8 (0.4)	27	0	0.0% (0.0%)
WWTP3	1.2 (0.2)	1.6 (0.1)	43	5	11.6% (4.9%)
WWTP4	1.2 (0.2)	1.6 (0.5)	47	6	12.8% (4.9%)
WWTP5	1.6 (0.2)	1.9 (0.3)	66	11	16.7% (4.6%)
WWTP6	1.0 (0.08)	2.0 (0.1)	28	2	7.1% (4.9%)
pond1	2.2 (0.7)	0.6 (0.3)	21	4	19.0% (8.6%)
beach1	0.3 (0.07)	-	23	8	34.8% (9.9%)
beach2 *	1.7 (0.007)	3.9 (0.008)	67	21	31.3% (5.7%)
beach3	0.5 (0.07)	-	37	13	35.1% (7.8%)
stream1	1.0 (0.1)	0.6 (0.3)	88	13	14.8% (3.8%)
stream2	0.7 (0.1)	1.8 (0.5)	89	37	41.6% (5.2%)
stream3	0.8 (0.1)	1.4 (0.4)	49	4	8.2% (3.9%)

Figures

Figure 1 Photoinactivation of enterococci from WWTP (left), stream (center, including pond1 in gray), and beach (left) sources. Data in gray (samples beach2 and pond1) are excluded from averaged photoinactivation rate constants as described in main text. $\ln(C/C_0)$ = natural log-transformed relative concentration of enterococci, measured as CFU/mL on mEI agar. Fluence was calculated in the UVB wavelengths. Different colors correspond to photoinactivation data from experiments that used indigenous, uncultured bacteria from different sources.
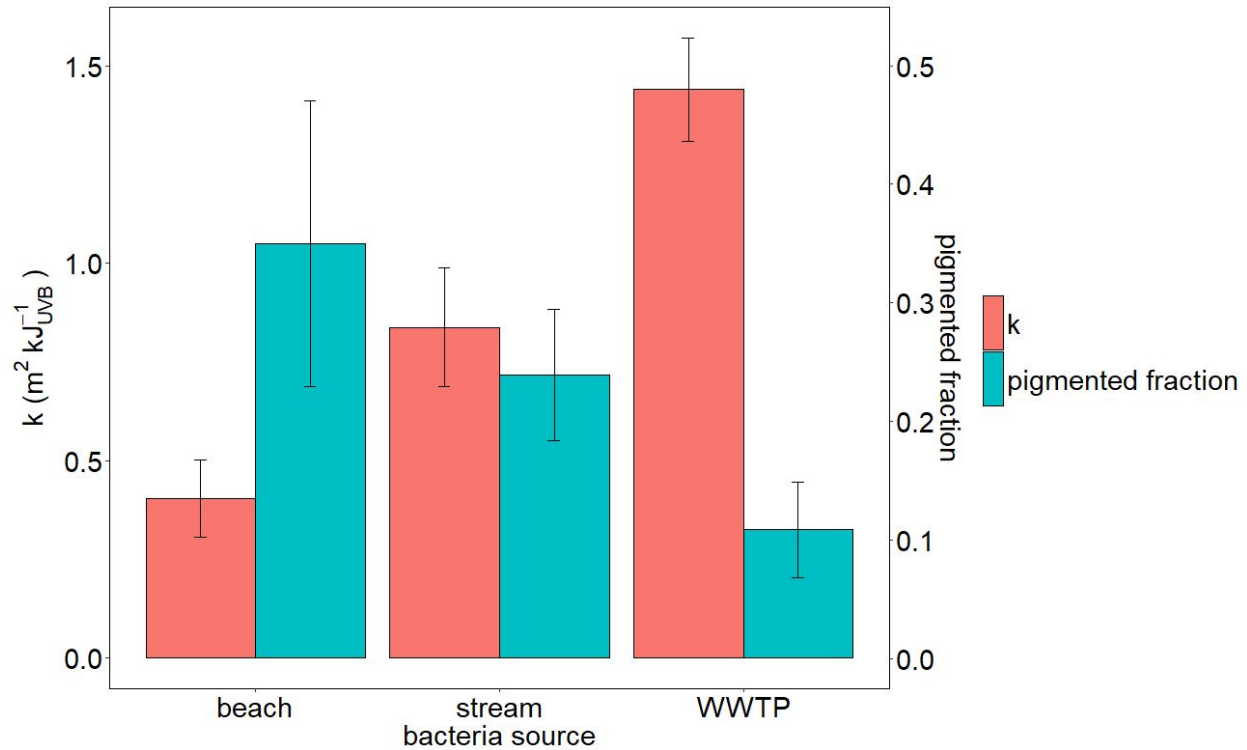
Figure 2 Correlation between fraction of pigmented enterococci isolates and $F_{90\%}$ (left) or k (right). Shaded region bounded by dotted lines is 95% confidence interval around a linear model fit.

Figure 3 Average photoinactivation rate constants (k) of enterococci sourced from wastewater (n = 6), urban streams (n = 3), and beach water (n = 2), and fraction of pigmented enterococci isolated from wastewater (n = 231), urban streams (n = 226), and beach water (n = 60). Error bars represent the 95% confidence interval.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table of Contents Entry

Indigenous enterococci communities photoinactivate at diverse rates that are associated with their source and their degree of pigmentation.