Diurnal variation in *Enterococcus* species composition in polluted ocean water: A potential role for the enterococcal carotenoid in protection against photoinactivation

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Abstract

Enterococcus species composition was determined each hour for 72 hours at a polluted marine beach in Avalon, Santa Catalina Island, California, USA. Species composition was significantly different during the day versus the night based on an analysis of similarity. *E. faecium* and *E. faecalis* were more prevalent at night than the day while *E. hirae* and other *Enterococcus* species were more prevalent during the day than the night. *Enterococcus* spp. containing a yellow pigment were more common during the day than the night suggesting that the pigmented phenotype may offer a competitive advantage during sunlit conditions. A laboratory microcosm experiment established that the pigmented *E. casseliflavus* and a pigmented *E. faecalis* isolated from the field site decay at a slower rate than a non-pigmented *E. faecalis* in a solar simulator in simulated, clear seawater. This further supports the idea that the yellow carotenoid pigment in *Enterococcus* provides protection during sunlit conditions. The findings are in accordance with previous work with other carotenoid-containing non-photosynthetic and photosynthetic bacteria that suggests the carotenoid is able to quench reactive oxygen species capable of causing photoinactivation and photostress. The results suggest that using enterococcal species composition as a microbial source tracking tool may be hindered by the differential environmental persistence of pigmented and non-pigmented enterococci.

Introduction

Enterococci are fecal indicator organisms used to assess coastal water quality around the world. Their concentrations in marine coastal waters correlate to health risk in swimmers based on data from epidemiology studies (Wade *et al*. 2003). There are dozens of enterococcal species (Facklam *et al*. 2002). Recently, it has been suggested that a specific subset of these species can be characterized as 'environmental' rather than 'fecal'; specifically *Enterococcus gallinarum*, *E. casseliflavus*, and *E. mundtii* have been proposed as environmental strains due to their documented associations with plants, soils, and non-human animal hosts (Bahirathan *et al*. 1998, Moore *et al*. 2008) while *E. faecalis* and *E. faecium* have been suggested to represent fecal strains due to their prevalence in human feces (Ferguson *et al*. 2005, Moore *et al*. 2008).

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Photoinactivation of enterococci in marine waters is well documented in both field settings (Boehm *et al*. 2002, Boehm *et al*. 2009, Sinton *et al*. 2007) as well as laboratory microcosms (Sinton *et al*. 1999, Sinton *et al*. 2002). Enterococci can be inactivated via several different mechanisms. Direct photoinactivation results from the direct damage of cellular chromophores by photons; the most common example is UVB damage of DNA (Malloy *et al*. 1997). Indirect photoinactivation results from the damage of cellular machinery and macromolecules via reactive species generated by photon-excited endogenous (intracellular) or exogenous (extracellular) sensitizers such as colored dissolved organic matter and humic acids, the most important of which are believed to be reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, superoxide, and hydroxyl radical. There is presently a lack of knowledge about the relative importance of direct versus indirect photoinactivation, and the relative importance of UVA, UVB, and visible radiation (Berney *et al*. 2006, Gourmelon *et al*. 1997, Malloy *et al*. 1997) in causing photodamage of fecal organisms in marine waters (Sinton *et al*. 2007).

Some enterococcal species, particularly those thought to be associated with soil and non-human hosts (Bahirathan *et al*. 1998) contain yellow pigments including *E. casseliflavus, E. mundtii, E. pallens, E. gilvus,* and *E. sulfureus* (Facklam et al. 2002, Martinez-Marcia and Collins 1991). The pigments of *E. casseliflavus* were investigated by Taylor *et al*. (1971) and determined to be carotenoids. Pigments in *E. sulfureus* and *E. mundtii* were also identified as carotenoids (Breithaupt *et al*. 2001). Carotenoids function as protectors against photodamage as they are able to quench ROS (Griffiths *et al*. 1995, Jensen 1965). At the present time, the effect of the carotenoid pigment in modulating photoinactivation of enterococci has not been studied. Experiments with Staphylococcus aureus, which also contains carotenoids, have shown that gene knock-out mutants that lack carotenoid synthesis capabilities are more susceptible to photodamage and death from ROS including hydrogen peroxide, superoxide, and hydroxyl radical (Clauditz *et al*. 2006). Thus, there is reason to suspect that carotenoids in enterococci may provide protection against photooxidative damage and subsequent death.

The present study investigates the potential role of pigments in modulating enterococcal photoinactivation using field and laboratory data. For the purpose of this study, we define inactivation as the loss of culturability of bacteria. Hourly enterococcal species composition data were collected for 72 hours at a polluted marine beach; these data include the proportion of pigmented enterococci. In addition, a microcosm experiment was carried out to test the relative photoinactivation rates of pigmented and non-pigmented Enterococcus strains. Results suggest that pigmented enterococci are protected from solar inactivation and can persist for extended periods of time in marine water relative to non-pigmented species, leading to differential environmental persistence of pigmented and non-pigmented enterococci.

Methods

Field Site

Avalon Beach is located on Santa Catalina Island in the Southern California Bight. Waterborne enterococci at the beach are thought to emanate mainly from leaking sewer lines (Boehm *et al.* 2003*)*. Other enterococcocal sources at the beach include sands and sediment, urban runoff, and wild animals including birds (Boehm *et al*. 2003).

Water Sampling

Water was collected every hour from 0400 hours 19 August to 0300 hours 22 August 2008 10 m north of the Pleasure Pier at Avalon Beach (33°20.9′ N, 118°19.5′ W). Samples were collected at ankle depth on an incoming wave in an ethanol-sterilized, triple-rinsed bucket and transferred to a 20 L 10% hydrochloric acid washed, triple-rinsed cube-container. The water was stored in the dark in a cooler and was processed within 6 hours of collection.

Irradiance and Tidal Predictions

Simple Model of the Atmospheric Radiative Transfer of Sunshine (SMARTS; Gueymard *et al*. 2005) was used to estimate UVB intensity each hour of the study. 'Day' was used to describe times when there was sunlight, and 'night' when there was no sunlight. Tidal predictions relative to mean sea level were obtained from online tidal calculators for Avalon, Santa Catalina Island, California (http://tbone.biol.sc.edu/tide/). When the tide relative to mean sea level was greater than or equal to 0.8 m, it was deemed 'high' and all other tides 'low'.

Enterococci Enumeration and Speciation

Enterococci were enumerated using EPA method 1600 (USEPA 2006). The method uses membrane filtration and mEI agar. 10 ml and 100 ml of each sample were filtered to obtain a countable number of colonies. Enterococci concentrations obtained with this method over the 72 hours are reported elsewhere (Boehm *et al*. 2005). Ten presumptive enterococci colonies from each time point were picked for speciation from mEI media and subcultured onto tryptic soy agar (TSA) agar with 5% sheep blood (Northeast Laboratory; hereafter referred to as blood agar plates, BAPs). In some cases less than 10 colonies were archived due to lack of colony availability. Of the 72 time points, 10 colonies were obtained at 66 time points, 6 colonies at 2 time points, 5 colonies at 3 time points, and 3 colonies at 1 time point. After 24 hours incubation at 35°C, BAPs were assessed for strain purity by confirming that colonies were morphologically identical. Pure strains were subcultured from BAPs onto TSA slants (Northeast Laboratory, Winslow, ME) and incubated at 35°C for 24 hours. TSA slants were subsequently stored at 4°C until speciation could be performed.

Isolates selected for speciation were subcultured from TSA slants onto BAPs 24 hours prior to species identification. All isolates were first tested using the Vitek microbial identification system (bioMérieux, St. Louis, MO). Isolates identified as *Enterococcus* species but at low discrimination (< 80% confidence level) were further tested using API 20S (bioMérieux, St. Louis, MO), an alternate species identification system used for Gram-positive cocci. Identifications by Vitek that were 'non-discriminatory' (e.g., *E. casseliflavus/E. gallinarum*) were tested using supplemental biochemical analyses including: motility, pigment production, and fermentation of arabinose, sucrose, and mannitol as per Facklam and Collins (1989) and Ferguson *et al*. (2005). These methods for enterococci speciation are well established and have been shown to provide good agreement (>90%) with species identified using partial 16s rRNA sequencing (Moore *et al*. 2006). The presence or absence of pigment was determined for each isolate. The tip of a sterile cotton-tip swab was touched to the surface of a single colony grown on BPA and held under a lamp. Yellow colonies were characterized as pigmented, as described by Facklam and Collins (1989).

Microcosm Experiments

The photoinactivation of non-pigmented *Enterococcus faecalis* (ATCC #19433), pigmented *Enterococcus casseliflavus* (ATCC #25788), and a lightly pigmented environmental isolate from Avalon Beach (identified as *Enterococcus faecalis* using the speciation procedures described previously, hereafter referred to as *E. faecalis* AB) was tested. Experiments were performed in simulated seawater at pH 8.1. Simulated seawater was prepared in deionized water containing all major anions and cations, including carbonates (US Department of Energy 1994, Grebel *et al*. 2009) and filter sterilized prior to use. All salts used were reagent grade or better.

Enterococcus spp. were grown in tryptic soy broth (*E. faecalis* and E. *faecalis* AB) or brain heart infusion broth (*E. casseliflavus*) to stationary phase at 37°C, as measured via spectrophotometry at a single wavelength of 650 nm. The density of cells at stationary phase was approximately 10^9 CFU/ml. Cells were then harvested by centrifuging and washed in phosphate buffer saline (PBS, pH = 7.32) three times. Cells were then resuspended in PBS (referred to as 'seed stock').

Fifty ml simulated seawater and 0.5 ml of *Enterococcus* seed stock were placed in 100 ml sterile beakers wrapped in black electrical tape. No exogenous sensitizers (e.g., humic acid) were added because previous research indicated that the seawater at Avalon Beach was quite clear with few sensitizers (Boehm *et al*. 2009). Beakers were placed in a recirculating water bath to maintain their temperature at 15°C, the temperature of seawater in Central California, in a solar simulator (Altas Suntest CPS+, Linsengericht-Altenhaßlau, Germany) equipped with a coated quartz filter and a UV special glass filter to block the transmission of wavelengths below 290 nm to simulate natural sunlight (passing wavelength, 290 nm < λ < 800 nm; Plumlee and Reinhard 2007). The irradiance was set at 400 W/m^2. The resulting light spectrum was measured using a spectroradiometer (RPS 200 and 380, International Light, Peabody, MA) and compared favorably to the light spectrum at Avalon Beach at 0900 hours 19 August 2008 as estimated using SMARTS. Stir bars in the beakers ensured that the solutions were well-mixed throughout the experiment.

One ml samples were withdrawn from the beakers every 15 to 30 minutes. Appropriate sampling time intervals were determined using data

from pilot experiments. Dark controls were run; seeded simulated seawater was stored in the dark at 15°C and samples were collected every 30 to 60 minutes.

Enterococcus colonies were enumerated in each sample by spread plating appropriate dilutions in duplicate on tryptic soy agar (TSA) for *E. faecalis* and *E. faecalis* AB and on brain heart infusion agar for *E. casselflavis*. Agar plates were incubated at 37°C and colonies were counted after 24 hours.

Photoinactivation experiments were performed in duplicate for *E. faecalis* and in triplicate for *E. faecalis* AB and *E. casseliflavus*. Dark controls were performed in duplicate for *E. faecalis* and once for *E. faecalis* AB and *E. casseliflavus*. Data from all replicate experiments are presented and used without averaging in the determination of decay rates (see below).

Statistical Methods

Species composition was compared between day and night, as well high and low tides using PRIMER version 5 (PRIMER-E Ltd, Ivybridge, United Kingdom). Bray-Curtis similarity matrices were created from square-root transformed standardized species composition data. Analysis of similarity (ANOSIM) was used to determine whether there were significant differences in species composition between samples collected during the day/night or high/low tides. Differences in the occurrence of specific enterococcal species, as well as pigmented versus non-pigmented enterococci during day versus night were assessed using χ^2 tests (IBM SPSS Statistics version 19.0, Chicago, IL).

For the inactivation experiment, concentrations of *Enterococcus* at each time point were normalized by the initial concentrations at time 0 (~10^7 CFU/ml for all experimental conditions) and the natural logarithm of normalized concentration was plotted as a function of time and fluence. Inactivation rate constants were calculated assuming Chick's law applied using linear-least squares regression on the portion of data where exponential decay was observed following the approach taken by others (Harm 1980, Plumlee and Reinhard 2007, Sinton *et al*. 1999). The decay rates (slopes of the lines) were compared for the three organisms using multiple linear regression (Neeter *et al*. 1990) to determine if they were significantly different. When replicate experiments were run, data were not averaged prior to curve fitting.

Results

Field Observations

The 72-hour time series of presumptive enterococci measured using mEI media was previously published in Boehm *et al*. (2009). The data showed a statistically significant diurnal trend with concentrations lower in the day relative to the night and subsequent modeling confirmed the influence of photoinactivation on their concentrations.

A total of 690 presumptive enterococci (~10 from each time point) were speciated as *E. faecium, E. faecalis, E. gallinarum, E. hirae, E. casseliflavus, E. cecorum, E. durans, Aerococcus viridans*, other enterococci, and non-enterococci (Table 1). The most common species was *E. faecalis* (308/690, 45%). The second most common species was the non-enterococcal *Aerococcus viridans* (119/690, 17%). Two of the isolates could not be identified and were designated as such. Of the speciated isolates (n=688), 82% were confirmed as members of the genus *Enterococcus*.

The time series of raw species composition relative to solar intensity and tide level is shown in Figure 1. To simplify data display, less common organisms (those where $n \leq 8$) were grouped into categories of 'other enterococci' or 'other non-enterococci' as specified in Table 1. A diurnal pattern in both presumptive and confirmed enterococci species composition was observed. When all identified species at the 72 time points were considered, there was a significant difference in species composition between day and night samples (ANOSIM Global R = 0.147, p = 0.002), but no difference in samples collected at high versus low tides (ANOSIM Global R = -0.007, p = 0.54). Results were similar when only confirmed enterococcal species were considered; enterococcal species composition was significantly different in samples collected during the day versus the night (ANOSIM Global R = 0.055, p = 0.02) but not different in samples collected during high versus low tide (ANOSIM Global R = -0.002, p = 0.45).

Variation in the occurrence of specific enterococcal species or groups (Table 1) during day and night was examined using χ^2 tests and contingency tables. *E. faecium* and *E. faecalis*

Table 1. Identification of isolates. The simplified designations are those used in Figure 1.

Species	# of Isolates	Simplified Designation
Enterococcus faecalis	308	*Enterococcus faecalis*
Aerococcus viridans	119	*Aerococcus viridans*
Enterococcus faecium	88	*Enterococcus faecium*
Enterococcus hirae	70	*Enterococcus hirae*
Enterococcus gallinarum	56	*Enterococcus gallinarum*
Enterococcus casseliflavus	17	other enterococci
Enterococcus species	8	other enterococci
Enterococcus durans	8	other enterococci
Enterococcus cecorum	6	other enterococci
Enterococcus mundtii	4	other enterococci
Unidentified	2	unidentified
Streptococcus mutans	1	other non enterococci
Streptococcus gallolyticus ssp gallolyticus	1	other non enterococci
Leuconostoc ssp	1	other non enterococci
Pediococcus acidilactici	1	other non enterococci

constituted a greater proportion of the confirmed enterococci isolated during the night versus the day (χ^2 test, $p < 0.01$ for both), while *E. hirae* and the 'other enterococci' group (see Table 1 for definition) made up a greater proportion of the enterococci isolated during the day compared to the night (χ^2 test, $p < 0.05$ for both).

The presence of a yellow pigment was assessed for each isolate and occurrence of pigmented species was compared between day and night. Of all 690 isolates examined, 151 were pigmented. Forty-two percent of these were subsequently speciated as *A. viridans*, 20% as *E. gallinarium*, 13% as *E. hirae*, 11% as *E. casseliflavus*, 7% as *E. faecalis*, 3% as *E. cecorum*, and 1% as *E. faecium*. Eighty-eight of the pigmented isolates were confirmed *Enterococcus*. The fraction of confirmed *Enterococcus* displaying a pigmented phenotype at each time point is provided in Figure 2. The proportion of pigmented *Enterococcus* was higher during the day (24% of confirmed *Enterococcus*) compared to the night (8% of confirmed *Enterococcus*; χ^2 test, $p < 0.0001$). If we include all isolates, even non-enterococcal ones, a greater proportion of isolates were pigmented during the day (31% of isolates) compared to the night (12% of isolates; χ^2 tests, $p < 0.0001$).

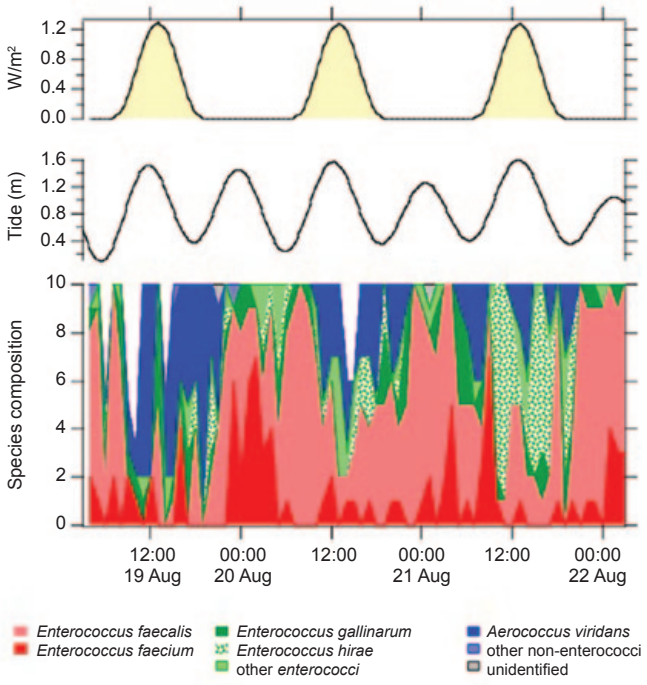

Figure 1. Tide level and UVB irradiance during the study are provided in the top two panels. The bottom panel provides the number of each species (height of colored section at each time point) of the total number of isolates examined (designated by the height of the stacked colors).

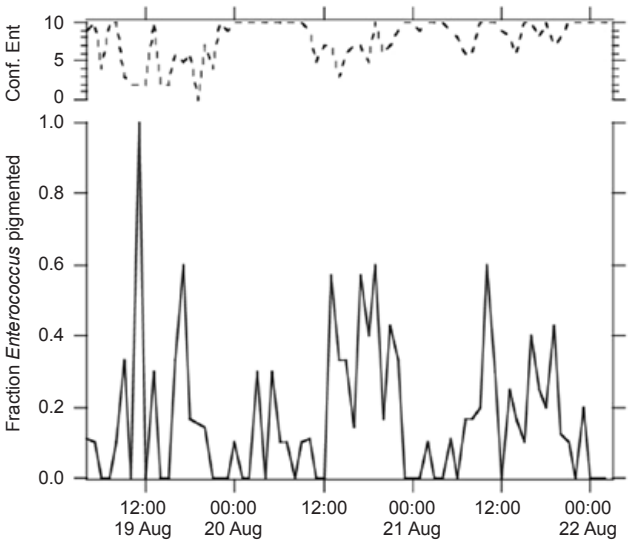

Figure 2. The fraction of confirmed pigmented *Enterococcus* at each time point. The top line is the total number of confirmed *Enterococococcus* of the ~10 isolates examined.

Photoinactivation Experiment

The photoinactivation of *E. faecalis, E. faecalis* AB, and *E. casseliflavus* in simulated seawater was compared using a solar simulator. When all three organisms were exposed to identical simulated sunlight, an initial period of limited inactivation ('shouldering') was observed when decline was similar to that observed in the dark controls (Figure 3). For the two *E. faecalis* strains, this lag period lasted 60 min; for the *E. casselflavis* strain, the lag period lasted 120 minutes. Thereafter, exponential decay was observed for all three organisms. *E. faecalis, E. faecalis* AB, and *E. casseliflavus* had first order decay rates (± standard deviation) of -0.1 ± 0.005, -0.08 ± 0.006, and -0.06 ± 0.007 /min, respectively. These rates may also be expressed as a function of fluence (Sinton *et al.* 1994, Thurston-Enriquez *et al.* 2003) as -0.04 ± 0.002, -0.03 ± 0.002, and -0.02 ± 0.003 cm^2/J, respectively. Multiple linear regression indicated that the decay rate of the non-pigmented *E. faecalis* was significantly faster than the decay rates of the pigmented strains ($p < 0.05$ for all comparisons). There was limited decline of the microorganisms in the dark controls over the course of the experiments (Figure 3).

Discussion

A diurnal pattern in enterococcal species composition was observed in hourly samples collected for 72 hours at a marine beach in Avalon, Santa Catalina Island, California, USA. 'Fecal' enterococcal species *E. faecalis* and *E. faecium* made up a greater fraction of the confirmed *Enterococcus* at night compared to the day, while *E. hirae* and the 'other enterococci' group (includes *Enterococcus* spp., *E. casseliflavus*, *E. durans, E. cecorum*, and *E. mundtii*) made up a greater fraction of the confirmed *Enterococcus* during the day compared to the night. Although tidal and diel cycles are synchronized over a short period of three days, the tide was not important controller of species composition. While there are certainly other controllers of species composition, including but not limited to temporally variable and differential sources, differential transport, grazing, and interaction with sediment, the results suggests that sunlight exposure influences the composition of enterococcal species at Avalon Beach.

A closer examination of enterococcal phenotypes indicates that a greater fraction of enterococci were pigmented during the day than the night, suggesting that the presence or absence of pigments could be one of the driving forces behind the observed diurnal pattern in species composition. Previous work with *Staphylococcus aureus* (Clauditz *et al*. 2006) and photosynthetic bacterial (Jensen 1965) mutants indicates that yellow carotenoid pigments, like those present in pigmented *Enterococcus* spp., are capable of quenching reactive oxygen species and play important roles in protecting organisms from photostress and photooxidation. Our experiments in the solar simulator indicate that a pigmented *E. casseliflavus* and a pigmented *E. faecalis* isolated from Avalon Beach decay more slowly than a non-pigmented *E. faecalis*. There are more differences between the tested organisms than just the presence or absence of a pigment. The non-pigmented and pigmented *E. faecalis* strains were not confirmed to be isogenic, and the pigmented *E. faecalis* was less pigmented than *E. casseliflavus* based on a qualitative evaluation of color. Regardless, the laboratory results suggest that the carotenoid pigment in the environmental *E. faecalis* AB and *E. casseliflavus* may be capable of providing protection against photoinactivation.

An initial shouldering was observed in the decay curves of all three tested *Enterococcus* spp. A shouldering effect during inactivation experiments has been observed by numerous researchers in experiments involving both viruses and bacteria and has been attributed to shielding of organisms from inactivation by other organisms or particles,

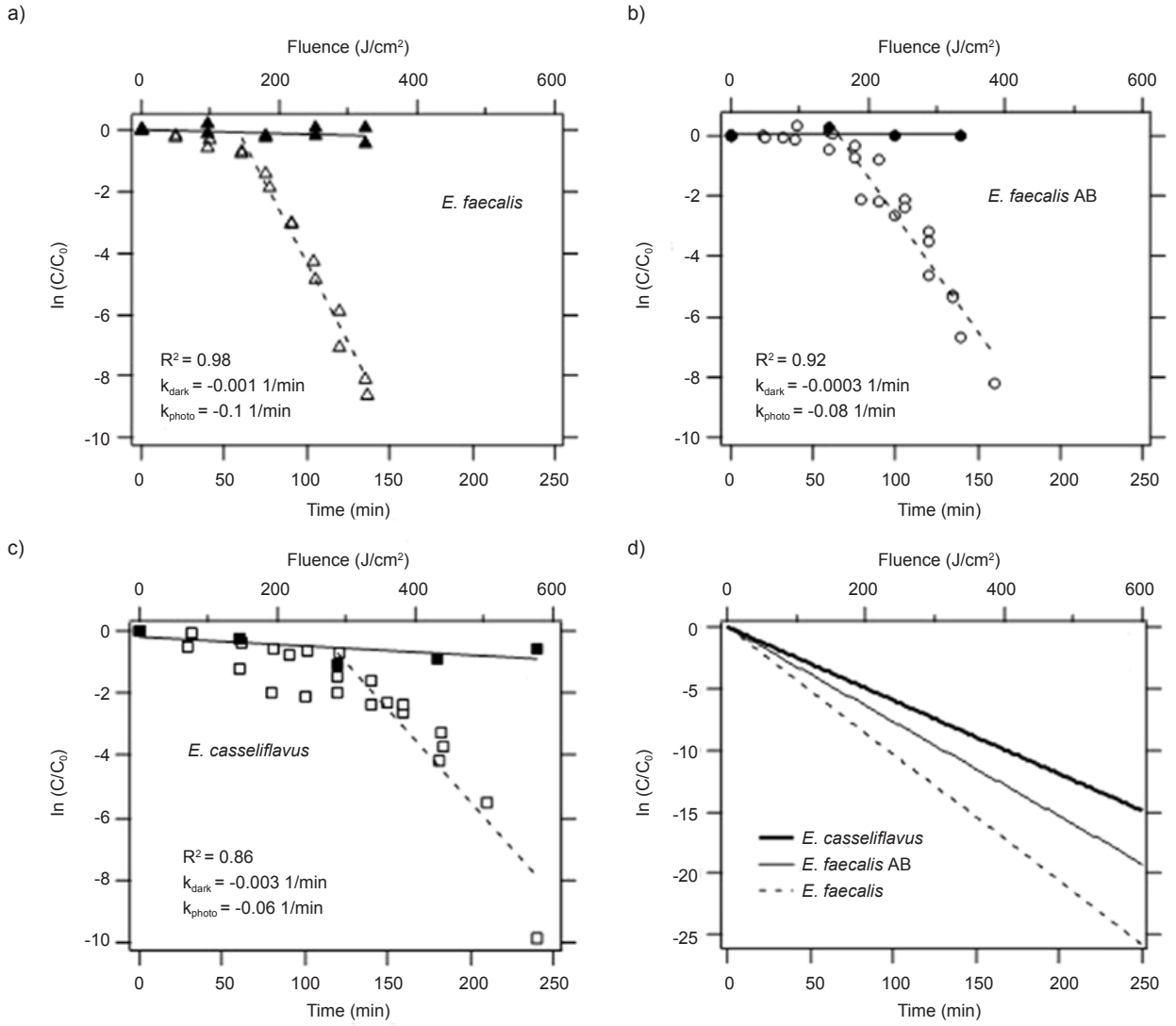

Figure 3. Decay of *Enterococcus faecalis* (A), *Enterococcus faecalis* AB (B), and *Enterococcus casseliflavus* (C) in simulated seawater in a solar simulator as a function of time (bottom axis) and fluence (top axis). Dark control data are provided. The best fit line is provided for the experimental data after the 'shoulder' (solid line – dark control, dashed line- photoinactivation treatment) and an r-square value is provided for the curve fit for the photoinactivation treatment. First order decay rates (in units of 1/min) for the dark control (k_{dark}) and photoinactivation treatment (k_{photo}) are provided. Panel D shows the best fit line for photoinactivation of the three organisms together on the same plot.

the requirement that multiple hits are needed for inactivation, and a threshold effect where a cell can withstand a certain level of stress before death occurs (Cebrián *et al*. 2007, Sinton *et al*. 1994, Sinton *et al*. 1995, Thurston-Enriquez *et al*. 2003). In the present experiment, the shouldering effect lasted one hour for the *E. faecalis* strains and two hours for the *E. casseliflavus* strain. Additional work will need to be done to investigate cause of the differential lag periods.

The laboratory study aids in the interpretation of the field results and suggests that the presence of pigments in enterococci affects the fate of these organisms in the environment. The field results are consistent with the following conceptual model. Exogenous or endogenous sources seed both pigmented and non-pigmented enterococci into seawater. Once the sun comes out, enterococci are inactivated due to either direct or indirect photoinactivation; however, the pigmented enterococci do not inactivate as quickly as the non-pigmented due to the protection provided by the carotenoid. The result is that during the day, pigmented enterococci have a competitive advantage over non-pigmented enterococci, and thus their relative abundance may be higher.

These findings have implications for using enterococci speciation for microbial source tracking. 'Environmental' and non-human enterococcal species are more likely to have pigments than human enterococcal species (Bahirathan *et al*. 1998, Mundt 1963). Assuming the differential fate of *E. faecalis* and *E. casseliflavus* observed in our microcosms is representative of the differential fate of non-pigmented and pigmented *Enterococcus* in general, the combined field and microcosm results suggest that species composition in sunlight-exposed water may not reflect that of the enterococcal source. During the dark, however, the ratio of pigmented to non-pigmented enterococci in water may better reflect the ratio of these organisms in the exogenous sources.

A secondary finding of this study is that mEI media used for the selective cultivation of enterococci allows for the growth of *Aerococcus viridans* at Avalon Beach. Seventeen percent of the 690 screened presumptive enterococci were *A. viridans*. This is consistent with a study by Moore *et al*. (2008) that also speciated enterococci from Avalon Bay during a different time period. However, they found 37% of their presumptive isolates to be *A. viridans*. The discrepancy in the percentages presented in Moore *et al*. and those presented herein might be explained by the fact that those authors sampled exclusively during sunlit hours. In the present study, 27% of isolates collected during sunlight hours were *A. viridans* while just 6% of those speciated from nighttime samples were *A. viridans*. The daytime percentage is closer to that reported by Moore *et al*. Interestingly, 63 (53% of the 119 *A. viridans* isolates) were pigmented which might indicate that this organism has a competitive advantage for surviving in sunlit waters.

There are several limitations of this study that should be acknowledged. First, only ten presumptive isolates per time point were included in the species analysis. It would have been preferable to include more. However, samples in the presented statistical analyses were aggregated (by time of day or tide) so that a large number of isolates (between 400 and 500) were used in exploring various hypotheses. The microcosm studies compared pigmented versus non-pigmented *Enterococcus* photoinactivation and just begin to explore the potential advantage for *Enterococcus* to express the carotenoid pigment. More work is warranted to fully understand the role of the pigment in *Enterococcus* ecology. Additionally, we measured inactivation as loss of culturability; unculturable organisms may be dead (due to cell lysis, for example), but also may remain viable and potentially undergo photorepair. The dynamics of the viable but non-culturable population of enterococci deserves future attention. The results described here apply to clear waters; the presence of particles in water may shield bacteria from sunlight and modulate photoinactivation rates. Finally, Ho *et al*. (2011) suggest that diurnal variation in enterococci concentrations at Avalon may be due to anthropogenic currents generated by the arrival of ferries in the harbor. Although several lines of evidence presented herein suggest that sunlight plays a role in modulating enterococcal species composition, the potential for diurnal currents or mixing to affect species composition remains a possibility.

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