Inactivation of indicator bacteria from various sources of fecal contamination in seawater and freshwater

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ABSTRACT - The survival of indicator microorganisms in aquatic systems is affected by both biotic and abiotic factors. Relatively little work in this field has been conducted on the wide variety of sources of microorganisms by using naturally occurring inoculants of microorganisms for study. Rates of inactivation of water quality indicators, total coliforms, *E. coli,* enterococci, and male-specific coliphage were studied in three week-long, factorially designed experiments using three different inoculants: sewage influent (to simulate a sewage spill), treated sewage effluent (to simulate chronic sewage discharges), and dry-weather urban runoff (to simulate non-point source discharges). Rates of inactivation were studied by examining the effects of temperature, nutrients, total suspended solids, initial bacterial load, and solar radiation in both natural fresh and seawater exposure matrices. Our results demonstrated that temperature and solar radiation had significant effects upon rates of inactivation (ANOVA, $p < 0.001$). These inactivation rates were similar, regardless of the inoculant type. Enterococci consistently degraded the slowest under dark conditions with a T90 of 115-121 h and 144-177 h at 20°C and 14°C, respectively. When incubated in sunlight, enterococci was inactivated significantly more rapidly than either *E. coli* or male-specific coliphage ($p < 0.001$). Enterococci T90s increased to 8-10 h when either low (wintertime) or high (summertime) solar radiation was applied. None of the other parameters that were examined had significant effects on inactivation rates ($p > 0.05$).

INTRODUCTION

Bacterial indicator testing is relatively inexpensive, and is one of the main modes of water quality management in use today. Fecal bacteria are considered to be good indicators of fecal contamination since they are present in the feces of humans and warm-blooded animals. Total coliforms (TC), fecal coliforms (FC), and enterococci (EC) are indicator bacteria that have been used for decades to infer the presence of other potentially harmful pathogens in recreational waters. When large numbers of indicator bacteria are present in the water, it is assumed that there is a greater likelihood that pathogens are present. Infection due to pathogen-contaminated recreational waters include gastrointestinal, respiratory, eye, ear, nose, throat, and skin disease (U.S. EPA 1986). Pathogens are a serious concern for managers of water resources, because excessive amounts of fecal bacteria in sewage and urban runoff have been known to indicate an increased risk of pathogen-induced illness in humans (Kay *et al.* 1994, Fleisher *et al.* 1998, Haile *et al.* 1999). Even though recent recommendations have been made for the use of *E. coli* for freshwater monitoring, and enterococci for marine water monitoring, TC and FC are still used in many states (and countries) as an integral part of water quality monitoring (U.S. EPA 1998).

Factors such as pH, temperature, solar (UV) radiation, predation, osmotic stress, nutrient deficiencies, particulate levels, turbidity, oxygen concentrations, and microbial community composition affect bacteria inactivation once they reach receiving waters (Mancini 1978, Davies-Colley *et al.* 1994, Fujioka *et al.* 1981, Berry and Noton 1976, Kapuscinski and Mitchell 1980, Auer and Niehaus 1992, Johnson *et al.* 1997, and Gerba and Bitton 1984). Although many studies have been conducted in the past to examine rates of indicator bacteria inactivation, most have focused upon laboratory-cultured inoculants, frequently with only a single indicator and one environmental factor (Barcina *et al.* 1997). Few of the studies have incorporated the inoculants of greatest concern to managers, such as sewage or urban

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runoff. This may be important since the flora in these inoculants can vary greatly from laboratory-cultured strains, which may lead to very different inactivation rates in natural systems. Even within sewage and urban runoff inoculants, the flora may differ, leading managers to make decisions that under- or over-protect receiving waters for human health concerns. In addition, this study analyzed the rates of loss of infectivity of male-specific coliphage, which are viruses that infect coliform bacteria. It has been postulated that these viruses can be used as models of human viral pathogen decay, because of their persistence characteristics.

A series of factorially designed experiments were performed using sewage influent, sewage effluent, and urban runoff as inoculants to determine inactivation rates of TC, *E. coli* (a subset of the Fc), EC, and male-specific coliphage. The goal of this study was to determine whether rates differ among each of the different inoculants, and to determine which of the environmental parameters were most important to inactivation.

METHODS

General experimental design

Three week-long experiments were conducted in December 1999, April 2000, and August 2000. The experiments were performed with seawater that was collected from either Santa Monica Bay (SMB) near Malibu Beach (34.04N, 118.69W), or with freshwater from Malibu Creek (Malibu Creek State Park, 34.01N, 118.54W). The experiments were factorially designed so as to permit analysis of the data by 2- and 3-way analysis of variance (ANOVA), allowing us to examine the relationship between rates of inactivation and various environmental parameters (Table 1). All experimental designs were conducted in duplicate. All three bacterial indicators were measured for each experiment: TC, *E. coli*, and EC. *E. coli* was used as a surrogate measure for the FC, based upon U.S. EPA recommendations for the use of *E. coli* for water quality determinations in freshwater (U.S. EPA 1986). The first experiment, in December 2000, examined the effects of temperature, nutrients, initial bacterial concentration, and total suspended solids (TSS) in a seawater matrix. The second experiment, in April 2000, examined the effects of temperature, inoculant type, and initial bacterial concentration in a seawater matrix. The third experiment, in August 2000, examined the effects of solar radiation, TSS, and inoculant type in both freshwater and seawater matrices.

Environmental Parameters

Temperature: Two temperatures were used for incubations, which approximated mean winter ocean surface temperatures in the SMB (14°C), and summer ocean surface temperatures in the SMB (20°C).

Inoculant Type and Initial Bacterial Concentration: Inoculant types were either advanced secondary treated sewage effluent from the Hyperion Wastewater Treatment Plant (HWWTP), raw sewage influent from HWWTP, or urban runoff effluent collected from a storm drain during dry weather that empties to the Los Angeles River at 8th

Table 1. Experimental design

Expt. #	Date	Matrix	Environmental Parameters	Number of treatments
1	December 1-7, 1999	Seawater	Nutrient loads (high and Low) Temperature (summer and winter) TSS* (ambient medium, and high) Bacterial concentration (high and low)	24
2	April 1-7, 2000	Seawater	Temperature (summer and winter) Bacterial concentration (high and low) Inoculant type (raw HWWTP** influent, HWWTP effluent, SD*** effluent	12
3	August 15-22, 2000	Seawater, Freshwater	Solar radiation (summer and winter) TSS (high and low) inoculant type (HWWTP raw influent, HWWTP effluent)	8 in seawater matrix 8 in freshwater matrix

*TSS: Total suspended solids
**HWWTP: Hyperion Wastewater Treatment Plant
***SD: Storm drain

Street in downtown Los Angeles, CA, USA. In all three experiments, inoculants were added to approximate two bacterial concentrations, high and low. The high bacterial concentration was chosen to approximate bacterial levels that would be observed during a raw sewage spill, and the low concentration was chosen at slightly above the State of California single-sample recreational water quality thresholds, so that during the course of the experiment, the threshold value would be crossed (Table 2).

Total Suspended Solids: The TSS addition was conducted using a silt/mud mixture taken from a local creek bed (Malibu Creek State Park, CA, USA). Material from a heavily forested and undeveloped site was used to reduce the chance of adding toxic chemicals to samples. The silt/mud was autoclaved, rinsed with di$H_2$0, sonicated, and resuspended in 0.02 µm filtered di$H_2$0. Large particles were screened and removed before addition to the sample bottles. In Experiment 1, three TSS concentrations were used: (1) ambient, the natural TSS load found in coastal seawater, (2) medium, which approximated the level of TSS found in urban runoff effluent during a low-flow period (Jones, personal communication), and (3) high, which approximated the level of TSS found in urban runoff effluent during a high-flow period (Jones, personal communication). In Experiment 3, two TSS levels were used: ambient and high.

Nutrient Levels: Nutrient levels were manipulated in Experiment 1 (nitrate, nitrite, ammonia, silicate, and phosphate) to approximate the ambient concentrations in seawater (low), and nutrient concentrations that would be found in storm drain effluent (high) (Jones, personal communication). Ultra-pure nutrient stocks were added to the seawater samples to reduce the chance of sample contamination.

Solar Radiation: Sample bottles were incubated outdoors under natural sunlight for Experiment 3. Solar radiation levels were measured with a Li-Cor LI-200SA Pyranometer light meter that records solar radiation received from a whole hemisphere. It is suitable for measuring global sun plus sky radiation, or the energy flux density of both direct beam and diffuse sky radiation passing through a horizontal plane of known unit area (Figure 1). Two solar radiation levels were used: high and low. High was ambient summertime light. Agricultural screens were used for creating the low solar radiation incubations. The screens reduced light levels to approximate solar radiation levels in southern California during winter months (ca. 30% of total, Figure 1). Bottles were incubated at the surface of a large shallow pool, so light attenuation due to depth of the incubation chambers was negligible.

Sampling and indicator microorganism analysis

For the three experiments, samples from each of the treatment bottles were collected at 0, 6, 12, 24, 48, 72, and 96 h. Sampling times were dependent upon logistical constraints and were sometimes adjusted slightly based upon rates of inactivation observed for earlier samples. Two-liter polycarbonate bottles were used for the experiments (Nalgene, Inc.). The bottles were triple 5% HCl acid-rinsed before use. At each time point, in duplicate, densities of TC, *E. coli*, and EC were determined using chromogenic substrate tests from IDEXX Laboratories, Inc. (Colilert® and Enterolert®) (APHA 1995). The study also analyzed the decay of infectivity of a male-specific coliphage, MS2 by loss of plaque forming units (PFU). Briefly, the *E. coli* F_{amp} host cells were grown overnight in 10.0 mL of tryptone broth containing 15.0 µg/mL ampicillin-streptomycin at 35.0 C. Three h prior to the start of the assays, 1 mL of host from the overnight culture was inoculated into 99.0 mL of fresh tryptone broth. At each time point, 5 mL of undiluted sample was taken from each sample bottle. One mL dilutions to 10^{-4} were conducted on all samples. In duplicate, 500 mL of each of these samples and 1 mL of host bacterial culture was added to 3.0 mL top agar that had been melted and cooled to 46.0-48.0 C. The top agar, host, and sample mixture was then poured over a plate of tryptone bottom agar (tryptone broth containing 1.5% agar and 15.0 µg/mL ampicillin-streptomycin), and the plates were incubated overnight at 35.0°C. Plates were counted the next day if they had at least 10 plaques.

In Experiment 3, the bottles inoculated with HWWTP sewage effluent did not have sufficient concentrations of indicator bacteria to calculate rates of inactivation. Therefore, we were only able to analyze our results for *E. coli* and EC in the seawater and freshwater samples that were inoculated with raw HWWTP influent.

Statistical Analysis

Linear regression was applied to the log-transformed indicator bacteria measurements in each experiment to derive the inactivation rate coefficient (k_D) in log units per hour.

Table 2. Nominal target values for experimental manipulations.

Expt. #	Manipulation	Concentration	Environmental Approximation
1,2	High temperature	20°C	Mean summer surface water temperature
1,2	Low temperature	14°C	Mean winter surface water temperature
1,3	Ambient TSS	0.0-0.5 mg/L	Ambient TSS typical of coastal seawater
1	Medium TSS	100 mg/L	Level of TSS found in dry-weather flows to beaches[a]
1,3	High TSS	400-500 mg/L	Level of TSS found in wet-weather flows to beaches[b]
1	Low nutrients	NO_3 0.5 uM NO_2 0.4 uM PO_4 0.4 uM SiO_4 0.5 uM NH_4^+ 3 uM	Ambient concentration in coastal seawater
1	High nutrients	NO_3 20.0 uM NO_2 1.0 uM PO_4 3.0 uM SiO_4 1.0 uM NH_4^+ 15.0 uM	Concentrations found in stormwater runoff [c]
1,2,3	Low initial bacterial concentration	TC 2-3 x 10^4 MPN/100 mL *E. coli* 1-2x10^4 MPN/100 mL EC 5-10x10^2 MPN/100 mL	Concentration of indicator bacteria barely above State of California single sample standard
1,2,3	High initial bacterial concentration	TC 1x10^5-1x10^6 E. coli 3x104-1x105 MPN/100 mL EC 1x10^3-1x10^4 MPN/100mL	Concentration of indicator bacteria found in typical sewage spill
2,3	HWWTP raw influent		Expected constituents from sewage spill
2,3	HWWTP effluent		Expected constituents from outfall pipe
2	Storm drain effluent		Expected constituents from surface stormwater runoff
3	High solar radiation		1200 W/m^2 (peak measure) Solar radiation typical of southern California summer day
3	Low solar radiation		300 W/m^2 (peak measure)Solar radiation typical of southern California winter day

[a]From Ballona Creek data reported in SCCWRP annual report.
[b]From Ballona Creek data reported in SCCWRP annual report.
[c]From B. Jones, *pers. comm.*

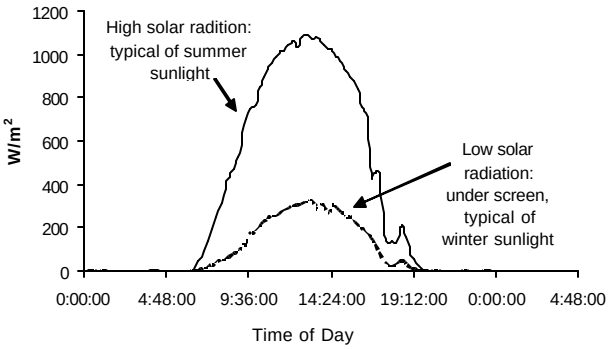

Figure 1. Solar irradiation profiles for southern California on August 20, 2000.

For comparison with other studies, we calculated the time taken to achieve a 90% reduction in the CFU or PFU count (T90 or S90, respectively). The T90 or S90 values were derived from the k_D value as 2.303/ k_D (given that ln (0.1) = -2.303).

RESULTS

Bacteria degraded significantly ($p < 0.0001$) more rapidly at 20°C than at 14°C (Figure 2). This pattern occurred across all three different indicator types. No significant interactions were observed among nutrients, TSS, or initial bacterial concentration and rates of bacterial indicator degradation ($p > 0.05$). Overall rates of degradation were highest for *E. coli*, and lowest for EC, regardless of the temperature at which the samples were incubated (Experiment 1 - Table 3). Inactivation rates of the indicator bacteria were significantly different from one another ($p < 0.0001$).

There were no significant differences ($p > 0.05$) among inactivation rates for indicator bacteria from the sewage influent, treated sewage effluent, and untreated urban runoff effluent (Experiment 2 - Figure 3, Table 4). There was also no significant effect due to the initial bacterial concentration in the samples ($p > 0.05$). Inactivation rates were significantly different among the different bacterial indicators ($p < 0.001$). Overall inactivation rates were highest for TC and lowest for EC. As in Experiment 1, temperature had a significant effect upon inactivation rates, with samples held at 20°C degrading more rapidly than those incubated at 14°C ($p < 0.0001$).

Solar radiation had a significant effect ($p < 0.0001$) upon inactivation rates for all indicators examined, including *E. coli*, EC, and F-specific coliphage (Table 5). Inactivation rates were significantly higher ($p < 0.0001$) when incubated at high-light versus low-light levels (Figure 4). Neither water matrix (freshwater or seawater), nor TSS levels were significant factors for inactivation rates of *E. coli*, EC, or male-specific coliphage. Inactivation rates were significantly different ($p < 0.001$) among the indicators, in decreasing order of EC > *E. coli* >

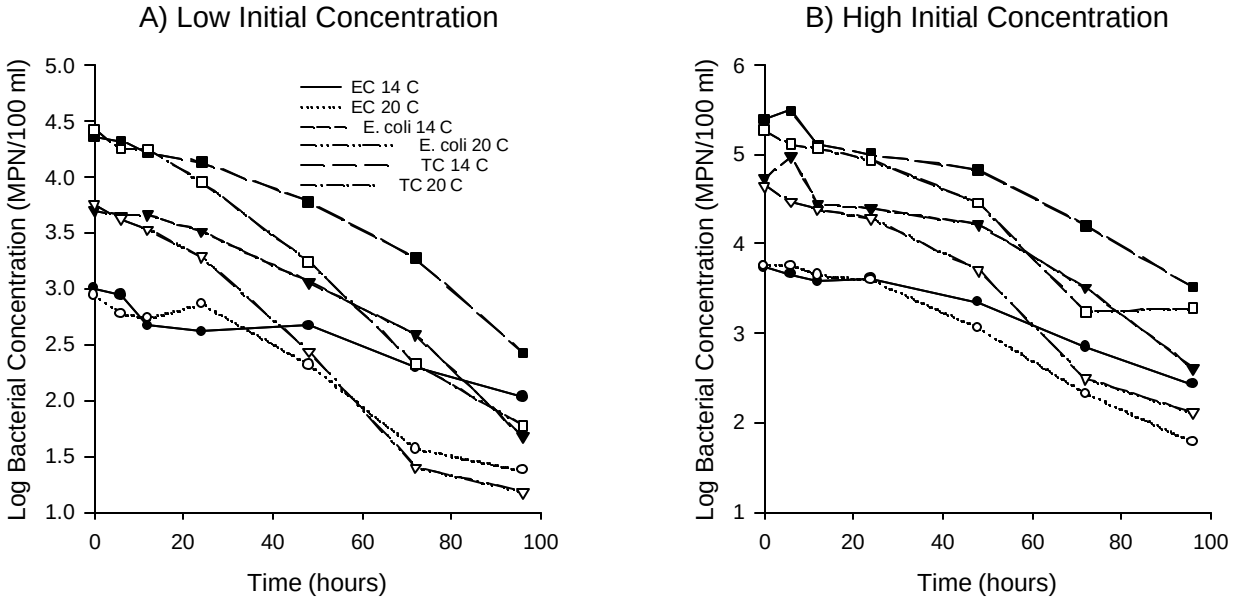

Figure 2. Bacterial concentration in sewage effluent versus time for enterococci (EC, circles), *E. coli* (triangles), and total coliforms (TC, squares) in seawater at 14° and 20°C at initially low (a) and high (b) concentrations.

Table 3. Inactivation coefficients (k_D) and time to reduce indicator density by 90% (T90) during Experiment 1.

Indicator Bacteria	k_D (SE/n) (at 20°C)	T90 (h) (at 20°C)	k_D (SE/n) (at 14°C)	T90 (h) (at 14°C)
Total Coliforms	0.027 (0.0011/12)	85.2	0.019 (0.0009/12)	121.2
E. coli	0.029 (0.0006/12)	79.4	0.021 (0.0010/12)	109.7
Enterococci	0.020 (0.0014/12)	115.1	0.013 (0.0017/12)	177.1

SE: standard error, n: sample size

male-coliphage. The high-solar radiation appeared to cause rapid inactivation, with inactivation rates of EC up to an order of magnitude higher than the inactivation rates observed in Experiments 1 and 2 (Tables 3, 4, and 5). Even under low-solar radiation conditions, inactivation rates of EC were still an order of magnitude higher than the inactivation rates measured in seawater during Experiments 1 and 2. Inactivation of *E. coli* was significantly higher at high levels as compared to low levels of solar radiation ($p < 0.001$). However, under low solar radiation, the *E. coli* samples exhibited only slighter higher inactivation rates than those seen in Experiments 1 and 2. Rates of loss of infectivity for male-specific coliphage were significantly lower than for *E. coli* or EC, under both high-solar and low-solar radiation conditions ($p < 0.001$). Rates of loss of infectivity for male-specific coliphage under high- and low-solar radiation conditions were significantly different from one another (Figure 4, $p < 0.01$). Rates of loss of infectivity for male-specific coliphage were not dependent on TSS levels, and were roughly similar to inactivation rates of EC and *E. coli* seen in Experiments 1 and 2 (Tables 3, 4, and 5).

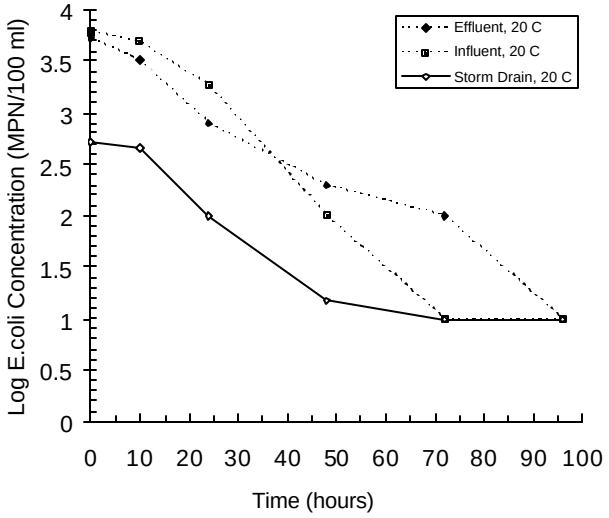

Figure 3. Bacterial concentration in sewage influent, sewage effluent, and urban storm drain runoff versus time for *E. coli* in natural seawater at 20°C.

DISCUSSION

Very limited documented work in the field of indicator bacteria inactivation has been conducted with varying inoculants. One of the few experiments using inoculants other than laboratory cultures was conducted by Sinton *et al.* (2002), who found significantly different inactivation rates for fecal coliforms between treated sewage effluent and raw sewage. In contrast, our results indicated that physical parameters overwhelm the phylogenetic differences among bacterial strains in treated sewage, untreated sewage, or untreated urban runoff. This demonstrates to water quality managers that similar actions to reduce human exposure could be taken, regardless of the source of indicator bacteria. Our experimental evidence is corroborated by the similar length of time beaches remained above regulatory thresholds following sewage spills or urban runoff events between 1995 and 2000 in SMB, California (Schiff *et al.* in press, Leecaster and Weisberg 2001).

The detrimental effect of sunlight on survival of enteric bacteria in aquatic systems has been recognized for decades (Fujioka *et al.* 1981). We found that sunlight increased inactivation rates by at least a factor five compared to experiments not conducted in natural sunlight. Other authors have also demonstrated that sunlight causes dramatic losses in the culturability of cells (Davies and Evison 1991). However, Barcina *et al.* (1990) reported that EC was more resistant to damage by sunlight than *E. coli*, but our results showed greater inactivation rates for EC than for *E. coli* even under low solar radiation levels.

Table 4. Inactivation coefficients (k_D) and time to reduce indicator de4nsity by 90% (T90) during Experiment 2.

Indicator Bacteria	k_D (SE/n) (at 20°C)	T90 (h) (at 20°C)	k_D(SE/n) (at 14°C)	T90 (h) (at 14°C)
Total Coliforms	0.037 (0.0033/6)	62.2	0.025 (0.0021/6)	92.1
E. coli	0.030 (0.0028/6)	76.7	0.021 (0.0030/6)	109.7
Enterococci	0.019 (0.0017/6)	121.2	0.016 (0.0021/6)	143.9

Table 5. Inactivation coefficients (k_D) and time to reduce indicator density by 90% (T90 or S90) during Experiment 3.

Indicator Bacteria	k_d (SE/n) High Solar Radiation	T90 (h) S90 (h)	k_d (SE/n) Low Solar Radiation	T90 (h) S90(h)
Seawater				
Total Coliforms	NA	NA	NA	NA
E. coli	0.1373 (0.0099/8)*	16.8	0.0480 (0.0035/8)*	47.9
Enterococci	0.2572 (0.0074/8)*	8.95	0.2434 (0.0221/8)*	9.46
F+ Specific Coliphage	0.0432 (0.0006/8)**	53.3	0.0256 (0.0002/8)**	89.9
Freshwater				
Total Coliforms	NA	NA	NA	NA
E. coli	0.1346 (0.0092/8)*	17.1	0.0542 (0.0039/8)*	42.5
Enterococci	0.2724 (0.0068/8)*	8.45	0.2434 (0.0133/8)*	9.46
F+ Specific Coliphage	0.0412 (0.0005/8)**	55.8	0.0250 (0.0004/8)**	92.1

*Calculated over 10 hr period
**Calculated over 72 hr period

Part of the difference may be due to the fact that we used IDEXX kits to determine concentrations of indicator bacteria, and therefore examined true loss of metabolic activity in bacterial cells rather than loss of culturability (Kapuscinski and Mitchell 1981). The effect of sunlight is important to note, especially in sub-temperate latitudes such as southern California, where fluctuations in solar radiation need to be considered. For example, we observed differential inactivation rates, particularly for *E. coli*, depending upon summertime or wintertime radiation levels.

Our experiments demonstrated that inactivation rates of indicator bacteria are also dependent upon temperature. Reported rates of inactivation or degradation, and/or cell loss of indicator bacteria have often been contradictory (Barcina *et al.* 1997). For example, we demonstrated a consistent difference between rates of inactivation at 20°C versus 14°C, but others have demonstrated that, in the absence of other microflora (grazers and other bacteria), a direct effect of temperature on survival is not detected (Barcina *et al.* 1991, Korhonen and Martikainen 1991). Other researchers have demonstrated lower survival rates in warmer waters due to increased grazer activity (Barcina *et al.* 1986). In stream waters, Beaudeau *et al.* (2001) demonstrated no

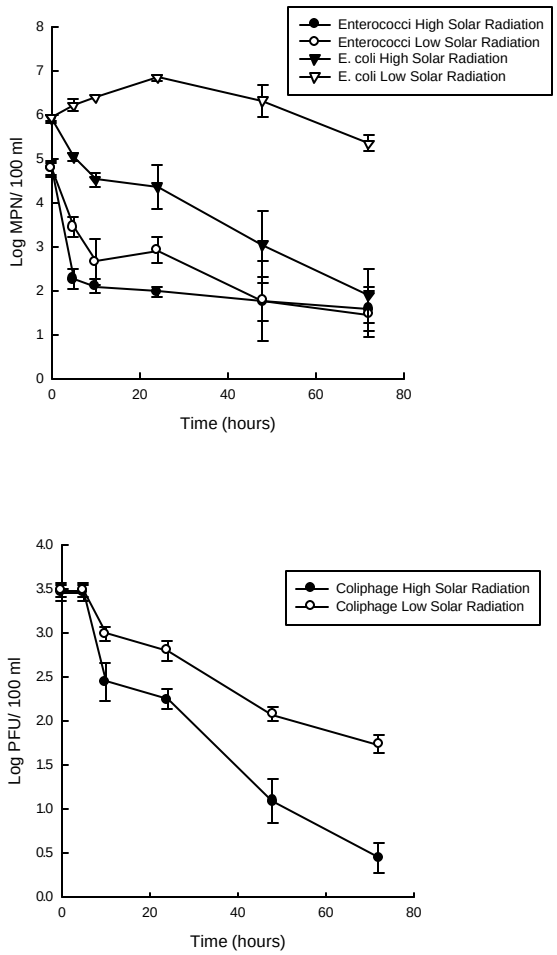

Figure 4. Enterococcus, *E. coli*, and F+ specific coliphage concentrations (MPN: most probable number, PFU: plaque forming units) versus time during experiment 3, exposure to high (summertime) and low (wintertime) solar irradiation.

significant relationship between rate of inactivation of *E. coli* and temperature. Because of the consistency of response among indicator bacteria regardless of exposure manipulation, we feel that temperature is an important mechanism to consider when evaluating inactivation in natural receiving waters.

Environmental parameters, such as TSS and nutrient levels, have been shown to have an effect on survival of heterotrophs and viruses in many aquatic systems (Häder *et al.* 1998, Morita 1992). However, we found no significant interactions of these factors with rates of inactivation in our experiments. Nutrient status has been reported to have strong effects on survival of allochthonous bacteria (Morita 1992). Conversely, it has been demonstrated that EC are capable of metabolically adapting to oligotrophic environments in order to prolong survival (Hartke *et al.* 1998). We originally hypothesized that TSS might present an environment for potential bacterial adsorption, nutrient microzones, and protection from detrimental effects of sunlight. Our results demonstrate, however, that the effects of TSS and nutrients on rates of inactivation were not statistically significant. These two environmental parameters, however, may play a more important role in other regions.

There was no apparent difference between inactivation rates for EC, male-specific coliphage, or *E. coli* in freshwater or seawater matrices during this experiment. This may not be surprising since EC are gram-positive, catalase negative bacteria that are able to grow in solutions at 6.5% NaCl, and 40% bile salts (Schleifer and Kilpper-Balz 1987). In addition, the seawater used from southern California is meso-oligotrophic, meaning that enzymatic degradation, secondary production, and grazing occur at lower rates than in eutrophic waters (Noble and Fuhrman 1999), causing lower rates of inactivation or removal of allochthonous bacteria. Once again, few investigators have examined the effect of varying receiving waters on bacterial inactivation. One such study by Gabutti *et al.* (2000) demonstrated different inactivation rates between saline and brackish waters; but due to a lag in inactivation in incubations from 0 to 48 h, the data are difficult to interpret in relation to our results.

Although inactivation rates for all three indicator bacteria (TC, EC, and *E. coli*) were within ranges previously reported by others, the reported ranges are quite large and appear to be system-specific. For example, published k_D values for *E. coli* in freshwater typically range from 0.03 to 0.06 h^{-1} (e.g., Barcina *et al.* 1986, Auer and Niehaus 1992, Menon 1993, and Mezrioui *et al.* 1995). However, other reports with k_D as low as 0.001 h^{-1} and as high as 0.29 h^{-1} have been reported by Davies and Evison (1991), and Sinton *et al.* (2002), respectively. Our results indicate longer persistence of EC in areas not influenced by sunlight (ca. 0.02 h^{-1}), but shorter persistence when sunlight is present (0.25 h^{-1}). While we attempted to make our experiment less system-specific by varying the exposure media (fresh and saltwater) and inoculant type (sewage influent, sewage effluent, urban runoff), the results generated herein are likely applicable to conditions seen in coastal and/or arid southwest regions of the United States.

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