Solar inactivation of four *Salmonella* serovars in fresh and marine waters

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ABSTRACT

Sunlight-mediated disinfection of water is of interest to both the drinking and recreational water quality community of researchers due to its potential to reduce microbial contamination and waterborne illness. Photo-inactivation of enteric bacteria has primarily been investigated using *Escherichia coli* and laboratory strains of model bacteria. The present study sought to document the photo-inactivation of environmental isolates of *Salmonella* in filter-sterilized natural seawater and freshwater and to test the hypothesis that diverse *Salmonella* serovars decay at similar rates both within and between water matrices. The inactivation of *Salmonella enterica* Typhimurium LT2, Typhimurium ST19, Heidelberg, and Mbandaka was examined in sunlit and dark microcosms. First order decay was observed in sunlit microcosms; the time until 90% inactivation was of the order of 10 min. A significant shoulder, of the order of 1 hr in length, was observed in the freshwater microcosms during which concentrations were stable. Serovar Mdandaka decayed more slowly than other serovars in both seawater and freshwater. The serovars were extremely stable in the dark microcosms showing little to no decay over 53 days. The results document intra-species variation in photo-inactivation, likely owing to differences in intracellular concentrations of photo-sensitizing molecules or molecules that quench reactive species.

Key words | disinfection, enteric pathogens, microbial pollution, photo-inactivation, *Salmonella*, sunlight

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INTRODUCTION

Photo-inactivation of enteric bacteria in natural waters has been well established using both field and laboratory studies (Boehm *et al.* 2009; Fisher *et al.* 2012). Bacterial photo-inactivation can result from direct and indirect damage caused by exposure to sunlight. Direct damage occurs when energetic photons are absorbed directly by cellular machinery or molecules. The most common example is UVB damage to DNA resulting in pyrimidine dimers that prevent DNA replication (Malloy *et al.* 1997; Ravanat *et al.* 2001; Sinha & Hader 2002). Indirect damage occurs when photons are absorbed by endogenous (intracellular) sensitizers such as porphyrins and chlorophylls or exogenous (extracellular) sensitizers such as humic compounds (Curtis *et al.* 1992). Excited sensitizers transfer energy or charge to other parts of the cell, damaging cellular components, or to molecular oxygen, producing reactive oxygen species that cause photo-oxidative damage (Maisch *et al.* 2007). Bosshard *et al.* (2009) and Berney *et al.* (2006) show through studies with *Escherichia coli*, *Salmonella*, and *Shigella*, that destruction of the respiratory chain appears to be the main cause of inactivation upon exposure to light in the UVA range.

The majority of work on sunlight inactivation of bacteria has been conducted with indicator organisms like *E. coli* or, to a lesser degree, enterococci. There are limited studies on sunlight inactivation of bacterial pathogens like *Campylobacter*, *Vibrio cholerae*, *Shigella*, and *Salmonella* (Kramer & Ames 1987; Berney *et al.* 2006; Sinton *et al.* 2007; Bosshard *et al.* 2009). Because indicator organisms are used to assess public health risk, there is a need to better understand how their inactivation in sunlight compares to the inactivation of pathogens.

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In most sunlight inactivation studies, a specific bacterial species or serovar is chosen as a representative for the genus or species, respectively, under study. There may be important differences in the photo-inactivation of different organisms in the same genus or species due to the presence of different photosensitizing molecules, stress responses, or the presence of pigments or other quenching molecules within the cell. For example, Maraccini *et al.* (2012) showed that *Enterococcus casseliflavus* decayed more slowly than *Enterococcus faecalis* likely owing to the former possessing a carotenoid pigment.

The present study investigated the photo-inactivation of four *Salmonella* serovars: one laboratory strain *Salmonella enterica* Typhimurium LT2, and three serovars isolated from natural waters in central California – *Salmonella enterica* Mbandaka, *Salmonella enterica* Heidelberg, and *S. enterica* Typhimurium sequence type (ST) 19. Experiments were conducted in filter-sterilized fresh and marine waters. The goals were to test whether photo-inactivation rates are serovar-specific and whether inactivation rates are different in marine versus freshwaters.

The work conducted herein was motivated in part by our previous study that investigated the distribution of *Salmonella* in natural waters of central California (Walters *et al.* 2011). We found *Salmonella* concentrations were higher in freshwater compared to estuarine or marine waters. We isolated a number of different serovars in the waters we studied. In particular, we found one serovar (*S. enterica* Typhimurium ST19) to be the most commonly isolated serovar. Thus, the experiments conducted herein explored the role of environmental persistence in explaining the field observations. In particular, we tested whether the *S. enterica* Typhimurium ST19 is more persistent in environmental waters than other serovars, and whether the four serovars are more persistent in fresh relative to marine waters.

MATERIALS AND METHODS

Seawater and freshwater matrices

Seawater was collected at Pacifica Beach, CA (salinity of 34.0 ppt) and freshwater collected at San Pedro Creek located in Pacifica, CA (salinity of 0.2 ppt) in 10% HCl-washed containers. Waters were transported to the laboratory and immediately filtered using a 0.1 μm pore size filtration system (Millipore Express PLUS, Billerica, MA) to remove predators and other bacteria. The filtered waters were stored at 4 °C and used for all experiments.

Salmonella serovars

Inactivation of four serovars was evaluated in this study. The laboratory strain *S. enterica* Typhimurium LT2 was used. Hereafter, this strain will be referred to as LT2. Also three environmental isolates were used: *S. enterica* Typhimurium subsp. 1 (ST19), *S. enterica* Mbandaka subsp. 1 (ST413), and *S. enterica* Heidelberg subsp. 1 (ST15). Hereafter, these isolates will be referred to as ST19, Mbandaka, and Heidelberg. ST19 and Mbandaka were isolated from Lagunitas Creek, and Heidelberg was isolated from Salinas River (Walters *et al.* 2011). Isolates were sequence-typed as described elsewhere (Walters *et al.* 2010). All serovars used in this study are pathogens and capable of causing human disease, except for LT2 which is an attenuated strain.

Salmonella cultivation and preparation of working solution

A loop of pure culture (frozen at −20 °C) was inoculated into 20 mL of tryptic soy broth (TSB) and incubated at 37 °C on a rotary shaker at 200 rpm. When the culture reached exponential phase, the culture was transferred to 30 mL of fresh TSB and incubated in the same manner for 9–12 hr until stationary phase was reached. The stationary phase was confirmed from five consecutive OD_{546} measurements in 2 hr.

Between ~100 and ~40 μL of stationary phase *Salmonella* culture was transferred to 25 mL (light experiments) or 10 mL (dark experiments), respectively, of filtered seawater or freshwater matrix to produce a concentration of about 10^7 colony forming units (CFU)/mL. This working solution was stirred at 15 °C for 20 min prior to beginning the experiments.

Light experiment

The photo-inactivation of each of the four serovars was tested in fresh and marine water in three or four replicate

experiments. For each experiment, 25 mL working solution was placed into a sterile 50 mL beaker wrapped with black tape. The beaker was placed in a recirculating water bath to maintain its temperature at 15 °C in a solar simulator (Altas Suntest CPS, Linsengericht-Altenhaßlau, Germany). This solar simulator is 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 (Maraccini *et al.* 2012). The solution was irradiated at 400 W/m^2 with continuous stirring. For a 2–5 hr period, 0.1 mL samples were withdrawn from the beaker every 15–30 min for *Salmonella* enumeration. No more than 1.5 mL was removed from the beaker over the course of the experiment.

Dark experiment

The dark inactivation of each serovar was tested in both fresh and marine water in a single experiment. Ten milliliters of working solution was placed in a sterile 20 mL culture tube and wrapped with aluminum foil. The tube was incubated in a 15 °C constant temperature room on a rotary shaker at 150 rpm. For a period of 53 days, 0.1 mL samples were withdrawn from the tube every 3–4 days for *Salmonella* enumeration. The culture tube was capped to prevent evaporation and uncapped every 2 days to allow the exchange of air. Approximately 1.5 mL was removed from the culture tube over the course of the experiment.

Salmonella enumeration

Salmonella was enumerated in each water sample by spread plating three consecutive decimal dilutions in triplicate on XLD agar (Xylose Lysine Deoxycholate Agar, EMD, Germany) that contained 20 mg/L of novobiocin (EMD, Germany). Plates were then incubated at 37 °C for 18–24 hr before enumeration. Plates with between 1 and 1,557 CFU were retained, and the corresponding concentration was calculated. The concentrations from all replicate plates for a water sample were averaged to obtain the concentration. Although the CFU counts for the three dilutions typically varied by orders of magnitude, the concentrations calculated were always quite consistent, so we felt it was appropriate to include all replicates in the calculation of a sample's concentration, even if they came from different dilutions.

Data analysis

Concentrations were normalized by the concentrations measured at time 0 for each experiment. Data from replicate experiments were combined. Photo-inactivation was modeled as first order with respect to time. A first order inactivation rate constant, k, was derived using a linear-least squares curve fit between natural logarithm (ln)-transformed normalized concentrations and time. k was set equal to the slope of the line; 95% confidence intervals were calculated for the slope, and an R^2 value for the curve fit was determined. If the decay data displayed a visible shoulder region, then data on the shoulder were not included in the curve fit or the calculation of the slope (Sinton *et al.* 1994). To test the hypothesis that the inactivation rates were different between serovars within the same matrix or between the same serovar in different matrices, the technique previously described by Walters *et al.* (2009) was used. In brief, concentration data from two conditions were concatenated and modeled as a function of time, with a dummy variable indicating each condition, and an interaction term between time and the dummy variable. If the interaction term was statistically significant ($p < 0.05$) in the model, then the difference between the slopes, and thus inactivation rates, for the two conditions was deemed statistically significant. k_s values with units per time obtained for light experiments were transformed to K_s values with units of m^2/MJ using dimensional analysis and taking into account the intensity of the solar simulator light source, which was set to 400 W/m^2.

RESULTS

In seawater, all four serovars declined exponentially when exposed to light while in the dark, the concentrations remained approximately constant over the same time period (Figure 1). The first order photo-inactivation rates (±95% confidence interval) were 5.3 ± 0.6, 3.9 ± 0.5, 5.7 ± 0.7, and 5.8 ± 0.4 hr^{-1} for LT2, Mbandaka, Heidelberg, and ST19, respectively (Table 1). Despite some scatter between experimental replicates, the R^2 values for the

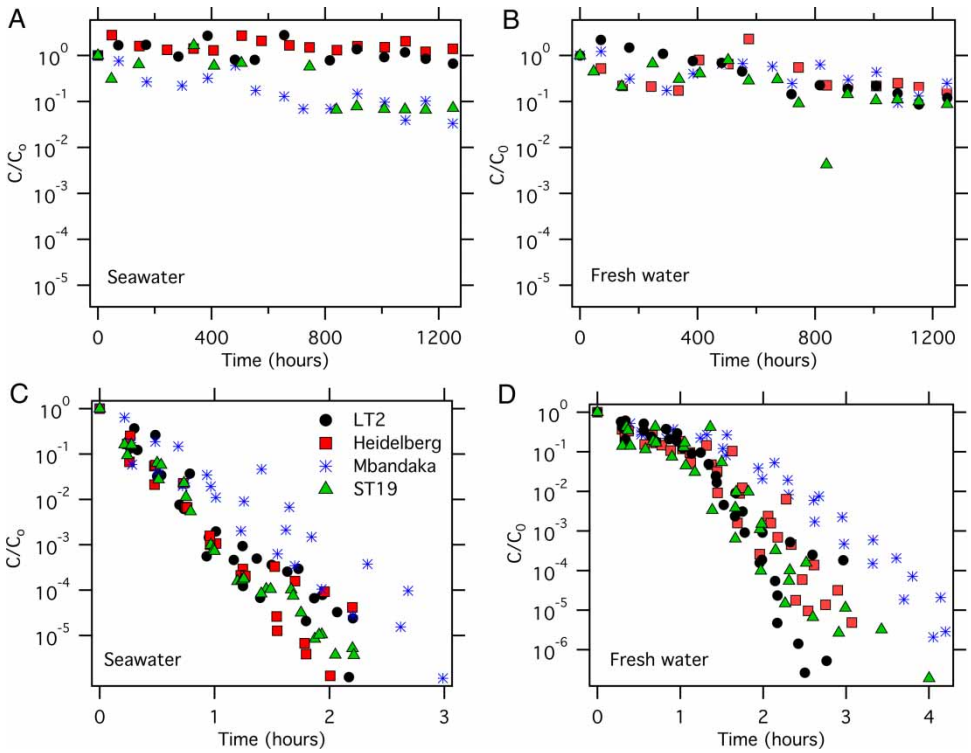

Figure 1 | Concentrations of the four serovars in dark (panels A and B) and light microcosms (C and D) carried out in seawater (A and C) and freshwater (B and D).

Table 1 | Dark (k_d) and light (k_s) inactivation rates with units per time, light inactivation rates in units per fluence (K_s), and associated times until 90% inactivation (T_{90}) and energy until 90% inactivation (F_{90}). 'na' indicates that decay was not significantly different from 0 or was not described by first order decay. T_{90d} and T_{90s} are for dark and sunlit experiments, respectively. Note that T_{90s} do not consider shouldering

Organism	Water type	k_d (hr^{-1})	T_{90d} (hr)	k_s (hr^{-1})	T_{90s} (hr)	K_s (m^2/MJ)	F_{90} (MJ/m^2)
LT2	Fresh	0.002	1150	6.2	0.37	4.3	0.53
	Marine	na	na	5.3	0.43	3.6	0.64
Mbandaka	Fresh	0.001	2300	3.9	0.59	2.7	0.85
	Marine	0.002	1150	3.9	0.59	2.7	0.85
Heidelberg	Fresh	na	na	5.6	0.41	3.9	0.59
	Marine	na	na	5.7	0.40	4.0	0.58
ST19	Fresh	0.002	1150	4.7	0.49	3.3	0.70
	Marine	0.002	1150	5.8	0.40	4.0	0.58

linear curve fits between the natural logarithm-transformed, normalized concentrations and time were greater than 0.90 for all four serovars. Pair-wise comparisons between the photo-inactivation rate constants using the multiple regression method indicated that serovar Mbandaka decayed significantly more slowly than the other three serovars ($p < 0.05$).

In the seawater dark experiments which lasted 53 days, the *Salmonella* serovars did not decay or decayed very slowly (Table 1). The decay rates of serovars Heidelberg and LT2 were not statistically different from 0 ($p > 0.05$). The first order decay rate of ST19 was $0.0025 \pm 0.0009\ hr^{-1}$ ($R^2 = 0.42$), and the first order decay rate of Mbandaka was $0.0022 \pm 0.0003\ hr^{-1}$ ($R^2 = 0.76$). These

rates are several orders of magnitude slower than the rates observed in the light experiments.

In freshwater, the serovars exhibited photo-inactivation in the light experiments and no to limited decay in the dark experiments. In contrast to the seawater light experiments, the decline in concentrations in the freshwater light experiments exhibited a shouldering effect for all four serovars. The shoulder period lasted approximately 1–1.5 hr. Thereafter, first order decay was observed. The decay rates during the period following the shoulder were 6.2 ± 1.5, 3.9 ± 0.5, 5.6 ± 1.2, and $4.7 \pm 0.9\ \mathrm{hr}^{-1}$ for LT2, Mbandaka, Heidelberg, and ST19, respectively (Table 1). The curve fits were slightly weaker for the freshwater experiments compared to the seawater experiments with R^2 values ranging from 0.76 (LT2) to 0.94 (Mbandaka). All curve fits were statistically significant ($p < 0.05$). The inactivation rates were compared pair-wise. Serovar Mbandaka decayed significantly slower than LT2 and Heidelberg ($p < 0.05$); it also decayed slower than ST19 at the alpha = 0.1 level ($p = 0.08$). Serovar LT2 decayed more quickly than ST19 at the alpha = 0.1 level ($p = 0.09$).

In the freshwater dark experiments, the serovar concentrations decreased minimally over the extended incubations. The dark decay rates were 0.002 ± 0.0002, 0.001 ± 0.0004, 0.0007 ± 0.0006, and $0.002 \pm 0.0007\ \mathrm{hr}^{-1}$ for LT2, Mbandaka, Heidelberg, and ST19, respectively (Table 1). The R^2 value for the linear curve fit for Heidelberg was not statistically significant ($R^2 = 0.08$, $p > 0.05$); however, the fits for the decay of the remaining serovars were statistically significant ($R^2 = 0.35$–0.89, $p < 0.05$).

The photo-inactivation rates of each serovar in seawater and freshwater were compared to determine if they were significantly different. When examining the derived first order rates only (and not considering the shouldering region), there were no differences in decay of LT2, Heidelberg, or Mbandaka in marine versus freshwater. However, ST19 decayed more quickly in seawater relative to freshwater ($p < 0.05$). Clearly, a large difference is the presence of the shoulders in freshwater and their absence in the seawater experiments.

The dark inactivation of each serovar in marine and freshwaters was compared. In marine water only ST19 and Mbandaka showed decline while in freshwater ST19, Mbandaka, and L2T showed decline. The decline of Mbandaka in freshwater was slower than its decline in seawater ($p < 0.05$). The decline of ST19 did not differ between fresh and marine water. Heidelberg did not decline in either water. LT2 declined in freshwater but not in marine water.

DISCUSSION

All four *Salmonella* serovars showed sensitivity to sunlight. The decay rates observed here (of the order of $5\ \mathrm{hr}^{-1}$ or $3.5\ \mathrm{m}^2/\mathrm{MJ}$ on a per fluence basis) are the same order of magnitude as those observed by Sinton *et al.* (2007) for *Salmonella enterica* Brandenburg environmental isolates exposed to natural sunlight; they reported decay rates of 5 and $10\ \mathrm{m}^2/\mathrm{MJ}$ for seawater and river water, respectively. Our photo-decay rates are an order of magnitude higher than that reported by Berney *et al.* (2006) for *S. enterica* Typhimurium ATCC 14028 suspended in drinking water; their $K = 0.7\ \mathrm{m}^2/\mathrm{MJ}$.

The mechanisms whereby *S. enterica* Typhimurium is inactivated by near UV light were investigated by Kramer & Ames (1987) who asserted that production of reactive oxygen species within the cell by photon-absorbing proteins is the cause of oxidative stress and death. They found that destruction of membranes as well as destruction of UV-absorbing macromolecules contributed to inactivation. The importance of photo-oxidative damage in *S. enterica* Typhimurium was further confirmed by Bosshard *et al.* (2009) who showed that the respiratory chain was damaged by sunlight leading to cell death. Only one study examined *Salmonella* photo-inactivation in a field setting (Sassoubre *et al.* 2011); *Salmonella* photo-inactivation was not observed potentially due to the low concentrations present during the study.

The photo-inactivation rates of the four serovars in fresh and marine waters were compared and Mbandaka consistently decayed more slowly than the other serovars. This indicates that intra-species differences in photo-decay are possible, as has been shown by Maraccini *et al.* (2012) for *Enterococcus*. It is possible that Mbandaka lacks a photosensitizing molecule that the other serovars possess, or that it contains a molecule, like a carotenoid, that quenches reactive oxygen species within the cell. There is a report of a *Salmonella* isolate containing a yellow pigment (Deskowitz &

Buchbinder 1935). Mbandaka was visually examined for the presence of the pigment (Facklam & Collins 1989) and found not to contain one.

The main difference in the photo-decay of the serovars in fresh versus marine waters is the shouldering observed in freshwater. Shouldering observed during the photo-decay of bacteria in microcosms has previously been observed and explained to be a result of 'multiple hits' being required in order for cells to become inactivated, or effective intracellular repair processes during the initial period of stress. Significant shouldering was observed in a previous study of *Salmonella* inactivation (Kramer & Ames 1987), but not in others (Berney *et al.* 2006; Sinton *et al.* 2007). Thus, shouldering might be affected by the experimental design (e.g., sampling regimes, light intensity, light source, preparation of bacterial seed, liquid matrix). In the present study, multiple hits might be required if the cells in the freshwater microcosms aggregated when they were added to the water. However, the initial number of CFU in the freshwater microcosm would have been lower than expected if cells had aggregated; this was not observed. It is possible that cells in both the marine and freshwater are injured to a similar extent by the light, but cells in the seawater were unable to withstand the stress imparted by high salinity water. In a previous study (Kramer & Ames 1987), *Salmonella* exposed to near UVA light lost culturability more quickly when exposed to increasingly saline waters, potentially a result of photo-oxidative membrane damage. Because the seawater and freshwater used in this study were natural waters, it is possible they contained different dissolved molecules that affected the mechanisms of inactivation. The absorbance of both waters was low (0.01 and 0.03 per cm at 300 nm for seawater and freshwater, respectively) but slightly higher for freshwater which could indicate more exogenous sensitizers.

The decay rate of *Salmonella* in the light microcosms was not different between the seawater and freshwater experiments for all serovars with the exception of ST19 which decayed more quickly in seawater compared to freshwater, although the difference in decay rate was not large. ST19 was the most common serovar isolated by Walters *et al.* (2011) in central California natural waters; they also found *Salmonella* was more likely to be detected in freshwater compared to seawater. The increased persistence of ST19 in freshwater could help explain the tendency to detect *Salmonella* in freshwater.

Salmonella showed remarkable persistence in the dark microcosms with decay rates on the order of 0.001 hr^{-1}. There was very little decay of all serovars over the 53 days of incubation in both seawater and freshwater. These results indicate that in the absence of light and predators, the bacteria may remain cultivatable for some time. Sinton *et al.* (2007) also examined the dark inactivation of *S. enterica* Brandenburg in seawater and river water and found quicker decay (~0.01 hr^{-1}), however, their experimental waters were not filtered prior to study so losses due to predation were possible.

We did not investigate dark repair of photo-inactivated *Salmonella*, although this has been examined by others. Whereas one set of researchers (Bosshard *et al.* 2009) found that *S. enterica* Typhimurium was not able to repair photo-damage caused by sunlight or UVA, another group (Sciacca *et al.* 2010) found that wild *Salmonella* sp. naturally present in drinking water exposed to sunlight were capable of dark regrowth with no additional nutrients added to the drinking water.

CONCLUSION

The photo-inactivation of four *S. enterica* serovars was investigated in marine and freshwaters. One serovar, Mbandaka, decayed more slowly than the others in both matrices pointing to intra-species differences in photo-decay. Photo-decay rates were generally the same for each serovar in marine and freshwater, but decay in freshwater was delayed by approximately an hour due to shouldering. ST19 decayed more slowly in freshwater compared to marine water in light microcosms, helping to explain field observations of Walters *et al.* (2011). *Salmonella* were extremely stable in the dark.

This study points to several subjects for future investigation. First, it would be interesting to determine the reason why Mbandaka decays more slowly that the other serovars. The presence or absence of intracellular quenching or photosensitizing molecules relative to the other serovars could be explored. Second, the reason for the shouldering in freshwater could be investigated by exploring whether

stress response genes are differentially upregulated in *Salmonella* in freshwater versus seawater, or whether sunlight-induced damage renders *Salmonella* more sensitive to high saline waters relative to low salinity waters.

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