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Effects of Exogenous Reactive Oxygen Species Scavengers on the Survival of *Escherichia coli* B23 during Exposure to UV-A radiation.

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Solar disinfection of water is a well-studied technique to cheaply and easily sterilize microbe-contaminated water through a combination of heat and UV-induced reactive oxygen species (ROS). Understanding the role of exogenous environmental compounds on the efficacy of the protocol as well as the optimization of an accurate bacterial enumeration strategy is integral to maximize the efficacy of this technique. A time course assay of *E. coli* B23 viability during UV-A irradiation was conducted, and the effects of L-histidine, formate, catalase and superoxide dismutase as scavengers of ROS were tested. Stepwise declines in bacterial cell numbers were observed in all UV-irradiated samples, and minimal effects on the decline were observed with the addition of formate or L-histidine. Catalase and superoxide dismutase appeared to decrease bacterial survival. Inability to effectively access internal cellular photosensitizers might have contributed to the inconclusive results with regards to formate and L-histidine. Changes in pH due to addition of excess enzyme may have played a role in the increase in bacterial cell death with catalase treatment. Exogenous compounds seem to play a role in the bactericidal efficacy of the SODIS protocol, however the results included here are only preliminary observations. Further studies of confounding variables such as pH, concentration, and temperature are required to fully characterize the impact of ROS quenching.

The minimal availability of clean drinking water in many developing countries is an important global health concern, as bacterial waterborne disease is thought to cause more than two million fatalities every year according to the World Health Organization website (http://www.who.int/water_sanitation_health/diseases/diarrhoea/en/). Efficient and inexpensive methods of sterilizing contaminated drinking water are greatly needed, and in recent years solar disinfection, or SODIS, has become a widely recognized protocol.

The main microbicidal components of SODIS are believed to be a combination of temperature and UV-A light. It has been theorized that although UV-B and UV-C components of sunlight have characteristic microbicidal activity based on direct DNA damage, UV-A works in a more indirect fashion (6). This action is mainly attributed to the production of reactive oxygen species (ROS) and the subsequent oxidative damage, which accounts for approximately 70% of the bactericidal effect of sunlight (5). However, previous studies may have overestimated the efficacy of UV-A irradiation alone based on inadequate growth conditions (5). It has been shown that aerobic growth conditions are sufficient for the growth of healthy, undamaged cells, whereas anaerobic growth conditions are required to resuscitate sublethally damaged cells (1). In SODIS, where cells may be damaged by UV radiation but not killed outright, it is essential to optimize growth conditions for an accurate enumeration process.

This study aimed to determine whether a direct correlation could be seen between bacterial viability and levels of ROS in the water, and whether specific ROS quenchers (5, 7) would increase bacterial survival during UV-A irradiation. Based on relative stability and the availability of an appropriate fluorescent assay (8), H_2O_2 was selected as an overall quantitative indicator of ROS. Both an anaerobic growth environment and the use of the oxygen scavenger sodium pyruvate in the growth medium were applied in this study in an effort to improve the enumeration accuracy by minimizing any further oxidative damage due to aerobic respiration.

MATERIALS AND METHODS

Preparation of *E. coli* culture: 20 mL of nutrient broth (5g/L peptone; 3g/L beef extract) were inoculated with *E. coli* B23 from the MICB 421 Culture Collection in the Microbiology and Immunology Department at the University of British Columbia, Vancouver BC. After incubating on a shaker for approximately 18 hours at 37 ^{o}C and 250 rpm, the culture was centrifuged for 10 minutes at 5,345 x g in a Biofuge 17R high-speed centrifuge. Two washes were conducted in which the pellet was resuspended in 2

mL of de-ionized water and microfuged at 16,000 x g for 5 minutes. A Spectronic 20 spectrophotometer was used to measure turbidity at a wavelength of 460 nm and a cell concentration assessed using a conversion factor of $O.D._{460}$ = 1.000 at $1x10^9$ cells/mL.

Inoculation of PET bottles: All PET bottles (500mL Nestle Natural Mineral Spring Water bottles emptied with wrapping removed) were filled with 400 mL of de-ionized water and inoculated with approximately $5x10^7$ cells. Bottles were shaken vigorously for 30 seconds to evenly distribute bacteria, and were subsequently topped up with de-ionized water to 500 mL.

Effect of UV-A exposure on bacterial survival: A 180 minute time-course viability assay was conducted exposing the PET bottles to UV-A radiation under a 20 W Sylvania Blacklight suspended 10 cm above the bottles. A control non UV-A treated bottle was wrapped in tinfoil. At 30 minute intervals, bottles were removed from the UV-A source, shaken vigorously for 30 seconds, and 100 uL aliquots were removed. Final plated dilutions of 10^{-2} and 10^{-3} were plated in duplicate on nutrient agar supplemented with 0.01% sodium pyruvate. All plates were incubated at 37 ^oC for 48 hours in anaerobic jars that had been vacuum purged and reconstituted with a CO_2/N_2 atmosphere.

Effect of exogenous quenching compounds on bacterial survival: 25 mL of stock solutions of L-Histidine (5 M), formate (3 M), as well as catalase (200 units/mL) from the UBC Department of Microbiology and Immunology MICB 421 laboratory stock and superoxide dismutase (Sigma-Aldrich; catalogue no. S7571-15KU) at 2 units/mL were added to separate 500 mL PET bottles, and the time course viability assay was performed as indicated above.

Fluorometric Assay for H_2O_2 Concentration: A pentafluorobenzenesulfonyl fluorescein (Cayman Chemicals; catalogue no. 10005983) was used as a fluorescent probe for detection of H_2O_2 as previously described (8). A 10 mM stock solution was prepared by dissolving 1 mg probe in dimethyl sulfoxide and stored at 4 ^oC. Daily working solutions were prepared by diluting 1:10 in HEPES buffer and adding 40 units of esterase (Sigma-Aldrich; catalogue no. E3019-3.5KU) per $2x10^{-5}$ mmol probe, as recommended by Maeda *et al* (8). The probe and esterase were allowed to react for 10 minutes, then added to water and varying concentrations of H_2O_2 to a final volume of 3 mL and working concentration of 25 uM. Samples were read in the Turner Quantech digital filter fluorometer with a 490 nm narrow band excitation filter and a 515 nm sharp cut emission filter.

RESULTS

Exposure to UV-A light caused a significant reduction in viable *E. coli* B23 cells in a time-dependent manner. The UV treated sample exhibited was approximately 70% following three hours of UV-A exposure. The control sample showed steady bacterial counts for the first hour, a steady increase in cell density for the second hour, culminating with an approximate 15% increase at the 120-minute mark, and a decline slightly below the starting concentration at 180 minutes (FIG. 1). a stepwise decline over the three-hour irradiation period, with each hour

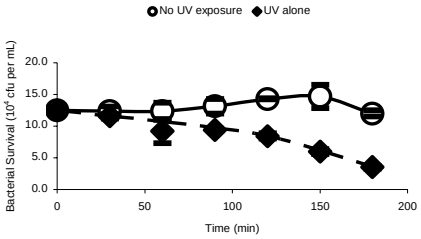

FIG. 1. Effect of UV-A exposure on *E. coli* B23 survival. The dotted line refers to data that has been normalized to the same starting concentration of *E. coli* B23 cells as the solid line. Each point represents the average of duplicate plate counts, of which are represented by error bars.

increment demarcating a change of slope and therefore a different death rate (FIG. 1). The total observed reduction of viable *E. coli* B23 cells.

***E. coli* B23 survival was not significantly affected by the addition of formate or L-histidine.** The treated samples did not appear to have the same stepwise decline as the untreated samples, but instead exhibited a period of steady decline but cell viability stabilized at 80 minutes for formate treated samples and 120 minutes for L-histidine treated samples (FIG. 2A and 2B).

Superoxide dismutase and catalase have similar effects as UV-A exposure alone on *E. coli B23* survival. The treated samples (FIG. 2C and 2D) followed a similar stepwise decrease in cell viability to the untreated samples however, there was more killing in the treated samples than the untreated samples with the catalase treated samples (FIG. 2C) initially undergoing most rapid decline in viability.

H_2O_2 fluorescence assay insufficiently quantitative for ROS measurement in samples: Unfortunately, we were unable to successfully generate a standard curve using the pentafluorobenzenesulfonyl fluorescein designed by Maeda *et. al* (8) (Data Not Shown) to measure hydrogen peroxide levels in the sample. The probe did react with H_2O_2, but was also very sensitive to the amount of time, the temperature and the concentration at which it reacted with the esterase and peroxide. Fluorescence of the probe alone increased with time after incubation with esterase with the upper limit surpassing the readable range of the fluorometer. Because of this, problems with the fluorometer, and time constraints we were unable to devise a procedure accurate enough for our purpose.

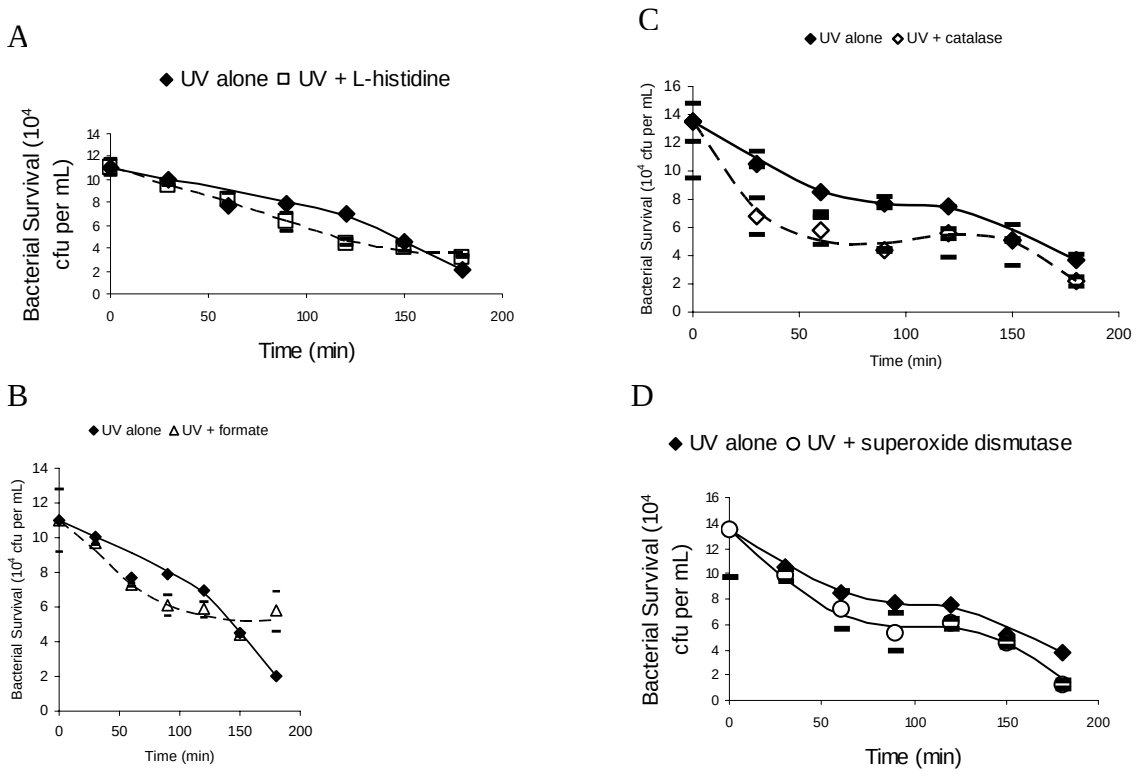

FIG. 2. Effect of ROS scavengers **(A)** L-histidine, **(B)** formate, **(C)** catalase **(D)** SOD, on survival of UV-A exposed *E. coli* B23. The dotted line refers to data that has been normalized to the same starting concentration of *E. coli* B23 cells as the solid line. Each point represents the average of duplicate plate counts, of which are represented by error bars.

DISCUSSION

Although the SODIS protocol according to the Swiss Federal Institute of Aquatic Science and Technology (http://www.sodis.ch/files/notes.pdf) was not followed exactly, there was a 70% reduction in cell viability observed for UV-A exposed *E. coli* by three hours (FIG.1). A more significant decrease would likely have been observed had the PET bottles achieved the recommended temperature of 55 ^{o}C (9). The dark control sample did not have the gradual decrease in bacterial cell numbers noted by Burgess *et al.* (2). The increase in cell counts of the dark control after approximately 120-150 minutes might have been due to some cells still slowly undergoing replication or simply experimental error as the shift is only approximately 15%. Also, some sampling error could be expected due to difficulty in thoroughly shaking and mixing each of the PET bottles as they were nearly full of water. Because of the relative consistency of the dark treated control, we expect that the osmotic shock was not a significant factor in our results.

The anaerobic growth conditions and the 0.01% sodium pyruvate supplement added to the nutrient agar were selected to achieve maximal recovery of cells that were sub-lethally damaged by the UV-A exposure. Anaerobic conditions and sodium pyruvate are believed to prevent any further oxidative damage due to ROS formed during aerobic respiration (5).

These growth conditions were considered more representative for the anaerobic growth of *E. coli* in the human gastrointestinal environment. These conditions may also have increased the accuracy in enumeration by helping to recover any cells that were sub-lethally damaged during the course of the experiment (5). Although no direct comparison to aerobic growth was performed during this series of experiments, the data obtained from Semenec *et al.* (10) in 2007 showed complete killing after 180 minutes of irradiation under the same conditions and initial *E. coli* concentration, using aerobic growth conditions. This hints that perhaps the conditions

described above more readily allow growth of damaged cells.

The noted stepwise decrease in cell viability of the sample exposed to UV-A alone (FIG. 1) could have been due to growth phase variability within the cell population. It has been found that cells in stationary phase were less susceptible to UV-A radiation (4). Therefore, the cells that had not achieved stationary phase after overnight culturing were potentially more susceptible to lethal damage by the UV-A light during the first 60 minutes. A decreased death rate was then observed until enough UV-A damage accumulated in the stationary phase cells at approximately 120-to-150 minutes, whereupon the cell counts resumed their decrease.

Several compounds and enzymes have been implicated with the neutralization of ROS, and the presence of these may diminish the efficacy of SODIS water treatment. Free radical scavengers L-histidine (acts to quench singlet oxygen) (5), and formate (acts to quench hydroxyl radicals) (7) were tested, along with catalase and superoxide dismutase which enzymatically remove ROS from a system. It was expected that the addition of these exogenous compounds would result in decreased bacterial killing and a lower detectable measurement of ROS in the irradiated water, thus providing insight into the interplay of many environmental factors for measuring the efficacy of SODIS as a whole. As can be seen in FIG. 2, none of the compounds added to the bacterial solutions offered significant protection from UV-A induced killing over the time assayed. A possible explanation could be that there were very low amounts, if any, of exogenous photosensitizers (i.e. humic acid) in the water. Therefore, most of the ROS were probably formed internally from endogenous photosensitizers such as porphyrins and flavins (3). To protect against internal ROS the formate and L-histidine would need to accumulate in sufficient intracellular concentration to impact the levels of ROS and consequently alter the rate of killing. Therefore, transport may have occurred, however accumulation may have been minimal over the three-hour time course.

The addition of formate (FIG. 2B) should have quenched hydroxyl radicals that may have formed due to UV-A exposure in the water (7). For most of the experiment, the formate did not appear to have a significant effect, however the final time point cell counts were higher for the UV-A and formate sample than for UV-A exposure alone. It is possible that later in the assay the lysis of UV-A killed *E. coli* cells allowed for the release of their internal sensitizers. This consequent accumulation of photosensitizers in the environment could have increased the induced extracellular ROS concentrations after three hours and allowed formate to offer some protection. A longer time-course would be necessary to test this possibility. The addition of L-histidine (FIG. 2A) should quench extracellular singlet oxygen (7). Examination of the survival curve of the L-histidine supplemented sample shows a continuous decline in bacterial viability during the first 2 hours followed by a plateau. L-histidine is a metabolite used by *E. coli* cells, therefore the bacteria have appropriate systems in place for histidine uptake. It is possible that the decline corresponded to the time required for uptake and accumulation of L-histidine to a threshold level. Attainment of this threshold would subsequently offer some protection from UV-A induced intracellular ROS, reflected by the plateau in FIG. 2A.

The enzymes used in FIG. 2C and FIG. 2D were unable to significantly protect the *E. coli* cells from UV-A induced killing. The superoxide dismutase (SD) treated sample showed similar rates of bacterial survival as the control, while the catalase treated sample showed a very rapid decline in viable cells followed by a period of relatively stable cell numbers before continuing to decline. It should be noted that the catalase was at a fairly high concentration (200 U/ml) compared to the SD (2 U/ml); a level high enough to cause the catalase sample to be cloudy. Since pH is a significant factor in determining UV-A killing efficiency (3), the rapid decline in cell numbers might have been due to a shift in pH (not measured) resulting from adding a large amount of protein. Certain residues on the proteins may also have acted as photosensitizers and increased the levels of ROS other than H_2O_2. It is also possible that the addition of a large amount of protein acted as a carbon source or stabilized the environment, which reduced the number of cells in stationary phase and therefore increased their susceptibility to the effects of UV-A exposure. Finally, a significant amount of bubbles were formed when the catalase treated sample was shaken. This could have increased the amount of available oxygen for the intracellular photosensitizers and thus increased ROS production early in the assay.

Exposure to UV-A light alone was sufficient to decrease cell numbers by approximately 70% over three hours. L-histidine and formate showed potential protection from intracellular ROS after 120 minutes and 150 minutes respectively, indicating the possibility for protection over an extended period of time. The enzymes used to scavenge exogenous ROS failed to offer any protection for UV-A induced killing, and in fact increased killing in this assay. It appears that the microbicidal activity of UV-A induced ROS might be an intracellular phenomenon

in this laboratory system using de-ionized water; extracellular ROS might play a more important role in environmental water samples containing exogenous photosensitizers such as humic acid. We were not able to determine whether a direct correlation existed between bacterial viability and the levels of ROS in the water as we were unable to optimize the assay using the pentafluorobenzenesulfonyl probe.

FUTURE EXPERIMENTS

It would be important to determine whether the formation of intracellular ROS is the actual basis for killing during the UV-A treatment through optimization of an appropriate quantitative assay. Also, as an indication of whether ROS scavengers can provide protection in the extracellular environment, addition of exogenous photosensitizers such as humic acid along with ROS scavengers could be used. This could also provide insight into the relative importance of intracellular versus extracellular generation of ROS in this system.

As an improvement to the protocols used above, it would be beneficial to improve the mixing efficiency by adding sterile beads or marbles to the PET bottles, and increasing the length of the time course assay to further characterize the trends observed after 120 minutes. Also, starting with cultures in different growth phases could evaluate whether there is variable susceptibility to UV-A radiation. It would also be valuable to measure the impact of temperature and pH change on the microbicidal activity of SODIS, as these variables can greatly impact the dynamic of a biological system.

It is clear that additional optimization is required to establish a sufficient assay for ROS. The assay described by Maeda *et al*. (8) needs to be optimized with controlled temperature, reaction time, and dilution to generate an appropriate standard curve and obtain quantitative data. However, under time constraints, an alternate assay for ROS detection is recommended.

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