

## The Role of Reactive Oxygen Species in the Solar Disinfection (SODIS) System of water contaminated with *Escherichia coli* and *Salmonella enterica* serovar *Typhimurium*

LEE-ANNA BURGESS, ALEKSANDRA GARA, BRIAN (VU) LE, AND STEPHANIE LETKEMAN

*Department of Microbiology and Immunology, UBC*

Solar disinfection (SODIS) is a technique that uses solar radiation and heat to inactivate microbial activity to the point where the water is safe for human consumption. Studies have confirmed that oxygen plays a critical role in solar disinfection and it is thought that ultraviolet-A (UV-A) radiation from sunlight induces the formation of reactive oxygen species (ROS) from dissolved organic carbon and oxygen. This study investigated the possibility that the UV-A bactericidal effects are due to photo-inactivation via the production of ROS. *Escherichia coli* B23 and *Salmonella enterica* serovar *Typhimurium* LT2 were subjected to UV-A irradiation and hydrogen peroxide levels, which are indicative of ROS generation and were measured using a fluorometric assay. A decline in viable bacteria was observed following UV-A exposure for all cell strains. The H<sub>2</sub>O<sub>2</sub> detection assay yielded inconclusive results.

In 2004, the World Health Organization (WHO) estimated that more than one billion people around the world, that is, one in six people, do not have access to a safe supply of drinking water. Unsafe drinking water can cause waterborne disease such as diarrhea killing 1.8 million per year, 90% of which are children under the age of 5 (18). The importance of ensuring and providing safe drinking water is evident, as it is the focus of three of the millennium development goals (16). The WHO endorses development of solutions that are simple, low cost and easily adapted for use in the developing world, which is the most affected region (13). SODIS is a well-recognized approach to water disinfection by WHO. The process uses solar radiation and heat to inactivate microbial activity and render contaminated water safe to consume (6, 7, 15). Solar light contains Ultraviolet-A, B and C (UV- A, B and C). UV-B and UV-C are known for their germicidal effects; they act by damaging the DNA of the microorganism. However, the majority of the light that reaches the earth's surface, UV-A (400-320 nm), does not have a well-described mechanism of microbial inactivation (9).

Numerous studies have hypothesized that the positive results of UV-A as a disinfectant is due to its ability to produce ROS (Khaengraeng; Wilton; WHO website). A study has confirmed that oxygen plays a critical role in solar inactivation, by showing that deoxygenated water had a dramatic decrease in the inactivation of the bacteria (12). Furthermore, Khaengraeng *et al.* (8) found that the addition of ROS scavengers in a photo-inactivation system resulted in significant decreases in germicidal effects. Much of the

evidence from previous studies has established a link between ROS and inactivation rates that are observed in the SODIS method. The study by Abele-Oeschger *et al.* demonstrated that both UV-A and UV-B contributed to H<sub>2</sub>O<sub>2</sub> formation (1). The mechanism of H<sub>2</sub>O<sub>2</sub> production required dissolved organic carbon (DOC) whereby UV-activation of DOC caused the carbon to reduce dissolved oxygen forming superoxide anions (1). Superoxide is highly unstable in aqueous solution and reacts with water to form H<sub>2</sub>O<sub>2</sub>. This mechanism may be responsible for the bactericidal activity observed with SODIS, which is why H<sub>2</sub>O<sub>2</sub> is a good target for investigation. Abele-Oeschger *et al.* (1) used indirect fluorometric analysis using scopoletin, which renders the peroxidase enzyme spectroscopically distinct.

With the development of fluorescent probes over recent years, it seems much more appealing to measure H<sub>2</sub>O<sub>2</sub> molecules directly. Soh (14) reviews conventional ROS probes and some of the newly developed molecules from which pentafluorobenzenesulfonyl fluorescein was selected for this investigation. Maeda *et al.* designed the pentafluorobenzenesulfonyl fluorescein probe (FIG. 2) for H<sub>2</sub>O<sub>2</sub> that relies on a deprotonation reaction, as opposed to a redox reaction (11).

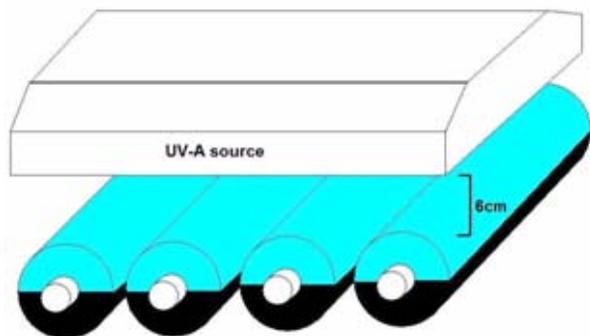
The purpose of this study was to correlate the germicidal effects associated with UV-A exposure in *Escherichia coli* B23 and *Salmonella enterica* serovar *Typhimurium* LT2 (*S. typhimurium* LT2) to the levels of hydrogen peroxide. As previously mentioned, H<sub>2</sub>O<sub>2</sub> levels are the preferred target, as it is the most stable

ROS and is commonly measured in surface waters (1). For the H<sub>2</sub>O<sub>2</sub> specific probe a detection assay must be developed, the preliminary stages of which are discussed in this paper. Though the detection assay has not been refined the sensitivity of *E. coli* and *S. typhimurium* to UV-A irradiation as demonstrated by a killing assay suggest the correlation of ROS with microbicidal effects.

## MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions** – Streaked plates of *E. coli* B23 and *S. typhimurium* LT2 were obtained from the MICB 421 culture collection in the Microbiology and Immunology Department at the University of British Columbia, Vancouver, BC. Overnight cultures were prepared by loop-full inoculations of bacteria in 15 ml of Luria-Bertani broth (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl and 0.2% w/v glucose adjusted to pH 7.0) and incubated at 37 °C for 18 hours in a shaking water bath to stationary phase. The cultures were collected, centrifuged in a Beckman J-21 high-speed centrifuge at 7,500xg for 10 minutes and resuspended in sterile distilled water. The cells were re-centrifuged at 13,500 x g for 5 minutes twice more to remove all traces of the growth medium. The cultures were then resuspended in an adequate volume of sterile distilled water to a final concentration of approximately 10<sup>7</sup> cells/ml.

**UV-A Exposure** – Two polyethylene terephthalate (PET) bottles were inoculated with the required volume of *E. coli* or *S. typhimurium* and filled with sterile distilled water to give an initial concentration of roughly 10<sup>6</sup> cells/ml in 500 ml. One bottle was inoculated with both bacterial species, each at 5x10<sup>5</sup> cells/mL in 500 ml of sterile distilled water. One bottle contained sterile distilled water only and served as a control. The bottles were exposed to a 20 W Sylvania Black light (UV-A light source) placed 6 cm from the top surface of the bottles for a 3-hour period at an intensity of 807.7 W/m<sup>2</sup>. The UV-A exposure simulated sunlight-mediated sterilization of the water by the SODIS method. An additional four bottles were prepared as those described above and were stored in the dark during the incubation times. These acted as non-UV-treated controls. Samples of UV-treated water were taken at 30 minutes intervals for a total of seven time points and control samples were taken at 0 and 180 minutes.



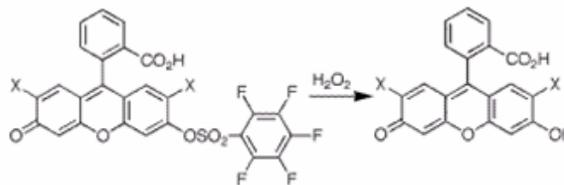
**FIG 1.** UV-A irradiation system setup. Four PET bottles painted half-black were placed 6cm below the UV-A lamp with the black side down.

A second trial was carried out with several modifications in an effort to optimize data. It was carried out in the same manner except the PET bottles were painted half-black in an effort to adhere to the

SODIS protocol (5) and maximize UV-A absorbance (see fig. 1). Furthermore, to allow for efficient thorough mixing of the bottles, only 400 ml of sterile distilled water was added to them. The extra airspace allowed for vigorous mixing. Additionally, time points were taken every 45 minutes rather than 30 for experimental samples, and at 0, 90, and 180 minutes for control samples to obtain a more complete data set.

**Enumeration** – For each time point the bottles were shaken vigorously and 500  $\mu$ l of the sample were obtained. Three 10-fold dilutions were performed in duplicate to provide an appropriate concentration where plate counts were between 30 and 300 colony forming units (cfu). Colonies were counted on the plates after incubation for 18 hours at 37 °C.

**Fluorometric H<sub>2</sub>O<sub>2</sub> Analysis** – A chemical fluorescence assay was used to measure ROS levels in the SODIS system. The assay is based on the fluorescent probe, pentafluorobenzenesulfonyl fluorescein (Cayman Chemical, Ann Arbor, MI, cat. # 10005983) that is specific to H<sub>2</sub>O<sub>2</sub> (FIG. 2) (14). Fluorescent emission was measured with the Turner Quantech Digital Filter Fluorometer using an emission filter of 490 nm and an excitation filter of 515 nm. Pentafluorobenzenesulfonyl fluorescein was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, then in the UV-A irradiated aqueous sample for a final concentration of 5  $\mu$ M. ROS measurements were taken at 30-minute intervals over 3 hours for the irradiated samples and at times 0 and 180 minutes for the control samples. The machines were not zeroed, but fluorescence was measured before the addition of probe to serve as a blank.



**FIG 2.** Chemical structure of pentafluorobenzenesulfonyl fluorescein and reaction with hydrogen peroxide.

Fluorometric Analysis was performed with both the Turner Quantech Digital Filter Fluorometer and the Perkin-Elmer Fluorescence Spectrophotometer (Model 651-10S). The fluorescence spectrophotometer used an emission filter of 485 nm and an excitation filter of 530 nm as recommended by Maeda *et al.* (11). As suggested by the manufacturer, the probe in the aqueous sample was diluted with 10X phosphate buffer to provide a 0.1 M phosphate buffered solution for optimal activity of the probe.

## RESULTS

**A decline in viable *E. coli* is observed with increasing periods of UV-A exposure.** When PET bottles were exposed to UV-A radiation, a steady decline by 56% in the number of viable *E. coli* was observed in trial two after a three hour period of irradiation (FIG. 3). The control sample showed the expected trend; bacterial counts remained higher than in the experimental condition at all times. However, the amount of killing observed in the control sample was 38%, which was a higher value than expected. It was expected that the osmotic factor would only minimally contribute to the killing. Trial two showed

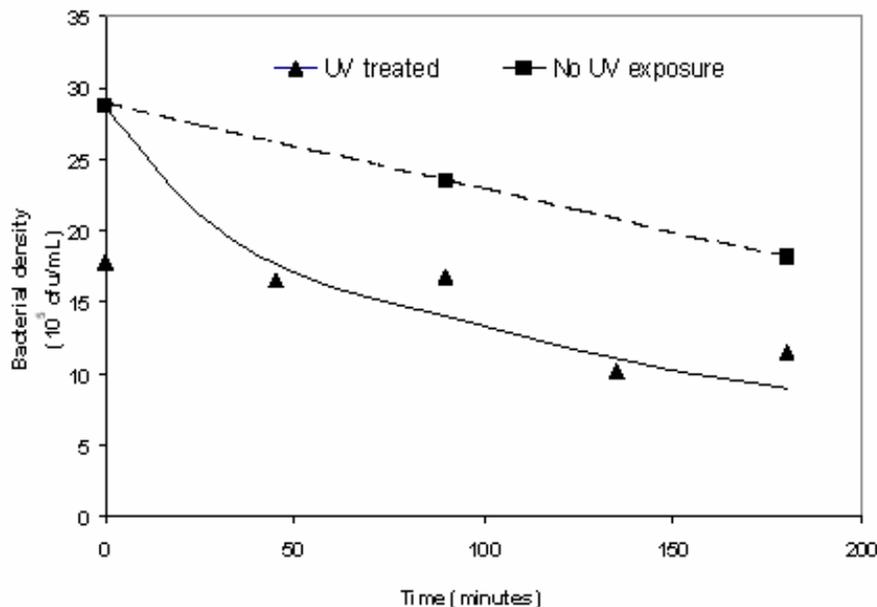


FIG 3. Photoinactivation of *E. coli* with UV-A irradiation in sterile distilled water at 22°C.

that *E. coli* dropped in numbers more rapidly in the UV-treated sample than in the non-UV-treated sample. It should be noted that initial bacterial densities of the SODIS system were expected to be equal to the control bottles, which was not the case.

***S. typhimurium* counts steadily declined over time when exposed to UV-A.** The bottle inoculated with *S. typhimurium* demonstrated a particular step-like inactivation. In summary, over the three hour period, a 95% reduction in viable counts was seen. The control dropped by 7% in the same interval. Figure 4 is based on data obtained from trial one. It should also be noted that although initial bacterial densities should have been equal between experimental and control conditions, they were not. Data from trial two showed similar effect, but the plateau effects became less evident as the collection time points were farther apart. It appears as though *S. typhimurium* is more sensitive to the germicidal effects of UV-A radiation compared to *E. coli*. Overall, *S. typhimurium* had a greater fraction of the original inoculation killed by UV-A exposure (FIG. 5).

Mixed cultures exhibited variable trends. MacConkey plates showed that concentrations of *S. typhimurium* in the mixed sample appears to exhibit a steady decrease over the 180 minute period (FIG. 6), with 37% cell death. The control showed 39% cell death. *E. coli* concentration, on the other hand, fluctuated throughout the 180 minutes (FIG. 6), with no obvious pattern of increase or decrease being detected.

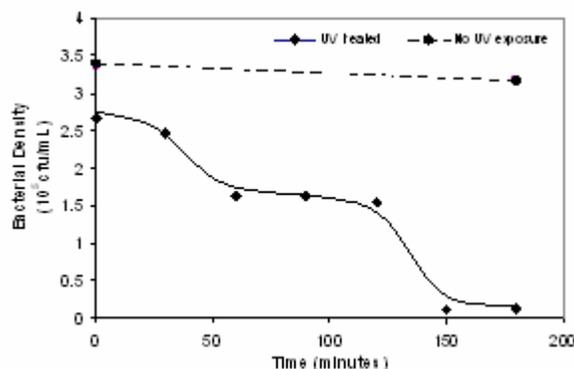


FIG 4. Photoinactivation of *S. typhimurium* with UV-A irradiation in sterile distilled water at 22°C.

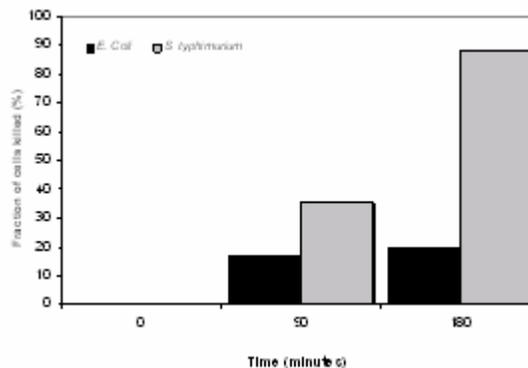
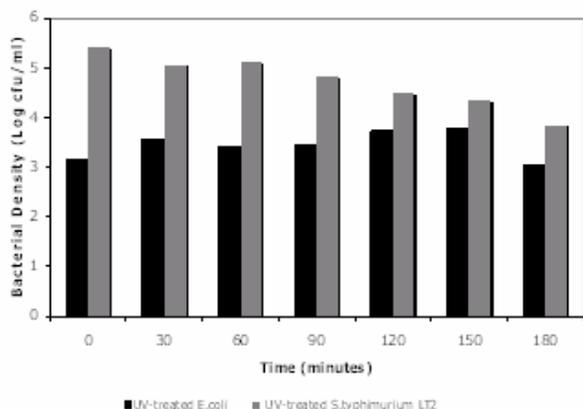


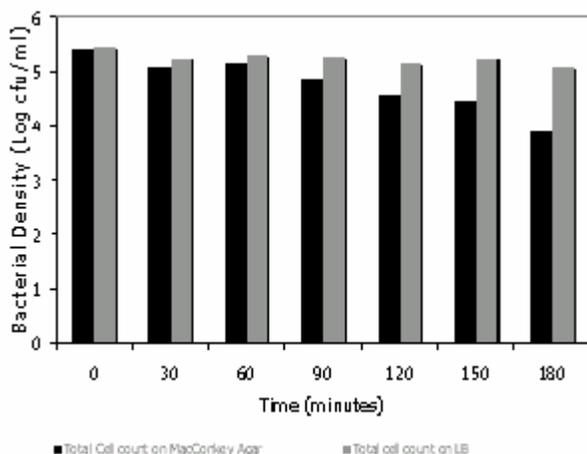
FIG 5. Percentage of bacteria killed due to UV-A exposure.

At the start and throughout the procedure, more *S. typhimurium* than *E. coli* was detected in the plate counts. This difference between *S. typhimurium* and *E. coli* concentrations appear to decrease with the decreasing concentration of *S. typhimurium* (FIG. 6).

The total cell count on MacConkey agar appeared to coincide with the decrease in *S. typhimurium* concentration (FIG. 6, 7), indicating that overall cell numbers are decreasing in the bottle as expected. However, total cell count on LB agar indicated the converse, as total cell numbers do not decrease with time when samples are plated (FIG. 7). The initial total cell count on LB coincided with the total cell count on MacConkey agar but did not decrease over the 180 minutes (FIG. 7). This was unexpected as total cell numbers were expected to decrease in the UV-treated sample with time.



**FIG 6.** Viable *E. coli* and *S. typhimurium* plate counts on MacConkey agar over 180 minutes from a mixed culture sample under UV-A irradiation at 22°C.



**FIG 7.** Total cell counts in the mixed water sample over 180 minutes of UV-A exposure at 22°C, detected on MacConkey and LB agar.

**Reactive Oxygen Species data shows a logical trend but is inconclusive.** The amount of hydrogen peroxide measured positively correlates with the fluorescence detected in arbitrary units of volts (FIG. 8). These results are difficult to equate to chemical concentrations as a hydrogen peroxide standard curve could not be generated with this method.

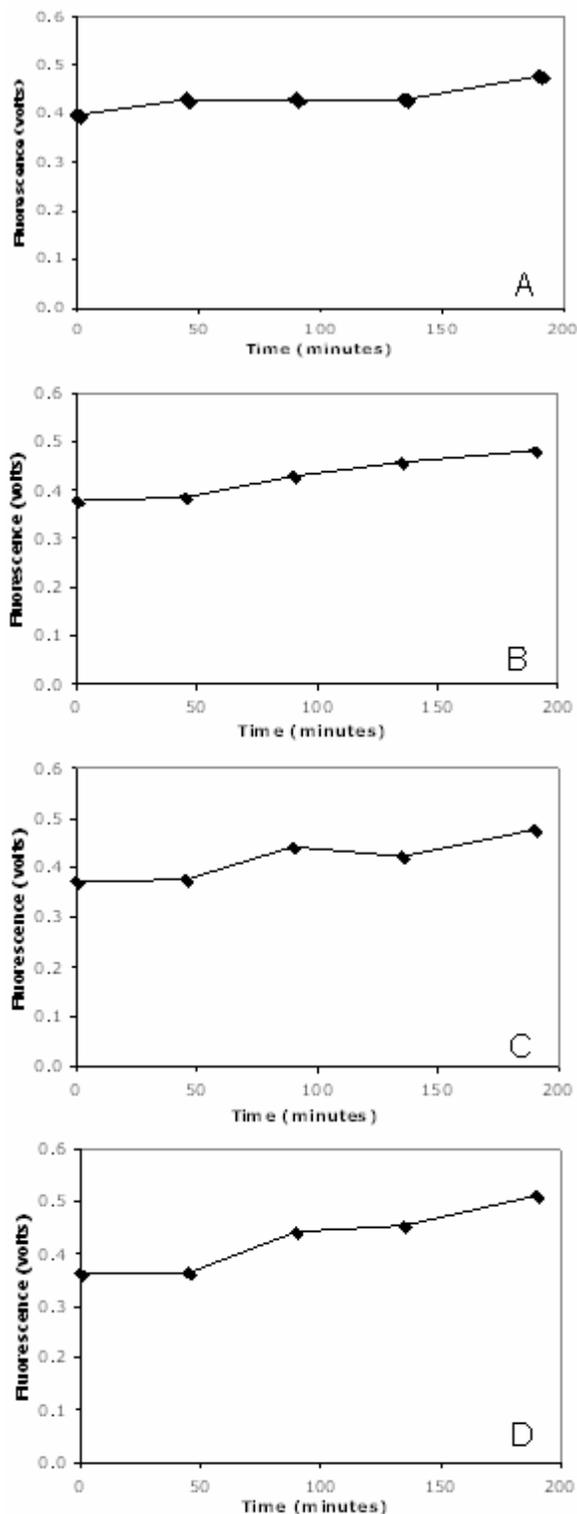
## DISCUSSION

The SODIS protocol was not reproduced identically, as the focus of this paper was to see the UV-A effects, and not thermal effects. Thus, inactivation rate was not maximal as the temperature did not reach optimal conditions of 50°C (5). Instead, the process was carried out at room temperature (22°C). Furthermore, the SODIS protocol calls for 1 to 2L PET bottles exposed to a full day's direct sunlight (5). In our experiment however, we used 500ml PET bottles, and the temperature of the water may have been below room temperature, as the water was stored in the refrigerator. Furthermore, the experiment was only 180 minutes in length when the protocol calls for an entire day. These changes could have severely impact the magnitude of the results obtained. Nevertheless, UV-A exposure showed the expected germicidal effects, to a lesser degree.

The control level of bacterial killing without UV-A exposure was likely due to osmotic effects of the water and starvation of the cells (FIG. 3, 4). It would be unreasonable to expect that all bacteria placed in sterile distilled water would survive after three hours in the absence of nutrients. Nonetheless, most cells are expected to survive. Under UV-A exposure however, it is expected that ROS will be formed in the water. The concentration of ROS will rise over time, and kill the bacteria. The bactericidal effect was more noticeable as the experiment proceeded, and this is indeed what is observed (FIG. 3, 4).

The photo-inactivation of *S. typhimurium* was novel and interesting to note. The step-like inactivation (FIG. 4) of *S. typhimurium* suggests a possible stage-like inactivation process. It appears as though there was a possible stress response responsible for reviving damaged cells. Thus, an accumulation of damage must occur before the cell is rendered unviable. This plateau effect could also be explained by an increase in ROS due to the addition of DOC derived from cellular debris over time.

The higher sensitivity of *S. typhimurium* was contrary to previous studies (17, 2). This trend was seen in the individual bacterial cultures (FIG. 3, 4) and the co-inoculation of both species (FIG. 6), and could be due to a difference in the magnitude of the stress response between *E. coli* and *S. typhimurium*. Though these species have the same stress response systems, it is



**FIG 8.** Hydrogen peroxide measurements using a fluorometric assay over a three-hour time course of (A) water only; (B) *E. coli* in water; (C) *S. typhimurium* in water; and (D) both *E. coli* and *S. typhimurium* in water. All values are adjusted for the net gain in fluorescence caused by the probe.

possible that the intensity of the stress required to induce a response is less in *E. coli* (2). An increase in the stress factor has been shown to correlate with an increase in the expression of sigma factor RpoS, a global stress regulator, as well as the expression of a DNA-binding protein, which protects cellular DNA from oxidative stress (4). Therefore, the *S. typhimurium* culture may have been expressing less stress regulators making it more sensitive to the UV exposure, while the *E. coli* culture may have up-regulated expression of stress factors (sigma-factor RpoS and DNA binding protein) rendering it less sensitive to UV stress.

Inoculation of the bottles with different initial concentrations of each culture, and the differences in nutrient requirements may have also contributed to the observed difference in sensitivity. For instance, *E. coli* is *lac+*, while *S. typhimurium* is *lac-*, showing that the bacteria are able to utilize different nutrients. It is possible that as UV exposure induced cell death, *E. coli* was able to utilize the limited nutrients released by the dying cells. *S. typhimurium*, being at a higher concentration (approximately 3% more), had more competition for the limited nutrients and thus exhibited a sharper decline in cell numbers (FIG. 4).

The lack of decrease in total cell numbers on LB agar compared to the notable decrease on MacConkey agar (FIG. 7) may have occurred due to the different components in the media. Berney *et al.* (3) have observed that 1500 kJ/m<sup>2</sup> of UV-A light is needed to permeabilize the outer membrane of *E. coli* cells with twice the dose being necessary to permeabilize the inner membrane. Thus, if the applied dose was not sufficient to permeabilize both membranes, only an intermediate state of injury would have been achieved. The cells could have recovered upon being plated on nutrient-rich LB agar, indicating no overall decrease in total cell number. However, when the damaged cells were plated on MacConkey agar, which contains bile acids and crystal violet, repair of the cell membrane and peptidoglycan could have been prevented. Bile acids have been shown to damage cellular membranes and eliminate peptidoglycan precursors while crystal violet may interfere with peptidoglycan synthesis (10).

To link the killing trends observed we measured H<sub>2</sub>O<sub>2</sub> in parallel over the course of the three hour UV-A irradiation. Though H<sub>2</sub>O<sub>2</sub> measurements were performed for the UV-irradiated samples a good standard curve could not be obtained for this assay. The standard curve was made using the method described above across a H<sub>2</sub>O<sub>2</sub> range of 1 nM to 1 mM (data not shown). Despite the huge range in H<sub>2</sub>O<sub>2</sub> concentrations the fluorometer readings only differed by a maximum 10 millivolts, whereas the experimental samples generated fluctuations up to 100 millivolts. Visible luminescence was attainable when a concentration of 1

M H<sub>2</sub>O<sub>2</sub> was probed, however, detection with the fluorescence spectrophotometer did not distinguish between this and the blank sample, which indicates an unoptimized detection system as opposed to a flaw in the chemical system. Though there was a marked increase in fluorescence after the addition of probe, the lack of resolution of the fluorescence generated by samples of different H<sub>2</sub>O<sub>2</sub> concentration meant this assay was unrevealing for the detection of H<sub>2</sub>O<sub>2</sub> levels.

Catalase experiments were performed and incubation with the enzyme prior to the addition of probe attenuated or abolished the initial fluorescence reaction (data not shown). There was a greater H<sub>2</sub>O<sub>2</sub> concentration range for the standard curve, thus the trend (FIG. 8) must have been due to some other effect.

The aberrance of the results determined by the standard curve experiments suggests a defect in the detection set up. The main target for optimization should be excitation and emission filters. The initial protocol for this investigation used the Turner spectrophotometer with rigid filters, however, the Perkin-Elmer fluorescence spectrophotometer was chosen due to its filters with a continuous range, which was meant to improve the sensitivity of detection.

The incorporation of a buffer also appeared to improve the activity of the probe as fluorescence levels increased more in the presence of buffer than in the absence (data not shown). Other experimental conditions, however, could be investigated for optimization. The manufacturers recommend dissolving the probe in an organic solvent and suggest DMSO or ethanol. Maeda *et al.* (11) dissolve the probe in ethanol instead of DMSO, which was done for this investigation,

Our experiments demonstrated the effectiveness of the SODIS method in disinfecting bacteria-contaminated water, however, the detection system failed to provide support linking this phenomenon to the production of ROS.

### FUTURE EXPERIMENTS

Detection of the probe should be optimized using the current excitation and emission wavelengths specific for fluorescein as it is crucial to duplicate the results observed by Maeda *et al.* (11) and produce a linear standard curve for which increasing H<sub>2</sub>O<sub>2</sub> concentrations results in increasing fluorescence. Generation of H<sub>2</sub>O<sub>2</sub> via UV-A irradiation can then be investigated using this assay.

In order to test if there are inhibitory effects due to the bile acids and crystal violet in MacConkey media, an experiment comparing CFU numbers of UV-treated cells on LB and MacConkey media should be performed for pure cultures of both *E.coli* and *S.typhimurium*. If issues with growth media are seen,

an improved method to circumvent the possible inhibitory effects of the McConkey Media, would be through the use of antibiotics. Two different antibiotic resistance strains of *E.coli* and *S.typhimurium* could be used in lieu of B23 and LT2. The antibiotic resistance can be exploited for selection and quantification of the respective species in the mixed culture.

To confirm the plateau trend observed a repeat of the experimental protocol along with more time points should be performed. Furthermore, to eliminate the discrepancies of initial concentrations in UV-treated and non-UV-treated cultures at the first time point the bacterial culture should be made up in a single volume and then partitioned into the designated bottles.

### ACKNOWLEDGEMENTS

We would like to thank Po-Yan Cheng for her assistance throughout our experimental development. We would also like to thank Jen Sibley for her expertise and know-how. A very special thanks to Dr. William Ramey for his continued guidance and advice. We would also like to acknowledge the media room staff for their invaluable contribution to this course and our investigation. This study was funded by the Department of Microbiology & Immunology at the University of British Columbia.

### REFERENCES

1. Abele-Oeschger, D., H. Tug, R. Rottgers. 1997. Dynamics of UV driven hydrogen peroxide formation on an intertidal sandflat. *Limnol. Oceanogr.* **42**:1406-1415.
2. Berney, M., H.U. Weilenmann, A. Simonetti, and T. Egli. 2006. Efficacy of solar disinfection of *Escherichia coli*, *Shigella flexneri*, *Salmonella typhimurium*, and *Vibrio cholerae*. *J. Appl. Microbiol.* **101**:828-836.
3. Berney, M., Hammes, F., Bosshard, Weilenmann H.U., and Egli, T. 2007. Intermediate injured state assessing and interpreting bacterial viability using LIVE/DEAD BacLight kit in combination with flow cytometry. *Appl. Environ. Micro.* published online ahead of print on 23 March 2007
4. Berney, M., Weilenmann H.U., Egli, T. 2007. Adaptation to UVA radiation of *E. coli* growing in continuous culture. *J. Photochem. Photobiol.* **86**: 149-159
5. Eawag Sandec. 25 April 2004. SODIS technical notes. [Online.] <http://www.sodis.ch/files/notes.pdf>.
6. Joyce, T.M., K.G. McGuigan, M. Elmore-Meegan, R.M. Conroy. 1996. Inactivation of fecal bacteria in drinking water by solar heating. *Appl. Environ. Microbiol.* **62**:399-402.
7. Kehoe, S.C., T.M. Joyce, P. Ibrahim, J.B. Gillespie, R.A. Shahar, and K.M. McGuigan. 2001. Effects of agitation, turbidity, aluminium foil reflectors and container volume on the inactivation efficiency of batch-process solar disinfectors. *Water. Res.* **35**:1061-1065.
8. Khaengraeng, R., and R.H. Reed. 2005. Oxygen and photoinactivation of *Escherichia coli* in UVA and sunlight. *J. Appl. Microbiol.* **99**:39-50.
9. Kielbassal, C., L. Roza, and B. Epe. 1997. Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis.* **18**:811-816.

10. **Madigan, M., and Martinko, J.** 2005. Brock Biology of Microorganisms, 11<sup>th</sup> Edition. Pearson Prentice Hall. Upper Saddle River, NJ
11. **Maeda, H., Y. Fukuyasu, S. Yoshida, M. Fukuda, K. Saeki, H. Matsuno, Y. Yamauchi, K. Yoshida, K. Hirata, and K. Miyamoto.** 2004. Fluorescent probes for hydrogen peroxide based on a non-oxidative mechanism. *Angew. Chem. Int. Ed. Engl.* **43**: 2389–2391.
12. **Reed, R.H.** 1997. Solar inactivation of faecal bacteria in water: critical role of oxygen. *Lett. Appl. Microbiol.* **24**: 276-280.
13. **Sobsey, M. D.** 2002. Managing Water in the Home: Accelerated Health Gains from Improved Water Supply. WHO. [http://www.who.int/water\\_sanitation\\_health/dwq/WSH02\\_07.pdf](http://www.who.int/water_sanitation_health/dwq/WSH02_07.pdf)
14. **Soh, N.** 2006. Recent advances in fluorescent probes for the detection of reactive oxygen species. *Anal. Bioanal. Chem.* **386**: 532-543.
15. **Sommer, B., A. Mariño, Y. Solarte, M.L. Salas, C. Dierolf, C. Valiente, D. Mora, R. Rechsteiner, P. Setter, W. Wirojanagud, H. Ajarmeh, A. Al-Hassan, and M. Wegelin.** 1997. SODIS - an emerging water treatment process. *J. Water. SRT-Aqua.* **46**:127-137.
16. **UNESCO.** 2007. The Millennium Development Goals and Water. accessed 9 April 2007. [http://www.unesco.org/water/wwap/facts\\_figures/mdgs.html](http://www.unesco.org/water/wwap/facts_figures/mdgs.html).
17. **Wegelin, M., S. Canoninca, K. Mechsner, T. Fleishmann, F. Pesaro, and A. Metzler.** 1994. Solar water disinfection: scope of the process and analysis of radiation experiments. *J. Water. SRT Aqua.* **43**:154-169.
18. **WHO.** 2004. Water, sanitation and hygiene links to health, Facts and figures updated November 2004. [http://www.who.int/water\\_sanitation\\_health/publications/facts2004/en/index.html](http://www.who.int/water_sanitation_health/publications/facts2004/en/index.html)
19. **Wilton, J., K. Jones, N. Kashani, S. Woo, and J. Yang.** 2006. Investigation into the germicidal effects of ultraviolet-A radiation and suggestions for improvement of solar water disinfections. *J. Exp. Microbiol. Immunol.* **9**:25-30.