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Investigation of the Potential UV-A Enhanced Toxicity and the Potential Inhibition of Catalase by Phthalate Exposed to UV-A

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Solar Water Disinfection (SODIS) is a simple process used in developing countries to improve the quality of bacteria-contaminated drinking water. A previous study found that phthalate, an aromatic compound released by polyethylene terephthalate (PET) bottles upon UV-A irradiation, enhanced the germicidal activity of SODIS. In the current study, two possible mechanisms behind this observation were examined: phthalate toxicity to bacteria and phthalate inhibition of catalase activity. The potential toxicity was studied by: 1) observing changes in growth rate in a 90-minute time course study in which irradiated and non-irradiated phthalate were added to *Escherichia coli* B23 cultures and 2) examining growth inhibition around phthalate saturated filter disks on *E. coli* B23 lawns. Since no differences in bacterial growth were observed between the control and the phthalate conditions, these experiments suggested that neither irradiated nor non-irradiated phthalate is toxic to *E. coli*. A third experiment tested the proposed inhibition of catalase by using a simple catalase assay. It was found that without UV-A irradiation, phthalate had no effect on catalase. In the presence of UV-A, when phthalate was at the same concentration as catalase (50 µg/mL), a protective effect on the enzyme was observed. However, at a concentration of 0.4 µg/L, the typical amount of phthalate released in a PET bottle, no protective effect was observed. Neither of the mechanisms proposed in this study proved to be the cause behind phthalate-enhanced germicidal activity of SODIS.

Inaccessibility to safe drinking water supplies has been one of the major factors contributing to numerous water-borne diseases (such as diarrhea) for at least one-third of the population in developing countries. There are approximately 4 billion cases of diarrhea worldwide each year and 2.2 million people in developing countries have died from diarrhea (15). According to the Swiss Federal Institute for Environmental Science and Technology (EAWAG), the Solar Water Disinfection Process (SODIS) is an effective, low-cost method of disinfecting contaminated water and thus able to improve the health of those who still lack safe drinking water (13). Recommended by the World Health Organization, SODIS is currently being used by about 2 million people in more than 20 countries globally for the daily treatment of drinking water at

household level (13). Several health impact studies have demonstrated that SODIS provides significant health benefits, including a 20~50% reduction in the incidence of diarrhea (13).

SODIS improves the quality of drinking water by inactivating pathogenic microorganisms via two synergetic mechanisms: sunlight raises water temperature and the reaction of UV-A with oxygen in water produces reactive oxygen species (ROS) in the form of singlet oxygen and hydrogen peroxide (14). In the SODIS protocol, contaminated water is filled in clear, transparent plastic bottles and exposed to full sunlight for six hours (13). The protocol recommends the use of polyethylene terephthalate (PET) bottles as they contain less UV-stabilisators than polyvinylchloride bottles (13). Phthalate, the plasticizer added to PET bottles for flexibility, has been shown to be released from the bottles into the water (9). A report from EAWAG found that PET bottles contain di-phthlate at a concentration of 0.10 – 0.71 µg/L (9).

In a previous study, Semenec *et al.* observed that the addition of phthalate to PET water bottles subjected to the SODIS treatment reduced the concentration of bacteria growing in the bottles (12). Two possible explanations were proposed for this observation (12): 1) the oxidation of phthalate by UV-A irradiation resulted in an increase of ROS in the water or 2) phthalate may have inhibitory effects on *Escherichia coli* catalase activity leading to an accumulation of ROS.

The first proposed mechanism was based on a study that described the oxidation of aromatic compounds in low oxygen conditions (10). However, theoretically, any chemical substance added to water in the presence of ROS would react with the ROS, thereby reducing the amount of ROS available. Thus, we hypothesized that instead of increasing ROS, phthalate is changed into a toxic compound after UV-A irradiation and thus

enhances the germicidal effects of SODIS. We also speculated that phthalate itself may be germicidal.

The second explanation proposed by Semenec *et al.* was supported by previous research showing that bovine liver catalase is strongly inhibited by 3,3'-diaminobenzidine, an aromatic compound (7). Catalase is an enzyme that protects bacteria living in aerobic environments from hydrogen peroxide (an ROS), which can damage DNA and proteins inside the cell. Since catalase catalyzes the conversion of hydrogen peroxide to oxygen and water, measuring the speed of oxygen production will serve as an indicator of the amount of active catalase present.

This study tested two potential mechanisms of phthalate-enhanced germicidal activity in SODIS by determining whether phthalate itself, either UV-A irradiated or not, has germicidal effects and whether phthalate serves as an inhibitory factor for catalase.

MATERIALS AND METHODS

Media. Luria Broth (LB) and LB agar plates were prepared (for 1 L: Tryptone 10 g; Yeast Extract 5 g; Sodium Chloride 5 g). Note that 2.0 g glucose was added to the media. For LB agar plates, 1.5% of agar (Invitrogen, catalogue number 30391-023) was used.

Time course study of the effect of phthalate on growth. The overnight culture was inoculated from one single colony of *E. coli* B23 into 20 mL of LB in sterilized 250 mL Erlenmeyer flask, and incubated in 37°C air-shaker (New Brunswick Scientific, model number Excella E 24) at 100 rpm for 24 hours. 100 mL of 8.0 µg/L phthalate solution was prepared and transferred to 100 mL Gibco glass bottles for incubation under UV-A irradiation eleven cm away or in the dark for 6 hours. The overnight culture was diluted in a 1/20 ratio in 90 mL of LB to give an OD_{660} reading of 0.15, as measured by the spectrophotometer (Spectronic 20+, Spectronic Instruments). 19 mL of the diluted overnight culture was transferred to each of the two sterilized 125 mL Erlenmeyer flask. 1 mL of each incubated phthalate sample was transferred to 125 mL Erlenmeyer flasks containing 19 mL of overnight culture which gave 0.4 µg/L as the final phthalate concentration in the flasks. A control condition with no phthalate was included and was subjected to all the same treatments as the phthalate conditions. The four Erlenmeyer flasks were incubated in a water bath shaker at 37°C at 200 rpm for 25 minutes to have *E. coli* B23 growing in the exponential phase; the timer was then reset. At 15 minutes intervals (15, 30, 45, 60, 75, 90 minutes), samples were taken out of the water bath shaker and turbidity was measured in OD_{660} units.

Zone of inhibition study of the effect of phthalate on growth. 0.0266g/L phthalate solution was prepared and incubated in the same conditions as described above. Filter papers (47mm Millipore filter paper media pad) were cut into uniform 5 mm disks using a 5 mm hole-puncher and autoclaved. From the *E. coli* B23 overnight culture described above, 1/20 dilutions with LB was performed to obtain an OD_{660} of 0.15 and 100 µL of the diluted overnight culture was spread-plated on five LB agar plates to have confluent lawns. The disks were saturated with 15 µL of the prepared phthalate solution to give a final amount of 4 µg of phthalate on each disk. Water without the addition of phthalate was used as the control. The spread-plated LB agar plate was divided into four quadrants, and four saturated filter disks containing each of the four incubated samples were placed on each quadrant. 10 µg streptomycin disk (Difco, catalogue number 716633) was put on the center of the spread plate as a positive control. The above steps were repeated to produce 4 more replicates. The plates were incubated in a 37°C incubator (New Brunswick Scientific, model number G76) for 20 hours and the zone of inhibition around each disk was measured.

Effect of phthalate on catalase. Enzymatic activity of catalase was measured with a simple catalase assay that records the time needed for catalase-solution-soaked filter paper disks to float to the surface of a 420mL 1% hydrogen peroxide solution. This procedure was adapted from the General Biology 111 Laboratory Manual from the University of Massachusetts in Boston (3). To create the standard curve relating the disk floating time to catalase concentration, standard catalase solutions were prepared by suspending catalase from bovine liver (Sigma-Aldrich, catalogue number C1345-1G) in de-ionized water to obtain concentrations of 5, 10, 25, 50, 100, 150 and 200 µg/mL. These solutions were kept in a 4°C fridge overnight and were used immediately after they warmed to room temperature on the day of the experiment. 5 mm filter paper disks were prepared by cutting the filter paper (#1 Whatman disk) using a 5 mm hole-puncher and the disks were soaked in the catalase solutions for 10 seconds. Dilutions were made to obtain 1% hydrogen peroxide solutions from stock solutions (30% Hydrogen Peroxide, VWR, catalogue number VW3742–1; 3% Hydrogen Peroxide, LIFE). Three replicates were performed for each standard catalase solution, and the 1% hydrogen peroxide solution was freshly prepared for each concentration of the catalase standards to ensure that hydrogen peroxide concentration was not a limiting factor in the reaction. In case of burping (the accumulation of oxygen bubbles in the disk that gets shed off, resulting in the disk sinking back down before more oxygen bubbles are produced), results were disregarded and additional trials were carried out. To investigate the effect of phthalate on catalase activity, the catalase solution was mixed with two different concentrations of phthalate solutions (prepared by dissolving phthalic acid potassium salt from Sigma-Aldrich, catalogue number P–6758, in de-ionized water). The catalase solutions were kept at a constant concentration of 50 µg/mL. The first phthalate concentration chosen, 0.4 µg/L, was based on the average of the values found in the literature described in the introduction (9). The second phthalate concentration, 50 µg/mL (presented in the figures as 50000 µg/L), was chosen because it equals the amount of catalase present in the reaction. De-ionized water, was used as control. 20 mL of each phthalate + catalase solution were transferred to 100 mL Gibco glass bottles, rather than PET bottles, in order to minimize the effects of additional phthalate or other compounds released from PET bottles. The bottles of the two different concentrations of phthalate + catalase solutions and the control were subjected to 6 hours of UV-A irradiation using the 20 W Sylvania Blacklight-blue 11 cm away. The other set of phthalate + catalase solutions were kept in the dark for 6 hours. However it should be noted that these solutions were prepared the day before the incubation and stored in 4°C fridge overnight. Then, these solutions were mixed together prior to the 6 hour incubation. The catalase assay described above was used to determine catalase activity after the incubation.

RESULTS

Phthalate had no effect on *E. coli* B23 growth. From the time course study on the effect of UV-A irradiated phthalate on bacterial growth, it was observed that phthalate at 0.4µg/L (irradiated or not, as final concentration in the media) does not have direct germicidal effect (Fig.1).

The growth curves of the two control conditions (addition of irradiated and non-irradiated water) were as expected, starting with an initial lag phase that moves into the log phase at around 45 minutes. The growth rate started to drop around 75 minutes indicating the onset of stationary phase. It should be noted that even

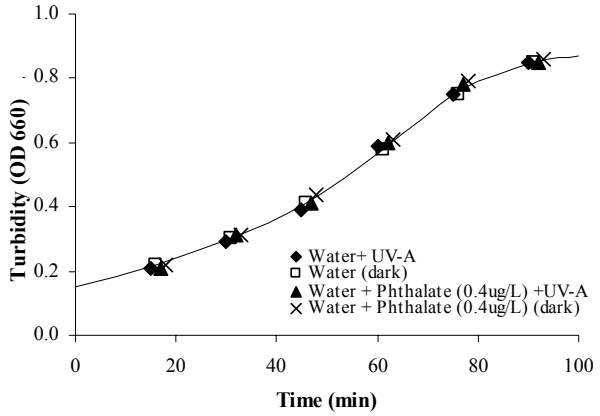

FIG. 1 Effect of phthalate on *E. coli* B23 growth. Phthalate solutions were incubated in 100 mL Gibco glass bottles for 6 hours under UV-A irradiation 11 cm away or kept in a dark drawer for 6 hours. 1 mL of each of the phthalate solutions was added to the *E. coli* culture to achieve a final concentration of 0.4 µg/L in the LB growth media. Using the same volume (1 mL) as the phthalate solutions added to the media, both irradiated and non-irradiated water were used as controls.

though ROS is normally produced when water is irradiated with UV-A, the amount of water used (1 mL) was added to 19 mL of LB medium making the ROS arising from the irradiated water insignificant. This was supported by our results where the growth rates of irradiated and non-irradiated water were the same.

If UV-A irradiated phthalate resulted in a toxic product, then the retardation of the growth rate for theculture with irradiated phthalate addition would be expected. However, when compared to the control

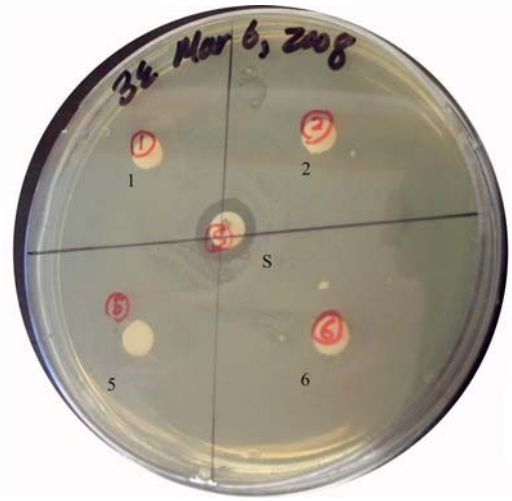

FIG 2. Effect of phthalate on *E. coli* B23 growth, as measured by zone of inhibition. Streptomycin placed at the centre of the plate served as a positive control disk, as compared to the negative control disks with irradiated and non-irradiated water and disks containing irradiated and non-irradiated phthalate. 1 = Water, 2 = UV-A, 3 = Water, 4 = Dark, 5 = Phthalate (0.4 µg) after UV-A, 6 = Phthalate (0.4 µg) kept in the dark, S = Streptomycin (10 µg)

conditions (water, both irradiated and kept in the dark), as well as the non-irradiated phthalate, no differences in growth rates were found around disks soaked with the UV-A irradiated phthalate solution should be seen. However, no zone of inhibition was detected around disks saturated with To test the possible long-term germicidal activity of phthalate, 0.4 µg of phthalate was put on filter disks (15 µl of 0.0266g/L phthalate solution). Streptomycin was used as a positive control. The appearance of bacterial growth around phthalate disks was the same as the negative control disks containing water (Fig.2).

The positive control disks containing streptomycin showed clear zones of inhibition between 1.1cm ~1.2cm and the negative controls with water showed uninhibited growth on the bacterial lawn. Again, if irradiated phthalate is toxic, then a zone of inhibition phthalate either UV-A irradiated or not. Five replicates of each condition were done on separate plates. No detectable zone of inhibition was observed around any of the disks containing water or phthalate in any of the plates even though the control disk with streptomycin had zones 1.1 to 1.2 cm across.

50 µg/mL of phthalate protected catalase from UV-A damage. Catalase activity was measured as the time that it took the catalase-saturated disks to float to the top of the hydrogen peroxide solution. Active catalase was calculated based on the line of best fit from the standard catalase curves. The equation of the standard catalase curve used for trial 1 was $y = 127.5x^{-0.7737}$ and for trials 2 and 3 was $y = 145.38x^{-0.837}$. Based on the standard curves created, the amount of active catalase present in each sample was determined by measuring the floating time to the top of the hydrogen peroxide solution of filter disks saturated in each condition. Figure 3 shows the relative amount of active catalase detected when catalase solutions were incubated under the different phthalate conditions. For ease of comparison, 50 µg/mL of phthalate was presented in the figures as 50000 µg/L.

In all three of the trials, the catalase control subjected to UV-A radiation had a significantly lower amount of active catalase present than its counterpart that was not subjected to UV-A radiation. The activity of non-irradiated catalase in the absence of phthalate was five times higher than that of the irradiated catalase in trial 1 and three times higher than the irradiated catalase in trial 2. In trial 3, where the UV-A lamp was placed closer to the samples and resulted in more heat, it was observed that non-irradiated catalase in the absence of phthalate had an active catalase concentration 30 times that of the irradiated catalase. Another similar trend observed in all 3 trials was that in the absence of UV-A irradiation, phthalate did not have a significant effect on catalase. The amount of active

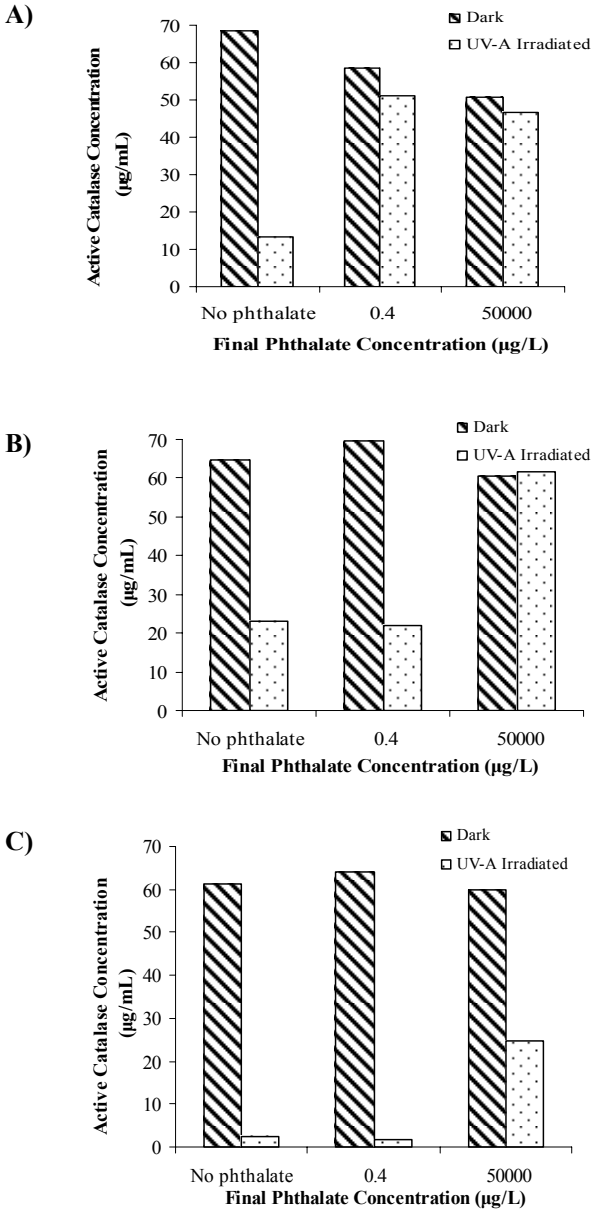

FIG 3. **The effect of phthalate on the active catalase concentration, as calculated using the catalase standard curve.** The initial catalase concentration used was 50 µg/mL. **A)** Trial 1-hydrogen peroxide used was from LIFE, height of UV-A lamp above samples was 11 cm; **B)** Trial 2-hydrogen peroxide used from VWR, height of UV-A lamp above samples was 11 cm; **C)** Trial 3-hydrogen peroxide used from VWR, height of UV-A lamp above samples was 6 cm**.**

catalase in the three dark conditions was similar in each trial. Although an initial catalase concentration of 50 µg/mL was used, a slightly higher concentration of catalase was found in the dark conditions.

In the first trial (Fig.3A), it was observed that the damaging effect of UV-A on catalase was moderated in solutions containing phthalate at either concentrations (0.4 µg/L and 50000 µg/L). Compared to the condition where catalase was irradiated with UV-A alone, the activity of catalase in both phthalate conditions were about four times higher. No significant difference was observed between the amounts of active catalase found in the two irradiated phthalate concentrations.

The second and third trials of this experiment used hydrogen peroxide from VWR. Here, a slightly different trend from the first trial was observed (Figures 3B and 3C). In the second and third trials, the 1:1 ratio of phthalate to catalase (both at 50000 µg/L) seems to have a protective effect on catalase activity when irradiated with UV-A. However, when phthalate concentration was at 0.4µg/L, no protective effect was observed. For both of these trials, the trend observed for catalase incubated with 0.4 µg/L was the same as the control with no phthalate addition. This result is different from the first trial of this experiment where both concentrations of phthalate were found to be protective of catalase irradiated with UV-A. Nevertheless, when phthalate is added at a concentration of 50000 µg/L, a reduction in the damaging effects of UV-A was still observed, although trial 2 showed complete protection and trial 3 showed partial protection.

DISCUSSION

This study looked at two possible mechanisms behind phthalate-enhanced, germicidal effects of SODIS. The first hypothesis speculated that the enhanced effect caused by the addition of phthalate was a result of phthalate being chemically changed due to UV-A and this new compound is toxic to the cells. The second hypothesis was that phthalate was inhibitory to catalase, and thus this compound indirectly kills bacteria by reducing the catalase mediated break down of ROS in the environment, so that the cells were more susceptible to the killing by ROS.

The results suggest that neither mechanism investigated was the cause of phthalate-enhanced germicidal effects of SODIS.

The two experiments that looked at the germicidal effect of phthalate found that phthalate did not have a direct germicidal effect. Even by the irradiation of UV-A, where the possibility of chemically changing phthalate existed, no apparent germicidal effect was observed. One explanation for the lack of effect of irradiated phthalate is that the new compound formed by irradiating phthalate may be reversible upon removal from UV-A irradiation. In order to focus solely on the two mechanisms that we were investigating, bacteria were not incubated together with phthalate under UV-A. This is a limitation to the current study as it did not take into account the formation of toxic intermediates

during phthalate irradiation that may very well have germicidal effect. In addition, the 90 min time course study may have been too short for phthalate to exert any effect on cell growth.

A previous study by Chelala *et al.* has found that the exposure of growth medium containing riboflavin and indole at low concentrations to visible light created photoproducts highly toxic to *Salmonella typhimurium* and other bacteria (5). The main photoproducts formed from phthalates subjected to UV-A irradiation are olefins, alcohols, and phthalic acid anhydride (1), which are much different from the aromatic compounds used in Chelala *et al.*'s study (5). The results of our current study suggest that UV-A irradiated phthalate, in particular, is not directly toxic to bacteria even though literature shows that other aromatic compounds had been found to be toxic after being exposed to visible light. However, it should be noted that the toxicity of UV-A irradiated phthalate might not have been observed at the high bacterial concentration used in this study.

It is possible that we did not observe a change in growth rate because the cells grown in LB were not exposed to high levels of ROS, which is normally produced when water is irradiated with UV-A (12). If phthalate alters growth rate by inhibiting bacterial catalase making the cells more susceptible to ROS killing, the lack of ROS in the growth media would mask any increased susceptibility caused by phthalate. However, no direct inhibitory effect of phthalate on catalase was observed, so phthalate probably would not affect bacterial growth rate through catalase inhibition.

Two catalases, hydroperoxidase I (HPI) and hydroperoxidase II (HPII), are normally produced in *E. coli* to respond to hydrogen peroxide (H_2O_2) found during oxidative stress and stationary phase (11). In general, catalase HPI, present in both the periplasmic space and the cytoplasm (8), protects *E. coli* cells against hydrogen peroxide by dismutating it to water and oxygen via a two electron transfer mechanism (2). It has been previously found that UV-A radiation can directly inactivate catalse by breaking down the blue and UV-A-absorbing chromophores as well as oxidizing tryptophan residues in the catalase, thus altering the chemical and spectral properties of catalase (16). UV-A radiation also induces the aggregation of the catalase peptides, resulting in a structural alteration as shown by a decline in the isoelectric point of the catalase (16). Therefore, as expected, in all three trials of the experiment examining the effect of phthalate on catalase, the catalase control subjected to UV-A irradiation had a much lower amount of active catalase than the corresponding control that was not subjected to UV-A irradiation.

The active catalase concentration measured was higher than the 50 μg/mL supplied initial concentration of catalase used in non-irradiated samples (Fig.3). As the relationship between floating time and concentration was extrapolated from the line of best fit on the standard curve, the slightly higher values reported were within the acceptable error range. Thus, the differences between catalase concentration used and active catalase concentration measured in the non-irradiated samples were insignificant. Another explanation to account for this is the fluctuation in human reflex when timing the floating of the disks. Even though the same person timed each trial of the catalase experiment, it is not certain whether the person's reflex was the same throughout the entire trial.

A discrepancy in trend was observed for the 0.4 μg/L phthalate condition among all three of the trials (Fig.3). In trial 1 (Fig.3A), phthalate at this concentration was as effective in protecting catalase against UV-A as phthalate at a concentration of 50 μg/mL. However, this effect was not observed in trials 2 and 3 (Fig.3B and 3C), where the phthalate concentration of 0.4 μg/L showed effects similar to that of the control condition instead. It is not completely understood why phthalate at a concentration of 0.4μg/L was protective of catalase in trial one. Errors in diluting the phthalate solutions may have occurred without the authors' knowledge. The results from trials 2 and 3 were consistent and therefore it is more likely these results reflect the true effect of phthalate, suggesting that the amount of phthalate typically found in the bottles used in the SODIS system (0.4 μg/L), does not have a protective effect on catalase.

On the other hand, in all 3 of the trials, phthalate added at a 1:1 ratio to catalase showed a protective effect, with significantly more catalase activity as compared to the control. Perhaps when incubated with catalase, phthalate (at a high enough concentration, such as 50 μg/mL used in this experiment) acts as a filter, preventing UV-A from breaking down the chromophores of catalase. This protective mechanism of phthalate can be explained by the presence of π-electrons in conjugated bonds in the aromatic configuration of phthalate. The π-electrons have absorption maxima at the UV region, and the electrons actively absorb UV light for excitation (6). Since π-electrons absorb energy from UV-A light, the presence of phthalate can lead to a decrease in the intensity of UV-A irradiation and therefore reduce the degree of catalase inactivation.

Trial 3 only showed a partial effect at 50 μg/mL of phthalate as compared to full catalase activity protection seen in trials 1 and 2, where the catalase activity at 50 μg/mL phthalate was comparable to that of the non-irradiated catalase control. This could be due to technical difficulties on the day of the third trial resulting in the UV-A lamp being placed closer to the samples (6 cm) than in the previous trials (11 cm). This

created more heat in the bottles of trial 3 that might have facilitated the damaging effect on catalase. Furthermore, the light intensity was increased fourfold as a result of reducing the distance of the light source to the bottle in half. This increase in UV-A intensity would also increase the damage to catalase. To confirm these speculations, more trials of this experiment should be run with a more stringent control of external variables.

Phthalate, as an inhibitory molecule of catalase, was not found to be the mechanism behind phthalate-enhanced germicidal activity. It should be taken into account that some strains of *E.coli* may not be able to transfer phthalate inside the cell due to the lack of OphD permease (4). Even if phthalate does inhibit catalase (which was not observed in our result) by itself or through its photoproducts, the lack of secreted catalase in *E. coli* would prevent the exposure of catalase to the damaging effects of phthalate.

The importance of compartmentalization of catalase was shown by a previous experiment where the antioxidants, α-tocopherol and de-feroxamine, could not protect water-dissolved purified catalase from UV-A irradiation (16). However, when lens epithelial cells from rabbits and squirrels were subjected to UV-A irradiation, pre-incubation of the epithelial cells in antioxidants provided protection on cellular catalases (16). Therefore, the location of catalase under UV-A irradiation may alter the activity of antioxidant and thus degree of damage on catalase by UV-A irradiation. Since this experiment only studied the effect of phthalate on free-floating purified catalase, not intracellular catalase, the experimental result obtained, which demonstrated phthalate's protective activity on free-floating catalase, must be evaluated carefully.

In conclusion, experiments carried out in this study did not provide supporting evidence that the two possible mechanisms investigated were the cause of phthalate-enhanced germicidal effects of SODIS.

FUTURE EXPERIMENTS

As this study showed that phthalate-enhanced germicidal activity of SODIS was not due to the inhibition of catalase or the creation of toxic by-products during UV-A irradiation, other mechanisms should be investigated in future studies. Possibly, the enhanced germicidal effect could be that phthalate interacts with a yet unknown compound released from the water bottle and together, these 2 compounds have a synergetic effect on killing the bacteria in the bottle. Other compounds found to be released from the bottle could be individually added to the experiments to see if they interact with phthalate.

Future research should also include experiments where phthalate is incubated with bacteria in glass bottles rather than in PET bottles, as done by Semenec *et al.* (12), and exposed to UV-A irradiation together, to see if the intermediate product of phthalate formed during irradiation has a toxic effect. Using the same methods and conditions, the experiments performed in Semenec *et al.*'s study should also be repeated, as the result of our current study do not provide evidence that UV-A irradiated phthalate is inhibitory of bacterial growth.

The amount of catalase used in this study was chosen because it is the middle value used in the standard curve. However, this may not represent the actual amount of catalase in the bacteria found in the SODIS system. Future experiments should use catalase purified from bacterial samples to see if the same effects can be observed.

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