

Speeding up solar disinfection (SODIS): effects of hydrogen peroxide, temperature, pH, and copper plus ascorbate on the photoinactivation of *E. coli*

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ABSTRACT

Solar disinfection, or SODIS, shows tremendous promise for point-of-use drinking water treatment in developing countries, but can require 48 h or more for adequate disinfection in cloudy weather. In this research, we show that a number of low-cost additives are capable of accelerating SODIS. These additives included 100–1000 μM hydrogen peroxide, both at room temperature and at elevated temperatures, 0.5 – 1% lemon and lime juice, and copper metal or aqueous copper plus ascorbate, with or without hydrogen peroxide. Laboratory and field experiments indicated that additives might make SODIS more rapid and effective in both sunny and cloudy weather, developments that could help make the technology more effective and acceptable to users.

Key words | drinking water, *E. coli*, Fenton, oxidative stress, point of use, solar disinfection

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TERMS AND ABBREVIATIONS:

CAP	copper, ascorbate and (hydrogen) peroxide
CFU	colony-forming unit: amount of a culturable organism required to form a single colony on culture media
FCRW	filtered Charles River water
LB	Luria-Bertani
LD ₅₀	lowest dose lethal to 50% of test organisms
LJ	lemon juice
MGRR	merry-go-round photoreactor
NGO	non-governmental organization
NUV	near-UV (290–400 nm)
PET	polyethylene terephthalate
POU	point-of-use
ROS	reactive oxygen species
SLJ	sweet lime juice
SODIS	solar disinfection
UV	ultraviolet
UVA	320–400 nm

UVB	290–320 nm
UVC	100–290 nm
DPD	N, N-diethyl-p-phenylene diamine

INTRODUCTION

Point-of-use (POU) drinking water treatment technologies can significantly reduce morbidity and mortality from diarrheal disease in developing countries (Sobsey 2002). A number of low-cost point-of-use technologies, including chlorination, sub-micron filtration, and solar disinfection (SODIS) are currently being promoted worldwide. While potentially effective, each technology has drawbacks in terms of reliability, convenience, or taste and odor. Thus, it is advantageous to have a variety of low-cost POU treatment technologies to choose from.

SODIS is inexpensive and simple to use, relying upon sunlight to inactivate pathogens in drinking water. Water of low turbidity (< 30 NTU) is poured into a clean, transparent polyethylene terephthalate (PET) bottle, shaken to increase

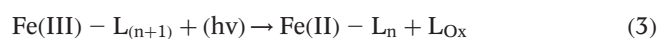
dissolved oxygen, and exposed to direct sunlight for at least 6 h (EAWAG/SANDEC 2006). However, on cloudy days, an exposure period of 48 h or more may be required to achieve inactivation of indicator bacteria to below detectable levels (Oates *et al.* 2003; EAWAG/SANDEC 2006) and sometimes even 2 d are not sufficient (Parsons 2002). Additionally, this technology is limited by the availability and useful lifetime of plastic bottles—typically a few months, (Wegelin *et al.* 2001; Altherr 2004) and by the availability of adequate space in direct sunlight, especially in dense urban slums. Finally, many users object to the amount of work required to perform solar disinfection (Rainey 2003). The effectiveness and acceptability of SODIS could therefore be improved if the process could be accelerated to achieve maximum disinfection within several hours, even in cloudy weather, and with minimal effort.

Several groups have already described modifications to accelerate SODIS. Sunlight exposure combined with water temperatures of 45–50°C resulted in rapid, synergistic inactivation, (Wegelin *et al.* 1994; Sommer *et al.* 1997; McGuigan *et al.* 1998) leading to the use of reflectors (Kehoe *et al.* 2001; Rijal & Fujioka 2003; Mani *et al.* 2006), bottles with the bottoms painted black (Sommer *et al.* 1997), and thermoregulated reactors (Sommer *et al.* 1997) to accelerate the process. Several groups have used TiO₂ as a suspended catalyst or coated onto a support to accelerate the photoinactivation of bacteria, (Ireland *et al.* 1993; Dunlop *et al.* 2002; Salih 2002; Rincon & Pulgarin 2004, 2006) while others have proposed the use of photosensitizers such as methylene blue in SODIS (Acher & Juven 1977; Wegelin *et al.* 1994). These modifications have proven effective and promising, but still pose problems. Reflectors, painted bottles, and thermoregulated reactors may still be inadequate to raise water temperatures to 45°C on cloudy days, while additives such as TiO₂ and methylene blue may be difficult to obtain in many areas and difficult to remove from drinking water prior to consumption; their long-term health effects must also be considered before they are used in drinking water. To the best of our knowledge, most of these modifications are not currently being widely used in the field.

In this study, we sought to identify additional safe, readily available, and inexpensive additives capable of accelerating solar disinfection. Choices of potential additives were guided by previous studies suggesting possible mechanisms of photoinactivation. A great deal of evidence suggests that

reactive oxygen species (ROS), such as hydroxyl radical, superoxide radical anion, hydrogen peroxide, and singlet oxygen play important roles. Researchers have shown that *E. coli* photoinactivation rates drop drastically in the absence of oxygen (Reed 1997), presumably because oxygen is required for the formation of ROS. *E. coli* photoinactivation rates likewise decrease in the presence of hydrogen peroxide scavengers (Sammartano & Tuveson 1984; Curtis *et al.* 1992; Curtis & Mara 1994; Gourmelon *et al.* 1994), while increasing in the presence of added H₂O₂ (Keenan 2003; Fisher 2004; Rincon & Pulgarin 2004). Because H₂O₂ is itself fairly unreactive, it has been suggested that an intracellular Fenton-like mechanism might be important in *E. coli* photoinactivation (Hoerter *et al.* 1996).

The Fenton reaction is defined as the iron-dependent decomposition of hydrogen peroxide (Equation 1). Reduction of Fe(III) perpetuates the reaction, and can occur either in the presence of a chemical reductant (Equation 2), or via photoreduction of ferric iron complexes (Equation 3) (Blough 1995; Halliwell & Gutteridge 1999).



In intracellular Fenton chemistry, the source of Fe(II) may be endogenous, either free or bound to protein moieties such as porphyrins (Tuveson & Sammartano 1986) or iron-sulfur clusters (Fridovich 1998). The resulting hydroxyl radical has the potential to oxidize DNA, cell membranes, and proteins, making it a source of oxidative stress and inactivation (Halliwell & Gutteridge 1999). Others have also proposed that at circumneutral pH, a Fenton-like mechanism may produce a highly reactive species other than hydroxyl radical (e.g. an Fe(IV) species such as (FeO)²⁺) with the potential to inactivate cells (Fridovich 1998; Hug & Leupin 2003; Sharpe *et al.* 2003). Analogous to this intracellular Fenton model, it has been shown that exogenously added Fe(III) and H₂O₂ are capable of dramatically enhancing *E. coli* photoinactivation (Rincon & Pulgarin 2006).

The putative role of Fenton's reaction in *E. coli* photoinactivation led to our investigation of H₂O₂ as a SODIS additive, both at 25°C and at elevated temperatures.

Likewise, the well-known ability of ascorbate to accelerate Fenton's reaction by reducing Fe(III) to Fe(II) led us to explore ascorbate plus iron as SODIS enhancers. When this combination proved ineffective, we explored analogous copper plus ascorbate systems, based in part on the known toxicity and phototoxicity of copper to aquatic organisms (Ragab-Depre 1982; Bjorklund & Morrison 1997; Halliwell & Gutteridge 1999), and on the ability of copper and ascorbate to disinfect wastewater in the dark (Ragab-Depre 1982). Finally, the difficulty of obtaining reagent-grade ascorbate in rural, developing country settings led to our investigation of citrus fruit juices as potential SODIS additives, resulting in the serendipitous finding that the low pH produced by these fruit juices was sufficient to accelerate inactivation.

METHODS

Materials

Chemicals and reagents

All chemicals used were reagent-grade and used as received unless stated otherwise. Unstabilized hydrogen peroxide (30%) was obtained from Fluka Chemie AG, CH-9470 Buchs.

Filtered Charles River Water (FCRW)

Between 02/2002 and 08/2004, water was periodically obtained from the Charles River at the MIT Boat House (Cambridge, MA), and filtered through a 0.2 μM Whatman Nuclepore filter before storage at 4°C. Some samples were prefiltered using glass fiber filters. Unless stated otherwise, filtered river water was used in each experiment.

Bacterial cultures

All laboratory experiments were performed using *E. coli* K12 MG1655 obtained from the laboratory of Dr. Martin Polz at MIT or *E. coli* K12 NCM3722, created in the lab of Dr. Sydney Kustu at UC Berkeley (Soupene *et al.* 2003). Colonies were maintained by continuous culture on Luria-Bertani (LB) agar plates. Liquid cultures were prepared fresh daily in LB broth,

incubated at 37°C to mid-log phase (OD_{600} 0.3–0.5, 2–3 h), harvested by centrifugation, and resuspended in filtered Charles River water (FCRW) or buffer (pH 7) at the desired concentration (typically 10^6 colony-forming units (CFU)/ml). Stationary phase cells were used in pH experiments performed at UC Berkeley. Bacteria were allowed to acclimate for 1 h prior to irradiation.

Analytical methods

Hydrogen peroxide

Hydrogen peroxide was analyzed colorimetrically using the method of Bader *et al.* (1988) as modified by Voelker & Sulzberger (1996), which relies on horseradish peroxidase to catalyze the oxidation of N,N-diethyl-p-phenylene diamine (DPD) by H_2O_2 to a colored product. Samples were quantified based on their absorbance at 552 nm, measured on a UV-Vis spectrophotometer with a 1-cm pathlength using a molar extinction coefficient of $22,000 \text{ M}^{-1}\text{cm}^{-1}$. Our detection limit was 500 nM with a linear range of 500 nM to 50 μM . Concentrations above 50 μM were diluted to within this range prior to measurement. Because *E. coli* cells degraded hydrogen peroxide during experiments, concentrations were reported as the average of initial and final concentrations unless stated otherwise.

Dissolved copper

Dissolved copper was analyzed colorimetrically using the method described by Bjorklund & Morrison (1997), modified as follows: to each 1.5 ml sample, hydroxylamine (150 $\mu\text{l} \times 0.035\text{M}$) was added to reduce Cu(II) to Cu(I), which was then chelated by bathocuproine (150 $\mu\text{l} \times 0.012\text{M}$), forming a bright yellow-orange complex. Total dissolved Cu was determined based on absorbance at 484 nm, using a 1-cm pathlength and a molar extinction coefficient of $14,500 \text{ M}^{-1}\text{cm}^{-1}$.

Bacterial enumeration

Laboratory. *E. coli* colony-forming units were enumerated in the laboratory using the spread-plate method on LB agar and standard plate-count techniques.

Field. In field studies, *E. coli* and total coliform bacteria present in environmental samples were exposed to sunlight in PET bottles and enumerated using the membrane filtration technique with 0.45 μm membrane filters on Hach mColiblu24 broth. This broth contained both a pH indicator to detect acid produced by lactose-fermenting bacteria (presumably coliforms), as well as the substrate 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, which enabled *E. coli* to be distinguished from other coliforms by its glucuronidase activity.

Inactivation rate coefficients

First-order inactivation rate coefficients (k) were determined by performing linear regressions on plots of $\ln(\text{concentration [CFU/ml]})$ vs. time irradiated. While most inactivation curves in this study were well described as first-order processes ($r^2 > 0.9$), the use of linear regressions and inactivation rate coefficients is a matter of operational convenience, rather than an assertion that first-order kinetics apply in all cases. To the contrary, some inactivation curves in our work (UC Berkeley lab experiments) and in the literature exhibited shoulders, bimodal decay rates, or sigmoidal shapes. By contrast, shoulders were rarely observed in field trials using indigenous bacteria. Use of threshold time or fluence and final slopes (Wegelin *et al.* 1994) or time/fluence required for three-log inactivation instead of first-order rate coefficients would not have affected our general conclusions. Error bars shown for first-order rate coefficients are the standard error of the slope calculated from the linear regression of a single experiment unless stated otherwise. Thus, a large error bar indicates either poor data quality (large scatter around the fitted line) or a poor fit of the data to a straight line, e.g. the presence of a large shoulder. In Figure 12 only, lag-corrected k values were calculated by removing data points in the shoulder period before performing regressions.

Experimental procedures

Laboratory irradiation experiments

MIT. Samples were irradiated using an ozone-free 450 W Xe arc lamp (Oriol Instruments, Stratford, CT). Infrared

(IR) radiation was eliminated with a liquid filter. Two Oriol airmass filters (AM-0 and AM-1) eliminated ultraviolet-C and most ultraviolet-B light and reproduced an approximate solar spectrum. The manufacturer's published spectrum for a 1000 W lamp with very similar relative intensities to the 450 W lamp used in our study is shown in Figure 1 with intensities at each wavelength multiplied by published transmittance values for the filters used (Oriol Instruments). Samples were irradiated from below in quartz test tubes in a merry-go-round photoreactor (MGRR) and remained at room temperature (22–25°C) throughout the irradiation. For experiments requiring temperatures above 25°C or the addition of copper metal, a continuously-stirred, quartz-bottomed water-jacketed batch reactor was used in combination with a recirculating water bath. Samples consisted of *E. coli* K12 ($\sim 10^6$ CFU/ml) in FCRW.

UC Berkeley. Experiments on the effects of pH were performed at UC Berkeley. Samples were irradiated using an ozone-free 1000 W Xe arc lamp housed in an Oriol Solar Simulator (Model 91194-1000), which projected an 8 × 8 inch beam of collimated light. Two different Oriol filter sets were used to approximate the solar spectrum on sunny (AM 1.5:G:A “global” and 8107 “atmospheric” filters) and cloudy days (AM 1.5:G:A “global” and AM 1.5:G:C “UV-BC-blocking” filters) (Figure 1). Solar simulator and Berkeley sunlight spectra were measured using International Light RPS 200 and RPS 380 portable UV-VIS spectroradiometers. Samples were maintained at 20°C in a recirculating water bath and continuously stirred in 55 × 100 mm black-painted glass beakers on a stir plate. The beakers were kept uncovered and irradiated from above. Samples consisted of *E. coli* K12 ($\sim 10^6$ CFU/ml) in buffers consisting of either approximately 20 mM total phosphate (mono + dibasic) + 10 mM NaCl or else phosphate + citrate (total buffer strength approximately 10 mM) with no added NaCl.

In all experiments, aliquots were removed at regular intervals and plated immediately. Dark controls were prepared and sampled at approximately the same time as irradiated solutions for all experiments. For room temperature studies, dark controls were stored in foil in a drawer to eliminate light. For elevated temperature studies, dark controls were immersed in the hot water bath providing

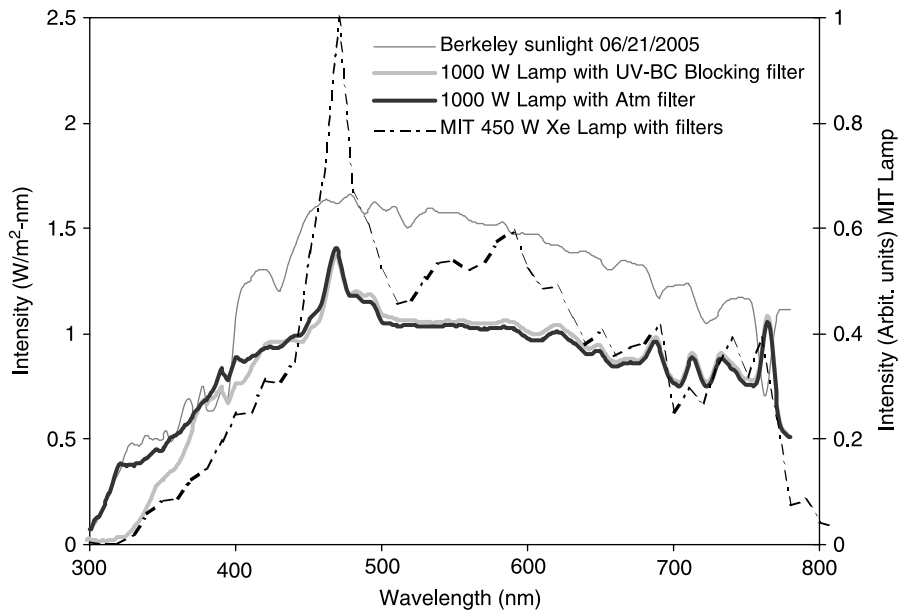


Figure 1 | Irradiance spectra for sunlight, UCB 1000W solar simulator with two different filter sets, and MIT 450W lamp with filters. Sunlight and UCB spectra were measured directly, whereas the spectrum shown for MIT was calculated from the manufacturer's published 1000W lamp spectrum and corrected for the filters used.

the water that circulated through the batch reactor's glass jacket to maintain constant reactor temperature. Due to heat losses in the lines connecting the water bath and reactor jacket, as well as to the insulating effects of the glass jacket, the reactor remained 2 to 4°C cooler than the water bath. As a result, dark controls experienced slightly higher temperatures than irradiated samples. Additives were added from stock solutions (1M hydrogen peroxide, 100 mM copper sulfate, and 100 mM ascorbate) and mixed immediately prior to irradiation to minimize pre-irradiation losses or reactions. Because Cu catalyzes the oxidation of ascorbate by oxygen, copper and ascorbate stock solutions were prepared immediately prior to each experiment and added to samples within 1 min of beginning irradiation. All tubes and reactors used in experiments involving copper or ascorbate were acid-washed before each use. Lengths of 18-gauge copper wire were used in copper metal experiments to ensure consistent surface area between trials.

Field trials

Jolivet, Haiti. Fieldwork was conducted at the Missions of Love clinic in Jolivet, Haiti, near Port de Paix. Water was collected from Les Trois Riviere, a contaminated local river

used widely as a community water source, by means of a *sous dlo*—a small hole dug in the sandy bank through which water infiltrates. Although it is locally believed that water collected from a *sous* is purer than that from the river, studies have suggested that there is no significant difference in concentrations of indicator bacteria in the two sources (Brin 2003). Water was exposed to sunlight in PET bottles as described by EAWAG (EAWAG/SANDEC 2006). Turbidity was field-checked according to the method outlined by EAWAG (EAWAG/SANDEC 2006) prior to exposure, and was acceptable in every case. Samples were exposed on a piece of corrugated steel on the roof of a jeep from 9:00 to 15:00, and the desired quantity of 3% hydrogen peroxide (over-the-counter, obtained in Cambridge, MA) was added to some samples. Water temperatures and weather conditions were recorded hourly, and aliquots were removed for bacterial analysis. Control bottles were stored in the dark.

Bombay, India. Field trials were performed in Bombay, India using water collected from a roadside drainage ditch in the Evershine Negar neighborhood of Bombay's Malad (West) district. Water was exposed to sunlight in 1-L PET bottles as described by EAWAG (EAWAG/SANDEC 2006) on the rooftop of an apartment building in Evershine Negar. The sample was diluted approximately four-fold with sterile

bottled water until it was possible to see clearly through the bottle (turbidity was not measured). pH and temperature were measured periodically using a Hanna HI-98103 digital pH meter and the temperature gauge of an Extech CL-200 chlorine meter. Sweet lime (nimbu) juice and 6% hydrogen peroxide were obtained from local markets and added to some samples. Controls were stored in the dark. Light intensity was measured using a TES-1332A Digital Illuminance Meter (TES Test Instruments, Taipei, Taiwan). This meter primarily measured intensity between 480 and 660 nm, with a relative sensitivity curve similar to the photopic vision sensitivity curve published by the International Commission on Illumination (Ohno 1997), making it a semi-quantitative field method for gauging differences in light intensity between trials.

RESULTS

No additives

Inactivation rate coefficients for *E. coli* observed for laboratory and sunny field trials in this study were comparable, ranging from 0.8–5.1, and from 1.3–5.3 h⁻¹, respectively (Table 1), and were within the range of published values for field inactivation rate coefficients, which ranged from 0.4– > 5.5 h⁻¹ (Table 2). In the absence of any additives, culturable *E. coli* cell concentrations were observed to decline at a mean rate of 2.0 h⁻¹ with a standard deviation of 0.1 h⁻¹ in MIT laboratory studies using a MGRR (Figure 2); batch reactor results were similar (Table 1). An inactivation rate coefficient of 5.1 h⁻¹ was observed using the UC Berkeley solar simulator with an atmospheric filter, compared to 0.82 h⁻¹ when the UV-BC-blocking filter was used instead.

Hydrogen peroxide

Laboratory results

Inactivation rate coefficients increased linearly with added [H₂O₂] (Figure 3). The slope of a linear regression of *k* values vs. average [H₂O₂] over the range 0–500 μM was 0.0037 h⁻¹μM⁻¹; the presence of 500 μM H₂O₂ (average concentration) approximately doubled the inactivation rate coefficient. [H₂O₂] decreased in the presence of *E. coli*

(Figure 4), presumably scavenged by peroxidase and catalase (Seaver & Imlay 2001a, b).

Field results

Haiti. Added H₂O₂ also accelerated *E. coli* and total coliform inactivation rates in field trials. In Haiti, increased H₂O₂ generally led to faster inactivation of both *E. coli* and total coliforms, relative to controls without added H₂O₂ (Figure 5a, b). Cloudy weather led to slower inactivation in both the nothing-added and 500 μM conditions on January 16, but the relative enhancement of H₂O₂ was still quite significant on this cloudy day. Water temperatures in these experiments ranged from 25 to 43°C.

India. In India, adding 200 μM hydrogen peroxide to contaminated ditchwater collected in Bombay's Malad (W) district dramatically enhanced the inactivation rate of environmental strains of *E. coli* (Figure 6a) and total coliforms (Figure 6b). Peak sunlight intensity occurred at the beginning of the experiment (Figure 6c) and water temperatures declined steadily from 29.5°C to 26.5°C between 13:00 and 18:30, with cloudy weather throughout.

Hydrogen peroxide and elevated temperatures

Temperatures of 35° and 45°C enhanced the effects of hydrogen peroxide and light. Compared to the more modest enhancements observed at room temperature (~25°C, Figure 3), at 35°C added hydrogen peroxide at concentrations as low as 100 μM dramatically increased inactivation rates in the light, while having no effect in the dark (Figure 7). At 45°C, added hydrogen peroxide at 100 and 1000 μM was able to produce significant inactivation in the dark, and even greater inactivation in the light. Hydrogen peroxide concentrations decreased over the course of the experiment at 25° and 35° C but seemed to increase slightly at 45°C (data not shown).

Copper sulfate and ascorbate

A combination of dissolved copper sulfate and ascorbate was found to increase the inactivation rate coefficient (*k*) with an approximately linear dependence on each reagent (Figures 8a, b). Increasing copper concentration from 0.1 μM to 2.5 μM in the

Table 1 | Inactivation rate coefficients for irradiation trials in the laboratory and field (no additives). Fields left blank correspond to data that were not reported or did not apply

k (h^{-1})	# of trials	Std. Dev. (h^{-1})	Light source	Condition (Max Temp)	Organism	Location, Date	Citation
2.0	4	0.09	450 W Xe Arc Lamp + Filters (Figure 1)	Laboratory MGRR (25°C)	<i>E. coli</i> K12	Cambridge, MA 2003	(Fisher 2004)
2.1	1		450 W Xe Arc Lamp + Filters (Figure 1)	Laboratory Batch Reactor (25°C)	<i>E. coli</i> K12	Cambridge, MA 2003	(Fisher 2004)
5.1	1		1000 W Xe Arc Lamp + atmospheric filter (Figure 1)	Laboratory Batch Reactor (25°C)	<i>E. coli</i> K12	Berkeley, CA 2005	Present Work
0.8	1		1000 W Xe Arc Lamp + UV-filter (Figure 1)	Laboratory Batch Reactor (25°C)	<i>E. coli</i> K12	Berkeley, CA 2005	Present Work
3.4	1		Sunlight (14:00-18:00)	Sunny (32.5°C)	<i>E. coli</i> K12	Cambridge, MA 6/24/2004	Present Work
2.8	1		Sunlight (13:00-17:00)	Sunny	<i>E. coli</i> K12	Bombay, India 07/30/2005	Present Work
0.1	1		Sunlight (13:00-19:00)	Cloudy (29.5°C)	<i>E. coli</i> (Wild)	Bombay, India 08/16/2005	Present Work
5.3	1		Sunlight (14:00-17:00)	Sunny (30.2°C)	<i>E. coli</i> K12	Berkeley, CA 09/28/2005	Present Work
0.5	1		Sunlight (15:00-19:00)	Partial Clouds	<i>E. coli</i> K12	Berkeley, CA 09/13/2005	Present Work
1.2	3	0.3	Sunlight (9:00-15:00)	Sunny (43°C)	<i>E. coli</i> (Wild)	Jolivert, Haiti 01/12-17/2003	(Keenan 2003)
0.7	1		Sunlight (9:00-15:00)	Partly Cloudy (36.9°C)	<i>E. coli</i> (Wild)	Jolivert, Haiti 01/16/2003	(Keenan 2003)
0.7	3	0.3	Sunlight (9:00-15:00)	Sunny (43°C)	Total Coliform (Wild)	Jolivert, Haiti 01/12-17/2003	(Keenan 2003)
0.6	1		Sunlight (9:00-15:00)	Partly Cloudy (36.9°C)	Total Coliform (Wild)	Jolivert, Haiti 01/16/2003	(Keenan 2003)

Table 2 | Inactivation rate coefficients from the literature. Fields left blank correspond to information that could not be found in the reference or did not apply

k (h^{-1})	# of trials	Std. Dev. (h^{-1})	Light Source	Condition (Max Temp)	Organism	Location, Date	Citation
1.4	1		Sunlight (9:30-9:30 (24h))		<i>E. coli</i>	Honolulu, HI. Date not reported	(Rijal and Fujioka 2003)
2.9	1		Sunlight (9:30-13:30)	43°C	<i>E. coli</i> ATCC11775	Kochi, India Date not reported	(Mani <i>et al.</i> 2006)
5.1	1		1000 W Oriol Solar Simulator with airmass filter	Lab	<i>E. coli</i> DH5 α	Cambridge, MA 06/24/2004	(Lonnen <i>et al.</i> 2005)
3.2	1		Sunlight (11:00-14:00)		<i>E. coli</i> (locally isolated strain)	Muscat, Oman Date not reported	(Salih 2002)
~1.4	1		Sunlight (13:00-19:00)		<i>E. coli</i> (locally isolated wild and laboratory strains)	Duebendorf, Switzerland	(Wegelin <i>et al.</i> 1994)
0.4	1		Sunlight	Sunny, 30°C	<i>E. coli</i> ATCC 11229	Ontario, Canada (June-Aug, year not reported)	(Shah <i>et al.</i> 1996)
>5.5			Sunlight (9:00-14:00)		<i>E. coli</i>	Beirut, Lebanon (1979-1981)	(Acra <i>et al.</i> 1984)
0.2	1		Sunlight	30°C	Total Coliforms (naturally present)	Cali, Colombia 1993 (exact date not reported)	(Sommer <i>et al.</i> 1997)
0.3	1		Sunlight	45°C	Total Coliforms (naturally present)	Cali, Colombia 1995 (exact date not reported)	(Sommer <i>et al.</i> 1997)
0.3	1		Sunlight	48°C	Total Coliforms (naturally present)	Cali, Colombia 1995 (exact date not reported)	(Sommer <i>et al.</i> 1997)
4.4			Sunlight (9:00-14:00)		Total Coliforms (naturally present in municipal sewage)	Beirut, Lebanon (1979-1981)	(Acra <i>et al.</i> 1984)
2.7	3	0.7	Sunlight (10:00-16:00)	Sunny, 26°C	Fecal Coliforms (naturally present)	Jaipur, India May-June 1997	(Reed <i>et al.</i> 2000)
1.6	3	0.1	Sunlight (10:00-16:00)	Sunny, 18°C	Fecal Coliforms (naturally present)	Mabopane, South Africa, Aug-Oct 1998	(Reed <i>et al.</i> 2000)

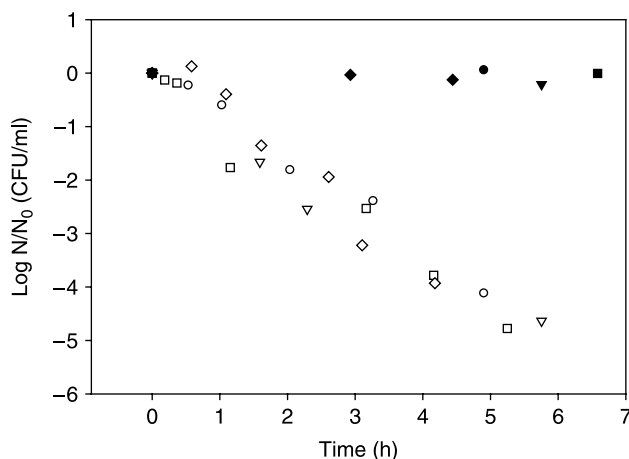


Figure 2 | Rate and reproducibility of inactivation of *E. coli* irradiated in FCRW on four separate dates. All trials were performed in quartz tubes. Open symbols: irradiated tubes; closed symbols: dark controls.

presence of 25 μM ascorbate raised the value of k from 0.9 to 5.3 h^{-1} . In the presence of 2.5 μM copper sulfate, increasing ascorbate from 0 to 37.5 μM raised k from 3.2 to 11.3 h^{-1} . The copper/ascorbate combination was also somewhat effective in inactivating *E. coli* in the absence of light. Ascorbate with little copper added had a protective effect in the light, since with 0.1 μM CuSO_4 and 25 μM ascorbate, the inactivation rate coefficient was a factor of 2.3 lower than the value of 2.0 h^{-1} determined for *E. coli* in the absence of any additives (Figure 2). By contrast, CuSO_4 appeared to have a slight enhancing effect on inactivation even in the absence of ascorbate. Thus, while increasing either copper or ascorbate in the presence of a fixed

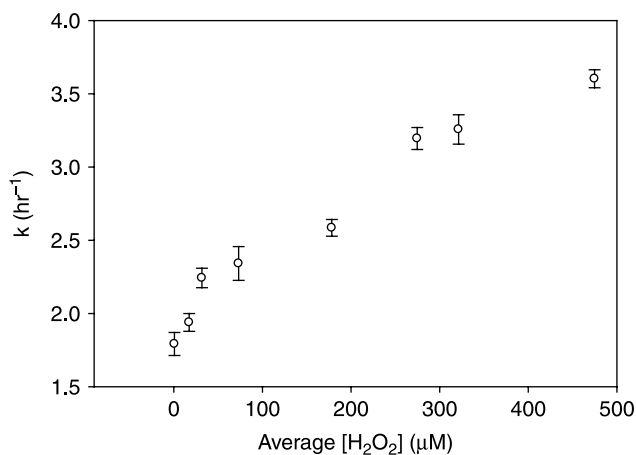


Figure 3 | Inactivation rate coefficients (k) for *E. coli* K12 vs. average hydrogen peroxide concentration. A linear regression of the data points gives a slope of 0.0037 $\text{h}^{-1}\mu\text{M}^{-1}$ with a y-intercept of 2.0 and an r^2 value of 0.95.

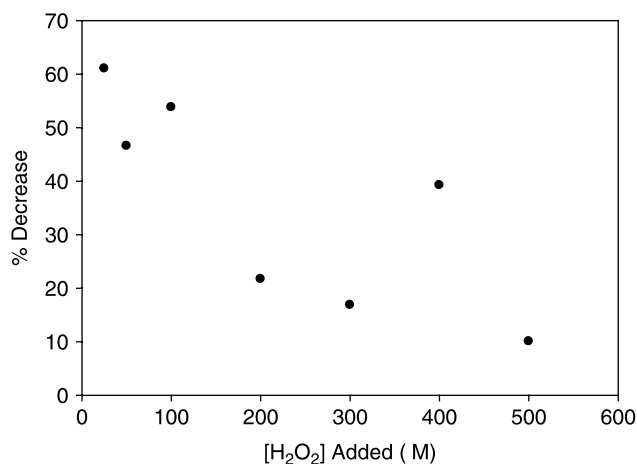


Figure 4 | Percent decrease in hydrogen peroxide concentration during inactivation experiments shown in Figure 3, each lasting 2–3 h. Initial *E. coli* concentrations were between 10^5 and 2.5×10^6 CFU/ml in all experiments.

concentration of the other species increased k in all cases, only the experiments with 2.5 μM Cu and 0–37.5 μM ascorbate produced faster inactivation than the nothing-added condition.

Day-to-day reproducibility of copper plus ascorbate (Cu + Asc) experiments was lower than that observed for experiments with only H_2O_2 or without additives. For example, the inactivation rate coefficients for four different irradiation trials of solutions of 2.5 μM CuSO_4 and 25 μM ascorbate, each with roughly 10^6 CFU/ml *E. coli*, averaged 6.3 h^{-1} with a standard deviation of 2.1 h^{-1} . The rapid disappearance of ascorbate in the presence of copper may contribute to this variability. However, lower variability was observed for experiments performed together on the same day, as shown in Figures 8a and 8b. In all experiments involving ascorbate, data plotted together were from experiments performed together on the same day unless otherwise indicated.

Copper metal and ascorbate

The difficulty of obtaining reagent-grade copper sulfate in rural settings prompted the investigation of a copper metal and ascorbate system, which was also found to substantially increase inactivation rates. In one trial, the addition of four 2.5-cm lengths of 18-gauge copper wire and 200 μM ascorbate to 100 ml of FCRW produced greater than 3-log inactivation in measured *E. coli* CFU concentration within 15 min following a 20-min shoulder, and within 1 h in the dark (data not shown). Aqueous

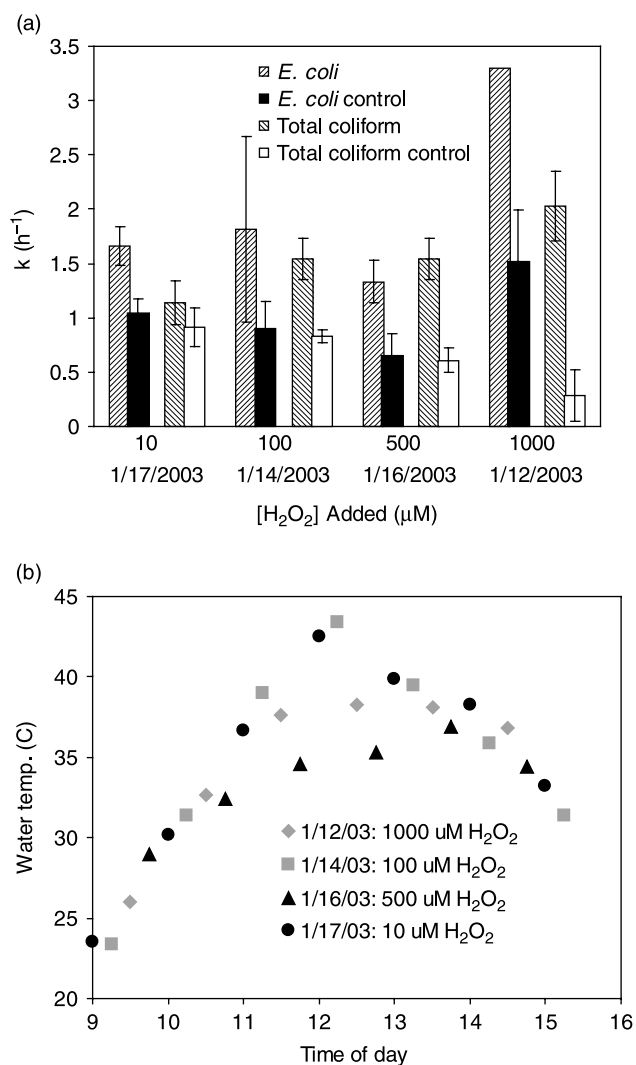


Figure 5 | (a) Inactivation rate coefficients (k) for indigenous *E. coli* and total coliforms in Haiti surface water samples exposed to natural sunlight with and without added hydrogen peroxide on different days. 1/16/2003 was cloudy while the other days were mostly sunny. No detectable *E. coli* remained after 1 h for the 1000 μM H₂O₂ added trial on 1/12/2003; therefore the minimum inactivation rate required to produce <1 detectable colony at 1 h is shown for this condition and an error bar could not be calculated. (b) Water temperature vs. time of day for each of the inactivation experiments plotted in (a).

copper concentrations steadily increased over the course of the light experiment to approximately 8 μM in 2 h. In the dark, no such increase in copper concentrations was observed.

Copper and H₂O₂

Since copper-catalyzed oxidation of ascorbate by oxygen is likely to result in H₂O₂ formation (Halliwell & Gutteridge 1999), we tested the hypothesis that the role of ascorbate in

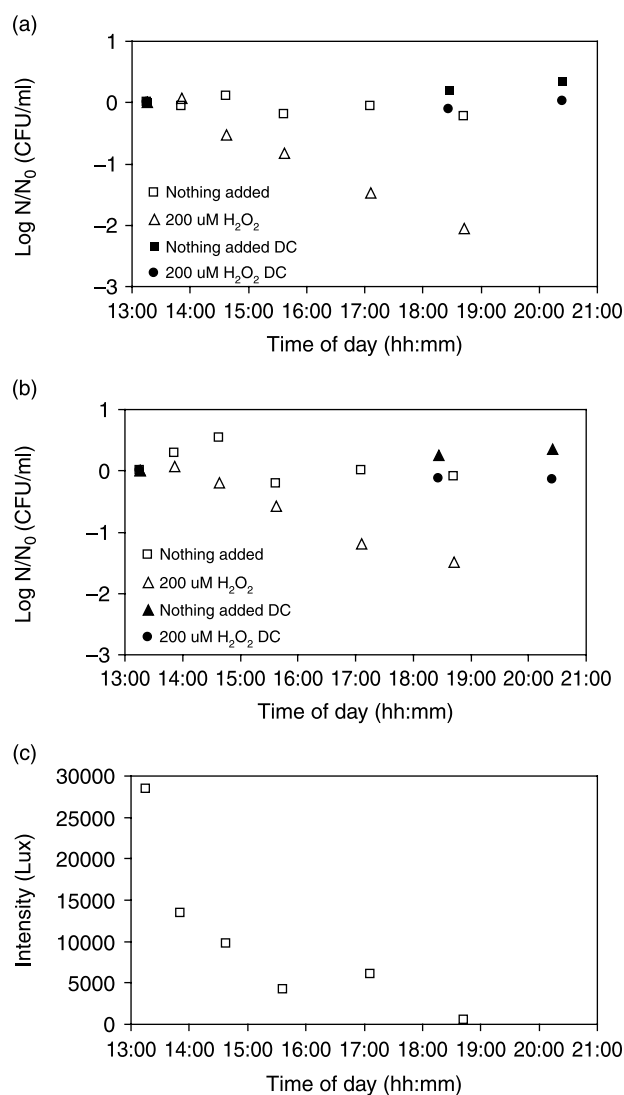


Figure 6 | Inactivation of naturally present (a) *E. coli* and (b) total coliforms vs. time in Bombay ditchwater with nothing or 200 μM H₂O₂ added. DC = dark control. Initial concentrations of both *E. coli* and total coliforms were on the order of 10³ CFU/ml (c).

the copper + ascorbate system was primarily as a source of hydrogen peroxide. H₂O₂ was added to CuSO₄ solutions at concentrations comparable to those expected from the autooxidation of ascorbate. The addition of up to 14 μM H₂O₂ did not significantly affect light or dark inactivation rates in the presence of 2.5 μM CuSO₄ (data not shown).

Copper + ascorbate + H₂O₂ (CAP)

Added H₂O₂ increased k synergistically in the presence of both CuSO₄ (2.5 μM) and ascorbate (25 μM). In the light,

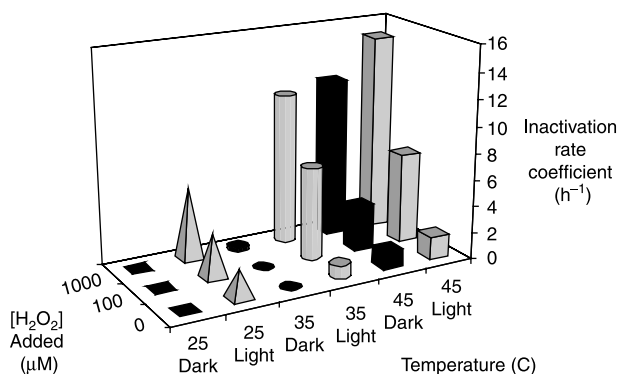


Figure 7 | Inactivation rate coefficient (k) versus temperature and $[H_2O_2]$ added for *E. coli* K12 irradiated in FCRW in a temperature controlled batch reactor. Initial *E. coli* concentrations were between 2×10^5 and 3×10^6 CFU/ml in all experiments; experiments ranged in duration from 2 to 6.5 h.

the presence of Cu + ascorbate amplified the effect of added H_2O_2 28 times (\circ versus dotted line in Figure 9a). While the combination of copper, ascorbate, and hydrogen peroxide (CAP) also produced significant inactivation in the dark, the k observed for CAP in the light (Figure 9a, \circ) was significantly greater than the sum (dashed line) of the rate coefficients for CAP in the dark and for H_2O_2 only in the light.

Citrus juice

Laboratory results

Because pure ascorbic acid may also be unavailable in some regions, citrus fruit juices were examined as a source of ascorbate. Surprisingly, while dramatic enhancement of

inactivation rates was observed in the presence of copper metal and lemon juice, even greater enhancement was observed when lemon juice was added alone, in the absence of copper (Figure 10). No significant inactivation was observed in dark controls for either condition (data not shown). Likewise, the addition of $20 \mu M$ $FeSO_4$ to *E. coli* in a lemon juice solution did not exhibit any significant enhancement, while 1000 mg/L Fe_2O_3 appeared to reduce the enhancing effect of the fruit juice, perhaps due to a light screening effect from the formation of a precipitate (Figure 10). The pH of lemon juice solutions in FCRW is summarized in Table 3.

Field results

Fieldwork conducted in Bombay, India also showed a pronounced effect of citrus juice. Juice from *Citrus limettioides* (Morton 1987), known locally as “sweet lime” or “nimbu,” showed a strong enhancing effect on the rate of SODIS on a cloudy rooftop when added to highly contaminated ditchwater from Bombay’s Malad (W) neighborhood (Figure 11a, b). Specifically, while no significant inactivation occurred in the absence of additives during the low-light, afternoon experiment, more than 2-log inactivation was measured in the presence of 0.5% sweet lime juice (SLJ). The initial and final pH values of 0.5% SLJ in ditchwater were measured as 3.5 and 4.3, respectively, over the course of the 6-h experiment. Previously discussed temperature and sunlight intensity data from other Bombay experiments (Figure 6c) are also applicable here.

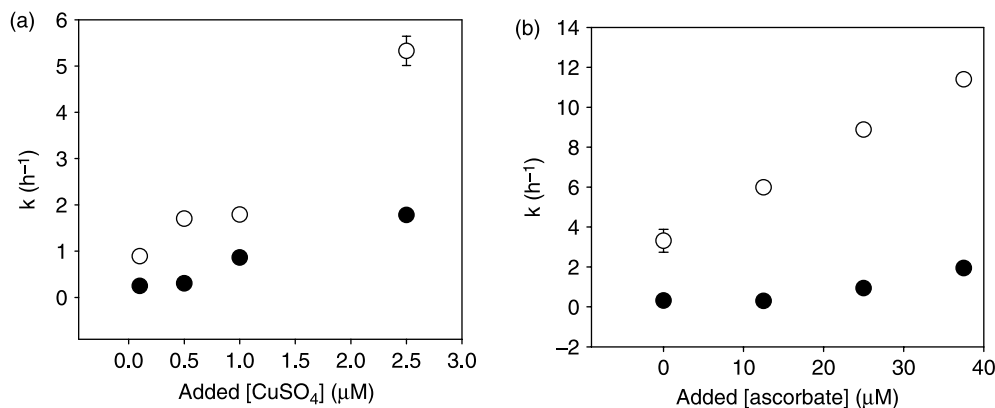


Figure 8 | Inactivation of *E. coli* K12 irradiated in FCRW with added ascorbate and aqueous $CuSO_4$. Inactivation rate coefficients (k) are shown for *E. coli* irradiated in quartz tubes (a) in the presence of $25 \mu M$ ascorbate with varying concentrations of added $CuSO_4$ and (b) for $2.5 \mu M$ $CuSO_4$ with varying concentrations of added ascorbate (b). Open symbols: irradiated tubes; closed symbols: dark controls. Error bars are shown for light inactivation data; where error bars are not visible they are smaller than the symbols used.

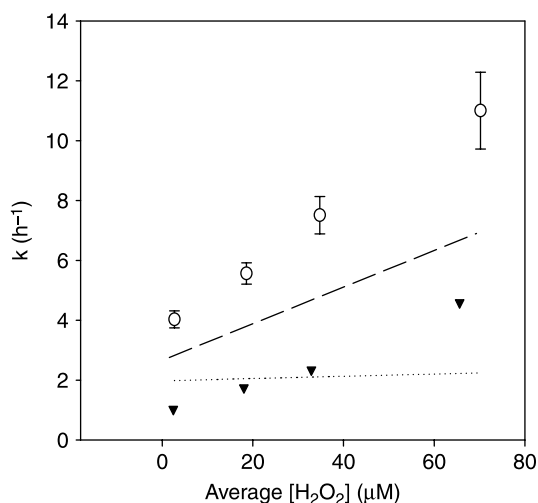


Figure 9 | Light (○) and dark (▼) inactivation rate coefficients (k) in the presence of 2.5 μM CuSO₄ and 25 μM ascorbate vs. [H₂O₂] added (M). These data points yield lines (not shown) with slopes of 0.10 and 0.058 h⁻¹μM⁻¹, respectively. Dotted line shows inactivation rate coefficients for H₂O₂ in the light with no copper or ascorbate (from Figure 3, slope: 0.0037 h⁻¹μM⁻¹). Also shown (dashed line, slope: 0.062 h⁻¹μM⁻¹), is the sum of the dark data from this experiment (▼) and the light data from Figure 3 (dotted line).

pH

The dramatic effects of citrus juice in the absence of copper led to the exploration of low pH as an enhancing factor in SODIS. A series of experiments performed in dilute buffer solutions demonstrated that low pH increased the inactivation rate of *E. coli* in sunlight, both with and without UV-B (Figure 12), while no significant inactivation was

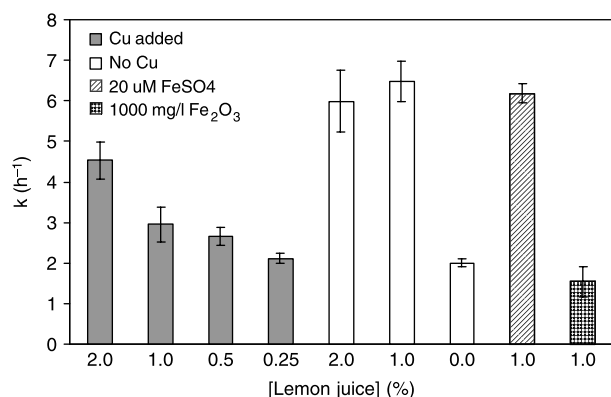


Figure 10 | Inactivation rate coefficient (k) for *E. coli* K12 in 2%, 1%, 0.5%, and 0.25% Lemon juice (LJ) with 0.5 cm of 18-gauge copper wire added (all irradiated together on 06/22/04). Also shown are k values for 2% and 1% LJ alone, 1% LJ with 20 μM FeSO₄, and 1% LJ with 1000 mg/l Fe₂O₃ (all irradiated together on 06/25/04). 0% LJ data are included from Figure 2 for comparison, and the error bar for this condition represents the standard deviation of four replicate trials.

Table 3 | Lemon juice concentration and pH

% Lemon Juice	pH
0.25	4.25
0.5	3.56
1	3.17
2	2.89

observed in the dark over the course of the experiments (data not shown). When the atmospheric filter was used, inactivation rate coefficients increased gradually as pH decreased from 7.0 to 3.0. In the presence of the UV-BC-blocking filter, which absorbed virtually all light below 320 nm and most light below 350 nm, inactivation was extremely slow at pH 7.0 and 5.5, increasing by factors of 2 and 8 at pH 4.0 and 3.0, respectively (Figure 12). While the overall trend of increased k with decreased pH was present

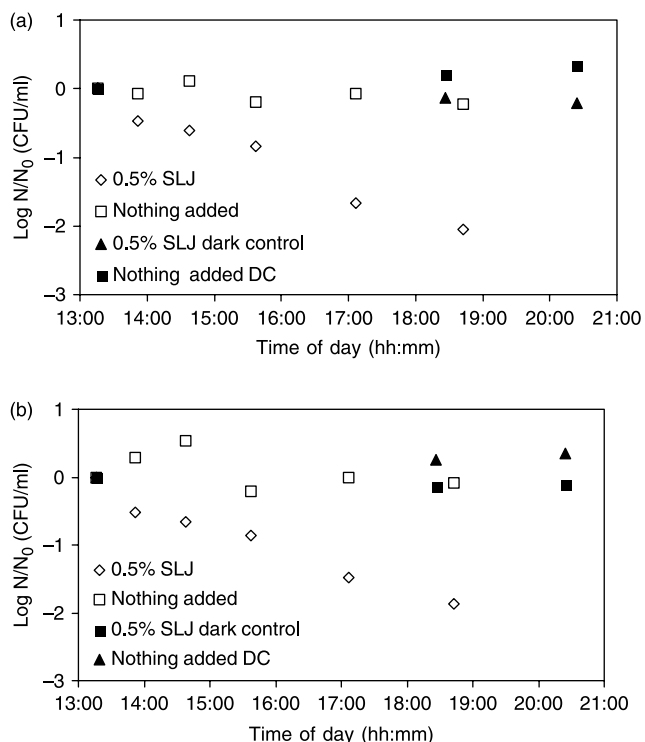


Figure 11 | Inactivation of (a) *E. coli* and (b) total coliforms in contaminated surface water on a cloudy Bombay rooftop with nothing added and with 0.5% sweet lime juice (SLJ). Initial concentrations were on the order of 10³ CFU/ml for both *E. coli* and total coliforms.

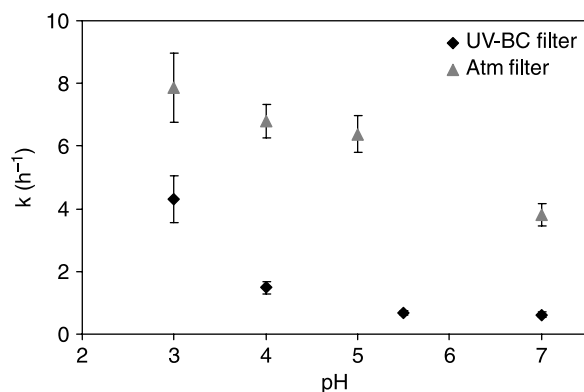


Figure 12 | Inactivation rate coefficient (k) vs. pH for *E. coli* K12 irradiated in 20 mM Phosphate Buffer with UV-BC blocking filter (◆) and lag-corrected k for *E. coli* K12 irradiated in 10 mM phosphate-citrate buffer with atmospheric filter (▲).

over both experiments, the relative enhancement of low pH values was more pronounced when the light below 320 nm was blocked. In all experiments, initial and final pH values differed by less than 0.1 units.

DISCUSSION

Use of additives for enhancing SODIS

The most significant result of our work was the finding that the rate of *E. coli* and total coliform inactivation can be significantly enhanced by the addition of simple chemical additives.

Hydrogen peroxide

The ability of hydrogen peroxide to enhance the photo-inactivation of *E. coli* at room temperature makes it an attractive SODIS additive. Furthermore, the synergistic enhancement of H_2O_2 and temperatures as low as 35°C make it a still more attractive option, since these temperatures are more likely to be reached in the field than the 45–50°C temperatures required for synergistic inactivation in the absence of additives (Wegelin *et al.* 1994; Sommer *et al.* 1997; McGuigan *et al.* 1998), especially in cold, cloudy weather or nontropical climates.

In exploring the effectiveness of H_2O_2 as a SODIS-accelerating additive further, health effects must also be considered. While the long-term effects of consuming low

levels (i.e. 1000 μM or less) of H_2O_2 in drinking water remain unclear, available information suggests that they are of little concern. H_2O_2 has been used as a drinking water additive in Europe (CSTEE 2001) and has been approved for this use by the National Sanitation Foundation (Weiner *et al.* 2000) at concentrations up to 30 mg/L, or 880 μM (US Peroxide 2006). It has been classified as “Generally Regarded As Safe” by the USFDA for use in food processing and as a food additive (Weiner *et al.* 2000).

However, although hydrogen peroxide itself may be safe, the presence of stabilizers in most commercially available H_2O_2 preparations is also a concern. While relatively benign additives such as colloidal stannate and sodium pyrophosphate are routinely found in H_2O_2 used for drinking water treatment (US Peroxide 2006), topical preparations of H_2O_2 may include toxic additives such as acetanilide which are not intended for human consumption (CSTEE 2001). The availability and shelf life of food-grade H_2O_2 preparations in SODIS regions and the ability of users to differentiate these from preparations unfit for consumption must therefore be considered.

Cu + ascorbate

Like H_2O_2 , copper and ascorbate show promise as potential SODIS additives (Figure 8). Studies have already proposed the use of copper and ascorbate without sunlight for the treatment of wastewater (Ragab-Depre 1982) and for therapeutic antiviral use in humans (Sagripanti *et al.* 1997). Both studies confirmed the effectiveness of this antimicrobial combination in the dark at concentrations higher than those considered in the current study (7.7 μM vs. 2.5 μM). Thus, copper and ascorbate could provide additional disinfecting power as SODIS additives on sunny days, as well as an alternative means of disinfection on cloudy days. Furthermore, the addition of very small quantities of H_2O_2 has the potential to dramatically enhance these benefits (Figure 9). However, the rapid disappearance of ascorbate in the presence of even trace amounts of copper may decrease its effectiveness as an additive, especially if added before peak sunlight or stored in an unstabilized aqueous form.

Another caveat of copper-ascorbate-assisted SODIS is the potential complexation of copper by humic substances or other ligands that might reduce its reactivity (Kogut & Voelker

2003). However, the fact that the copper and ascorbate system effectively disinfected wastewater in the dark (Ragab-Depre 1982) and our observation that the irradiated copper-ascorbate system performed efficiently in a natural surface water containing humic ligands (FCRW) suggests that these concerns may not present major problems in the field. Strong interactions between Cu and natural ligands (e.g. citrate) in lemon juice may, however, explain the inability of the ascorbate contained in lemon juice to accelerate SODIS in the presence of copper metal (Figure 10). A more thorough investigation of the effects of complexation on the copper-ascorbate system may help identify more appropriate natural sources of ascorbate for accelerated SODIS.

To the extent that copper and ascorbate are practical and available, health data suggest that these additives should be safe at concentrations that significantly enhance SODIS. Copper is efficiently chelated and sequestered in the human body (Halliwell & Gutteridge 1999), and no effect was observed when copper and ascorbate were fed to mice at many times the doses (in mg/kg body weight) considered in the present work (Sagripanti *et al.* 1997). Finally, the USEPA's secondary drinking water standard (MCLg) for copper, 1 mg/L or roughly 15 μM (USEPA 2003), is well above the highest copper concentration measured in our experiments (7.7 μM). Nonetheless, final concentrations of dissolved copper may be difficult to control when copper metal is the source, and the health effects of concentrations greater than 15 μM should be considered.

While obtaining copper sulfate and ascorbic acid in SODIS regions could be difficult, the finding that copper metal is effective in the presence of ascorbate increases the feasibility of this method considerably. However, readily available sources of ascorbate or an effective alternative must still be identified before copper/ascorbate-assisted SODIS is likely to find practical applications.

Acidic fruit juice

While they were not effective sources of copper-reactive ascorbate, citrus juices proved to be SODIS enhancers on their own. The ability of 1% lemon juice and 0.5% sweet lime juice to accelerate SODIS is perhaps the most readily applicable result of our studies. Moreover, to the extent that it is affordable and acceptable to SODIS users, adding

lemon juice might provide an immediately apparent method of distinguishing between SODIS-treated water and untreated water in the household. Finally, these concentrations of citrus juices are low enough to produce an acceptable or even pleasant taste. Ingestion of dilute citrus juices should not pose health concerns, since stomach acid is far more acidic. However, acidic beverages may pose some risk of tooth decay.

Mechanisms

Added hydrogen peroxide

The observation that k appears to increase linearly with $[\text{H}_2\text{O}_2]$ over the 0–500 μM range suggests that if a Fenton-like reaction occurs, Fe(II) is not the limiting reagent over this H_2O_2 concentration range. This would then imply that Fe(III) is rapidly reduced in irradiated cells. However, future work is required to confirm that the Fenton reaction (Equation 1) is part of the mechanism by which light and H_2O_2 interact synergistically to damage cells.

Also worth exploring is the observation that added hydrogen peroxide decayed over the course of experiments at 25 and 35°C, but not at 45°C. This difference may reflect the more rapid inactivation of cells at 45°C, resulting in diminished opportunity for them to scavenge H_2O_2 , but it is also possible that catalase and peroxidase, the enzymes responsible for scavenging H_2O_2 under normal conditions, are inactivated at 45°C (Ghademarzi & Moosavi-Movahedi 1996). If so, this may be one mechanism by which elevated temperatures and light produced synergistic inactivation.

Low pH

Comparison of the effects of acidic phosphate buffers and citrus juices (Figures 10 and 12) strongly suggests that low pH alone is sufficient to explain the enhanced inactivation by citrus juices and light. Low pH may increase inactivation rate by presenting a significant additional stress to the cell, for example by requiring it to expend energy maintaining pH homeostasis, thus accelerating the depletion of ATP and/or reducing equivalents. This metabolic stress might then reduce the rate at which energy-consuming proteins in the cell can scavenge ROS and/or repair damaged DNA, facilitating more rapid photoinactivation.

These findings differ from those of Rincon and Pulgarin, who found that initial pH values between 4 and 9 did not affect *E. coli* inactivation rates (Rincon & Pulgarin 2004). This difference may be due to the fact that this group used a different light source in their work, and only tested pH values of 4 and above, while the most dramatic effects observed in this study were at pH 3. Furthermore, while Rincon and Pulgarin's constant pH experiments did not exhibit an effect of pH, they did report enhanced inactivation when pH was gradually decreased from 7 to 4 over the course of an experiment (Rincon & Pulgarin 2004). Taken together, the differences in light source, pH values tested, and experimental conditions may account for much of the disparity between this work and theirs. Given the different effects of low pH on inactivation rates by different light sources (Figure 12), a comparison of photoaction spectra at different pH values may be informative.

Copper and ascorbate

While the ability of copper, ascorbate, and hydrogen peroxide to inactivate cells has been previously demonstrated, the ability of light to enhance these systems is of interest and has not, to our knowledge, been previously reported. Previous groups have suggested a variety of mechanisms for the CAP system in the dark, the most convincing of which is that ascorbate reduces Cu(II) to Cu(I), allowing it to react more rapidly with H₂O₂ in an Udenfriend-like mechanism ("Udenfriend" refers to the analogous reaction with Fe, ascorbate, and H₂O₂). In such a mechanism the presumed target would be extracellular, and the role of light in enhancing toxicity is unclear, since ascorbate already acts as a reductant. Further work is required to determine whether the action of the CAP system is primarily extra- or intracellular, and what role light plays in enhancing its toxicity.

Sources of variability in field trials

While inactivation curves for *E. coli* K12 irradiated in the lab were found to be quite reproducible for each combination of light source, filters, and reactor, our fieldwork results varied more between trials and locations. First and foremost, the inactivation rates for *E. coli* and total coliforms in the absence of additives were found to be

essentially 0 in some Bombay experiments, while some inactivation was always observed in Haiti. This difference may be due to the fact that Bombay studies were performed on cloudier, cooler days, and begun later in the day than Haiti trials. Other possible sources of variability include growth stage of cells (Berney *et al.* 2006), the use of indigenous strains, and differences in turbidity between trials. Furthermore, while the mColiblu24 medium enables the specific identification of *E. coli*, which appear as blue colonies, it does not distinguish between species of non-*E. coli* coliforms, which all appear as red colonies, and differences in the species and strains of non-*E. coli* coliforms from one environmental sample to the next could have had a significant effect on inactivation rates. All of these factors, combined with variations in season and latitude, could explain why our and other groups' published rates can vary by an order of magnitude or more.

CONCLUSIONS

While this study demonstrates the effectiveness of SODIS interventions in accelerating the inactivation of indicator organisms by simulated sunlight, additional research with actual pathogens under a variety of conditions will be required before these findings can be generalized to realistic field conditions. However, the proven effectiveness of conventional SODIS against a broad spectrum of pathogens suggests that factors able to accelerate the inactivation of one organism may also be effective against others. If so, the potential benefits could be considerable, including not only rapid disinfection under cloudy conditions, but also routine disinfection in larger, more practical containers. Further work elucidating the mechanisms of SODIS in the presence and absence of enhancing additives may contribute to the realization of these benefits by suggesting economical means of optimizing the performance of SODIS in both favorable and unfavorable weather conditions.

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