Synergistic effect of heat and solar UV on DNA damage and water disinfection of *E. coli* and bacteriophage MS2

Dana Jennifer Theitler, Abid Nasser, Yoram Gerchman, Abraham Kribus and Hadas Mamane

ABSTRACT

The response of a representative virus and indicator bacteria to heating, solar irradiation, or their combination, was investigated in a controlled solar simulator and under real sun conditions. Heating showed higher inactivation of *Escherichia coli* compared to the bacteriophage MS2. Heating combined with natural or simulated solar irradiation demonstrated a synergistic effect on the inactivation of *E. coli*, with up to 3-log difference for 50 °C and natural sun insolation of 2,000 kJ m^{-2} (compared to the sum of the separate treatments). Similar synergistic effect was also evident when solar-UV induced DNA damage to *E. coli* was assessed using the endonuclease sensitive site assay (ESS). MS2 was found to be highly resistant to irradiation and heat, with a slightly synergistic effect observed only at 59 °C and natural sun insolation of 5,580 kJ m^{-2}. Heat treatment also hindered light-dependent recovery of *E. coli* making the treatment much more effective.

Key words | DNA damage, endonuclease sensitive site (ESS), heat inactivation, insolation, recovery, solar disinfection

Dana Jennifer Theitler
Abraham Kribus
Hadas Mamane (corresponding author)
School of Mechanical Engineering,
Faculty of Engineering, Tel-Aviv University,
Tel Aviv, 69978,
Israel
E-mail: *hadasmg@post.tau.ac.il*

Abid Nasser
Water Quality Research Laboratory,
Ministry of Health,
Tel-Aviv, 61082,
Israel

Yoram Gerchman
Department of Biology and Environment,
University of Haifa at Oranim,
Tivon, 36006,
Israel

INTRODUCTION

Point-of-use (POU) technologies used in developing countries to improve water quality via inactivation of bacteria and viruses include chlorine tablets, solar disinfection, ceramic filters, combined flocculation and disinfection, and boiling (WHO 2007). Solar Water Disinfection (SODIS) is a simple, effective and low-cost POU technology for the treatment of drinking water in developing countries. Solar disinfection is based on UV energy, heating, or an additive or synergistic effect of these two processes. Effective combination of solar UV and thermal radiation can produce a greater level of microorganism inactivation than either treatment used separately. Wegelin *et al.* (1994) and Sommer *et al.* (1997) found that the water temperature has to reach at least 50 °C to achieve a synergistic effect of UV radiation and heating on bacterial inactivation. Although much research has been done in the field of solar disinfection, there is still room for improvement in terms of increasing volumes of treated water and reducing inactivation time. Enhancement technologies for SODIS include addition of TiO$_2$ (Gelover *et al.* 2006), addition of riboflavin (Alotaibi & Heaselgrave 2011), use of compound parabolic concentrators (CPCs) (Ubomba-Jaswa *et al.* 2009) and use of SODIS bags (Saladin 2010).

The mechanism underlying inactivation by SODIS is not fully understood, and it might be related to protein damage, DNA damage and/or increased cell-wall permeability. Bosshard *et al.* (2010) indicated that solar UV radiation probably damages proteins via oxidative stress. They showed that under a simulator UVA lamp set to 1,000 kJ m^{-2}, many different proteins were aggregated (suggesting protein oxidation), among them Dps, which is responsible for DNA protection and repair. To assess DNA damage, an endonuclease sensitive site (ESS) analysis can be performed, to determine the number of induced pyrimidine dimers in the genomic DNA. Recent studies using the ESS method are used to assess UVC disinfection.

For example, Oguma *et al.* (2001) examined the pyrimidine dimers in *E. coli* and *Cryptosporidium parvum* following UVC exposure, photoreactivation and dark repair. Eischeid & Linden (2007) investigated the formation of pyrimidine dimers in *E. coli* under different UVC doses and wavelengths. Yet, DNA damage by solar irradiance was rarely investigated (Kuluncsics *et al.* 1999) and DNA damage by combined heat and solar irradiation has not been previously evaluated.

SODIS is more efficient at inactivating bacteria than other types of microorganisms, such as phages, protozoa or fungi (Lonnen *et al.* 2005; McGuigan *et al.* 2006). Sinton *et al.* (2002) examined sunlight inactivation of bacteriophages and fecal indicator bacteria in fresh water, seawater and a 50:50 mixture of waters. Both a somatic coliphage and an F-RNA phage were found to be more resistant to sunlight than bacteria, suggesting F-RNA phages as a better model for virological water quality monitoring in fresh water than *E. coli*. The bacteriophage MS2, which is relatively resistant to heat and UV irradiation (Walker *et al.* 2004), is a popular model microorganism for UV disinfection studies, and was selected for the current work.

The response of the representative virus MS2 and the indicator bacteria *E. coli* to heating, solar irradiation and their combination was investigated, using both simulated and natural sunlight, with emphasis on influence of the season for *E. coli* with natural sunlight. The impact of these conditions with the simulated sun was examined by quantitative ESS assay for *E. coli*. Finally, recovery after heat treatment was also examined.

MATERIALS AND METHODS

The experiments were conducted under various conditions of heat and solar irradiation to estimate the contribution of each factor to inactivation rates.

Model organisms

Escherichia coli K-12 (ATCC 2363) was used as the test bacterium in this study, due to its widespread use as a fecal indicator (US-EPA 2006). The bacterium was maintained in the laboratory by passing once a week on mFC selective agar (Difco, cat. no. 267720) and cultivating to a high concentration in nutrient broth. Prior to each experiment, a single colony of *E. coli* was inoculated into 5 mL sterile liquid tryptone broth (BD, cat. no. 211705) and incubated overnight at 37 °C, then transferred into a 500-mL Erlenmeyer flask containing 45 mL of tryptone broth and shaken at 37 °C and 125 rpm for 4–6 h to obtain approximately 10^9 CFU mL^{-1}.

Bacteriophage MS2 (ATCC 15597-B1) was used as a model virus. MS2 is a single-stranded polyhedral RNA virus with a diameter of 0.026 μm. MS2 was selected since it belongs to genotype I of the F-specific RNA bacteriophage, which has been found to be the most environmentally resistant serogroup (Schaper *et al.* 2002), and because it has been shown that there is a positive correlation between its presence and the presence of pathogenic viruses in water (Borrego *et al.* 1987). MS2 bacteriophages were propagated as needed on its *E. coli* K12 host. Prior to each experiment, bacteriophages were cultivated and purified as previously described (Nasser *et al.* 2002). Studies showed that MS2 is very resistant, and was unaffected by solar UV wavelengths (above 295 nm), while *E. coli* inactivation by these wavelengths was very rapid compared to viruses (Mamane *et al.* 2007) indicating the suitability of using both organisms for evaluating two sensitivity extremes of solar treatment processes.

Cultivation and enumeration of the microorganisms

Bacterial inactivation: *E. coli* K12 grown in tryptone broth (10^9 CFU mL^{-1}) was diluted to a final concentration of 10^6 CFU mL^{-1} in deionized (DI) water at ambient temperature or in deionized water that was preheated to a designated temperature. In the preheated water samples, *E. coli* was enumerated to determine the impact of heating only. In other treatments, water-bacteria mixtures were exposed to solar irradiation as indicated to determine the effect of irradiation only or exposed to preheated water to obtain the combined heating and solar irradiation effect. Control samples were kept in the dark at ~22–23 °C. In all experiments water samples were collected at the designated times, diluted with sterile DI water, and the viable *E. coli* was enumerated by spreading 0.1 mL on mFC selective agar. The dishes were incubated overnight at 37 °C and colonies were counted the following day.

Virus inactivation: concentrated MS2 suspension (10^9 PFU mL^{-1}) was diluted to an initial concentration of 10^6 PFU mL^{-1} into deionized water at ambient temperature or deionized water that was preheated to a designated temperature. Similarly to *E. coli* treatments, water-phage mixtures were exposed to solar irradiation as indicated to determine the response of MS2 virus to heating, solar irradiation, or their combination. Enumeration of MS2 coliphage was accomplished by the double agar layer method (Adams 1959). Briefly, 500 µL of *E. coli* K-12 at the logarithmic phase and 500 µL of the test sample (water-MS2 mixture) were added to a test tube (kept at 55 °C) containing 4 mL of semi-solid tryptone broth amended with 0.003% (w/v) calcium chloride dehydrate, 0.01% glucose (w/v), and 0.075% agar. The mixture was vortexed and the contents spilled onto solid tryptone agar. The samples were incubated overnight at 37 °C and plaques were counted the following day (Nasser *et al.* 2002). The *E. coli* host strain for MS2 bacteriophage was also the *E. coli* test strain for bacterial inactivation. In the control experiments no *E. coli* or MS2 inactivation was observed in DI water, under dark condition and room temperature.

Calculation of inactivation factor

Three types of treatments were examined: (1) heat only, (2) solar irradiation, either natural sunlight or simulated sunlight, and (3) combined heat and irradiation. Microorganisms seeded into the test samples were enumerated as the initial concentration, N_0. For each treatment at various time points, the mean of the test microorganism (from at least three separate experiments) were calculated, and taken as N_t. The $\log_{10}$ transformation for N_t/N_0 for both *E. coli* and MS2 were plotted as a function of the exposure time or solar dose (insolation).

Heat treatment

Samples of 500 mL were transferred into a 500-mL quartz glass bottle and placed in a temperature-controlled water bath (Digisystem Lab, DSB-1000D BT-150) at designated temperatures between 30 and 60 °C. Bottles were entirely submerged to prevent temperature gradients. Temperature was controlled using a type T thermocouple submerged in

the water and connected to a dual-input thermometer (Extech Instruments 421508). Once the water reached the designated temperature, the test microorganisms were seeded. Glass/quartz bottles are a preferred choice compared to plastic containers for heat experiments, especially at temperatures up to 60 °C and above, since the glass is inert, no leaching of chemicals are formed (as ethylene glycol from plastic bottles during heating, McGuigan *et al.* (1999)) or container degradation by heat.

Solar treatment: field natural sunlight exposure

These experiments were performed on the roof at Tel Aviv University's Solar Laboratory (32.1° latitude north, 34.8 longitude east, and +15 m altitude) from 13 July to 2 November 2010. Figure 1 shows the field experiment set-up. The solar radiation was continuously measured during the experiments and recorded every 5 s. Direct radiation was measured by a sun tracking pyrheliometer (The Eppley Laboratory Inc., Model 6537-5, RI, USA) with a field of view limited to 5°. Global radiation (direct and diffuse radiation) was measured by a horizontal pyranometer (Kipp & Zonen, Model 031101, Delft, The Netherlands) on a plane surface, normal to the solar radiation, in this manner total direct and diffuse radiation is being measured. Additionally, to obtain the spectral UV and VIS irradiation (irradiance as a function of wavelength from 250 to 950 nm), during each experiment, solar radiation was also measured by a spectroradiometer (International Light, Model ILT 900R, USA) with a horizontal detector (Figure 1(b)). Other climatic conditions, such as wind speed, relative humidity and air temperature, were also recorded every 5 s. Water temperature was continuously measured by a thermocouple and recorded every 1 min (Figure 1(a)).

For each condition 200-mL of deionized water was poured into Pyrex glass dishes. The glass dishes were wrapped with a hollow copper tube connected to a chiller (BL-30, MRC Israel) circulating water at the desired temperature (Figure 1(d)). To reduce heat loss, an insulation layer was wrapped around the silicone tube that connects the chiller to the copper tube (Figure 1(e)). Once the water in the dishes reached the desired water temperature, the microorganisms were seeded into the deionized water, and the temperature was maintained fixed throughout the

Figure 1 | Field experiment set-up: (a) thermocouple, (b) spectroradiometric detector, (c) glass dishes exposed to solar radiation only, (d) test dishes exposed to solar radiation and heat via water flowing through the copper tube, (e) insulation layer around the silicone tube that connects the copper tube to the chiller.

experiment using the hollow copper tubes. In the radiation-only treatments, the glass dishes were wrapped in aluminum foil to prevent any radiation input from the sides of the dish (Figure 1(c)), so that exposure would be similar to the controlled heat and irradiation experiments where the copper coil blocked side-radiation. The dishes filled with deionized water sample were left to reach ambient temperature before microorganism addition. All experimental dishes for the heated water and solar irradiations (with or without heating) were placed on a magnetic stirrer to facilitate mixing of the water and uniform radiation. To evaluate the inactivation of the microorganisms in the glass dishes, samples were taken at designated intervals.

Solar treatment: solar simulator

Samples of 40 mL deionized water were transferred into 50-mL Pyrex dishes and placed underneath the solar simulator light. A 150 W ozone-free xenon arc lamp (Sciencetech Inc.,

SS150W, Canada) was used with a maximum optical output irradiance given by the manufacturer of 1,000 W m^{-2}, after the light beam was filtered with a 1.5 Global air mass (AM) filter. In reality, other losses in our lab system and design around the target, resulted in irradiance of ~750 W m^{-2}. Optical irradiance was measured using the calibrated spectroradiometer and an irradiation detector (Nova-23A-P-FS, Ophir, Israel). The experiments were conducted at either ambient temperature or at a controlled temperature (similar to the field sunlight exposure).

The solar simulator was calibrated by comparing the simulated spectrum to the natural solar spectrum (Figure 2), and by photo analysis (Parretta *et al.* 2006) using a linear-response camera (body:u-eye 4002724050, Germany; lens: computer 09F, Japan) which photographs the irradiated area from a target with nearly Lambertian reflection properties (a diffuse white paper). The acquired image was corrected for geometric image distortion due to camera view angle, and analyzed with MATLAB software to
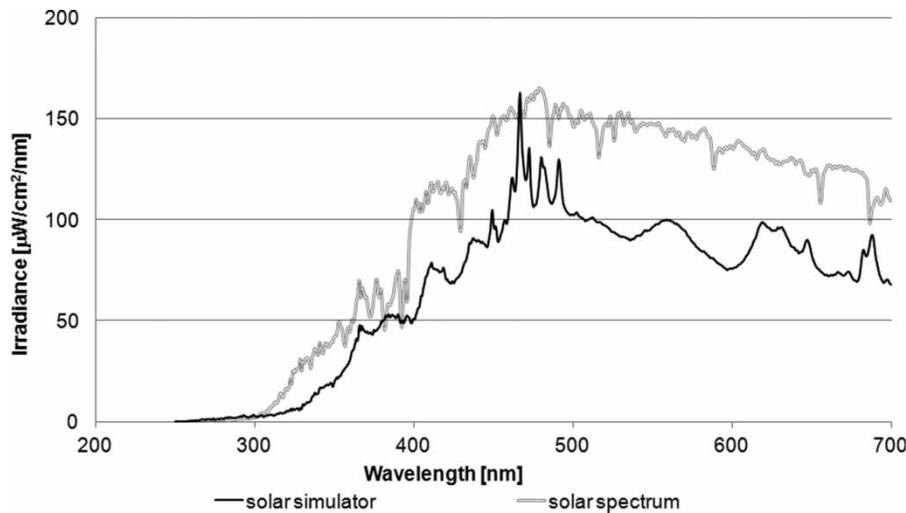
Figure 2 | Comparison of spectra obtained from the solar simulator with 1.5 G filter and the natural sun.

produce an additional estimation of the irradiance flux distribution and non-uniformity over the target area.

Although the spectral match under 400 nm is not part of the ASTM standard, examination on a randomly chosen mid-summer day (16 August 2010 at 12:00 noon) gave a spectrum similar to that achieved by the simulator (especially below 400 nm, Figure 2).

A 280-nm longpass (lp) filter was used as a precaution for ensuring that there is no UVC output from the solar simulator. After installing all filters (280-nm lp filter and the 1.5 AM Global filter), the irradiance integrated between 220 and 2,100 nm was 680 W m^{-2}, including UVA irradiance of 26 W m^{-2} and UVB irradiance of 1 W m^{-2}.

Endonuclease sensitive site (ESS) assay

The ESS assay was used to obtain a quantitative evaluation of DNA damage in *E. coli* exposed to irradiation, heating at 50 °C, or their combination. A higher concentration of *E. coli* was used for the ESS assay (10^9 CFU mL^{-1}) compared to inactivation experiments (10^6 CFU mL^{-1}) in order to obtain adequate amount of DNA. Cells were harvested by centrifugation (8,000 g; 5 min) and genomic DNA isolated from the pellet using a commercial kit (Bacterial DNA Microprep, Zymo Research). The samples were incubated with Exonuclease 5', an enzyme which cuts at the site of a pyrimidine dimer. After nicking the DNA, NaOH was added and the DNA was separated by

electrophoresis on agarose-NaOH gels according to molecular lengths (Oguma et al. 2001). Under these conditions double-stranded DNA is denatured fragmented according to the number of nicks created by Exonuclease 5'. By analyzing the migration patterns of the DNA fragments relative to standard size markers, the median molecular length of the DNA can be determined. A non-fractured DNA (control) will appear as a focused band at the top of the picture, while damage to the DNA results in more incisions, reflected as a downward spread and smears.

RESULTS AND DISCUSSION

Effect of heating on microorganism disinfection

The influence of heating on *E. coli* in DI water was examined: exposure of *E. coli* for 2 h to a temperature 52 °C or higher was needed to achieve significant bacterial inactivation (Figure 3), with immediate total inactivation at 55 °C. Berney et al. (2006) investigated the sensitivity of *E. coli* and another three pathogenic enteric strains in mineral water to mild heat, and also found 52 °C to be the specific temperature that inactivates the bacteria. The influence of heating on MS2 in DI water was also examined: exposure of MS2 for 2 h to a temperature of 55 °C did not result in significant viral inactivation, demonstrated that indeed MS2 is less sensitive than *E. coli* to heating; even heating to 60 °C
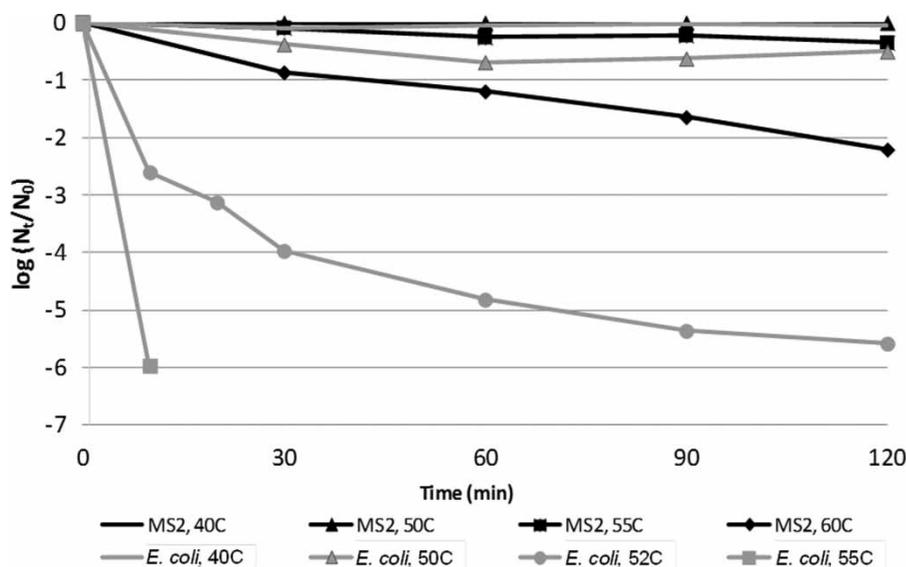
Figure 3 | Thermal inactivation curves of *E. coli* and MS2 at initial concentrations of 10^6 CFU mL^{-1} and 10^6 PFU mL^{-1}, respectively, in DI water at various temperatures.

did not produce total inactivation of MS2, but only 2-log inactivation. Gomez-Couso *et al.* (2010) demonstrated the important role of temperature in the inactivation of *Cryptosporidium* relative to exposure time: 8 and 12 h exposure produced similar results because in both experiments, the maximum temperature reached nearly 45 °C. Heaselgrave *et al.* (2006) examined the heat resistance of *Acanthamoeba polyphage* in DI water and found a maximum 2-log inactivation at 55 °C. Together, these results emphasize the importance of investigating heat resistant microorganisms, such as the bacteriophage MS2 studied here.

Insolation (dose)-response curves

The solar irradiance can be presented as global irradiance dose (direct and diffuse), direct normal irradiance (DNI) dose, global UVA dose or global UVB dose. Insolation is defined as the cumulative global solar irradiation and is the measure of the sunlight exposure or 'dose' (Sinton *et al.* 2002). Figure 4 gives some examples of these different dose-response curves (experiment conducted on 18 July 2010 at 35 °C).

Each presentation of the required dose for 6-log inactivation is different: ~6,000 kJ m^{-2} for global insolation, ~4,300 kJ m^{-2} for direct insolation, ~300 kJ m^{-2} for UVA insolation and ~11 kJ m^{-2} for UVB insolation. The

global insolation was chosen for the dose-response curves in this study for several reasons: first, the pyranometer monitors global irradiation every 5 s, whereas UVA and UVB irradiation are recorded once at every sampling (between 10 and 30 min), providing less accurate total dose values. Second, the experimental glass dishes were covered from the sides and were placed vertically on a horizontal surface (table), thus the water surface was exposed from the top to both direct and diffuse irradiation; making global insolation a more appropriate variable than direct insolation. Third, although most biological damage to DNA or proteins occurs due to exposure to UV radiation, inactivation dose is presented in terms of global irradiation, due to a good correlation between UV and global dose (as UV is found in the diffuse component and its value may be low). Fourth, similar studies performed in the field have also presented the dose-response curve as a function of global insolation (e.g. King *et al.* 2008), facilitating comparisons between our data and those reported previously.

Effect of natural solar irradiation

The influence of solar irradiation on *E. coli* inactivation in DI water was examined at ambient temperature. On 16 August 2010, ~3,000 kJ m^{-2} of global insolation was
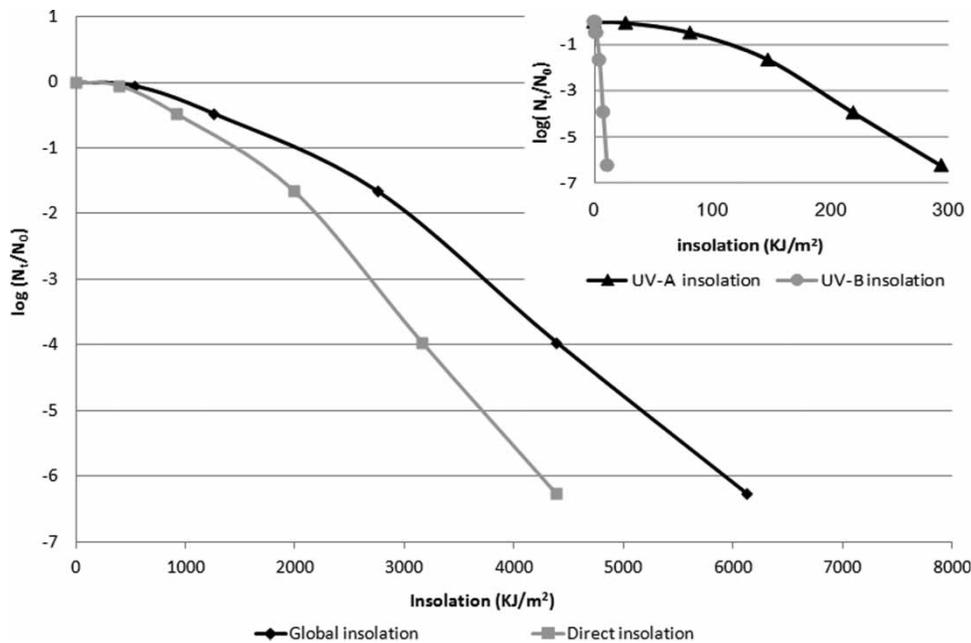
Figure 4 | Example of *E. coli* inactivation vs. global, direct, UVA and UVB insolation at 35 °C.

required for 4-log inactivation of *E. coli* with a k (inactivation rate constant) of $-0.0018 \text{ m}^2 \text{ kJ}^{-1}$. In all cases, a lag phase was observed at low insolation (data not shown). The lag appeared up to a global insolation of 1,000 kJ m^{-2} (similarly to Figure 4). During those experiments the maximum measured ambient temperature was 36.8 °C. The covered control samples were heated to the same maximum temperature but no inactivation occurred.

Most previous studies investigating inactivation of microorganisms under SODIS used a different set-up, i.e. polyethylene terephthalate (PET) bottles or tubes that filter out or reduce solar irradiation, mainly the UV portion of the spectrum. This makes comparisons difficult as the water samples in the present study were exposed to direct sunlight with no filtering.

Dejung *et al.* (2007) examined the inactivation kinetics for various microorganisms, including non-spore-forming bacteria, spores, phages, and protozoan cysts. The inactivation kinetics of the vegetative bacteria was remarkably similar under SODIS conditions (in PET bottles). A 45–90 W h m^{-2} dose of UVA was necessary to achieve 3-log (99.9%) inactivation, corresponding to 113–226 kJ m^{-2} of UVA before the ~30% loss in the PET bottles, and an intensity of 12 W m^{-2} UVA was enough to inactivate the different bacteria. Those results are in accordance with the current study results, in which exposure to 100–200 kJ m^{-2} of UVA resulted in inactivation of 99.9% of *E. coli* (data not shown). Boyle *et al.* (2008) examined inactivation of five different species of microorganisms using SODIS technique under real solar irradiation in untreated groundwater and showed different inactivation kinetics for the different species. Results for enteropathogenic *E. coli* inactivation were very similar to those obtained in this study, with a minimum 90 min needed for complete inactivation under strong natural sunlight. The shape of the inactivation curve also resembled that presented in the current study, with three different rate trends.

Seasonal influence on *E. coli* inactivation

A widely used means for comparisons among studies and for representing the exponential decay component of the inactivation process is to calculate T_{90} and S_{90} values (e.g. Sinton *et al.* 2002; King *et al.* 2008) which refer, respectively, to the time and level of insolation necessary to reduce the plate count by 90%. T_{90} was calculated by interpolation and S_{90} was calculated directly from the pyranometer data. As shown in Table 1, T_{90} and S_{90} values increased consistently with time from summer.

Table 1 | Values of T_{90} and S_{90} for (*E. coli*) irradiated in DI water under ambient temperature

Date (2010)	T_{90}(mm)	S_{90}(kJ m^{-2})
16 Aug	24	1275
1 Sep	32	1546
18 Oct	53	1905
2 Nov	–	>2500

Ubomba-Jaswa *et al.* (2010) also showed seasonal variation in inactivation rate: they used a parabolic collector-enhanced batch reactor to purify 25 L of water and in the winter months, they did not achieve full inactivation (within 5 h), in comparison to the summer months when solar irradiance was high enough to reach complete inactivation (they indicated that the effect was not due to the temperature). The increase in time to inactivation as the seasons advance from summer to winter suggests that the insolation parameter does not accurately predict the inactivation rate and cannot be used as a standardized variable, similar to Rincon & Pulgarin's (2004) observation. Equivalent insolation at high intensity for a short time and low intensity for a longer time should theoretically result in the same inactivation rate. However, Sommer *et al.* (1998) reported a greater efficiency of disinfection with high UVC intensity for a short time vs. a lower intensity for a longer time. This may be caused by the negative effect of high UV intensity on the activity of repair enzymes in the cell (Sommer *et al.* 1998). In addition, Rincon & Pulgarin (2004) determined that the changing characteristics of UV and VIS intensities in each season significantly affect solar photoinactivation, and suggested effective disinfection time (EDT) as a parameter to standardize solar disinfection. To compensate for low solar UV intensity in the winter, it was recommended to increase the water temperature (Heaselgrave *et al.* 2006; Ubomba-Jaswa *et al.* 2010), for instance, by using a foil backing (Kehoe *et al.* 2001). Another way to overcome low irradiation is to increase radiation-collection efficiency with a CPC (Navntoft *et al.* 2008).

Influence of combined irradiation and heating on microorganisms – field experiments with *E. coli*

Previous studies in field set-up examined synergy under changing temperature (such as Sommer *et al.* 1997; Dejung *et al.* 2007). The current study examined synergy of solar irradiation and heating on *E. coli* in DI water under controlled temperature, in order to elucidate clearly the impact of temperature on synergy under natural sunlight. Several experiments were carried out at different temperatures (from 30 to 50 °C) to assess the critical temperature for a potential synergistic effect. No dramatic differences were observed in *E. coli* inactivation (less than 0.5 log) at temperatures of 30, 40 and 45 °C, and no inactivation occurred in the covered control samples. Thus there was no synergetic effect of heat and irradiation at temperatures below 45 °C, in agreement with other studies reporting no synergistic effect at temperatures of less than 50 °C (Wegelin *et al.* 1994; McGuigan *et al.* 1998). Reed (2004) suggested that this might be due to a decrease in dissolved oxygen with increasing temperature, which cancels out the influence of temperature on inactivation. To determine synergy with natural sunlight at a fixed temperature, the log inactivation for the synergy experiments were compared to the sum of the log inactivation of the irradiation-only experiment and the log inactivation of the heating-only experiment. This sum, used as reference for comparison, is an artificial combination of the two separate experiments, as if the two effects were independent and cumulative. The log differences between synergy and summation determined the net synergy effect.

In the current study, when the water was heated to 50 °C, there was an obvious synergistic effect with irradiation (Table 2). For up to 20 min, there was no significant difference between the artificial summation of heat and irradiation effects and their combined effect. However, the natural sunlight synergy became more noticeable with time, with up to 3-log inactivation difference observed for 60 min exposure to the combined treatment.

Influence of combined irradiation and heating on microorganisms – field experiments with MS2

The effect of solar irradiation and heating on MS2 inactivation in DI water was examined under controlled temperature, as illustrated in Figure 5. Solar irradiation alone had a negligible effect on MS2, even at 5,580 kJ m^{-2} global insolation for 120 min. Heating for 120 min produced only 1.23 log inactivation at a temperature of 59 °C. The

Table 2 │ A comparison of *E. coli* log inactivation in DI, of irradiated and heated (at 50 °C) samples compared to an artificial summation of the log inactivation of samples that were only heated (at 50 °C) and only irradiated, on 18 October 2010, under natural sunlight

Exposure time (min)	Insolation, global (kJ m^{-2})	Insolation, UVA (kJ m^{-2})	Insolation, UVB (kJ m^{-2})	Synergy experiment log (N_t/N_0)	Artificial summation log (Nu_t/Nu_0)	Log differences log (N_t/N_0)
10	387	17	0.8	−0.49	−0.62	−0.12
20	763	32	1.5	−1.59	−0.88	0.71
30	1126	47	2.2	−2.97	−1.60	1.36
60	2129	71	2.2	−5.70	−2.62	3.08

combination of heating at temperatures lower than 59 °C and solar irradiation (experiments held on 19 October 2010 with 5,000 kJ m^{-2} global insolation after 120 min) did not result in any significant inactivation. However, combining irradiation and heating to 59 °C resulted in over 2-log inactivation, which is almost 1 log higher than the artificial summation of heating and irradiation (experiments held on 29 August 2010 with 5,580 kJ m^{-2} global insolation after 120 min). Although the experiments were conducted on different dates, the differences between global insolation were small and the differences between the inactivation caused by irradiation only were negligible; thus, the difference in inactivation observed at high temperature is likely to be the result of real synergy. It should be noted that although significant inactivation was achieved when combining irradiation and heating to 59 °C, this might not be applicable under

ambient conditions, due to the low probability of obtaining such high temperatures under natural non-concentrated sunlight.

To conclude, there is a synergistic effect of heat and irradiation for MS2 inactivation but it does not yield a dramatic effect such as that found with *E. coli*. Many studies, however, attempted various methods to increase the inactivation effect of sunlight-resistant species as MS2, not necessarily with a combination of heat and sunlight. For example, Walker *et al.* (2004) developed a 'second generation' SODIS system, termed SODIS-pouch, which is constructed by fusing a UV-transmitting upper layer to metalized plastic, which reflects light (effectively doubling the dose passing through the water). Using this pouch, the team was able to inactivate MS2 by 4 logs after 6 h of exposure to sunlight, showing the important role of light
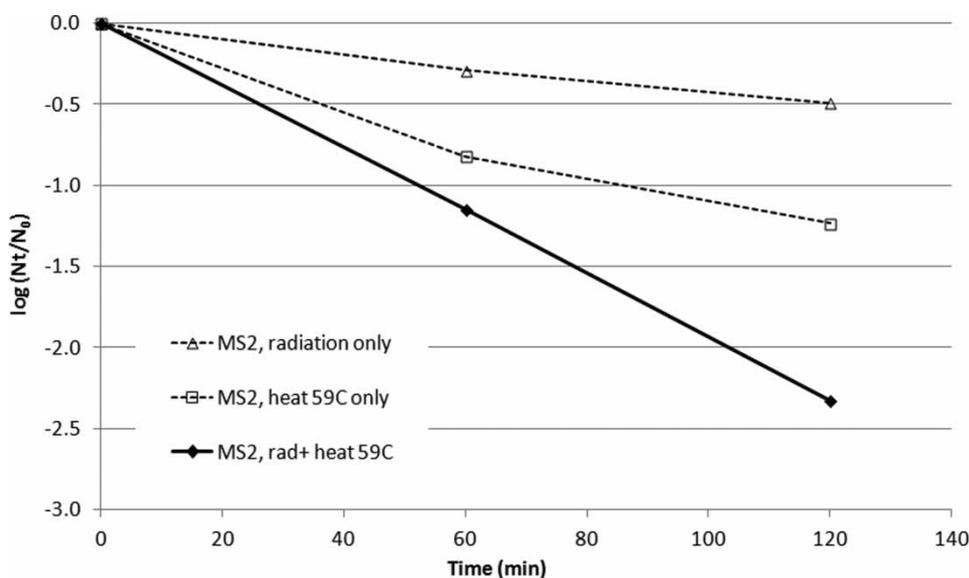
Figure 5 │ MS2 log inactivation in DI, of irradiated and heated (at 59 °C) samples compared to combined heat and irradiation.

reflection rather than the modest temperature (maximum 52 °C).

Kohn et al. (2007) showed that singlet oxygen ($^1O_2$) produced within a natural organic matter (NOM) macro-molecule or supramolecular assembly by sunlight is responsible for exogenous inactivation of MS2 and this interaction can be enhanced by lowering pH or by adding Mg^{2+}. In another study, a stronger synergistic effect under sunlight was obtained in the presence of H_2O_2 (Kohn & Nelson 2007).

Solar simulator experiments with E. coli

The effect of solar simulator irradiation and heating on E. coli inactivation in DI water was examined under controlled temperatures. Three sets of experiments were carried out: (1) heating at 50 °C only: the sample was covered from any illumination; (2) irradiation only (ambient temperature) using a 280-nm lp filter to ensure that no UVC wavelengths reach the water sample from the lamp; (3) irradiation and heating at 50 °C using the 280-nm lp filter. The resultant inactivation rates for E. coli are presented in Table 3.

The synergistic effect observed with the sun's irradiation was also observed under the solar simulator. In the first few minutes, no differences were observed between heat exper-iments under 'heating only' compared to experiments that combined irradiation and 50 °C heating. However, after a few more minutes, the synergistic effect of heat and irradiation began to develop, to an over 4-log difference in inactivation after 30 min compared to the artificial sum-mation of heat and irradiation effects alone, similar to the synergy effect under real sunlight. A higher effect of synergy was obtained under simulated light compared to natural sunlight, probably due to the inaccuracy in calculating the cumulative effect of UVA and UVB photons. The values in Tables 2 and 3 are based on UVA and UVB irradiation measurements that are recorded only once at every sampling and thus do not represent well the true cumulative effect of these wavelengths for the natural sunlight.

Synergy was observed by other researches that have examined E. coli inactivation under solar simulator or under UVA or UVB lamps (Wegelin et al. 1994; Berney et al. 2006), although the conditions such as water type, light intensity or temperature were different, still synergy was shown above 50 °C.

ESS analysis

ESS analysis enabled investigating the pyrimidine dimers in the DNA of microorganisms (reflecting DNA damage) induced by heat, solar irradiation or both. To the best of our knowledge, ESS analysis has not been performed pre-viously to analyze inactivation by solar irradiance, heat or their combination. Figure 6 shows ESS analysis of E. coli DNA after heating at 50 °C, irradiation, and their combi-nation, under the solar simulator.

Undamaged DNA appears as a white line at the top of the picture, whereas the more the DNA is damaged, the more it spreads downwards. The undamaged DNA of the control sample can be seen at the top of lane 1. Moreover, no significant DNA damage was observed after heating only (lanes 2 and 3). DNA damage from irradiation occurred only after an extended period of time (20 min, lane 5). The synergistic effect of the two treatments, pre-viously seen in the plate count, was also reflected here by producing the most smearing of the bands (lanes 6 and 7) compared to heating or irradiation alone (summation).

The molecular mechanisms that damage cells by solar disinfection process are not fully understood. Bosshard et al. (2010) showed that proteins are damaged under solar

Table 3 | A comparison of E. coli log inactivation in DI, of irradiated and heated (at 50 °C) samples compared to an artificial summation of the log inactivation of samples that were only heated (at 50 °C) and only irradiated under solar simulator (ND, not detected)

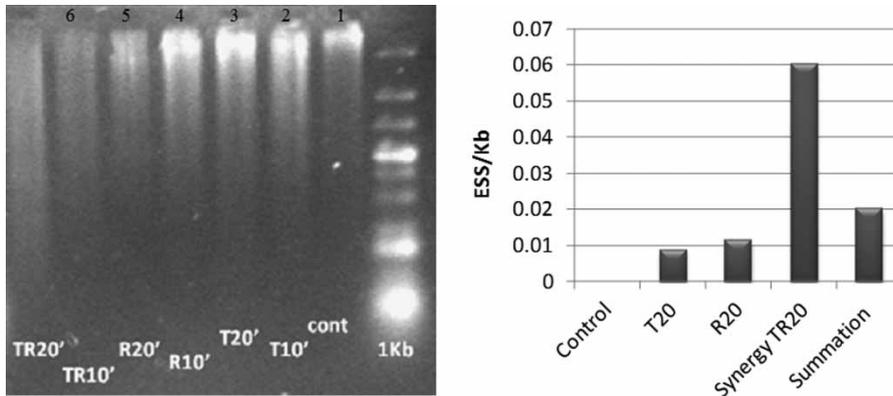| Exposure time (min)	Insolation, global (kJ m^{-2})	Insolation, UVA (kJ m^{-2})	Insolation, UVB (kJ m^{-2})	Synergy experiment log (N_t/N_0)	Artificial summation log (N_t/N_0)	Log differences log (N_t/N_0)
10	372	ND	ND	−1.30	−0.82	−0.48
20	744	21.42	0.65	−3.60	−1.16	−2.44
30	1116	42.84	1.30	−5.60	−1.55	−4.06

Figure 6 | ESS analysis of *E. coli* DNA after heating at 50 °C (T), irradiation (R) or combined heating at 50 °C and irradiation (TR). cont, control non-treated sample; 10′, 10 min treatment; 20′, 20 min treatment; 1 Kb, DNA size ladder (New England Biolabs cat # N3232).

UVA irradiated *E. coli* cells. In this study, the ESS assay showed that DNA is a clear target of the combined solar and heat effect. These results provide a better understanding of the inactivation mechanism and prove that the production of pyrimidine dimers in the DNA of *E. coli* plays a role in the synergistic effect. To summarize, synergy was also confirmed on a molecular level using the ESS assay, where the heat accelerated the DNA damage process in the combined treatment compared to irradiation alone.

E. coli recovery

Microorganisms' ability to recover from harmful irradiation by DNA repair is only manifested under certain conditions: DNA repair depends on the intensity of the photobiological damage, and can occur in the dark or with exposure to VIS wavelengths (Sinton *et al.* 2002). The ability to recover in the light is due to the photolyase enzyme which functions under VIS light and repairs thymine dimers (Jagger & Stafford 1965; Sancar 2000).

The ability of *E. coli* to recover from inactivation by irradiation was examined by subjecting the bacteria to either heat, lethal irradiation or heat combined with lethal irradiation. After obtaining a certain level of inactivation, the water sample was exposed to solar simulator with 400-nm lp for an additional hour. The 400-nm lp filter allows only VIS and IR wavelengths to pass through which is necessary for photo-repair. *E. coli* succeeded in recovering from the irradiance effect by more than 0.7 logs after 60 min exposure (Figure 7). No recovery was observed

when the sample was held in the dark. Moreover, no recovery was observed after exposing the *E. coli* samples to 50 °C or irradiation combined with 50 °C heating (the combined treatment). This suggests that the inactivation targets by heating and irradiation are different.

As recommended by the SODIS project, after irradiation water should be stored in a dark place to prevent light recovery (http://www.sodis.ch). Alternatively, if storage under dark conditions is not available, recovery can be prevented by subjecting the water to temperatures that are high enough to eliminate recovery, as previously shown. These results confirm the importance of treatment including heating to 50 °C, which may avoid the need for dark storage.

CONCLUSIONS AND RECOMMENDATIONS

A significant impact of heating was found on *E. coli* inactivation at 52 °C, while some inactivation was found for MS2 only at 60 °C, demonstrated that MS2 is less sensitive than *E. coli* to heating. In this study, a significant synergistic effect of combined heating and solar irradiation was observed for *E. coli* inactivation, with an up to 3-log difference for 50 °C and insolation of 2,000 kJ m^{-2} compared to simple summation of the separate treatment effects with natural sunlight and more than 4-log difference for the same temperature and insolation of 1,116 kJ m^{-2} compared to a simple summation of the separate treatment effects with simulated sunlight. This synergy was initially
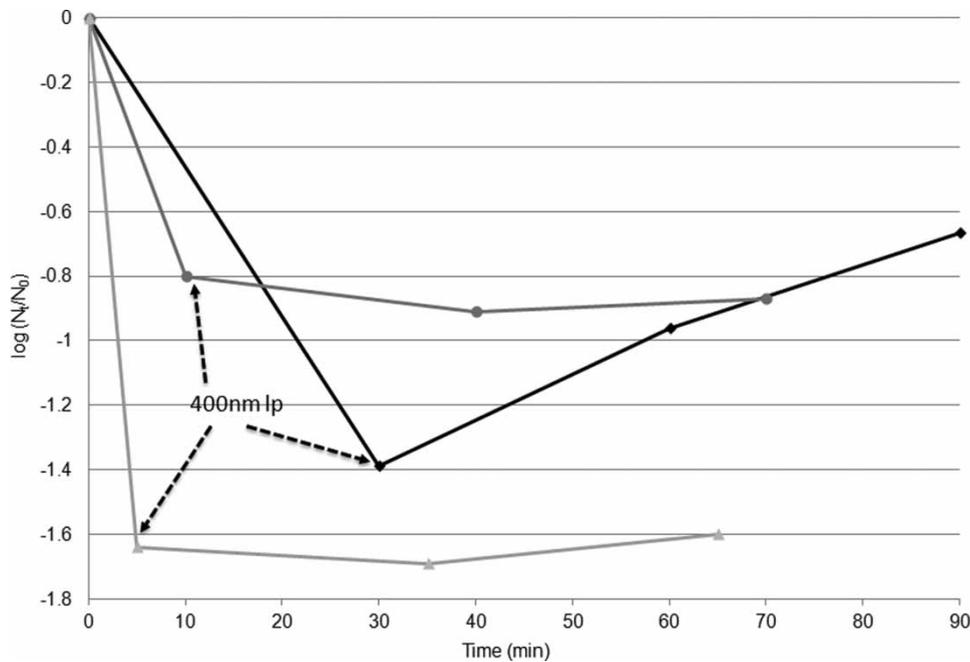
Figure 7 | *E. coli* recovery ability under VIS light: (a) after pre-exposure to 50 °C only for 10 min (•), (b) irradiance with 280-nm lp filter for 30 min (♦), and (c) irradiance with 280-nm lp filter combined with heating at 50 °C for 5 min (▲). The arrow shows when the water sample was exposed to a 400-nm lp filter for an additional hour. VIS light refers to wavelengths above 400 nm; 280-nm lp is a long-pass filter that transmits wavelengths above 280 nm.

indicated by Wegelin *et al.* (1994) and by forthcoming studies. MS2, however, was found in this study to be highly resistant to irradiation and heat, with a slightly synergistic effect observed for 59 °C (and above) at an insolation of 5,580 kJ m^{-2}.

MS2 represents a worst case microorganism because it is more resistant for use in evaluating SODIS as a drinking water treatment process, and should be considered where and when it can be applied, and especially the limitations of only using *E. coli*. Various researchers criticized the use of *E. coli* as an indicator for water quality (as Savichtcheva & Okabe 2006). Furthermore, it is important to determine the parameters of heat and irradiation and their combination that would achieve significant inactivation, per organism examined. Understanding the inactivation response may lead to design of a solar disinfection apparatus that would be efficient also for more resistant microorganisms.

A seasonal climatic influence was found on the inactivation process: a longer lag in the inactivation curve was observed with time from summer. This finding should also be considered when designing a solar disinfection system.

The ESS assay, in this study, proved that this synergy results in DNA damage (proven for *E. coli*), even after 10 min. The heat accelerated the DNA damage process in the combined treatment compared to irradiation alone. *E. coli* recovery was observed with irradiation, however, no recovery was observed after exposing the *E. coli* samples to 50 °C or irradiation combined with 50 °C heating (the synergistic effect). Heaselgrave *et al.* (2006) suggested that increasing temperature leads to increased permeability of the cyst walls and may facilitate the transport of UV radiation products (ROS) into the interior of the cysts, where they can result in a greater biocide effect. Our findings of DNA damage, which is accelerated by heat (ESS analysis) in addition to the observation of the non-recovery of *E. coli* after heating to 50 °C, reinforce this hypothesis. We believe that the heat increases the cell membrane permeability and by that the generated reactive products formed by irradiation can easily penetrate to the cell and produce intracellular damage. Thus, we recommend using the ESS assay as a quantitative evaluation of DNA damage which may lead to better understanding of the synergetic inactivation mechanism.

It is possible, however, that in the SODIS treatment, a temperature of 50 °C and above will not be achieved in many regions of the world, therefore, the SODIS method is known to be geographically dependent. If the water is to be heated up to 50 °C, then the demand of dark storage in the SODIS treatment may be more flexible and dark storage may be avoided or reconsidered. Of course more experiments are needed (comparing different microorganisms) to confirm this finding.

By understanding the importance of the combined heat and irradiation in solar inactivation, we propose designing a simple system, open to the sky, for enhancing the microbial inactivation based on increasing the following parameters:

1. Absorbing surface: placing a black absorbing surface to increase the water temperature.
2. Front mirror surface: placing a mirror with a special coating (such as aluminum and quartz) that reflects also the solar UV radiation. Such a mirror can increase the irradiance from '1 sun' to more than '1.8 suns' (e.g. SEA-UV, Praezisions Glas & Optik, Germany).

This system could be at a large scale for rural use in third world countries and should ideally be simple and inexpensive. Using these simple methods can lead to more efficient inactivation.

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