

# Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS)

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The effectiveness of solar disinfection (SODIS), a low-cost household water treatment method for developing countries, was investigated with flow cytometry and viability stains for the enteric bacterium *Escherichia coli*. A better understanding of the process of injury or death of *E. coli* during SODIS could be gained by investigating six different cellular functions, namely: efflux pump activity (Syto 9 plus ethidium bromide), membrane potential [bis-(1,3-dibutylbarbituric acid)trimethine oxonol; DiBAC<sub>4</sub>(3)], membrane integrity (LIVE/DEAD BacLight), glucose uptake activity (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; 2-NBDG), total ATP concentration (BacTiter-Glo) and culturability (pour-plate method). These variables were measured in *E. coli* K-12 MG1655 cells that were exposed to either sunlight or artificial UVA light. The inactivation pattern of cellular functions was very similar for both light sources. A UVA light dose (fluence) of <math>< 500 \text{ kJ m}^{-2}</math> was enough to lower the proton motive force, such that efflux pump activity and ATP synthesis decreased significantly. The loss of membrane potential, glucose uptake activity and culturability of >80% of the cells was observed at a fluence of  $\sim 1500 \text{ kJ m}^{-2}$ , and the cytoplasmic membrane of bacterial cells became permeable at a fluence of  $> 2500 \text{ kJ m}^{-2}$ . Culturable counts of stressed bacteria after anaerobic incubation on sodium pyruvate-supplemented tryptic soy agar closely correlated with the loss of membrane potential. The results strongly suggest that cells exposed to  $> 1500 \text{ kJ m}^{-2}$  solar UVA (corresponding to  $530 \text{ W m}^{-2}$  global sunlight intensity for 6 h) were no longer able to repair the damage and recover. Our study confirms the lethal effect of SODIS with cultivation-independent methods and gives a detailed picture of the 'agony' of *E. coli* when it is stressed with sunlight.

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## INTRODUCTION

Waterborne diarrhoeal diseases are prevalent in many countries where sewage and drinking water treatment are inadequate. Over 1.2 billion people are at risk because they lack access to safe drinking water (World Health Organization, 1996). Every day, diarrhoeal diseases such as cholera (Fewtrell *et al.*, 2005) claim the lives of  $\sim 5000$  young children throughout the world and most of the cases could be easily prevented (Hinrichsen *et al.*, 1997). The World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) have recently claimed that improvement of drinking water quality and basic sanitation can cut this toll, and simple, low-cost, household water treatment has the potential to save further lives because it cuts the primary transmission route for

diarrhoeal diseases (World Health Organization/United Nations Children's Fund, 2005).

Solar disinfection (SODIS) is such a water treatment method. Through exposure of drinking water in poly(ethylene terephthalate) (PET) bottles to sunlight ( $\geq 6$  h), enteric bacteria in the water are inactivated (Acra *et al.*, 1984; Wegelin *et al.*, 1994). Both UVA light and mild heat have been shown to have inimical potential and, if the water temperature rises above  $50^\circ\text{C}$ , a synergistic effect is observed (Wegelin *et al.*, 1994). Field trials in different geographic regions, carried out by the Swiss Federal Institute of Aquatic Science (EAWAG) Department of Water and Sanitation in Developing Countries (SANDEC), have shown that temperatures  $> 50^\circ\text{C}$  are rarely reached (R. Meierhofer, personal communication). Acra *et al.* (1984) have proposed that solar UVA irradiation accounts for  $> 70\%$  of the negative effects of sunlight. Today, SODIS is one of the recommended methods for household drinking water disinfection (World Health Organization/United Nations Children's Fund, 2005). Nevertheless, the mechanism(s) of disinfection are not yet known precisely. The two primary sources for bacterial inactivation in this method are

Abbreviations: 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; DiBAC<sub>4</sub>(3), bis-(1,3-dibutylbarbituric acid)trimethine oxonol; EB, ethidium bromide; PEP, phosphoenolpyruvate; PEP-PTS, phosphoenolpyruvate-phosphotransferase system; PI, propidium iodide; SODIS, solar disinfection.

believed to be mild heat and UVA light (Wegelin *et al.*, 1994). The first report on the inimical effect of sunlight on bacteria was published by Downes (1886) and, 100 years later, Acra *et al.* (1984) proposed the use of sunlight to disinfect oral rehydration solutions. Subsequently, it was demonstrated to be effective against bacteria and higher organisms (Joyce *et al.*, 1996; Lonnen *et al.*, 2005; Wegelin *et al.*, 1994), and its applicability has been shown in a recent health impact study (Hobbins, 2004). The effectiveness of SODIS has been shown using classical cultivation of micro-organisms on solid agar substrates, which represents the reference procedure for estimating the number of viable bacteria. This method, however, only enumerates culturable bacteria that are able to initiate cell division and replication on agar plates. By using conventional culturability assays, there is a risk of overestimation of effectiveness of disinfection processes. For example, bacteria that are injured can fail to proliferate on plates and may appear dead (Aldsworth *et al.*, 1999). It has been shown that injured bacteria do not grow due to the stress of high nutrient concentrations, which causes a free radical burst (Dodd *et al.*, 1997). This behaviour has been referred to as a suicide response (Aldsworth *et al.*, 1999). Postgate had already observed this behaviour much earlier and called it substrate-accelerated death (Postgate & Hunter, 1964), but did not link it to oxidative stress. Furthermore, in view of the discussion about the viable but non-culturable (VBNC) state, a careful analysis of bacterial viability after SODIS treatment is of great importance (Barer & Harwood, 1999; Colwell, 2000; Nystrom, 2001). It is still an ongoing debate if such a state exists at all and how this can be proven.

Multi-parameter flow cytometry has enjoyed increasing popularity in microbiology, particularly in biotechnological processing, food preservation and chemical disinfection (Hewitt *et al.*, 1999; Nebe-von-Caron *et al.*, 2000; Porter *et al.*, 1997). Hewitt & Nebe-Von-Caron (2004) have shown that multi-parameter flow cytometry allows a functional classification of the physiological state of single-celled micro-organisms beyond that of culturability alone.

In the work presented here, the SODIS method was assessed with flow cytometry and viability stains. A set of essential bacterial functions of *E. coli*, namely, membrane integrity, membrane potential, efflux pump activity, glucose uptake activity and culturability were measured.

## METHODS

**Bacterial strains.** Wild-type *E. coli* K-12 MG1655 (ATCC 700926) was used for all experiments.

**Growth media and cultivation conditions.** Luria-Bertani (LB) broth (10 g tryptone l<sup>-1</sup>, 5 g yeast extract l<sup>-1</sup>, 10 g NaCl l<sup>-1</sup>) that was filter-sterilized with membrane filters (Millex GP, 0.22 µm pore size; Millipore), and diluted to 33% (v/v) of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon-treated), was used for batch cultivation (Miller, 1972). Precultures were prepared for each individual batch experiment from the same cryo-vial stored at -80 °C by streaking the

stock solution onto LB agar plates. After 15–18 h of incubation at 37 °C, one colony was picked, loop-inoculated into a 125 ml Erlenmeyer flask containing 20 ml diluted LB broth, and incubated at 37 °C on a rotary shaker at 200 r.p.m. At an OD<sub>546</sub> between 0.1 and 0.2 (measured spectrophotometrically at 546 nm in glass cuvettes with a 1 cm light path using a Jasco V550 UV/VIS spectrophotometer), an aliquot of the culture was transferred into 500 ml Erlenmeyer flasks containing 50 ml prewarmed LB broth to obtain an OD<sub>546</sub> of 0.002. With this procedure, no lag phase was observed. These flasks were then shaken at 200 r.p.m. in a temperature-controlled water bath (SBK 25D; Salvis AG) at 37 °C for ~18 h until stationary phase (specific growth rate,  $\mu=0 \text{ h}^{-1}$ ) was reached. The specific growth rate  $\mu$  was calculated from five consecutive OD<sub>546</sub> measurements. In drinking water, cells grow very slowly or not at all; therefore, we used stationary-phase cells, which were shown to be more resistant to SODIS than cells in the growth phase (Berney *et al.*, 2006; Reed, 1997).

**Sample preparation and plating.** Cells were harvested by centrifugation from batch culture (at 13 000 g, Biofuge Fresco; Kendro), washed three times with filter-sterilized (Nuclepore Track-Etch Membrane, 0.22 µm pore size; Sterico) commercially available bottled water (Evian) and diluted to an OD<sub>546</sub> of ~0.01 (corresponding to 1–5 × 10<sup>7</sup> cells ml<sup>-1</sup>). During exposure, aliquots were withdrawn at different time points and diluted in decimal steps (10<sup>-1</sup>–10<sup>-5</sup>) with sterile-filtered (0.22 µm), bottled mineral water (Evian). Volumes of 1 ml of appropriate dilutions were withdrawn and mixed with 7 ml liquid tryptic soy agar (TSA) (Biolife) at 40 °C (pour-plate method). After 20 min, the solidified agar was covered with another 4 ml liquid TSA (40 °C). Plates were incubated for 48 h at 37 °C until further analysis. For cultivation under reduced oxidative stress conditions, a 1 ml aliquot of the appropriate test solution was mixed with 7 ml TSA supplemented with 0.05% sodium pyruvate, covered after 5 min with the same agar, and then placed in an anaerobic jar (Oxoid; Khaengraeng & Reed, 2005). Just before closure of the jars, an AnaeroGen sachet (Oxoid) was placed in the container to generate an anaerobic gas phase. The jars were incubated for up to 72 h at 37 °C. Plate counts were determined with an automatic plate reader (Acolyte; Synbiosys).

**Sunlight exposure.** Samples of 10 ml bacterial suspension (see above) were exposed to solar light in 30 ml quartz tubes, which were placed into a temperature-controlled, acrylic glass container with a quartz front glass, holding 25 tubes in total. A water bath was used to control the temperature of the sample tubes in the container. The container was adjusted regularly so that sunlight met the tubes at an angle of 90 ± 2°. At each time point, one tube was withdrawn and its content immediately processed as described above. Irradiation intensity data were obtained from a weather station, which was located 300 m away from the exposure site (BUWAL/NABEL, EMPA Dübendorf, Switzerland). The fluence rates for sunlight irradiation given in this work refer to the wavelength range 350–450 nm, which reflects that of the UVA lamp used (see below). Conversion factors and calculations were used as described by Wegelin *et al.* (1994). The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-COR), 8 nm bandwidth, fitted with a model 1800-10 detector head.

**UVA exposure.** Samples of 10 ml bacterial suspension (see above) were exposed to UVA light in 30 ml quartz tubes placed in a carousel reactor (holding 10 tubes) (adapted from Wegelin *et al.*, 1994) equipped with a medium-pressure mercury lamp (Hanau TQ718 Z4), which was operated at 500 W (for wavelength spectrum, see Berney *et al.*, 2006). The lamp was placed in a cooling jacket (Duran 50 borosilicate glass) in the centre of the carousel reactor. The light emitted from the lamp passed through the glass jacket and through 35 mm of filter solution before reaching the cells in the quartz tubes. The temperature of the filter solution was maintained at

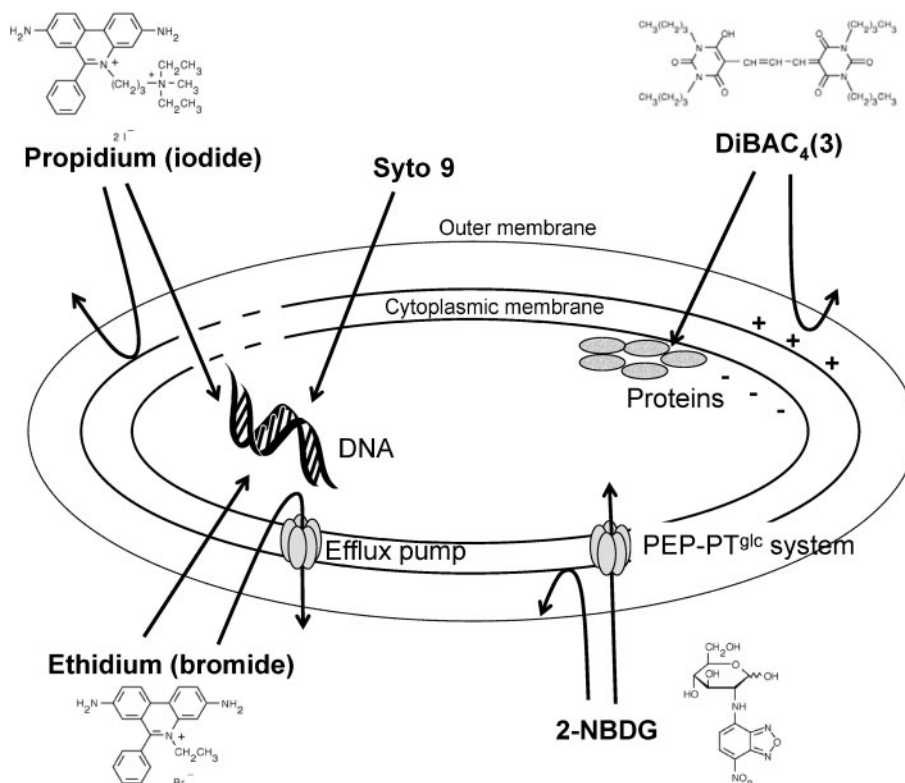
37 °C, and the solution consisted of 12.75 g sodium nitrate  $l^{-1}$  with a cut-off at 320 nm and a half maximum at 340 nm. The transmission property of the filter solution was measured before each experiment. Chemical actinometry with *p*-nitroanisole/pyridine was used to determine the fluence rate at the tube position (Wegelin *et al.*, 1994). Bacterial solutions were mixed intermittently on a magnetic stirrer. At each time point, one tube was withdrawn and its contents immediately processed as described above. UVA and light spectra were recorded as outlined above.

**Flow-cytometric measurements.** Flow-cytometric measurements were made using a Partec PAS III flow cytometer with 488 nm excitation from an argon ion laser at 20 mW. Five fluorescent dyes were used alone or in different combinations: Syto 9 (Molecular Probes), propidium iodide (PI; Molecular Probes), bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC<sub>4</sub>(3); Molecular Probes], ethidium bromide (EB; Fluka Chemie) and 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG; Invitrogen). Samples taken from irradiation experiments (sunlight and artificial UVA) were divided into five subsamples and immediately stained with two mixtures of fluorescent dyes (Syto 9/PI and Syto 9/EB) and three single fluorescent dyes [DiBAC<sub>4</sub>(3), Syto 9 and 2-NBDG]. Samples were incubated in the dark at 37 °C for 5 min (2-NBDG) or at 20 °C for 10 [DiBAC<sub>4</sub>(3)], 15 (Syto 9/EB), 20 (Syto 9/PI) and 25 min (Syto 9), respectively, before analysis. Prior to flow-cytometric analysis, cell samples were diluted with sterile-filtered bottled water (Evian) to 1% (v/v) of the initial concentration ( $\sim 1 \times 10^5$  cells  $ml^{-1}$

final concentration). Stock solutions of the dyes were prepared as follows: PI and Syto 9 were used from the LIVE/DEAD BacLight kit (Invitrogen), EB was prepared at 25 mM in distilled and filtered water, DiBAC<sub>4</sub>(3) was prepared in dimethylsulfoxide at 10 mM, and 2-NBDG was dissolved in distilled and filtered water at 5 mM. All stock solutions were stored at -20 °C. The working concentrations of Syto 9, PI, EB, DiBAC<sub>4</sub>(3) and 2-NBDG were 5, 30, 30, 10 and 5  $\mu M$ , respectively. 2-NBDG was added in combination with 2,4-dinitrophenol (final concentration 2 mM) (Natarajan & Srienc, 2000). In the flow cytometer, optical filters were set up such that PI and EB were measured above 590 nm and Syto 9, DiBAC<sub>4</sub>(3) and 2-NBDG at 520 nm. The trigger was set for the green fluorescence (520 nm) channel FL1.

The 2-NBDG uptake kinetics of *E. coli* K-12 MG1655 were measured prior to the irradiation experiment by incubating a bacterial sample with a mixture of 2-NBDG and 2,4-dinitrophenol at 37 °C for 40 min (Natarajan & Srienc, 1999). Every 5 min, a sample was withdrawn, diluted with sterile-filtered mineral water (Evian) to 1% (v/v) of the initial concentration and analysed on the flow cytometer. The peaks of the bacterial population were gated (range-gate RN1) and the geometric mean of green fluorescence intensity was used for data analysis.

**Fluorescence stains and their function.** The cellular properties and physiological functions indicated by the fluorescent stains used here are visualized in Fig. 1. Syto 9, a green fluorescent nucleic



**Fig. 1.** Viability indicators (fluorescence stains) applied in combination with flow cytometry and their function in *E. coli* (simplified). All stains have to pass the cytoplasmic membrane to be detected by flow cytometry. Functions: Syto 9 (green fluorescence) for total counts; propidium iodide (red) for membrane integrity; ethidium bromide (red) for efflux pump activity; 2-NBDG (green) for glucose uptake activity (PEP-PT<sup>glc</sup>, the PTS component for glucose transport); DiBAC<sub>4</sub>(3) for membrane potential.

acid stain, has been shown to stain living and dead Gram-positive and Gram-negative bacteria (Haugland, 2002). Therefore, it can be used for total count measurements. EB binds to nucleic acid, and can cross the intact cytoplasmic membrane, but is actively pumped out of the cell via a non-specific, proton antiport transport system in active cells (Midgley, 1987). It has been shown that the ethidium efflux system is dependent on a transmembrane proton electrochemical gradient, but is ATP independent. PI is a red fluorescent dye that intercalates with dsDNA and only enters permeabilized cytoplasmic membranes. If EB or PI is combined with Syto 9, a quenching effect on the green fluorescence intensity is observed as soon as the red fluorescent dye enters the cells. It is important to mention that only the cation (ethidium or propidium) enters the cells; nevertheless, the stains are always referred to together with their anionic constituent (bromide or iodide). DiBAC<sub>4</sub>(3) is a lipophilic and anionic bis-oxonol. The uptake of this membrane potential-sensitive dye is restricted to depolarized cells or cells with disrupted cytoplasmic membranes. The fluorescent dye accumulates inside the cells by binding to intracellular proteins and membranes. For further information about the dyes described above, refer to Hewitt & Nebe-Von-Caron (2004). 2-NBDG is a fluorescent glucose analogue, which is taken up by the glucose-specific phosphoenolpyruvate-phosphotransferase system (PEP-PTS) (Natarajan & Srienc, 1999; Yoshioka *et al.*, 1996). The system consists of five proteins and uses PEP as an energy source.

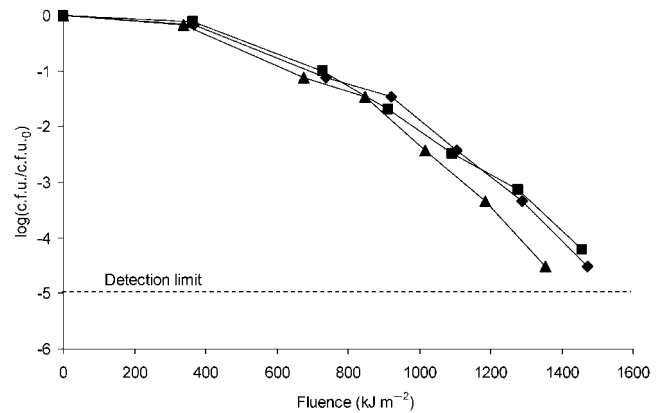
**Total ATP.** For the determination of total ATP, the BacTiter-Glo system (Promega) was used. The BacTiter-Glo buffer was mixed with the lyophilized BacTiter-Glo substrate and equilibrated at room temperature. The mixture was stored overnight at room temperature to ensure that all ATP was hydrolysed ('burned off') and the background signal had decreased. A cell suspension of 100 µl was mixed in a 2 ml Eppendorf tube with an equal volume of the previously prepared BacTiter-Glo reagent (stored on ice). The sample was then briefly vortexed and put into a water bath at 37 °C for 30 s. The luminescence of the sample was measured in a luminometer (model TD-20/20; Turner BioSystems) immediately after incubation. A calibration curve with dilutions of pure rATP (Promega, P1132) was measured before each experiment. ATP concentration per cell was then calculated using this calibration curve and the total count measurements (Syto 9) from flow cytometry.

## RESULTS

We investigated the potential of sunlight to inactivate different cellular functions in stationary-phase *E. coli* cells and compared it to artificial UVA light. The application of multi-parameter flow cytometry allows the measurement of cellular functions at the single-cell level (as opposed to bulk parameters), thus, one can acquire a more detailed picture of the physiological state of bacterial cells than that with plating alone.

### Culturability and total counts

Three independent experiments were conducted to measure all cellular functions during artificial UVA irradiation. Culturability on TSA was measured in all three experiments to ensure comparability of results. The inactivation based on this parameter was reproducible (Fig. 2). Exposure to sunlight was repeated twice (on two different days) while, in the experiment presented here, triplicate measurements (three independent cultures were grown, diluted and exposed to the same sunlight) of culturability were



**Fig. 2.** Inactivation curves of *E. coli* K-12 MG1655 exposed to artificial UVA light in three independent experiments (▲, ■, ◆). Bacterial cells were harvested from a stationary-phase LB batch culture, washed and diluted in mineral water (Evian). Initial cell numbers in these experiments were  $1.7\text{--}1.9 \times 10^7$  cells ml<sup>-1</sup>. Culturability of bacterial cells was measured with the pour-plate method using TSA.

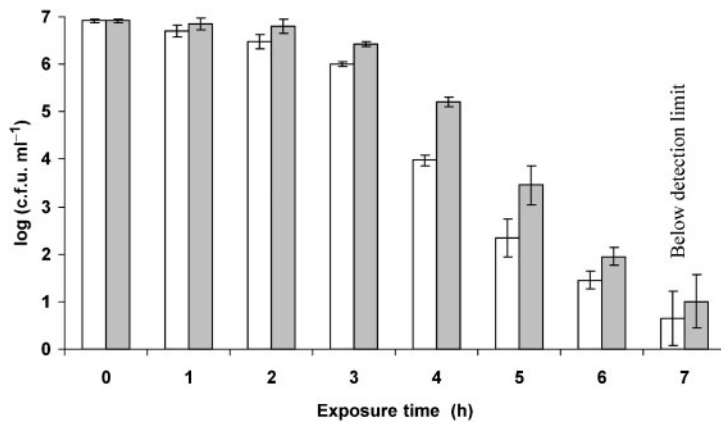
made (Fig. 3). To prevent oxidative bursts in the cells during aerobic incubation, samples were also incubated on pyruvate-supplemented agar in anaerobic jars, which resulted in higher c.f.u. In all experiments, total counts remained constant over the whole irradiation period (Fig. 4a). UVA light appeared to be slightly more effective in inactivating *E. coli* cells than sunlight when assessed with plating (Fig. 4b).

### ATP and efflux pump activity

Soon after the start of exposure (400–600 kJ m<sup>-2</sup>) to either UVA light or sunlight, the ATP concentration per cell dropped significantly and levelled out to ~5 % of the initial value (Fig. 4c). The uptake of EB (non-pumping cells) closely followed the decline in ATP concentration per cell (Fig. 4d). EB-positive cells (non-pumping cells) showed a decrease in green fluorescence intensity (quenching effect) and an increase in red fluorescence intensity on the flow cytometer (Fig. 5a–c). The loss of culturability of bacterial cells appeared to be time-delayed when compared to the decrease in ATP.

### Membrane potential and membrane integrity

The loss of membrane potential, which was measured with the green fluorescent bis-oxonol DiBAC<sub>4</sub>(3), was very similar for both light sources (Fig. 4e). In the flow cytometer, *E. coli* cells with polarized cytoplasmic membranes did not appear, because the signal trigger was set on the green fluorescence channel (FL1, 520 nm; Fig. 5d), therefore, only depolarized (green fluorescent) cells were counted (Fig. 5e, f). The percentage of depolarized cells was calculated using the total count measurements. In our experiment, depolarization of the bacterial cells occurred later than



**Fig. 3.** Culturability of *E. coli* K-12 MG1655 during sunlight exposure for 7 h. Bacterial cells were harvested from a stationary-phase LB batch culture, washed and diluted in mineral water (Evian). Samples were plated either on unsupplemented TSA (pour-plate method) and incubated under aerobic conditions (open bars), or on TSA with 0.05% sodium pyruvate and incubated in anaerobic jars (grey bars). Error bars represent SD from triplicate measurements.

the cessation of efflux pump activity but before membrane integrity was lost (Fig. 4f). PI, a dye which only enters cells with permeabilized cytoplasmic membranes, was only detected after a dose of 2000–2500 kJ m<sup>-2</sup> had been applied. The permeabilized cell population showed a typical increase in red fluorescence and decrease in green fluorescence intensity because of quenching (Fig. 5a–i). Interestingly, green fluorescence intensity increased by a factor of 4.3 before PI was able to enter the cells significantly. At the end of the sunlight experiment, after a fluence of 2088 kJ m<sup>-2</sup>, ~50% of the cell population had lost membrane integrity. From the UVA light experiment, we learned that, after ~2500 kJ m<sup>-2</sup> of UVA light, most of the *E. coli* cell population (>95%) had lost membrane integrity. Artificial UVA light seemed to be slightly more effective in permeabilizing *E. coli* cells than sunlight.

### Glucose uptake activity

The 2-NBDG uptake kinetics of healthy *E. coli* cells followed a typical pattern (Fig. 6a). The mean green fluorescence intensity of the cells increased linearly with time during the first 10 min of incubation with 2-NBDG. Therefore, irradiated cells were always incubated for 5 min to ensure that the mean fluorescence intensity correlated with the uptake rate. The mean 2-NBDG uptake rate of the cells remained constant over the first 2 h of sunlight exposure (800 kJ m<sup>-2</sup>), thereafter, it dropped along with the number of fluorescent cells, indicating that the PEP-PTS system was compromised (Fig. 6b). Bacterial cell size did not change significantly during exposure, as was indicated by the forward angle light scatter (data not shown). This loss of the ability to take up glucose correlated well with the loss of membrane potential.

The data acquired during artificial UVA irradiation for the different cellular functions strongly resembled those of the sunlight experiments. Although the light intensity applied in the laboratory system was much higher than that during sunlight exposure, the time-dependent pattern of the different viability stains was similar. With the UVA lamp it was possible to apply a higher fluence, which eventually led to the loss of membrane integrity (PI staining). This was

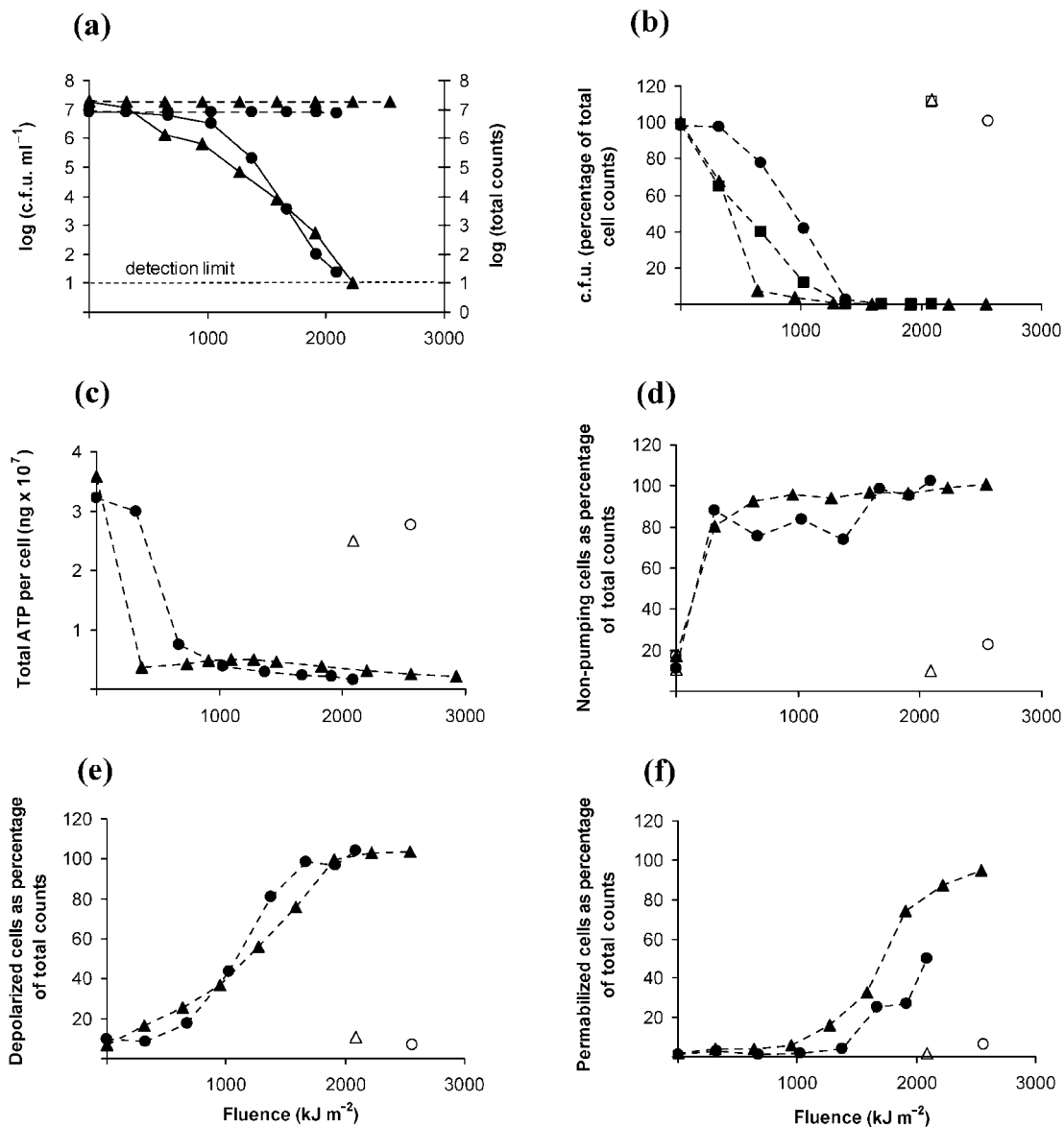
the most apparent and essential difference between the two different light sources.

### Syto 9 staining characteristics

The analysis of membrane integrity with the Syto 9/PI mixture during UVA irradiation revealed a unique fluorescence pattern for the bacterial cell population (Fig. 5g–i). On a two-dimensional dot plot, the whole population moved in a circle from low green and red fluorescence intensity (Fig. 5g) to an ~4.3-fold increased green fluorescence intensity (Fig. 5h), and then to a state of increased red and decreased green fluorescence intensity (Fig. 5i). The increase of green fluorescence intensity coincided with the loss of culturability of >97% of the cell population and, only after prolonged irradiation, was PI able to enter the cells and quench Syto 9 fluorescence. Hence, membrane integrity was lost long after the culturability of the population dropped below the detection limit. An increase in green fluorescence intensity was also observed when *E. coli* was exposed to sunlight. In these experiments, the geometrical mean of green fluorescence intensity of stained cells increased ~3.7-fold, while culturability was lost by >98% of the population. This observation is probably related to an increased uptake of Syto 9. This was tested by using the Syto 9/PI mixture and Syto 9 alone in the same irradiation experiment (Fig. 7). Using Syto 9 alone also led to a significant 3.7-fold increase in green fluorescence intensity. Furthermore, staining with DiBAC<sub>4</sub>(3) revealed that the loss of membrane potential coincided with the increase in green fluorescence intensity in samples that were stained with the Syto 9/PI mixture (Fig. 5e, h). This implies that Syto 9 is also subject to active dye exclusion and accumulates intracellularly when efflux pump activity and membrane potential are lost.

## DISCUSSION

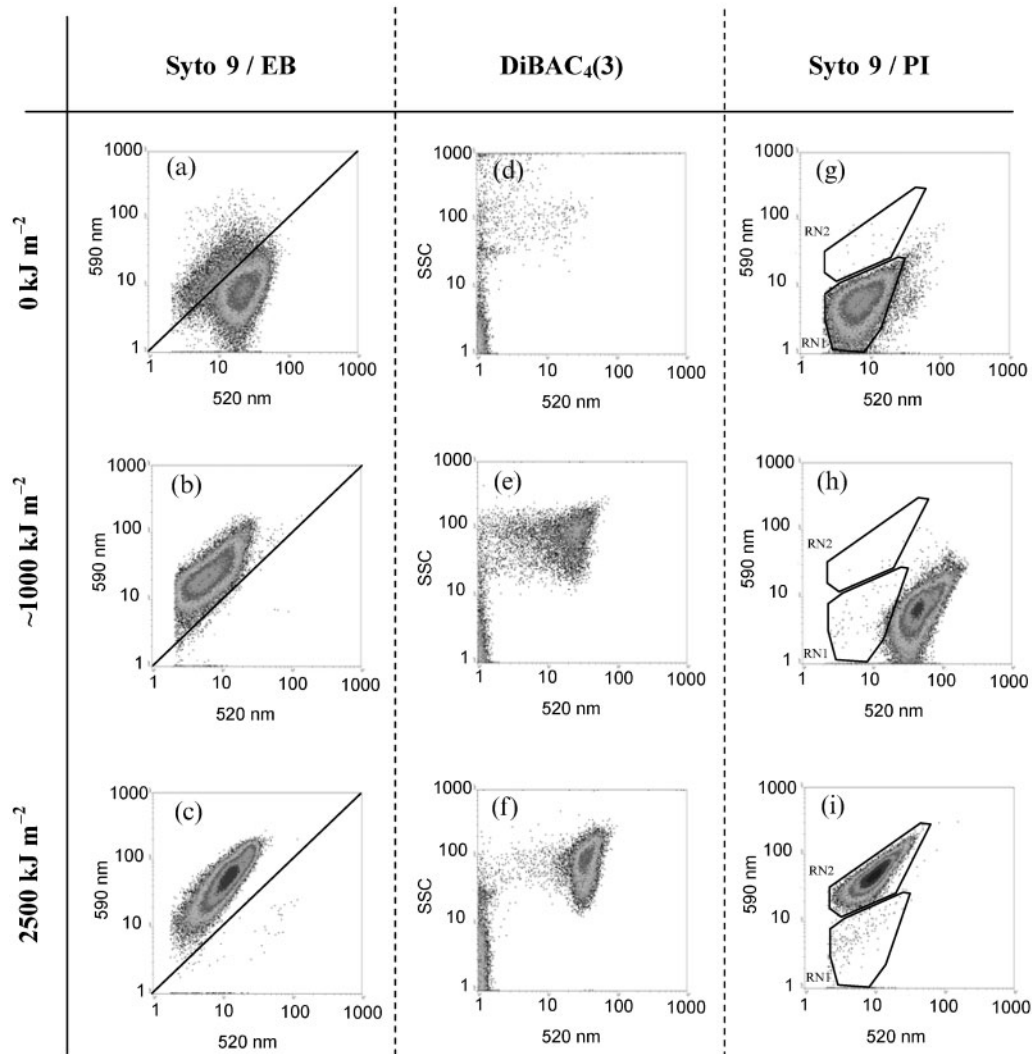
This work enables us to present an inactivation pattern of the essential cellular functions in *E. coli* during exposure to both sunlight and artificial UVA light. Our finding that artificial UVA light produces an almost identical



**Fig. 4.** Analysis of cellular functions of *E. coli* K-12 MG1655 exposed to sunlight (circles) or artificial UVA light (triangles) with five viability indicators. Bacterial cells were harvested from a stationary-phase LB batch culture, washed and diluted in mineral water (Evian). Results were calculated as a percentage relative to the total cell count (Syto 9-stained cells) at a given sampling point. Unstressed control samples (in the dark at 37 °C) are displayed as open symbols. (a) Total counts (Syto 9-stained cells, dashed lines) measured flow-cytometrically compared to c.f.u. (solid lines). (b) Culturability of UVA- (▲) and sunlight-irradiated cells [aerobic (■) and anaerobic (●) incubation] as a percentage of total counts. (c) Total ATP concentration per cell. (d) Non-pumping cells; loss of efflux pump activity measured with Syto 9/EB staining. (e) Depolarized cells; loss of membrane potential measured with DiBAC<sub>4</sub>(3). (f) Permeabilized cells; loss of membrane integrity measured with Syto 9/PI staining by flow cytometry. All experiments were done at least twice. Data from one representative experiment are displayed.

inactivation pattern to that of sunlight indicates that there is a similar inactivation mechanism for both light sources. UVA light seems to affect the functioning of the electron transport chain, because ATP synthesis and efflux pump activity (both functions are dependent on a trans-membrane proton gradient) are inactivated quickly. The loss of culturability correlated well with the loss of

membrane potential and glucose uptake activity. We also describe a yet-unreported feature of Syto 9 uptake, namely its dependence on membrane potential and efflux pump activity. Furthermore, our results stress the importance of using several viability indicators, apart from culturability, to characterize the physiological state of a stressed bacterial cell.



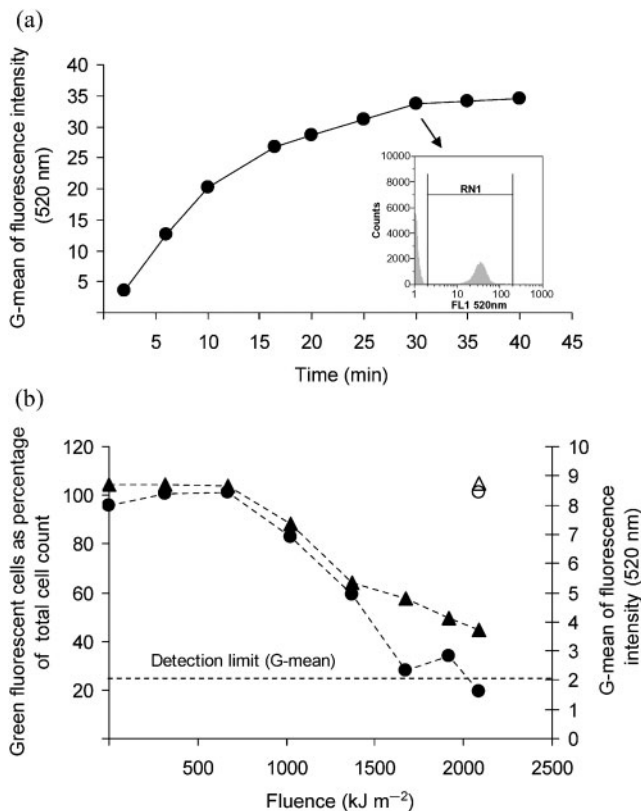
**Fig. 5.** Flow-cytometric analysis of *E. coli* K-12 MG1655 irradiated with artificial UVA light. Bacterial cells were harvested from a stationary-phase LB batch culture, washed and diluted in mineral water (Evian). Bacterial cell samples were stained with Syto 9/EB, DiBAC<sub>4</sub>(3) or Syto 9/PI and analysed on a flow cytometer after being exposed to different fluences (irradiation intensity  $\times$  time). After 1000 kJ m<sup>-2</sup>, >95% of the cells were non-pumping (b), 40% were depolarized (e), and <1% permeabilized (upper polygon-gate RN2) (h). After 2500 kJ m<sup>-2</sup>, 100% of the cells were non-pumping (c), 100% depolarized (f), and >90% permeabilized (polygon-gate RN2) (i). SSC, side scatter.

### Inactivation pattern of vital cellular functions

The inactivation of cellular functions in *E. coli* measured during exposure to light followed a typical inactivation pattern. Shortly after the start of exposure, the total ATP concentration decreased rapidly, indicating the cessation of ATP synthesis by ATPases. At the same time, efflux pump activity stopped as well. Both functions are dependent on the proton motive force. Gradually, the membrane potential was also lost and the glucose uptake rate diminished accordingly. Finally, the cytoplasmic membrane of bacterial cells became permeable. The loss of culturability was dependent on the incubation method and the growth medium. Anaerobic incubation on sodium

pyruvate-supplemented agar, a procedure known to also recover injured cells (Bromberg *et al.*, 1998; Czechowicz *et al.*, 1996), showed a decrease in culturability that closely correlated with the loss of membrane potential ( $r^2=0.96$ ).

The rapid decrease of total ATP during irradiation implies a direct or indirect inhibition of ATP synthesis and the utilization of the remaining ATP by ATP-dependent functions. Nevertheless, a certain level of ATP was still measured after prolonged irradiation. This implies that cells could either maintain alternative ways of ATP synthesis, such as glycolysis, or that the remaining ATP could not be used due to damage to systems which require ATP. It has been shown that the activity of ATPase from *Saccharomyces*



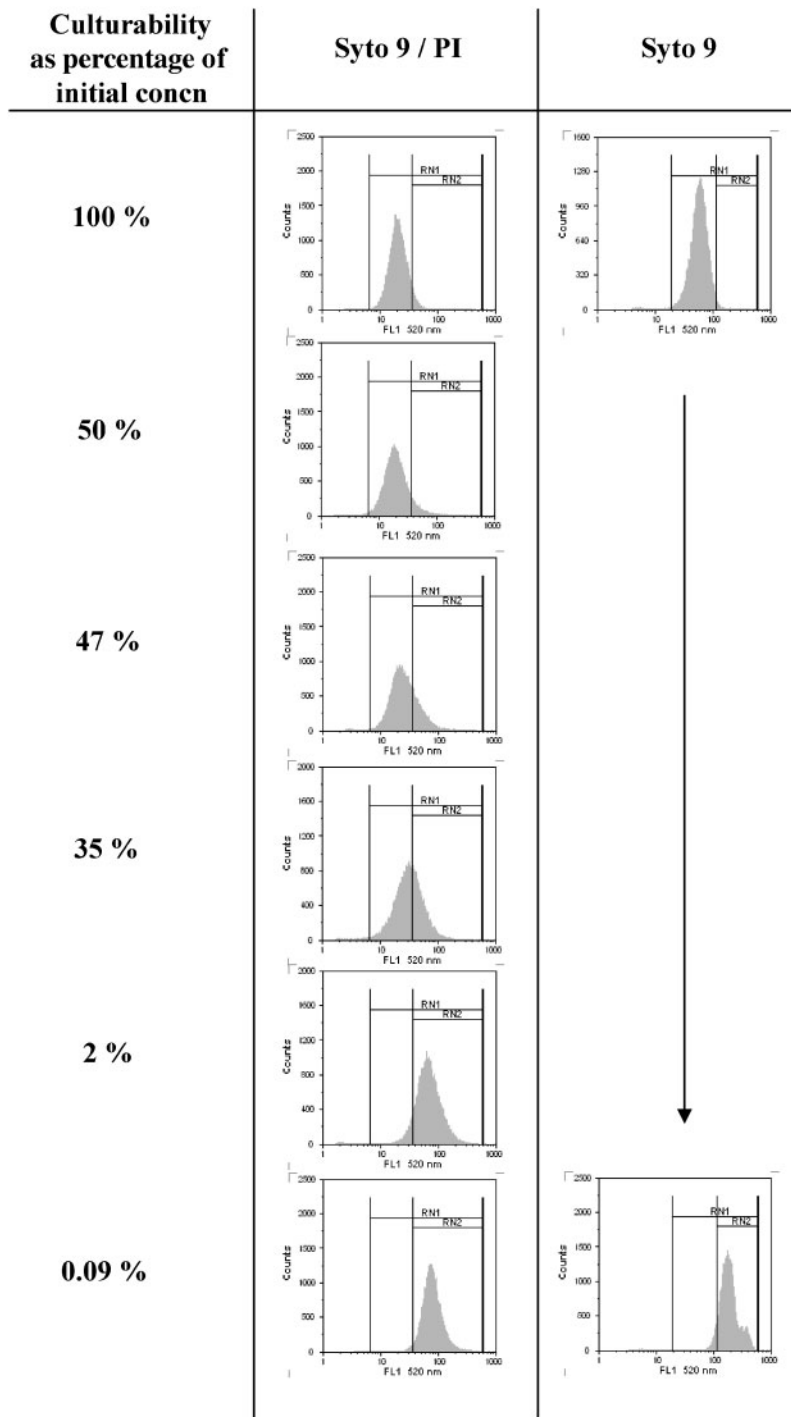
**Fig. 6.** (a) 2-NBDG uptake by *E. coli* K-12 MG1655 harvested from a stationary-phase LB batch culture, washed and diluted in sterile-filtered, bottled mineral water (Evian). A bacterial cell sample was incubated with 2-NBDG and 2,4-dinitrophenol at 37 °C. Green fluorescence intensity (520 nm) of sub-samples was analysed every 5 min in a flow cytometer. On the graph, the geometrical means (G-means) of green fluorescence intensity (measured in range-gate RN1) are displayed in the inset. (b) 2-NBDG uptake of *E. coli* K-12 MG1655 (pre-treatment as above) exposed to sunlight. Bacterial cell samples were incubated with 2-NBDG and 2,4-dinitrophenol for 5 min at 37 °C and analysed immediately in a flow cytometer. (●) 2-NBDG-positive cells as a percentage of total cell counts; (▲) G-mean of green fluorescence intensity (520 nm) of the cell population. Unstressed control samples are displayed with open symbols.

*cerevisiae* solubilized from the plasma membrane and exposed to UVA light remains constant irrespective of dosage, indicating that the ATPase molecule itself is not damaged by UVA irradiation (Arami *et al.*, 1997a, b). The authors proposed that the reduction of ATPase activity in the membrane by UVA irradiation is attributable to conformational changes resulting from an alteration in the higher-order structure of the membrane due to photochemical decomposition of ergosterol. For *E. coli* K-12 growing on succinate, it has been shown that ubiquinone Q-8 is a chromophore for inhibition of ATP synthesis (Lakchaura *et al.*, 1976). Nevertheless, the same authors concluded that the oxidative phosphorylation system is

not a primary factor in the induction of growth inhibition in *E. coli* by UVA light, because the doses required for inhibition of growth are only one-sixth of those required for inhibition of ATP synthesis. This contradicts our findings that the inhibition of ATP synthesis and efflux pump activity occurs earlier than the loss of culturability. However, it has to be mentioned that, in their experiments, stressed cells were plated on unsupplemented complex media. Such media can enhance an oxidative burst in bacterial cells, which eventually leads to cell death (Aldsworth *et al.*, 1999; Dodd *et al.*, 1997; George *et al.*, 1998). Furthermore, the strain used in their study (AB2480) was *recA*-deficient and, therefore, was probably more susceptible to UVA light. These circumstances can lead to an overestimation of the negative effects of UVA light on the culturability of *E. coli*.

It has been hypothesized that when a cell is stressed energetically, the active transport systems will cease first, then the cytoplasmic membrane will depolarize, and finally permeabilization will occur, indicating cell death (Nebe-von-Caron *et al.*, 2000). These authors observed that *Salmonella typhimurium*, which was stored for 25 days on nutrient agar at 4 °C and resuspended in growth medium, could be differentially stained with a combination of EB, DiBAC<sub>4</sub>(3) and PI. In their case, 35 % of the depolarized cells could still form colonies when sorted onto nutrient agar plates, while all permeabilized cells were unable to grow. In our experiments, depolarization of bacterial cells correlated well with the loss of culturability. In contrast to their measurements, we did not use EDTA to facilitate permeabilization of the outer membrane. Still, the uptake of DiBAC<sub>4</sub>(3) seemed to be specific without the addition of EDTA, because permeabilization of the cytoplasmic membrane was observed at a much higher fluence. Without disrupting the outer membrane with EDTA, one probably overestimates the fluence needed to depolarize and permeabilize the cytoplasmic membrane of *E. coli*. In the case of SODIS this is rather favourable, because an underestimation could have severe consequences for the user of SODIS.

The specific glucose uptake rate by the PEP-PTS decreased simultaneously with the loss of membrane potential, although the cause of inhibition remains unclear. Changes in uptake rates due to cell size can be ruled out, because bacterial cell size remained constant during exposure (data not shown). Also, the loss of membrane potential cannot be a direct cause, because 2-NBDG uptake is measured in combination with an uncoupler (2,4-dinitrophenol) which itself causes a collapse of the bacterial membrane potential (by preventing ATP synthesis, hence the degradation of 2-NBDG) (Natarajan & Srienc, 1999; White, 1995). One can speculate that enzymes of the PEP-PTS are inactivated by UVA light, and that glucose is taken up only until all PEP is used up. It has been proposed that components of the electron transport chain, possibly menaquinone or dehydrogenases, are damaged by UVA light and that this inhibits permeases such as the lactose permease (Jagger, 1981). Therefore, the high-affinity glucose permease could be an



**Fig. 7.** Flow-cytometric analysis of *E. coli* K-12 MG1655 irradiated with artificial UVA light. Bacterial cells were harvested from a stationary-phase LB batch culture, washed and diluted in mineral water (Evian). Staining with Syto 9/PI or Syto 9 alone was compared to culturability on TSA (pour-plate method). The loss of culturability coincided with an increase in green fluorescence intensity (range-gate RN2).

indirect target of UVA. Recently, it has been revealed by two-dimensional PAGE analysis that pyruvate kinase (PykF) and elongation factor G (EF-G) (FusA) of *S. typhimurium* specifically increased in cells just after heat treatment and recovery in tryptic soy broth (TSB) (Kobayashi *et al.*, 2005). PykF catalyses the synthesis of pyruvate from PEP in the sugar phosphotransferase system. Kobayashi and coworkers suggest that the increase of PykF in cells just after heating and recovery of cells might facilitate the production of

ATP in the electron transport system, because pyruvic acid is a key substrate for the citric acid cycle and subsequent electron transport system (Kobayashi *et al.*, 2005). The same authors state that ATP seems to be particularly important, because many reactions involved in recovery require ATP as an energy source. If PykF were a direct or indirect target of UVA light, it would prevent recovery by the process described above. This is in line with our finding that cells which are inhibited in ATP synthesis can still be recovered

on sodium pyruvate-supplemented agar. Furthermore, inhibition of PykF would not directly inhibit glucose uptake by the PEP-PTS, because PEP is not converted to pyruvate and is still available for the uptake of glucose molecules, as discussed above.

All viability indicators were applied again to the irradiated cells 5 days after irradiation. No regrowth or recovery of injured bacterial cells was observed (data not shown). Moreover, a significant decrease in all measured cellular functions, especially in cells which received  $> 1500 \text{ kJ m}^{-2}$  solar UVA (corresponding to  $530 \text{ W m}^{-2}$  global sunlight intensity for 6 h), was observed. This indicates that the damage in *E. coli* caused by UVA light is irreversible. It has to be mentioned, however, that resuscitation attempts with sterile supernatant, as carried out in previous studies (Kaprelyants & Kell, 1993; Mukamolova *et al.*, 1998), were not performed in this study. However, to our knowledge, the resuscitation of permeabilized cells has never been shown and seems highly unlikely.

### Differences in viability stains

The death of a bacterial cell has long been defined as the inability of a cell to grow to a visible colony on bacteriological media. With culturability methods, one can only observe bacterial death in retrospect (Postgate, 1989). Today, several viability indicators can be assessed without culturing cells, and each method is based on criteria that reflect different levels of cellular integrity or functionality. Consequently, the interpretation of viability is often ambiguous. Hewitt & Nebe-Von-Caron (2004) have shown that multi-parameter flow cytometry allows a functional classification of the physiological state of single-celled micro-organisms. They claim that, with this technique, it is possible to resolve a cell's physiological state beyond culturability and to determine population heterogeneity. Our study confirms their findings and shows that the use of only one viability indicator, as often applied, is not sufficient to describe the physiological status of a bacterial cell under stress. Therefore, these viability indicators or indirect methods do not provide short cuts by which viability may be determined (Postgate, 1967). We propose that only the sum of all these methods, including the detection of culturability, can give us some certainty about the physiological state of a bacterium.

### Implications for the SODIS method

Our results show that a UVA fluence of  $> 1700 \text{ kJ m}^{-2}$  is needed to depolarize the cytoplasmic membrane of  $> 95 \%$  of the cell population, while  $2500 \text{ kJ m}^{-2}$  is needed for permeabilization. This corresponds to about  $600\text{--}880 \text{ W m}^{-2}$  of sunlight intensity (global intensity) over a period of 6 h, a threshold which can be achieved in most developing countries (Martin-Dominguez *et al.*, 2005). The same study also confirms that temperature is not a predominant factor in the elimination of bacteria with sunlight, but that it is mainly radiation which determines the efficiency of

the method. Furthermore, our culturability data fitted well with those of Khaengraeng & Reed (2005), in which a solar simulator at  $410 \text{ W m}^{-2}$  for a period of 6 h was used to irradiate stationary-phase *E. coli* cells. A one log reduction was achieved with a UVA fluence of  $\sim 1200 \text{ kJ m}^{-2}$ . This corresponds well with the  $1400 \text{ kJ m}^{-2}$  measured in our work.

Important for the user of SODIS is the loss of infectivity of pathogenic bacteria in the treated water. It has been shown that *S. typhimurium* C5Nx<sup>r</sup> exposed to simulated sunlight (probably  $\sim 700 \text{ W m}^{-2}$ ) for 8 h and a temperature regime temporarily reaching  $55^\circ\text{C}$  fail to produce detectable infections in BALB/c mice (Smith *et al.*, 2000). Even culturable cells that have been irradiated for 1.5 h are less infective (virulent) than their non-irradiated counterparts. Although infectivity of target organisms is the ultimate parameter to measure in disinfection processes, it is not a feasible alternative to viability measurements, because appropriate models for certain pathogens are rare and difficult to perform. Our results suggest that cells that (i) have lost culturability under anaerobic conditions in sodium pyruvate-supplemented agar, (ii) are not able to take up glucose, and (iii) have lost membrane potential, will not be able to regain viability in the human intestine. The highest certainty about cell death, however, will be achieved if fluences are applied that lead to membrane permeability. It is likely that these results also apply to other enteric bacteria, but this still has to be investigated. Also, the effect of sunlight on eukaryotes, such as *Cryptosporidium* or *Amoeba*, can be assessed with the methods used in this study.

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