Inactivation of Enteric Bacteria
by Solar Disinfection

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Summary

The World Health Organization (WHO) estimated in 1996 that more than five million people die per year from illnesses linked to consumption of unsafe drinking water or inadequate sanitation. In developing countries, population growth and the lack of money make it almost impossible to provide the needed infrastructure for safe drinking water. Alternative approaches suitable for rapid implementation are needed to meet present needs. Self-sustaining, decentralized approaches like Solar Disinfection of drinking water (SODIS) have been proposed and also tested in the field. SODIS is a simple water treatment method using solar UVA radiation and mild heat to inactivate pathogens causing diarrhea. SODIS is used at household level under the responsibility of the user. Although SODIS has already been widely used there are still many unanswered questions, some of which are addressed in this thesis.

Most of the experiments concerning SODIS have been conducted using E. coli, the fecal indicator organism in drinking water surveillance. However, experimental data about the effect of SODIS on pathogenic bacteria in drinking water are still scarce. It is demonstrated in this thesis that resistance to sunlight of four enteric strains was in the following order: S. Typhimurium > E. coli > S. flexneri > V. cholerae. Additionally it is demonstrated that the reciprocity law (i.e., total applied fluence produces the same response regardless of the fluence rate) is applicable for solar disinfection of E. coli. Furthermore, we showed that sensitivity to mild heat of V. cholerae starts above 40 °C and, therefore, the mild heat effect in SODIS experiments should not be neglected since a small increase in temperature can immensely increase disinfection efficacy for certain strains.

Also, the influence of the preparation of test cells and the way of exposure on stress-sensitivity has often been neglected in SODIS studies. Here it is demonstrated that sensitivity of E. coli to SODIS-relevant stresses like UVA light and mild heat are specific growth rate-dependent. Bacterial cells that grew fast before washing and subsequent exposure were more susceptible to SODIS than slow or non-growing cells. A correlation between sensitivity to SODIS and RpoS, a global stress regulator in E. coli, was demonstrated. The study emphasizes the
Summary

need in disinfection studies for accurate reporting of specific growth rates and
detailed culture conditions for test organisms and provides a procedure to
conduct experiments with reproducible results.

The efficacy of SODIS has been shown using the classical cultivation of micro-
organisms on solid agar substrates. In recent years increasing concern about the
validity of such methods arose, because it was shown that they can lead to an
overestimation of effectiveness of disinfection processes. Therefore, the
disinfection process during SODIS was analyzed for E. coli at the single cell level
with the latest culture-independent methods (multi-parameter flow cytometry
and others). The inactivation pattern of life-supporting functions, namely efflux
pump activity, membrane potential, membrane integrity, glucose uptake activity,
total ATP concentration and culturability is described for E. coli under sunlight
and artificial sunlight radiation. The results strongly suggest that cells exposed
to more than 1500 kJ/m² of solar UVA (corresponding to 530 W/m² global
sunlight intensity for 6 h) were not anymore 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.

It is still unclear, which measures cells can take to withstand solar irradiation,
which nutritional conditions are more favorable for resistance or even survival,
and how this is influenced by irradiation intensity, irradiation dose and
wavelengths distribution. While all investigations described above were carried
out with starved cells the question remained whether cells have increased
chances to survive sunlight exposure if supplemented with the appropriate
amount of nutrients. It is shown here that E. coli cells growing in continuous
culture (at a dilution rate ≥ 0.3 h⁻¹) were able to maintain growth under UVA
irradiation at an intensity of 50 W/m² after a transient phase of adaptation. In
contrast, slow growing cells (D = 0.05 h⁻¹) could not induce enough "protection
capacity" to maintain growth under UVA irradiation. This suggests that fast
growing E. coli cells have a higher adaptive capacity to UVA light-stress than
slow-growing cells. Although such adaptation is rather unlikely in the natural
aquatic environment due to low temperatures and substrate concentrations, an
investigation of the nature of this adaptation can elucidate the inactivation
mechanism of UVA light and sunlight. Hence, global gene expression analysis was carried out at different time points during the adaptive response. It is shown that UVA light induces stringent response and an additional stress response that includes the upregulation of amino acid synthesis of valine, isoleucine, leucine, phenylalanine, histidine and glutamate. Furthermore, our results corroborate earlier reports about the induction of the SOS response in UVA irradiated cells and the involvement of oxidative stress. Taken together our findings support the hypothesis that production of reactive oxygen species by UVA light, damages DNA, enzymes or other life-supporting cell structures. Additionally, in the UVA-adapted population a strong repression of the acid tolerance response was found. Also, a number of enzymes (e.g., PheA, MgtA, and PyrBI) was differentially expressed and can now be further investigated to elucidate their role in protection of *E. coli* against UVA light.

**Zusammenfassung**

immer noch offene zentrale mikrobiologische Fragen, welche in dieser Dissertation behandelt werden.


ein genaueres Bild über den Absterbe-Prozess der Enterobakterien zu gewinnen. Dafür wurde die Inaktivierung von lebenswichtigen Funktionen wie die Aktivität der Membranpumpen, das Membranpotential, die Membranintegrität, die Aktivität der Glukoseaufnahme, der totale ATP-Gehalt und die Kultivierbarkeit während der Sonnenbestrahlung untersucht und eine Inaktivierungs-Abfolge wurde bestimmt. Die Resultate zeigen, dass Zellen, die mit einer UVA-Dosis von mehr als 1500 kJ/m² (entspricht ca. 530 W/m² Globalstrahlung während 6 Stunden) bestrahlt werden, die Beschädigungen nicht mehr reparieren können. Die Arbeit attestiert SODIS eine tödliche Wirkung auf E. coli und zeichnet ein detailliertes Bild des „Todeskampfes“ von E. coli-Bakterien unter Sonnenbestrahlung.

1. **General introduction**

**Introductory remark**

In this work, solar disinfection (SODIS) of drinking water, a low-cost household water treatment method for developing countries, was investigated for its effectiveness on *E. coli* and different enteric pathogens. The response of *E. coli* to sunlight and artificial sunlight was analyzed on the physiological and molecular level. A comprehensive description and discussion of SODIS follows below. As an introduction to the subject the published physical, microbiological and molecular information, which is relevant to the SODIS method, is reviewed and summarized.

**Solar radiation**

Solar radiation, in particular the ultraviolet (UV) part, is one of the major stresses organisms have to face on the surface of this planet. It affects microorganisms, plants, animals and humans by either catalyzing or modulating biochemical reactions in the cell (Eisenstark, 1989; Phillipson *et al.*, 2002). Commonly, the UV radiation spectrum is arbitrarily subdivided into three wavelength bands designated as UVC (190 - 290 nm), UVB (290 - 320 nm) and UVA (320 - 400 nm) (Jagger, 1985). The range from 320 - 400nm is also referred to as near-UV (NUV) radiation / light, because its wavelengths are close to the visible spectrum of light and consequently the lower range (190 - 290 nm) is defined as far-UV (FUV) light (Jagger, 1985). Quite often the term NUV light is used for the range 290 - 400 nm (e.g., Eisenstark (1989). The part of the solar UV radiation that reaches the surface of the earth consists mainly of NUV light, since the ability of radiation to penetrate the atmosphere drops dramatically for wavelengths below 320 nm due to the ozone layer in the stratosphere. On the following pages NUV and UVA light are used as synonyms for the wavelength range
Chapter 1

between (320-400 nm) (according to Jagger). I also tried, if possible, to provide wavelength ranges in cases where confusion between definitions might occur.

**The pioneering work**

Solar radiation had long been recognized to contribute to bacterial die-off in natural waters. The first systematic study into the inhibitory effects of solar radiation on bacteria was presented by Downes (1886), who reported that the development of bacteria in nutrient broth and urine was halted by exposure to the sun. He further demonstrated that the spores of mycelial fungi are more resistant to the inhibitory action of sunlight than bacterial cells and also showed that short-wavelength solar radiation had the strongest antimicrobial effect.

About 50 years later Alexander Hollaender (1943) performed remarkable experiments to establish the lethal action of FUV and NUV radiation, and his work was fundamental for UV photobiology. In carefully controlled irradiation experiments he exposed *E. coli* to UV radiation of 265 nm and 350 - 490 nm. He found a significant difference in survival behavior after exposure to FUV compared to NUV light. Stationary cells exposed to 265 nm UV light showed a straight exponential inactivation pattern (assessed by determining colony-forming units (CFU)), while at 350 - 490 nm a shoulder effect was observed during the first 40 minutes of exposure where little or no inactivation occurred. Furthermore, cells irradiated at wavelengths above 300 nm showed increased UV-sensitivity with increasing temperatures between 15-40 °C. Incubation of cells after irradiation in dilute beef broth resulted in growth retardation, indicating that cells had to recover from damage before resuming growth. Additionally, when irradiated bacteria were suspended in physiological salt solution, a substantially greater dying rate was observed compared to the control. Hollaender hypothesized that wavelengths below 300 nm directly damage nucleic acids (before these were actually recognized as the carrier of genetic information!) and that near UV radiation either produces some toxic substances that inhibit growth, or destroys essential compounds in the cell.
Additionally, he suggested that the presence of a temperature effect indicates the existence of some secondary process. This could be the inhibition of material needed for repair or some morphological changes resulting in increased permeability. He was proven right in most of his ideas by later investigations. His ideas will be discussed below whilst putting them also into context with the progress made in NUV light research.

"The toxic substances" and reactive oxygen species (ROS)

In the early seventies it was demonstrated that NUV light-inactivation is oxygen-dependent (Eisenstark, 1970; Webb & Lorenz, 1970). This indicated that oxygen might play an important role in the unknown mechanism of inactivation. Others proposed that H₂O₂ was an important photoproduct of NUV light, from which subsequently other reactive oxygen molecules were generated. This suggestion was supported by later reports (Ahmad, 1981; Hartman & Eisenstark, 1978; Hartman et al., 1979; McCormick et al., 1976). It was also confirmed again by Webb and Brown who observed no oxygen dependence of damage in E. coli WP2s at 313 nm and shorter wavelengths, and strong oxygen dependence at wavelengths longer than 313 nm (Webb & Brown, 1979). Their data suggested the participation of non-DNA cell constituents in aerobic lethality.

Essentially, any molecule in the cell, with an absorption band between 320-400 nm can absorb NUV radiation. Such molecules are referred to as chromophores or photosensitizers. Photosensitization either inactivates the photosensitizer itself, as reported for thiolated tRNA (Favre & Hajnsdorf, 1983; Jagger, 1985), or the absorbed energy is transferred to other molecules, particularly molecular oxygen, producing reactive oxygen species (ROS). The main forms of reactive oxygen species include singlet oxygen (¹O₂), the superoxide radical anion (O²⁻), hydrogen peroxide (H₂O₂), and the hydroxyl radical (OH⁻). Superoxide is involved in the production of hydrogen peroxide, which can produce oxidizing species in the presence of iron. Hydroxyl radicals are highly reactive and can oxidize virtually any organic compound in the cell, including macromolecules (for more
details see Whitelam & Codd (1986)). Consequently, (aerobic) bacteria have developed sophisticated ways for quenching ROS. Enzymes involved in the detoxification of ROS are superoxide dismutase (specific for \( \text{O}_2^\cdot \)), catalase and peroxidase (both for \( \text{H}_2\text{O}_2 \)) (Madigan et al., 2000). However, it has been shown that in bacteria catalase plays only a minor role in protection against NUV radiation damage (Eisenstark & Perrot, 1987) probably because it acts as a chromophore itself and is easily inactivated (Kramer & Ames, 1987; Zigman et al., 1996). The last extensive review about oxidative stress responses in \( \text{E. coli} \) and \( \text{Salmonella Typhimurium} \) was presented more than ten years ago (Farr & Kogoma, 1991). Oxygen-dependent photoreactions were proposed to be the major component of solar inactivation as a result of ROS-induced membrane lipid peroxidation (Bose & Chatterjee, 1994) and DNA damage (Yonezawa & Nishioka, 1999).

**Changes in morphology – increased permeability**

Yet, the endogenous production of toxic substances during NUV light exposure was not the only prediction of Hollaender that was confirmed in later research. In the 1960ies it was found that broad-spectrum NUV light can block the electron transport chain by photochemical decomposition of aromatic cofactors, predominately the membrane-associated quinones (see review by Jagger (1972)). Koch and coworkers (1976) presented evidence that NUV light can alter galactoside transport in \( \text{E. coli} \) in several independent ways. It can inactivate transport systems, interfere with metabolic energy production, and it can cause a general increase in permeability of the membrane. Subsequently, membrane damage by NUV light was investigated by a number of scientists mainly during the 1980’s (Chamberlain & Moss, 1987; Kelland et al., 1983a; Kelland et al., 1983b; Kelland et al., 1984; Klamen & Tuveson, 1982; Leven et al., 1990; Mandal & Chatterjee, 1980; Moss & Smith, 1981; Pizarro, 1995; Pizarro & Orce, 1988; Sammartano & Tuveson, 1987; Tuveson et al., 1988; Wagner et al., 1980). It was in that time when a controversy arose about the relative importance of DNA
damages versus membrane damages in the inactivation mechanisms in bacteria induced by NUV radiation.

The bacterial cytoplasmic membrane (inner membrane in Gram negative bacteria) is of complex nature. It incorporates "dissolved" organic molecules such as cytochromes and quinones, enzymes of the electron transfer system, transport systems, and enzymes involved in biosynthetic processes. Additionally, the membrane integrity is essential in providing a permeability barrier. Therefore, there are a number of possible target molecules within the membrane that can be damaged by UV light. Mandai and Chatterjee (1980) were the first to show that the leakiness of liposomal membrane particles was directly caused by exposure to either UV light or sunlight. Others observed that the survival of the wild-type *E. coli* K-12 strain JG139 after irradiation with broad-band NUV radiation was significantly influenced by the concentration of inorganic salts present in the minimal medium used for plating (Moss & Smith, 1981). Increased inorganic salt concentrations in the medium caused an increase in cell lethality after NUV light but not after FUV irradiation. This was confirmed later in studies where $^{86}$Rb$^+$ was used to detect leakage of the cytoplasmic membrane (Kelland et al., 1983a; Kelland et al., 1983b; Kelland et al., 1984). Others were able to link NUV light-induced membrane damage with lipid peroxidation (Chamberlain & Moss, 1987). They proposed that lipid hydroperoxides can lead to a disorder in the structure of the membrane lipid bilayer, causing increased permeability for metabolites and inorganic ions and that this may impair the function of transport enzymes localized in the membrane. Furthermore, with experiments performed in D$_2$O, these authors presented data indicating that singlet oxygen might play an important role in lipid peroxidation.

Parallel to research in NUV light-induced membrane damage numerous genes have been identified in *E. coli* that may influence the response to NUV light. This includes several genes influencing catalase and superoxide dismutase expression (Loewen & Triggs, 1984; Touati, 1988), genes encoding DNA repair
proteins like exonuclease III (Eisenstark & Perrot, 1987) and endonucleases IV (Weiss & Cunningham, 1985) and other genes like nuvA (Favre & Hajnsdorf, 1983) that seems to influence 4-thiouridine formation in tRNA. Furthermore, regulons like the SOS regulon (Walker, 1996), the oxidative stress regulon controlled by the oxyR gene (Kramer & Ames, 1987), the stringent response regulon’s main effector ppGpp (Hajnsdorf & Favre, 1986), the “starvation” sigma factor RpoS (Eisenstark, 1998), and the heat-shock regulon with the sigma factor σ^32 (Yura & Nakahigashi, 1999) were reported to be linked to NUV light and temperature stress response. Abraham Eisenstark (Eisenstark, 1989; Eisenstark, 1998) comprehensively reviewed these molecular studies. However, although the studies mentioned above could bring some insights about the genetic response to NUV light-stress, we are far from understanding the process. The controversy about the primary inactivation mechanism of NUV light is not yet resolved.

**Growth inhibition, sublethal, lethal effects and adaptive response induced by NUV light**

Browsing through the literature on effects of NUV light on bacteria and solar disinfection it is striking how many different experimental procedures and growth conditions were applied to cultivate and recover the cells used in such studies. Bacterial cells were irradiated while growing in batch culture in different growth phases, but also after washing and dilution in different buffers such as PBS, M9 or HPLC grade water. Furthermore, irradiation intensity varied between 0.5 and 1000 W/m² and wavelength spectra of the lamps differed significantly. This all makes comparison of results from different studies very difficult but on the other hand also reveals that the cellular physiological state of bacteria can greatly influence the response to NUV light or sunlight. It is still unclear, which measures cells can take to withstand solar irradiation, which physiological states are more favorable for either increased resistance or survival, and how this is influenced by irradiation intensity (fluence-rate), irradiation dose
General Introduction

(fluence) and wavelengths distribution. The damages caused in *E. coli* from NUV light and sunlight at different doses are commonly split into lethal and sublethal injury or actions (Eisenstark, 1989; Jagger, 1985). Two different phenomena of sublethal injury in bacteria can be found in the literature: Firstly, bacterial cells were referred to as sublethally injured when they did not grow on selective media after irradiation but could be recovered on catalase- and pyruvate-supplemented media (Kubitschek & Peak, 1980); secondly, bacterial cells grown in batch culture and irradiated with broad-band UVA light for a restricted time period were found to exhibit a lag time (also called growth delay, here referred to as growth inhibition) before resuming growth. Unfortunately, in these experiments it is not clear whether the observed growth inhibition is due to inhibition of growth of the whole population or if part of the population died and the other part resumed growth after removing the stress. In the very first report growth inhibition in *E. coli* caused by NUV irradiation with a medium-pressure mercury arc lamp (350-490 nm) was shown to be a direct function of dose and occurred already at sublethal doses (Hollaender, 1943). Some 20 years later an action spectrum for the induction of growth inhibition with a maximum at 340 nm was presented for *E. coli* B (Jagger et al., 1964). Subsequently, a number of studies were initiated to characterize the response of *E. coli* to NUV light. It was shown that growth inhibition was largely due to absorption of light by 4-thiouridine (s4U), an unusual base occurring in the 8-position in 65 % of tRNAs species of *E. coli* (Jagger, 1985). The base 4-thiouridine absorbs light optimally at about 340 nm resulting in the production of a cross-link with a cytidine at position 13 in the tRNA (Favre et al., 1985). These cross-linked tRNAs were found to be poor substrates for amino acid charging and, therefore, amino acid availability is lowered and causes a shut-off of net RNA synthesis (stringent response) (Favre & Hajnsdorf, 1983; Ramabhadran & Jagger, 1976). Others proposed a close relationship between membrane damage and the UVA light-induced growth inhibition (Koch et al., 1976; Kubitschek & Doyle, 1981; Pizarro & Orce, 1988). Other authors again suggested that the growth inhibition might help the cell to induce repair of damage (Favre & Hajnsdorf, 1983), but it was not until 1988 when it was proposed that induction of growth inhibition triggered by tRNA cross-linking could be part of an adaptive response to UVA
light (Kramer et al., 1988). The same authors pointed out that the use of only monochromatic UVA light at the $\mu_{\text{max}}$ of s'U (as opposed to broad-band UVA light) leads to an overestimation of this base as a photosensitizer and, hence, of its importance under environmental conditions, and suggested a model where s'U acts as a sensor for UVA light stress that mediates the induction of specific proteins (some of them are oxidative stress-related) and the synthesis of dinucleotides. Therefore, what was commonly referred to as sublethal injury might just describe the reaction of the cell to NUV light damages. It seems very likely that UVA light, regardless of the fluence-rate, always creates the same kind of damages in the cells and the terms sublethal and lethal injury are only dependent on fluence and the speed of repair processes. Recently, it was shown that continuous UVA irradiation of E. coli growing in batch culture with a very low intensity (7.4 W/m²) leads to an adaptive response that includes the expression of some oxidative stress-related enzymes (Hoerter et al., 2005b). This means that the adaptive effect allowed the cells to sustain a certain dose of fluence-rate but how this is achieved is still unknown. The nature and mechanism of this adaptive response is still poorly understood at the physiological as well as the molecular level. It is clear that E. coli can trigger a response to NUV light stress but it is unknown, under which nutritional conditions and in which physiological state this response leads to adaptation or to death of the cells. Furthermore, data on the genetic response to NUV light stress are still very scarce.

**Temperature effect and the idea of solar water disinfection**

Hollaender’s results were confirmed with the observation of a strong positive interaction (synergy) of radiation dose and mild heat (52 °C) in irradiation experiments with FUV (254 nm), NUV (334 and 365 nm) and violet visible (405 nm) light (Tyrell, 1976). Tyrell proposed that in addition to DNA damage, both heat treatment and NUV light interfere with DNA recovery mechanisms so that their combination leads to the strong synergistic effect observed. The synergy of temperature and NUV radiation was not further investigated until 1984, when
Acra and coworkers carried out field tests to assess the effect of solar radiation on the biological quality of water used in oral rehydration solution (Acra et al., 1984). He and his coworkers also investigated the feasibility of water disinfection in small quantities that would satisfy the daily needs of individuals or a family. They exposed artificially contaminated water in small, transparent containers of 1 to 3 liters volume to direct sunlight for varying periods of time. Under their test conditions E. coli strains were slightly more resistant to the damaging effects of sunlight than other bacteria such as Pseudomonas aeruginosa, Shigella flexneri, Salmonella typhi and Salmonella enteritidis, but less resistant than Salmonella paratyphi B. They concluded that E. coli strains could serve as an indicator to assess the effect of sunlight on the most important pathogenic enteric bacteria. Between 1986/1987 Acra and his coworkers carried out field experiments using continuous-flow solar disinfection units and reactor volumes of 5-18 L (Acra et al., 1990). These studies revealed that Streptococcus faecalis was only slightly more resistant to solar radiation than E. coli and, therefore, confirmed the use of the latter bacterium as an indicator organism to quantify the survival of microbial pathogens. Furthermore, they found that at higher bacterial concentrations (10⁶ CFU/ml) cells were less susceptible to solar radiation than at low or moderate concentrations (1-10³ CFU/ml). In 1985 the Integrated Rural Energy Systems Association (INRESA), an associated program of the United Nations' University, initiated a network project in which they encouraged local institutions to start research on the applicability of solar water disinfection. The results were many but they were hardly conclusive because the methods used were not standardized. It became apparent that additional research was necessary to develop guidelines for the design and operational requirements of solar water disinfection.

This was when researchers at Eawag (The Swiss Federal Institute for Aquatic Research) started research with the goal to elucidate the processes of solar water disinfection and promote the technology once the potential of its treatment efficiency was assessed (Wegelin et al., 1994). This project was called SODIS (an abbreviation for Solar Disinfection). As stated earlier, the results from Acra's group were promising but hardly conclusive. It was inferred that further
laboratory and field work was necessary to establish minimum solar radiation intensity and required exposure time for the inactivation of different types of pathogens (bacteria, viruses, protozoa and helminths), as well as to identify the most lethal range of the light spectrum. In 1994 Wegelin and coworkers (Wegelin et al., 1994) were able to show that UVA light is mainly responsible for the inactivation of microorganisms. In their experiment violet light alone ($\geq 400$ nm) was hardly bactericidal. But, along with UVA light, its inactivation rate on *E. coli* was increased by a factor of three. Furthermore, they found that a fluence (dose of solar radiation integrated over the 350-450 nm wavelength range) of $\sim 2000 \text{ kJ/m}^2$ was required to achieve a 3-log reduction of *E. coli* at water temperatures between 20 °C and 40 °C. The viruses tested were about twice as resistant as *E. coli*. Additionally, water temperatures between 20-40 °C did not affect the inactivation of bacteria by UVA and visible light irradiation but above 50 °C a synergistic effect was observed (see Figures 1.1 and 1.2). Compared to lower water temperatures, the fluence required to inactivate *E. coli* was more than three-times smaller at this temperature. Within this temperature range viruses were even more sensitive.

![Inactivation of fecal coliforms](image-url)
From these results, Wegelin and his coworkers (Wegelin et al., 1994) derived the prerequisites, which have to be met in order to guarantee the effectiveness of solar water disinfection. This boils down to the following recommendations: "SODIS, Solar water disinfection, is a simple method to improve the quality of drinking water by using sunlight to inactivate pathogens causing diarrhea. Contaminated water is filled into transparent plastic bottles and exposed to the full sunlight for 5 hours. During the exposition the sun destroys the pathogens. The sunlight is disinfecting the water through two effects: Radiation in the spectrum of UVA light and increased water temperature. A solar radiation intensity of at least 500 W/m² is required during 5 hours for SODIS to be efficient. This is equivalent to 5 hours mid-latitude sunshine in summer. A synergy of radiation and temperature occurs if the water temperature raises above 50 °C, then the disinfection process only requires a third of the solar radiation intensity. After one hour of exposition to sunlight at 50 °C, the water is safe for consumption" (Wegelin et al., 1994).

Consequently practical experience had to be gained in order to evaluate the method and to check its applicability. Therefore, socio-cultural acceptance,
application procedure and financial viability were studied in demonstration projects in local communities in several South American, African and East Asian countries. The evaluation of the demonstration projects revealed that users appreciate the sustainable and simple water treatment method (Table 1). Over 2000 persons took part in this evaluation. On average more than four out of five users stated that they certainly would continue to use SODIS after the end of the demonstration project. Only a minority refused to use SODIS as they found their health not affected by the original water quality.

Table 1.1. Results of the SODIS acceptance evaluation (numbers in %) (SODIS Technical Notes)

<table>
<thead>
<tr>
<th>Country</th>
<th>certainly</th>
<th>maybe</th>
<th>probably not</th>
<th>definitely not</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colombia</td>
<td>90</td>
<td>8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bolivia</td>
<td>93</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Burkina Faso</td>
<td>70</td>
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<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Thailand</td>
<td>97</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>China</td>
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<td>45</td>
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</tr>
<tr>
<td>average</td>
<td>84</td>
<td>12.6</td>
<td>0.4</td>
<td>3</td>
</tr>
</tbody>
</table>

In addition, the influence of SODIS of several more parameters was investigated such as; container material (glass or plastic), aging effects on PET bottles (Wegelin et al., 2000), climatic conditions, seasonal effects and weather changes, turbidity, water volume and oxygen concentration. These results are comprehensively summarized in the SODIS Technical Notes #1-17 on the SODIS Webpage (www.sodis.ch).

Health improvements of SODIS users have been investigated in four studies so far (Conroy et al., 1996; Conroy et al., 1999; Conroy et al., 2001; Hobbins et al., 2003). The initial study was conducted in Kenya with Maasai children of 6-15 years of age. Subsequently, two more studies followed focusing on children under 6 years. They revealed that a 16 % reduction in cases of diarrhea could be
achieved by using SODIS compared to a control group. This number is impressive, because it corresponds to an 80% reduction if we consider that only about 20% of diarrheal cases can be clearly attributed to infection by drinking contaminated water.

In parallel with the research at Eawag two other research groups reported valuable data about the properties of solar water disinfection (Joyce et al., 1996; Reed, 1997; Reed et al., 2000). In recent years McGuigan and coworkers (1998) investigated effects of temperature and NUV radiation separately in E. coli. The latter authors developed a solar simulation apparatus that allowed heating and irradiation of the sample independently. In their experiments they simulated climate conditions that were recorded during a series of solar water disinfection measurements carried out in Kenya (Joyce et al., 1996) and reported for a temperature regime constantly below 45 °C (without applying irradiation) a negligible inactivating effect on E. coli, whereas inactivation rates increased with temperatures above this value. One important conclusion was that the relative importance of thermal inactivation depends very much on both initial water quality (turbidity) and prevailing weather conditions. Indications for a "viable but non culturable" state of E. coli cells were not found, since the inactivation in bacterial populations detected in the simulation experiments was always found to be of permanent nature (i.e., no re-activation after 24 h was observed).

Somewhat in opposition to Acra and McGuigan, Reed argued that the importance of temperature in the process of solar water disinfection was overestimated (Reed, 1997; Reed et al., 2000). He proposed that dissolved oxygen concentration in the water was more important than temperature. He presented data indicating that solar disinfection of water will only be fully effective under aerobic conditions. Both, Enterococcus faecalis and E. coli showed an increased inactivation rate under aerobic conditions (air saturated water) compared to anaerobic conditions. Cells grown to early log phase were slightly more sensitive to sunlight than cells in stationary phase. Decreases in CFU ml⁻¹ of exponential phase cells roughly followed first order kinetics, while
inactivation of stationary phase cells showed an initial shoulder of about 30min, where the bacteria seemed to be resistant to solar inactivation, followed by an exponential decrease in CFUs. The mean inactivation rates were slightly lower for stationary phase cells when compared with their growing counterparts, reflecting the decreased sensitivity of stationary phase bacterial cells to physicochemical stress. Also here, no regrowth or recovery was observed after 24h. Reed pointed out that the response of stationary phase cells are more likely to be representative of enteric bacteria in natural waters where they exist under conditions of restricted growth and nutrient availability.

Some larger scale treatment methods using sunlight as the only energy source were also proposed (Acra & Ayoub, 1997; Joergensen et al., 1998; Rijal & Fujioka, 2001; Sommer et al., 1997). These methods lack applicability because they are much more cost intensive than SODIS. Nevertheless, these solutions could be applied in regions where money and maintenance for large-scale disinfection facilities can be provided. Furthermore, it is also worth mentioning that sunlight is an important factor responsible for inactivation of fecal bacteria in many natural environments, including freshwater and seawater (Barcina et al., 1990; Davies & Evison, 1991; Rozen & Belkin, 2001; Sinton et al., 2002) bathing water (Mascher et al., 2003) and wastewater (Lindenauer & Darby, 1994; Sinton et al., 1999).

**Dead, alive, injured, or “viable but non-culturable” (VBNC)?**

The effectiveness of SODIS has been shown using the 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 includes cultivable bacteria that are able to initiate cell division and replication on agar plates. There are a number of eventualities, though, which could lead to an overestimation of effectiveness by using conventional culturability assays in disinfection processes. For example, bacteria that are only injured can fail to proliferate on plates and may appear “dead”. Dawe and Penrose (1978) showed that coliform bacteria exposed to sunlight in surface
seawater were unable to form colonies on a selective medium (eosin-methylene blue agar) but were still cultivable on non-selective nutrient agar. These observation were confirmed by Fujioka and Narikawa (2002). Taken together, these studies suggested that conventional selective media for fecal indicator bacteria may result in false-negative counts when used with sunlight-illuminated cells, which puts some of the earlier data obtained with selective media in doubt.

Furthermore, it was shown that injured bacteria do not grow when transferred into a nutrient-rich environment due to the stress of excessive nutrient concentrations (Kapuscinski & Mitchell, 1981), which causes an intracellular oxidative burst. This behavior has also been referred to as suicide response (Aldsworth et al., 1999; Dodd et al., 1997) and adds a new dimension to Postgate’s conclusion that with cultivation methods one can only observe bacterial death in retrospect (Postgate, 1989). Postgate himself already observed this behavior much earlier and called it substrate accelerated death (Postgate & Hunter, 1964) but did not link it to oxidative stress. Also, 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). The VBNC hypothesis proposes a response to environmental stress in some bacteria, where they remain alive but do not form surface colonies on conventional agar-based media (Roszak & Colwell, 1987). The same authors suggested that such bacteria could only be detected via methods based on metabolic activity. Although the oxidative burst theory might explain the observation of a VBNC state in some cases, it still does not answer the question how to detect the cell’s true physiological state. This calls for culture-independent methods. Such techniques have long been available in the form of viability stains (Postgate, 1967), but are now even more powerful by using them in combination with single-cell-analysis tools like flow cytometry. Lately, multi-parameter flow cytometry has enjoyed increasing popularity in microbiology, particularly in biotechnological processing, food preservation and chemical disinfection processes (Nebe-von-Caron et al., 2000). Hewitt and 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 culturability. Hence, this method could help to analyze the physiological state of individual enteric bacterial cells during SODIS.

**Objectives of this thesis**

In the preceding paragraphs, I have documented that although a lot of experimental data are available on the subject, there are still a lot of questions. Although a lot of puzzle pieces seem to be available, there are many missing links for a broader understanding of the inactivation mechanism and of all processes involved in the response and adaptation to UVA light of bacterial cells in general and of *E. coli* in particular. Based on theses considerations, the following objectives were set for this thesis:

(i) to investigate if *E. coli* is indeed a good indicator bacterium to quantify the effectiveness of SODIS for pathogenic enteric bacteria;

(ii) to investigate the influence of growth conditions and physiological growth state on the sensitivity of different enteric bacteria to solar radiation and to ensure data reproducibility and comparability;

(iii) to explore the agony of enteric bacteria during solar disinfection by assessing the activity of life-supporting functions with culture-independent methods at the single cell level (flow cytometry);

(iv) to investigate the ability of *E. coli* to respond or adapt to NUV irradiation;

(v) and to examine global gene expression and life-supporting functions of *E. coli* in response to NUV light to obtain indirect clues about the inactivation mechanisms of SODIS.
## Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Fluence</td>
<td>Integrated light intensity during a specific time period (kJ/m²) (SI units)</td>
</tr>
<tr>
<td>Fluence-rate</td>
<td>Light intensity (W/m²) (SI units)</td>
</tr>
<tr>
<td>Dose</td>
<td>Synonym for Fluence</td>
</tr>
<tr>
<td>Intensity</td>
<td>Synonym for Fluence-rate</td>
</tr>
<tr>
<td>Reciprocity</td>
<td>says that total applied fluence produces the same response regardless of the fluence-rate</td>
</tr>
<tr>
<td>UVA</td>
<td>Ultraviolet A, wavelength range 320 - 400 nm, also called near-UV or NUV</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B, wavelength range 290 - 320 nm, also called mid-UV or MUV</td>
</tr>
<tr>
<td>UVC</td>
<td>Ultraviolet C, wavelength range 190 - 290 nm, also called far-UV or FUV</td>
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2. Specific growth rate determines the sensitivity of *E. coli* to thermal, UVA and solar disinfection

Abstract

Knowledge about the sensitivity of the test organism is essential for the evaluation of any disinfection method. In this work we show that sensitivity of *Escherichia coli* MG1655 to three physical stresses (mild heat, UVA light and sunlight) that are relevant in the disinfection of drinking water with solar radiation is determined by the specific growth rate of the culture. Batch- and chemostat-cultivated cells from similar specific growth rates showed similar stress sensitivity. Generally, fast-growing cells were more sensitive to the stresses than slow growing cells. For example, slow growing chemostat-cultivated cells (D = 0.08 h⁻¹) and stationary phase bacteria from batch culture that were exposed to mild heat had very similar T₉₀ values (T₉₀,chemostat = 2.66 h, T₉₀,batch = 2.62 h), whereas T₉₀ for cells growing at μ = 0.9 h⁻¹ was 0.2 h. We present evidence that the stress-sensitivity of *E. coli* is correlated with the intracellular level of the alternative sigma factor RpoS. This is also supported by the fact that *E. coli rpoS* mutant cells were more stress-sensitive than the parent strain by a factor of 4.9 (mild heat), 5.3 (UVA light) and 4.1 (sunlight), respectively. Furthermore, modeling of inactivation curves with GInaFiT revealed that the shape of inactivation curves changed depending on the specific growth rate. Inactivation curves of cells from fast growing cultures (μ = 1.0 h⁻¹) that were irradiated with UVA light showed a tailing effect, while for slow growing cultures (μ = 0.3 h⁻¹), shouldered inactivation curves were obtained. Our findings emphasize the need for accurate reporting of specific growth rates and detailed culture conditions in disinfection studies, to allow a comparison of data from different studies and laboratories and sound interpretation of the data obtained.

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Introduction

Effective disinfection of water is extremely important in developing countries, where safe access to drinking water is often difficult. In such regions, enteric bacteria such as *Salmonella*, *Shigella* or *Vibrio cholerae* are still the major drinking water health threats (Hinrichsen *et al.*, 1997). An estimated 4 billion cases of diarrheal disease occur every year worldwide, causing 3 to 4 million deaths, mostly among children. This situation can only be improved by effective household water treatment systems that meet local financial and practical needs. Solar disinfection (SODIS) is such a method (Acra *et al.*, 1984; Wegelin *et al.*, 1994). Exposing drinking water in PET bottles to sunlight (ca. 6 h) inactivates enteric bacteria present in the water. The two primary causes for bacterial inactivation in this method are believed to be mild heat and UVA light (Wegelin *et al.*, 1994).

Disinfection methods like SODIS are assessed with living test microorganisms in order to ensure efficacy and sound application. In most cases the resistance of a particular indicator organism to different quantities of chemical disinfectant or doses of physical stresses is investigated. However, a key point often overlooked is that the resistance of bacteria can vary significantly as a function of growth conditions. Bacteria change their physiology or even their phenotype under different growth conditions (Berg, 1987; Bremer & Dennis, 1987; Wanner & Egli, 1990). For example, the expression of heat shock proteins in *E. coli* was shown to be affected by the composition of the growth medium and growth conditions (Wick & Egli, 2004). Moreover, it was shown that induction of the general stress response in *E. coli*, which is controlled by the global regulator RpoS, increases with decreasing specific growth rate (Ihssen & Egli, 2004; Notley & Ferenci, 1996). Recently, it was found that the concentration of the DNA-binding protein Dps in *E. coli*, which is believed to protect DNA from oxidative stress, increases with decreasing specific growth rate (Ali Azam *et al.*, 1999). Therefore, it is very important to define precisely the growth conditions and physiological state of bacteria used for disinfection experiments. In most disinfection studies the
growth conditions of the test organisms are not properly specified and, where mentioned, mostly ill-defined states like "late mid-log" or "early stationary phase" are given. This makes proper comparisons among different studies difficult if not impossible.

In the light of the wide-spread use of disinfection methods, it is surprising how few studies have addressed the dependence of disinfection (or stress) resistance on the physiological state of bacterial cells. In most studies the scope of different physiological states investigated is confined to exponential and stationary phase. Sensitivity of E. coli to disinfectants like ClO₂, UVC, mild heat and sunlight were shown to be higher during exponential phase than during stationary phase (Gourmelon et al., 1997; Lisle et al., 1998; Morton & Haynes, 1969; Reed, 1997), but only Berg (1987) showed a correlation of specific growth rate and the sensitivity to ClO₂ for E. coli and Legionella pneumophila.

In the present study we tested the hypothesis that sensitivity of E. coli K12 MG1655 to physical stresses like mild heat, UVA light and sunlight increases with increasing specific growth rate (μ) and that μ can be used as a key parameter to compare different disinfection experiments and ensure reproducibility. Furthermore, the role of RpoS as a possible key link between specific growth rate and sensitivity to the respective stresses was investigated by using an rpoS mutant of E. coli and by measuring the RpoS-dependent catalase activity.

Materials and Methods

Bacterial strains

For most experiments, E. coli K-12 MG1655 [genotype F λ rph1] was used (Wick et al., 2001). For selected experiments an isogenic rpoS mutant of E. coli K12 MG1655 (rpoS13::Tn10) (Wick et al., 2002) was used for comparisons.
Growth media and cultivation condition

**Batch.** Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) was used for batch cultivation (Miller, 1972). LB medium was always filter-sterilized with Millipore syringe filters (Millex GP, 0.22 μm) and diluted to 25 % volume/volume (v/v) of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon filtered). Precultures were prepared for each individual batch experiment from the same cryo-vial stored at -80 °C by streaking onto LB agar plates. After 15-18 h of incubation at 37 °C one colony was picked and loop-inoculated into a 125 ml Erlenmeyer flask containing 20 ml of LB broth. This was incubated at 37 °C on a rotary shaker at 200 revolutions per minute (rpm). At an OD$_{546}$ between 0.1 and 0.2 (measured spectrophotometrically in glass cuvettes with 1 cm light path using a JASCO V550 UV/VIS spectrophotometer; Tokyo, Japan) cells were transferred into 500 ml Erlenmeyer flasks containing 50 ml of LB broth. This way no lag phase was observed. The culture volume to be inoculated into the fresh medium was calculated beforehand to attain an initial OD$_{546}$ of 0.002. These flasks were then shaken at 200 rpm in a temperature-controlled water bath (SBK 25D, Salvis AG, Reussbühl, CH) at 37 °C and OD$_{546}$ was monitored every 20 minutes. Specific growth rate $\mu$ was calculated from five consecutive OD$_{546}$ measurements ($\mu = \frac{\Delta \ln OD_{546}}{\Delta t}$). For the measurement of dissolved oxygen concentration (pO$_2$) in batch culture experiments a pO$_2$ probe (Ingold Electrodes Inc., Wilmington, MA, USA) with a Bioengineering amplifier was used (Bioengineering, Wald, Switzerland).

**Chemostat cultivation.** Precultures were prepared as described above. For most continuous culture experiments, temperature-controlled and air-sparged one liter glass reactors (Schmizo AG, Zofingen, Switzerland) were used. For particular experiments computer-controlled glass and stainless steel bioreactors (MBR, Wetzikon, Switzerland) were employed. The pH was kept constant at 7.0 ± 0.1 by automatic addition of 0.5 M NaOH/0.5M KOH, and the temperature was maintained at 37±0.1 °C. Oxygen concentration was kept between 95 and 100 %
of air saturation at 37 °C and the stirrer speed was set to 800 rpm. For glucose-limited chemostat cultures the medium was prepared as described earlier (Ihssen & Egli, 2004) and for LB chemostats cultures 10 % v/v LB broth was used.

**Sample preparation and plating.** Cells were harvested by centrifugation from batch or chemostat cultures (at 13000 g, Biofuge fresco; Kendro, Zürich, Switzerland), washed three times with filter-sterilized (Nuclepore Track-Etch Membrane, 0.22 μm, Sterico AG, Dietikon, Switzerland) commercially available bottled water (EVIAN, Danone, Paris, France) and diluted to an OD<sub>546</sub> of 0.01 (corresponding to 1-5 x 10<sup>7</sup> cells/ml). Exposure of bacterial suspensions was started immediately after dilution, to prevent cells from adjusting to low nutrient conditions. Aliquots were withdrawn at different time points and diluted in decimal steps (10<sup>-1</sup> to 10<sup>-5</sup>) with sterile-filtered (0.2 μm) bottled water (EVIAN). Following dilution 1 ml of test solution was withdrawn and mixed with 7 ml of liquid tryptic soy agar (TSA) (Biolife, Milano, Italy) at 40 °C (pour plate method). After 20 minutes the solidified agar was covered with another 4 ml of liquid TSA (40 °C). Plates were incubated for 48 hours at 37 °C until further analysis. Plate counts were determined with an automatic plate reader (acolyte, SYNBIOSIS, Cambridge, UK).

**Mild heat exposure.** A bacterial suspension of 20 ml (see above) was transferred into 30 ml quartz glass tubes (WISAG AG, Zürich, Switzerland) and placed into a temperature-controlled water bath at 48 °C. Care was taken that vials were entirely submersed to prevent temperature gradients in the vials. The final temperature was reached in ≤ 2 min. During exposure samples were stirred on a magnetic stirrer. Unstressed control samples were kept in the dark at 37 °C.

**Sunlight exposure.** Samples of 10 ml of bacterial suspension (see above) were exposed to solar light in 30 ml quartz glass tubes, which were placed into a temperature-controlled acrylic glass container with a quartz front glass, holding 25 tubes in total (Fig. 2.1a). A circulating 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 degree. At each
time point one tube was withdrawn and its contents immediately processed as
described above. Irradiation intensity data were obtained from a weather
station, which is located 300m 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 of 350-450 nm, which reflects the
wavelength range of the UVA lamps also used (see below). Conversion factors
and calculations were used as in Wegelin and co-workers (1994). The light
spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-
COR, Lincoln, Nebraska, USA), 8 nm bandwidth, fitted with a model 1800-10
detector head (FIG. 2.1b).

**UVA exposure.** Samples of 10 ml of 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. (38)) equipped with medium-pressure
mercury lamps (Hanau TQ718 or TQ718 Z4 (doped)), which were operated at 700
W and 500 W, respectively. The doped lamp TQ718 Z4 exhibits a broader UVA
spectrum and is more effective in disinfection than the undoped lamp (Fig. 2.1b).
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 consisted of 12.75 g/L sodium nitrate 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.
Fig. 2.1. (a) Field reactor for sunlight exposure of bacterial samples. The container is built from acrylic glass and has a quartz glass front window. Up to 25 tubes can be fitted into the container. Samples are stirred magnetically and the temperature is controlled by a circulating water bath. The apparatus can be swiveled and turned in any direction. (b) Wavelength spectra of medium pressure mercury lamps Hanau TQ71S (dashed line) and TQ718 Z4 (thin solid line) both corrected for filter solution NaNO₃ and the sunlight spectrum on a mid-summer day measured at 1 pm (fat solid line).
Hydroperoxidase assay. The two *E. coli* hydroperoxidases (catalases) were used as “reporter genes” for RpoS-dependent transcription because both, hydroperoxidase I (HPI, *katG* gene product, cytoplasmic membrane associated) and hydroperoxidase II (HPII, *katE* gene product, present in the cytosol), were shown to be regulated by σ^8^ (Ivanova *et al.*, 1994), with hydroperoxidase II expression being entirely dependent on RpoS (Visick & Clarke, 1997). HPI and HPII specific activities were measured in whole cell assays. The activity of the two enzymes can be separated by a heat inactivation treatment step because HPII is heat-stable whereas HPI is heat-labile (Visick & Clarke, 1997). Briefly, samples from chemostats were washed and diluted in PBS (140 mM NaCl, 2.5 mM KCl, 1.6 mM KH₂PO₄, 15 mM Na₂HPO₄) to an OD₅₄₆ of 0.1. To measure total catalase activity 1 ml of this solution was mixed with 1μl of chloramphenicol solution (25 mg/ml) transferred into a quartz cuvette and placed into a temperature-controlled (37 °C) spectrophotometer. Before starting the array the cuvette was incubated for 2 min for the sample to adjust to the temperature. Absorption at 240 nm was recorded for 2 minutes after starting the assay by the addition of 2 μl of H₂O₂ (37 %) and thorough mixing. For heat-stable catalase activity measurements samples were incubated for 15 min in a heating block at 55 °C to inactivate HPI prior to analysis (Visick & Clarke, 1997). Catalase activity was calculated from the first 60 s of recorded absorption where the rate of ΔE₂₄₀/Δtime decrease was linear.

Modeling with GlnaFIT. The Geeraerd and Van Impe Inactivation Model Fitting Tool (GlnaFIT) was used for testing six different types of microbial survival models on our data (Geeraerd *et al.*, 2005). The following models were used: Log-linear regression (Bigelow & Esty, 1920), Log-linear + Tail (Geeraerd *et al.*, 2000), Log-linear + Shoulder (Geeraerd *et al.*, 2000), Log-linear + Shoulder + Tail (Geeraerd *et al.*, 2000), Biphasic model (Cerf & Metro, 1977), Biphasic + shoulder (Geeraerd *et al.*, 2005). All models were run for each inactivation curve and the values of the Root Mean Sum of Squared Errors (RMSE) were compared. The RMSE is considered to be the most simple and most informative measure of goodness-of-fit for linear as well as non-linear models (Geeraerd *et al.*, 2005). The model with the smallest RSME was considered the best fit for the respective
inactivation curve. When the magnitude of RMSE was much smaller than experimental precision the model was considered to overfit the data and a simpler model was chosen. If two models had the same or very similar RMSE value the simpler model was considered to fit best. \( T_{90} \) values (time until 90% of the population is inactivated) were calculated using the best-fit model of GlnaFIT.

**Reproducibility.** To ensure reproducibility selected experiments were repeated. Analysis of inactivation behavior with GlnaFit-modelling showed nearly identical results for both replicates in all cases. This was based on comparison of specific parameters of the best-fitting model (e.g. the specific inactivation rate constants \( k, k_{max1} \) and \( k_{max2} \) for the model “Biphasic” (Geeraerd et al., 2005)). Standard deviation of model parameters was below ± 5%. In graphs, only data from one representative experiment are displayed. Periodically, precision of pour-plating with subsequent colony counting was analyzed. The standard deviation for the standardized plating and colony counting was ± 15% at the maximum (calculated from triplicates). Catalase activity was measured in triplicates in all cases. In all experiments an unstressed control sample was measured in the beginning and at the end of data acquisition. For convenience, these control samples are always displayed with the same x-axis value as the last data point of the respective curve.

**Results**

**Influence of cultivation conditions on batch growth pattern**

Growth history of the test organism has a strong influence on the reproducibility of disinfection experiments. Therefore, for our laboratory set-up the influence of different cultivation variables on the bacterial growth pattern was investigated first. Because aeration is known to be a critical factor in shake flask cultures, oxygen concentration for different aeration regimes during batch
culture cultivation was measured (Fig. 2.2). Cultures were grown in either stirred or shaken Erlenmeyer flasks and in a fully aerated and stirred bioreactor using the same growth medium. The pO₂ decreased rapidly in stirred Erlenmeyer flasks as the cell density increased (Fig. 2.2) and an abrupt decline in specific growth rate (μ) was observed as soon as the culture became oxygen-limited. In contrast, cultivation of *E. coli* in a fully aerated and stirred bioreactor resulted in O₂ levels ≥ 60 % and a continuous decrease of specific growth rate with time.

![Graph](image)

**Fig. 2.2.** *E. coli* growth curves and corresponding dissolved oxygen tension for different aeration systems in batch cultivation. The effect of stirring in Erlenmeyer flasks on the development of cell density (OD₅₄₆) and pO₂ was investigated and compared to growth in a fully aerated and stirred bioreactor. Erlenmeyer flask: OD₅₄₆ (●), pO₂ (dashed line). Bioreactor: OD₅₄₆ (▲), pO₂ (solid line).

The same growth pattern and O₂ levels as for the bioreactor resulted in Erlenmeyer flask cultures that were shaken on a rotating shaker at 200 rpm (data not shown). This indicates that in our set-up shaking prevents oxygen depletion during growth and, therefore, it was employed as the preferred method for all further experiments. Furthermore, we found that a typical batch growth curve of *E. coli* cultivated in LB medium shows no true exponential phase (i.e. a straight line of the growth curve in a semi-logarithmic plot) but in fact,
specific growth rate decreases constantly until stationary phase is reached (Fig. 2.3). This stands in contrast to the common assumption that during LB batch culture an exponential phase exists where specific growth rate remains constant over an extended time period. Examination of the sterilization method of the growth medium revealed two interesting aspects. During the first two hours after inoculation from a exponentially growing inoculum ($\mu \geq 1.5h^{-1}$), filter-sterilized LB medium allowed a higher initial specific growth rate (~ 2.4 h$^{-1}$) compared to autoclaved LB medium (~ 2 h$^{-1}$) (data not shown). Additionally, we observed that prolonged autoclaving lowered the initial $\mu$. Therefore, only filter-sterilized LB medium was used for all experiments.

Fig. 2.3. Growth curve of *E. coli* K12 MG1655 in LB batch culture (shaken Erlenmeyer flask) at 37 °C (• lnOD$_{546}$). Specific growth rates $\mu$ (h$^{-1}$) were calculated as the slope of five adjacent points (▲). Error bars indicate standard deviation of triplicate measurement.

**Specific growth rate and stress resistance**

Comprehensive and detailed information about cultivation conditions are rarely given in the disinfection literature although it has been known that cultivation
conditions and growth phase can affect stress sensitivity of bacteria. This calls for a general growth parameter, which allows reproducibility and comparability among different disinfection experiments. Here we test the hypothesis that specific growth rate of _E. coli_ correlates with sensitivity to mild heat treatment, UVA light and sunlight.

**Mild heat.** _E. coli_ cells were harvested from LB batch cultures at three different specific growth rates and exposed to mild heat (48 °C) (Fig. 2.4). Inactivation rates of bacterial cells harvested at different time points differed markedly. Cells with slow specific growth rates were significantly less sensitive to thermal stress than faster growing cells.

![Fig. 2.4. Sensitivity to mild heat (48 °C) of _E. coli_ K12 MG1655 harvested at three different specific growth rates from LB batch culture (● 0 h⁻¹, ▲ 0.44 h⁻¹, • 2 h⁻¹). Unstressed control samples are displayed with open symbols. Sensitivity was determined as cfu/cfu at time zero.](image)

The correlation between sensitivity to mild heat and specific growth rate was confirmed when _E. coli_ cells cultivated in the same medium in chemostat cultures, operated at five different dilution rates (D = 0.08 h⁻¹, 0.2 h⁻¹, 0.3 h⁻¹, 0.6 h⁻¹, 0.9 h⁻¹), were exposed to the same stress (Fig. 2.5). Batch- and chemostat-grown cells from similar specific growth rates exhibited similar sensitivity. For example,
chemostat-cultivated cells growing very slowly \((D = 0.08 \text{ h}^{-1})\) exhibited the same sensitivity as stationary phase cells from batch culture. Curve-fitting with GlnaFIT gave a good fit with the traditional log linear decay model for low specific growth rates \((0.08 \text{ h}^{-1}, 0.2 \text{ h}^{-1}, 0.3 \text{ h}^{-1})\). The inactivation pattern of cells with a higher specific growth rate \((0.6 \text{ h}^{-1}, 0.9 \text{ h}^{-1})\) showed a true biphasic behavior (fast inactivation followed by a slower part), suggesting that the population was heterogeneous with respect to sensitivity.

![Graph showing log transformation of CFU/cfu0 against time (h)](image)

**Fig. 2.5.** Sensitivity to mild heat \((48^\circ \text{C})\) of *E. coli* K12 MG1655 cells cultivated in filter sterilized LB broth in chemostat culture operated at different dilution rates \((\square 0.08 \text{ h}^{-1}, \bigtriangleup 0.2 \text{ h}^{-1}, \bigstar 0.3 \text{ h}^{-1}, \bigtriangledown 0.6 \text{ h}^{-1}, \bullet 0.9 \text{ h}^{-1})\). Curves were fitted with GlnaFIT (Geeraerd et al., 2005). The best fit is displayed for each curve. Inactivation curves from Fig. 2.4 are displayed as dashed lines (--) for comparison. Unstressed control samples are displayed with open symbols. Sensitivity was determined as cfu/cfu at time zero.

Changes in initial inactivation rates displayed as \(T_{90}\) values became more pronounced especially towards slower dilution rates (Fig. 2.6). Total catalase
activity (HPI and HPII) and T₉₀ values followed a similar pattern in relation to
dilution rates of chemostat cultures. Activities of the RpoS-dependent catalase
HPII correlated well with T₉₀ values (R² = 0.988) while with HPI activities the
correlation was very poor (R² = 0.727). This result points to an important
possible role of RpoS in the thermal stress resistance of E. coli.

Mild heat treatment of E. coli rpoS mutant cells grown at three different specific
growth rates (1.5 h⁻¹, 0.3 h⁻¹, 0 h⁻¹) revealed that RpoS indeed regulates protection
from mild heat stress to some extent (Fig. 2.7). Although bacteria were sampled
at different specific growth rates, differences in inactivation rates were very
small compared to wild type cells. Nevertheless, the pattern of decreasing
sensitivity towards slower growth rates remained.

Fig. 2.6. Catalase specific activity (●HPI, ▲ HPII) of E. coli cells grown at different dilution
rates in LB chemostat culture. Inactivation values T₉₀ (○) (time when 90 % of the population are
inactivated measured with plate counting), derived from data in Fig. 2.5, are displayed. Catalase
activity was not measured at dilution rate 0.2 h⁻¹. Error bars represent standard deviations of
triplicate measurements. HPI activity was calculated as the difference between total catalase
activity (HPI&HPII) minus HPII activity and therefore, no error bars are displayed.
Growth rate and disinfection efficiency

**FIG. 2.7.** Sensitivity to mild heat (48 °C) of *rpoS*-minus *E. coli* cells harvested at different specific growth rates and cultivation conditions (▲ 0 h⁻¹ LB batch, ● 0.3 h⁻¹ glucose mineral medium chemostat, ◆ 1.5 h⁻¹ LB batch,) compared to wild type *E. coli* from stationary phase (● 0 h⁻¹ LB batch). Unstressed controls are displayed with open symbols. Sensitivity was determined as cfu/cfu at time zero.

**UVA light.** UVA sensitivity of *E. coli* cells from chemostat cultures operated at three different dilution rates also showed a dependence on specific growth rate (Fig. 2.8). Stationary phase cells and chemostat cells that were grown at a low dilution rate of D = 0.3 h⁻¹ exhibited a log-linear inactivation behavior with a typical shoulder. This was confirmed with GlnaFIT modeling. For bacterial cells with a specific growth rate of 0.7 h⁻¹ no shoulder effect was observed and the model Log-Linear Regression fitted best. Cells cultivated at a D = 1.0 h⁻¹ exhibited a true biphasic behavior. A small proportion of the culture (0.05 %) seemed to be less sensitive to UVA stress. We speculated that this might be due to physiological changes during exposure or during sample preparation resulting in increased protection against UVA injury. To test this hypothesis *E. coli* cells from the same chemostat culture (D = 1.0 h⁻¹) were left in Evian for 4 h before exposure (Fig. 2.9). The best fit for this curve was now obtained with the Biphasic + Shoulder model. The appearance of a shoulder indicates that physiological changes took place during sample preparation and bacterial cells became more resistant to UVA irradiation. Obviously, growth at μ = 1.0 h⁻¹ and a
subsequent change from a nutrient rich medium (LB) to a nutrient poor environment (EVIAN) forces *E. coli* to enter a stationary phase-like state. Still, the culture that was left to adapt to EVIAN for 4 h in addition also displays a biphasic inactivation pattern indicating that we see a combination of cellular adaptation effects during preparation and exposure. Again, *E. coli* rpoS mutants from stationary phase batch culture were much more sensitive to UVA light than the parent wt-strain (Fig. 2.10a).

**Fig. 2.8.** Sensitivity to UVA irradiation (doped lamp) at 37 °C of *E. coli* K12 MG1655 harvested from LB chemostat cultures run at different dilution rates, (▲ 0.3 h⁻¹, □ 0.7 h⁻¹, ● 1.0 h⁻¹) compared to cells from stationary phase batch cultures (■ μ = 0.0 h⁻¹). Unstressed control samples are displayed with open symbols. Curves were fitted with GlnaFIT (Geeraerd et al., 2005). The best fit is displayed for each curve. Sensitivity was determined as cfu/cfu at time zero.
Fig. 2.9. Sensitivity to UVA irradiation (doped lamp) at 37 °C of *E. coli* K12 MG1655 from LB chemostat culture run at a dilution rate of $D = 1.0$ h$^{-1}$. Cells were either irradiated immediately after harvesting (●) or left to adjust to the new environment for 4 h before exposure (▲). Unstressed control samples are displayed with open symbols. Curves were fitted with GlnaFIT (Geeraerd et al., 2005). The best fit is displayed for each curve. Sensitivity was determined as cfu/cfu at time zero.

**Sunlight.** Sensitivity to sunlight was also specific growth rate-dependent (Fig. 2.10b). A 4-log inactivation of stationary phase cells was observed at a 3-times higher fluence compared to fast-growing *E. coli* cells. Exposure to sunlight also resulted in inactivation curves exhibiting a shoulder effect. Interestingly, also fast growing cells exhibited a small shoulder effect. *E. coli rpoS* mutants from stationary phase demonstrated similar sensitivity as fast growing cells from the parent wt-strain.
Fig. 2.10. Sensitivity of *E. coli* K12 w.t. (▲) and *rpoS* mutant (●) to UVA light (undoped lamp) (A) and sunlight (B). Bacterial cells were harvested from stationary phase LB batch cultures. In (B) an inactivation curve for *E. coli* cells harvested at $\mu = 2 \, \text{h}^{-1}$ (●) was added for comparison. Unstressed control samples are displayed with open symbols. Sensitivity was determined as cfu/cfu at time zero.
Discussion

This work clearly demonstrates that sensitivity of *E. coli* to stresses like mild heat, UVA light and sunlight is determined to a large part by the specific growth rate (Figs. 2.4-2.8). Decreasing sensitivity of progressively slower growing bacterial cells strongly depends on RpoS, a global stress regulatory protein, which itself is increasingly expressed with decreasing specific growth rate (Ihssen & Egli, 2004). Furthermore, our results imply that detailed information on growth conditions and specific growth rate of bacterial cells must be given in disinfection experiments to allow comparison and correct interpretation of reported data. We suggest that for testing a disinfection method it is not enough to choose the apparently most resistant organism but that one also has to produce cells of the most resistant state or of reproducible resistance. For solar disinfection this would be cells from stationary phase ($\mu = 0 \text{ h}^{-1}$).

There have been a number of reports in the past where sensitivity to disinfection was related to culture age or growth phase but almost all of them showed only differences between bacterial cells from stationary phase and (an insufficiently defined) exponential phase. To our knowledge the only systematic study on specific growth rate and sensitivity to disinfection was conducted by Berg (1987), who showed that the sensitivity of *E. coli* and *L. pneumophila* to ClO₂ increases with increasing dilution rate in chemostat experiments. Concerning sensitivity to UVA light conflicting results have been reported. In one study stationary phase *E. coli* cells were more resistant than exponentially growing cells to both lethal and sub-lethal doses of UVA radiation (Dantur & Pizarro, 2004). On the contrary, Reed (1997) showed only very minor differences between inactivation rates for stationary phase *E. coli* cells and their growing counterparts. In seawater microcosm experiments *E. coli* cells from exponential phase were shown to be more susceptible to artificial visible light than stationary phase cells. However, in all three studies the exponential phase was not well defined. For thermal inactivation it was demonstrated that culture age of *E. coli* and *Streptococcus faecalis* affects heat resistance and death kinetics (Elliker & Frazier, 1938; Hansen
& Rieman, 1963; Lemcke, 1959; White, 1953). During exponential phase these bacteria exhibited high sensitivity to mild heat (different temperatures) while in stationary phase and during lag phase they were less sensitive. For UVC-irradiated *E. coli* it was demonstrated that UVC resistance is varying during growth in batch culture, however, it is not clear from the published data how specific growth rate relates to UVC sensitivity (Martiny *et al.*, 1990; Morton & Haynes, 1969). Our results show that sensitivity of *E. coli* to three different physical stress conditions relevant to the SODIS disinfection method (UVA light, sunlight and mild heat) is correlating strongly with specific growth rate. Hence, the results presented here indicate a general pattern for bacterial sensitivity to these stresses.

RpoS, the α subunit of RNA polymerase, seems to link stress response and specific growth rate (Fig. 2.6). $T_{90}$ values correlate well with HPII activity, which increases twelve-fold towards slower specific growth rates, while HPI activity only doubles. These results confirm published reports of a mild increase in HPI expression as cells approach stationary phase and a major increase in the synthesis of the RpoS-dependent HPII (Visick & Clarke, 1997). Another indication for the importance of RpoS is demonstrated in experiments with *E. coli rpoS* mutants that show largely increased sensitivity to all three physical stresses virtually independent of specific growth rate. RpoS is a global regulator that enables *E. coli* to adapt to various stress conditions like osmotic stress, pH differences or oxidative stress (Hengge-Aronis, 2002). It was reported to regulate over 480 different genes (Weber *et al.*, 2005). Ihssen and Egli (2004) proposed that as an intracellular signal (p)ppGpp might link RpoS and specific growth rate because RpoS expression is positively regulated by this factor. In our experiments, HPII activity was measured as an indicator for RpoS levels because HPII is primarily regulated by RpoS (Visick & Clarke, 1997). Although our results strongly suggest RpoS to be a main reason for differences in stress resistance, other mechanisms could still be important. The small differences in inactivation rates for *rpoS* mutants from different specific growth rates (Fig. 2.7) suggests that additional regulatory mechanisms (also specific growth rate-
Growth rate and disinfection efficiency

dependent) are involved in stress protection against mild heat, and this might be the case also for sensitivity towards UVA light and sunlight.

A distinct shoulder effect is observed only for UVA and sunlight irradiated samples. In contrast, no such effect was detected during exposure to mild heat. Two models have been proposed in the literature to explain the shoulder effect: the multi-target and the multi-hit scenario (Harm, 1980). The multi-target scenario suggests that an organism contains "n" distinct (usually identical) targets, each of which must receive at least one hit before the cell is inactivated, while the multi-hit model is based on the assumption that a single target must be hit at least "n"-times. In the case of sunlight and UVA, a combination of the two theories seems most likely. In contrast to UVC disinfection (254 nm), where only one target (DNA) exists, the broad wavelength spectrum of UVA can harm the cells in many different ways such as membrane damage, DNA damage or indirect damages by reactive oxygen species (Eisenstark, 1989). Cells from chemostats run at higher dilution rates also exhibited initial first-order inactivation kinetics when irradiated with UVA light. When fast growing cells were exposed to high intensity UVA irradiation, probably enough targets are hit at once for the population to show first order inactivation kinetics without a (visible) shoulder effect. In sunlight experiments also for faster growing cells a shoulder effect was observed. This was probably due to longer incubation time before exposure was started, because the vials with the bacterial solution had to be carried outside. In contrast, E. coli cells exposed to mild heat seem to follow first order inactivation kinetics. The mechanism of thermal inactivation of microorganisms has been widely investigated. Cellular sites of heat injury like membranes, nucleic acids and certain enzymes have been identified (Allwood & Russell, 1970; Gould, 1989; Lee & Kaletunc, 2002; Mackey et al., 1991). Evaluation of heat inactivation by differential scanning calorimetry (DSC) suggested that a strong relationship exists between thermal death of bacteria and the first major peak in DSC thermograms, which is attributed to the unfolding of ribosomes (Lee & Kaletunc, 2002). It should however be pointed out that these studies were conducted with cells pre-exposed to heat, which are generally known to be more resistant to thermal stress (Katsui et al., 1981).
date, we are unable to bring forward a coherent explanation for the first order inactivation pattern during mild heat treatment.

The biphasic nature of the inactivation curves in UVA and mild heat disinfection experiments seem to occur only when bacterial cells from growing cultures are used. It indicates that two discrete populations were present during exposure as has been reported for heat stressed *Salmonella eneritidis* PT4 over a range of lethal temperatures (Humpheson et al., 1998). These authors suggested that heat shock protein (HSP) production occurs in a small proportion of the culture and that this results in tailing of survivor curves during lethal heating. Allen and coworkers (1988) demonstrated that a temperature shift from 30 to 45 °C induced synthesis of at least 17 HSPs in *Pseudomonas aeruginosa* within approximately 1 minute. In our mild heat experiments the sample temperature increased from 37 °C to 48 °C in 2 minutes. During this time some HSP production could have occurred. In the UVA light experiments we have seen that already the handling of cells until exposure can influence their sensitivity. The change from a nutrient rich to a nutrient poor environment puts *E. coli* cells under starvation stress. This stress must be less pronounced for stationary phase cells because they already went through a starvation adaptation period. Apparently, the starvation stress imposed on growing bacteria leads to a general stress response similar to stationary phase bacteria, which can include the induction of protective mechanisms against UVA stress. But the cells do not become as resistant as stationary phase cells because the adaptation process needs energy and this requires changes in cell composition, intracellular degradation of components etc. Still, the induction of a stress-specific protection during exposure can not be ruled out. Such a response would be more specific on the stress imposed and therefore could produce cells, which are more resistant than stationary phase cells (as seen in Fig. 2.8). The mechanism of inactivation by UVA light is still not fully understood. Recent studies suggest that UVA light mediates its biological effects primarily via oxidative mechanisms that lead to reactive oxygen species (Hoerter *et al.*, 2005a).
In batch culture with defined media where only one carbon source is present, the microbial growth pattern can be clearly separated into an exponential and a stationary phase. However, in complex medium, where many different carbon sources are present, the change from exponential to stationary phase is not clear-cut (Fig. 2.3). Specific growth rate is steadily decreasing, which means that physiological changes take place throughout the whole growth cycle. This is supported by the work of Schaechter et al. (1958) who examined the variations in growth and composition of Salmonella Typhimurium cultures in different media and found that the cellular contents of DNA, RNA and protein at a given temperature are highly dependent on specific growth rate. Therefore, expressions such as early exponential, late exponential or early stationary phase, as often used in literature, are inadequate to describe the physiological state of cells. Also the statement of harvesting time and optical density of a culture is insufficient because the growth history is a very important factor when it comes to reproducibility. For example, if bacterial cells are harvested at an OD of 0.3, a culture that was inoculated to an initial OD of 0.1 will be in a different physiological state compared to a culture that started from an OD of 0.001. If a high bacterial cell concentration is present at the start, easily accessible nutrients in LB like amino acids will be used up very fast and the development of specific growth rate will differ greatly from that of highly diluted cultures. Also, small changes in cultivation conditions like changing the aeration method (Fig. 2.2), or autoclaving the medium can lead to a big change for the development of the culture (Barry et al., 1956).

Our results corroborated previous reports (e. g. Berg (1987)) on the direct link between increasing specific growth rate and increasing susceptibility to stress for bacteria. We confirmed our hypothesis that this concept also applies to physical stresses like mild heat, UVA light and sunlight and that it is strongly related to cellular RpoS levels. This emphasizes the need for accurate reporting of specific growth rates and detailed culture conditions during disinfection studies, which would enable better comparison of data from different studies, as well as sound interpretation of the data obtained.
Chapter 2

Acknowledgments

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3. Efficacy of solar disinfection of *E. coli*, *S. flexneri*, *S. Typhimurium* and *V. cholerae*

**Abstract**

The efficacy of solar disinfection (SODIS), a low-cost household water treatment method for developing countries, was investigated for *Escherichia coli* MG1655, *Salmonella Typhimurium* ATCC 14028, *Shigella flexneri* ATCC 12022 and *Vibrio cholerae* 01 Ogawa biotype El Tor. Lethality of sunlight and mild heat were investigated separately for cells suspended in bottled mineral water. Resistance to sunlight at 37 °C based on $F_{99}$ values (fluence needed to reduce plate counts by 99%) was in the following order: *S. Typhimurium* > *E. coli* > *S. flexneri* > *V. cholerae*. While $F_{50}$ values of *S. Typhimurium* and *E. coli* were very similar, $F_{99}$ values differed by 60% because of the different shape of inactivation curves. This emphasizes that in this case extrapolation from $T_{90}$ or $F_{90}$ values is not possible and that these values are inadequate for comparing results from different studies. We demonstrate that the reciprocity-law is applicable for stationary phase *E. coli* suspended in mineral water and irradiated with sunlight or artificial sunlight. Sensitivity to mild heat was observed above a temperature of 45 °C for *E. coli*, *S. Typhimurium* and *S. flexneri*, while *V. cholerae* was already susceptible above 40 °C.

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Introduction

The World Health Organization (WHO) estimated in 1996 that every eight seconds a child dies from a water-related disease and that each year more than five million people die from illnesses linked to consumption of unsafe drinking water or inadequate sanitation (WHO, 1996). Daily, there are numerous reports of outbreaks worldwide due to the consumption of untreated or improperly treated drinking water contaminated with bacterial, viral or parasitic microorganisms (Ford & Colwell, 1996; Hunter, 1997; WHO, 1993; WHO, 1996; WHO, 2001). WHO and the United Nations Children’s Fund (UNICEF) recently claimed that improvement of drinking water quality and basic sanitation can cut this toll and, furthermore, that simple, low-cost household water treatment can save lives because it cuts the primary transmission route for diarrhoeal diseases (WHO/UNICEF, 2005). Solar disinfection (SODIS) is such a water treatment method where by 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). The two primary sources for bacterial inactivation in this method are believed to be mild heat and UVA light (Wegelin et al., 1994). SODIS was demonstrated to be effective against bacteria and higher organisms (Joyce et al., 1996; Lonnen et al., 2005; McGuigan et al., 1998; Wegelin et al., 1994) and its applicability was shown in a recent health impact study (Hobbins et al., 2003). Today, SODIS is one of the recommended methods for household drinking water disinfection (WHO/UNICEF, 2005).

The inactivation mechanism of SODIS is complex and not yet fully understood. The central hypothesis is that UVA light produces reactive oxygen species, which can damage nucleic acids, proteins or other life-supporting cell structures (Eisenstark, 1989; Jagger, 1985). It was also found that broad-spectrum UVA light can block the electron transport chain, inactivates the permease system, interferes with metabolic energy production and can cause a general increase in permeability of the membrane (Berney et al., 2006a; Jagger, 1981; Koch et al.,
Efficacy of SODIS of enteric pathogens

1976). Furthermore, direct inhibition of certain enzymes (e.g. catalase) has also been observed (Eisenstark, 1998).

Although solar disinfection is a recommended and regionally used drinking water treatment method, experimental data about its effect on pathogenic bacteria in drinking water are still scarce. The lethal action of mild heat on enteric bacteria, which adds another inimical component to solar disinfection, was mostly investigated in complex media and at temperatures above 52 °C, but for bacteria in water challenged with continuous heating at temperatures between 40-52 °C very little data are available (Benito et al., 1999; Humpheson et al., 1998). Also, reciprocity (i.e. that total applied fluence produces the same response regardless of the fluence rate) was studied only for monochromatic UVA irradiation (365nm) but not broad band UVA or for sunlight (Lang et al., 1986; Peak & Peak, 1982). Reciprocity is very important for solar disinfection because solar irradiation can vary considerably during a day due to clouds or other factors. Hence, in this study, we investigated the sensitivity of E. coli and three pathogenic enteric strains, namely Salmonella enterica serovar Typhimurium, Shigella flexneri and Vibrio cholerae, to the lethal actions of sunlight and mild heat. Furthermore, we tested whether or not the reciprocity-law is valid for solar disinfection.

Materials and Methods

Bacterial strains

The following strains were used in this study: Escherichia coli K-12 MG1655 (ATCC 700926), Salmonella enterica serovar Typhimurium ATCC 14028, Shigella flexneri ATCC 12022 and Vibrio cholerae 01 Ogawa biotype El Tor (Nent 720-95). For selected experiments, an isogenic rpoS mutant of E. coli K12 MG1655 (rpoS13::Tn10) (Wick et al., 2002) was used.
Growth media and cultivation condition

**Batch.** Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) was used for batch cultivation (Miller, 1972). LB medium was always filter-sterilized with Millipore syringe filters (Millex GP, 0.22 μm) and diluted to 33 % volume/volume (v/v) of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon filtered). Precultures were prepared for each individual batch experiment from the same cryo-vial stored at -80 °C by streaking out a loop-full onto LB agar plates. After 15-18 h of incubation at 37 °C, one colony was picked and loop-inoculated into a 125 ml Erlenmeyer flask containing 20 ml of LB broth. This was incubated at 37 °C on a rotary shaker at 200 revolutions per minute (rpm). At an OD<sub>546</sub> between 0.1 and 0.2 (measured spectrophotometrically in glass cuvettes with 1 cm light path using a JASCO V550 UV/VIS spectrophotometer; Tokyo, Japan) cells were transferred into 500 ml Erlenmeyer flasks containing 50 ml of LB broth. In this way, no lag phase of the culture was observed. The culture volume to be inoculated into the fresh medium was calculated beforehand to attain an initial OD<sub>546</sub> of 0.002. These flasks were then shaken at 200 rpm in a temperature-controlled water bath (SBK 25D, Salvis AG, Reussbühl, CH) at 37 °C for about 18 h until stationary phase (specific growth rate, μ = 0 h<sup>-1</sup>) was reached. The specific growth rate μ was calculated from five consecutive OD<sub>546</sub> measurements (Berney et al., 2006c).

**Sample preparation and plating.** Cells were harvested by centrifugation from batch culture (at 13000 g, Biofuge fresco; Kendro, Zürich, Switzerland), washed three times with filter-sterilized (Nuclepore Track-Etch Membrane, 0.22 μm, Sterico AG, Dietikon, Switzerland) commercially available bottled water (EVIAN, Danone, Paris, France) and diluted to an OD<sub>546</sub> of 0.01 (corresponding to 1-5 x 10<sup>7</sup> cells/ml). Exposure of bacterial suspensions was started 1 hour after dilution, to let the cells adapt to the mineral water. Aliquots were withdrawn at different time points and diluted in decimal steps (10<sup>-1</sup> to 10<sup>5</sup>) with sterile-filtered (0.2 μm) mineral water (EVIAN). Following dilution, 1 ml of test solution was withdrawn and mixed with 7 ml of liquid tryptic soy agar (TSA) (Biolife,
Milano, Italy) at 40 °C (pour plate method). After 20 minutes, the solidified agar was covered with another 4 ml of liquid TSA (40 °C). Plates were incubated for 48 hours at 37 °C until further analysis. Plate counts were determined with an automatic plate reader (acolyte, SYNBIOSIS, Cambridge, UK).

**Mild heat exposure.** A bacterial suspension of 20 ml (see above) was transferred into 30 ml quartz glass tubes (WISAG AG, Zürich, Switzerland) and placed into a temperature-controlled water bath at defined temperatures between 40 and 52 °C as indicated later. Care was taken that vials were entirely submersed to prevent temperature gradients in the vials. The final temperature of the liquid was reached in ≤ 2 min. During exposure, samples were stirred with a magnetic stirrer. Unstressed control samples were kept in the dark at 37 °C.

**Sunlight exposure.** Samples of 10 ml of 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 (Berney et al., 2006c). A circulating 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 degree. At each time point one tube was withdrawn and its contents immediately processed as described above. Irradiation intensity data were obtained from a weather station, which is 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 of 350-450 nm, which reflects the wavelength range of the UVA lamps also used (Berney et al., 2006c). Conversion factors and calculations were used as in Wegelin et al. (1994). The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-COR, Lincoln, Nebraska, USA), 8 nm bandwidth, fitted with a model 1800-10 detector head (Berney et al., 2006c).

**Artificial UVA exposure.** Samples of 10 ml of 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 medium-
pressure mercury lamps Hanau TQ 150, TQ718 or TQ718 Z4 (doped) (WISAG AG, Zürich, Switzerland), which were operated at 150, 500 - 700 W, and 500 - 700 W, respectively. The fluence rates varied from 56 to 734 W/m². The lamps TQ 150 and TQ 718 exhibit the same wavelength spectrum but differ in power output. The doped lamp TQ 718 Z4 exhibits a broader wavelength spectrum (see Berney et al. (Berney et al., 2006c)). 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 consisted of 12.75 g/L sodium nitrate 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.

**Modeling with GInaFIT.** The Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFIT) was used for testing six different types of microbial survival models on our data (Geeraerd et al., 2005). The following models were used: Log-linear regression (Bigelow & Esty, 1920), Log-linear + Tail (Geeraerd et al., 2000), Log-linear + Shoulder (Geeraerd et al., 2000), Log-linear + Shoulder + Tail (Geeraerd et al., 2000), Weibull model (Mafart et al., 2002), Biphasic model (Cerf & Metro, 1977), Biphasic + shoulder (Geeraerd et al., 2005). All models were run for each inactivation curve and the values of the Root Mean Sum of Squared Errors (RMSE) were compared. The RMSE is considered to be the most simple and most informative measure of goodness-of-fit for linear as well as non-linear models (Geeraerd et al., 2005). The model with the smallest RMSE was considered the best fit for the respective inactivation curve. When the magnitude of RMSE was much smaller than experimental precision the model was considered to overfit the data and a simpler model was chosen. If two models had the same or very similar RMSE value the simpler model was
considered to fit best. $T_{90}$ and $F_{90}$ values (time or fluence to reduce plate counts by 90 %) were calculated using the best-fit model of GInaFIT (Table 1).

**Results**

Solar disinfection works on the basis of two major facts, the lethal action of solar UVA light, mild heat, and the synergistic effect, which is created when water temperature rises above 50 °C (Wegelin et al., 1994). Unfortunately, it is not always possible to reach such high water temperatures in the bottles. SODIS field trials in different geographic regions carried out by SANDEC (Department of Water and Sanitation in Developing Countries, Eawag, Switzerland) have shown that temperatures above 50 °C are rarely reached (personal communication R. Meierhofer). Therefore, we investigated the effectiveness of sunlight (factoring out the temperature effect) to inactivate *E. coli* and three pathogenic strains, namely *S. Typhimurium*, *S. flexneri* and *V. cholerae*. In order to estimate a possible synergistic effect between solar irradiation and mild heat, we also measured temperature sensitivities for all four strains at temperatures relevant to the SODIS method between 40 and 52 °C.

**Sunlight sensitivity**

Sensitivity to sunlight was investigated at a constant temperature of 37 °C. For each strain three experiments were conducted on three different days (Fig. 3.1). The fluences in the sunlight experiments were calculated for the wavelength range between 350 and 450 nm (Wegelin et al., 1994) in order to compare results with experiments carried out with the medium pressure lamps. A fluence of 2400 kJ/m² (350-450 nm) corresponds to about 6-7 hours of sunlight exposure in Switzerland from June to August. *E. coli* and *S. flexneri* exhibited a very similar inactivation behaviour with *S. flexneri* being more sensitive than *E. coli* (see also Table 3.1). The inactivation curves of these two strains both showed a distinct shoulder effect as indicated from the best fitting models when analysed
with GlnaFiT. Both *S. Typhimurium* and *E. coli* had very similar $F_{90}$-values but the shape of the curve was totally different. This discrepancy is also indicated by the $F_{99}$ values (Table 3.1). The slow inactivation of *S. Typhimurium* indicates low sensitivity of this strain to sunlight. On the other hand, *V. cholerae* was a lot more sensitive to sunlight. For the data of *V. cholerae* the Log-linear model fitted best.

![Inactivation curves](image)

**Fig. 3.1.** Inactivation curves of *V. cholerae*, *S. Typhimurium*, *S. flexneri* and *E. coli* exposed to sunlight (350-450 nm) on three different days. Temperature was held constant at 37°C. Bacterial cells were harvested from stationary phase LB batch cultures, washed three times and diluted in bottled mineral water before exposure. Colony forming units were measured by pour-plating and sensitivity was recorded as cfu/(cfu at time zero). Dashed lines represent modelling results obtained with the software GlnaFiT (Geeraerd et al., 2005).
Mild heat sensitivity

Sensitivity to mild heat was investigated in order to elicit the role of temperature in SODIS experiments (Fig. 3.2). Again the pathogenic enteric bacterial strains (S. Typhimurium, S. flexneri and V. cholerae) were compared to E. coli. Stationary phase bacteria (μ=0) were exposed to temperatures between 41-52 °C.

**E. coli**

**S. Typhimurium**

**S. flexneri**

**V. cholerae**

![Graphs showing inactivation curves of different bacteria exposed to mild heat]

**Fig. 3.2.** Inactivation curves of *V. cholerae*, *S. Typhimurium*, *S. flexneri* and *E. coli* exposed to mild heat between 40-52°C. Bacterial cells were harvested from stationary phase LB batch cultures, washed three times and diluted in bottled mineral water before exposure. Colony forming units were measured by pour plating and sensitivity was recorded as cfu/(cfu at time zero). Error bars represent standard deviations of triplicate measurement. Dashed lines represent modelling results with the program GInaFiT (Geeraerd et al., 2005). Control samples were held at 37 °C (○).
Chapter 3

*S. Typhimurium, S. flexneri and E. coli* reacted similarly to mild heat with *E. coli* being less sensitive at higher temperatures. However, *V. cholerae* exhibited sensitivity already at temperatures above 40°C. When inactivation curves were modelled with the Program GlnaFIT, the model Log-linear + shoulder or Log-linear model fitted best.

**Reciprocity**

Up to now it was assumed that fluence (dose) is the appropriate parameter to compare different irradiation experiments. This is based on the multihit and multitarget theory (Harm, 1980). As far as we know it has not been shown yet that this assumption holds true for *E. coli* exposed to sunlight or simulated sunlight.

![Inactivation curves of E. coli exposed to eleven different UVA intensities.](image)

**Fig. 3.3.** Inactivation curves of *E. coli* exposed to eleven different UVA intensities. (TQ150: 56 & 91 W/m²; TQ718 Z1: 352-569 W/m²; TQ718 Z4: 282, 305, 314 and 734 W/m²). Bacterial cells were harvested from stationary phase LB batch cultures, washed three times and diluted in bottled mineral water. Colony forming units were measured by pour plating and sensitivity was recorded as cfu/(cfu at time zero). Lines represent modelling results with the program GInaFiT for experiments with undoped (dashed line) and doped (solid line) lamps (Geeraerd et al., 2005)
Therefore, we performed irradiation experiments at ten different fluence rates with three different medium pressure mercury lamps (Fig. 3.3) (see also "Materials and Methods" section). The two lamps with the same wavelength spectrum showed the same inactivation efficiency with an average $F_{90}$-value of $1123 \pm 121 \text{ kJ/m}^2$ for E. coli cells in 6 experiment with different fluence-rates (56, 91, 352, 483, 542, 569 W/m$^2$).

![Graph](a)

![Graph](b)

**Fig. 3.4.** (a): E. coli rpoS-minus exposed to sunlight on a cloudy (▲) and on a sunny day (●). For comparison the inactivation curve of wild type E. coli on a sunny day was added (●). Bacterial cells were harvested from stationary phase LB batch cultures, washed three times and diluted in bottled mineral water. Colony forming units were measured by pour plating and sensitivity was recorded as cfu/(cfu at time zero). Error bars represent standard deviations of triplicate measurement. Unstressed control samples are displayed with open symbols. (b): Average sunlight intensities (10 min average) on the corresponding days during the experiment.
Also the doped lamp showed no difference in inactivation efficiency when operated at different intensities (four experiments, 282, 305, 314, 734 W/m²) but compared to the undoped lamp it seemed to be more effective for inactivation of *E. coli* \( (F_{90} = 759 \pm 39 \text{ kJ/m}^2) \). Interestingly, *E. coli* cells exhibited similar sensitivity to irradiation with the undoped lamps as with sunlight \( (F_{90} = 1210 \pm 188 \text{ kJ/m}^2) \). In the field, *rpoS*-minus mutants of *E. coli* were exposed on a sunny and on a cloudy day (Fig. 3.4a). On the sunny day plate counts of *E. coli rpoS*-minus mutants were below detection limit already after 4 hours. During these 4 hours, the solar irradiation intensity was always above 600 W/m². On the cloudy day, average intensity was mostly below 300 W/m² except during a short spell of sunlight in the first hour of irradiation (Fig. 3.4b). When inactivation curves were compared regarding fluence, no significant difference was observed. Furthermore, as expected, the *rpoS*-minus mutant was more sensitive to sunlight irradiation than the parent strain (Berney *et al.*, 2006c). *RpoS* is an alternative sigma factor in *E. coli* and *S. Typhimurium* and is involved in global stress response to various environmental stresses (Hengge-Aronis, 1999).

### Table 3.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>( T_{90} ) (min)</th>
<th>( F_{90} ) (kJ/m²)</th>
<th>( F_{99} ) (kJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> MG1655</td>
<td>182 ± 15</td>
<td>1210 ± 188</td>
<td>1530 ± 70</td>
</tr>
<tr>
<td><em>S. typhimurium</em> ATCC 14028</td>
<td>187 ± 37</td>
<td>1238 ± 341</td>
<td>2431 ± 425</td>
</tr>
<tr>
<td><em>S. flexneri</em> ATCC 12022</td>
<td>136 ± 37</td>
<td>932 ± 233</td>
<td>1194 ± 142</td>
</tr>
<tr>
<td><em>V. cholerae</em> 01 Ogawa biotype El Tor</td>
<td>24 ± 5</td>
<td>165 ± 32</td>
<td>305 ± 35</td>
</tr>
</tbody>
</table>

* Fluence was calculated from solar irradiation data for the wavelength range between 350 and 450 nm
Discussion

For a sound application of the solar disinfection method it is vital to know whether the reciprocity-law applies for bacterial inactivation by sunlight. The method is partly based on the assumption that each bacterial strain is inactivated above a certain threshold fluence. For example, it is assumed that a 3-log reduction of *E. coli* requires a fluence of about 2000 kJ/m² (dose of solar radiation integrated in the 350-450 nm wavelength range), which corresponds to about 5 h of mid-latitude midday summer sunshine (Wegelin et al., 1994).

The reciprocity-law states that the effect of radiation is the function of the total radiant energy, and is independent of intensity and time (Zetterberg, 1964). Although this assumption holds true for cells irradiated with monochromatic UVC (254 nm) (Zetterberg, 1964) it was shown to be incorrect for *E. coli* cells irradiated with monochromatic UVA light at wavelength of 365 nm (Peak & Peak, 1982). These authors demonstrated that *E. coli* WP2 followed the reciprocity-law only above fluences of about 750 W/m² and that sensitivity increased with lower fluence-rates (if the same fluence is applied). However, our results show that for stationary phase *E. coli* diluted in mineral water and irradiated with artificial sunlight the reciprocity-law holds true (Fig. 3.3). We did not find any tendency of increased sensitivity of *E. coli* towards lower fluence-rates. Also the field experiments do not indicate otherwise. Although the average fluence-rate was considerably lower on the cloudy day (250 W/m²) than on the sunny day (756 W/m²) *E. coli* rpoS-minus mutants did not show a different inactivation behaviour. Furthermore, we found that the lamp with the broad wavelength spectrum (TQ 718 Z4) was slightly more effective in inactivating *E. coli* cells. This is probably due to the increased wavelength intensities in the UVA range (300-400 nm), which cause more damage to the cells than the longer wavelength (Kramer & Ames, 1987).

Most studies about sensitivity of enteric bacteria to solar or artificial UV-light have been conducted with *E. coli*. Apparently, *E. coli* is the most suitable bacterium for such experiments. It is accepted worldwide as an indicator.
bacterium for detecting a potential contamination of drinking water with feces (WHO, 1996). Despite this, its use as the indicator bacterium for SODIS should be considered carefully because the inactivation mechanism in this particular treatment is not known yet and may be different in *E. coli* than in other enteric pathogens.

In this study, we compared the inactivation efficiency of SODIS for the three different entero-pathogenic strains *S. Typhimurium*, *S. flexneri* and *V. cholerae* and compared it to *E. coli*. Resistance to sunlight at a constant water temperature of 37 °C of these four organisms based on $F_{90}$ values (fluence needed to reduce plate counts by 90%) was in the following order: *Salmonella Typhimurium* ATCC 14028 > *E. coli* MG1655 > *Shigella flexneri* ATCC 12022 > *Vibrio cholerae* 01 Ogawa biotype El Tor. Although the $F_{90}$ values reflected the observed order, it has to be mentioned that due to the different shape of the inactivation curves, some are shouldered, others are linear, one can not extrapolate from these values to, e.g, $F_{99}$ values (Table 3.1). For example, the inactivation curve of *S. Typhimurium* is log-linear during the time measured as opposed to clearly shouldered curves for *E. coli*. The different curve shape can either indicate different sensitivity to the same damaging effect, different inactivation mechanisms in the two strains or the capability of *S. Typhimurium* to adapt to sunlight stress. Even less appropriate are $T_{90}$ values because they do not take into account different irradiation intensities. Unfortunately, the display of $T_{90}$ values has become very common in solar disinfection publications, which makes a comparison among different studies very difficult.

Evison (1988) also found that *S. Typhimurium* had an increased resistance to sunlight (artificial source) compared to *E. coli*. If this is true for all *Salmonella* strains, especially also for *S. typhi*, this species should be considered as an indicator organism for SODIS effectiveness. However, it was shown that *S. Typhimurium* C5Nx exposed to artificial sunlight (probably ~700 W/m²) for 8 h and a temperature regime temporarily reaching 55 °C failed to produce detectable infections in BALB/c mice (Smith et al., 2000). Even culturable cells that had been irradiated for 1.5 h were less infective (virulent) than their non-

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Efficacy of SODIS of enteric pathogens

irradiated counterparts. Furthermore, *V. cholerae* seems to be very susceptible to solar disinfection. This might be due to its preference for water with higher salt concentrations. Its natural niche is believed to be in brackish water (Louis *et al.*, 2003). The influence of salt concentrations on the sunlight sensitivity of *V. cholerae* still has to be tested. In another study it was shown that *V. cholerae* was more resistant to simulated sunlight than *S. Typhimurium* or *S. flexneri* (Kehoe *et al.*, 2004). When one deduces from these data $F_{90}$ values for the UVA light range, one finds slightly higher values for *V. cholerae* ($\approx 230 \text{ kJ/m}^2$), but more than ten-times lower values for *S. Typhimurium* and *S. flexneri* ($\leq 100 \text{ kJ/m}^2$) compared to our study (see Table 1). The higher resistance of *V. cholerae* in the study of Kehoe and coworkers could be due to the use of PBS, which probably had a higher salt concentration than our dilution medium, while the much lower resistance of *S. Typhimurium* and *S. flexneri* in their study might due to different cultivation conditions or again the dilution medium, which might be less favorable for these organisms. A list of $T_{90}$ values collected from different studies for inactivation of various bacteria by sunlight or artificial sunlight was provided by Reed (2004). The two values given for *V. cholerae* 35 min and 171 min, respectively, differ by a factor of five. Unfortunately, it is not possible to deduce the reasons for these discrepancies between different studies; it might be either due to the reasons discussed above, different experimental methods, or differences between strains. In a recent study, we demonstrated the influence of cultivation condition, particularly specific growth rate, on the sensitivity of bacteria to sunlight. Perhaps much of the discrepancies might be due to using cells cultivated differently. Furthermore, our results for *E. coli* compared well to those of previous reports (Evison, 1988; McGuigan *et al.*, 1998; Wegelin *et al.*, 1994).

*V. cholerae* was more susceptible to mild heat than the other three strains. This might be due to the same reason as described above. For the other three strains temperatures above 47 °C seemed to become lethal over a period of 5 hours. Very striking was the fact that already a temperature increase of 1 °C led to a measurable difference in sensitivity. Wegelin and coworkers (1994) reported earlier that a synergistic effect between sunlight and mild heat can be observed.
for *E. coli* at temperatures above 50 °C. Our results confirm this observation and suggest a slight synergistic effect even at 48°C. McGuigan et al. (1998) reported a synergy between light and thermal inactivation already above 45°C for *E. coli*. The latter authors used HPLC grade water for dilution, which might have "weakened" the cells before exposure. The synergistic effect between mild heat and sunlight is not well studied. Temperature regime can vary greatly depending on irradiation intensity, ambient temperatures and underlying surface. A recent field study confirmed 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 (Martin-Dominguez et al., 2005). However, we propose that the mild heat effect should not be neglected in SODIS experiments since a very small increase in temperature can immensely increase SODIS effectiveness for certain strains. Therefore, all possible and available measures should be taken to increase water temperatures in the bottles during SODIS.

**Acknowledgments**

This project was financially supported by the Velux Foundation (project number 119) and by Eawag-internal funding. We thank Martin Wegelin, Regula Meierhofer and Silvio Canonica for valuable discussions.
4. Flow-cytometric study of vital cellular functions in *E. coli* during solar disinfection (SODIS)

Abstract

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 solar disinfection could be gained by investigating six different cellular functions, namely efflux pump activity (Syto®9 plus ethidium bromide), membrane potential (DiBAC₄(4)), membrane integrity (LIVE/DEAD® BacLight™), glucose uptake activity (2-NBDG), total ATP concentration (BacTiter-Glo™) and culturability (pour-plate method). These variables were measured in *E. coli* K12 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. An UVA light dose (fluence) of less than 500 kJ/m² 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 more than 80 % of the cells was observed at a fluence of approximately 1500 kJ/m², and the cytoplasmic membrane of bacterial cells became permeable at a fluence of >2500 kJ/m². Culturable counts of stressed bacteria after anaerobic incubation on sodium pyruvate-supplemented TSA closely correlated with the loss of membrane potential. The results strongly suggest that cells exposed to more than 1500 kJ/m² of solar UVA (corresponding to 530 W/m² 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.
Chapter 4

Introduction

Water-borne 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 (WHO, 1996). Every day, diarrhoeal diseases claim the lives of approximately 5000 young children throughout the world and most of the cases could be easily prevented (Hinrichsen et al., 1997). The World Health Organisation (WHO) and the United World Children’s Fund (UNICEF) 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 (WHO/UNICEF, 2005).

Solar disinfection (SODIS) is such a water treatment method. Through exposure of drinking water in poly(ethylene terephthalate) (PET) bottles to sunlight (≥ 6 h), enteric bacteria in the water are inactivated (Acra et al., 1984; Wegelin et al., 1994). Both, UVA light and mild heat were shown to have inimical potential and, if the water temperature rises above 50 °C, a synergistic effect was observed (Wegelin et al., 1994). Field trials in different geographic regions carried out by Eawag’s (Swiss Federal Institute for Aquatic Science) Department of Water and Sanitation in Developing Countries (SANDEC) have shown that temperatures above 50 °C are rarely reached (R. Meierhofer, personal communication). (Acra et al., 1984) proposed that solar UVA irradiation accounts for more than 70 % of the negative effects of sunlight. Today, SODIS is one of the recommended methods for household drinking water disinfection (WHO/UNICEF, 2005). Nevertheless, the mechanism(s) of disinfection are not yet known precisely. The two primary sources for bacterial inactivation in this method are 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 (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
E. coli inactivation by sunlight tracked by flow cytometry

against bacteria and higher organisms (Joyce et al., 1996; Lonnen et al., 2005; Wegelin et al., 1994) and its applicability was shown in a recent health impact study (Hobbins, 2004). The effectiveness of SODIS has been shown using the 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 was shown that injured bacteria do not grow due to the stress of high nutrient concentration, which causes a free radical burst (Dodd et al., 1997). This behavior has been referred to as a suicide response (Aldsworth et al., 1999). Postgate already observed this behavior 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 processes (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 culturability.

In the work presented here, the solar disinfection 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.


Chapter 4

**Materials and 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, 5 g yeast extract, 10 g NaCl per liter) that was filter-sterilized with membrane filters (Millipore GP, 0.22 μm, Millipore, Tullagreen, Ireland) and diluted to 33 % volume/volume (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 of diluted LB broth and incubated at 37 °C on a rotary shaker at 200 revolutions per minute (rpm). At an optical density (OD$_{546}$) between 0.1 and 0.2 (measured spectrophotometrically at 546 nm in glass cuvettes with 1 cm light path using a Jasco V550 UV/VIS spectrophotometer; Tokyo, Japan) an aliquot of the culture was transferred into 500 ml Erlenmeyer flasks containing 50 ml of prewarmed LB broth to obtain an OD$_{546}$ of 0.002. In this way no lag phase was observed. These flasks were then shaken at 200 rpm in a temperature-controlled water bath (SBK 25D, Salvis AG, Reussbühl, CH) at 37 °C for about 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$_{546}$ 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 solar disinfection than during growth (Berney et al., 2006c; Reed, 1997)

**Sample preparation and plating.** Cells were harvested by centrifugation from batch culture (at 13000 g, Biofuge fresco; Kendro, Zürich, Switzerland), washed three times with filter-sterilized (Nuclepore Track-Etch Membrane, 0.22 μm, Sterico AG, Dietikon, Switzerland) commercially available bottled water
E. coli inactivation by sunlight tracked by flow cytometry

(Evian, Danone, Paris, France) and diluted to an OD_{465} of approximately 0.01 (corresponding to 1-5 x 10^7 cells/ml). During exposure, aliquots were withdrawn at different time points and diluted in decimal steps (10^{-1} to 10^{-5}) with sterile-filtered (0.22 μm) bottled mineral water (Evian). A volume of 1 ml of appropriate dilutions were withdrawn and mixed with 7 ml of liquid tryptic soy agar (TSA) (Biolife, Milano, Italy) at 40 °C (pour plate method). After 20 minutes the solidified agar was covered with another 4 ml of liquid TSA (40 °C). Plates were incubated for 48 hours 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 minutes with the same agar and then placed into an anaerobic jar (Oxoid, AG, Basel, Switzerland) (Khaengraeng & Reed, 2005). Just before closure of the jars an AnaeroGen sachet (Oxoid, Basel, Switzerland) was placed into the container to generate an anaerobic gas phase. The jars were incubated for up to 72 hours at 37 °C. Plate counts were determined with an automatic plate reader (acolyte, Synbiosys, Cambridge, UK).

Sunlight exposure. Samples of 10 ml of 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 degrees. 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 is 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 of 350-450 nm, which reflects the wavelength range of the UVA lamp used (see below). Conversion factors and calculations were used as in (Wegelin et al., 1994). The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-COR, Lincoln, Nebraska, USA), 8 nm bandwidth, fitted with a model 1800-10 detector head.
**UVA exposure.** Samples of 10 ml of 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 (wavelength spectrum see (Berney et al., 2006c)). 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 it consisted of 12.75 g/l sodium nitrate 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 above.

**Flow-cytometric measurements.** Flow-cytometric measurements were made with a Partec PAS III flow cytometer (Partec GmbH, Münster, Germany) with 488 nm excitation from an argon-ion laser at 20mW. Five fluorescent dyes were used alone or in different combinations: Syto®9 (Molecular Probes, Eugene, USA), propidium iodide (PI) (Molecular Probes, Eugene, USA), bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC₄(3)) (Molecular Probes), ethidium bromide (EB) (Fluka Chemie AG) and 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) (Invitrogen AG, Basel, Switzerland). 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 (DiBAC₄(3), Syto®9 and 2-NBDG). Samples were incubated in the dark at 37 °C for 5 minutes (2-NBDG) or at 20 °C for 10 (DiBAC₄(3)), 15 (Syto®9/EB), 20 (Syto®9/PI) and 25 minutes (Syto®9), respectively, before analysis. Prior to flow-cytometric analysis cell samples were diluted with sterile filtered Evian to 1 % v/v of the initial concentration (approx. 1 x 10⁵ cells/ml final concentration). Stock solutions of
E. coli inactivation by sunlight tracked by flow cytometry

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, DiBAC₄(3) was prepared in dimethylsulfoxide (DMSO) 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 concentration of Syto®9, PI, EB, DiBAC₄(3) and 2-NBDG were 5, 30, 30, 10 and 5 μM, 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, DiBAC₄(3) and 2-NBDG at 520 nm. Trigger was set for the green fluorescence (520 nm) channel FL1.

The 2-NBDG uptake kinetics of E. coli K12 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 minutes (Natarajan & Srienc, 1999). Every 5 minutes 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. 4.1. Syto®9, a green fluorescent nucleic acid stain, has been shown to stain living and dead Gram-positive and Gram-negative bacteria (Product manual. Molecular Probes, USA). Therefore, it can be used for total count measurements. Ethidium bromide (EB) binds to nucleic acid, 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 was shown that the ethidium efflux system is dependent on a transmembrane proton electrochemical gradient (Δµ_H+.) but is ATP-independent. Propidium iodide (PI) is a red fluorescent dye that intercalates with double stranded DNA and it only enters permeabilized cytoplasmic membranes. If EB or PI is combined
with Syto\textsuperscript{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\textsubscript{4}(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.

Fig. 4.1. Viability indicators (fluorescence stains) that were 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 FCM. Syto\textsuperscript{9} (green fluorescent) for total counts; propidum iodide (red) for membrane integrity; ethidium bromide (red) for efflux pump activity; 2-NBDG (green) for glucose uptake activity (PEP-PT system); DiBAC\textsubscript{4}(3) for membrane potential.
For further informations 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 phosphoenolpyruvate as an energy source.

**Total ATP:** For the determination of total ATP, the BacTiterGlo™ System (Promega, WI, USA) was used. The BacTiterGlo™-Buffer was mixed with the lyophilized BacTiterGlo™-Substrate and equilibrated at room temperature. The mixture was stored over night 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 BacTiterGlo™-reagent (stored on ice). The sample was then briefly vortexed and put in to a water bath at 37 °C for 30 seconds. The luminescence of the sample was measured in a Luminometer (Model TD-20/20, Turner BioSystems, WI, USA) immediately after incubation. A calibration curve with dilutions of pure rATP (Promega, Madison, USA) 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 *E. coli* cells from stationary phase and compared it to artificial UVA light. The application of multi-parameter flow cytometry allows the measurement of cellular functions on the single cell level (as opposed to bulk parameters) and thus, one can acquire a more detailed picture of the physiological state of bacterial cells as opposed to plating alone.
Chapter 4

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. 4.2). 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 made (Fig. 4.3). To prevent oxidative burst in the cells during aerobic incubation, samples were also incubated on pyruvate supplemented agar in anaerobic jars, which resulted in higher colony forming units. In all experiments, total counts remained constant over the whole irradiation period (Fig. 4.4a). UVA light appeared to be slightly more effective in inactivating E. coli cells than sunlight when assessed with plating (Fig. 4.4b).

Fig. 4.2: Inactivation curves of E. coli K12 MG1655 exposed to artificial UVA light from 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 between 1.7-1.9x10^7 cells/ml. Culturability of bacterial cells was measured with the pour-plate method using tryptic soy agar (TSA).
E. coli inactivation by sunlight tracked by flow cytometry

**Fig. 4.3.** Culturability of *E. coli* K12 MG1655 during sunlight exposure for 7 hours. 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 standard deviations from triplicate measurements.

**ATP and efflux pump activity**

Soon after the start of exposure (between 400-600 kJ/m²) to either UVA light or sunlight the ATP concentration per cell dropped significantly and leveled out on about 5% of the initial value (Fig. 4.4c). The uptake of ethidium bromide (non-pumping cells) closely followed the decline in ATP concentration per cell (Fig. 4.4d). Ethidium bromide 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. 4.5a-c). The loss of culturability of bacterial cells appeared to be time-delayed when compared to the decrease of ATP.
Fig. 4.4. Analysis of cellular functions of E. coli K12 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 in % relative to the total cell count (Syto®9-stained cells) at the 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 colony-forming units (solid lines). (b) Culturability of UVA (△) and sunlight-irradiated cells (aerobic (●) and anaerobic (○) incubation) in % of total counts. (c) Total ATP concentration per cell. (d) Non-pumping cells: Loss of efflux pump activity measured with Syto®9 plus EB-staining. (e) Depolarized cells: Loss of membrane potential measured with DiBAC₄(3). (f) Permeabilized cells: Loss of membrane integrity measured with Syto®9 plus PI-staining on a FCM. All experiments were done at least twice. Data from one representative experiment are displayed.
**Fig. 4.5.** Flow-cytometric analysis of *E. coli* K12 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 a mixture of Syto®9 plus EB, DiBAC₄(3) or a mixture of Syto®9 plus PI and analysed on a flow cytometer after having exposed to different fluences (irradiation intensity × time). After 1000kJ/m²: >95 % of the cells are non-pumping (b), 40% are depolarized (e), and < 1% permeabilized (RN2) (h). After 2500kJ/m²: 100% of the cells are non-pumping (c), 100% depolarized (f), and > 90% permeabilized (polygon-gate RN2) (i).
Membrane potential and membrane integrity

The loss of membrane potential, which was measured with the green fluorescent bis-oxonol DiBAC$_4$(3), was very similar for both light sources (Fig. 4.4e). On 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, 520nm) (Fig. 4.5d), therefore, only depolarized (green fluorescent) cells were counted (Fig. 4.5e-f). The percentage of depolarized cells was calculated using the total count measurements. In our experiment depolarization of the bacterial cells occurred later than the cessation of efflux pump activity but before membrane integrity was lost (Fig. 4.4f). Propidium iodide, a dye which only enters cells with permeabilized cytoplasmic membranes, was only detected after a dose of 2000-2500 kJ/m$^2$ had been applied. The permeabilized cell population showed a typical increase in red fluorescence and decrease in green fluorescence intensity because of quenching (Fig. 4.5a-i). Interestingly, green fluorescence intensity increased by 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$^2$ almost 50 % of the cell population had lost membrane integrity. From the UVA light experiment we learned that after around 2500 kJ/m$^2$ of UVA light most of the *E. coli* cell population (> 95 %) has lost membrane integrity. Artificial UVA light seems 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 uptake pattern (Fig. 4.6a). The mean green fluorescence intensity of the cells linearly increased with time during the first ten minutes of incubation with 2-NBDG. Therefore, irradiated cells were always incubated for 5 minutes 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 two hours of sunlight exposure (800 kJ/m$^2$), thereafter it dropped along with the number of fluorescent cells, indicating that the PEP-PTS system was compromised (Fig. 4.6b).
E. coli inactivation by sunlight tracked by flow cytometry

(a)

![Graph showing the inactivation of E. coli by sunlight tracked by flow cytometry]

(b)

![Graph showing the effect of sunlight on E. coli K12 MG1655 harvested from a stationary phase LB batch culture]

Fig. 4.6.  (a) 2-NBDG uptake of *E. coli* K12 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 (520nm) of subsamples was analyzed every 5 minutes on a flow cytometer. In the graph the geometrical means (G-mean) of green fluorescence intensity (measured in range-gate RN1) are displayed in the inset. (b) 2-NBDG uptake of *E. coli* K12 MG1655 (pre-treatment like above) exposed to sunlight. Bacterial cell samples were incubated with 2-NBDG and 2,4-dinitrophenol for five minutes at 37 °C and analyzed immediately on a flow cytometer. (●) 2-NBDG positive cells in % of total cell counts, (▲) geometrical mean of green fluorescence intensity (520nm) of the cell population. Unstressed control samples are displayed with open symbols.
Bacterial cell size did not change significantly during exposure as was indicated by the forward angle light scatter (FALS) (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 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 Syto®9 and PI mixture during UVA irradiation revealed a unique fluorescence pattern of the bacterial cell population (Fig. 4.5g-i). On a two-dimensional dot plot the whole population moved in a circle from low green and red fluorescence intensity (Fig. 4.5g) to a (approximately 4.3 fold) increased green fluorescence intensity (Fig. 4.5h) and then to a state of increased red and decreased green fluorescence intensity (Fig. 4.5i). The increase of green fluorescence intensity coincided with the loss of culturability of more than 97 % of the cell population and only after prolonged irradiation PI was able to enter the cells and quenched Syto®9 fluorescence. Hence, membrane integrity was lost long after 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 approximately 3.7-fold while culturability was lost by more than 98 % of the population. This observation is probably related to an increased uptake of Syto®9. This was tested by using the Syto®9 and PI mixture and Syto®9 only in the same irradiation experiment (Fig. 4.7). Using only Syto®9 led to a significant increase (3.7-fold) in green fluorescence intensity as well.
**E. coli** inactivation by sunlight tracked by flow cytometry

<table>
<thead>
<tr>
<th>Culturability % of initial conc.</th>
<th>Syto®9 plus Propidium iodide</th>
<th>Syto®9</th>
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*Fig. 4.7.* Flow-cytometric analysis of *E. coli* K12 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 plus PI or Syto®9 alone was compared to culturability on TSA (pour-plate method). The loss of culturability coincides with an increase in green fluorescence intensity (range-gate RN2).
Furthermore, staining with DiBAC$_4$(3) revealed that the loss of membrane potential coincided with the increase in green fluorescence intensity in samples that were stained with Syto®9 and PI mixture (Fig. 4.5e, 5h). 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 inactivation pattern indicates 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 transmembrane 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.

**Inactivation pattern of vital cellular functions**

The inactivation of cellular functions in *E. coli* measured during light exposure 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, also the membrane potential was lost and glucose uptake rate diminished accordingly. At last, the cytoplasmic membrane of bacterial cells became permeable. The loss of culturability was dependent on the incubation method and the growth
E. coli inactivation by sunlight tracked by flow cytometry

Anaerobic incubation on sodium pyruvate supplemented agar, a procedure known to recover also 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 quick 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 like glycolysis or that the remaining ATP could not be used due to damage to systems, which require ATP. It was shown that the activity of ATPase from Saccharomyces cerevisiae solubilized from the plasma membrane and exposed to UVA light remained constant irrespective of dosage, indicating that the ATPase molecule itself was not damaged by UVA irradiation (Arami et al., 1997a; Arami et al., 1997b). 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 was 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. It has to be mentioned though that in their experiments, stressed cells were plated on unsupplemented complex media. Such media can enhance an oxidative burst in the 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, is 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.
Chapter 4

It was hypothesized that when a cell is stressed energetically, firstly the active transport systems will cease, then the cytoplasmic membrane will depolarise 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, DiBAC₄(3) and PI. In their case 35 % of the depolarized cells could still form colonies when sorted on to 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 DiBAC₄(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 solar disinfection this is rather favorable, because an underestimation could have severe consequences for the user of SODIS.

The specific glucose uptake rate by the PEP-PT system decreased simultaneously with the loss of membrane potential. The cause of inhibition though remains unclear. Changes in uptake rates due to cells size could 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 (to prevent ATP synthesis, hence the degradation of 2-NBDG) (Natarajan & Srienc, 1999; White, 1995). One can speculate that enzymes of the PT-system are inactivated by UVA light, and that glucose is taken up only until all PEP is used up. It was proposed that components of the electron transport chain, possibly menaquinone or dehydrogenases, are damaged by UVA light and this inhibits permeases such as the lactose permease (Jagger, 1981). Therefore, the high affinity glucose permease could be an indirect target of UVA. Recently, it was revealed by 2D-PAGE analysis that pyruvate kinase (PykF) and EF-G (FusA) of S. Typhimurium
specifically increased in cells just after heat treatment and recovery in TSB (Kobayashi et al., 2005). Pyruvate kinase PykF catalyzes the synthesis of pyruvate from phosphoenolpyruvate in the sugar phosphotransferase system. Kobayashi and coworkers suggested 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 stated that ATP seems most important because many reactions involved in recovery require ATP as an energy source. If PykF was 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, an inhibition of PykF would not directly inhibit glucose uptake by PEP-PTS because PEP is not converted to pyruvate but would still be available for the uptake of glucose molecules as discussed above.

All viability indicators were applied again on 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 more than 1500 kJ/m² of solar UVA (corresponding to 530 W/m² global sunlight intensity for 6 hours) was observed. This indicates that the damages in E. coli caused by UVA light are irreversible. It has to be mentioned, though, that resuscitation attempts with sterile supernatant like they were done 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 retrospective (Postgate, 1989). Today,
several viability indicators can be assessed without culturing cells 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 microorganisms. 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 show that the use of only one viability indicator, as it is 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 solar disinfection method (SODIS)**

Our results show that a UVA fluence of more than 1700 kJ/m² is needed to depolarize the cytoplasmic membrane of more than 95 % of the cell population, while 2500 kJ/m² is needed for permeabilization. This corresponds to about 600-880 W/m² of sunlight intensity (global intensity) over a period of 6 hours, a threshold which can be achieved in most developing countries (Martin-Dominguez et al., 2005). The same study also confirmed 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 W/m² for a period of 6 hours was used to irradiate stationary phase E. coli cells. A one-log reduction was achieved with a UVA fluence of about 1200 kJ/m². This corresponds well with the 1400 kJ/m² measured in our work.

Important for the user of SODIS is the loss of infectivity of pathogenic bacteria in the treated water. It was shown that S. Typhimurium C5Nxr exposed to
simulated sunlight (probably ~700 W/m²) for 8 h and a temperature regime temporarily reaching 55 °C failed to produce detectable infections in BALB/c mice (Smith et al., 2000). Even culturable cells that had been irradiated for 1.5 h were 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 death, though, will be achieved if fluences are applied that lead to membrane permeability. If these results also apply to other enteric bacteria is likely, but still has to be investigated. Also the effect of sunlight on higher organisms like Cryptosporidium or Amoeba can be assessed with the methods used in this study.

Acknowledgments

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Adaptation of E. coli in continuous culture to artificial sunlight

5. Adaptation of E. coli growing in continuous culture to artificial sunlight

Abstract

Adaptive responses of bacteria to physical or chemical stresses in the laboratory or in the environment are of great interest. Here we investigated the ability of E. coli growing in continuous culture to adapt to artificial sunlight (primarily UVA light). It was shown that E. coli indeed induces an adaptive response during UVA irradiation at an intensity of 50 W/m². Cells grown in continuous culture with complex medium (diluted Luria Bertani broth) at dilution rates of 0.7 h⁻¹, 0.5 h⁻¹ and 0.3 h⁻¹ were able to maintain growth under UVA irradiation after a transient reduction of specific growth rate and recovery. In contrast, slow-growing cells (D = 0.05 h⁻¹) were unable to induce enough protection capacity to maintain growth under UVA irradiation. We propose that faster growing E. coli cells have a higher adaptive flexibility to UVA light-stress than slow-growing cells. Furthermore it was shown with flow cytometry and viability stains that at a dilution rate of 0.3 h⁻¹ only a small fraction (≤ 1%) of the initial cell population survived UVA light-stress. Adapted cells were significantly larger (30 %) than unstressed cells and had a lower growth yield. Furthermore, efflux pump activity was diminished in adapted cells. In a second irradiation period (after omitting UVA irradiation for 70 hours) adapted cells were able to trigger the adaptive response twice as fast. Additionally, this study shows that continuous cultivation with direct stress application allows reproducible investigation of the physiological and possibly also molecular mechanisms during adaptation of E. coli populations to UVA light.

This chapter was submitted for publication; Authors: Michael Berney, Hans-Ueli Weilenmann and Thomas Egli
Introduction

Sunlight has long been recognized as a harmful radiation capable of damaging microorganisms (Downes, 1886). Solar radiation is believed to be a physiologically relevant stress also for enterobacteria since transmission between hosts can involve environmental exposure (Calkins & Thordardottir, 1980). For example, sunlight has been proposed to be the most important agent inactivating sewage microorganisms (e.g., Enterococci or E. coli) in shallow seawater or fresh water (Rozen & Belkin, 2001; Sinton et al., 2002). Furthermore, the deleterious effect of sunlight on enteric bacteria has been used to develop simple drinking water disinfection methods based on the action of sunlight (SODIS) (Acra et al., 1984; Wegelin et al., 1994).

The main fraction of solar ultraviolet radiation reaching the earth’s surface is UVA light (320-400 nm), also called near UV light (NUV). UVA light, depending on the applied dose (fluence), is believed to have both lethal and sublethal effects on E. coli. Sublethal effects in Escherichia coli were shown to occur at fluences of 100 kJ/m² at 366 nm; such fluences are approximately an order of magnitude lower than lethal fluences (Jagger, 1981). Two different phenomena of sublethal injury in bacteria can be found in the literature: Firstly, bacterial cells were referred to as sublethally injured when they do not grow on selective media after irradiation but can be recovered on catalase- and pyruvate-supplemented media (Kubitschek & Peak, 1980); secondly, bacterial cells grown in batch culture and irradiated with broad-band UVA light for a restricted time period were found to exhibit a lag time (also called growth delay) before resuming growth. In the very first report growth delay in E. coli caused by near-UV irradiation with a medium-pressure mercury arc lamp (350-490 nm) was shown to be a direct function of dose and that it occurred already at “non-lethal” doses (Hollaender, 1943). Some 20 years later an action spectrum for induction of growth delay with a maximum at 340 nm was presented for E. coli B (Jagger et al., 1964). Subsequently, a number of studies were initiated to characterize the response of E. coli to NUV. It was shown that growth delay is...
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largely due to cross-linking of cytidine with 4-thiouridine (s^4U) (λ_{max} = 340 nm) an unusual base occurring in the 8-position in 65% of tRNAs species of E. coli (Favre et al., 1985; Jagger, 1985). The same authors proposed that due to the lack of tRNA-charging activity stringent response with subsequent growth inhibition is triggered. Others proposed a close relationship between membrane damage and the UVA-induced growth delay (Koch et al., 1976; Kubitschek & Doyle, 1981; Pizarro & Orce, 1988). Other authors again suggested that the growth delay might help the cell to induce repair of damage (Favre & Hajnsdorf, 1983), but it was not until 1988 when it was proposed that induction of growth delay triggered by tRNA crosslinking could be part of an adaptive response to UVA light (Kramer et al., 1988). The same authors suggested a model where s^4U acts as a sensor for UVA stress that mediates the induction of specific proteins (some of them are oxidative stress-related) and the synthesis of dinucleotides. Recently, it was shown that continuous sublethal UVA irradiation of E. coli growing in batch culture exposed to a very low intensity (7.4 W/m²) leads to an adaptive response that includes the expression of some oxidative stress-related enzymes (Hoerter et al., 2005b). Despite all these suggestions the nature and mechanism of this adaptive response is still poorly understood at the physiological as well as the molecular level.

To our knowledge, all studies conducted so far on UVA light-stress in E. coli were done in batch culture with complex medium and no control of specific growth rate. Here, we investigated the ability of E. coli growing in continuous culture to adapt to UVA light-stress. The influence of specific growth rate on E. coli's ability to adapt to UVA irradiation was tested and physiological changes during adaptation were investigated with single cell analysis (flow cytometry) and other methods.

**Materials and Methods**

**Bacterial strains.** In all experiments wild-type E. coli K-12 MG1655 (ATCC 700926) was used.
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**Cultivation conditions.** Used for batch cultivation was Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) (Miller, 1972) that was filter-sterilized with membrane filters (Millex GP, 0.22 μm, Millipore, Tullagreen, Ireland) and diluted to 33 % volume/volume (v/v) of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon-treated). Precultures were prepared for each individual experiment from the same cryo-vial stored at -80 °C by streaking out the stock culture 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 of diluted LB broth and incubated at 37 °C on a rotary shaker at 200 revolutions per minute. At an optical density (OD$_{546}$) between 0.1 and 0.2 (measured spectrophotometrically at 546 nm in glass cuvettes with 1 cm light path using a JASCO V550 UV/VIS spectrophotometer; JASCO, Tokyo, Japan) an aliquot of the culture was transferred into the bioreactor with 50 ml of prewarmed diluted LB broth (5% v/v) to obtain an OD$_{546}$ of 0.002. The bioreactors for continuous culture experiments consisted of temperature-controlled and air-sparged cylindrical quartz glass tubes of 100 ml total and 50 ml working volume (WISAG, Oerlikon, Switzerland). The feed-medium consisted of 5% v/v LB broth. Before starting exposure, at least 10 volume changes were allowed for the culture to reach steady-state (based on OD$_{546}$).

**UVA exposure.** The bioreactors were installed in a incubation device (holding up to six bioreactors) (Fig. 5.1) equipped with a medium-pressure mercury lamp (Hanau TQ150) operated at 150 W (wavelength spectrum see (Berney et al., 2006c), TQ150 has the same spectrum as TQ718). The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-COR, Lincoln, Nebraska, USA), 8 nm bandwidth, fitted with a model 1800-10 detector head. The lamp was placed in a cooling jacket (Duran 50 borosilicate glass) in the centre of the incubation device and the bioreactors were arranged around the lamp at equal distance from the lamp. 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 it consisted of 12.75 g/l sodium nitrate 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 bioreactor position (Wegelin et al., 1994). The light intensity applied was 50 W/m², which represents the average mid-day sunlight intensity in the NUV range. In all experiments one aluminum foil-wrapped control bioreactor was added. All bioreactors were fed with medium from the same reservoir.

![Fig. 5.1. Picture of two bioreactors during irradiation.](image-url)
Throughout the experiments, OD$_{546}$ was measured as described above. Specific growth rate $\mu$ was calculated from three consecutive OD$_{546}$ measurements ($\mu = \frac{\Delta \ln OD_{546}}{\Delta t} + D$) (above $D$ is the dilution rate and $\Delta t$ the time between two OD$_{546}$ measurements) and the division rate was calculated from total cell count measurements accordingly.

**Flow-cytometric measurements.** Flow-cytometric measurements were made with a Partec PAS III flow cytometer (Partec GmbH, Münster, Germany) with 488 nm excitation from an argon-ion laser at 20 mW. Three fluorescent dyes were used alone or in different combinations: Syto®9 (Molecular Probes, Eugene, USA), propidium iodide (PI) (Molecular Probes) and ethidium bromide (EB) (Fluka Chemie AG, Buchs, Switzerland). Culture samples withdrawn from irradiation experiments were washed once with 0.22 μm membrane-filtered mineral water (EVIAN) to remove residual nutrients from LB and then divided into three subsamples (100 μl) and immediately stained. One subsample was stained with a mixture of Syto®9/PI, the second subsample was stained with Syto®9/EB and the third subsample with Syto®9 only. Before analysis, subsamples were incubated in the dark at room temperature for 15 (Syto®9/EB), 20 (Syto®9/PI) and 25 minutes (Syto®9), respectively. Prior to flow-cytometric analysis cell samples were diluted with 0.22 μm membrane-filtered EVIAN to yield a concentration suited for flow cytometry (approx. 1 x 10$^5$ cells/ml). Stock solutions of the dyes were prepared as follows: PI and Syto®9 were used from the LIVE/DEAD® BacLight™ kit (Molecular Probes) and EB was made up at 25 mM in 0.22 μm membrane-filtered deionized water. All dye stock solutions were stored at -20 °C. The working concentration of Syto®9, PI, and EB was 5 μM, 30 μM, and 30 μM, respectively. In the flow cytometer, optical filters were set up such that PI and EB emitted fluorescence was measured above 590 nm and Syto®9 at 520 nm. The trigger was set for the green fluorescence channel FL1 (520 nm), i.e., only green-fluorescing particles are registered.

**Fluorescence stains and their function.** A detailed and comprehensive description of the stains and their functions is given elsewhere (Berney et al., 88.
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2006a). Syto®9, a green fluorescent nucleic acid stain was used for total count measurements. Ethidium bromide is actively pumped out of the cell via a non-specific proton antiport transport system in active cells (Midgley, 1987). EB enters cells if the transmembrane proton gradient ($\Delta \mu_{\text{H}+}$) is lacking. Propidium iodide (PI) is a red fluorescent dye that only enters cells with a permeabilized cytoplasmic membrane. When 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. For further information about the dyes described above the reader is referred to the literature (Hewitt & Nebe-Von-Caron, 2004; Joux & Lebaron, 2000).

**Total ATP.** For the determination of total ATP, the BacTiterGlo™ System (Promega, Madison, WI, USA) was used. The BacTiterGlo™-Buffer was mixed with the lyophilized BacTiterGlo™-Substrate and equilibrated at room temperature. This reagent was stored over night at room temperature to make sure, 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 BacTiterGlo™-reagent (stored on ice). The sample was then briefly vortexed and incubated in a water bath at 37 °C for 30 seconds. The luminescence of the sample was measured in a Luminometer (Model TD-20/20, Turner BioSystems, WI, USA) immediately after the 30 second incubation. A calibration curve with dilutions of pure rATP (Promega) was measured before each experiment. ATP concentration per cell was then calculated using this calibration curve and the total cell count measurements (Syto®9) from flow cytometry.

**Reproducibility.** Continuous culture experiments at dilution rates of 0.3 h⁻¹ and 0.5 h⁻¹ were repeated three times. Flow-cytometric measurements were repeated twice to ensure reproducibility. Representative results are displayed in the graphs. The continuous cultures at dilution rates of 0.7 h⁻¹ and 0.05 h⁻¹ were run only once.
Chapter 5

Results

Although the effect of UVA light on bacteria in batch culture has been intensively studied by many groups, the influence of UVA light on a continuously growing culture has not been tested yet. Since cultivation conditions and specific growth rate play an important role in irradiation experiments with UVA light (Berney et al., 2006a), irradiating bacterial cells in a continuous culture offers a lot of advantages to batch culture irradiation. As indicated earlier, specific growth rate can be maintained constant at a set value in a continuous culture, allowing high reproducibility of experiments. In this study we investigated the influence of specific growth rate on the response and adaptation process of *E. coli* to UVA irradiation and recorded physiological changes primarily at the single cell level with flow cytometry.

Specific growth rate and cell size

*E. coli* K12 MG1655 was grown to steady-state in continuous culture at a dilution rate of 0.3 h⁻¹ and was then irradiated with UVA light (50 W/m²). An aluminum foil-wrapped continuous culture was run with the same feed medium at the same D as a control. Immediately after irradiation started, optical density as well as total cell count decreased significantly in the continuous culture, indicating that the bacteria had stopped growing and were washed out (Fig. 5.2). This decrease in optical density closely followed the theoretical wash-out curve. After about 20 hours of irradiation optical density and total cell count started to increase again. Interestingly, the total count did not increase to the same degree as optical density, indicating that cell size varied during the irradiation experiment. A repetition of this experiment combined with flow-cytometric analysis revealed that indeed the average cell size changed significantly (Fig. 5.3b). A steady increase of average cell size was observed until 20 hours, thereafter bacterial cells seemed to become smaller again. ATP concentration per cell (Fig. 5.3c) initially increased by 20-30% but dropped suddenly 2 hours after starting irradiation to a cellular level of ca. 30 % of the starting
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cellular ATP levels recovered again and transiently reached even higher concentrations than in unstressed cells.

![Graph showing OD vs time and total counts vs time for E. coli K12 MG1655 growing in continuous culture exposed to UVA light.](image)

**Fig. 5.2.** E. coli K12 MG1655 growing at a dilution rate of 0.3 h⁻¹ in “LB-limited” continuous culture was exposed to UVA light at 50 W/m². Optical density (○) and total counts (▲), measured with flow cytometry, are compared. A control bioreactor (aluminum foil-wrapped) was run at D = 0.3 h⁻¹ (●) with the same feed medium. Theoretical wash-out is indicated (solid line).

Interestingly, this coincided with an increase in specific growth rate (Fig. 5.3d) and also cell size. Specific growth rate and the division rate followed a very similar pattern (Fig. 5.3d). Both, specific growth rate and division rate initially dropped almost to zero, then increased to a D = 0.4 h⁻¹ and, subsequently, leveled out in a new steady-state at D = 0.3 h⁻¹. Both, optical density and total counts of the continuous culture in the new steady-state were significantly lower than before irradiation.

**Physiological changes during exposure**

Efflux pump activity (Syto®9 plus EB) and membrane integrity (Syto®9 plus PI) determined with flow cytometry indicated that during the first two hours of
irradiation the cells stayed intact with all measured functions active (Fig. 5.4). The ATP concentration per cell increased by 35% during this period (Fig 5.3c).

After about 6 hours more than 80% of the remaining population was EB-positive (non-pumping), while only 20% of the population had lost membrane integrity. After 10 hours, almost all remaining cells were EB-positive (96%) and more than 70% had lost membrane integrity. These bacterial cells had received a UVA fluence of approximately 1800 kJ/m² if one assumes that the cells had stopped growing right after the start of irradiation. Between 10 and 48 hours a
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A less light-sensitive bacterial population evolved, which showed no sign of permeabilization of the cytoplasmic membrane. Interestingly, these cells were unable to pump out ethidium bromide to the same extent as their non-irradiated predecessors.

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**Fig. 5.4.** *E. coli* K12 MG1655 growing at a dilution rate of 0.3 h⁻¹ in "LB-limited" continuous culture was exposed to UVA light at 50 W/m². Bacterial cell samples were withdrawn from the bioreactor, washed and diluted in filter sterilized mineral water (EVIAN) and analysed on the flow cytometer. Results were calculated in % relative to the total cell count (Syto®9-stained cells) at the given sampling point. Total counts (Syto®9-stained cells) (}
between non-pumping (upper left; Fig 5.5b,c) and pumping (lower right, Fig. 5.5a) or intact (Fig. 5.5d,f) and permeabilized (Fig. 5.5e) cells, respectively (see also Berney et al. (2006a)).

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**Fig. 5.5.** Flow-cytometric analysis of *E. coli* K12 MG1655 growing in "LB-limited" continuous culture at a dilution rate of \(D = 0.3 \text{ h}^{-1}\) irradiated with artificial UVA light. Bacterial cell samples were withdrawn from the bioreactor at different time points of irradiation (0 h, 10 h 48 h), washed and diluted in mineral water (EVIAN) and stained with a mixture of Syto®9 plus EB or a mixture of Syto®9 plus PI and analysed on a flow cytometer. After 10 h: (b) >95 % of the cells are non-pumping (RN2) and (e) > 70% are permeabilized (RN2). After 50 h: (c) 65% of the cells are non-pumping (RN2), and (f) 4% are permeabilized (RN2).
After 50 hours almost all bacterial cells appeared intact again (Fig. 5.f). The fluorescence pattern for EB plus Syto9 had changed but epi-fluorescence microscopy of these cells showed that most of them were still EB-positive.

**Dilution rate and fluence**

The influence of the dilution rate on the sensitivity of *E. coli* to UVA light was investigated (Fig. 5.6). One aluminum foil-wrapped control bioreactor (D = 0.5 h⁻¹) and four bioreactors run at different dilution rates (D = 0.7 h⁻¹, 0.5 h⁻¹, 0.3 h⁻¹, 0.05 h⁻¹) were operated simultaneously with the same feed medium. At dilution rates of 0.7 h⁻¹, 0.5 h⁻¹ and 0.3 h⁻¹ the bacterial population adapted to the UVA light-stress and reached a new steady-state after about 6, 30 and 48 hours, respectively (Fig. 5.7).

![Graph showing the influence of dilution rate on bacterial population](image)

**Fig. 5.6.** *E. coli* K12 MG1655 growing in continuous cultures with four different dilution rates (● 0.05 h⁻¹, △ 0.3 h⁻¹, ● 0.5 h⁻¹, □ 0.7 h⁻¹) was exposed to UVA light at 50 W/m². A control bioreactor (aluminum foil wrapped) was run at D = 0.5 h⁻¹ (∺). When steady-state was reached the lamp was switched on (time = 0 h) and OD₅₄₀ was measured during 50-80 hours.
At dilution rates of 0.7 h\(^{-1}\) and 0.5 h\(^{-1}\) *E. coli* cells triggered their adaptive response very quickly, which was indicated by the fast turnaround of specific growth rate (after about 1.5 hours) (Fig. 5.7). *E. coli* cells, which were cultured in the bioreactor with the lowest dilution rate (0.05 h\(^{-1}\)) showed no sign of adaptation during the time span of the experiment (75 hours).

Furthermore, it was observed that optical density of the culture in the newly established steady-state was always lower than before irradiation indicating that growth yield was lower under irradiation. The average residence time of a cell in the new steady-state of the three bioreactors (D = 0.7, 0.5, 0.3 h\(^{-1}\)) was 1.4, 2 and 3.33 hours respectively. Hence, in the new steady-state these cells received an average UVA fluence of 260, 360, and 600 kJ/m\(^2\), respectively.

**Fig. 5.7.** Specific growth rate of *E. coli* K12 MG1655 during continuous UVA irradiation (50 W/m\(^2\)) of the “LB-limited” continuous cultures with different dilution rates (\(\bullet\) 0.05 h\(^{-1}\), △ 0.3 h\(^{-1}\), \(\bullet\) 0.5 h\(^{-1}\), □ 0.7 h\(^{-1}\)) (see Fig. 5.6). In case of the slowest bioreactor specific growth rate was only calculated until 24 hours because after that time OD\(_{546}\) was below the detection limit.
Adaptive response

The nature of the adaptation of *E. coli* growing in continuous culture under continuous UVA light irradiation was investigated. *E. coli* was grown in a bioreactor with a dilution rate of 0.3 $h^{-1}$ and irradiated with UVA light at 50 W/m² (Fig. 5.8). After the new steady-state with respect to OD$_{546}$ was reached the lamp was switched off and the culture was allowed to grow back to its original steady-state. After switching off the lamp the culture immediately grew with a much higher specific growth rate (0.62 $h^{-1}$). After about 70 hours (21 generations) and establishment of the new steady-state with respect to OD$_{546}$ the lamp was switched on again. It was assumed that after this time cells containing proteins only expressed under UVA stress, had been diluted out. Also, in the second period of irradiation a reduction in specific growth rate of the culture was observed, however, it recovered much faster and the time to reach new steady-state was only 24 hours (compared to the 50 hours needed before).

![Graph showing the adaptive response of E. coli to UVA light](image)

**Fig. 5.8.** *E. coli* K12 MG1655 growing at a dilution rate of 0.3 $h^{-1}$ in “LB-limited” continuous culture was exposed to UVA light at 50 W/m². OD$_{546}$ was measured throughout the experiment (●). At the new steady-state was reached after about 50 h. After 75 hours the lamp was switched off. In the second irradiation period steady-state was reached after 24 hours already.
Chapter 5

Discussion

In this study we show for the first time that *E. coli* cells cultivated in continuous culture and irradiated with UVA light can adapt to this stress. The adaptive response seems to be dependent on specific growth rate. With flow cytometry and viability stains the physiological state of the bacteria in the bioreactor was followed and we saw indications that only a very small part of the initial population was able to divide under UVA irradiation. Our results indicate that an adaptation of *E. coli* to UVA light is possible at a much higher fluence-rate (50 W/m²) than previously derived from batch culture experiments (7.4 W/m²) (Hoerter et al., 2005b).

Physiological changes during irradiation of a continuous culture

During the first 10 hours of UVA irradiation of *E. coli* in continuous culture (D = 0.3 h⁻¹) more than 90% of the initial cell population was washed out due to growth inhibition. Interestingly, in the first 2 hours of irradiation the average ATP concentration per cell increased significantly, indicating that more ATP was produced or that ATP-dependent functions were inactivated while ATP production remained constant. The slight increase in cell size in the first ten hours of irradiation suggests that some cells were still able to grow. The data for specific growth rate and division rate indicated that the cells became larger and still divided. Others have shown that *E. coli* AB1157 cells, growing in batch culture with a specific growth rate of 0.83 h⁻¹ were able to adapt to illumination with 366-nm light (25 W/m²) by lowering their specific growth rate to 0.57 h⁻¹ (Caldeira de Araujo & Favre, 1985). The same authors showed that the reduction in cell mass-doubling time occurred without change in the rate of cell division. Hence, they proposed that cell size decreased during the first 2 hours of irradiation, which is in contrast to our finding. However, they provided no data about the behavior of cells that were irradiated for more than 2 hours.
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Efflux pump activity had ceased in the remaining cells after 10 hours of irradiation and about 75% of the cell population was permeabilized. We have shown earlier that permeabilized cells were not able to recover from UVA injury and that fluences of about 2000-2500 kJ/m² of UVA irradiation are needed for stationary phase E. coli cells to become permeabilized (Berney et al., 2006a). Therefore, the permeabilized cells detected in the continuous culture after 10 hours irradiation (1800 kJ/m²) are probably cells that have stopped growing immediately after irradiation started and were not washed out. About 20% of the remaining population (this corresponds to about 1% of the initial population), which appeared still intact after 10 hours was probably the surviving fraction that was able to adapt to UVA light. Between 10 and 20 hours of irradiation specific growth rate of the cells increased significantly and was higher (0.37 h⁻¹) after 20 hours than the dilution rate (0.3 h⁻¹). The increase in ATP production per cell is probably coupled with the observed increase in cell size. A larger cell is expected to have more total ATP. This is also supported by the fact that in the new steady-state a 30% increase in ATP concentration per cell compared to the initial steady-state paralleled an increase in cell size by 30% as measured with the flow cytometer. In contrast, cell size stayed constant in the non-irradiated control bioreactor.

In the new steady-state after 50 hours more than 99% of the cells were intact (PI-negative), while most of the cells were still non-pumping (EB-positive). The efflux pump activity is dependent on the electrochemical proton gradient, which might be lower in the UVA irradiated cells. It has been shown earlier that the loss of efflux pump activity is not lethal for stationary phase E. coli (Berney et al., 2006a; Nebe-von-Caron et al., 2000).

Adaptive response and its dependence on growth conditions and growth state

Up to now typical “growth delay” experiments were performed by irradiating bacterial cells growing in batch culture with UVA light for a restricted time period. After irradiation was stopped a lag time was observed before the
culture resumed growth. Evidence that *E. coli* can resume growth during irradiation with UVA light is very scarce. It was shown that at a light intensity of 25 W/m² at 366 nm the specific growth rate of *E. coli* cells in batch culture decreased and a new "stable" exponential mode of growth was reached (Caldeira de Araujo & Favre, 1985; Favre, 1980). However, irradiation of cultures never exceeded 2 hours. Therefore, it is not known if this adaptation was of stable nature or not. Others showed that when *E. coli* was subjected to continuous low-fluence UVA irradiation at 7.4 W/m² (emission peak at 365 nm) while growing in batch culture it responded by increasing the activity level of certain oxidative stress-related proteins, which led to an attenuation of the growth delay response and an increased resistance to lethal UVA irradiation (Hoerter et al., 2005b). Whether these changes of enzyme activity are due to changes in the expression profile or simply the activity level is not known. The same authors proposed that a UVA fluence of 135 kJ/m² delivered at a fluence rate of 50 W/m² eventually results in cell death. Our results show that *E. coli* cells growing in continuous culture at dilution rates between 0.3 to 0.7 h⁻¹ were able to adapt to broad band UVA light with a fluence-rate of 50 W/m² while at a dilution rate of 0.05 h⁻¹ the cells were not able to adapt during the time measured.

In the irradiated continuous culture bacterial cells are subject to selective pressure. Only cells that are able to initiate an appropriate stress response and can maintain cell division under the applied stress will be able to maintain themselves in the bioreactor during continuous irradiation. Hence, our results indicate that the adaptive flexibility of *E. coli* is dependent on specific growth rate. Faster growing cells seemed to have a much higher adaptive flexibility than slow-growing cells. This is somewhat surprising because we have shown that *E. coli* cells grown in continuous culture at a low dilution rate (0.1 h⁻¹), after washing and suspending them in mineral water before irradiation, were less sensitive to UVA light or sunlight than cells grown at higher dilution rates (0.7 h⁻¹) (Berney et al., 2006c). In contrast to the experiments presented here those bacterial cells were irradiated without addition of substrate. Therefore, the constant supplementation with substrate in the bioreactor experiments
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presented here seems to be essential for an adaptive response. This is in line with the knowledge that de novo protein synthesis is hindered during slow growth or growth arrest, which makes it difficult for the bacterial cell to respond to an additional stress quickly and effectively (Wick & Egli, 2004). It was shown that RpoS levels in *E. coli* increased with decreasing growth rate and that at specific growth rates around 0.1 – 0.2 h⁻¹ RpoS concentrations are similar to stationary phase bacteria (Ihssen & Egli, 2004; Notley & Ferenci, 1996). Hence, it can be followed that the general stress response, which was expressed in the slow continuous culture (D = 0.05 h⁻¹) under the regulation of RpoS, was not enough to withstand UVA irradiation at 50 W/m².

Furthermore, the adaptation of *E. coli* in continuous culture seems not to be dependent on generation time. In all bioreactors a significant decrease in specific growth rate was observed immediately after starting irradiation (Fig. 5.7) and biomass was washed out. This wash-out was more pronounced in slow-growing continuous cultures. In a continuous culture a wash-out of biomass is normally paralleled by an accumulation of non-utilized substrate, which temporary would allow bacteria to grow at a higher specific growth rate than the dilution rate. In the continuous culture with the slowest dilution rate (0.05 h⁻¹), the bacterial cells seemed not to be able to take advantage of the higher substrate concentration during the time measured. This indicates that the adaptive response of the bacteria might have been to slow due to their inability to quickly induce *de novo* protein synthesis (see above). With time the accumulated UVA light intensity (fluence) becomes lethal. A fluence of 2500 kJ/m² (corresponding to 14 hours mean residence time in the irradiated bioreactor) is enough to permeabilize stationary phase *E. coli* cells (Berney et al., 2006a). In all other bioreactors the cells could adapt to the UVA light-stress, which was indicated with a sudden increase in specific growth rate after the initial decrease (Fig. 5.7). In all three bioreactors (D = 0.7, 0.5 and 0.3 h⁻¹) specific growth rate transiently increased above the dilution rate demonstrating a temporary accumulation of substrates (see above). Interestingly, the bacterial cells from the bioreactor run at dilution rates of 0.7 h⁻¹, 0.5 h⁻¹ and 0.3 h⁻¹ were able to withstand an average fluence of 260, 360 and 600 kJ/m² in their new
steady-state. This is significantly above the proposed lethal fluence of 135 kJ/m² (Hoerter et al., 2005b). OD₅₄₆ in the new steady-state though, was below the initial value in all continuous culture experiments suggesting growth yield was lower. In a UVA-irradiated batch culture this metabolic shift would result in a slower specific growth rate. It was shown earlier that the specific growth rate of UVA-irradiated E. coli cells in batch culture inversely correlated with the fluence-rate applied (12 - 40 W/m², 365 nm) (Favre, 1980). It has to be mentioned, though, that the bacterial cells were only exposed up to 2 hours. Therefore, it is not known whether these cells eventually would have died or adapted under continuous irradiation.

When the lamp was switched off the adapted bacterial culture started to grow immediately at a much higher specific growth rate (0.62 h⁻¹) in a batch-like manner until the initial (before irradiation) optical density was reached. This supports the idea that UVA light triggers the production of a stress response leading to a lower growth yield. In a second irradiation period a new steady-state was reached already after 24 hours. Although the continuous culture was running for 21 volume changes between the two irradiation periods the bacterial cells seemed to “remember” the stress response to UVA light. This indicates that either stress related proteins were still expressed to some extent or that a mutant population has been selected. The nature of this adaptation will now be subject to further investigation. Proteome and transcriptome analysis are currently undertaken in our lab.

Environmental relevance

Sunlight (and especially solar UVA light) is one of the most common environmental stresses that microorganisms are subjected to. Enteric bacteria like E. coli or Salmonella are exposed to solar irradiation during transmission between hosts. The inimical effect of sunlight on enteric bacteria is also used for drinking water disinfection (SODIS) (Wegelin et al., 1994). Hence, the adaptive capabilities of these organisms to sunlight are of high interest. In the environment carbon and energy sources are scarce and it is not clear under
which circumstances enteric bacteria are able to multiply on the available nutrients. Many studies about the fate of enteric bacteria in aquatic environments and about the physical, chemical and biological factors affecting their survival in such habitats have been conducted (Bogosian et al., 1996; Davies & Evison, 1991; Rozen & Belkin, 2001; Santo Domingo et al., 2000). It is generally believed that enteric bacteria may survive in natural waters but are not able to multiply. Recently, it was shown that populations of fecal coliforms and enterococci did not grow in freshwater mesocosms (Anderson et al., 2005). Our study shows that with a constant flux of substrate E. coli is able to adapt and grow in UVA light-irradiated bioreactors at dilution rates above 0.3 h⁻¹. In natural aquatic environments carbon availability and temperatures are much lower and therefore, the expected specific growth rates of enteric bacteria are probably below 0.1 h⁻¹ (Ihssen, 2005). Even in the colon, E. coli's primary habitat, specific growth rates of 0.3 h⁻¹ are very unlikely (Ihssen, 2005). Therefore, it is unlikely that the observed adaptation of E. coli to UVA light may occur in a natural aquatic environment. Nevertheless, in further experiments we can now investigate the nature of this adaptation, which might give us a clue about the inactivation mechanism of UVA light and sunlight.

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6. Gene expression of *E. coli* in continuous culture during adaptation to artificial sunlight

Abstract

*E. coli* growing in continuous culture under continuous UVA irradiation exhibits growth inhibition with a subsequent adaptation to the stress. Transcriptome analysis was performed during transient growth inhibition and in the UVA light-adapted growth state. The results indicate that UVA light induces stringent response and an additional response that includes the upregulation of the synthesis of valine, isoleucine, leucine, phenylalanine, histidine and glutamate. The induction of several SOS response-genes strongly points to DNA damage as a result of UVA exposure. The involvement of oxidative stress was observed with the induction of *ahpCF*. Taken together it supports the hypothesis of the production of reactive oxygen species by UVA light. In the UVA-adapted cell population strong repression of the acid tolerance response was found. We identified the enzyme chorismate mutase as a possible chromophore for UVA light-inactivation and found strong repression of the *pyrB* operon and the gene *mgtA* encoding for an ATP dependent Mg²⁺ transporter. Furthermore, our results indicate that the role of RpoS may not be as important in the adaptation of *E. coli* to UVA light as it was implicated by previous results with starved cells, but that RpoS might be of crucial importance for the resistance under transient light exposure.

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Chapter 6

**Introduction**

Sunlight has long been recognized as a harmful radiation that is capable of damaging microorganisms (Downes, 1886). In the environment, during transmission between hosts, enteric bacteria are often exposed to sunlight (Calkins & Thordardottir, 1980). The deleterious effect of sunlight on enteric bacteria has been used to develop simple drinking water disinfection methods like Solar Disinfection (SODIS) (Acra et al., 1984; Wegelin et al., 1994). Today, SODIS is one of the recommended methods for household drinking water disinfection (WHO/UNICEF, 2005). However, the exact mechanism(s) of disinfection are not yet known. The two primary factors for bacterial inactivation in this method are believed to be mild heat and UVA light.

The spectrum of solar UV light is divided into three wavelength ranges called UVC (200-290nm) or far-UV (FUV), UVB (290-320nm) or mid-UV, and UVA (320-400nm) or near-UV (NUV) (Jagger, 1985). In other publications UVB and UVA is collectively referred to as NUV (290-400nm) (Eisenstark, 1989). On the following pages NUV and UVA are used as synonyms for the wavelength range between 320-400 nm (according to Jagger). The response of enteric bacteria, particularly *Escherichia coli*, to irradiation with solar UV light has been investigated for more than 70 years. A first physiological study on the lethal action (no recovery on standard agar) of NUV light on starved bacterial cultures was conducted by Hollaender (1943) followed by studies on the growth kinetics of *E. coli* under sublethal (recovery on standard or non-standard agar) NUV irradiation intensities (Jagger et al., 1964; Phillips et al., 1967). A well examined type of sublethal effects in *E. coli* is the phenomenon of growth delay (Jagger et al., 1964). It was shown that growth delay is largely due to absorption ($\lambda_{max} = 340$ nm) by 4-thiouridine ($s^U$), an unusual base occurring in the 8-position in 65% of tRNA species of *E. coli* (Favre et al., 1985; Jagger, 1985). The relative importance of DNA and membrane damage in the lethal and sublethal actions of NUV light on *E. coli* are still unclear. In the early seventies it was demonstrated that NUV inactivation is oxygen-dependent (Eisenstark, 1970; Webb & Lorenz, 1970). This
Transcriptome analysis of *E. coli* during UVA adaptation

Finding was followed by many studies showing that NUV light produces reactive oxygen species (ROS) via sensitization of endogenous photosensitizers (e.g., flavin and heme groups) and that intracellular iron pools may be involved as well (Ahmad, 1981; Eisenstark, 1998; Hartman & Eisenstark, 1978; McCormick et al., 1976; Webb & Brown, 1979). ROS can harm proteins as well as nucleic acids and, therefore, the lethal actions of NUV light are complex. It was found that broad-spectrum NUV light blocks the electron transport chain, inactivates the permease system, interferes with metabolic energy production and causes a general increase in permeability of the membrane (Berney et al., 2006c; Jagger, 1981; Koch et al., 1976). Numerous proteins, e.g., catalases (HPI and HPII), dihydroxyacid dehydratase (DHAD) and ribonucleotide reductase were identified to be NUV-sensitive (Eisenstark, 1989; Eisenstark, 1998). The same author listed more than 20 different genes, which might be involved in protection against NUV light. These studies were conducted either with strains carrying a mutation or a reporter gene fusion at the specific gene location. In a recent study, Qui and coworkers (Qiu et al., 2005) found that *Shewanella oneidensis* expressed twice as many genes after UVA light exposure than after UVC treatment. This result points to a high complexity of UVA light-induced stress response.

Although many pieces of the puzzle seem to be available, a comprehensive understanding of all processes involved in the response of *E. coli* to UVA light is still lacking. Recent advances in molecular microbiology allow analysis of differential gene expression of the whole genome in a number of microorganisms. Therefore, we used the micro-array technology, to screen global gene expression in *E. coli* in response to UVA light. Recently, we have shown that *E. coli* growing in continuous culture initially showed growth inhibition but then adapted to UVA irradiation with a fluence-rate of 50 W/m² (Berney et al., 2006b). In order to investigate the process of initial growth inhibition and subsequent adaptation of these cells, we analyzed the gene expression pattern in an unadapted (after 1 hour of irradiation) and in an adapted population of *E. coli* (after 50 hours of irradiation).
Chapter 6

Materials and Methods

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

**Cultivation conditions.** Used for batch cultivation was Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) (Miller, 1972) that was filter-sterilized with membrane filters (Millex GP, 0.22 µm, Millipore, Tullagreen, Ireland) and diluted to 33 % volume/volume (v/v) of its original strength (unless indicated otherwise) with ultrapure water (deionized and activated carbon-treated). Precultures were prepared for each individual experiment from the same cryo-vial stored at -80 °C by streaking out the stock culture 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 of diluted LB broth and incubated at 37 °C on a rotary shaker at 200 revolutions per minute. At an optical density (OD$_{546}$) between 0.1 and 0.2 (measured spectrophotometrically at 546 nm in glass cuvettes with 1 cm light path using a JASCO V550 UV/VIS spectrophotometer; JASCO, Tokyo, Japan) an aliquot of the culture was transferred into the bioreactor with 50 ml of prewarmed diluted LB broth (5% v/v) to obtain an OD$_{546}$ of 0.002. The bioreactors for continuous culture experiments consisted of temperature-controlled and air-sparged cylindrical quartz glass tubes of 100 ml total and 50 ml working volume (WISAG, Oerlikon, Switzerland). The feed-medium consisted of 5% v/v LB broth. Before starting exposure, at least 10 volume changes were allowed for the culture to reach steady-state (based on OD$_{546}$).

**UVA exposure.** The bioreactors were installed in a incubation device (holding up to six bioreactors) (adapted from (Wegelin et al., 1994)) equipped with a medium-pressure mercury lamp (Hanau TQ150) operated at 150 W (same wavelength spectrum as for Hanau TQ718 (Berney et al., 2006c)). The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-COR, Lincoln, Nebraska, USA), 8 nm bandwidth, fitted with a model 1800-10
detector head. The lamp was placed in a cooling jacket (Duran 50 borosilicate glass) in the centre of the incubation device and the four bioreactors were arranged around the lamp at equal distance. 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 it consisted of 12.75 g/l sodium nitrate with a cut-off at 320 nm and a half maximum at 340 nm. The transmission property of the filter solution was checked before each experiment. Chemical actinometry with p-nitroanisole/pyridine was used to determine the fluence rate at the bioreactor position (Wegelin et al., 1994). The light intensity applied was 50 W/m², which represents the average mid-day sunlight intensity in the NUV range.

Throughout the experiments, OD₅₄₆ was measured as described above. After 1 hour and after 50 hours of exposure one UVA-exposed and one non-irradiated control bioreactor were stopped and the entire bacterial culture (50 ml) of each tube was immediately mixed with 30 g of crushed ice to cool down the samples. After centrifugation for 10 min at 15000 rpm at 4 °C (Centrikon T-324, Kontron Instruments, Schlieren, Switzerland) the supernatant was discarded and total RNA was isolated (see below).

RNA isolation, synthesis of cDNA. Total RNA from E. coli cells was isolated with the QIAGEN RNeasy Midi Kit (QIAGEN, Basel, Switzerland) according to the manufacturer's manual. RNA in samples was quantified spectrophotometrically by measuring extinction at 260 nm and purity was checked by gel electrophoresis. Synthesis of cDNA from RNA was performed with the CyScribe First-Strand cDNA Labelling Kit (Amersham Bioscience, Little Chalfont, England). In the following description all ingredients are contained in the CyScribe kit unless otherwise noted. Reverse transcription was performed using 25 µg of total RNA (maximally in 10 µl) and 1 µl of random nonamer primers. The volume of the assay mixture was adjusted to 11 µl with RNase-free water, then the assay mixture was incubated for 5 min at 70 °C, followed by incubation for 10 min at room temperature to allow the primers to anneal with the RNA. After cooling down to room temperature the reagents for the labelling reaction were added. After the addition of 4 µl of 5x CyScript buffer, 2 µl of 0.1 M DTT, 1 µl of
deonucleoside triphosphate (dNTP) mixture, 1 μl of either 0.5 mM Cy3-labeled or Cy5-labeled DCTP (Amersham Bioscience), and 1 μl of CyScript reverse transcriptase (100 U/μl), the final reaction volume was 20 μl. The control cDNA (from the unirradiated bioreactor) and the probe cDNA (from either the 1 hour or the 50 hours irradiated bioreactor culture) were labelled differently, with the control cDNA always labelled with Cy5. The labelling reaction was performed at 42 °C for 1.5 h, followed by RNA degradation and cDNA purification. The RNA was degraded by addition of 2 μl of 2.5 M NaOH, the mixture was then heated at 65 °C for 10 min and subsequently neutralized with 10 μl of 2 M HEPES buffer. Control and probe cDNA obtained were pooled and purified on the same column of MinElute Gel Extraction Kit (QIAGEN, Basel, Switzerland) to avoid differences in extraction yields.

DNA microarray, hybridization and washing. Slide microarrays were purchased from MWG-Biotech AG (Ebersberg, Germany). The MWG E. coli Array contains 4,288 gene specific oligonucleotide probes representing the complete E. coli K12 genome. The purified cDNA was concentrated to 5 μl and was mixed with 120 μl of hybridization buffer (MWG-Biotech AG), heated to 95 °C for 3 min and cooled down on ice for 3 min. The hybridization mixture was then added to the microarray slide and covered with a coverslip. The hybridisation slide was incubated over night at 42 °C. After the hybridization step the slide was washed three times, the first time for 5 min in 2x (times concentrated) SSC - 0.1 % SDS, the second time for 5 min in 1 x SSC, and finally for 5 min in 0.1 x SSC. SSC buffer was prepared as 20 x solution containing 0.3 M Na-citrate and 3 M NaCl at pH 7.0. The slides were dried by centrifugation at room temperature for 2 min at 500 g.

Image and data analysis. Microarray slides were scanned using the Affymetrix 428™ Array Scanner (High Wycombe, UK). Spot intensities and corresponding background signals were quantified with the Affymetrix Jaguar™ software version 2.0. Further data analysis was performed with the program GeneSpring from Silicon Genetics (Redwood City, CA, USA). Induction factors (IF) were calculated from the Cy3 and Cy5 signal intensities of the spot. Spots with
signal intensity below a value of 50 were excluded from the analysis and the minimal induction factor was set to 0.01. The normalization was performed with the 50th percentile distribution of remaining spots after background correction. The mean value of the IF of a specific gene was calculated from three replicates. Biological experiments were carried out three times, which provided three biological repeats. Data from the independent experiments were combined, genes that were differentially regulated ≥ 2 and ≤ 0.5 (t-test, p ≤ 0.05) were defined as being statistically significant. Inductions factors of downregulated genes are displayed as -1/IF for the convenience of the reader (e.g., an IF of 0.5 is displayed as -2). For two different sampling time points (1 hour and 50 hours) IF, and IF_{50} were defined as abbreviations for the induction factors.

**Real Time PCR.** cDNA was synthesized with the SuperScript reverse transcriptase (Invitrogen, Basel, Switzerland). The mixture for RNA transcription was prepared for 40 reactions, mixing 0.8 μg of total RNA (DNase-digested) with 2 μl of random hexamer primers (Invitrogen). The total volume was adjusted to 9 μl with RNase-free water, then incubated for 5 min at 65 °C to melt secondary structures, and then cooled down for 2 min on ice to anneal the primers. Subsequently, reverse transcriptase reaction components were added, consisting of 0.5 μl RNase Inhibitor (Sigma, Buchs, Switzerland), 4 μl of dNTP mixture (2.5 mM each), 2 μl 0.1 M DTT, 4 μl 5x reaction buffer, and 0.5 μl SuperScript reverse transcriptase. The reaction mixture was mixed and spun down shortly and was then incubated for 10 min at 25 °C for annealing (90 min at 39 °C) reverse-transcribed mRNA. The reaction was stopped by incubation at 95 °C for 5 min. The total volume was adjusted to 40 μl before starting Real Time PCR. Real Time PCR was conducted according to the manufacturer, and the assay contained 12.5 μl of SYBR® Green PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 9.5 μl of RNase-free water, 1 μl forward primer, 1 μl reverse primer, and 1 μl cDNA (20 ng), or the same amount of RNA as negative control. Primers (Microsynth, Balgach, Switzerland) were designed with the software Primer Express® v2.0 (Applied Biosystems) for 8 genes (Appendix B). Primer optimization was performed by mixing different concentrations of forward and reverse primers. The optimized primer concentration was used to analyze RNA samples.
The Real Time PCR reaction was conducted using ABI Prism® 7000 (Applied Biosystems). The results were normalized using the gene *rrsB* as endogenous control (Table 6.1). Real Time PCR data were analysed with ABI Prism® 7000 SDS software version 1.0 according to the System User Bulletin #2 Relative Quantitation of Gene Expression (P/N 4303859) from Applied Biosystems.

**Table 6.1.** Comparison of mRNA expression levels of selected genes measured with either the microarray or the Real-Time PCR method. Only genes with p-values ≤ 0.05 were selected.

<table>
<thead>
<tr>
<th>Gene-#</th>
<th>Name</th>
<th>Induction Factor Microarray</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3942</td>
<td>katG</td>
<td>1.9</td>
<td>2.9</td>
</tr>
<tr>
<td>B0605</td>
<td>ahpC</td>
<td>2.2</td>
<td>3.3</td>
</tr>
<tr>
<td>B2699</td>
<td>recA</td>
<td>6.7</td>
<td>3.7</td>
</tr>
<tr>
<td>B2573</td>
<td>rpoE</td>
<td>-1.1</td>
<td>-1.9</td>
</tr>
<tr>
<td>B0417</td>
<td>thil</td>
<td>-1.2</td>
<td>-1.2</td>
</tr>
<tr>
<td>B1749</td>
<td>xthA</td>
<td>-1.4</td>
<td>-1.2</td>
</tr>
<tr>
<td>B2741</td>
<td>rpoS</td>
<td>-1.1</td>
<td>-1.8</td>
</tr>
<tr>
<td>B1732</td>
<td>katE</td>
<td>-2.3</td>
<td>-3.3</td>
</tr>
</tbody>
</table>

**Results & Discussion**

*E. coli* K12 MG1655 was grown in continuous culture in two bioreactors at a dilution rate of 0.5 h⁻¹ and was directly irradiated with UVA light (50 W/m²) (Fig. 6.1). Two aluminum foil-wrapped control bioreactors were run with the same feed medium at the identical dilution rate. Immediately after starting irradiation, optical density in the bioreactor decreased significantly indicating that bacteria stopped growing and were washed out (Fig. 6.1). Between 10-20 hours of irradiation optical density in the culture stabilized and then started to increase again and after approximately 50 hours specific growth rate was back on the initial level (0.5 h⁻¹) indicated by the new steady-state that was reached.
Optical density of the continuous culture in the new steady-state was significantly lower than before irradiation. A thorough discussion of the physiological changes during this adaptive response was presented recently (Berney et al., 2006b).

**Fig. 6.1.** *E. coli* K12 MG1655 growing at a dilution rate of 0.5 h\(^{-1}\) in continuous culture was exposed to UVA light at 50 W/m\(^2\) and optical density was measured throughout the experiment (▲). A control bioreactor (aluminum foil-wrapped) was run at 0.5 h\(^{-1}\) (△) with the same feed medium. Samples for gene array analysis were taken after 1 h and 50 h. Here only the data from the irradiated continuous culture running for 50 hours are shown.

**Global gene expression of *E. coli* during adaptation to UVA irradiation**

The global transcriptional profile of the *E. coli* population harvested from the continuous cultures that were irradiated with UVA light for either 1 hour or 50 hours were analyzed with the microarray technology. After 1 hour a total of 312 genes were differentially expressed in the irradiated cells compared to the non-irradiated control population. Upregulated were 163 genes (induction factor (IF) ≥ 2, t-test p-value ≤ 0.05) and 149 genes were downregulated (IF ≤ -2).
Fig. 6.2. Classification of genes differentially expressed in *Escherichia coli* MG1655 grown in "LB-limited" continuous culture at a dilution rate of $D = 0.5 \text{ h}^{-1}$ and irradiated with UVA light (50 W/m²) for 1 hour (A) or 50 hours (B). Only genes that were upregulated by a factor $\geq 2$ or downregulated by a factor $\leq -2$ are displayed. Only genes with p-values $\leq 0.05$ were selected.

After 50 hours a total of 193 differentially expressed genes (100 upregulated, 93 downregulated) were detected, and 52 of these genes were differentially expressed at both time points (appendix A). All differentially expressed genes
Transcriptome analysis of *E. coli* during UVA adaptation

detected after 1 hour or 50 hours of irradiation were distributed into 9 functional groups according to the EcoCyc annotation (http://ecocyc.org/) (Fig. 6.2). The tables shown in this paper list selected genes collected from different functional groups.

**Amino acid biosynthesis**

One of the most striking results of this analysis was perhaps that after 1 hour of irradiation 37 genes involved in amino acid metabolism, mostly amino acid biosynthesis, were upregulated while only 4 genes in this category were downregulated (Fig. 6.2). The same pattern was also observed after 50 hours with 21 up- and 2 downregulated genes. Among these, genes involved in biosynthesis of histidine, valine, isoleucine, leucine, glutamate and phenylalanine were upregulated in both physiological states while the superpathway of cysteine biosynthesis and sulfate assimilation was not induced after 50 hours (Table 6.2). The increased mRNA levels for amino acid biosynthesis in growth arrested (after 1 hour irradiation) as well as in the adapted cells (after 50 hours) indicate that either the uptake or/and the biosynthesis of these amino acids is hampered. However the situation is confusing. On one hand, the inhibition of both the leucine and the leucine-isoleucine-valine (LIV) systems in *E. coli* K-12 by UVA light was shown by Robb and colleagues (Robb *et al.*, 1978). The same authors provided a UV action spectrum for the two uptake systems showing a distinct peak at 365 nm. On the other hand, it was found that the addition of branched-chain amino acids (leucine, isoleucine and valine) to the medium when exposing *thiI*-minus mutants of *Salmonella* Typhimurium to NUV greatly delayed the entry into the growth lag, whereas other amino acids (methionine, arginine and glutamate) had no effect (Kramer *et al.*, 1988). These authors proposed that the NUV-induced growth delay in this strain may involve damage to branched-chain amino acid biosynthesis. Furthermore, the initial target of hyperbaric O₂ toxicity has been shown to be leucine-isoleucine-valine biosynthesis, i.e., the addition of these amino acids protected *E. coli* from this stress (Boehm *et al.*, 1976; Brown & Seither, 1983).
Table 6.2. *E. coli* genes involved in amino acid synthesis whose expression levels were increased by a factor $\geq 2$ or decreased by a factor $\leq -2$ in cultures exposed for either 1 or 50 hours to UVA light in "LB-limited" continuous culture at $D = 0.5 \, h^{-1}$. Genes were only listed if the p-value of the three replicates was $\leq 0.05$.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Name</th>
<th>Gene product</th>
<th>Ind. Factor (IF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
<td>50h</td>
</tr>
<tr>
<td><strong>Histidine biosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2024</td>
<td>hisA</td>
<td>n-(5'-phospho-l-ribosyl-formimino)-5-amino-1-(5'- phosphoribosyl)-4-imidazolecarboxamide isomerase</td>
<td>5.1 3.9</td>
</tr>
<tr>
<td>B2022</td>
<td>hisB</td>
<td>imidazoleglycerolphosphate dehydratase and histidinol-phosphate phosphatase</td>
<td>3.4 2.8</td>
</tr>
<tr>
<td>B2021</td>
<td>hisC</td>
<td>histidinol-phosphate aminotransferase</td>
<td>4.0 2.4</td>
</tr>
<tr>
<td>B2020</td>
<td>hisD</td>
<td>1-histidinol:na d+ oxidoreductase, 1-histidinol:na d+ oxidoreductase</td>
<td>5.6 3.0</td>
</tr>
<tr>
<td>B2025</td>
<td>hisF</td>
<td>imidazole glycerol phosphate synthase subunit in heterodimer with hish = imidazole glycerol phosphate synthase holoenzyme</td>
<td>4.0 2.9</td>
</tr>
<tr>
<td>B2019</td>
<td>hisG</td>
<td>atp phosphoribosyltransferase</td>
<td>3.8 2.4</td>
</tr>
<tr>
<td>B2023</td>
<td>hisH</td>
<td>glutamine amidotransferase subunit of heterodimer with hisf = imidazole glycerol phosphate synthase holoenzyme</td>
<td>2.5</td>
</tr>
<tr>
<td>B2026</td>
<td>hisI</td>
<td>phosphoribosyl-amp cyclohydrolase, phosphoribosyl-amp pyrophosphatase</td>
<td>3.9 2.9</td>
</tr>
<tr>
<td>B2018</td>
<td>hisL</td>
<td>his operon leader peptide</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Valine, Isoleucine, leucine biosynthesis</strong></td>
<td></td>
</tr>
<tr>
<td>B0071</td>
<td>leuD</td>
<td>isopropylmalate isomerase subunit</td>
<td>2.5</td>
</tr>
<tr>
<td>B0072</td>
<td>leuC</td>
<td>3-isopropylmalate isomerase (dehydratase) subunit</td>
<td>2.4</td>
</tr>
<tr>
<td>B0073</td>
<td>leuB</td>
<td>3-isopropylmalate dehydrogenase</td>
<td>6.0</td>
</tr>
<tr>
<td>B3670</td>
<td>ilvN</td>
<td>acetylactate synthase 1, valine sensitive, small subunit</td>
<td>7.7 6.7</td>
</tr>
<tr>
<td>B3671</td>
<td>ilvB</td>
<td>acetylactate synthase 1, valine-sensitive, large subunit</td>
<td>18.1 6.0</td>
</tr>
<tr>
<td>B3672</td>
<td>ilvL</td>
<td>ilvb operon leader peptide</td>
<td>5.6 2.6</td>
</tr>
<tr>
<td>B3766</td>
<td>ilvL</td>
<td>ilvgeda operon leader peptide</td>
<td>2.4</td>
</tr>
<tr>
<td>B3767</td>
<td>ilvG</td>
<td>acetylactate synthase ii, large subunit</td>
<td>10.5 2.5</td>
</tr>
<tr>
<td>B3769</td>
<td>ilvM</td>
<td>acetylactate synthase ii, valine insensitive, small subunit</td>
<td>4.7</td>
</tr>
<tr>
<td>B3770</td>
<td>ilvE</td>
<td>branched-chain amino-acid amidotransferase</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>superpathway of sulfate assimilation and cysteine biosynthesis</strong></td>
<td></td>
</tr>
<tr>
<td>B2752</td>
<td>cysD</td>
<td>atp:sulfurylase (atp:sulfate adenylyltransferase), subunit 2</td>
<td>6.8</td>
</tr>
<tr>
<td>B2763</td>
<td>cysI</td>
<td>sulfite reductase, alpha subunit</td>
<td>6.9</td>
</tr>
<tr>
<td>B2764</td>
<td>cysJ</td>
<td>sulfite reductase (nadph), flavoprotein beta subunit</td>
<td>3.3</td>
</tr>
<tr>
<td>B2421</td>
<td>cysM</td>
<td>cysteine synthase b, o-acetylserca sulphydrolase b</td>
<td>2.1</td>
</tr>
<tr>
<td>B2751</td>
<td>cysN</td>
<td>atp-sulfurylase (atp:sulfate adenylyltransferase), subunit 1, probably a gipase</td>
<td>2.3</td>
</tr>
<tr>
<td>B2425</td>
<td>cysP</td>
<td>thiosulfate binding protein</td>
<td>2.3</td>
</tr>
<tr>
<td>B2423</td>
<td>cysW</td>
<td>abc-type sulfate transport system, permease component</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>glutamate biosynthesis</strong></td>
<td></td>
</tr>
<tr>
<td>B3212</td>
<td>gltB</td>
<td>glutamate synthase, large subunit</td>
<td>5.2 4.7</td>
</tr>
<tr>
<td>B3213</td>
<td>gltD</td>
<td>glutamate synthase, small subunit</td>
<td>2.1 2.3</td>
</tr>
<tr>
<td>B3214</td>
<td>glf</td>
<td>regulator of gltD operon, induction of ntr enzymes</td>
<td>-22.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>phenylalanine biosynthesis</strong></td>
<td></td>
</tr>
<tr>
<td>B2599</td>
<td>pheA</td>
<td>chorismate mutase-p and prephenate dehydratase</td>
<td>9.4 19.2</td>
</tr>
</tbody>
</table>

116
This indicates that although amino acid uptake systems are compromised by UVA light, this seems not to affect all systems, and bacterial cells can still take up a certain amount of amino acids. LB medium that was used in our experiments contains yeast extract, which itself was shown to contain enough amino acids to protect E. coli E26 from oxygen toxicity (Boehm et al., 1976). Hence, our results probably indicate both, an inhibition of amino acid biosynthesis and an inhibition of amino acid uptake systems with subsequent over-expression of the biosynthesis genes. After 1 hour of irradiation the gene brnQ was downregulated by a factor of -2.2. It codes for a branched-chain amino acid transport system II carrier protein, corresponding to the Liv-II branched-chain amino acid transport system in E. coli, which has been shown to transport leucine, valine, and isoleucine. On the other hand livK, which codes for the periplasmic binding protein of the high-affinity leucine-specific transport system was upregulated by a factor of 3.1. In the adapted population though (after 50 hours), theses systems were not differentially expressed indicating that this response is only transient and perhaps secondary.

The pheA gene encodes two enzymes, chorismate mutase and prephenate dehydratase, both involved in phenylalanine biosynthesis. It was found that prephenate bound in the active site of chorismate mutase absorbs light around 340 nm (Roitberg et al., 2000). This might inactivate the enzyme and explains the strong upregulation during adaptation (IF1h = 9.4; IF50h = 19.2). Furthermore, histidine was shown to be sensitized in UVA exposed S. Typhimurium (Rahman et al., 1995). These authors suggest that histidine first reacts with singlet oxygen to produce an endoperoxide, which decomposes to a complex mixture of products, some of which may be genotoxic. The expression of the his operon of S. Typhimurium is governed by at least two independent mechanisms: I) an operon-specific "activator-attenuator" mechanism and, II) a mechanism involving ppGpp as a positive effector (Stephens et al., 1975; Venetianer, 1969). The same authors showed that an intracellular upshift of ppGpp concentration resulted in the induction of the his operon and proposed that ppGpp is a component of a sensing mechanism for adjusting the synthesis of histidine biosynthetic enzymes linking the need for histidine with the availability of all of...
the amino acids from the external environment. So far, the suggestion that this mechanism is similar for other amino acids has not been proven. Indeed, the contrary seems more likely because Chang and coworkers (Chang et al., 2002) measured a general downregulation of amino acid biosynthesis genes in *E. coli* during growth arrest after *H₂O₂* addition in batch culture. In this respect our results indicate that the upregulation of biosynthesis of isoleucine, leucine, valine, phenylalanine and glutamate is indeed important in the UVA light-triggered stress response and is not only a result of the stringent response. Also the role of histidine could be important in the UVA stress response because the *his* operon was still over-expressed in the adapted cell population (Table 6.2). So here, in contrast to "growth arrest" experiments we see an upregulation of amino acid biosynthesis genes despite the fact that the cells slowed down growth. Hence, this is a strong indication that it is a distinctively different stress response.

The NADH-dependent glutamate synthase (encoded by *gltB*: IFₜₕ = 5.2, IFₜₕₐ = 4.2 and *gltD*: IFₜₕ = 2.1, IFₜₕₐ = 2.3) converts L-glutamine and alpha-ketoglutarate into two L-glutamate molecules (Castano et al., 1988). L-glutamate is an essential amino acid in the assimilation of ammonia and for the biosynthesis of other amino acids (e.g., phenylalanine), for NAD biosynthesis or purine and pyrimidine *de novo* biosynthesis. Surprisingly, GltF, which is thought to be a regulator for the *gltBDF* operon, was downregulated 22-fold but the genes *gltB* and *gltD* were upregulated. However, it was shown that the expression of *gltB* and *gltD* are not directly regulated by GltF and that the function of GltF is still elusive (Goss et al., 2001). Our results rather suggest that GltF might be involved in the acid stress response. The induction of glutamate synthase after 1 hour as well as after 50 hours of irradiation (Table 6.2) indicates that glutamate is an important amino acid in UVA stress resistance and, therefore, glutamate decarboxylase (encoded by *gadA*, IFₚₒ = -14.9) might be repressed. Glutamate synthase together with glutamine synthetase (encoded by *glnA*, IFₜ = -3.2) is part of the high affinity ammonia assimilation pathway (GS-GOGAT) in *E. coli* (Reitzer, 2003). Its upregulation indicates cell internal N-limitation.
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Table 6.3. Selected *E. coli* genes involved in stress response, whose expression levels were increased by a factor ≥ 2 or decreased by a factor ≤ -2 in cultures exposed for either 1 or 50 hours to UVA light in "LB-limited" continuous culture at \( D = 0.5 \) h'. Genes were only listed if the p-value of the three replicates was ≤ 0.05.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Name</th>
<th>Product and function</th>
<th>Ind. Factor 1h</th>
<th>Ind. Factor 50h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dna damage inducible</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3645</td>
<td>dinD</td>
<td>dna-damage-inducible protein</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>B1061</td>
<td>dinI</td>
<td>damage-inducible protein</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>B2699</td>
<td>recA</td>
<td>dna strand exchange and renaturation, dna-dependent atpase, dna- and atp-dependent coprotease</td>
<td>3.1</td>
<td>6.7</td>
</tr>
<tr>
<td>B2616</td>
<td>recN</td>
<td>protein used in recombination and dna repair</td>
<td>5.7</td>
<td>6.8</td>
</tr>
<tr>
<td>B1863</td>
<td>ruvC</td>
<td>Holliday junction nuclease, resolution of structures, repair</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>B2009</td>
<td>sbmC</td>
<td>sbmC protein, Gene expression is increased at stationary phase and by treatment with compounds that cause DNA damage</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>B0958</td>
<td>sulA</td>
<td>suppressor of lon, inhibits cell division and ftsz ring formation</td>
<td>3.8</td>
<td>5.6</td>
</tr>
<tr>
<td>B1183</td>
<td>umuD</td>
<td>sos mutagenesis, error-prone repair, processed to umud, forms complex with umuc</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>B1848</td>
<td>yeBG</td>
<td>hypothetical protein; gene is part of SOS regulon (YeBG)</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td><strong>Cold/Heat shock</strong></td>
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<tr>
<td>B3556</td>
<td>cspA</td>
<td>cold shock protein 7.4, transcriptional activator of hns</td>
<td>-2.6</td>
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<tr>
<td>B1558</td>
<td>cspF</td>
<td>cold shock protein</td>
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<tr>
<td>B0990</td>
<td>cspG</td>
<td>homolog of salmonella cold shock protein</td>
<td>-3.4</td>
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<tr>
<td>B0015</td>
<td>dnaJ</td>
<td>chaperone with dnaK, heat shock protein</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>B1967</td>
<td>hchA</td>
<td>heat shock protein (Hsp) 31 (HchA)</td>
<td>-2.1</td>
<td></td>
</tr>
<tr>
<td>B3687</td>
<td>ibpA</td>
<td>heat shock protein</td>
<td>-2.2</td>
<td></td>
</tr>
<tr>
<td>B3686</td>
<td>ibpB</td>
<td>heat shock protein</td>
<td>-2.6</td>
<td>-2.2</td>
</tr>
<tr>
<td><strong>oxidative stress</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0605</td>
<td>ahpC</td>
<td>alkyl hydroperoxide reductase, c22 subunit, detoxification of hydroperoxides</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>B0606</td>
<td>ahpF</td>
<td>alkyl hydroperoxide reductase, f52a subunit, detoxification of hydroperoxides</td>
<td>2.3</td>
<td>4.2</td>
</tr>
<tr>
<td>B1732</td>
<td>katE</td>
<td>catalase, hydroperoxidase hpi(iii)</td>
<td>-2.3</td>
<td></td>
</tr>
<tr>
<td>B3908</td>
<td>sodA</td>
<td>superoxide dismutase, manganese</td>
<td>-2.7</td>
<td></td>
</tr>
<tr>
<td><strong>Stringent response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0029</td>
<td>ispH</td>
<td>control of stringent response, involved in penicillin tolerance</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>B3779</td>
<td>gppA</td>
<td>guanosine pentaphosphatase, exopolyporphatase, requires Mg2+</td>
<td>-2.4</td>
<td></td>
</tr>
<tr>
<td><strong>UV response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1895</td>
<td>uspC</td>
<td>universal stress protein with a role in resistance to UV irradiation</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td><strong>General stress response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2741</td>
<td>rpoS</td>
<td>rna polymerase, sigma s (sigma38) factor, synthesis of many growth phase related proteins</td>
<td>2.8</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6

Hence, inhibition of nitrogen-assimilation might be another reason for the upregulation of amino acid biosynthesis because nitrogen is an essential molecule in amino acids.

**Adaptation to stress**

Eisenstark (1989) proposed to divide the complex mechanism in *E. coli* for coping with UVA light into two parts: (1) detoxification of reactive molecules that result from photooxidation (reactive oxygen species) and (2) repair of DNA damage and re-synthesis of damaged tRNA. We suspect that this is not the whole story and that mechanisms like (3) the repair or the reaction to the inhibition of components like transport of amino acids (and perhaps other) is an additional aspect. The DNA damage caused by UVC radiation is known to induce a cellular protective response known as SOS response. The *recA* gene plays a central role in the regulation of the SOS response. Whether UVA radiation can also induce a similar SOS response in bacterial cells has been a subject of controversy. Neither monochromatic light (335nm or 365nm) nor broad-spectrum solar UV resulted in derepression of the *recA* promoter and subsequent SOS function in *E. coli* (Turner & Eisenstark, 1984). These authors proposed that the specific NUV-induced DNA lesions, and particularly single-strand DNA breaks, are unable to trigger the SOS response because they bind the RecA protein but fail to activate it. Others reported that the inducibility of the SOS response under NUV stress was 10-20 times higher in the *thiI* mutant than in the parent strain (*thiI*) of *E. coli* AB1157 (Caldeira de Araujo & Favre, 1986). These same authors suggested that the growth delay effect resulting from exposure to UVA light was actually responsible for reducing the SOS response. Similar results were obtained with *S. Typhimurium* TA 1535 containing multiple copies of plasmid psK1002 carrying a *umuC-lacZ* fusion gene (Rahman et al., 1995). Our results show a clear induction of the *recA* gene in both populations (*IF*$_{th} = 3.1$, *IF*$_{soh} = 6.7$) (Table 6.3). Accordingly, *recN* (*IF*$_{th} = 5.7$, *IF*$_{soh} = 6.8$) was upregulated at both time points. The induction of *dinD* (*IF*$_{soh} = 5.4$) and *dinI* (*IF*$_{soh} = 3.2$) genes in the adapted culture is another indication that the SOS response is involved in the UVA stress response. Both genes encode for DNA damage inducible proteins and are part of the SOS
Transcriptome analysis of *E. coli* during UVA adaptation

response in *E. coli* (Quillardet et al., 2003). Another expressed gene product of the SOS response in *E. coli* is SulA (IF$_{5h}$ = 3.8, IF$_{50h}$ = 5.6), a cell division inhibitor that inhibits septation by interacting with FtsZ, a component of the cell division apparatus (Huisman & D’Ari, 1981; Jones & Holland, 1985). In wild-type cells, SulA is very unstable. This normal instability of SulA permits the cell to resume cell division once the environmental stress has been alleviated (Trempy & Gottesman, 1989). In our previous paper we observed a cell size increase in UVA light-stressed *E. coli* cells, which was measured with flow cytometry (Berney et al., 2006b). Even the UVA light-adapted cells showed increased cell size although they had resumed cell division. This indicates that SulA does not entirely inhibit cell division under continuous UVA light-exposure, but it may transiently do so or lead to bigger cells when its concentration is higher.

There are some indications in our data supporting the hypothesis that the UVA inactivation mechanism includes oxidative stress. The *ahpCF* operon was upregulated especially in the adapted cells after 50 hours of irradiation (IF$_{ahpC}$ = 2.2, IF$_{ahpF}$ = 4.2). This operon encodes alkylhydroperoxidase reductase (Ahp) in *E. coli* as well as in *S. Typhimurium* (Jacobson et al., 1989). The enzyme converts lipid hydroperoxides and other alkyl hydroperoxides to the corresponding alcohols, using either NADH or NADPH as the reducing agent. It was shown that Ahp is induced by oxidative stress in *E. coli* and *S. Typhimurium* (Storz et al., 1989). Interestingly, hydroperoxidase II (KatE) was downregulated in adapted cells (IF$_{50h}$ = -2.2). Catalase is normally expressed under oxidative stress with H$_2$O$_2$ (Loewen & Triggs, 1984) but was shown to be directly inactivated by UVA light (Zigman et al., 1996). This is supported by the fact that *E. coli* mutants carrying an insertion in the *katE* or *katG* locus were not more sensitive to UVA light than the parent strain (Sammartano et al., 1986). The expression of the *katE* gene is regulated by RpoS (Visick & Clarke, 1997). RpoS, or σ$^7$, is a sigma subunit of RNA polymerase in *E. coli* that is induced in batch culture during transition from fast to slow growth (Hengge-Aronis, 2002) or in continuous culture as a function of specific growth rate (Ihssen & Egli, 2004). More than 400 genes are under RpoS control, many of them involved in responses to various environmental stresses (Weber et al., 2005). In our experiment the *rpoS* gene was upregulated during
growth inhibition at the start of irradiation (IF = 2.8). This was expected since intracellular RpoS concentration is dependent on specific growth rate and increases as specific growth rate decreases (Ihssen & Egli, 2004; Notley & Ferenci, 1996). The repression of katE in the UVA-adapted cell population might indicate a repression of rpoS. However, the regulation of RpoS levels in E. coli is very complex because it is also regulated at the translationary level (Hengge-Aronis, 2002). When comparing the list of differentially expressed genes in our experiments (appendix A) with the core group of 140 genes, which were reported to be $\sigma^A$-controlled under three different growth and stress conditions (Weber et al., 2005), only 13 of these genes were upregulated and one was downregulated after 1 hour of UVA irradiation. In the adapted cell population (50 hours) even 15 $\sigma^A$-controlled genes were downregulated. The control with RT-PCR showed an induction factor of -1.8 for the rpoS gene compared to -1.1 for the microarray. This indicates that the global stress regulator RpoS is repressed rather than induced in UVA light-adapted cells. Starved cultures of an E. coli rpoS-minus strain were more sensitive to UVA, NUV and sunlight (Berney et al., 2006a; Sammartano et al., 1986). Starved or slow growing cells, though, have a very low protein turnover and, therefore, might not be able to rapidly adjust to UVA stress (Berney et al., 2006b; Trempy & Gottesman, 1989). Hence, the general stress response initiated by RpoS before the entrance of the cells into stationary phase makes them more resistant to UVA light than the rpoS-minus cells by an unknown mechanism. However, in a culture growing under UVA light-stress the role of RpoS might be different form that in stationary phase cells. Among the downregulated genes under RpoS control in the adapted cell population, we found 12 genes that are involved in acid stress resistance (Table 6.4). These genes are crucial for acid resistance and include gadA, gadC, and the hde genes (as well as other less-well-characterized genes), together with their regulatory genes gadE, gadX, and gadW. The regulatory network of acid tolerance is large, which might reflect the importance of acid stress resistance in the live cycle of E. coli (Hommais et al., 2004). Table 6.4 is almost identical with the list of $\sigma^A$-controlled acid resistance genes published by Weber and coworkers (Weber et al., 2005). Surprisingly, these genes were clearly stationary-phase-induced in a $\sigma^A$-dependent manner but exhibited only a minor
or no σ8-dependence upon acidic shift (Weber et al., 2005). In our study these acid resistance genes showed no σ8-dependent upshift after 1 hour although the rpoS gene was upregulated (IF = 2.8). On the other hand, the same genes were strongly downregulated in the UVA-adapted cell population after 50 hours of irradiation although rpoS expression was not affected. The pH in the UVA light-irradiated bioreactors was 7.2 ± 0.1 after 50 hours while in the non-irradiated control bioreactor we measured pH = 7.6 ± 0.1. This are clearly not acidic conditions. Hommais and colleagues (Hommais et al., 2001) reported strong derepression of acid resistance genes in hns mutants. Nevertheless, in our experiments the induction factor of the hns gene was IF = 1 in both cases. One could speculate that the strong repression of acid tolerance genes seen in our experiment is a result from an upregulation in the control culture. However, no induction of acid tolerance genes was observed in cells from a glucose-limited culture having been cultivated under such conditions for 40 hours (Franchini & Egli, submitted). The GadE regulator was reported to be essential for the expression of gadA, gadBC, and the hde genes (Hommais et al., 2004) and, therefore, the high repression of the gadE gene is consistent with the observed repression of other acid stress-related genes.

Table 6.4. E. coli genes involved in acid stress response, whose expression levels were increased by a factor ≥ 2 or decreased by a factor ≤ -2 in cultures exposed for either 1 or 50 hours to UVA light in “LB-limited” continuous culture at D = 0.5 h\(^{-1}\). Genes were only listed if the p-value of the three replicates was ≤ 0.05.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Name</th>
<th>Product and function</th>
<th>Ind. Factor</th>
<th>1h</th>
<th>50h</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3517</td>
<td>gadA</td>
<td>glutamate decarboxylase isozyme</td>
<td>-14.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1492</td>
<td>gadC</td>
<td>acid sensitivity protein, putative transporter</td>
<td>-14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3512</td>
<td>gadE</td>
<td>GadE transcriptional activator, GadE overproduction causes resistance to low pH</td>
<td>-13.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3515</td>
<td>gadW</td>
<td>GadW transcriptional repressor; involved in acid resistance</td>
<td>-11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3516</td>
<td>gadX</td>
<td>GadX transcriptional activator (YhiX)</td>
<td>-6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3510</td>
<td>hdeA</td>
<td>acid-resistance periplasmic protein, possible chaperone (HdeA)</td>
<td>-8.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3509</td>
<td>hdeB</td>
<td>10K-L protein, related to acid resistance protein of Shigella flexneri</td>
<td>-6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3511</td>
<td>hdeD</td>
<td>protein involved in acid resistance (HdeD)</td>
<td>-7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3514</td>
<td>yhiUV multidrug transporter</td>
<td>component of YhiUV multidrug transporter</td>
<td>-2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3506</td>
<td>slp</td>
<td>outer membrane protein induced after carbon starvation, involved in the resistance</td>
<td>-5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>to low pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3508</td>
<td>yhiD</td>
<td>putative transport atpase</td>
<td>-2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3504</td>
<td>yhiS</td>
<td>orf, hypothetical protein</td>
<td>-6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3513</td>
<td>yhiU</td>
<td>subunit of YhiUV multidrug transporter</td>
<td>-6.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 6

**Nucleotide metabolism and tRNA synthesis**

In the experiments presented here we investigated the global regulatory profile of two different physiological states of *E. coli* growing in continuous culture under UVA irradiation. The first physiological state (after 1 hour of irradiation) shows strong similarities with the state of growth inhibition during batch culture exposure to NUV light. It has been shown that growth inhibition is largely due to absorption by 4-thiouridine (s4U) (Jagger, 1985) with the resultant production of a cross-link (Favre et al., 1985). These crosslinked tRNAs have been shown to be poor substrates for amino acid-charging and, therefore, the amino acid availability is lowered and causes a shut-off of net RNA synthesis, resulting in the so-called stringent response (Favre & Hajnsdorf, 1983; Ramabhadran & Jagger, 1976). The stringent response serves as a control mechanism that reduces the cellular protein synthesis capacity when substrates for protein synthesis get scarce (Wick & Egli, 2004). The stringent response is mediated by the alarmones pppGpp and ppGpp, the intracellular levels of which are regulated by the enzymes RelA, SpoT, Gpp and Ndk. All these genes were not significantly upregulated in our experiments. The primary characteristic of the stringent response is the decrease in stable RNA levels. Further effects of (p)ppGpp accumulation are the induction of RpoS, inhibition of active transport of several metabolites and especially the enhanced transcription of some amino acid biosynthesis enzymes (Wick & Egli, 2004). Several of these effects were also observed in our experiments whereas others were lacking. Transcription of the *rpoS* gene was induced (only after 1 hour), biosynthesis of several amino acids (see above) was enhanced and transport systems like the phosphate ABC transporter (encoded by the *pstABC* operon) were repressed (Appendix A). In fact, transcription of 25 genes that are associated with transport processes were downregulated compared to only 8 upregulated genes in this category. Also the *de novo* biosynthesis of purine, pyridine and pyrimidine ribonucleotides was strongly repressed (Table 6.5). Particularly, strong repression was observed for the genes of the pyrimidine ribonucleotide biosynthesis pathway *carA, carB, pyrB, pyrC, pyrD, pyrH, pyrI and pyrL* (Table 6.5) whereas the repression of purine biosynthesis was not as apparent. An almost identical result was found
by Chang et al. (2002) in their growth arrest experiment with *E. coli* triggered by the addition of \( \text{H}_2\text{O}_2 \). Others have shown that the *pyrBI* operon, which encodes aspartate carbamoyltransferase, was repressed during stringent response induced upon addition of a high concentration of valine (Turnbough, 1983). This author showed that ppGpp induction was very short and after a transient increase the molecule was degraded again. In our experiment the stringent response was probably induced right after starting irradiation and, therefore, induction of stringent response genes like *relA* and *spoT* was not observed in our analysis. What we did detect, though, were the consequences of the stringent response induction (see above). In the adapted cell population only the *pyrBI* operon was still repressed, indicating that this system was not necessary for the survival of the adapted cells.

### Table 6.5. *E. coli* genes involved in de novo biosynthesis of purine, pyridine and pyrimidine ribonucleotides, whose expression levels were increased by a factor \( \geq 2 \) or decreased by a factor \( \leq -2 \) in cultures exposed for either 1 or 50 hours to UVA light in continuous culture at \( D = 0.5 \text{ h}^{-1} \). Genes were only listed if the p-value of the three replicates was \( \leq 0.05 \).

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Name</th>
<th>Product and function</th>
<th>Ind. Factor 1h</th>
<th>Ind. Factor 50h</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0032</td>
<td><em>carA</em></td>
<td>carbamoyl-phosphate synthetase, glutamine (small) subunit</td>
<td>-17.6</td>
<td></td>
</tr>
<tr>
<td>B0033</td>
<td><em>carB</em></td>
<td>carbamoyl-phosphate synthase large subunit</td>
<td>-7.0</td>
<td></td>
</tr>
<tr>
<td>B2507</td>
<td><em>guaA</em></td>
<td>gmp (guanosine monophosphate) synthetase (glutamine-hydrolyzing)</td>
<td>-2.1</td>
<td></td>
</tr>
<tr>
<td>B2508</td>
<td><em>guaB</em></td>
<td>imp dehydrogenase (This is the first reaction unique to GMP biosynthesis)</td>
<td>-2.5</td>
<td></td>
</tr>
<tr>
<td>B0522</td>
<td><em>purK</em></td>
<td>phosphoribosylaminomimidazole carboxylase = air carboxylase, co(2)-fixing subunit</td>
<td>-2.2</td>
<td></td>
</tr>
<tr>
<td>B4245</td>
<td><em>pyrB</em></td>
<td>aspartate carbamoyltransferase, catalytic subunit</td>
<td>-28.2</td>
<td>-6.1</td>
</tr>
<tr>
<td>B1062</td>
<td><em>pyrC</em></td>
<td>dihydro- orotase</td>
<td>-4.6</td>
<td>-2.3</td>
</tr>
<tr>
<td>B0945</td>
<td><em>pyrD</em></td>
<td>dihydro- orotate dehydrogenase</td>
<td>-6.6</td>
<td></td>
</tr>
<tr>
<td>B0171</td>
<td><em>pyrH</em></td>
<td>uridylate kinase</td>
<td>-2.0</td>
<td></td>
</tr>
<tr>
<td>B4244</td>
<td><em>pyrI</em></td>
<td>aspartate carbamoyltransferase, regulatory subunit</td>
<td>-20.7</td>
<td>-18.9</td>
</tr>
<tr>
<td>B4246</td>
<td><em>pyrL</em></td>
<td>pyrbi operon leader peptide</td>
<td>-4.4</td>
<td>-4.5</td>
</tr>
<tr>
<td>B2498</td>
<td><em>upp</em></td>
<td>uracil phosphoribosyltransferase</td>
<td>-2.9</td>
<td></td>
</tr>
</tbody>
</table>

The expression of isoleucine (*ileS*), phenylalanine (*pheS*), hisitidine (*hisS*) and valine tRNA synthase (*valS*) genes was also upregulated after 1 hour of irradiation (Table 6.6). Interestingly, only the phenylalanine tRNA synthase (*pheS*) gene was still upregulated in the adapted cells. In fact, Blondel and Favre (1988) showed that most tRNA species were slightly affected by UVA light but
only those acylated by Phe and Pro were strongly affected in vivo. Both acylation levels decreased to less than 10% of their initial value during the illumination period, remained stable all along the growth lag and increased with cell mass when growth resumed. Hence, tRNA(Phe) and tRNA(Pro) are the UVA light molecular targets triggering growth delay and possibly also photoprotection and protection against UVA mutagenesis. Kramer and coworkers (1988) came to a similar conclusion stating that the inactivation of tRNA could also mediate a stringency-independent stress response possibly via adenylated dinucleotides but that also a direct interaction of crosslinked tRNA on the transcription level seems to be possible. Our results strongly support this idea, since many genes induced during growth inhibition seem to be stringency-independent. Recently, it was found that the biosynthesis of 4-thiouridine (s4U) in E. coli tRNA requires the action of both the thiamine pathway enzyme Thil and the cysteine desulfurase IscS. IscS catalyzes sulfur transfer from L-cysteine to Thil, which utilizes Mg-ATP to activate uridine 8 in tRNA and transfers a sulfur group to give s4U (Lauhon et al., 2004). In our experiments the thil and iscS genes were neither differentially expressed after 1 hour nor after 50 hours. This indicates that the adapted cells are not thil mutants like those that were isolated by others (Kramer et al., 1988; Ramabhadran et al., 1976).

Table 6.6. E. coli genes involved in tRNA synthesis, whose expression levels were increased by a factor ≥ 2 or decreased by a factor ≤ -2 in cultures exposed for either 1 or 50 hours to UVA light in "LB-limited" continuous culture at D = 0.5 h⁻¹. Genes were only listed if the p-value of the three replicates was ≤ 0.05.

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Name</th>
<th>Product and function</th>
<th>Ind. Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>B0026</td>
<td>ileS</td>
<td>isoleucine tRNA synthetase</td>
<td>2.1</td>
</tr>
<tr>
<td>B1714</td>
<td>pheS</td>
<td>phenylalanine tRNA synthetase, alpha-subunit</td>
<td>2.5 5.4</td>
</tr>
<tr>
<td>B2114</td>
<td>metG</td>
<td>methionine tRNA synthetase</td>
<td>2.0</td>
</tr>
<tr>
<td>B2518</td>
<td>hisS</td>
<td>histidine tRNA synthetase</td>
<td>4.2</td>
</tr>
<tr>
<td>B2791</td>
<td>truC</td>
<td>tRNA pseudouridinc 65 synthase</td>
<td>-2.4</td>
</tr>
<tr>
<td>B3166</td>
<td>truB</td>
<td>tRNA pseudouridinc 5s synthase</td>
<td>2.6</td>
</tr>
<tr>
<td>B4258</td>
<td>valS</td>
<td>valine tRNA synthetase</td>
<td>2.5</td>
</tr>
<tr>
<td>B2392</td>
<td>trmD</td>
<td>tRNA methyltransferase, tRNA (guanine-7-)-methyltransferase</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Interestingly, the gene mgtA encoding for an ATP dependent Mg\textsuperscript{2+} transporter was strongly repressed by a factor of -16.4 in the adapted cells.

Concluding remarks

Protein turnover serves as a regulatory strategy for cells during periods of changing environmental conditions or in cases of physiological emergencies. When a crisis is encountered, the cell may require the rapid synthesis or elimination of specific gene products to allow survival. *E. coli* grown in continuous culture and exposed to UVA irradiation exhibits a growth inhibition with a subsequent adaptation to UVA stress. This study is the first report that uses transcriptome analysis to characterize global gene expression in *E. coli* under continuous UVA irradiation. Our analysis suggests that the adapted cells are not *thil* mutants like they were isolated by others (Kramer *et al.*, 1988; Ramabhadran *et al.*, 1976). It was shown that indeed the stringent response is initiated, most likely via the crosslinking and inhibition of 4-thiouridine and cytidine in tRNA, but that also an additional stress response is induced including the upregulation of amino acid biosyntheses of valine, isoleucine, leucine, phenylalanine, histidine and glutamate. This induction might be due to direct inhibition of enzymes involved in the uptake or/biosynthesis of these amino acid or results from the inhibition of N-assimilation. The reason for the enhanced biosynthesis of these amino acids can now be further investigated. Furthermore, our results corroborate earlier reports about the induction of the SOS response in UVA-irradiated cells. The induction of genes in Table 6.3, as for example recA, recN, dinD, dinI and umuD, strongly point to DNA damage as a result from UVA light exposure. Also the involvement of oxidative stress was confirmed with the induction of alkylhydroperoxidase reductase (Ahp). In the UVA light-adapted cell population we found a strong repression of the acid tolerance response. This result might imply that the cells are shutting down unneeded biosynthesis (e.g., acid resistance proteins) and can lead us to a yet unknown survival mechanism in UVA light-adapted cells. Furthermore, the role of the global stress regulator RpoS may not be as important in the adaptation *E. coli* to UVA light-stress as it was implicated by previous results with starved cells,
but it might be important for the resistance under transient conditions during exposure. The role of certain enzymes as sites of direct damage or for essential protective function (e.g., PheA, MgtA, PyrBl) can now be further investigated to elucidate their role in the stress response of *E. coli* to UVA light. Also a comparison of the transcription profile with the proteome pattern of adapted cells and the specific measurement of RpoS and ppGpp concentrations during adaptation could give us new details about the inactivation mechanism of UVA light in enteric bacteria.

**Acknowledgments**

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<table>
<thead>
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<th>Gene #</th>
<th>Name</th>
<th>Product and function</th>
<th>Ind. Factor</th>
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<tr>
<td></td>
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Amino acid metabolism

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<td>3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (dahp synthetase, phenylalanine repressible)</td>
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<td>B3433</td>
<td>asd</td>
<td>aspartate-semialdehyde dehydrogenase</td>
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<td>asparagine synthetase b</td>
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<td>sulfite reductase, alpha subunit</td>
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<td>cysJ</td>
<td>sulfite reductase (nadph), flavoprotein beta subunit</td>
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<td>cysM</td>
<td>cysteine synthase b, o-acetylserine sulfhydrolase b</td>
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<td>B2024</td>
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<td>n-(5'-phospho-L-ribosyl-formiminoo)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide isomerase</td>
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<td>hisB</td>
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<td>hisC</td>
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<td>B2020</td>
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<td>l-histidinol:nad+ oxidoreductase, l-histidinol:nad+ oxidoreductase</td>
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<td>B2025</td>
<td>hisF</td>
<td>imidazole glycerol phosphate synthase subunit in heterodimer with hisf = imidazole glycerol phosphate synthase holoenzyme</td>
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<td>glutamine amidotransferase subunit of heterodimer with hisf = imidazole glycerol phosphate synthase holoenzyme</td>
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<td>hisI</td>
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<td>ilve</td>
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<td>B2599</td>
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<td>selD</td>
<td>selenophosphate synthase, h(2)se added to acrylyl-trna</td>
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<td>subunit of agmatinase</td>
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<td>B1263</td>
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<td>anthranilate synthase component ii, glutamine amidotransferase and phosphoribosylanthranilate transferase</td>
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**Transport**

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<td>alsA</td>
<td>subunit of D-allose ABC transporter, AlsA is the ATP-binding component</td>
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<td>brnQ</td>
<td>branched chain amino acid transport system ii carrier protein, probably corresponds to the Liv-II branched chain amino acid transport system in <em>E. coli</em>, which has been shown to transport <strong>leucine, valine, and isoleucine</strong></td>
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<td>B4132</td>
<td>cadB</td>
<td>transport of lysine/cadaverine</td>
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<td>codB</td>
<td>cytosine permease/transport</td>
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<td>B3336</td>
<td>corA</td>
<td>mg2+ transport, system i</td>
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<td>ctsA</td>
<td>peptide transporter induced by carbon starvation</td>
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<td>cysW</td>
<td>abc-type sulfate transport system, permease component</td>
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<td>dctA</td>
<td>uptake of c4-dicarboxylic acids</td>
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<td>exbD</td>
<td>uptake of enterochelin, tonb-dependent uptake of b colicins</td>
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<td>gabP</td>
<td>transport permease protein of gamma-aminobutyrate</td>
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<td>B1492</td>
<td>gacC</td>
<td>acid sensitivity protein, putative transporter</td>
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<td>gntX</td>
<td>glucosamine periplasmic binding protein</td>
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<td>predicted membrane-bound protein that is involved in high-affinity gluconate transport</td>
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<td>livK</td>
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<td>Component of: YhiUV multidrug transporter</td>
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<td>mgtA</td>
<td>mg2+ transport atpase, p-type 1, Transcription is regulated by Mg2+</td>
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<td>mscS</td>
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<td>anaerobically inducible l-threonine, l-serine permease, TdeC is a member of the STP family of amino acid transporters</td>
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<td>ugpA</td>
<td>sn-glycerol 3-phosphate transport system, integral membrane protein</td>
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<td>ugpE</td>
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<td>uracil transport</td>
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<td>periplasmic vitamin B12 binding protein (YadT)</td>
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Transcriptome analysis of *E. coli* during UVA adaptation

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<td><em>ybeX</em></td>
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<td>B0841</td>
<td><em>ybfG</em></td>
<td>putative permease (YbfG)</td>
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<td>YeiO is a probable efflux transporter for sugars such as lactose and IPTG</td>
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<td>B2258</td>
<td><em>yfbJ</em></td>
<td>putative transport/receptor protein (same operon as amnT)</td>
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<td><em>yfhD</em></td>
<td>putative periplasmic binding transport protein</td>
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<td><em>ygaY</em></td>
<td>MFS transporter (major facilitator superfamily)</td>
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<td>YgcZ probably functions as a glucarate/proton transporter.</td>
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<td>YhaO STP transporter</td>
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<td>YifK APC transporter, putative amino acid/amine transport protein</td>
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<td>Ynal is a putative member of the small mechanosensitive ion channel (MscS) family.</td>
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<td><em>yffR</em></td>
<td>putative ATP-binding component of a transport system</td>
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**Cell structure**

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<td>B1903</td>
<td><em>fbbG</em></td>
<td>3-oxoacyl-[acyl-carrier-protein] reductase</td>
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<td>B4317</td>
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<td>outer membrane protein, export and assembly of type 1 fimbriae, interrupted</td>
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<td><em>flhE</em></td>
<td>flagellar protein</td>
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<td>flagellar biosynthesis</td>
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<td><em>fsbE</em></td>
<td>ATP-binding component of a membrane-associated complex involved in cell division</td>
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<td>involved in lipopolysaccharide biosynthesis</td>
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<td>proton conductor component of motor, no effect on switching</td>
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<td>rod shape-determining membrane protein, sensitivity to radiation and drugs, cell</td>
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<td>udp-n-acetylenolpyruvoylglucosamine reductase</td>
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<td><em>ompC</em></td>
<td>outer membrane porin protein, locus of qsr prophage</td>
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<td>outer membrane protein 1b (ib,c), OmpC is a member of the GMP family.</td>
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<td>heptosyl transferase 1, lipopolysaccharide core biosynthesis</td>
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<td>protein export - membrane protein</td>
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### Chapter 6

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<td><em>tatC</em></td>
<td>TatC, subunit of TatABCE protein export complex</td>
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<td>B0739</td>
<td><em>tolA</em></td>
<td>membrane spanning protein, required for outer membrane integrity</td>
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<td><em>wbbH</em></td>
<td>o-antigen polymerase</td>
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<td><em>ycO</em></td>
<td>putative membrane protein with possible relationship to novobiocin and deoxycholate resistance (YicO)</td>
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<td><em>ygX</em></td>
<td>KpLE2 phage-like element; putative transmembrane protein</td>
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<td>B4364</td>
<td><em>yjIP</em></td>
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#### Energy and carbon metabolism

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<tr>
<td>B4016</td>
<td><em>aceK</em></td>
<td>isocitrate dehydrogenase kinase/phosphatase</td>
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<tr>
<td>B0118</td>
<td><em>acaB</em></td>
<td>aconitate hydrase b</td>
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<tr>
<td>B0980</td>
<td><em>appA</em></td>
<td>phosphoanhydride phosphorlyase, pH 2.5 acid phosphatase, periplasmic</td>
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<td>B0979</td>
<td><em>appB</em></td>
<td>cytochrome bd-II terminal oxidase subunit II = AppB</td>
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<tr>
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<td><em>appC</em></td>
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<td>cytochrome d terminal oxidase polypeptide subunit ii</td>
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<td><em>edd</em></td>
<td>6-phosphogluconate dehydratase</td>
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<td>B2779</td>
<td><em>eno</em></td>
<td>enolase, Products: Eno, subunit of degradosome. The degradosome is a large, multiprotein complex involved in RNA degradation</td>
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<td><em>fadB</em></td>
<td>4-enzyme protein: 3-hydroxyacyl-coa dehydrogenase, 3-hydroxybutyryl-coa epimerase, delta(3)-cis-delta(2)-trans-enoyl-coa isomerase, enoyl-coa hydratase</td>
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<td><em>folL</em></td>
<td>formate dehydrogenase, cytochrome b556 (fdo) subunit</td>
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<td>fructose-1-phosphate kinase</td>
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<td>glyceraldehyde-3-phosphate dehydrogenase a</td>
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<td>maltodextrin phosphorylase</td>
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### Transcriptome analysis of *E. coli* during UVA adaptation

**Appendix A continued**

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<th>Ind. Factor 50h</th>
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<td>B3176</td>
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<td></td>
<td></td>
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<td>B2278</td>
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<td>B1020</td>
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<td><em>sdhB</em></td>
<td>succinate dehydrogenase, iron sulfur protein</td>
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<td><em>sfaA</em></td>
<td>Subunit of soluble pyridine nucleotide transhydrogenase: SfA</td>
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<td><em>sucA</em></td>
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<td><em>yagH</em></td>
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<td><em>yghE</em></td>
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### Nucleotide metabolism

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<td>apt</td>
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<td>B0032</td>
<td>carA</td>
<td>carbamoyl-phosphate synthetase, glutamine (small) subunit</td>
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<td>B0033</td>
<td>carB</td>
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<td>B3162</td>
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<td>B0849</td>
<td>grxA</td>
<td>glutaredoxin I redox coenzyme for glutathione-dependent ribonucleotide reductase</td>
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<td>guaA</td>
<td>gmp (guanosine monophosphate) synthetase (glutamine-hydrolyzing)</td>
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<td>B2508</td>
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<td>mcrC</td>
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### Protein processing

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<td>dsbC</td>
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<td>eco</td>
<td>ecotin, a serine protease inhibitor</td>
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<td>folA</td>
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Transcriptome analysis of *E. coli* during UVA adaptation

<table>
<thead>
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<td>plays structural role in maturation of all 3 hydrogenases</td>
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<td>lipoil-protein ligase LipB</td>
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<td>aminopeptidase a/i</td>
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<td>b4021</td>
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<td>peptidase e, a dipeptidase where amino-terminal residue is aspartate</td>
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<td><em>rplT</em></td>
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<td><em>rpsC</em></td>
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<td><em>rpsO</em></td>
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<td>l-serine dehydratase (deaminase), l-sd2</td>
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<td><em>sohB</em></td>
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<td><em>yfdl</em></td>
<td>putative ligase, the OxyR protein interacts with a site within the yfdl gene</td>
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<td><em>ygdD</em></td>
<td>putative o-sialoglycoprotein endopeptidase, the protein is essential for growth</td>
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<td><em>yjhH</em></td>
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### Adaptation to stresses

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<td><em>dinI</em></td>
<td>damage-inducible protein i</td>
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<td>B1183</td>
<td><em>umuD</em></td>
<td>sos mutagenesis, error-prone repair, processed to umud', forms complex with umuc</td>
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<td>alkyl hydroperoxide reductase, c22 subunit, detoxification of hydroperoxides</td>
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<td>alkyl hydroperoxide reductase, f52a subunit, detoxification of hydroperoxides</td>
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<td>nad+-dependent betaine aldehyde dehydrogenase, glycine betaine biosynthesis l, osmoprotectant</td>
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<td><em>cbpA</em></td>
<td>curved dna-binding protein functions closely related to dnaJ</td>
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<td>B0999</td>
<td><em>cbpM</em></td>
<td>purified CbpM protein specifically inhibits both the DNA binding and co-chaperone activity of CbpA</td>
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<td><em>clpS</em></td>
<td>specificity factor for ClpA-ClpP chaperone-protease complex</td>
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<td>B3556</td>
<td><em>cspA</em></td>
<td>cold shock protein 7.4, transcriptional activator of hns</td>
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<td><em>cspF</em></td>
<td>cold shock protein</td>
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<td>homolog of salmonella cold shock protein</td>
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<td>B3779</td>
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<td>guanosine pentaphosphatase, exopolyporphosphate, requires Mg2+</td>
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<td><em>hchA</em></td>
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<td>acid-resistance protein, possible chaperone (HdeA). HdeA is a periplasmic protein that plays a role in resistance to low pH, which is involved in pathogenesis</td>
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<td>B3511</td>
<td>hdeD</td>
<td>protein involved in acid resistance (HdeD)</td>
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<td>ibpA</td>
<td>heat shock protein</td>
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<td>B3686</td>
<td>ibpB</td>
<td>heat shock protein</td>
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<td>B0029</td>
<td>ispH</td>
<td>control of stringent response, involved in penicillin tolerance</td>
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<td>katE</td>
<td>catalase, hydperoxidase hpi(iii)</td>
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<td>peptidyl-prolyl cis-trans isomerase. PpiD activity is a chaperone required for wild-type outer membrane protein folding</td>
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<td>dna strand exchange and renaturation, dna-dependent apase, dna- and atp-dependent coprotease</td>
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<td>protein used in recombination and dna repair</td>
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<td>Holliday junction nuclease, resolution of structures, repair</td>
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<td>sbmC</td>
<td>sbmc protein, Gene expression is increased at stationary phase and by treatment with compounds that cause DNA damage</td>
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<td>B3908</td>
<td>sodA</td>
<td>superoxide dismutase, manganese</td>
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<td>B3862</td>
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<tr>
<td>B1895</td>
<td>uspC</td>
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### Regulatory functions

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<td>allR</td>
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<td>B4401</td>
<td>arcA</td>
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<td>B1422</td>
<td>b1422</td>
<td>putative transcriptional regulator lyst-type</td>
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<td>B4366</td>
<td>bglI</td>
<td>2-component transcriptional regulator</td>
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<td>B0034</td>
<td>caiF</td>
<td>transcriptional regulator of cai operon</td>
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<td>B2143</td>
<td>cdl</td>
<td>cytidine/deoxycytidine deaminase</td>
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<td>dgoR</td>
<td>regulator protein for dgo operon</td>
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<td>d-serine dehydratase (deaminase) transcriptional activator</td>
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<td>B4293</td>
<td>fecI</td>
<td>sigma19 factor , subunit of RNA Polymerase sigma19</td>
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<td>B4292</td>
<td>fecR</td>
<td>regulator for fec operon, periplasmic</td>
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<td>B3898</td>
<td>frxX</td>
<td>frx operon protein</td>
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<td>B3512</td>
<td>gadE</td>
<td>GadE transcriptional activator, GadE overproduction causes resistance to low pH</td>
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<td>B3515</td>
<td>gadW</td>
<td>GadW transcriptional repressor, involved in acid resistance</td>
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<td>B3516</td>
<td>gadX</td>
<td>GadX transcriptional activator (YhiX)</td>
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<td>B3214</td>
<td>glf</td>
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<tr>
<td>B2706</td>
<td>gutM</td>
<td>glucitol operon activator</td>
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Transcriptome analysis of *E. coli* during UVA adaptation

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**Miscellaneous**

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<td>gidB</td>
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<td>B0773</td>
<td>yhhB</td>
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<td>ycdO</td>
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<td>yeaG</td>
<td>orf, hypothetical protein, The yeaG gene of Salmonella enterica serovar Typhimurium is regulated by sigma S (RpoS)</td>
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<td>Putative competence-damage protein</td>
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## Transcriptome analysis of *E. coli* during UVA adaptation

### Appendix A continued

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<th>Ind. Factor 50h</th>
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<td><em>ygaU</em></td>
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<td><em>yijO</em></td>
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<td>B3848</td>
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<td>B3889</td>
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<td>B4243</td>
<td><em>yjGF</em></td>
<td>YjGF homotrimer</td>
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<td>B1330</td>
<td><em>ynal</em></td>
<td>Ynal (polypeptide)</td>
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<td>B1448</td>
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<td>B2646</td>
<td><em>yphF</em></td>
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**Appendix A** *E. coli* genes whose expression levels were increased by a factor ≥ 2 or decreased by a factor ≤ -2 in cultures exposed for either 1 or 50 hours to UVA light in “LB-limited” continuous culture at D = 0.5 h⁻¹. Genes were only listed if the p-value of the three replicates was ≤ 0.05.
### Appendix B: Primers used in the Real Time PCR experiments.

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<td></td>
<td>K1 rev</td>
<td>5'-ACCCACTCCCCATGGGTGGTA-3'</td>
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<td>katE</td>
<td>K2 fw</td>
<td>5'-TCCCTTGCGCAATATCG-3'</td>
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<td>K2 rev</td>
<td>5'-AAGGTGTGTTGTTAGGCTTCCATCAG-3'</td>
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<td>5'-CCGGAGCTGGTGCCCAA-3'</td>
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<td>rtrpoS rev</td>
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<td>rtkatG rev</td>
<td>5'-CAGTGACTCGGTGGTGGAAAC-3'</td>
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<td>rtxthA rev</td>
<td>5'-ATGCAGACCCAGGTTATC-3'</td>
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7. **Implications for the SODIS method and outlook**

The missing puzzle pieces

The SODIS method has a high potential of bringing relief to people in regions where the access to safe drinking water is not self-evident. In the last 40 years many field and laboratory studies showed the efficacy and applicability of solar disinfection not only for the inactivation of bacteria but also of protozoa, fungi and viruses (Joyce et al., 1996; Lonnen et al., 2005; Wegelin et al., 1994). Browsing through the literature, it quickly becomes obvious that comparison of results among different SODIS studies is hampered by the many different methods used and particularly also the lack of information given by researchers on the cultivation, preparation and exposure of test bacteria. However, as shown in this work, depending on growth stage, bacteria can exhibit very different stress sensitivity. The stress response of bacteria is also greatly influenced by the buffer medium and the availability of substrate during the disinfection process, a fact, which has not yet been thoroughly investigated for the SODIS method.

The mechanistic view of the solar disinfection process has been adopted mainly from studies concerning the reaction of fecal indicator bacteria *E. coli* or *S. Typhimurium* to UVA irradiation (of different wavelength distributions) under laboratory conditions. Taken together, these studies imply that solar UV and visible light cause indirect damage to DNA, proteins and other cellular structures via the production of reactive oxygen species (ROS). Also the inhibition of several intracellular (e.g., catalase) or membrane-bound structures (e.g., ubiquinone) have been proposed (Jagger, 1985). Injury is believed to be either of lethal or sublethal nature but definitions of these expressions are manifold, not very precise and depend on the ability of the cells to repair damages (Eisenstark, 1989; Jagger, 1985). Especially the presence or absence of substrate could have an immense influence on repair or adaptive capabilities of the cells.
The stress response of SODIS treated bacteria is not well characterized and it is not known under which circumstances it turns out to be lethal or sublethal for the bacteria.

There is a general consensus that effective solar disinfection requires around 3-5 hours of strong sunlight at an intensity above 500 Wm\(^{-2}\) (Oates et al., 2002). This recommendation, though, is based on the classical cultivation on solid agar substrates. In recent years increasing concern about the validity of such methods arose, because it was shown that they can lead to an overestimation of effectiveness of disinfection processes. Also, traditional plate counting does not give us a coherent answer about the physiological state of the bacteria (e.g. alive, dead, dormant, viable but non-culturable) during solar disinfection. For a conclusive interpretation other approaches had to be chosen.

**Preparation of the test organism in SODIS experiments**

Commonly, the fecal indicator bacterium *E. coli* was used as a test organism for solar disinfection studies. In most studies the bacteria were grown in batch culture with complex media, harvested at a certain optical density, washed subsequently and diluted into buffers like PBS, M9 or HPLC grade water before exposure. It is a long standing knowledge that bacteria growing in batch culture on complex media go through various physiological states from the starting point of the culture until all utilizable substrate is consumed. The sensitivity to various stresses (e.g., UVC, chlorine) has been shown to be different for cells in different physiological states and, therefore, it is an absolute necessity to clearly describe the physiological state of the cells tested in disinfection studies (Gourmelon et al., 1997; Lisle et al., 1998; Morton & Haynes, 1969; Reed, 1997). In this thesis we showed that sensitivity of *E. coli* to mild heat and artificial sunlight is a direct function of the specific growth rate of the cells when harvested before exposure. We show evidence that this dependence is linked to intracellular RpoS concentration, a global stress regulator found in several enteric bacteria. Furthermore, we found the shape of inactivation curves to be very variable and demonstrated that growing cells undergo a time-
Implications for the SODIS method and outlook

dependent physiological change when washed and diluted in buffer solution, resulting in a change of their sensitivity to UVA light stress. Hence, our results suggest that specific growth rates of the test bacteria and the time and procedure from harvesting until exposure should always be clearly stated in solar disinfection studies. To make things simple, we recommend using only stationary bacteria because we demonstrated for E. coli that this is (so far) the most resistant state regarding solar disinfection.

Efficacy of SODIS in enteric pathogens

Although solar disinfection is a recommended and regionally used drinking water treatment method, experimental data about its effect on pathogenic bacteria in drinking water are still scarce. The data available often lack precise description of cell preparation (see above) and, thus, shows very different inactivation efficacies. Also the use of E. coli as an indicator organism for SODIS efficacy is not cast in stone because the inactivation mechanism is not yet well characterized. Our study clearly showed that among the enteric strains tested S. Typhimurium is the most resistant strain (order of decreasing resistance: S. Typhimurium > E. coli > S. flexneri > V. cholerae). This might be due to different ways or abilities to deal with this stress and suggests that E. coli is not necessarily the best indicator bacterium for testing solar disinfection. Hence, analysis of S. Typhimurium’s stress response and a comparison with e.g. V. cholerae could lead to a better understanding of the inactivation mechanism(s). Our study also emphasizes that extrapolation from T_{90} or F_{90} values (Time or Fluence needed to reduce plate counts by 90%) are difficult and that these values are inadequate for comparing results from different studies. It is recommended to always supply the original data in the form of inactivation curves. Furthermore, our data suggests that the mild heat effect should not be neglected in SODIS experiments, since a very small increase in temperature can strongly increase SODIS effectiveness for certain strains (here documented particularly for V. cholerae). Therefore, all possible and available measures should be taken to increase water temperatures in the bottles during SODIS.
Chapter 7

**Process of injury and death**

In addition to the classic plating method we used viability stains combined with flow cytometry to follow vital cellular functions in *E. coli* during SODIS. This helped us to better understand the process of injury and death of bacteria that are treated with solar disinfection and enabled use to determine the light-dose needed for (probably) lethal injury of enteric bacteria to occur.

An UVA light dose (fluence) of less than 500 kJ/m² was enough to lower the proton motive force such that efflux pump activity and ATP synthesis decreased significantly. Such cells, though, were still able to grow on pyruvate supplemented agar. The loss of membrane potential, glucose uptake activity and culturability of more than 95% of the cells was observed at a solar UVA fluence of more than 1700 kJ/m² (corresponding to 550 W/m² global sunlight intensity for 6 h). These cells were unable to recover right after treatment as well as 4 days later, indicating that injury was lethal. The highest certainty about death, though, will be achieved if fluences are applied that lead to membrane permeability (≥ 2500 kJ/m²). This corresponds to about 600-880 W/m² of global sunlight intensity over a period of 6 hours, a threshold which can be achieved in most developing countries. Recently, average sunlight intensities between 700 and 828 W/m² over a period of 8 hours starting at 8 o’clock in the morning were measured all year round in a study on the efficiency of solar disinfection in Mexico (Martin-Dominguez et al., 2005). 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. It remains to be clarified, if other enteric bacteria, especially *S. Typhimurium*, are inactivated with a similar pattern.

**Can enteric bacteria adapt to sunlight stress during SODIS or in the environment?**

Sunlight (and especially solar UVA light) is one of the most common environmental stresses that microorganisms are subjected to. Enteric bacteria
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like *E. coli* or *Salmonella* are exposed to solar irradiation during transmission between hosts, and the inimical effect of sunlight on enteric bacteria is also utilized for solar disinfection. Hence, the adaptive capabilities of these organisms to sunlight are of high interest. In the environment carbon and energy sources are scarce and it is not clear under which circumstances enteric bacteria are able to multiply on the available nutrients. Many studies about the fate of enteric bacteria in aquatic environments and the physical, chemical and biological factors affecting their survival in such habitats have been conducted (Bogosian et al., 1996; Davies & Evison, 1991; Rozen & Belkin, 2001; Santo Domingo et al., 2000). It is generally believed that enteric bacteria may survive in natural waters but are not able to multiply. Recently, it was shown that populations of fecal coliforms and enterococci did not grow in freshwater mesocosms (Anderson et al., 2005). Our study showed that with a constant flux of substrate *E. coli* is able to adapt and grow in continuous culture under continuous irradiation with artificial sunlight at dilution rates above 0.3 h⁻¹. In contrast, slow-growing cells (D=0.05 h⁻¹) were unable to induce enough protection capacity to maintain growth under UVA irradiation. This suggests that faster growing *E. coli* cells have a higher adaptive flexibility to UVA light-stress than slow growing cells, which is in line with the knowledge that fast growing cells have increased ability to induce *de novo* protein synthesis in times of sudden stress (Wick & Egli, 2004). In natural aquatic environments carbon availability (assimilable organic carbon, AOC) and temperatures are much lower and, therefore, the expected growth rates of enteric bacteria are probably below 0.1 h⁻¹ (Ihssen, 2005). Even in the colon, *E. coli*’s primary habitat, specific growth rates of 0.3 h⁻¹ are very unlikely (Ihssen, 2005). Therefore, it is unlikely that the observed adaptation of *E. coli* to UVA light does occur in a natural aquatic environment. In SODIS experiments, a defined amount of water is filled into a bottle and no additional flux of substrates is possible. Under these circumstances an adaptation is unlikely to take place but with the right amount of substrates enteric bacteria might be able to repair damages for a certain time period. In our SODIS experiments with starved cells (see above) we used bottled mineral water (EVIAN) as buffer medium. The level of AOC in EVIAN is very low, at least 10 times lower than in natural waters. This is because available AOC is
used up right after bottling by a natural consortium (Leclerc & Moreau, 2002). The only carbon source left for repair and recovery would originate from dead cells that disintegrate or from polymeric organic carbon that is broken down to AOC during exposure. In water collected from natural sources, the consumption of AOC may take up to half a day for the natural consortium (personal communication F. Hammes, Eawag). However, AOC concentrations in natural waters vary with time of the day. A peak can be expected after midday when photosynthetic activity is highest (Munster, 1993). The same author suggests that the collection time of the water could be relevant as well. Commonly though, water is collected in the morning when AOC is low, in order to catch a full day of exposure. Nevertheless, the influence of AOC on the sensitivity and adaptive flexibility of enteric bacteria in SODIS experiments has to be further investigated.

**Inactivation mechanism, lethal or sublethal injury?**

The inactivation mechanism of SODIS is still open to debate. Our results from gene array analysis of artificial sunlight-adapted cells corroborate earlier reports about the induction of the SOS response in UVA-irradiated cells and the involvement of oxidative stress. Taken together it supports the hypothesis of the production of ROS by UVA light, which can damage DNA, proteins or other life supporting cell structures. But also direct inhibition of enzymes and important cellular functions (e.g. electron transport chain) could lead to lethality. We see indications for inhibition of amino acid uptake and biosynthesis, of specific enzymes like chorismate mutase, and our flow cytometric analysis showed that functions like ATP synthesis or efflux pump activity are inhibited rapidly in starved cells. This suggests that damages due to sunlight happen simultaneously at different levels and that the sum of all these effects eventually turns out to be lethal already in the first SODIS phase when temperature is low. The lethal fluence is dependent on repair capability and adaptive flexibility, which both are dependent on the physiological state and substrate availability. From this point of view a differentiation between lethal and sublethal actions seems inadequate. It is more likely that damages due to sunlight in enteric
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bacteria are always similar but that the capability of the cells to deal with them are different, depending on factors like growth state and availability of nutrients. As pointed out earlier, the influence of different AOC concentrations in SODIS experiments remains to be investigated. Also, a comparison of the transcription profile with the proteome pattern of adapted cells and during adaptation could give us new details about the inactivation mechanism of sunlight in enteric bacteria.
References


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