

1 **Adaptation to UVA radiation of *E. coli* growing in continuous**
2 **culture**

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4 Michael Berney, Hans-Ulrich Weilenmann and Thomas Egli*

5

6 Swiss Federal Institute of Aquatic Science and Technology, Eawag, P. O. Box 611, CH-8600

7 Dübendorf, Switzerland

8

9 *Author for correspondence:

10 Thomas Egli, Swiss Federal Institute of Aquatic Science and Technology (Eawag),

11 Environmental Microbiology, P. O. Box 611, CH-8600 Dübendorf, Switzerland, Tel: +41 1

12 823 5158, Fax: +41 1 823 5547, E-mail: egli@eawag.ch

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16

17 Abbreviations:

18

19 D Dilution rate in h⁻¹

20 OD₅₄₆ Optical density measured spectrophotometrically at 546 nm

21 PI Propidium iodide

22 EB Ethidium bromide

23 FL1 Fluorescence channel 1

24 $\Delta\bar{\mu}_{H^+}$ Transmembrane proton gradient

1 **Abstract**

2

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

1 **1. Introduction**

2

3 Sunlight has long been recognized as a harmful radiation capable of damaging
4 microorganisms [10]. Solar radiation is believed to be a physiologically relevant stress also
5 for enterobacteria since transmission between hosts can involve environmental exposure [8].
6 For example, sunlight has been proposed to be the most important agent inactivating sewage
7 microorganisms (e.g., *Enterococci* or *E. coli*) in shallow seawater or fresh water [34,37].
8 Furthermore, the deleterious effect of sunlight on enteric bacteria has been used to develop
9 simple drinking water disinfection methods based on the action of sunlight (SODIS) [1,39].

10

11 The main fraction of solar ultraviolet radiation reaching the earth's surface is UVA light
12 (320-400 nm), also called near-UV light (NUV). UVA light, depending on the applied dose
13 (fluence), is believed to have both lethal and sublethal effects on *E. coli*. Sublethal effects in
14 *Escherichia coli* were shown to occur at fluences of 100 kJ/m² at 366nm; such fluences are
15 approximately an order of magnitude lower than lethal fluences [19]. Two different
16 phenomena of sublethal injury in bacteria can be found in the literature: Firstly, bacterial
17 cells were referred to as sublethally injured when they do not grow on selective media after
18 irradiation but can be recovered on catalase- and pyruvate-supplemented media [26];
19 secondly, bacterial cells grown in batch culture and irradiated with broad-band UVA light for
20 a restricted time period were found to exhibit a lag time (also called growth delay) before
21 resuming growth. In the very first report growth delay in *E. coli* caused by near-UV
22 irradiation with a medium-pressure mercury arc lamp (350-490 nm) was shown to be a direct
23 function of dose and occurred already at “non-lethal” doses [16]. Some 20 years later an
24 action spectrum for induction of growth delay with a maximum at 340 nm was presented for
25 *E. coli* B [21]. Subsequently, a number of studies were initiated to characterize the response

1 of *E. coli* to NUV. It was shown that growth delay is largely due to cross-linking of cytidine
2 with 4-thiouridine (s^4U) ($\lambda_{max} = 340$ nm) an unusual base occurring in the 8-position in 65%
3 of tRNAs species of *E. coli* [13,20]. The same authors proposed that due to the lack of
4 tRNA-charging activity stringent response with subsequent growth inhibition is triggered.
5 Others proposed a close relationship between membrane damage and the UVA-induced
6 growth delay [23,25,32]. Other authors again suggested that the growth delay might help the
7 cell to induce repair of damage [12], but it was not until 1988 when it was proposed that
8 induction of growth delay triggered by tRNA crosslinking could be part of an adaptive
9 response to UVA light [24]. The same authors suggested a model where s^4U acts as a sensor
10 for UVA stress that mediates the induction of specific proteins (some of them are oxidative
11 stress-related) and the synthesis of dinucleotides. Recently, it was shown that continuous
12 sublethal UVA irradiation of *E. coli* growing in batch culture exposed to a very low intensity
13 (7.4 W/m^2) leads to an adaptive response that includes the expression of some oxidative
14 stress-related enzymes [15]. Adaptive response in *E. coli* to low levels of mutagens is a
15 known phenomenon[27,35]. Despite all these suggestions the nature and mechanism of this
16 adaptive response to UVA radiation is not fully understood at the physiological as well as the
17 molecular level.

18

19 To our knowledge, all studies conducted so far on UVA light-stress in *E. coli* were done in
20 batch culture with complex medium and no control of specific growth rate. Here, we
21 investigated the ability of *E. coli* growing in continuous culture to adapt to UVA light-stress.
22 This has several advantages. In continuous culture bacteria can be held at a specific growth
23 rate for infinite time. Parameters like substrate availability and aeration can be held constant
24 and the culture can be run at such a low optical density that UVA light does not get
25 significantly scattered or absorbed by the medium or culture. The influence of specific

- 1 growth rate on *E. coli*'s ability to adapt to UVA irradiation was tested and physiological
- 2 changes during adaptation were investigated with single cell analysis (flow cytometry) and
- 3 other methods.

1 **2. Materials and Methods**

2

3 **2.1 Bacterial strains.** In all experiments wild-type *E. coli* K-12 MG1655 (ATCC
4 700926) was used.

5

6 **2.2 Cultivation conditions.** Used for batch cultivation was Luria-Bertani (LB) broth (10
7 g tryptone, 5 g yeast extract, 10 g NaCl per liter) [29] that was filter-sterilized with membrane
8 filters (Millex GP, 0.22 μm , Millipore, Tullagreen, Ireland) and diluted to 33 %
9 volume/volume (v/v) of its original strength (unless indicated otherwise) with ultra pure
10 water (deionized and activated carbon-treated). Precultures were prepared for each individual
11 experiment from the same cryo-vial stored at $-80\text{ }^{\circ}\text{C}$ by streaking out the stock culture onto
12 LB agar plates. After 15-18 h of incubation at $37\text{ }^{\circ}\text{C}$ one colony was picked, loop-inoculated
13 into a 125 ml Erlenmeyer flask containing 20 ml of diluted LB broth and incubated at $37\text{ }^{\circ}\text{C}$
14 on a rotary shaker at 200 revolutions per minute. At an optical density (OD_{546}) between 0.1
15 and 0.2 (measured spectrophotometrically at 546 nm in glass cuvettes with 1 cm light path
16 using a JASCO V550 UV/VIS spectrophotometer; JASCO, Tokyo, Japan) an aliquot of the
17 culture was transferred into the bioreactor with 50 ml of prewarmed diluted LB broth (5%
18 v/v) to obtain an OD_{546} of 0.002. The bioreactors for continuous culture experiments
19 consisted of temperature-controlled and air-sparged cylindrical quartz glass tubes of 100 ml
20 total and 50 ml working volume (WISAG, Oerlikon, Switzerland). The feed-medium
21 consisted of 5% v/v LB broth. Before starting exposure, at least 10 volume changes were
22 allowed for the culture to reach steady-state (based on OD_{546}).

23

24 **2.3 UVA exposure.** The bioreactors were installed in a incubation device (holding up to
25 six bioreactors) (Fig. 1) equipped with a medium-pressure mercury lamp (Hanau TQ150)

1 operated at 150 W (wavelength spectrum see [5], TQ150 has the same spectrum as TQ718).
2 The light spectra were recorded with a calibrated LI-1800 portable spectroradiometer (LI-
3 COR, Lincoln, Nebraska, USA), 8 nm bandwidth, fitted with a model 1800-10 detector head.
4 The lamp was placed in a cooling jacket (Duran 50 borosilicate glass) in the centre of the
5 incubation device and the bioreactors were arranged around the lamp at equal distance from
6 the lamp. The light emitted from the lamp passed through the glass jacket and through 35
7 mm of filter solution before reaching the cells in the quartz tubes. The temperature of the
8 filter solution was maintained at 37 °C and it consisted of 12.75 g/l sodium nitrate with a cut-
9 off at 320 nm and a half maximum at 340 nm. The transmission property of the filter
10 solution was measured before each experiment. Chemical actinometry with *p*-
11 nitroanisole/pyridine was used to determine the fluence rate at the bioreactor position [39].
12 The light intensity applied was 50 W/m², which represents the average mid-day sunlight
13 intensity in the NUV range. In all experiments one aluminum foil-wrapped control bioreactor
14 was added. All bioreactors were fed with medium from the same reservoir. Throughout the
15 experiments, OD₅₄₆ was measured as described above. Specific growth rate μ was calculated
16 from three consecutive OD₅₄₆ measurements ($\mu = \frac{\Delta \ln OD_{546}}{\Delta t} + D$) (above D is the
17 dilution rate and Δt the time between two OD₅₄₆ measurements) and the division rate was
18 calculated from total cell count measurements accordingly.
19 To check for toxic effects caused by the irradiated LB medium two reactors running at D =
20 0.5 h⁻¹ were connected. The first reactor was irradiated but not inoculated. The irradiated
21 medium from the first bioreactor then passed to the second aluminum foil wrapped
22 bioreactor, which was inoculated with *E. coli*.

23
24

1 **2.4 Flow-cytometric measurements.** Flow-cytometric measurements were made with a
2 Partec PAS III flow cytometer (Partec GmbH, Münster, Germany) with 488 nm excitation
3 from an argon-ion laser at 20 mW. Three fluorescent dyes were used alone or in different
4 combinations: Syto[®]9 (Molecular Probes, Eugene, USA), propidium iodide (PI) (Molecular
5 Probes) and ethidium bromide (EB) (Fluka Chemie AG, Buchs, Switzerland). Culture
6 samples withdrawn from irradiation experiments were washed once with 0.22 µm membrane-
7 filtered mineral water (EVIAN) to remove residual nutrients from LB and then divided into
8 three subsamples (100µl) and immediately stained. One subsample was stained with a
9 mixtures of Syto[®]9/PI, the second subsample was stained with Syto[®]9/EB and the third
10 subsample with Syto[®]9 only. Before analysis, subsamples were incubated in the dark at
11 room temperature for 15 (Syto[®]9/EB), 20 (Syto[®]9/PI) and 25 minutes (Syto[®]9), respectively.
12 Prior to flow-cytometric analysis cell samples were diluted with 0.22 µm membrane-filtered
13 EVIAN to yield a concentration suited for flow cytometry (approx. 1×10^5 cells/ml). Stock
14 solutions of the dyes were prepared as follows: PI and Syto[®]9 were used from the
15 LIVE/DEAD[®] BacLight[™] kit (Molecular Probes) and EB was made up at 25 mM in 0.22 µm
16 membrane-filtered deionized water. All dye stock solutions were stored at -20 °C. The
17 working concentration of Syto[®]9, PI, and EB was 5 µM, 30 µM, and 30 µM, respectively. In
18 the flow cytometer, optical filters were set up such that PI and EB emitted fluoresce was
19 measured above 590 nm and Syto[®]9 at 520 nm. The trigger was set for the green
20 fluorescence channel FL1 (520 nm), i.e., only green-fluorescing particles are registered.

21

22

23 **2.5 Fluorescence stains and their function.** A detailed and comprehensive description
24 of the stains and their functions is given elsewhere [3]. Syto[®]9, a green fluorescent nucleic
25 acid stain was used for total count measurements. Ethidium bromide is actively pumped out

1 of the cell via a non-specific proton antiport transport system in active cells [28]. EB enters
2 cells if the transmembrane proton gradient ($\Delta\bar{\mu}_{H^+}$) is lacking. Propidium iodide (PI) is a red
3 fluorescent dye that only enters cells with a permeabilized cytoplasmic membrane. When EB
4 or PI is combined with Syto[®]9 a quenching effect on the green fluorescence intensity is
5 observed as soon as the red fluorescent dye enters the cells. For further information about the
6 dyes described above the reader is referred to the literature [14,22].

7
8 **2.6 Total ATP.** For the determination of total ATP, the BacTiterGlo[™] System
9 (Promega, Madison, WI, USA) was used. The BacTiterGlo[™]-Buffer was mixed with the
10 lyophilized BacTiterGlo[™]-Substrate and equilibrated at room temperature. This reagent was
11 stored over night at room temperature to make sure, that all ATP was hydrolysed (“burned
12 off”) and the background signal had decreased. A cell suspension of 100 μ l was mixed in a 2
13 ml Eppendorf tube with an equal volume of the previously prepared BacTiterGlo[™]-reagent
14 (stored on ice). The sample was then briefly vortexed and incubated in a water bath at 37 °C
15 for 30 seconds. The luminescence of the sample was measured in a Luminometer (Model
16 TD-20/20, Turner BioSystems, WI, USA) immediately after the 30 second incubation. A
17 calibration curve with dilutions of pure rATP (Promega) was measured before each
18 experiment. ATP concentration per cell was then calculated using this calibration curve and
19 the total cell count measurements (Syto[®]9) from flow cytometry.

20
21 **2.7 Reproducibility.** Continuous culture experiments at dilution rates of 0.3 h⁻¹ and 0.5
22 h⁻¹ were repeated three times. Flow-cytometric measurements were repeated twice to ensure
23 reproducibility. Representative results are displayed in the graphs. The continuous cultures at
24 dilution rates of 0.7 h⁻¹ and 0.05 h⁻¹ were run only once.

1 **3. Results**

2

3 Although the effect of UVA light on bacteria in batch culture has been intensively studied by
4 many groups, the influence of UVA light on a continuously growing culture has not been
5 tested yet. Since cultivation conditions and specific growth rate play an important role in
6 irradiation experiments with UVA light [5], irradiating bacterial cells in a continuous culture
7 offers a lot of advantages to batch culture irradiation. As indicated earlier, specific growth
8 rate can be maintained constant at a set value in a continuous culture, allowing high
9 reproducibility of experiments. In this study we investigated the influence of specific growth
10 rate on the response and adaptation process of *E. coli* to UVA irradiation and recorded
11 physiological changes primarily at the single cell level with flow cytometry.

12

13 **3.1 Specific growth rate and cell size**

14

15 *E. coli* K12 MG1655 was grown to steady-state in continuous culture at a dilution rate of 0.3
16 h⁻¹ and was then irradiated with UVA light (50 W/m²). An aluminum foil-wrapped
17 continuous culture was run with the same feed medium at the same D as a control.
18 Immediately after irradiation started, optical density as well as total cell count decreased
19 significantly in the continuous culture, indicating that the bacteria had stopped growing and
20 were washed out (Fig. 2). This decrease in optical density closely followed the theoretical
21 wash-out curve. After about 20 hours of irradiation optical density and total cell count started
22 to increase again. Interestingly, the total count did not increase to the same degree as optical
23 density, indicating that cell size varied during the irradiation experiment. A repetition of this
24 experiment combined with flow-cytometric analysis revealed that indeed the average cell size
25 changed significantly (Fig. 3b). A steady increase of average cell size was observed until 20

1 hours, thereafter bacterial cells seemed to become smaller again. ATP concentration per cell
2 (Fig. 3c) initially increased by 20-30% but dropped suddenly 2 hours after starting irradiation
3 to a cellular level of ca. 30 % of the starting concentration. Subsequently, cellular ATP levels
4 recovered again and transiently reached even higher concentrations than in unstressed cells.
5 Interestingly, this coincided with an increase in specific growth rate (Fig. 3d) and also cell
6 size. Specific growth rate and the division rate followed a very similar pattern (Fig. 3d).
7 Both, specific growth rate and division rate initially dropped almost to zero, then increased to
8 a $D = 0.4 \text{ h}^{-1}$ and, subsequently, leveled out in a new steady-state at $D = 0.3 \text{ h}^{-1}$. Both, optical
9 density and total counts of the continuous culture in the new steady-state were significantly
10 lower than before irradiation.

11 To rule out toxic effects of possible metabolites of the LB medium, a control bioreactor was
12 fed with UVA-irradiated medium. This control culture showed no difference to normal
13 control cultures, which received nonirradiated LB medium (data not shown). Additionally,
14 the transmission properties in the UVA range of 20 times diluted LB was checked with a
15 spectrophotometer. The transmission at 366 nm was about 95 % compared to ultra-pure
16 water. Full strength LB, though, showed only about 20 % transmission at 366 nm.

17

18 **3.2 Physiological changes during exposure**

19

20 Efflux pump activity (Syto[®]9 plus EB) and membrane integrity (Syto[®]9 plus PI) determined
21 with flow cytometry indicated that during the first two hours of irradiation the cells stayed
22 intact with all measured functions active (Fig. 4). The ATP concentration per cell increased
23 by 35 % during this period (Fig 3.c). After about 6 hours more than 80 % of the remaining
24 population was EB-positive (non-pumping), while only 20 % of the population had lost
25 membrane integrity. After 10 hours, almost all remaining cells were EB-positive (96 %) and

1 more than 70 % had lost membrane integrity. These bacterial cells had received a UVA
2 fluence of approximately 1800 kJ/m² if one assumes that the cells had stopped growing right
3 after the start of irradiation. Between 10 and 48 hours a less light-sensitive bacterial
4 population evolved, which showed no sign of permeabilization of the cytoplasmic membrane.
5 Interestingly, these cells were unable to pump out ethidium bromide to the same extent as
6 their non-irradiated predecessors. In Figure 5.a-f two-dimensional dot-plots from flow-
7 cytometric measurements of the different cellular function are depicted. After 10 hours, EB
8 and PI entered the cells, leading to a typical quenching effect on the green fluorescence
9 intensity (emission of green fluorescence is diminished by fluorescence resonance energy
10 transfer [38]), which allowed an easy discrimination between pumping (upper left; Fig 5.b,c)
11 and non-pumping (lower right, Fig. 5a) or intact (Fig. 5d,f) and permeabilized (Fig. 5e) cells,
12 respectively (see also Berney *et al.* [3]). After 50 hours almost all bacterial cells appeared
13 intact again (Fig. 5f). The fluorescence pattern for EB plus Syto9 had changed but epi-
14 fluorescence microscopy of these cells showed that most of them were still EB-positive.

15

16 **3.3 Dilution rate and fluence**

17

18 The influence of the dilution rate on the sensitivity of *E. coli* to UVA light was investigated
19 (Fig. 6). One aluminum foil-wrapped control bioreactor ($D = 0.5 \text{ h}^{-1}$) and four bioreactors
20 run at different dilution rates ($D = 0.7 \text{ h}^{-1}$, 0.5 h^{-1} , 0.3 h^{-1} , 0.05 h^{-1}) were operated
21 simultaneously with the same feed medium. At dilution rates of 0.7 h^{-1} , 0.5 h^{-1} and 0.3 h^{-1} the
22 bacterial population adapted to the UVA light-stress and reached a new steady-state after
23 about 6, 30 and 48 hours, respectively (Fig. 7). At dilution rates of 0.7 h^{-1} and 0.5 h^{-1} *E. coli*
24 cells triggered their adaptive response very quickly, which was indicated by the fast
25 turnaround of specific growth rate (after about 1.5 hours) (Fig. 7). *E. coli* cells, which were

1 cultured in the bioreactor with the lowest dilution rate (0.05 h^{-1}) showed no sign of adaptation
2 during the time span of the experiment (75 hours). Furthermore, it was observed that optical
3 density of the culture in the newly established steady-state was always lower than before
4 irradiation indicating that growth yield was lower under irradiation. The average residence
5 time of a cell in the new steady-state of the three bioreactors ($D = 0.7, 0.5, 0.3 \text{ h}^{-1}$) was 1.4 , 2
6 and 3.33 hours respectively. Hence, in the new steady-state these cells received an average
7 UVA fluence of 260, 360, and 600 kJ/m^2 , respectively.

8

9

10 **3.4 Adaptive response**

11

12 The nature of the adaptation of *E. coli* growing in continuous culture under continuous UVA
13 light irradiation was investigated. *E. coli* was grown in a bioreactor with a dilution rate of 0.3
14 h^{-1} and irradiated with UVA light at 50 W/m^2 (Fig. 8). After the new steady-state was
15 reached with respect to OD_{546} the lamp was switched off and the culture was allowed to grow
16 back to its original steady-state. After switching off the lamp the culture immediately grew
17 with a much higher specific growth rate (0.62 h^{-1}). After about 70 hours (21 generations) and
18 establishment of the new steady-state with respect to OD_{546} the lamp was switched on again.
19 It was assumed that after this time cells containing proteins only expressed under UVA stress,
20 had been diluted out. Also, in the second period of irradiation a reduction of specific growth
21 rate of the culture was observed, however, it recovered much faster and the time to reach new
22 steady-state was only 24 hours (compared to the 50 hours needed before).

23

1 **4. Discussion**

2

3

4 In this study we show for the first time that *E. coli* cells cultivated in continuous culture and
5 irradiated with UVA light can adapt to this stress. The adaptive response seems to be
6 dependent on specific growth rate. With flow cytometry and viability stains the physiological
7 state of the bacteria in the bioreactor was followed and we saw indications that only a very
8 small part of the initial population was able to divide under UVA irradiation. Our results
9 indicate that an adaptation of *E. coli* to UVA light is possible at a much higher fluence-rate
10 (50 W/m^2) than previously derived from batch culture experiments (7.4 W/m^2) [15].

11

12

13 **4.1 Physiological changes during irradiation of a continuous culture**

14

15 During the first 10 hours of UVA irradiation of *E. coli* in continuous culture ($D = 0.3 \text{ h}^{-1}$) more
16 than 90% of the initial cell population was washed out due to growth inhibition.

17 Interestingly, in the first 2 hours of irradiation the average ATP concentration per cell
18 increased significantly, indicating that more ATP was produced or that ATP-dependent
19 functions were inactivated while ATP production remained constant. The slight increase in
20 cell size in the first ten hours of irradiation suggests that some cells were still able to grow.

21 The data for specific growth rate and division rate indicated that the cells became larger and
22 still divided. Others have shown that *E. coli* AB1157 cells, growing in batch culture with a
23 specific growth rate of 0.83 h^{-1} were able to adapt to illumination with 366-nm light (25
24 W/m^2) by lowering their specific growth rate to 0.57 h^{-1} [7]. The same authors showed that
25 the reduction in cell mass-doubling time occurred without change in the rate of cell division.

1 Hence, they proposed that cell size decreased during the first 2 hours of irradiation, which is
2 in contrast to our finding. However, they provided no data about the behavior of cells that
3 were irradiated for more than 2 hours.

4

5 Efflux pump activity had ceased in the remaining cells after 10 hours of irradiation and about
6 75% of the cell population was permeabilized. We have shown earlier that permeabilized
7 cells were not able to recover from UVA injury and that fluences of about 2000-2500 kJ/m²
8 of UVA irradiation are needed for stationary phase *E. coli* cells to become permeabilized [3].

9 Therefore, the permeabilized cells detected in the continuous culture after 10 hours
10 irradiation (1800 kJ/m²) are probably cells that have stopped growing immediately after
11 irradiation started and were not washed out. About 20 % of the remaining population (this
12 corresponds to about 1 % of the initial population), which appeared still intact after 10 hours
13 was probably the surviving fraction that was able to adapt to UVA light. Between 10 and 20
14 hours of irradiation specific growth rate of the cells increased significantly and was higher
15 (0.37 h⁻¹) after 20 hours than the dilution rate (0.3 h⁻¹). The increase in ATP production per
16 cell is probably coupled with the observed increase in cell size. A larger cell is expected to
17 have more total ATP. This is also supported by the fact that in the new steady-state a 30 %
18 increase in ATP concentration per cell compared to the initial steady-state paralleled an
19 increase in cell size by 30 % as measured with the flow cytometer. In contrast, cell size
20 stayed constant in the non-irradiated control bioreactor.

21

22 In the new steady-state after 50 hours more than 99 % of the cells were intact (PI-negative),
23 while most of the cells were still non-pumping (EB-positive). The efflux pump activity is
24 dependent on the electrochemical proton gradient, which might be lower in the UVA

1 irradiated cells. It has been shown earlier that the loss of efflux pump activity is not lethal
2 for stationary phase *E. coli* [3,30].

3

4

5 **4.2 Adaptive response and its dependence on growth conditions and growth state**

6

7 Up to now typical “growth delay” experiments were performed by irradiating bacterial cells
8 growing in batch culture with UVA light for a restricted time period. After irradiation was
9 stopped a lag time was observed before the culture resumed growth. Evidence that *E. coli*
10 can resume growth during irradiation with UVA light is very scarce. It was shown that at a
11 light intensity of 25 W/m² at 366 nm the specific growth rate of *E. coli* cells in batch culture
12 decreased and a new “stable” exponential mode of growth was reached [7,11]. However,
13 irradiation of cultures never exceeded 2 hours. Therefore, it is not known if this adaptation
14 was of stable nature or not. Others showed that when *E. coli* was subjected to continuous
15 low-fluence UVA irradiation at 7.4 W/m² (emission peak at 365 nm) while growing in batch
16 culture it responded by increasing the activity level of certain oxidative stress-related
17 proteins, which led to an attenuation of the growth delay response and an increased resistance
18 to lethal UVA irradiation [15]. Whether these changes of enzyme activity are due to changes
19 in the expression profile or simply the activity level is not known. The same authors
20 proposed that a UVA fluence of 135 kJ/m² delivered at a fluence rate of 50 W/m² delivered to
21 stationary phase *E. coli* in M9 eventually results in cell death. Our results show that *E. coli*
22 cells growing in continuous culture at dilution rates between 0.3 to 0.7 h⁻¹ were able to adapt
23 to broad band UVA light with a fluence-rate of 50 W/m² while at a dilution rate of 0.05 h⁻¹
24 the cells were not able to adapt during the time measured.

25

1 In the irradiated continuous culture bacterial cells are subject to selective pressure. Only cells
2 that are able to initiate an appropriate stress response and can maintain cell division under the
3 applied stress will be able to maintain themselves in the bioreactor during continuous
4 irradiation. Hence, our results indicate that the adaptive flexibility of *E. coli* is dependent on
5 specific growth rate. Faster growing cells seemed to have a much higher adaptive flexibility
6 than slow-growing cells. This is somewhat surprising because we have shown that *E. coli*
7 cells grown in continuous culture at a low dilution rate (0.1 h^{-1}), after washing and
8 suspending them in mineral water before irradiation, were less sensitive to UVA light or
9 sunlight than cells grown at higher dilution rates (0.7 h^{-1}) [5]. These bacterial cells were
10 irradiated without addition of substrate, which is in contrast to the experiments presented
11 here. Therefore, the constant supplementation with substrate in the bioreactor experiments
12 presented here seems to be essential for an adaptive response. This is in line with the
13 knowledge that de novo protein synthesis is hindered during slow growth or growth arrest,
14 which makes it difficult for the bacterial cell to respond to an additional stress quickly and
15 effectively [40]. It was shown that RpoS levels in *E. coli* increased with decreasing growth
16 rate and that at specific growth rates around $0.1 - 0.2 \text{ h}^{-1}$ RpoS concentrations are similar to
17 stationary phase bacteria [18,31]. Hence, it can be followed that the general stress response,
18 which was expressed in the slow continuous culture ($D = 0.05 \text{ h}^{-1}$) under the regulation of
19 RpoS, was not enough to withstand UVA irradiation at 50 W/m^2 .

20

21 Furthermore, the adaptation of *E. coli* in continuous culture seems not to be dependent on
22 generation time. In all bioreactors a significant decrease in specific growth rate was observed
23 immediately after starting irradiation (Fig. 7) and biomass was washed out. This wash-out
24 was more pronounced in slow-growing continuous cultures. In a continuous culture a wash-
25 out of biomass is normally paralleled by an accumulation of non-utilized substrate, which

1 temporary would allow bacteria to grow at a higher specific growth rate than the dilution rate.
2 In the continuous culture with the slowest dilution rate (0.05 h^{-1}), the bacterial cells seemed
3 not to be able to take advantage of the higher substrate concentration during the time
4 measured. This indicates that the adaptive response of the bacteria might have been too slow
5 due to their inability to quickly induce *de novo* protein synthesis (see above). With time the
6 accumulated UVA light intensity (fluence) becomes lethal. A fluence of 2500 kJ/m^2
7 (corresponding to 14 h mean residence time in the irradiated bioreactor) is enough to
8 permeabilize stationary phase *E. coli* cells [3]. In all other bioreactors the cells could adapt to
9 the UVA light-stress, which was indicated with a sudden increase in specific growth rate after
10 the initial decrease (Fig. 7). In all three bioreactors ($D = 0.7, 0.5$ and 0.3 h^{-1}) specific growth
11 rate transiently increased above the dilution rate demonstrating a temporary accumulation of
12 substrates (see above). The bacterial cells from the bioreactor run at dilution rates of 0.7 h^{-1} ,
13 0.5 h^{-1} and 0.3 h^{-1} were able to withstand an average fluence of 260, 360 and 600 kJ/m^2 in
14 their new steady-state. OD_{546} in the new steady-state, though, was below the initial value in
15 all continuous culture experiments suggesting growth yield was lower. In a UVA-irradiated
16 batch culture this metabolic shift would result in a slower specific growth rate. It was shown
17 earlier that the specific growth rate of UVA-irradiated *E. coli* cells in batch culture inversely
18 correlated with the fluence-rate applied ($12 - 40 \text{ W/m}^2$, 365 nm) [11]. It has to be mentioned,
19 though, that the bacterial cells were only exposed up to 2 hours. Therefore, it is not known
20 whether these cells eventually would have died or adapted under continuous irradiation.
21
22 When the lamp was switched off the adapted bacterial culture started to grow immediately at
23 a much higher specific growth rate (0.62 h^{-1}) in a batch-like manner until the initial (before
24 irradiation) optical density was reached. This supports the idea that UVA light triggers the
25 production of a stress response leading to a lower growth yield. In a second irradiation

1 period a new steady-state was reached already after 24 hours. Although the continuous
2 culture was running for 21 volume changes between the two irradiation periods the bacterial
3 cells seemed to “remember” the stress response to UVA light. This indicates that either stress
4 related proteins were still expressed to some extent or that a mutant population has been
5 selected. A recent transcriptome analysis suggested that the UVA-adapted cells were not *thiI*
6 mutants like they were isolated by others [4,24,33]. A comparison of these results with
7 proteome analysis will further elucidate the regulatory network behind this adaptation.

8
9

10 **4.3 Environmental relevance**

11

12 Sunlight (and especially solar UVA light) is one of the most common environmental stresses
13 that microorganisms are subjected to. Enteric bacteria like *E. coli* or *Salmonella* are exposed
14 to solar irradiation during transmission between hosts. The inimical effect of sunlight on
15 enteric bacteria is also used for drinking water disinfection (SODIS) [39]. Hence, the
16 adaptive capabilities of these organisms to sunlight are of high interest. In the environment
17 carbon and energy sources are scarce and it is not clear under which circumstances enteric
18 bacteria are able to multiply on the available nutrients. Many studies about the fate of enteric
19 bacteria in aquatic environments and about the physical, chemical and biological factors
20 affecting their survival in such habitats have been conducted [6,9,34,36]. It is generally
21 believed that enteric bacteria may survive in natural waters but are not able to multiply.
22 Recently, it was shown that populations of fecal coliforms and enterococci did not grow in
23 freshwater mesocosms [2]. Our study shows that with a constant flux of substrate *E. coli* is
24 able to adapt and grow in UVA light-irradiated bioreactors at dilution rates above 0.3 h^{-1} . In
25 natural aquatic environments carbon availability and temperatures are much lower and

1 therefore, the expected specific growth rates of enteric bacteria are probably below 0.1 h^{-1}
2 [17]. Even in the colon, *E. coli*'s primary habitat, specific growth rates of 0.3 h^{-1} are very
3 unlikely [17]. Therefore, it is unlikely that the observed adaptation of *E. coli* to UVA light
4 may occur in a natural aquatic environment. Nevertheless, in further experiments we can
5 now investigate the nature of this adaptation, which might give us a clue about the
6 inactivation mechanism of UVA light and sunlight.

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1 **References**

2

- 3 [[1] A. Acra, Z. Raffoul, Y. Karahagopian, Solar disinfection of drinking water and oral
4 rehydration solutions (UNICEF (extract), 1984).
- 5 [2] K.L. Anderson, J.E. Whitlock, V.J. Harwood, Persistence and differential survival of
6 fecal indicator bacteria in subtropical waters and sediments, *Appl Environ Microbiol*
7 71 (2005) 3041-3048.
- 8 [3] M. Berney, H. Weilenmann, T. Egli, Flow-cytometric study of vital cellular functions
9 in *E. coli* during solar disinfection (SODIS), *Microbiology* 152 (2006) 1719-1729.
- 10 [4] M. Berney, H.U. Weilenmann, T. Egli, Gene expression of *Escherichia coli* in
11 continuous culture during adaptation to artificial sunlight, *Environ Microbiol* 8 (2006)
12 1635-1647.
- 13 [5] M. Berney, H.U. Weilenmann, J. Ihssen, C. Bassin, T. Egli, Specific growth rate
14 determines the sensitivity of *Escherichia coli* to thermal, UVA, and solar disinfection,
15 *Appl Environ Microbiol* 72 (2006) 2586-2593.
- 16 [6] G. Bogosian, L.E. Sammons, P.J. Morris, J.P. O'Neil, M.A. Heitkamp, D.B. Weber,
17 Death of the *Escherichia coli* K-12 strain W3110 in soil and water, *Appl Environ*
18 *Microbiol* 62 (1996) 4114-4120.
- 19 [7] A. Caldeira de Araujo, A. Favre, Induction of size reduction in *Escherichia coli* by
20 near-ultraviolet light, *Eur J Biochem* 146 (1985) 605-610.
- 21 [8] J. Calkins, T. Thordardottir, Ecological significance of solar UV-radiation on aquatic
22 organisms, *Nature* 283 (1980) 563-566.
- 23 [9] D.J.G. Davies, L.M. Evison, Sunlight and the survival of enteric bacteria in natural
24 waters, *J Appl Bacteriol* 70 (1991) 265-274.

- 1 [10] A. Downes, On the action of sunlight on micro-organisms, &c., with a demonstration
2 of the influence of diffused light, Proc Ro Soc (London) 40 (1886) 14-22.
- 3 [11] A. Favre, Croissance de la bactérie *E. coli* sous illumination à 366 nm., Comptes
4 Rendus Acad. Sci. Paris 290 (1980) 1111-1114.
- 5 [12] A. Favre, E. Hajnsdorf, Photoregulation of *E. coli* growth and the near ultraviolet
6 photochemistry of tRNA, in: B.F. Erlanger, G. Montagnoli (Eds.), Molecular Models
7 of Photoresponsiveness (Plenum, New York, 1983) 75-94.
- 8 [13] A. Favre, E. Hajnsdorf, K. Thiam, A. Caldeira de Araujo, Mutagenesis and growth
9 delay induced in *Escherichia coli* by near-ultraviolet radiations, Biochimie 67 (1985)
10 335-342.
- 11 [14] C.J. Hewitt, G. Nebe-Von-Caron, The application of multi-parameter flow cytometry
12 to monitor individual microbial cell physiological state, Adv Biochem Eng Biotechnol
13 89 (2004) 197-223.
- 14 [15] J.D. Hoerter, A.A. Arnold, D.A. Kuczynska, A. Shibuya, C.S. Ward, M.G. Sauer, A.
15 Gizachew, T.M. Hotchkiss, T.J. Fleming, S. Johnson, Effects of sublethal UVA
16 irradiation on activity levels of oxidative defense enzymes and protein oxidation in
17 *Escherichia coli*, J Photochem Photobiol B 81 (2005) 171-180.
- 18 [16] A. Hollaender, Effect of long ultraviolet and short visible radiation (3500 to 4900A)
19 on *Escherichia coli*, J Bacteriol 46 (1943) 531-541.
- 20 [17] J. Ihssen, Adaptation of *Escherichia coli* to growth with low concentrations of carbon
21 and energy substrates, Diss. ETH No. 16019, ETHZ, 2005.
- 22 [18] J. Ihssen, T. Egli, Specific growth rate and not cell density controls the general stress
23 response in *Escherichia coli*, Microbiology 150 (2004) 1637-1648.
- 24 [19] J. Jagger, Near-UV radiation effects on microorganisms, Photochem Photobiol 34
25 (1981) 761-768.

- 1 [20] J. Jagger, Solar-UV actions on living cells (Praeger Publishers, New York, 1985).
- 2 [21] J. Jagger, W. Curtis Wise, R.S. Stafford, Delay in growth and division induced by
3 near ultraviolet radiation in *Escherichia coli* B and its role in photoprotection and
4 liquid holding recovery, *Photochem Photobiol* 3 (1964) 11-24.
- 5 [22] F. Joux, P. Lebaron, Use of fluorescent probes to assess physiological functions of
6 bacteria at single-cell level, *Microbes Infect* 2 (2000) 1523-1535.
- 7 [23] A.L. Koch, R.J. Doyle, H.E. Kubitschek, Inactivation of membrane transport in
8 *Escherichia coli* by near-ultraviolet light, *J Bacteriol* 126 (1976) 140-146.
- 9 [24] G.F. Kramer, J.C. Baker, B.N. Ames, Near-UV stress in *Salmonella typhimurium*: 4-
10 thiouridine in tRNA, ppGpp, and ApppGpp as components of an adaptive response, *J*
11 *Bacteriol* 170 (1988) 2344-2351.
- 12 [25] H.E. Kubitschek, R.J. Doyle, Growth delay induced in *Escherichia coli* by near-
13 ultraviolet radiation: relationship to membrane transport functions, *Photochem*
14 *Photobiol* 33 (1981) 695-702.
- 15 [26] H.E. Kubitschek, M.J. Peak, Action spectrum for growth delay induced by near-
16 ultraviolet light in *E. coli* B/r K, *Photochem Photobiol* 31 (1980) 55-58.
- 17 [27] T. Lindahl, B. Sedgwick, M. Sekiguchi, Y. Nakabeppu, Regulation and expression of
18 the adaptive response to alkylating agents, *Annu Rev Biochem* 57 (1988) 133-157.
- 19 [28] M. Midgley, An efflux system for cationic dyes and related compounds in
20 *Escherichia coli*, *Microbiol Sci* 4 (1987) 125-127.
- 21 [29] J. Miller, Experiments in Molecular Genetics (Cold Spring Harbour Laboratory Press,
22 Cold Spring Harbour, NY, 1972).
- 23 [30] G. Nebe-von-Caron, P.J. Stephens, C.J. Hewitt, J.R. Powell, R.A. Badley, Analysis of
24 bacterial function by multi-colour fluorescence flow cytometry and single cell sorting,
25 *J Microbiol Methods* 42 (2000) 97-114.

- 1 [31] L. Notley, T. Ferenci, Induction of RpoS-dependent functions in glucose-limited
2 continuous culture: what level of nutrient limitation induces the stationary phase of
3 *Escherichia coli*?, J Bacteriol 178 (1996) 1465-1468.
- 4 [32] R.A. Pizarro, L.V. Orce, Membrane damage and recovery associated with growth
5 delay induced by near-UV radiation in *Escherichia coli* K-12, Photochem Photobiol
6 47 (1988) 391-397.
- 7 [33] T.V. Ramabhadran, J. Jagger, Mechanism of growth delay induced in *Escherichia coli*
8 by near ultraviolet radiation, Proc Natl Acad Sci U S A 73 (1976) 59-63.
- 9 [34] Y. Rozen, S. Belkin, Survival of enteric bacteria in seawater, FEMS Microbiol Rev 25
10 (2001) 513-529.
- 11 [35] L. Samson, J. Cairns, A new pathway for DNA repair in *Escherichia coli*, Nature 267
12 (1977) 281-283.
- 13 [36] J.W. Santo Domingo, S. Harmon, J. Bennett, Survival of *Salmonella* species in river
14 water, Curr Microbiol 40 (2000) 409-417.
- 15 [37] L.W. Sinton, C.H. Hall, P.A. Lynch, R.J. Davies-Colley, Sunlight inactivation of fecal
16 indicator bacteria and bacteriophages from waste stabilization pond effluent in fresh
17 and saline waters, Appl Environ Microbiol 68 (2002) 1122-1131.
- 18 [38] S.M. Stocks, Mechanism and use of the commercially available viability stain,
19 BacLight, Cytometry A 61 (2004) 189-195.
- 20 [39] M. Wegelin, S. Canonica, K. Mechsner, T. Fleischmann, F. Pesaro, A. Metzler, Solar
21 water disinfection: scope of the process and analysis of radiation experiments, J Water
22 SRT-Aqua 43 (1994) 154-169.
- 23 [40] L.M. Wick, T. Egli, Molecular components of physiological stress responses in
24 *Escherichia coli*, Adv Biochem Eng Biotechnol 89 (2004) 1-45.

25

1 Fig. 1: Picture of two bioreactors during irradiation.

2

3 Fig. 2: *E. coli* K12 MG1655 growing at a dilution rate of 0.3 h^{-1} in “LB-limited” continuous
4 culture was exposed to UVA light at 50 W/m^2 . Optical density (\blacklozenge) and total counts
5 (\blacktriangle), measured with flow cytometry, are compared. A control bioreactor (aluminum
6 foil-wrapped) was run at $D = 0.3 \text{ h}^{-1}$ (\square) with the same feed medium. Theoretical
7 wash-out is indicated (solid line).

8

9 Fig. 3: *E. coli* K12 MG1655 growing at a dilution rate of 0.3 h^{-1} in “LB-limited” continuous
10 culture was exposed to UVA light at 50 W/m^2 . (a) Optical density, (b) cell size
11 (forward scatter signal) of irradiated (\bullet) and control bioreactor (\circ) and (c) total ATP
12 concentration per cell were measured throughout the experiment. Specific growth
13 rate (Δ) and division rate (\bullet) were calculated from OD_{546} measurements and total
14 counts respectively (d).

15

16 Fig. 4: *E. coli* K12 MG1655 growing at a dilution rate of 0.3 h^{-1} in “LB-limited” continuous
17 culture was exposed to UVA light at 50 W/m^2 . Bacterial cell samples were
18 withdrawn from the bioreactor, washed and diluted in filter sterilized mineral water
19 (EVIAN) and analysed on the flow cytometer. Results were calculated in % relative
20 to the total cell count (Syto[®]9-stained cells) at the given sampling point. Total
21 counts (Syto[®]9-stained cells) (Δ), non-pumping cells (ethidium bromide positive)
22 (\blacksquare), permeabilized cells (propidium iodide positive) (\bullet).

23

24 Fig. 5: Flow-cytometric analysis of *E. coli* K12 MG1655 growing in “LB-limited”
25 continuous culture at a dilution rate of $D = 0.3 \text{ h}^{-1}$ irradiated with artificial UVA

1 light. Bacterial cell samples were withdrawn from the bioreactor at different time
2 points of irradiation (0 h, 10 h 48 h), washed and diluted in mineral water (EVIAN)
3 and stained with a mixture of Syto[®]9 plus EB or a mixture of Syto[®]9 plus PI and
4 analysed on a flow cytometer. After 10 h: (b) >95 % of the cells are non-pumping
5 (RN2) and (e) > 70% are permeabilized (RN2). After 50 h: (c) 65% of the cells are
6 non-pumping (RN2), and (f) 4% are permeabilized (RN2).

7
8 Fig. 6: *E. coli* K12 MG1655 growing in continuous cultures with four different dilution
9 rates (\blacklozenge 0.05 h⁻¹, \blacktriangle 0.3 h⁻¹, \bullet 0.5 h⁻¹, \square 0.7 h⁻¹) was exposed to UVA light at 50
10 W/m². A control bioreactor (aluminum foil wrapped) was run at D = 0.5 h⁻¹ (\diamond).
11 When steady-state was reached the lamp was switched on (time = 0 h) and OD₅₄₆
12 was measured during 50-80 hours.

13
14 Fig. 7: Specific growth rate of *E. coli* K12 MG1655 during continuous UVA irradiation (50
15 W/m²) of the “LB-limited” continuous cultures with different dilution rates (\blacklozenge 0.05
16 h⁻¹, \blacktriangle 0.3 h⁻¹, \bullet 0.5 h⁻¹, \square 0.7 h⁻¹) (see Fig. 6). In case of the slowest bioreactor
17 specific growth rate was only calculated until 24 hours because after that time OD₅₄₆
18 was below the detection limit.

19
20 Fig. 8: *E. coli* K12 MG1655 growing at a dilution rate of 0.3 h⁻¹ in “LB-limited” continuous
21 culture was exposed to UVA light at 50 W/m². OD₅₄₆ was measured throughout the
22 experiment (\bullet). At the new steady-state was reached after about 50 h. After 75
23 hours the lamp was switched off. In the second irradiation period steady-state was
24 reached after 24 hours already.

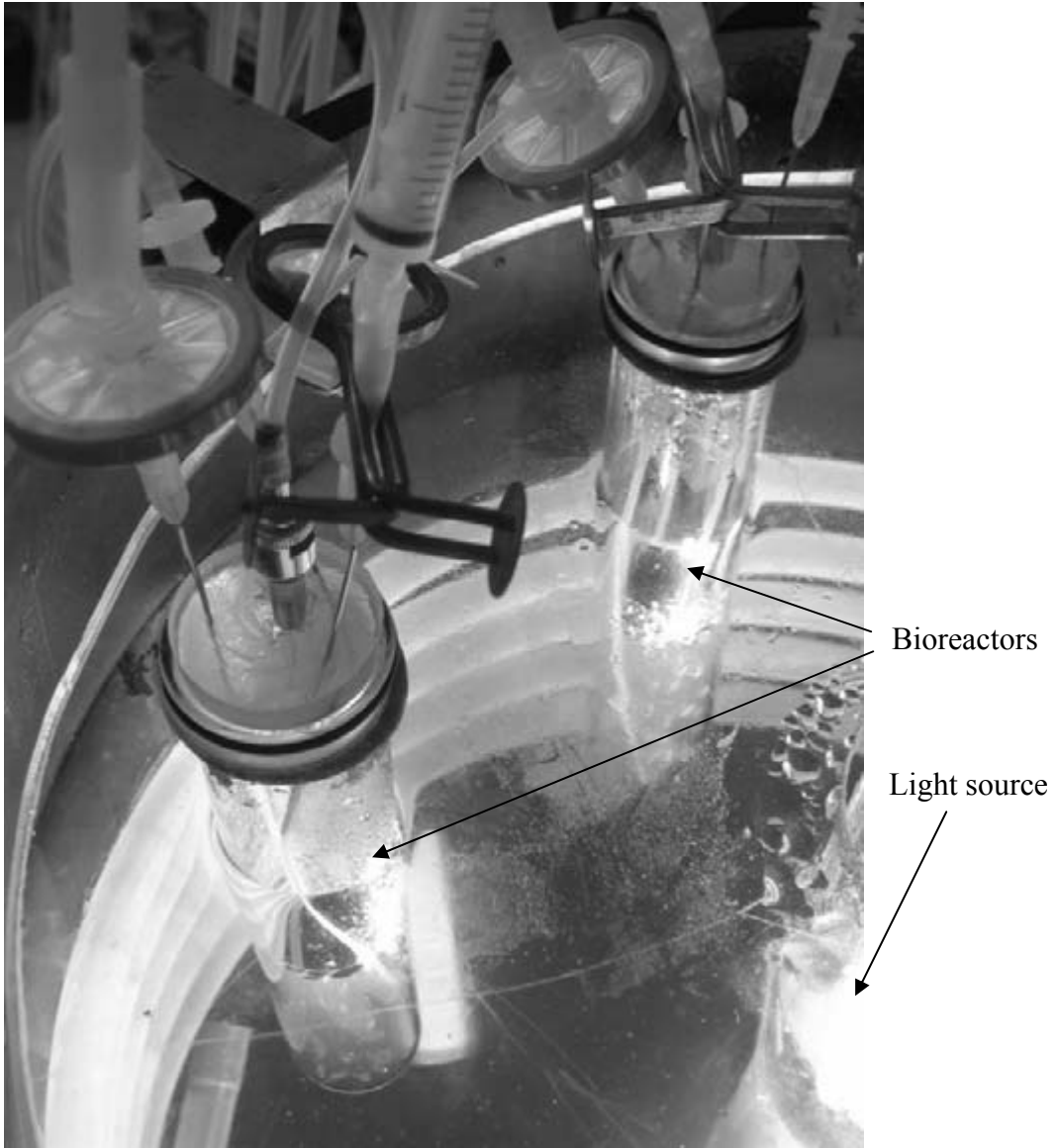


Fig. 1

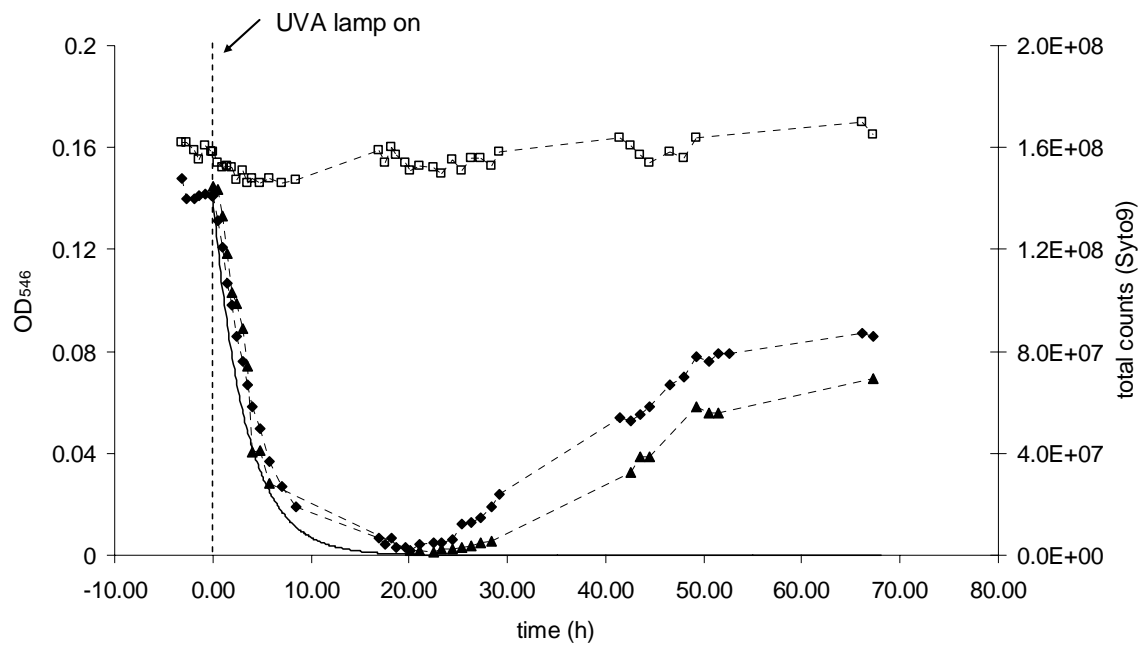


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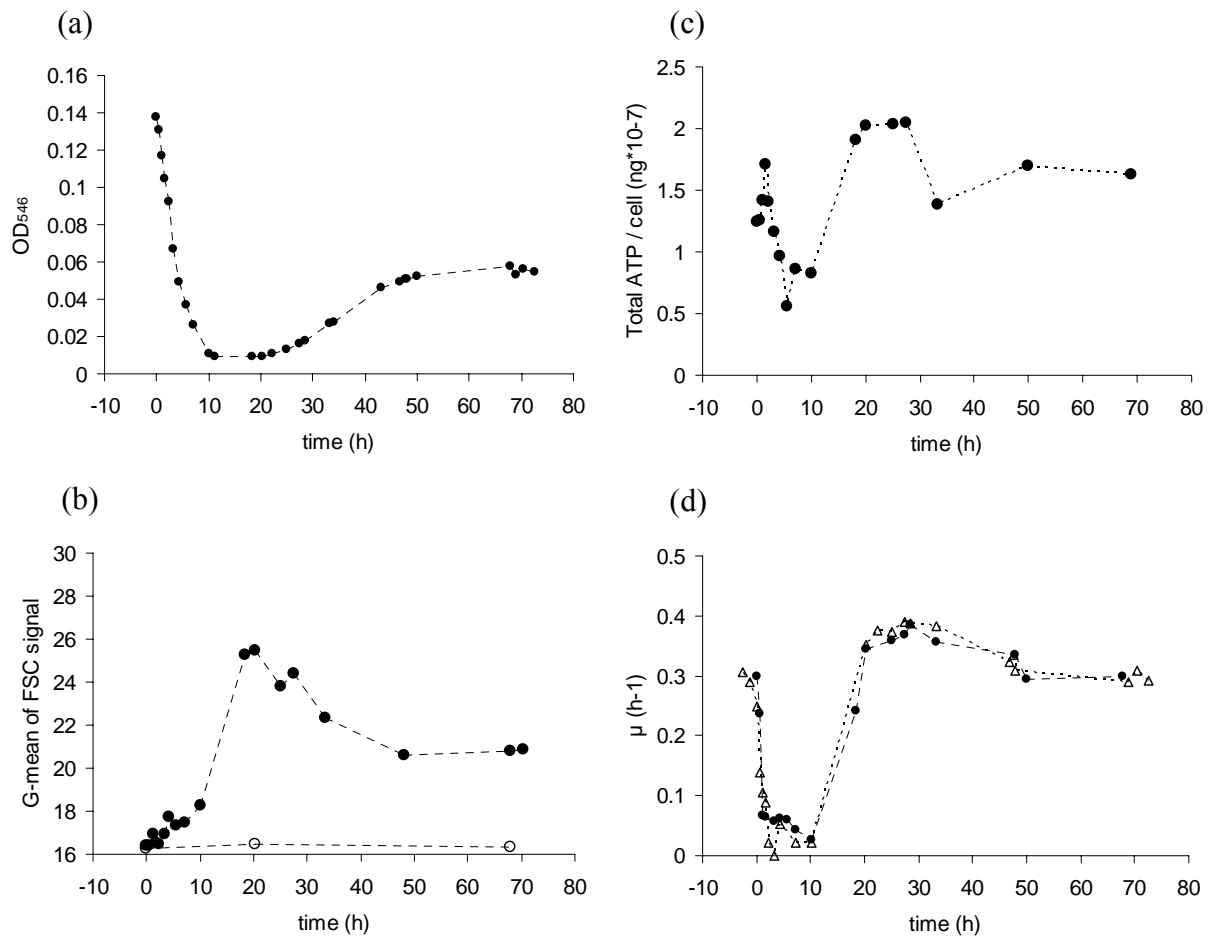


Fig. 3

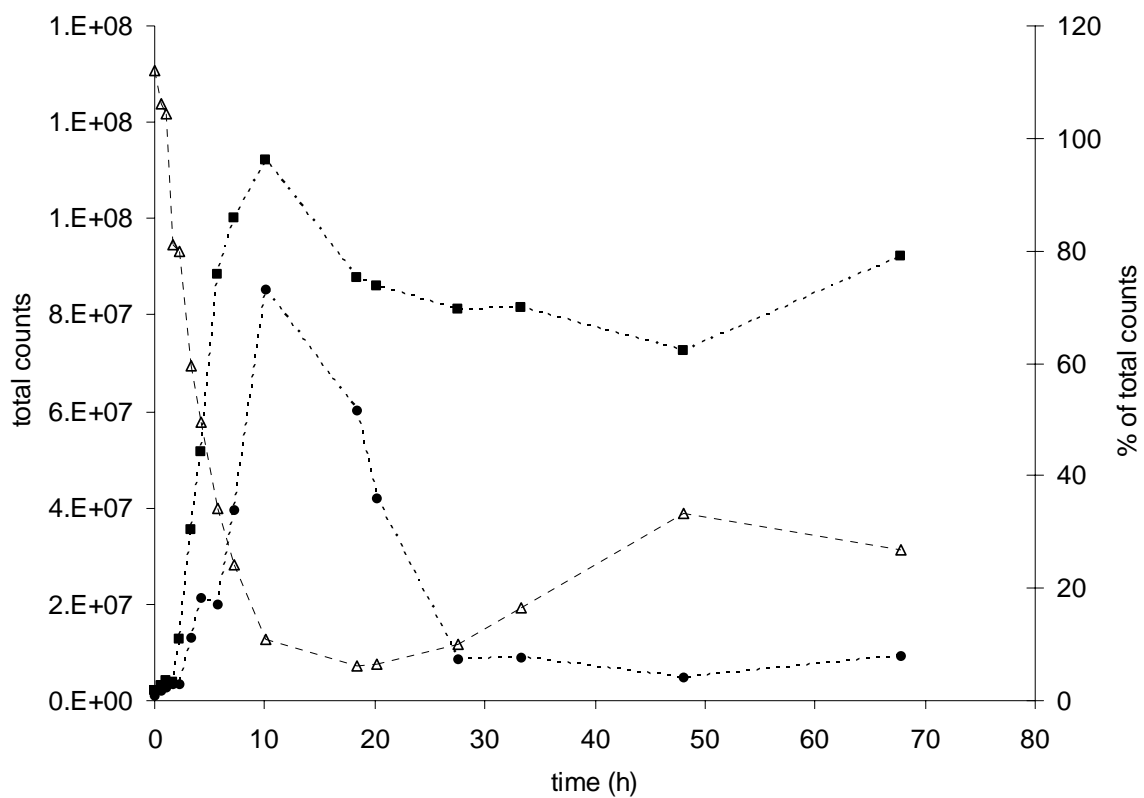


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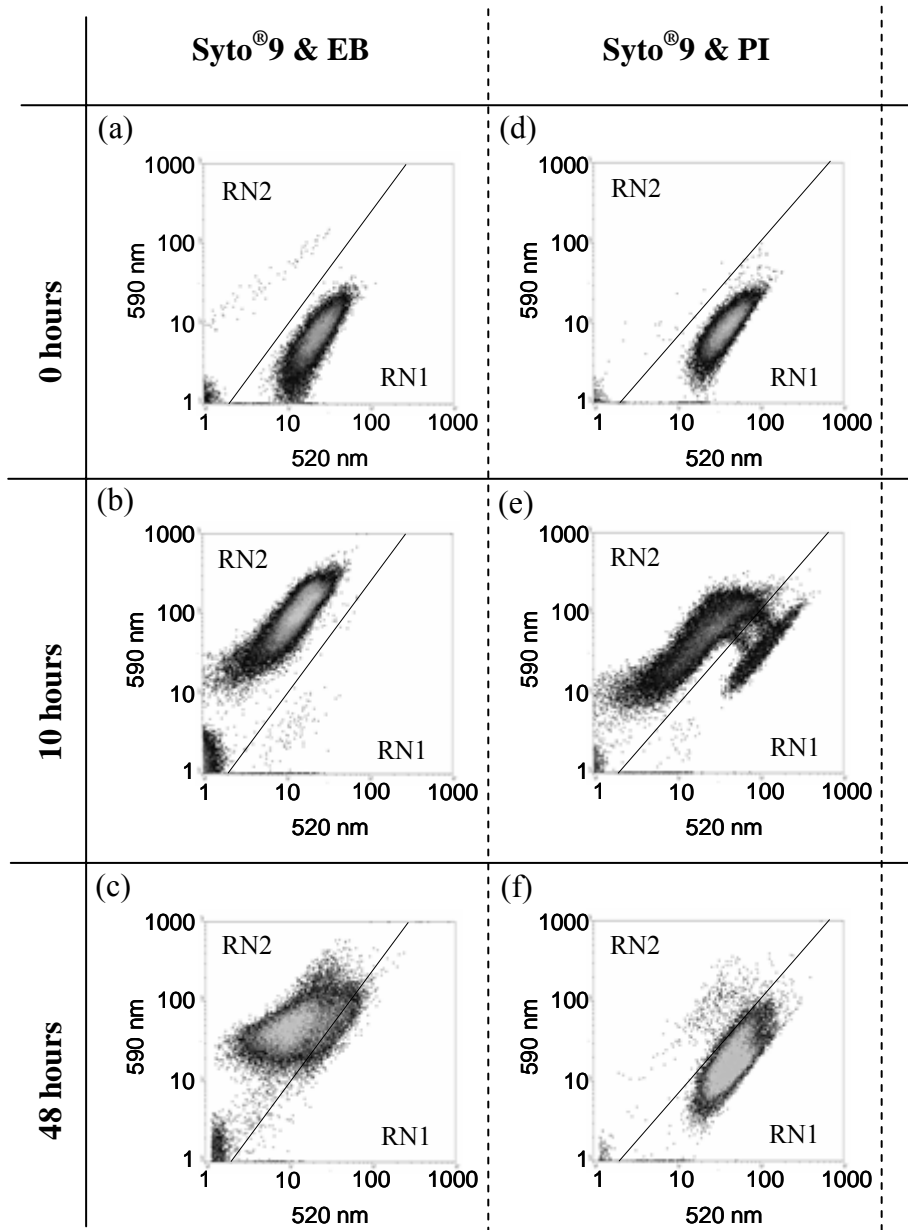


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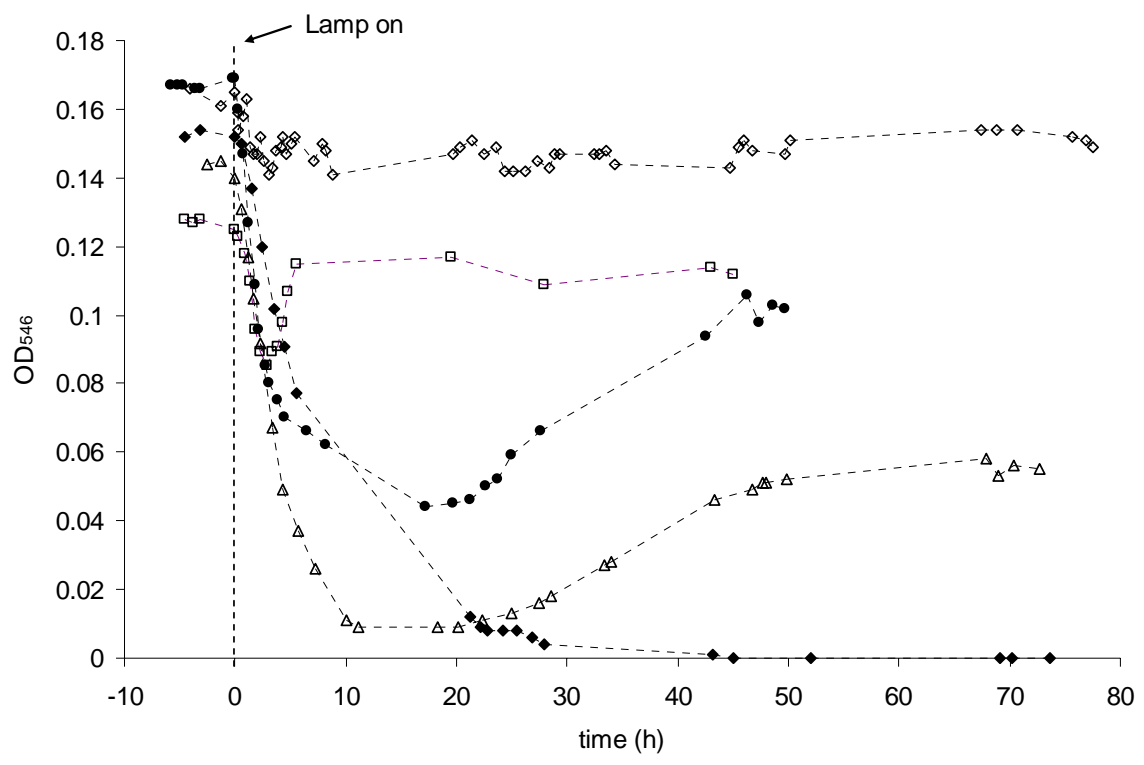


Fig. 6:

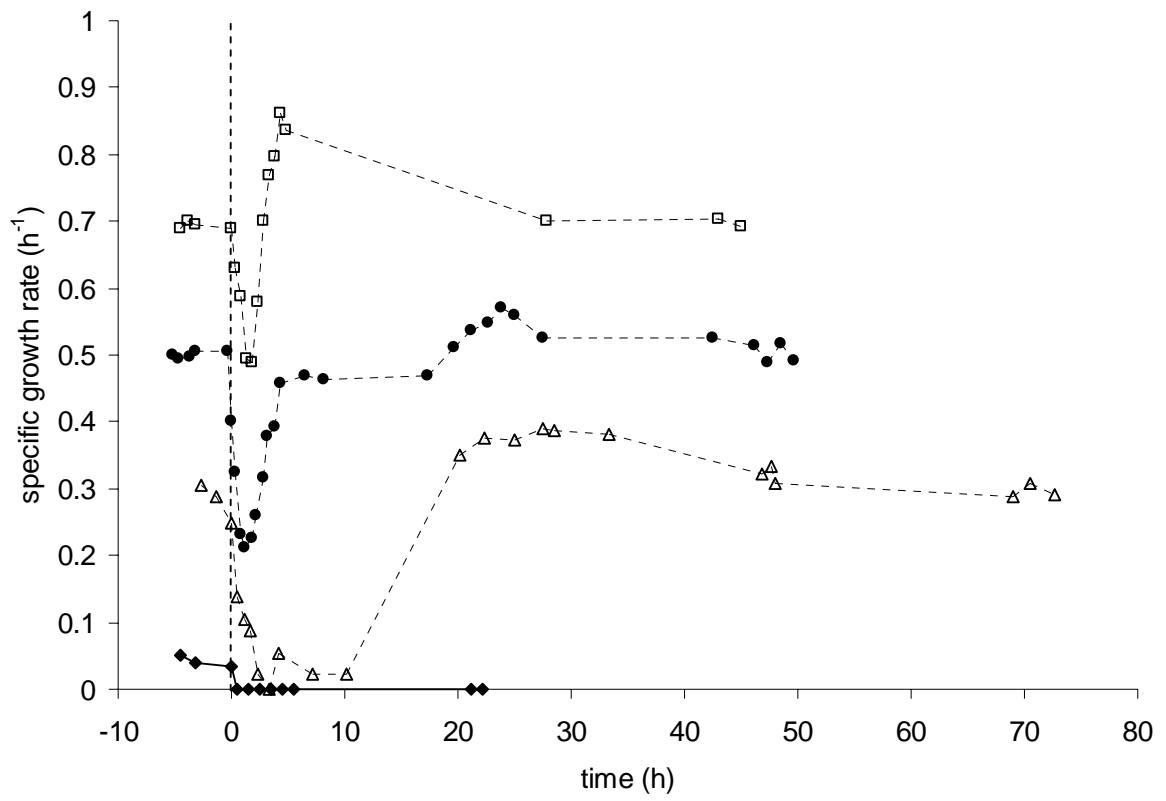


Fig. 7:

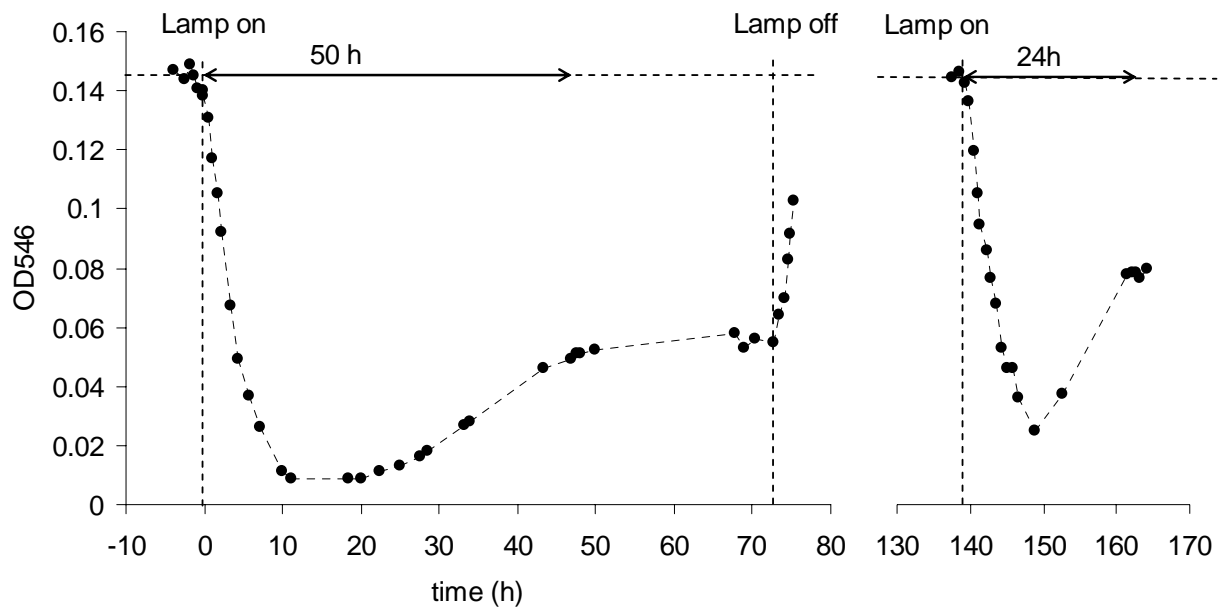


Fig. 8: