Elucidating bacterial regrowth: Effect of disinfection conditions in dark storage of solar treated secondary effluent

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Abstract

In this study, we systematically investigate solar disinfection of synthetic secondary wastewater, with the effort to decrypt the effects disinfection conditions have on post-irradiation bacterial regrowth in the dark. A full factorial design of 240 experiments was employed to investigate the effects of i) exposure time (1, 2, 3 and 4 h), ii) treatment temperature (20, 30, 40, 50 and 60°C), iii) initial bacterial concentration (10^3, 10^4, 10^5 and 10^6 CFU/mL) and iv) sunlight intensity (0, 800 and 1200 W/m^2) on *Escherichia coli* survival for a subsequent 48-h dark control period. The decisive implications treatment temperature inflicted in regrowth were monitored and interpreted within two temperature ranges, from 20-40°C and 40-60°C. In dark tests, bacterial populations presented initial moderate growths at 20-40°C range, followed by intense regrowth. At 40-60°C range, acute thermal inactivation without long-term regrowth predominated at 50°C and was total at 60°C, within the 4-h treatment period. Introduction of light resulted in higher removal rates or permanent inactivation for 800 and/or 1200 W/m^2, respectively. No post-treatment regrowth in the dark was observed after 24 and 48 h, in completely inactivated samples, and its demonstration, when observed, was well correlated to the bacterial numbers at the end of the disinfection period. Statistical observations on the transferred bacterial populations from day to day are also discussed in this paper.

Keywords: Solar disinfection, wastewater, full factorial design, E. coli, regrowth, dark repair

Research Highlights

- 240 solar disinfection experiments were performed, focusing on regrowth
- The effects treatment conditions on bacterial dark repair were evaluated.
- No regrowth was observed in samples with null counts.
- Regrowth was more intense in low-temperature treatment.
- The live fraction at the end of treatment influences short and long-term regrowth.

Graphical Abstract
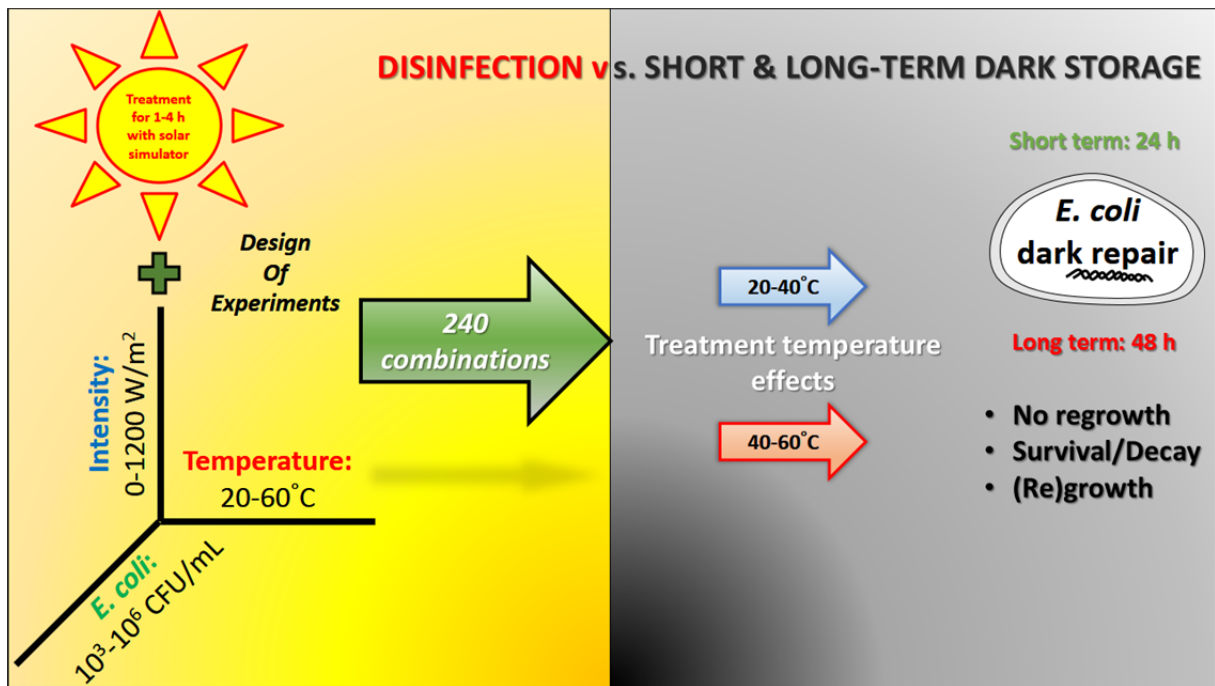
1. INTRODUCTION

The greatest disadvantage of UV disinfection of wastewater, regardless of the source, i.e. either mechanical (UV-C lamps) or physically induced (solar UV disinfection), is its point efficiency, which lacks residual effect (White, 2010). In any UV disinfection unit, the effluent of the process will include inactive (completely decayed microorganisms), injured (not lethally damaged, potentially dangerous if healed) and a fraction of microorganisms that escaped the process. The absence of the residual disinfecting factor could possibly allow the reactivation of injured microorganisms, if favorable downstream conditions are presented (Hijnen et al., 2006; Hallmilch and Gehr, 2010). The remaining bacteria could increase their numbers while being in the treated effluent, due to a variety of reasons; for example, the existence of nutrients and related chemicals in wastewater could provide an abundant food source for the bacteria, allowing them to metabolize and reproduce (Marugan et al, 2010). Hence, the main two factors that are responsible for bacterial regrowth are (Guo et al, 2011): i) the growth of injured microorganisms ii) the reactivation and regrowth of the reactivated microorganisms.

Long after regrowth as a phenomenon was observed, the ''viable but non-cultivable'' (VNC) hypothesis was developed to explain the repopulation of a sample, although appearing microorganism-free at the end of the treatment; this statement provided explanations to similar findings and was adopted by various researchers (Xu et al, 1982; Roszak and Colwell, 1987). This hypothesis suggests that not all the bacteria are destroyed by the action of light, but there is a significant number that is alive, but unable to reproduce.

DNA is one of the main targets of both direct and indirect actions of UV light, through the direct dimerization of thymines or indirect attacks by reactive oxygen species, (ROS) (Pigeot-Remy et al., 2012). The generated ROS have a well-explained action mode, especially hydroxyl radicals; they interact with the intracellular components of the microorganism. Bacteria possess the ability to repair a number of their DNA damages through two main mechanisms: light-dependent ones, namely photoreactivation, and light-independent (dark repair), which help them recover from during photo-exposure.

Photoreactivation is completed by a two-step mechanism. First, there is the formation of a complex between a photoreactivation enzyme (PRE) and the dimer to be repaired (Nebot Sanz et al, 2007) and afterwards, release of PRE and repaired DNA. The restoration of the dimer to its original monomerized form is absolutely dependent upon light energy intensity (Nebot Sanz et al, 2007); the energy needed to repair the damage is provided by visible light (310-480 nm) (Hijnen et al, 2006; Guo et al, 2011).

The dark repair methods are regulated by the expression of recA, a critical gene in the bacterial cell, with well-known properties (Sinha and Hader, 2002; Jungfer et al., 2007). The nucleotide and base

excision repair, includes numerous molecular steps, including identification of the damage, assimilation of a repair complex, incision and removal of the damaged strand and filling with DNA polymerase, finalized by attaching the replaced DNA with the rest of the strand with a ligase (Britt, 1996; Amsler, 2008; Shang et al, 2009).

There is extensive literature on the genetic interpretation of regrowth, as well as experimental findings on the factors that affect this process; among the most common factors affecting regrowth are the effects of temperature (Chan and Killick, 1995; Shang et al., 2009), the salt and nutrient contents of the treated water (Munshi et al., 1991; Rincon and Pulgarin 2004a), the effect of UV dosage and light intensities (Lindenauer and Darby, 1994; Nebot Sanz et al., 2007), the pre-illumination with non-coherent visible and infrared wavelengths (Lage et al., 2000), the initial bacterial population (Craik et al., 2001; Gomes et al., 2009b) and the type of bacterial strain (Rincon and Pulgarin, 2004b). However, most of the works either focus on photoreactivation, employ artificial UVC irradiation, focus on drinking water or treat regrowth exclusively as added value on the evaluation of a treatment method. This occurs due to the fact that dark repair tests offer a good evaluation of the durability of a process, namely the ability to handle post-treatment events.

The present study focuses clearly on bacterial dark repair of previously solar irradiated of secondary effluent. After the extensive works for drinking water in developing regions (Wegelin et al., 1994; McGuigan et al., 1998; Martin-Dominguez, 2005), there is an interest in introducing low-cost treatment methods in developing countries, in order to efficiently help controlling contagious diseases (McGuigan et al., 2012); solar disinfection of wastewater could offer a solution, under certain conditions. A system that could treat the effluent, for instance a series of shallow ponds, and could drastically reduce microbial load, would be of great interest in these areas, where the number of sunny days per year is an order of hundreds (Meichtry et al., 2005). In that manner, there would be an extra source of water, maybe not for direct consumption, but potentially able to enrich local availability, intended for secondary use (Gamage and Zhang, 2010). Such a practice would be of equal interest in both developed and developing countries, since a considerable amount of water could be recovered.

Considering the application point of view, a preliminary approach has been done (Giannakis et al. 2014), in terms of complexity of factors involved, but there are few statistical findings and experimental processes verifying the effect of basic parameters of treatment, for instance, treatment time (Polo-Lopez et al. 2011) and temperature conditions with regard to the dark repair potential of the target bacterial population. Bacterial regrowth has been observed to occur in water samples (Rincon and Pulgarin, 2004b; Sciacca et al., 2010). Wastewater is a rich in nutrients matrix which could support bacterial growth, and given the time treated water could spend in the dark, due to the storage times potentially required to further use reclaimed water, regrowth is rendered as a primary problem in water disposal in natural water bodies or the reuse.

Therefore, in this study we recreate the conditions of solar treatment of secondary effluent and perform a multilevel, full factorial design of experiments (DOE), in order to fully investigate the

122 effects of the treatment conditions, during solar disinfection, on bacterial regrowth. With the

123 application of an experimental design valuable information can be acquired that are not evident due to

124 interaction of the parameters (Montgomery, 2001); the factorial experimental design has been proven

125 an efficient method in bacterial inactivation studies (Rodriguez-Chueca et al., 2012; Giannakis et al.,

126 2014). The parameters under investigation are i) exposure time, ii) temperature, iii) initial population

127 and iv) intensity of the solar simulated light, on *E. coli*-spiked synthetic wastewater, as a model

128 microorganism. After the measurements of the process efficiency, post-treatment control in the dark

129 was made, to estimate the bacterial regrowth/survival capabilities of the treated samples.

2. MATERIALS AND METHODS

2.1. Preparation of the synthetic secondary effluent

The pre-experimental processes involved with the preparation of the synthetic wastewater included two significant parts, the preparation of the *E. coli* solution and the actual wastewater, as follows.

2.1.1. Bacterial culture preparation

E. coli K12 (MG 1655) was acquired from "Deutsche Sammlung von Mikroorganismen und Zellkulturen". A colony was loop-inoculated in pre-sterilized 5 mL Luria-Bertani broth; for each L of sterile distilled water, 10 g Bacto^{TM} Tryptone, 5 g Yeast extract and 10 g NaCl were added. 25 mL sterile plastic falcons, containing the spiked LB, were incubated for 8 h and another 1/100 dilution to LB solution (2.5 mL sample into 250 mL LB) was incubated for another 15 h. Bacterial cells were then centrifuged (5000 rpm for 15 min) and washed 3 times with sterilized saline solution (8 g/L NaCl and 0.8 g/L KCl). The bacterial pellet was dispersed in fresh, sterilized saline solution, forming a solution with 10^9 CFU/mL initial population.

2.1.2. Synthetic wastewater composition

The employed wastewater was a 1/10 dilution of the presented in Table 1, instructed by OECD (1999). 1 mL of the prepared (10^9) bacterial solution was added per liter to obtain a bacterial concentration of 10^6 CFU/mL. In order to obtain 10^3, 10^4 and 10^5 CFU/mL, dilution of the same proportion (wastewater/distilled water = 1/10) were done.

2.2. Suntest solar simulator

The artificial solar simulator employed in our experiments employed was a Suntest, acquired from Hanau. It bears a 1500 W air-cooled Xenon lamp, and provides 560 cm^2 effective illumination surface. 0.5% of the emitted photons belong to the UVB area and 7% in UVA. Cut-off filter ensures no UVC is emitted and IR as well. The spectrum above 400 nm follows the natural solar one. The intensity levels were measured by a Kipp & Zonen Mod. CM3 and CUV3 radiometer.

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2.3. Batch reactors

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All tests were performed in cylindrical glass reactors, with double walls that allow recirculation of thermostated water, for temperature control. The effective irradiation surface was 20.41 cm^2. Also, mild stirring took place during all the experiments with a magnetic stirrer; sampling was always done while stirring, from the body of the sample.

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2.4. Sampling and post-experimental handling of samples

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Sampling was performed in hourly manner and irradiated microorganisms were kept in plastic vials in the dark, covered by aluminum foil, in room temperature (20°C). Regrowth tests were conducted exactly after 24 and 48 hours from the sampling time. An important point is that the samples were kept in sterile vials for the said period to avoid enhanced bacterial regrowth (Sciacca et al., 2010).

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2.5 Bacterial enumeration

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Viable bacterial counts after solar treatment were assessed by pour-plating on non-selective agar as suggested by Reed (2004) and Rizzo (2004), in order to obtain all viable counts, after proper dilution in sterile saline solution to achieve measurable counts on the dishes (15-150 colonies). Experiments were performed with duplicate plating in three consecutive dilutions. Difference was less than 5% and maximum 10% in undiluted samples, therefore, error bars will be omitted for reasons of clarity, only the average counts.

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2.6. Experimental design set-up

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A multilevel, full factorial DOE was employed to assess the influence of i) treatment time, ii) temperature, iii) initial bacterial population and iv) light intensity. The full factorial design allows measuring the response (i.e. disinfection and/or regrowth after 24 and 48 h) in all different levels and combinations (Rodriguez-Chueca et al., 2012). MINITAB for Windows was used to analyze the data. Table 2 summarizes the selected parameters, as well as their respective levels of study.

3. RESULTS AND DISCUSSION

3.1. Disinfection Experiments

Figure 1 summarizes the results obtained through the DOE focused on the study of treatment time, temperature during treatment and initial bacterial population. Their effects on disinfection efficiency, are grouped by the three intensity levels, for clarity. A detailed study on the antagonistic and synergistic effects of temperature was previously performed (Giannakis et al., 2014), whose summary is presented here. Figure 1a summarizes the results in absence of light, 1b the 800-W/m^2 results and 1c the 1200-W/m^2 ones, respectively. The accompanying Table 3 is also grouped in three distinct areas, according to the applied irradiation intensity and presents the percentage of removal only at the end of the 4-h period of treatment, excluding the cases of 0 W/m^2, temperatures 20, 30 and 40˚C; removal rate was always 0 and growth rates are presented instead.

From Figure 1a and Table 3, we draw the information that when no irradiation is applied the disinfection process is temperature-driven. However, *E. coli* are mesophilic microorganisms that demonstrate their maximum growth in the most comfortable temperature for them, around 37˚C (Fotadar et al., 2005). Therefore, taking into account the favorable existence of nutrients and salts in the system (Marugan et al., 2010) a different (increasing) growth rate for each temperature range is observed, until 40˚C, when it reaches its peak. After this point, at 50˚C and even more at 60˚C, thermal inactivation dominated the outcome of the experiment, near-total and total inactivation after 4h of exposure to heat. This is somewhat expected, since the thermal stress applied to the cells is denaturizing proteins and alters cell membrane significantly, up to a fatal point (Blaustein, 2013). For the study of both disinfection and regrowth, this will be considered as a boundary condition and all cases will be studied separately.

When light is applied to the system, there is an extra stress inflicted on the system. The solar simulator emits photons within the UVB, UVA and visible light region. Literature suggests the mode of action of light against bacteria, summarized in direct DNA strand damage (Hallmilch and Gehr, 2010; Matalana-Surget, 2012) and indirect damage through reactive oxygen species (ROS) production (Regensburger et al., 2011), due to UVB light. UVA damages the cells indirectly, also through ROS generation inside and outside the cell (Spuhler et al., 2010; Pigeot-Remy et al., 2012). Also, synergy between light and temperature is reported (McGuigan et al., 1998; Rincon and Pulgarin, 2004c), which enhances the disinfecting action.

This is also observed in our case, where we notice elevated removal rates when 800 W/m^2 irradiance was applied, for all temperature levels, although higher for the higher temperatures (Figure 1b, Table 3). Normally, the maximum irradiance value reaching Earth's outer layers of atmosphere is 1360 W/m^2 and around the equator, the normal values fluctuate around 1120 W/m^2 (McGuigan et al., 2012).

229   However, in low temperatures, the growth rate is disrupting the expected inactivation behavior, with
230   this mitigation effect increasing towards 40˚C. This intensity level was proven enough to control
231   excess growth, but did not provide proper disinfection in this timeframe. However, when 1200 W/m^2
232   were inflicted, the balance between the growth and the inactivation coming from the light actions has
233   turned to the disinfection side, demonstrating total inactivation in 4 h for all temperatures and initial
234   population levels. The synergy between light and temperature is reflected in disinfection times, where
235   4 h were required for low temperatures, a little less for 50˚C and 0.5 h for 60˚C (Figure 1c, Table 3).

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237   ## 3.2. Parameters affecting survival and regrowth after 0 W/m^2 irradiation experiments

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239   As far as the post-treatment events are concerned, we divide the behavior of *E. coli* into two groups:
240   treated under mild temperatures (20-40˚C) or treated in higher temperatures than 40˚C. The first group
241   of graphs presenting the experiments performed in lower temperatures (Figure 2a), demonstrates a
242   high increase of the bacterial population, influenced by the pre-treatment conditions. It is clear that the
243   samples treated at 40˚C, present higher dynamics of growth and relatively higher final counts after 24
244   and 48h. Also, there is visible influence of the initial population, by which higher initial populations
245   result in higher reproduction rates after 48 h. In addition, we can notice a gradual decrease in growth
246   rates between the 1st and the 2nd day of storage, probably interpreted by the stress caused by some
247   initial nutrient shortage, due to the overgrown bacterial numbers.

248   Figures 2b and 2c are the contour plots that visualize all regrowth tests, performed by hourly sampling
249   in all temperatures and initial population rates. They reveal that there is a correlation between the
250   treatment temperature and the regrowth after 24 or 48 h (expressed by C_{24}/C_0 and C_{48}/C_0). These
251   fractions reveal the regrowth of the bacterial numbers higher than the initial one; if the ratio is <1, then
252   we observe survival, instead. Lower temperatures present suppressed rates, compared to higher ones.
253   Also, we notice the difference between the bacterial number after 24 h and 48 h, being influenced by
254   the disinfection conditions, which is expressed in orders of magnitude. Plus, temperatures that initially
255   seemed safer against regrowth (around 25˚C), demonstrate equally high rates. In figures 2d and 2e, the
256   correlation between treatment time and regrowth is presented; the prolongation of the experiment has a
257   profound effect in the bacterial numbers observed after 2 days. However, initial concentration cannot
258   be attributed to a direct effect. In the last sub-graphs which present the main effects of the temperature
259   on regrowth, elevating temperature during treatment is observed to have a strong and rather linear
260   impact only over 30˚C for the regrowth after one day, and stronger for after two days.

261   The samples treated under higher temperatures (Figure 3a) do not present any recovery of the
262   population; the population, if any bacteria still existed, continued the decay during dark storage. For
263   the bacterial samples treated at 50˚C, although total inactivation was not observed, after 24h no viable
264   counts were observed. As it seems, the thermal damage rendered bacteria unable to reproduce; no

265 repair mechanism was observed to act. The remaining samples, after their treatment at 60˚C, presented

266 the same behavior. Higher temperatures accelerated inactivation, which was total within the 4-h

267 timespan, and no regrowth was observed thereafter.

268 Contour plots 3b and 3c, present the survival rates after 24 and 48 hours, for all hourly samples taken

269 during disinfection. First of all, high regrowth risk (C_{24}/C_0 and $C_{48}/C_0 \geq 1$) is observed around 50˚C and

270 for 60-90 min of treatment. The survival pattern for the rest of temperatures and time is consistent, for

271 the two post-treatment days, and slightly more elevated numbers are observed after 2 days. The main

272 effects plots (figures 3d and 3e) demonstrate the inverse effect that high-temperature treatment has on

273 regrowth; as time passes, survival capability is diminishing, and as temperature increases, we observe

274 the same effect. However, initial population follows a similar pattern from the first to the second day.

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276 ## 3.3. Effects of 800 W/m^2 irradiance on the parameters affecting survival and regrowth

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278 Figures 4 and 5 present the extension of monitoring the bacterial population for 48 more hours after

279 800-W/m^2 intensity irradiation is complete. Results are grouped per temperature range (20-40˚C and

280 50-60˚C) and initial concentration of bacteria. It can be deduced that post-irradiation survival is more

281 complex, compared to the experiments in absence of light.

282 The first temperature range (20-40˚C, Figure 4) demonstrates very low inactivation rates, and as a

283 consequence, presents elevated (re)growth/survival rates; since there is no total inactivation taking

284 place (i.e. zero viable counts), the recovery of the bacterial numbers could be attributed to i) alive

285 bacteria that continued replicating, ii) bacteria that recovered their DNA lesions by dark repair

286 methods, and growth of the revived bacteria (Guo et al., 2011).

287 The contour plots (Figure 4b and 4c) demonstrating the bacterial population after 24 or 48 h, reveal an

288 interesting behavior, as far as the influence temperature is concerned. Although 40˚C is a breaking

289 point, where bacterial disinfection is drastically changing, it appears that 30˚C is the most critical

290 value for regrowth. First of all, after 24 h, regrowth is not probable, and only occurred from samples

291 treated around 3-4 h and 30-40˚C. On the contrary, samples that were treated in low temperatures and

292 for short time, present low counts after 24 h.

293 Normally, bacteria in samples that remain for longer time under illumination tend to get more

294 inactivated, as it is shown in figure 4a. However, prolonging their treatment in this favorable

295 temperature promotes multiplication and therefore, new strains, that gain resistance against solar

296 irradiation in conditions of exposure to (visible) light (Hijnen et al, 2006; Nebot Sanz et al, 2007;

297 Shang et al, 2009). This bacterial ability is a heritage of evolution through time, to protect themselves

298 from the natural ultraviolet rays from the sun (Quek and Hu, 2008).

As a consequence, higher remaining populations led to higher survival rates from the bacteria. Although Lindenauer and Darby (1994) supported that no significant correlation exists between regrowth and the initial number of coliforms in wastewater, at any dose, they found out that in low doses, the surviving coliforms affected the reactivation rates. Craik et al (2001) explained this noting that if the initial population is high, there is a big chance that there will be a part of it going through unharmed due to shielding (by each other) and bad mixing.

After 48 h, we notice a change in the effect; in figure 1c, we observe that samples treated in lower temperatures and for shorter times, demonstrate higher regrowth rates and samples that presented regrowth show 5-fold suppressed rates, instead. This is clearly demonstrated in the main effects plot, where treatment times reveal inverse action, and 30˚C reveal their statistical significance in regrowth. This can be explained, mostly by the action of light; samples that were treated for a short time accumulated a relatively low dose, and were able to recover their cultivability, whereas samples that were treated in high temperatures (and showed high regrowth), remained for a long time under illumination, and their repair capabilities were diminished.

The behavior of bacteria that were treated in high temperatures is more straightforward. First of all, almost no regrowth is observed; all values for C_{24}/C_0 and C_{48}/C_0 are <1. Hence, we can deduce that it is crucial to obtain null bacterial counts at the end of the experiments (total inactivation) in order to avoid their re-appearance. The combined action of light and temperature, and the joint actions are proven to be not only more efficient (faster), but hinder re-population as well. Among the figures 5b and 5c, that picture bacterial survival after 24 and 48 hours, the highest survival rates have appeared around 1.5-2 h, but are still low ones. This peak is explained by the influence of the type of concurring actions in the batch tests employed in this study: we mentioned that there is an equilibrium of growth and inactivation, and it appears to bend, in favor of inactivation, at this time point, for 50˚C. Beyond this time mark, inactivation is higher, and as inactivation negatively influences regrowth, lower rates are observed. Finally, in the main effects plot in figures 5d and 5e, temperature and time have a straightforward effect, where prolongation of treatment equals to regrowth suppression; this is considered normal, since higher experimental times assists both bacterial protein damage and light inactivation.

3.4 Effects of 1200 W/m^2 irradiance on the parameters affecting survival and regrowth

In Table 3, the total inactivation achieved after 4 h in all samples has been demonstrated, in all temperature ranges and initial population, at 1200 W/m^2. As it seems, apart from the contribution of temperature we have verified the beneficial effect for switching from thermal to light/thermal treatment, now it is evident that light has a significant, additional role in bacterial inactivation (Ubomba-Jaswa et al., 2009); for the same temperature levels and initial bacterial population in the

samples, the outcome was altered, when intensity was increased from 800 to 1200 W/m^2. The synergy of light and temperature has reached the maximum inactivating action (among our cases), leading to null bacterial counts, at the end of the treatment, for another 2 days.

When moderate light (800 W/m^2) was applied and the conditions favored disinfection (all cases of 60˚C treatment and 10^3–10^4 at 50˚C), no regrowth was observed. Common denominator in all cases was a null bacterial count active at the end of the process. Therefore, it is expected that no regrowth will be observed. Figure 6a demonstrates the post-treatment phenomena, after the illumination of the varied population samples subjected to the different process temperatures.

In the previous cases, only the outcome after the end of the treatment is plotted, for clarity. However, the contour plots of C_{24}/C_0 and C_{48}/C_0 (figures 6a, 6b and 7a, 7b) contain information, for the fate of the microbial population at each hour and level of population and temperature. We observe that there are only two combinations that led to regrowth, deriving from samples that were irradiated for only 1 h, between 20 and 40˚C and of high risk are the next 30 min for all temperatures. In this case, there is shortage of dose accumulation from the cells, so the reactivation is highly probable. This is reflected in the regrowth rates in day 2, with the excess growth effects around 40˚C playing the most important role in regrowth appearance.

The effect of time, demonstrated in the main effects plots (figures 6 c and 6d) is in favor of bacterial inactivation; firstly, prolonging the samples in such high intensities renders bacteria unable to recover or deploy defense mechanisms, because the incoming photonic rate is very high to cope with, and secondly, we observe that after 2 h of treatment, C_{24}/C_0 and C_{48}/C_0 are less than 1, and therefore, no regrowth is observed. Finally, temperature produces the same obstacles stated in the previous section, against inactivation, but high intensities overcome this effect.

The most effective combination, of high intensity and elevated temperatures, is demonstrated in figure 7, and shows a very low survival potential and also, for the first time, it is decreasing from day to day. The surviving populations are very low in and in condition unable to recover neither their numbers nor their cultivability and decay day by day. The main effects plots (figures 7c and 7d) demonstrate the negligible differences time and temperature have in survival. However, both main effects plot between 20˚C-40˚C and 40˚C-60˚C allow a good comparison on the effect of light intensity, if compared with the respective ones of 800 W/m^2 and 0 W/m^2. It is clear that although temperature has a strong effect, it affects (re)growth indirectly, through cell growth effects and thermal inactivation. Temperature on the other hand shows that it is the main active force leading to suppressed risk of bacterial re-appearance. For 800 W/m^2, repair was possible, whereas for 1200 W/m^2, even after 1-2 h of exposure, bacteria have lost their ability to perform dark repair of their damage.

3.5. Bacterial regrowth vs. disinfection efficiency

371   Our study has employed direct plating to measure cultivable bacteria, therefore regrown or surviving

372   bacteria are treated as one, cultivable entity. Also, we have rather avoided suggesting an influence of

373   the initial bacterial population, because of the lack of a straightforward correlation or tendency. Each

374   population level withholds its own special effect; for instance, initial population of 10^3 bacteria

375   encounter more available nutrients per cell and initial population 10^6 offer higher chances of

376   aggregation and shielding; in both cases, surviving bacteria are offered an enhanced possibility of

377   (re)growth. Therefore, in order to be able to correlate the influence of starting bacterial population in

378   the regrowth period, some statistical indicators were used. A main target was to homogenize results,

379   regardless of initial population, to aid the overall robustness of the treatment.

380   Figures 8a and 8b demonstrate the correlation between the efficiency of the disinfection process, for

381   all possible treatment times (1 to 4 h) and the consequent regrowth, for samples that have been treated

382   in low (20˚C≤T≤40˚C) or high temperatures (40˚C<T≤60˚C). The ▲ traces reveal the population after

383   24 h while the ▲ traces, after 48 h, expressed as the fraction of bacteria/initial population, for

384   homogenization of the 20˚C≤T≤40˚C results, regardless of initial bacterial numbers. We observe that

385   in overall, the population after 48 h is tending to be higher than the population after 24 h. It also

386   appears that as efficiency increases, the samples without regrowth are increasing (line indicating

387     $C_{24,48}/C_0$ ratio=1), and a tendency to reduce their regrowth potential, according to the percentage of

388   efficiency increase. However, for higher temperatures, we notice the significant absence of regrowth

389   after 24 h (trace:✚) (line indicating $C_{24,48}/C_0$ ratio=1) and the suppression of growth after 48 h

390   (trace:✚), compared to the lower temperatures. Hence, treating in higher temperatures is detrimental

391   in both short and long-term storage of the treated samples.

392   Furthermore, we calculated the alive (cultivable) number of bacteria left at the end of the process, and

393   plotted with the population after 24 and 48 h, for both low (figure 8c) and high temperatures of pre-

394   treatment (figure 8d). Figure 8c demonstrates a constant live bacteria/initial population ratio

395   fluctuating around 1 after 24 h of treatment (trace:●), but the bacterial numbers after 48 days (trace:●)

396   seem to decrease, as the live fraction increases; lower populations would be expected when the live

397   fraction is lower. This indicates that the correlation between the pre-treatment and regrowth is not

398   limited to the live fraction at the end of the given treatment time (1 to 4 h), but is linked to the

399   treatment method. For instance, a low surviving fraction, deriving from a short-treatment time in low

400   intensity is very susceptible to regrowth. The opposite statement, for higher light intensities and low

401   temperatures to expect low regrowth, is validated as well. Special mention should be made at the non-

402   treated samples (live fraction = 1) that always present (re)growth. In contrast, in figure 8d, plotting the

403   higher temperature experiments, we do not find live bacteria at 100%, but we observe less regrowth

404   after 24 (trace:■) and 48h (trace:■). Also, a higher number of experiments present near-zero regrowth,

405   compared with the low-temperature experiments. Even samples that presented 90% live bacterial

406   fraction present diminished numbers, with obvious positive effects of high temperature in suppressing

407   regrowth.

408   Finally, figure 9 presents an estimation of the bacteria transferred from the end of the treatment time to
409   the first day and from these ones, in the second day. On X axis, we plot the final live fraction of
410   bacteria after 24 h, due to the bacteria at the end of treatment time i (i=1-4 h) per initial concentration
411   and on Y axis the respective ones for 48 h storage. This ratio assesses the transferability of bacterial
412   growth from day 1 to day 2 and expresses the fate at the end of the treatment time; i.e. values >1
413   indicate higher numbers after 48 h, due to the live fraction in 24 h. Mathematically, this ratio is
414    $\frac{C_{24}/C_0}{C_i/C_0}$ or $\frac{C_{48}/C_0}{C_i/C_0}$, and is expressed as C_{24}/C_i or C_{48}/C_i, respectively. As it seems, the transferability from
415   day 1 to day 2 is strongly influenced by the treatment temperatures during the experiment; for low
416   temperatures 20˚C≤T≤40˚C, we observe that the same fraction of live bacteria after 1 day can yield
417   higher fractions after 48h (trace:●) than the respective 40˚C<T≤60˚C ones (trace:●). For example, 24-
418   h ratios of 1 or 10 can result in much higher ratios (up to 1000) after 48 h. It is shown that i) there is no
419   repair on the damages inflicted by temperature and ii) the synergistic action of light and temperature
420   ensures low transferability from the surviving fraction. The dominant trend existing in regrowth is also
421   expressed by the logarithmic equations and the possibility of increased appearance after 2 days is
422   reflected by the constants of the equations which describe that trend.

423   In overall, there is a lighter regrowth risk when high temperatures of treatment are applied. However,
424   this condition is not always applicable, when it comes to the existing solar disinfection techniques. In
425   that case, either higher light intensities must be accounted for, low (around 20˚C) ambient
426   temperatures or maybe, prolongation of the exposure time can compensate the risk of remaining
427   bacteria in the solution. In this manner, either light action will be enhanced, bacterial division will not
428   be favored or extended damage will be inflicted, to ensure low live fractions at the end of the
429   treatment; it was proved that this condition, regardless the pre-treatment condition, is a precursor of
430   the bacterial numbers in short or long term storage of water.

431

5. CONCLUSIONS

433

434   •   Non-irradiated samples of secondary effluent treated at 20-40°C showed slight growth during
435   treatment, and high post-treatment regrowth (ratios of 250-1000). Significantly, thermal inactivation
436   with no regrowth predominated at 50°C and was total at 60°C.

437   •   At 800 W/m^2, bacterial regrowth only occurred in incompletely disinfected samples, which are
438   linked to lower irradiation, shorter times or high initial microorganism populations. No regrowth was
439   observed in samples presenting no bacterial counts at the end of the treatment. An erratic behavior was
440   observed when treatment temperature was among 20-40˚C, where prolongation of treatment resulted
441   in higher long term re-appearance of bacteria in the samples, related to growth issues after 30˚C.

- High intensities revealed almost no regrowth (special cases: 1-h treatment), for low temperatures, revealing the detrimental effect of elevated light intensities, whereas the combination of high temperatures with high intensity resulted in no regrowth and survival diminishing, as well, due to the very high levels of synergetic action between light and temperature.

- When present, regrowth was directly connected to the enumerated leftover bacteria. The lower temperature region promoted bacterial regrowth (max. in 30°C) and high temperatures suppressed the reappearance, both in short and long term storage. Also, the lower temperature set demonstrated higher rate of transferring their live bacteria from the end of the treatment time towards the next days, than high temperatures.

- The temperature range for light-temperature synergy (40-60°C) is well above the common temperatures in shallow ponds, even in tropical countries, while a normal sustained intensity lies around 800-900 W/m^2.

- Our study suggests that contact times longer than the 4 h observed here would be required at field conditions. Other field factors should be investigated, like shielding by particles (residual suspended solids, algae), for they would extend required exposure time to days.

6. ACKNOWLEDGEMENTS

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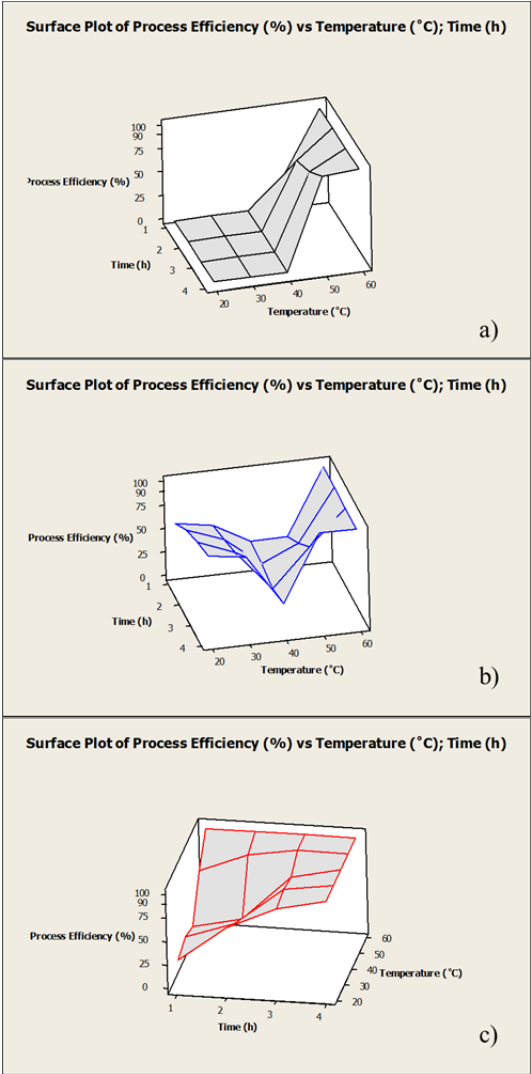
Figure 1 – Overview of disinfection experiments. Process efficiency vs. treatment time and temperature is plotted. a) 0 W/m^2. b) 800 W/m^2. c) 1200 W/m2
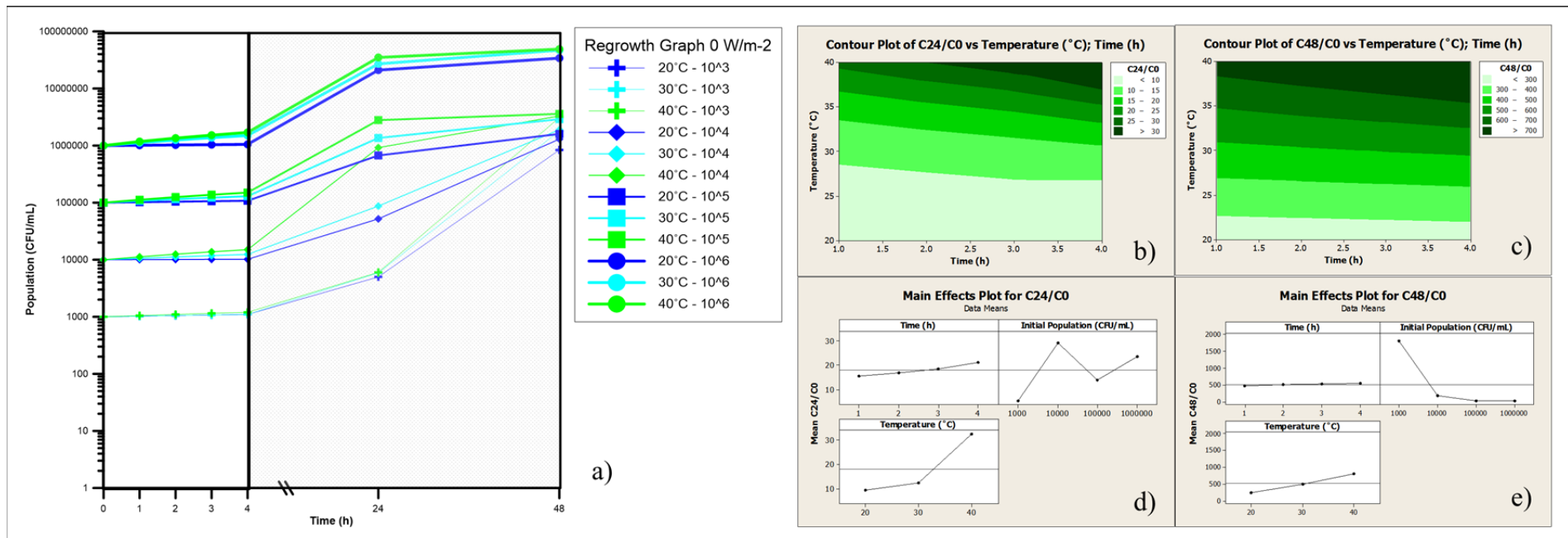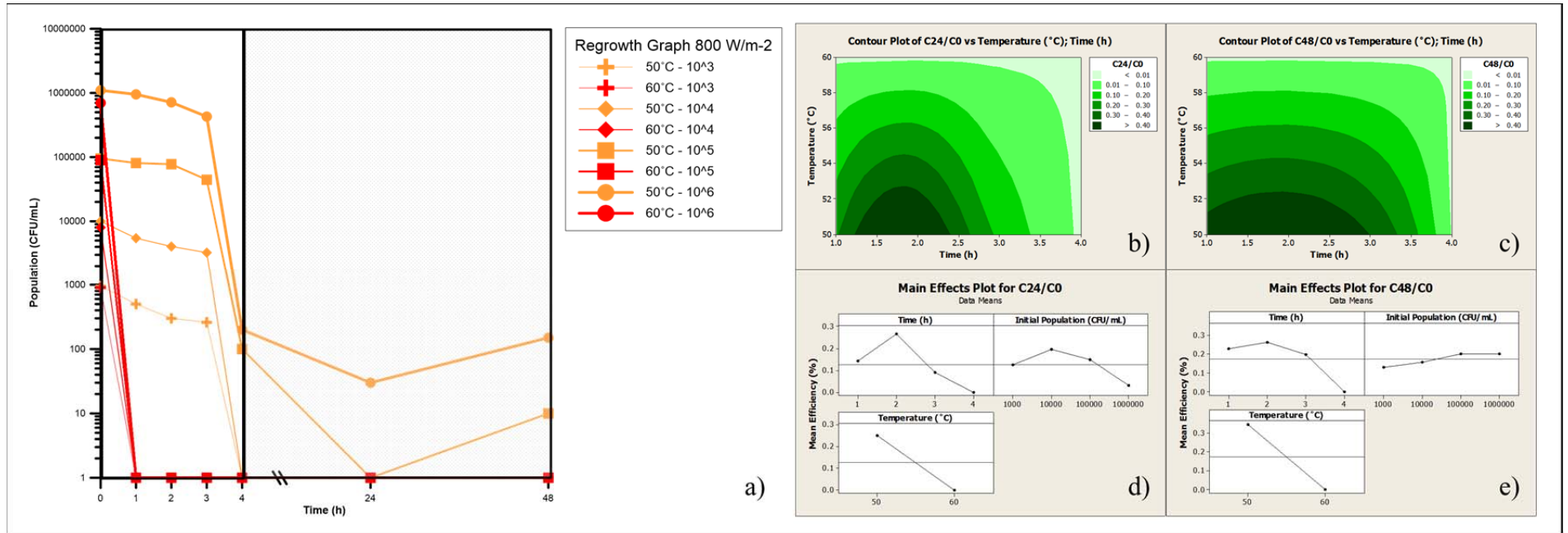
Figure 2 - Main results of non-irradiation experiments for synthetic secondary effluent at among 20-40°C and all initial E. coli populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: Regrowth after 1 day). (e) Main effects plot (control variable: Regrowth after 2 days).
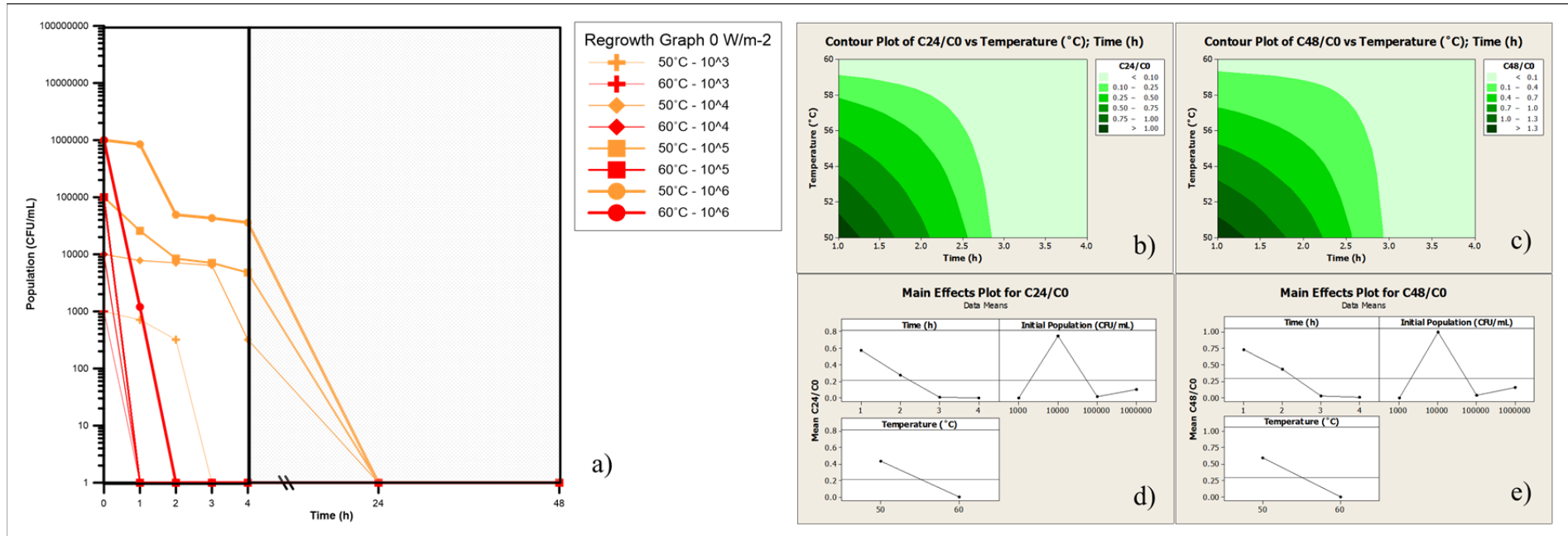
Figure 3 - Main results of non-irradiation experiments for synthetic secondary effluent at among 50-60°C and all initial E. coli populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: Regrowth after 1 day). (e) Main effects plot (control variable: Regrowth after 2 days).
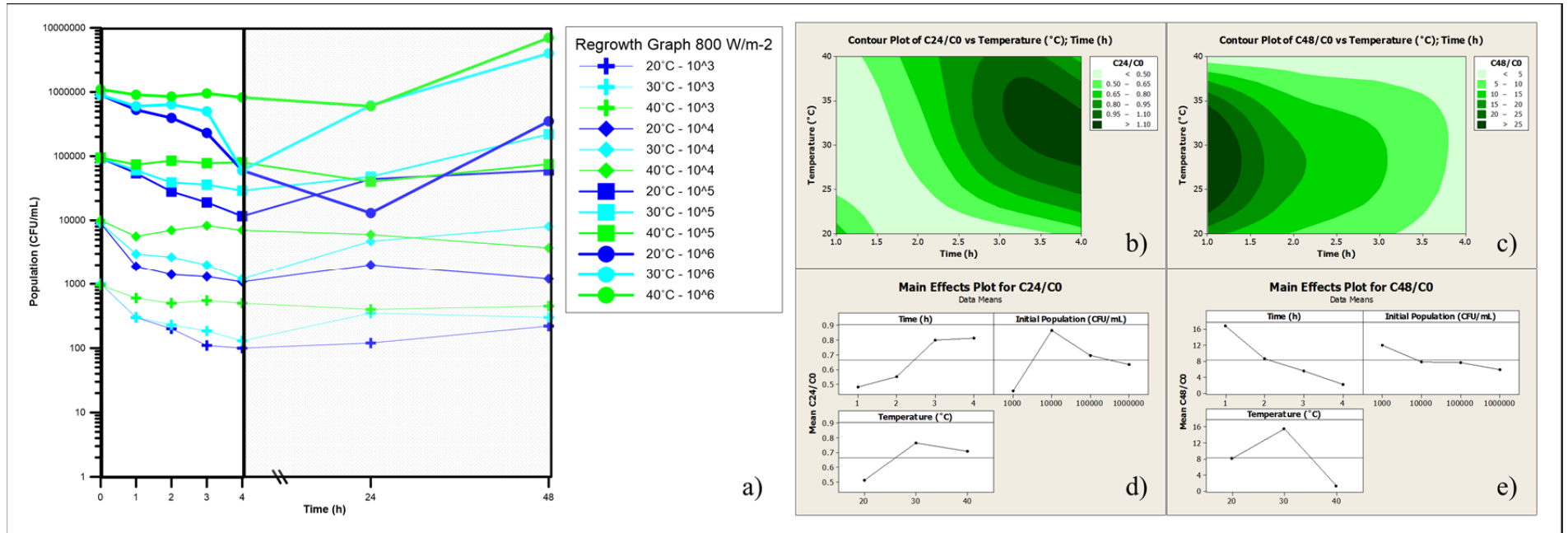
Figure 4 - Main results of 800 W/m^2-irradiated experiments for synthetic secondary effluent at among 20-40°C and all initial E. coli populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: Regrowth after 1 day). (e) Main effects plot (control variable: Regrowth after 2 days).

Figure 5 - Main results of 800 W/m²-irradiated experiments for synthetic secondary effluent at among 50-60°C and all initial E. coli populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: Regrowth after 1 day). (e) Main effects plot (control variable: Regrowth after 2 days).
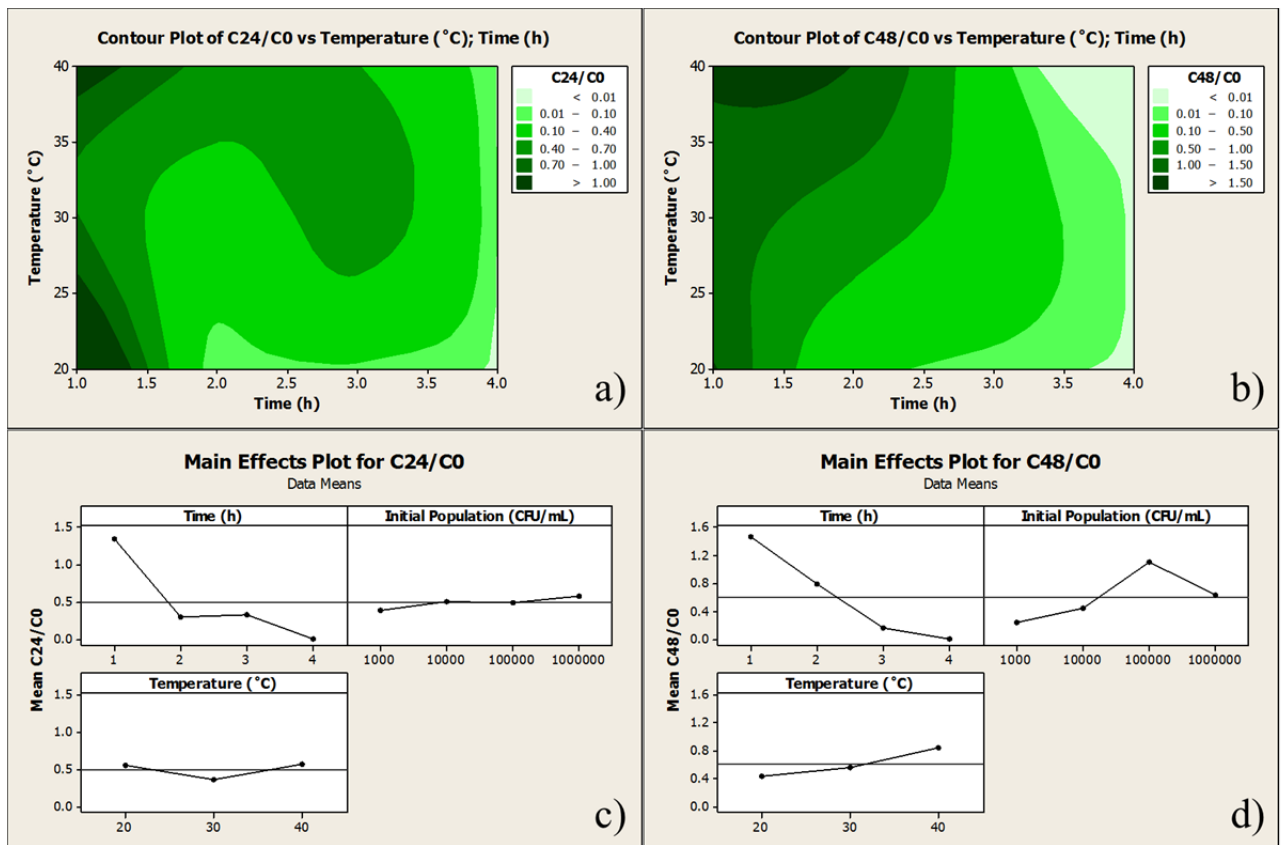
Figure 6 – Overview of the 1200 W/m^2-irradiation experiments, among 20-40°C and all initial E. coli populations. (a) Contour plot of regrowth after 1 day vs. temperature and time. (b) Contour plot of regrowth after 2 days vs. temperature and time. (c) Main effects plot (control variable: Regrowth after 1 day). (d) Main effects plot (control variable: Regrowth after 2 days).
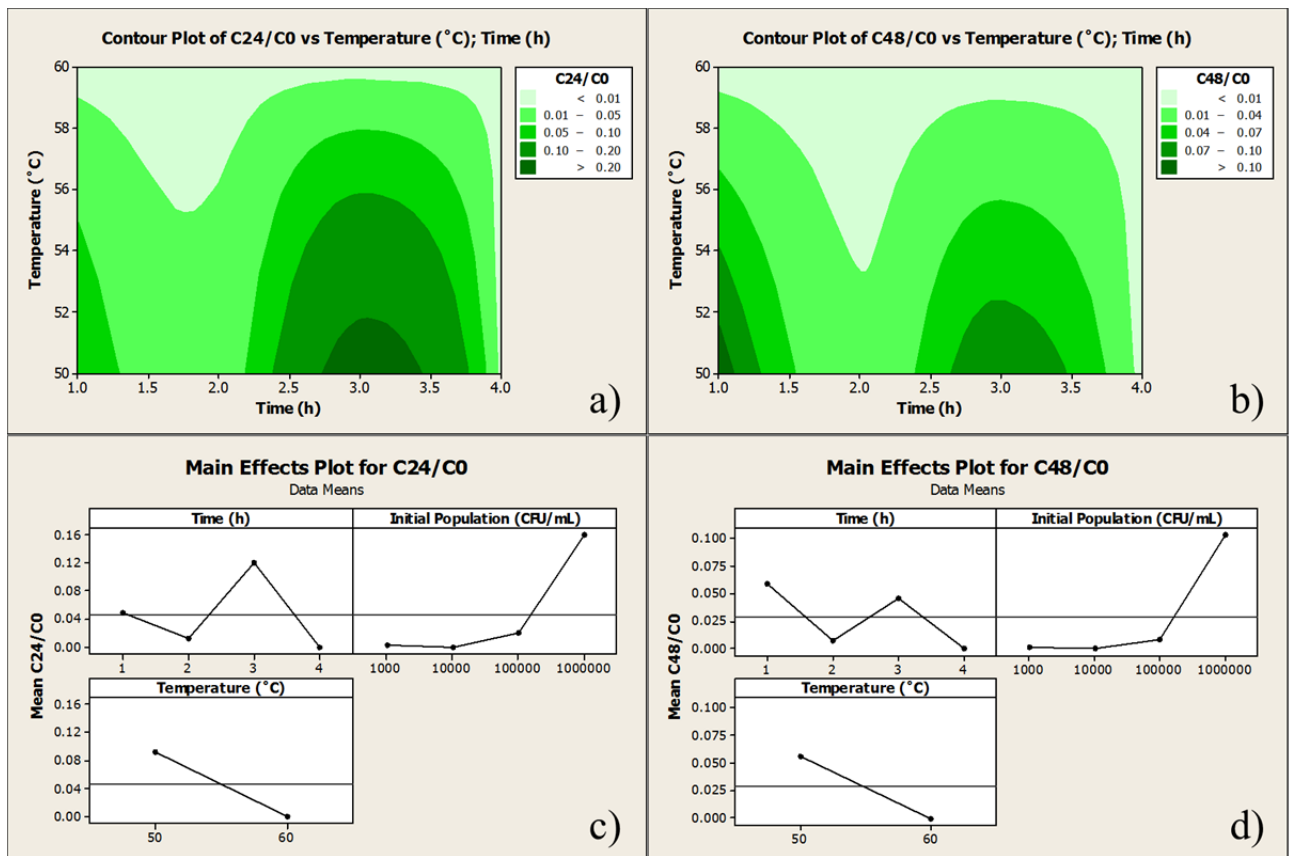
Figure 7 - Overview of the 1200 W/m^2-irradiation experiments, among 50-60°C and all initial E. coli populations. (a) Contour plot of regrowth after 1 day vs. temperature and time. (b) Contour plot of regrowth after 2 days vs. temperature and time. (c) Main effects plot (control variable: Regrowth after 1 day). (d) Main effects plot (control variable: Regrowth after 2 days).
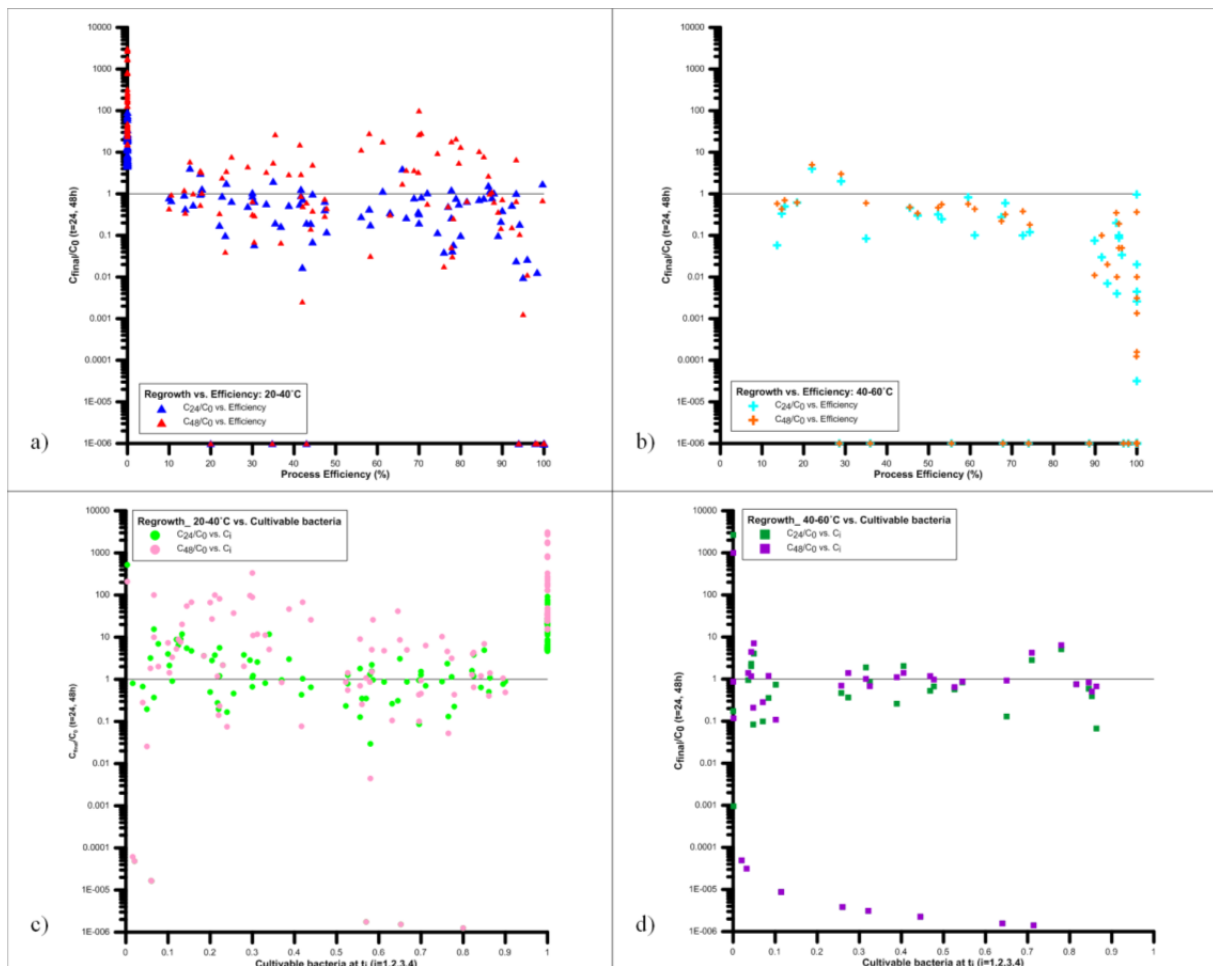
Figure 8 – Statistical interpretation of regrowth vs. disinfection efficiency. (a) Efficiency vs. Regrowth after 1 day. (b) Efficiency vs. Regrowth after 2 days. (c) Live fraction at the end of the treatment period (1-4 h) vs. Regrowth after 1 day. (d) Live fraction at the end of the treatment period (1-4 h) vs. Regrowth after 2 days.
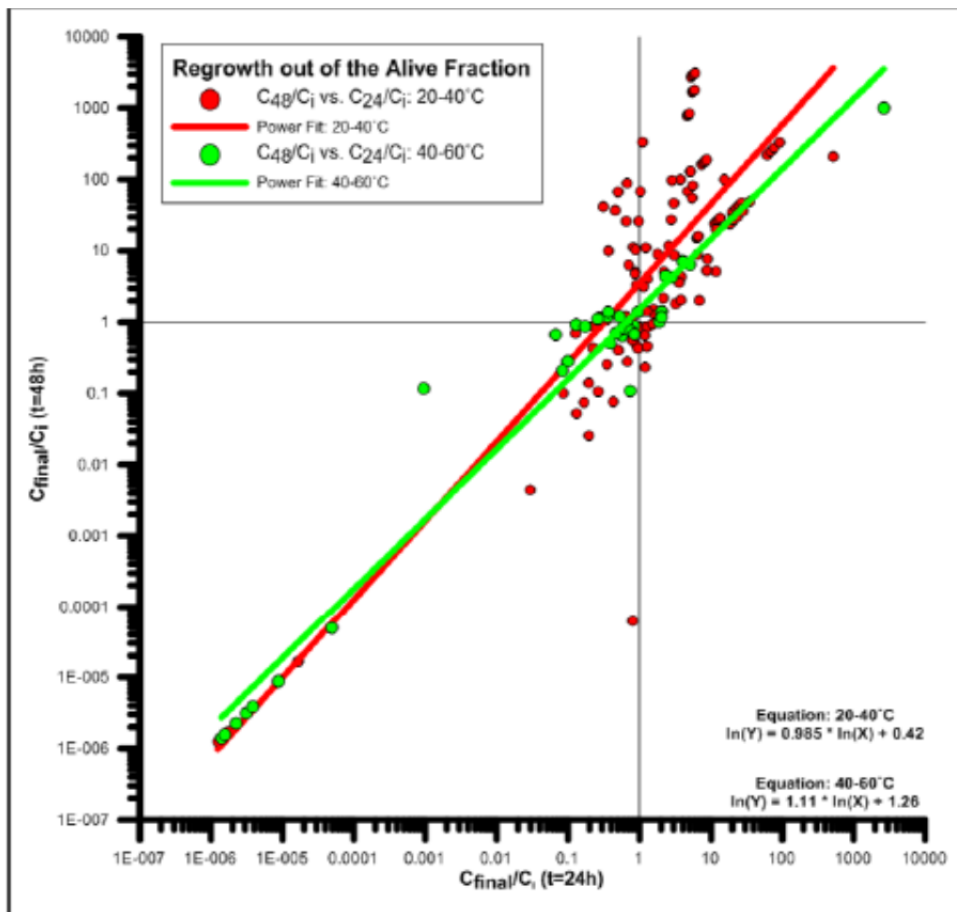
Figure 9 – Transferability of live bacteria through the post-irradiation treatment period. Regrowth after 24 h out of the live fraction subjected to i hours of treatment (i=1-4 h) vs. Regrowth after 48 h.

Table 1 – Synthetic wastewater composition

Chemical composition of the synthetic municipal wastewater before dilution

Chemicals Concentration (mg/L)	
Peptone	160
Meat extract	110
Urea	30
K_2HPO_4	28
NaCl	7
$CaCl_2 \cdot 2H_2O$	4
$Mg_2SO_4 \cdot 7H_2O$	2

Table 2 – Disinfection conditions employed in the DOE.

Parameters	Levels
Time (h)	1, 2, 3, 4
Initial Population (CFU/mL)	10^3, 10^4, 10^5, 10^6
Temperature (°C)	20, 30, 40, 50, 60
Light Intensity (W/m^2)	0, 800, 1200

Table 3 – Inactivation efficiency % after 4 h (at the end of each treatment method) for 0, 800 and 1200 W/m^2.

Intensity	Population (CFU/mL) / Temperature (˚C)	10^3	10^4	10^5	10^6
0 W/m^2	20˚C (% growth)	*10*	*2*	*8*	*5*
	30˚C (% growth)	*10*	*24*	*30*	*50*
	40˚C (% growth)	*20*	*50*	*50*	*70*
	50˚C	100	96.8	95.2	95
	60˚C	100	100	100	100
800 W/m^2	20˚C	90	88	87.5	93.3
	30˚C	87	86.7	68.8	93.3
	40˚C	47.4	30	15.8	25
	50˚C	100	100	99.9	99.9
	60˚C	100	100	100	100
1200 W/m^2	20˚C	100	100	100	100
	30˚C	100	100	100	100
	40˚C	100	100	100	100
	50˚C	100	100	100	100
	60˚C	100	100	100	100