

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/m²) 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 to 40 °C and 40 to 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/m², 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.

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1. Introduction

The greatest disadvantage of UV disinfection of wastewater, regardless of the source, i.e. either UV-C lamps or solar UV disinfection, is its point efficiency, which lacks residual effect [41]. 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 [13,12]. 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 [18]. Hence, the main two factors that are responsible for bacterial regrowth are [11]: (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 [42,35]. 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) [25]. The generated ROS have a well-explained action mode, especially hydroxyl radicals;

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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 [23] 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 [23]; the energy needed to repair the damage is provided by visible light (310–480 nm) [13,11].

The dark repair methods are regulated by the expression of *recA*, a critical gene in the bacterial cell, with well-known properties [38,14]. 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 [4,1,37].

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 [5,37], the salt and nutrient contents of the treated water [22,30], the effect of UV dosage and light intensities [16,23], the pre-illumination with non-coherent visible and infrared wavelengths [15], the initial bacterial population [6,10] and the type of bacterial strain [31]. 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 [20,17,45], there is an interest in introducing low-cost treatment methods in developing countries, in order to efficiently help controlling contagious diseases [21]; 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 [3]. 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 [8]. 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 [9], 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 [26] and temperature conditions with regard to the dark repair potential of the target bacterial population. Bacterial regrowth has been observed to occur in both in water [31,36] and wastewater samples [43]. 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, regrowth is rendered as a primary problem. Since the goal is to increase the water supplies of a specific region, regrowth of bacteria in the natural environment could possibly mean a re-contamination of downstream water supplies. In both cases of aquifers used for drinking water, or, water reuse for irrigation, the limits set by the World Health

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 ₂ HPO ₄	28
NaCl	7
CaCl ₂ ·2H ₂ O	4
MgSO ₄ ·7H ₂ O	2

Organization could be exceeded a posteriori [44]; either result in dangerous conditions for the end-users.

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 effects of the treatment conditions, during solar disinfection, on bacterial regrowth. With the application of an experimental design valuable information can be acquired that are not evident due to interaction of the parameters [46]; the factorial experimental design has been proven an efficient method in bacterial inactivation studies [34,9]. The parameters under investigation are (i) exposure time, (ii) temperature, (iii) initial population and (iv) intensity of the solar simulated light, on *E. coli*-spiked synthetic wastewater, as a model microorganism. After the measurements of the process efficiency, post-treatment control in the dark 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™ 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 10⁹ 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 [24]. 1 mL of the prepared (10⁹) bacterial solution was added per liter to obtain a bacterial concentration of 10⁶ CFU/mL. In order to obtain 10³, 10⁴ and 10⁵ 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

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

air-cooled Xenon lamp, and provides 560 cm² 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.

2.3. Batch reactors

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 cm². 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.

2.4. Sampling and post-experimental handling of samples

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 h 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 [36].

2.5. Bacterial enumeration

Viable bacterial counts after solar treatment were assessed by pour-plating on non-selective agar as suggested by Reed [28] and Rizzo [33], 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.

2.6. Experimental design set-up

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 [34]. 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

Fig. 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 [9], whose summary is presented here. Fig. 1a summarizes the results in absence of light, 1b the 800-W/m²

Table 3Inactivation efficiency % after 4 h (at the end of each treatment method) for 0, 800 and 1200 W/m².

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

results and 1c the 1200-W/m² 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/m², temperatures 20, 30 and 40 °C; removal rate was always 0 and growth rates are presented instead.

From Fig. 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 [7]. Therefore, taking into account the favorable existence of nutrients and salts in the system [18] 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 4 h 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 [2]. 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 [12,19] and indirect damage through reactive oxygen species (ROS) production [29], due to UVB light. UVA damages the cells indirectly, also through ROS generation inside and outside the cell [39,25]. Also, synergy between light and temperature is reported [20,32], which enhances the disinfecting action.

This is also observed in our case, where we notice elevated removal rates when 800 W/m² irradiance was applied, for all temperature levels, although higher for the higher temperatures (Fig. 1b, Table 3). Normally, the maximum irradiance value reaching Earth's outer layers of atmosphere is 1360 W/m² and around the equator, the normal values fluctuate around 1120 W/m² [21]. However, in low temperatures, the growth rate is disrupting the expected inactivation behavior, with this mitigation effect increasing toward 40 °C. This intensity level was proven enough to control excess growth, but did not provide proper disinfection in this timeframe. However, when 1200 W/m² were inflicted, the balance between the growth and the inactivation coming from the light actions has turned to the disinfection side, demonstrating total inactivation in 4 h for all temperatures and initial population levels. The synergy between light and temperature is reflected in

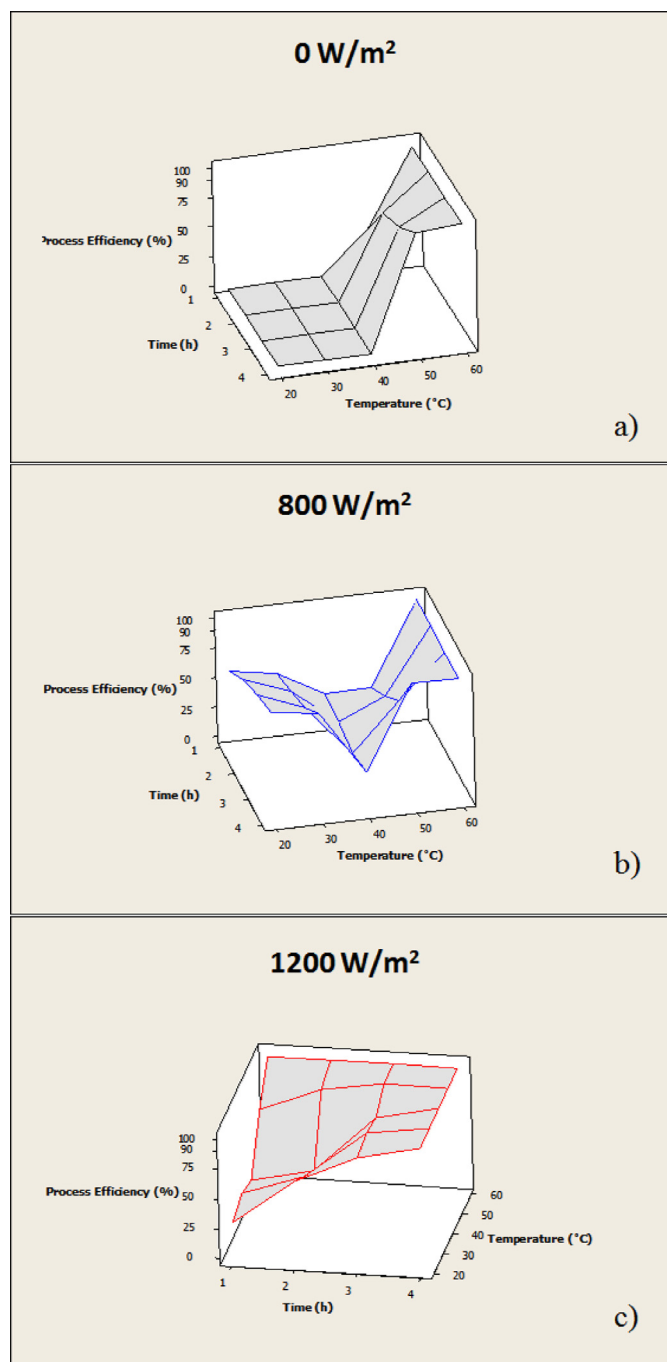


Fig. 1. Overview of disinfection experiments. Process efficiency vs. treatment time and temperature is plotted. (a) 0 W/m². (b) 800 W/m². (c) 1200 W/m².

disinfection times, where 4 h were required for low temperatures, a little less for 50 °C and 0.5 h for 60 °C (Fig. 1c, Table 3).

3.2. Parameters affecting survival and regrowth after 0 W/m² irradiation experiments

As far as the post-treatment events are concerned, we divide the behavior of *E. coli* into two groups: treated under mild temperatures (20–40 °C) or treated in higher temperatures than 40 °C. The first group of graphs presenting the experiments performed in lower temperatures (Fig. 2a), demonstrates a high increase of the bacterial population, influenced by the pre-treatment conditions. It is clear that the samples treated at 40 °C, present higher dynamics of growth and relatively higher final counts after 24 and 48 h.

Also, there is visible influence of the initial population, by which higher initial populations result in higher reproduction rates after 48 h. In addition, we can notice a gradual decrease in growth rates between the 1st and the 2nd day of storage, probably interpreted by the stress caused by some initial nutrient shortage, due to the overgrown bacterial numbers.

Fig. 2b and c are the contour plots that visualize all regrowth tests, performed by hourly sampling in all temperatures and initial population rates. They reveal that there is a correlation between the treatment temperature and the regrowth after 24 or 48 h (expressed by C_{24}/C_0 and C_{48}/C_0). These fractions reveal the regrowth of the bacterial numbers higher than the initial one; if the ratio is <1, then we observe survival, instead. Lower temperatures present suppressed rates, compared to higher ones. Also,

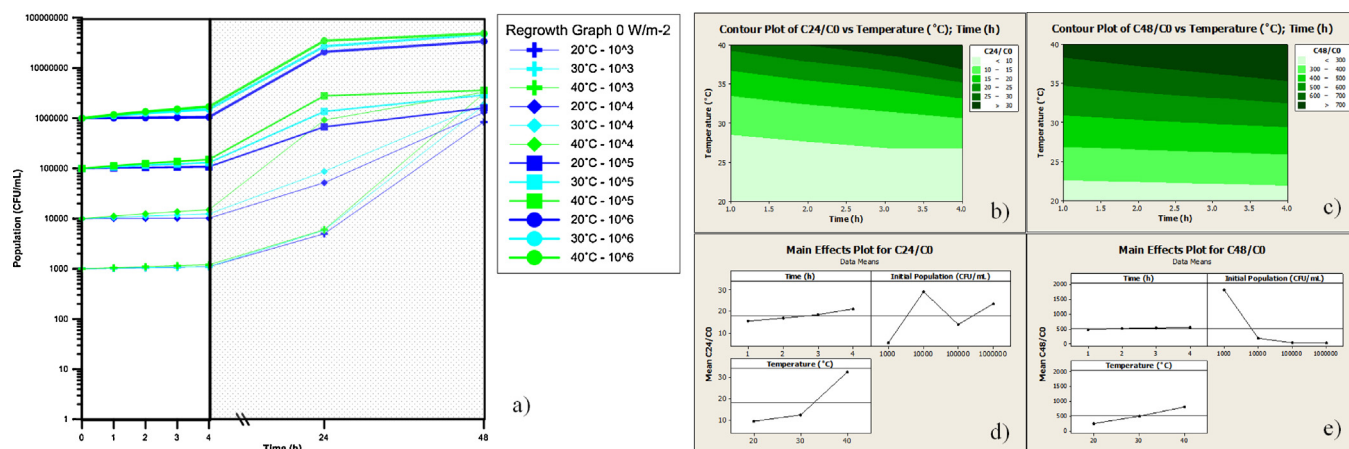


Fig. 2. Main results of non-irradiation experiments for synthetic secondary effluent at among 20–40 °C and all initial *E. coli* populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: regrowth after 1 day). (e) Main effects plot (control variable: regrowth after 2 days).

we notice the difference between the bacterial number after 24 h and 48 h, being influenced by the disinfection conditions, which is expressed in orders of magnitude. Plus, temperatures that initially seemed safer against regrowth (around 25 °C), demonstrate equally high rates. In Fig. 2d and e, the correlation between treatment time and regrowth is presented; the prolongation of the experiment has a profound effect in the bacterial numbers observed after 2 days. However, initial concentration cannot be attributed to a direct effect. In the last sub-graphs which present the main effects of the temperature on regrowth, elevating temperature during treatment is observed to have a strong and rather linear impact only over 30 °C for the regrowth after one day, and stronger for after two days.

The samples treated under higher temperatures (Fig. 3a) do not present any recovery of the population; the population, if any bacteria still existed, continued the decay during dark storage. For the bacterial samples treated at 50 °C, although total inactivation was not observed, after 24 h no viable counts were observed. As it seems, the thermal damage rendered bacteria unable to reproduce; no repair mechanism was observed to act. The remaining samples, after their treatment at 60 °C, presented the same behavior. Higher temperatures accelerated inactivation, which was total within the 4-h timespan, and no regrowth was observed thereafter.

Contour plots 3b and 3c, present the survival rates after 24 and 48 h, for all hourly samples taken 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 for 60–90 min of treatment. The survival pattern for the rest of temperatures and time is consistent, for the two post-treatment days, and slightly more elevated numbers are observed after 2 days. The main effects plots (Fig. 3d and e) demonstrate the inverse effect that high-temperature treatment has on regrowth; as time passes, survival capability is diminishing, and as temperature increases, we observe the same effect. However, initial population follows a similar pattern from the first to the second day.

3.3. Effects of 800 W/m² irradiance on the parameters affecting survival and regrowth

Figs. 4 and 5 present the extension of monitoring the bacterial population for 48 more hours after 800-W/m² intensity irradiation is complete. Results are grouped per temperature range (20–40 °C and 50–60 °C) and initial concentration of bacteria. It can be deduced that post-irradiation survival is more complex, compared to the experiments in absence of light.

The first temperature range (20–40 °C, Fig. 4) demonstrates very low inactivation rates, and as a consequence, presents elevated

(re)growth/survival rates; since there is no total inactivation taking place (i.e. zero viable counts), the recovery of the bacterial numbers could be attributed to (i) live bacteria that continued replicating, (ii) bacteria that recovered their DNA lesions by dark repair methods, and growth of the revived bacteria [11].

The contour plots (Fig. 4b and c) demonstrating the bacterial population after 24 or 48 h, reveal an interesting behavior, as far as the influence temperature is concerned. Although 40 °C is a breaking point, where bacterial disinfection is drastically changing, it appears that 30 °C is the most critical value for regrowth. First of all, after 24 h, regrowth is not probable, and only occurred from samples treated around 3–4 h and 30–40 °C. On the contrary, samples that were treated in low temperatures and for short time, present low counts after 24 h.

Normally, bacteria in samples that remain for longer time under illumination tend to get more inactivated, as it is shown in Fig. 4a. However, prolonging their treatment in this favorable temperature promotes multiplication and therefore, new strains, that gain resistance against solar irradiation in conditions of exposure to (visible) light [13,23,37]. This bacterial ability is a heritage of evolution through time, to protect themselves from the natural ultraviolet rays from the sun [27].

As a consequence, higher remaining populations led to higher survival rates from the bacteria. Although [16] 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. [6] 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 Fig. 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

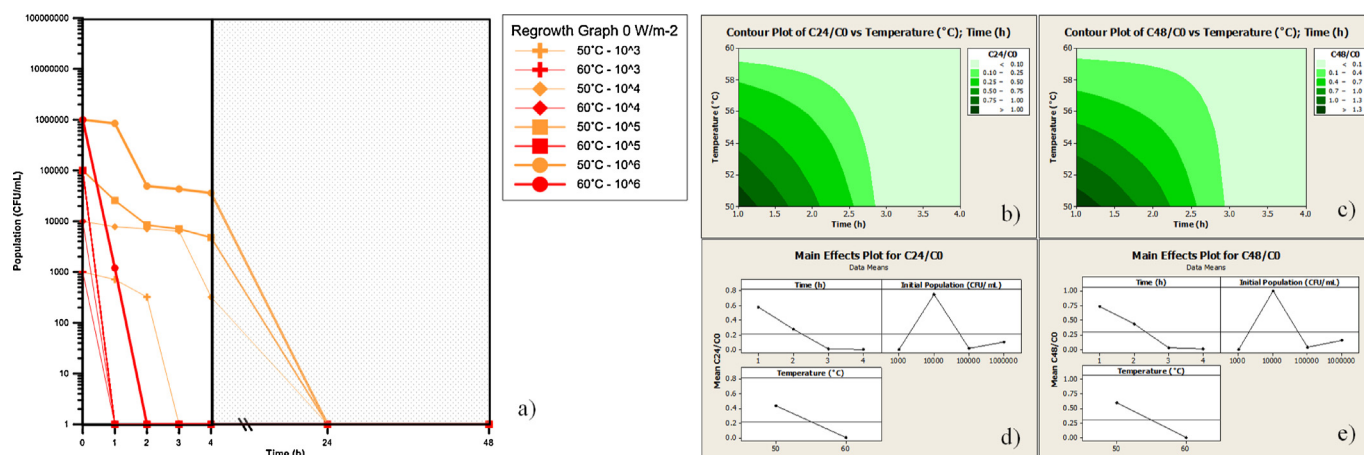


Fig. 3. Main results of non-irradiation experiments for synthetic secondary effluent at among 50–60 °C and all initial *E. coli* populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: regrowth after 1 day). (e) Main effects plot (control variable: regrowth after 2 days).

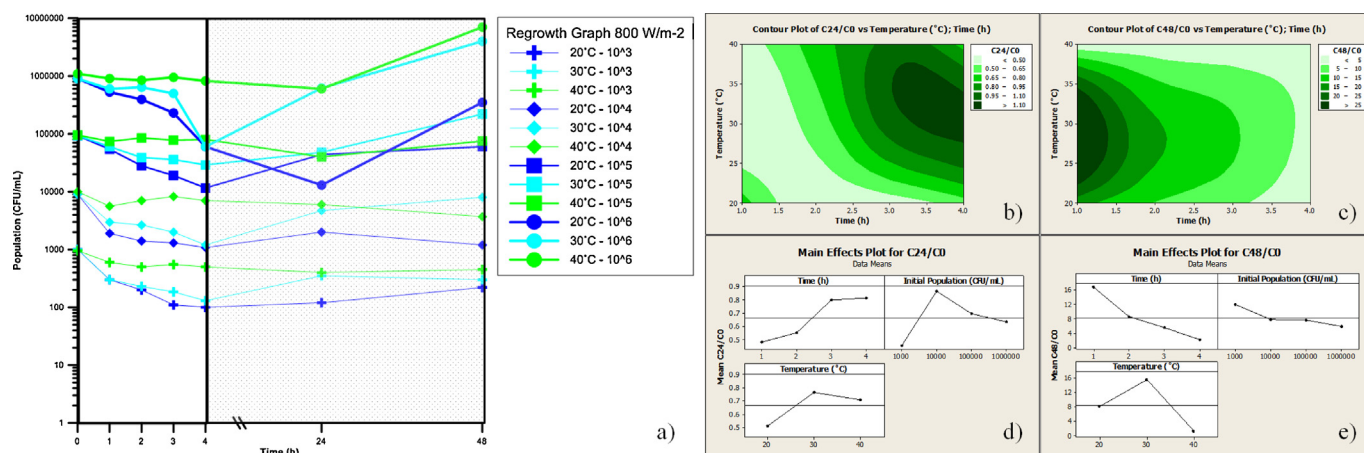


Fig. 4. Main results of 800 W/m²-irradiated experiments for synthetic secondary effluent at among 20–40 °C and all initial *E. coli* populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: regrowth after 1 day). (e) Main effects plot (control variable: regrowth after 2 days).

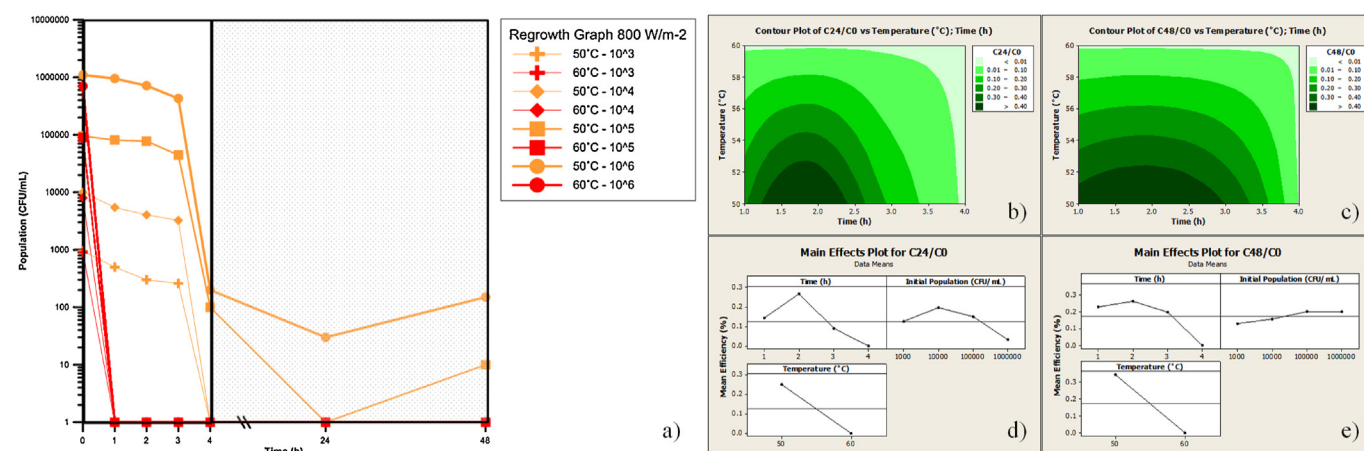


Fig. 5. Main results of 800 W/m²-irradiated experiments for synthetic secondary effluent at among 50–60 °C and all initial *E. coli* populations. (a) Post-treatment regrowth curves. (b) Contour plot of regrowth after 1 day vs. temperature and time. (c) Contour plot of regrowth after 2 days vs. temperature and time. (d) Main effects plot (control variable: regrowth after 1 day). (e) Main effects plot (control variable: regrowth after 2 days).

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 Fig. 5b and c, that picture bacterial survival after 24 and 48 h, the highest survival rates have appeared around 1.5–2 h, but are still low ones. This peak is

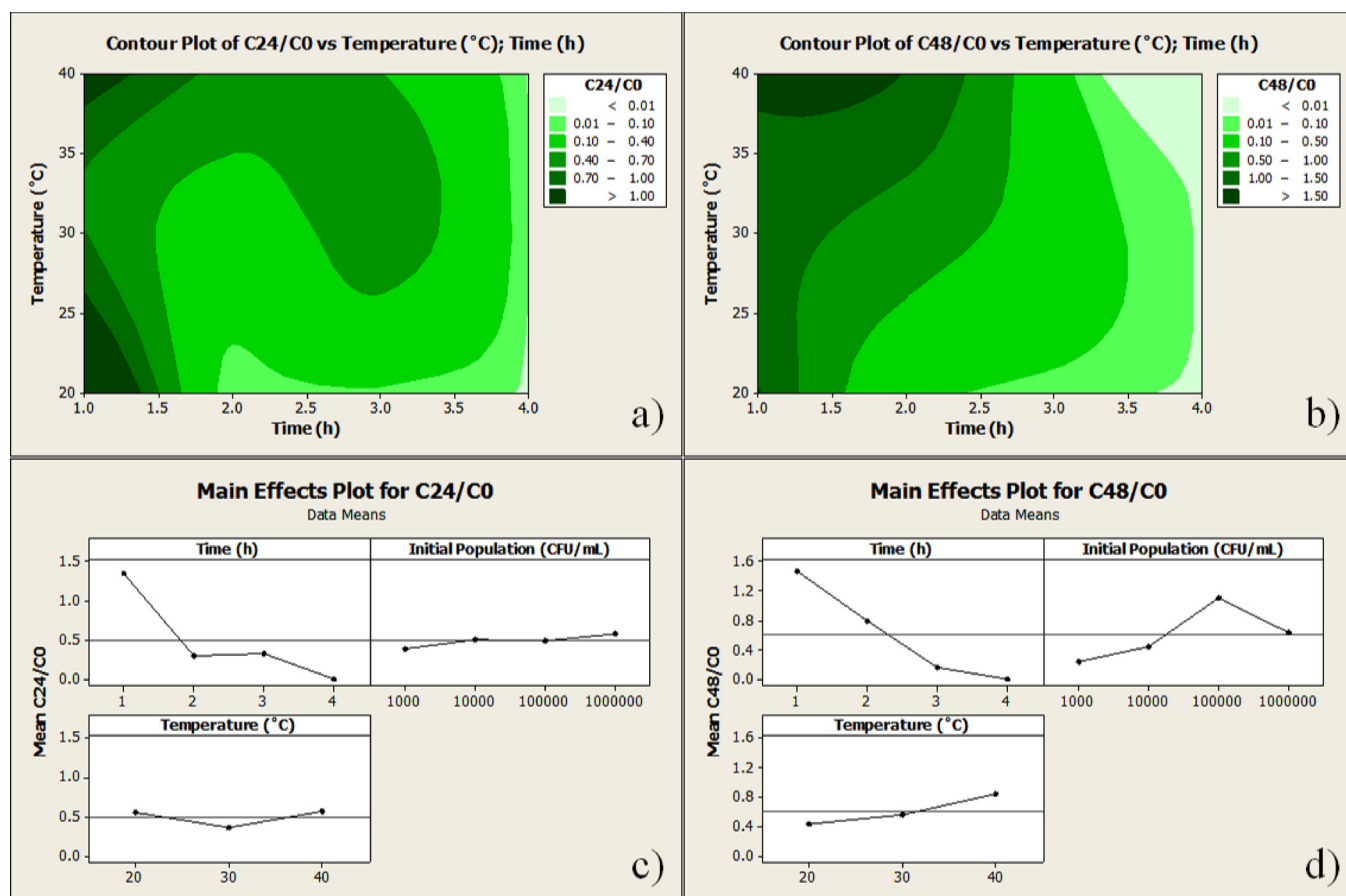


Fig. 6. Overview of the 1200 W/m²-irradiation experiments, among 20–40 °C and all initial *E. coli* populations. (a) Contour plot of regrowth after 1 day vs. temperature and time. (b) Contour plot of regrowth after 2 days vs. temperature and time. (c) Main effects plot (control variable: regrowth after 1 day). (d) Main effects plot (control variable: regrowth after 2 days).

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 Fig. 5d and e, 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/m² 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/m². 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 [40]; 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/m². 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/m²) was applied and the conditions favored disinfection (all cases of 60 °C treatment and 10³–10⁴ 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. Fig. 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 C₂₄/C₀ and C₄₈/C₀ (Figs. 6a and b and 7a and b) 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 were 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 (Fig. 6c and d) 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, C₂₄/C₀ and C₄₈/C₀ 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 Fig. 7, and shows a very low survival potential and also, for the first time, it is decreasing from day

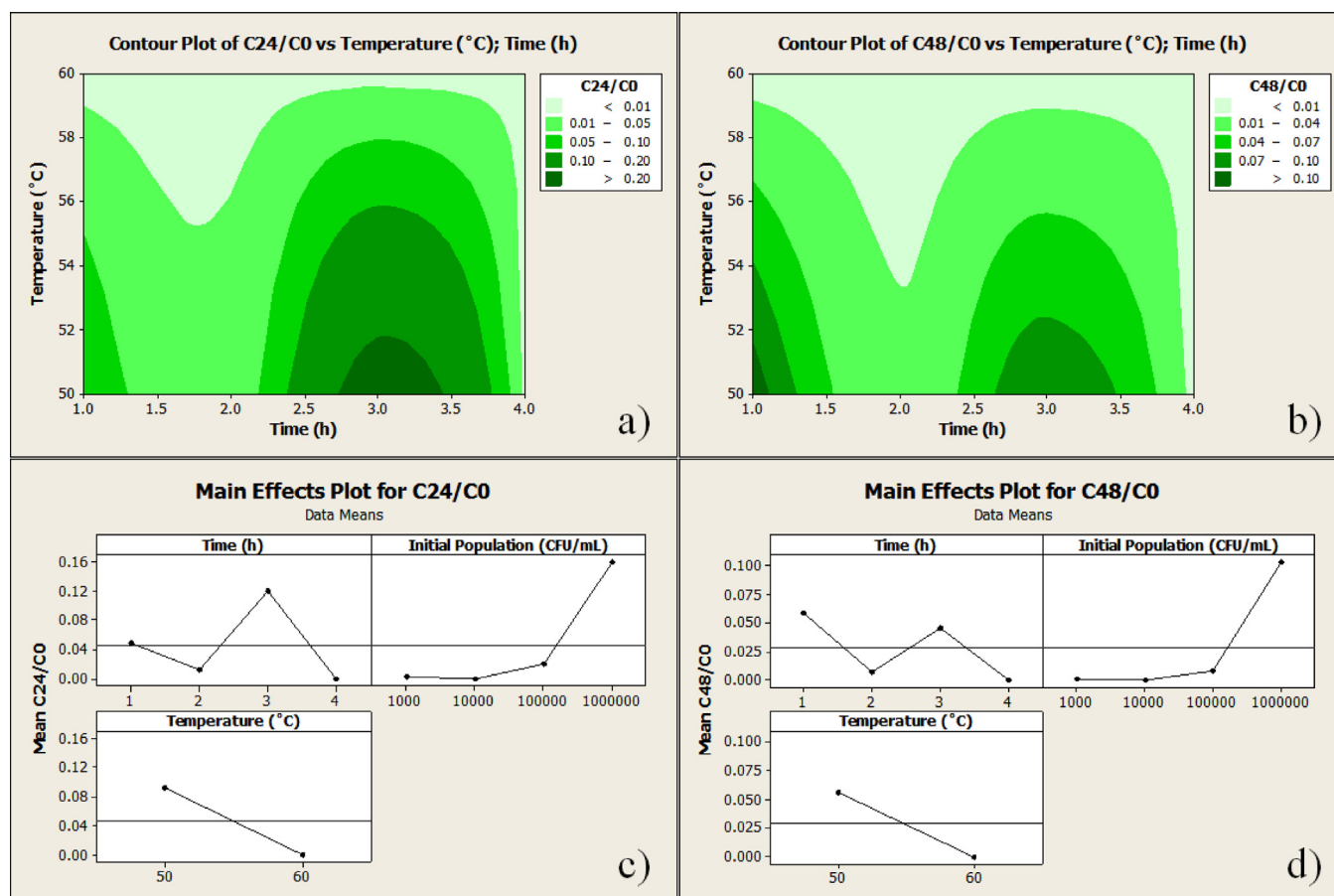


Fig. 7. Overview of the 1200 W/m²-irradiation experiments, among 50–60 °C and all initial *E. coli* populations. (a) Contour plot of regrowth after 1 day vs. temperature and time. (b) Contour plot of regrowth after 2 days vs. temperature and time. (c) Main effects plot (control variable: regrowth after 1 day). (d) Main effects plot (control variable: regrowth after 2 days).

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 (Fig. 7c and d) demonstrate the negligible differences time and temperature have in survival. However, both main effects plot between 20–40 °C and 40–60 °C allow a good comparison on the effect of light intensity, if compared with the respective ones of 800 W/m² and 0 W/m². 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/m², repair was possible, whereas for 1200 W/m², 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

Our study has employed direct plating to measure cultivable bacteria, therefore regrown or surviving bacteria are treated as one, cultivable entity. Also, we have rather avoided suggesting an influence of the initial bacterial population, because of the lack of a straightforward correlation or tendency. Each population level withholds its own special effect; for instance, initial population of 10³ bacteria encounter more available nutrients per cell and initial population 10⁶ offer higher chances of aggregation and shielding; in both cases, surviving bacteria are offered an enhanced possibility of (re)growth. Therefore, in order to be able to correlate the influence of starting bacterial population in the regrowth period, some statistical indicators were used. A main target was to homogenize

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

Fig. 8a and b demonstrates the correlation between the efficiency of the disinfection process, for all possible treatment times (1–4 h) and the consequent regrowth, for samples that have been treated in low (20 °C ≤ T ≤ 40 °C) or high temperatures (40 °C < T ≤ 60 °C). The ▲ traces reveal the population after 24 h while the ▲ traces, after 48 h, expressed as the fraction of bacteria/initial population, for homogenization of the 20 °C ≤ T ≤ 40 °C results, regardless of initial bacterial numbers. We observe that in overall, the population after 48 h is tending to be higher than the population after 24 h. It also appears that as efficiency increases, the samples without regrowth are increasing (line indicating C_{24,48}/C₀ ratio = 1), and a tendency to reduce their regrowth potential, according to the percentage of efficiency increase. However, for higher temperatures, we notice the significant absence of regrowth after 24 h (trace: +) (line indicating C_{24,48}/C₀ ratio = 1) and the suppression of growth after 48 h (trace: ●), compared to the lower temperatures. Hence, treating in higher temperatures is detrimental in both short and long-term storage of the treated samples.

Furthermore, we calculated the live (cultivable) number of bacteria left at the end of the process, and plotted with the population after 24 and 48 h, for both low (Fig. 8c) and high temperatures of pre-treatment (Fig. 8d). Fig. 8c demonstrates a constant live bacteria/initial population ratio fluctuating around 1 after 24 h of treatment (trace: ●), but the bacterial numbers after 48 h (trace: ●) seem to decrease, as the live fraction increases; lower populations would be expected when the live fraction is lower. This indicates that the correlation between the pre-treatment and

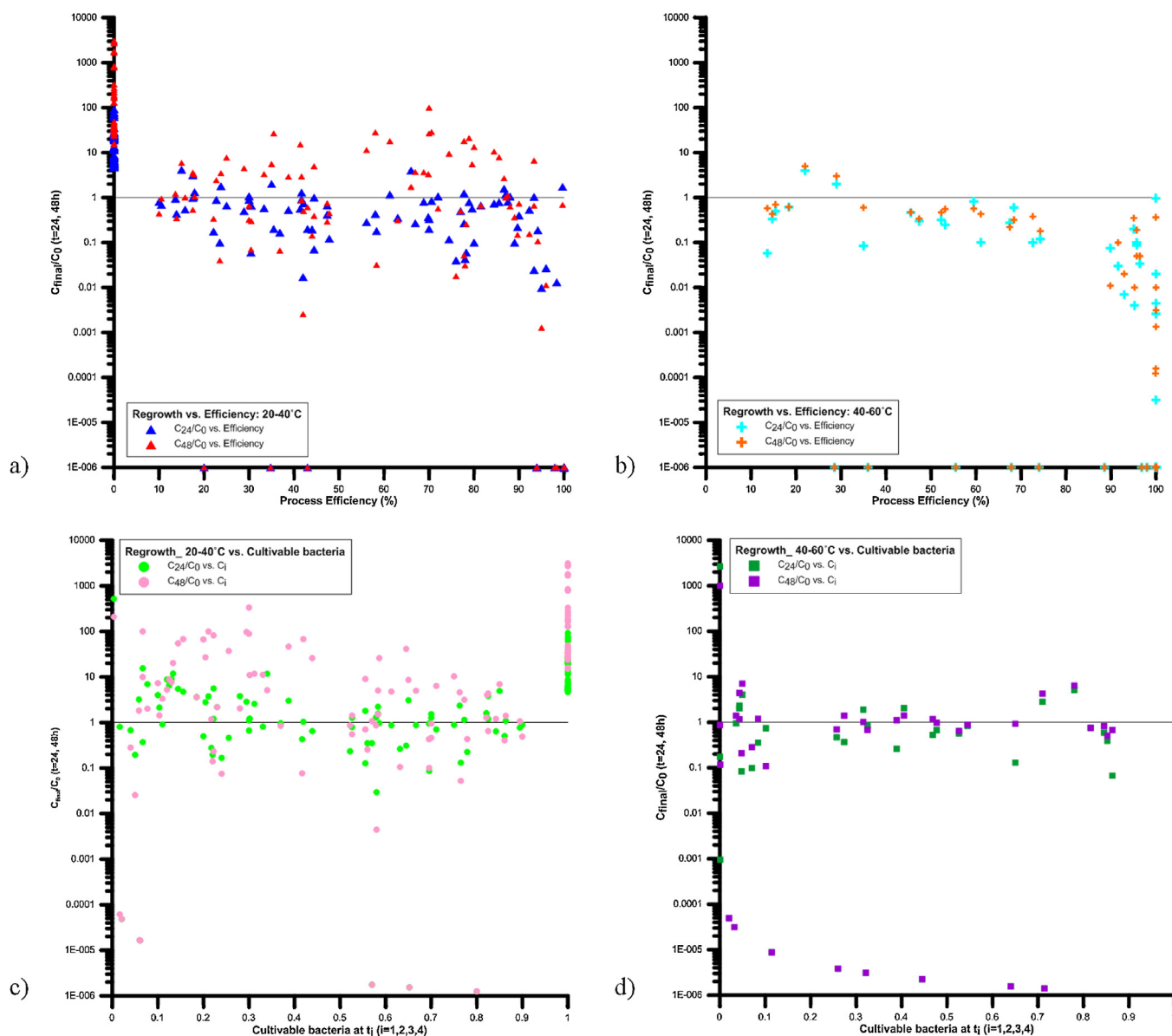


Fig. 8. Statistical interpretation of regrowth vs. disinfection efficiency. (a) Regrowth vs. efficiency after 1 day. (b) Regrowth vs. efficiency after 2 days. (c) Regrowth after 1 day vs. concentration of cultivable bacteria at the end of the treatment period (1–4 h). (d) Regrowth after 2 days vs. concentration of cultivable bacteria at the end of the treatment period (1–4 h).

regrowth is not limited to the alive fraction at the end of the given treatment time (1–4 h), but is linked to the treatment method. For instance, a low surviving fraction, deriving from a short-treatment time in low intensity is very susceptible to regrowth. The opposite statement, for higher light intensities and low temperatures to expect low regrowth, is validated as well. Special mention should be made at the non-treated samples (live fraction = 1) that always present (re)growth. In contrast, in Fig. 8d, plotting the higher temperature experiments, we do not find live bacteria at 100%, but we observe less regrowth after 24 (trace:■) and 48 h (trace:■). Also, a higher number of experiments present near-zero regrowth, compared with the low-temperature experiments. Even samples that presented 90% live bacterial fraction present diminished numbers, with obvious positive effects of high temperature in suppressing regrowth.

Finally, Fig. 9 presents an estimation of the bacteria transferred from the end of the treatment time to the first day and from these ones, in the second day. On X axis, we plot the final live fraction of bacteria after 24 h, due to the bacteria at the end of treatment

time i ($i = 1–4$ h) per initial concentration and on Y axis the respective ones for 48 h storage. This ratio assesses the transferability of bacterial growth from day 1 to day 2 and expresses the fate at the end of the treatment time; i.e. values >1 indicate higher numbers after 48 h, due to the live fraction in 24 h. Mathematically, this ratio is $((C_{24}/C_0)/(C_i/C_0))$ or $((C_{48}/C_0)/(C_i/C_0))$, and is expressed as C_{24}/C_i or C_{48}/C_i , respectively. As it seems, the transferability from day 1 to day 2 is strongly influenced by the treatment temperatures during the experiment; for low temperatures $20^\circ\text{C} \leq T \leq 40^\circ\text{C}$, we observe that the same fraction of live bacteria after 1 day can yield higher fractions after 48 h (trace:●) than the respective $40^\circ\text{C} < T \leq 60^\circ\text{C}$ ones (trace:●). For example, 24-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 repair on the damages inflicted by temperature and (ii) the synergistic action of light and temperature ensures low transferability from the surviving fraction. The dominant trend existing in regrowth is also expressed by the logarithmic equations and the possibility of increased appearance after 2 days is reflected by the constants of the equations which describe that trend.

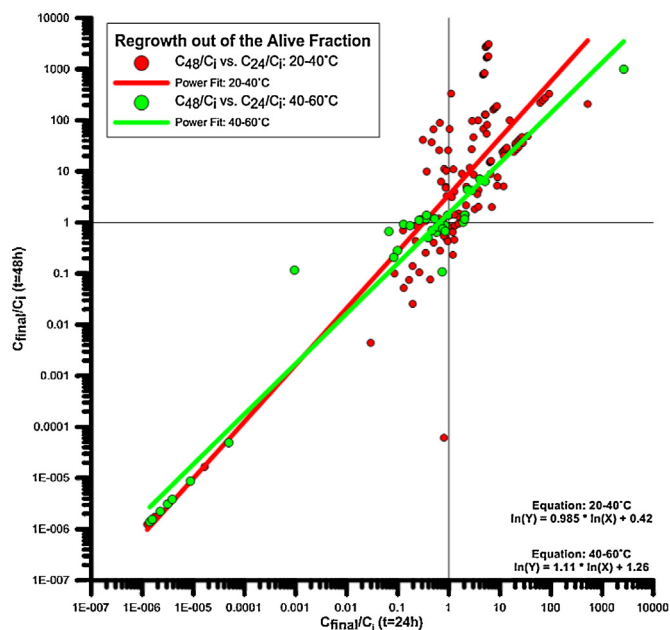


Fig. 9. Transferability of live bacteria through the post-irradiation treatment period. Regrowth after 24 h out of the live fraction subjected to i hours of treatment ($i = 1\text{--}4$ h) at X axis and regrowth after 48 h in Y axis.

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

4. Conclusions

- Non-irradiated samples of secondary effluent treated at 20–40 °C showed slight growth during treatment, and high post-treatment regrowth (ratios of 250–1000). Significantly, thermal inactivation with no regrowth predominated at 50 °C and was total at 60 °C.
- At 800 W/m², bacterial regrowth only occurred in incompletely disinfected samples, which are linked to lower irradiation, shorter times or high initial microorganism populations. No regrowth was observed in samples presenting no bacterial counts at the end of the treatment. An erratic behavior was observed when treatment temperature was among 20–40 °C, where prolongation of treatment resulted 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 toward 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/m². Our study suggests that contact times longer than the 4 h observed here would be required at field conditions.
- For a holistic view on the potentials of the application implications, other field factors should be also investigated, like shielding by particles (residual suspended solids, algae), for they would extend required exposure time to days.

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