PHOTOCATALYTIC DISINFECTION TREATMENTS: VIABILITY, CULTIVABILITY AND METABOLIC CHANGES OF *E. coli* USING DIFERENT MESUREMENTS METHODS

DESINFECCION CON FOTOCATALISIS: VIABILIDAD, CULTIVABILIDAD Y CAMBIOS METABOLICOS DE *E. coli* USANDO DIFERENTES METODOS DE MEDICION

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ABSTRACT: In this work, the oxidative stress was produced by the illumination of either fixed TiO_2 or suspended TiO_2, and the relation between cultivability and viability under oxidative conditions was studied using a Direct Viable Count (DVC) and Fluorescence In Situ Hybridisation (FISH) methods. Under certain oxidative conditions simulating incomplete treatments, viable cells that had lost their cultivability were observed to recover all of their physiological functions when transferred to fresh nutrient media. Moreover, alterations in the nutrient assimilation processes were studied by 1H-NMR during and after the photocatalytic treatment. This allowed us to shed some light on how the damage chain propagates during the treatment. This work demonstrate that the Standard Plate Count (SPC) method commonly used to estimate the cell populations during disinfection processes is ill adapted, whereas the FISH-DVC pair offers a reliable alternative.

KEYWORDS: Photocatalysis, drinking water, E. coli, oxidative stress, titanium dioxide, cultivability, disinfection, NMR

RESUMEN: En este trabajo, el estrés oxidativo producido con radiación en presencia TiO_2 fijo o suspendido TiO_2, y la relación entre cultivabilidad y viabilidad se han realizado, utilizando recuento directo de células viables (CDV) y fluorescencia de hibridación in situ (FISH). Se encontró que cuando el tratamiento es incompleto, las células viables que habían perdido su cultivabilidad pueden recuperar todas sus funciones fisiológicas, cuando son transferidas a un nuevo medio nutritivo. Por otra parte, las alteraciones en los procesos de asimilación de nutrientes fueron estudiados por 1H-RMN durante y después del tratamiento fotocatalítico. Esto permitió tener indicios sobre cómo se propaga la cadena de daños durante el tratamiento. Se demostró que el recuento en placa comúnmente usado para estimar la población de E coli en los procesos de desinfección no sería confiable, mientras que el método combinado FISH-DVC ofrece una alternativa fiable.

PALABRAS CLAVES: Fotocatálisis, agua potable, E. coli, estrés oxidativo, dióxido de titanio, cultivabilidad, desinfección, RMN

1. INTRODUCTION

The presence of microorganisms in water is an important issue for drinking water production. The most widely used procedure for water disinfection is chlorination. It has been shown that in combination with organic compounds, chlorination produces disinfection by-products (DBPs) such as trihalomethanes that are harmful for human health. In some cases, such products can even restrict the use of treated water for other purposes; therefore alternative disinfection processes such as photocatalysis are currently under development. Photocatalysis is based on heterogeneous catalytic reactions involving UV-photons and a semi-conductor; usually titanium dioxide (TiO_2). Photocatalysis is well suited for the inactivation of *E. coli* and other pathogenic bacteria, since it produces highly oxidizing radicals, a chemical specie can either inactivated or cause severe damage to bacteria. These oxidative species are unselective and can target virtually all types of macromolecules including lipids; proteins; DNA and RNA leading to metabolic pathway disruption, spontaneous mutation, bacteriostatic, and bactericidal effects [5, 10]. As a consequence, the evaluation of the dead fraction of the bacterial population is not trivial.

To the best of our knowledge, the standard plate count (SPC) has often been used to evaluate photocatalytic disinfection. However, under photocatalytic treatment, bacteria were seen to have lost their cultivability before suffering irreversible damages, both in natural and laboratory conditions [11, 20]. Growth was observed during and after photocatalytic treatment when applied to *E. coli* and other bacteria in natural waters [3]. The oxidative stress induced by irradiation with UV-light, photocatalysis or by chlorination has been shown to increase the number of cells found in a viable but not cultivable state (VBNC) [7, 17, 18]. The cultivability of bacteria strains was also observed to depend on the characteristics of the solid media used for SPC [3, 16, 19].

Clearly, the loss of cell cultivability could lead to errors in the estimation of the optimal conditions for the complete elimination of bacteriological risk during disinfection processes (viability).

In this paper, resuscitation techniques and Direct Viable Counting methods (DVC) using fluorescent dyes were used to monitor the viable and culturable cell populations under photocatalytic conditions.

2. MATERIALS AND METHODS

2.1 Microorganisms, culture media and culture conditions

Bacterial strain of *E. coli* K12 (ATCC 23716) was obtained from DSM, (German Collection of Microorganisms and Cell Cultures). An initial volume of 1 mL of bacteria from an overnight culture was re-suspended in 100 mL of Luria Broth (LB) liquid medium and further incubated at 37 ºC under shaking at 176 rpm reaching the stationary state, and concentration of OD_{650nm} 1.74-1.79 or 10^8-10^9 UFC mL^{-1}. Cells were harvested by centrifugation at 5000 x g for 10 min and the resulting pellets were washed two times in a tryptose solution to ensure the complete removal of culture media. Finally, each pellet was re-suspended in tryptose solution and completed to reach the initial volume, from which 1 mL was used to inoculate the photocatalytic reactors previously filled with 100 mL of isotonic solution (NaCl 8 g L^{-1} KCl 0.8 g L^{-1}). This ensures initial concentrations ranging from 10^6 to 10^7 colony forming units per mL (CFU mL^{-1}).

2.2 The photocatalytic reactor

The reactor consisted of a Pyrex glass cylinder connected to a peristaltic pump to maintain a continuous flow of 150 mL/min at a temperature of 32°C (see Fig. 1a). A cylindrical plastic block was placed at the centre of the reactor to keep the total illuminated volume at 43 mL in all of the experiments. This volume, filled with inoculated TiO_2 slurry (0.2 TiO_2 P-25 g L^{-1}), was irradiated for 1.5 hours. Every 15 minutes, 1 mL was sampled to monitor the effects of photocatalytic treatment on cultivability, viability, and cell physiology during the illumination. At the end of the irradiation, 10 mL samples were stored in the dark without shaking at 32º C during 24 hours to evaluate possible post-irradiation effects. The illumination was produced with a Hanau Suntest (Heraeus, Germany) solar simulator, using a lamp that irradiates 5 Wm^{-2} at 54 nm. This lamp simulates the solar spectrum, and its overall intensity was set to 1000 Wm^{-2} for the entire experiment. We refer to these procedures as suspended-TiO_2 experiments. These experiments were repeated using a paper impregnated with TiO_2 of 65*94 mm^2 kindly provided by Ahlstrom Company (12g /cm2 of TiO_2 P-25 NW10) that was fixed to the central cylinder. The volume was filled with a salty solution, inoculated, and irradiated during 5 hours. In this case the solution was sampled each hour. This procedure is referred to as fixed-TiO_2 experiments.

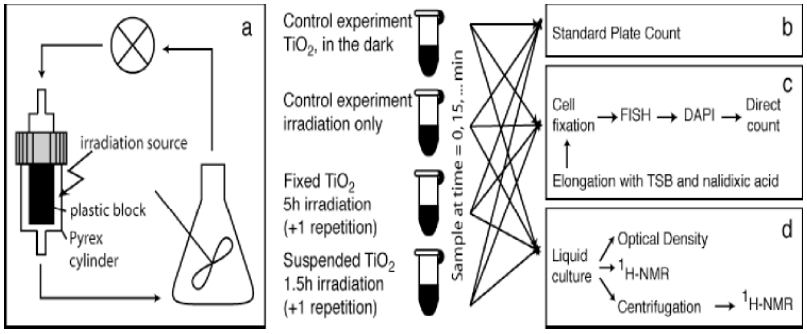

Figure 1(a). The Photocatalytic reactor. The total illuminated volume was 43 mL, the temperature was maintained at 32°C and a peristaltic pump ensured a flow of 150 mL min^{-1}. Irradiation was at about 40 W/m^2 of UV-light at 254 nm. (b-d) Flow diagram of the experiments

2.3 Enumeration of cultivable cells

The number of cultivable cells was determined using a standard plate count method (See Fig. 1a). Samples of 1 mL were collected to perform dilutions in Tryptose solution. Aliquots of 100 μl were plated in Agar Plate Count Media (MERCK), incubated at 37°C for 24 hours, and counted. Additional verification counts were done after 48 hours.

2.4 Enumeration of total and viable cells

The FISH-DAPI coloration procedure suggested by Amman et al. [2] was used for the total cell enumeration, while viable cells were enumerated using the DVC-FISH method proposed by Kogure, [9] and Renault [14], with the following modifications.

DVC-FISH: Samples (See Fig. 1a) of 3 mL were poured into 7 mL of a nutrient/antibiotic solution (20μg mL^{-1} of Nalidixic acid in TSB 20%) and incubated at room temperature without shaking. After 12 hours, cells were harvested by centrifugation at 5000 x g. Depending on the expected concentration, 100 or 300 μl were re-suspended in 10 x PBS to reach 1 mL and fixated with a *para*-formaldehyde fixation buffer 30% (v/v), stored overnight at 4ºC, and harvested by centrifugation at 1000 x g for 10 min. The pellets were washed with 1 x PBS solution. This washing process was repeated two times to ensure the complete removal of para-formaldehyde, and the final pellets were resuspended in 500μL of 1 x PBS and 500μL of ethanol 100%. 10 μL of the resulting cell suspension was transferred to each well of a 8-well slide, previously covered with gelatine (0.1%w/v) and $KCr(SO_4)_2$ (0.01% w/v). The loaded slides were desiccated with ethanol and incubated for 2 hours in the dark with 10μl of hybridisation solution pre-warmed at 45ºC and containing 24 ng μL^{-1} of *E. coli* probe [6], marked with 5' Cy3 (prepared by Microsynth GmbH). Finally, cells were washed with a washing solution at 45 ºC and then rinsed with distilled water.

DAPI: After hybridisation, all samples were washed in distilled water and stained with 9 μl of DAPI (4',6'-diamidino-2-phenylindole) 0.0001% (w/v) for 10 minutes, washed again in distilled water and dried (to aid cell localization, slides were cover with antifade citifluor solution). Microscopic cell count (DAPI) and oligonucleotide probe-positive count (Cy3) were performed using a Nikon Eclipse E800 microscope equipped with a specific DAPI filter and G-2A filter sets. A minimum of 20 view squares were enumerated for each well; two wells were examined in total. For samples taken at the end of the treatment, the whole wells were enumerated.

2.5 Assays to evidence metabolic changes

Four tubes containing 20 mL of mineral media (ref. 382 DSMZ German Collection of Microorganisms and Cell Cultures for *E. coli* strains), and 20% of Glucose, Lactose, Mannitol, or Peptone, respectively, were inoculated with 1 mL of irradiated bacteria. The turbidity at 650 nm was measured after 18, 24, and 72 h of incubation and the increase in 0.1 or more turbidity units was reported as positive growth. For each tube, ^{1}H-NMR was used to determine the amount of the initial sugar present in the media and inside the cells. Two samples, A and B, of 1 mL each, were taken from the resulting 20 mL tubes after incubation (See Fig. 1a). Sample A was centrifuged and 700 μL aliquots of the supernatant were transferred to an NMR-tube. 700 μL of sample B were directly transferred to an NMR-tube. 100 μL of D_2O were added to both samples. One-dimensional proton spectra of A and B were recorded using 8 scans (ca. 30 s). The water signal was attenuated using appropriate water-suppression techniques. Such spectra were acquired for the four nutrients *before* and *after* photocatalytic treatment.

3. RESULTS AND DISCUSSION

3.1 Comparison between fixed and suspended TiO_2 using SPC

In suspended TiO_2, the decrease of the bacterial population followed a first-order kinetic, and after 90 minutes empty plates were found. Different results were obtained for bacteria treated with fixed TiO_2. In this case, no decrease in cell population was observed after 1.5 hours, and a reduction of about two orders of magnitude was achieved after 5 hours of treatment. No significant reduction was observed when irradiation was performed without TiO_2, and control samples kept in the dark did not show any variation in their populations (See Fig. 2). The higher efficiency observed with suspended TiO_2 can be explained by the larger contact surface available in suspended TiO_2. Since oxidative radicals are very reactive they have to be produced close to the cell in order to produce damages. It was observed under the microscope that in a solution of suspended TiO_2, cells and catalyst particles tend to aggregate. These results are consistent with those reported by Goniat and collaborators using optical microscopy [6].

3.2 Relationship between cultivability (SPC) and viability (DVC-FISH) on bacteria

Cells were considered as viable if their sizes after the DVC procedure (incubation with nutrients and 20g mL^{-1} of Nalidixic acid in TSB 20%) exceeded twice the regular size, while the total of the cells present was obtained by counting all of the fluorescent cells (without DVC). The nalidixic acid acts as a specific inhibitor of DNA synthesis and prevents cell division without affecting other cellular metabolic activities [4].

After inoculation, (time 0) a difference of more than one order of magnitude was observed between cultivable and viable cell concentration, even in control samples without titanium dioxide. Although the photocatalytic treatment was expected to induce a loss in cultivability, such a difference at the beginning of the treatment was not expected. A possible explanation is that the DVC-FISH method allows for the counting of the individual cells while with the SPC method, several cells might be counted as a single colony. Indeed, the initial cell populations were high and the plates were crowded. It has been estimated that in some cases, only 10% of the total cells were detected by SPC [1]. Finally, the difference between fixed and suspended TiO_2 can be explained with the formation of cell agglomerates often observed in this process, although the centrifugation and dilution of the sample into a salty solution did not lead VBNC cells.

Suspended catalyst. During the first hour of the treatment, the concentration of viable cells remains constant (See Fig. 3), while the cultivable cell concentration slightly decreased to reach approximately 10^6 CFU mL^{-1}. At that point, the difference between cultivable and viable cell population reached 6 orders of magnitude. These results confirmed the fact that the photocatalytic treatment might lead bacteria to a VBNC state. Between 60 and 75 minutes of treatment, the concentration of both viable and cultivable bacteria abruptly decreased in four orders of magnitude. More generally, during the whole irradiation period, a difference of at least two orders of magnitude was observed between cultivable and viable cell concentration. As can be seen from Figs. 2 and 3, control experiments did not show any variation in cell concentration.

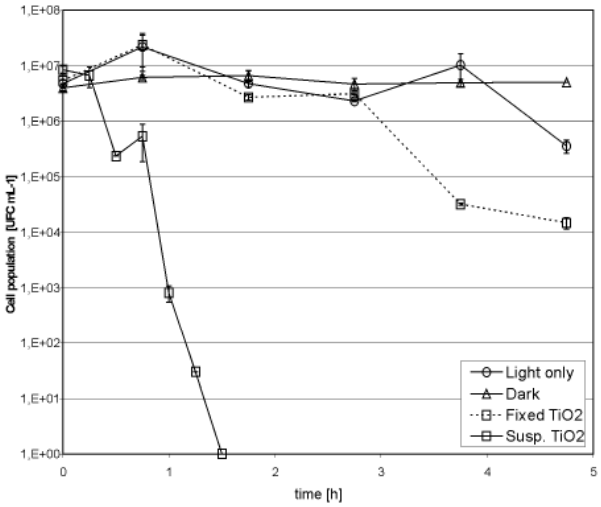

Figure 2. UFC mL^{-1} comparison between suspended and fixed TiO_2: Triangles represent the control experiment, without light and without TiO_2. Circles represent the experiment with irradiation only, while squares with a dashed and full line represent experiments with fixed and suspended TiO_2, respectively

Here we would like to point out that, during the whole treatment, the total cell concentration and the viable cell concentration remain equal. This suggests that photocatalysis not only kills the bacteria, but also destroys their membranes.

Fixed catalyst. A difference of at least one order of magnitude was found between the plate count and DVC-FISH during the first 3 hours of treatment. After five hours, the amount of cultivable cells had decreased in about 3 logarithmic units; Meanwhile, the amount of viable cells decreased only in one order of magnitude. As already mentioned, these results showed that the experiment using suspended titanium dioxide is more effective than the one using fixed TiO_2 (See Fig. 3). The lower efficiency clearly showed that the photocatalyticoxidative stress induced VBNC cells.

The results obtained in experiments with fixed TiO_2 are similar to those obtained previously in real waters treated with TiO_2 in suspension, which.

could indicate that the decrease in the availability of OH radicals (in natural waters with high levels of turbidity, organic matter and other chemical compounds, as the efficiency of disinfection diminishes, due to the competition for •OH radical) induces the occurrence of VBNC cells. Finally the results of this comparison (SPC vs. FISH) show that the plate count is a limited method for estimating the removal efficiency of bacteria in water and that great care should be taken in evaluating photocatalytic bacterial elimination in real conditions.

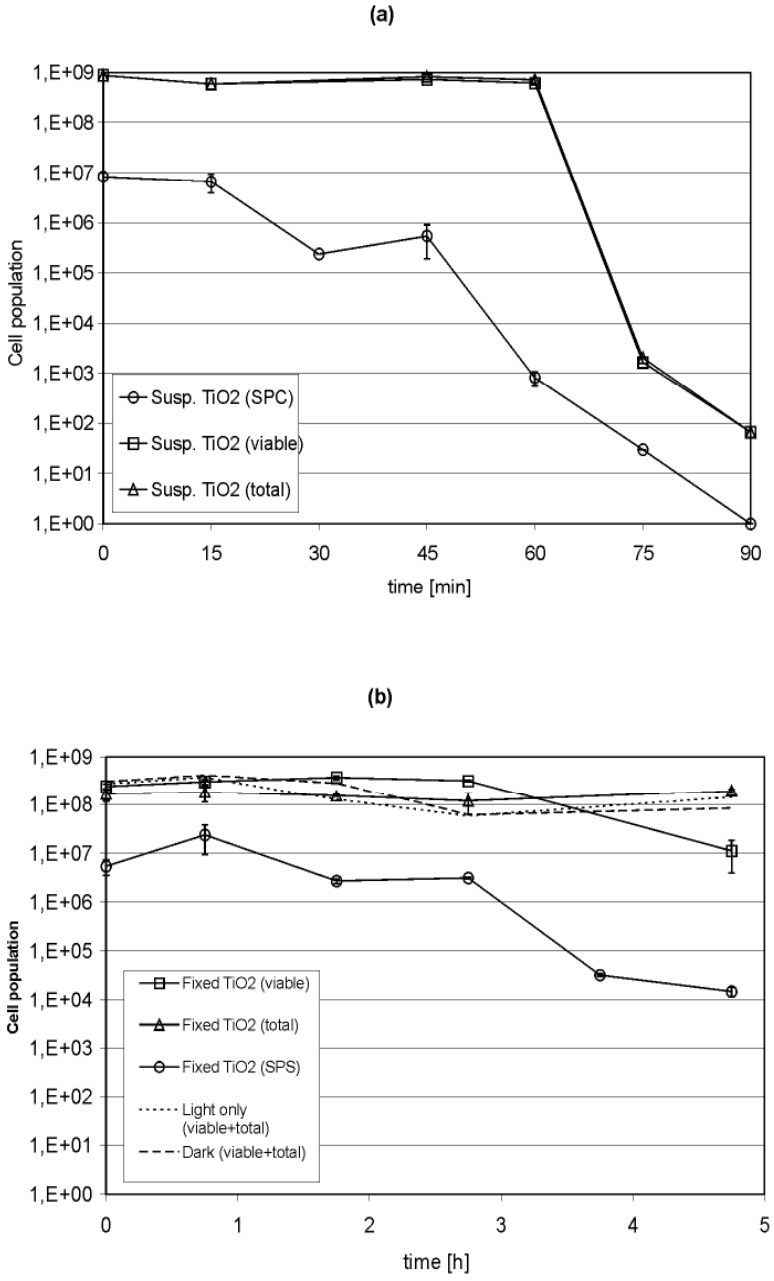

Figure 3. Evolution of the cell population during the photocatalytic treatment. **a)** cell population during irradiation with suspended TiO_2. Filled triangles, rectangles, and circles stand for the total of cells (FISH), the population of viable cells (FISH-DVC), and the population of cultivable cells (SPC-UFC mL^{-1}), respectively. **b)** cell population during the irradiation of fixed TiO_2. Filled triangles, rectangles, and circles stand for the total of cells (FISH), the population of viable cells (FISH-DVC), and the population of cultivable cells (SPC- UFC mL^{-1}), respectively

3.3 Assays to evidence how irradiation affects the metabolism

Irradiated bacteria were immediately transferred to a solution of mineral media enriched with glucose, lactose, mannitol, or peptone, in order to determine how their ability to grow was affected by the treatment. After the slurry had been illuminated, bacterial growth was only evidenced in peptone media. As illustrated in Table 1, cell growth was observed for samples irradiated between 15 to 75 minutes, in agreement with the results observed using SPC. However, after 105 minutes of illumination, while SPC reported no colony-forming unit, cell growth was still observed in peptone media, as expected from FISH-DVC analysis.

Table 1. Cell growth ability monitored during photocatalytic treatment with suspended TiO2. During the treatment, samples were collected and transferred to a mineral media solution to evidence their ability to grow. The column (a) represents the samples that were directly re-suspended in nutrient solution, while the column (b) shows the results when the samples were stored in the dark during 24 hours before their transfer into mineral media

Energy source	GLUCOSE						LACTOSE					
Culture Time	0 hours		18 hours		72 hours		0 hours		18 hours		72 hours	
Irradiation [min]	a	b	a	b	a	b	a	b	a	b	a	b
0	-	-	+	na	+	+	-	-	+	na	+	+
15	-	-	-	na	+	+	-	-	-	na	+	+
30	-	-	-	na	+	+	-	-	-	na	+	+
45	-	-	-	na	+	-	-	-	-	na	+	+
60	-	-	-	na	+	-	-	-	-	na	+	-
75	-	-	-	na	+	-	-	-	-	na	+	-
105	-	-	-	na	+	-	-	-	-	na	+	-
Energy source	MANNITOL						PEPTONE					
Culture Time	0 hours		18 hours		72 hours		0 hours		18 hours		72 hours	
Irradiation [min]	a	b	a	b	a	b	a	b	a	b	a	b
0	-	-	+	na	+	+	-	-	+	na	+	+
15	-	-	-	na	+	+	-	-	+	na	+	+
30	-	-	-	na	+	-	-	-	+	na	+	-
45	-	-	-	na	+	-	-	-	+	na	+	-
60	-	-	-	na	+	-	-	-	+	na	+	-
75	-	-	-	na	+	-	-	-	+	na	+	-
105	-	-	-	na	+	-	-	-	-	na	+	-

Interestingly, the cell growth was observed when the bacteria were suspended in peptone media, but not when using sugars as a nutrient source. This suggests that the membrane is affected by the treatment and thereby the metabolic pathways of assimilation of sugars (Embden-Meyerhof [EM] and Entner-Doudoroff). In turn, the peptone assimilation process is less affected by membrane damages, since the peptone mainly consists of amino acids that are metabolized differently. Indeed, it is known that most of the amino acids are metabolized in TAC (Thricarboxylic acids cycle) via fumarate. Thus, when the treatment period is not long enough to cause the death of the bacteria, they can recover their ability to grow.

Again the comparison between experiments performed with fixed and suspended TiO_2 leads to the conclusion that the former are less efficient, as can be seen from Table 2.

Table 2. Cell growth ability monitored during photocatalytic treatment with fixed TiO_2. During the treatment, samples were collected and transferred to a mineral media solution to evidence their ability to grow. Dark recovery experiments were not carried out since SPC never reported the complete elimination of the bacteria

Energy source	GLUCOSE			LACTOSE		
Culture Time	0	18	72	0	18	72
Irradiation [min]	hours	hours	hours	hours	hours	hours
0	-	+	+	-	+	+
60	-	+	+	-	+	+
120	-	-	+	-	-	+
180	-	-	+	-	-	+
240	-	-	-	-	-	-
Energy source	MANNITOL			PEPTONE		
Culture Time	0	18	72	0	18	72
Irradiation [min]	hours	hours	hours	hours	hours	hours
0	-	+	+	-	+	+
60	-	+	+	-	+	+
120	-	-	+	-	+	+
180	-	-	+	-	+	+
240	-	-	-	-	+	+

3.3.1 Effect of storage in the darkness

When samples were stored in the darkness 24 hours before their transfer to the solution of mineral media, the cell growth was found to be less frequent and was not observed after 45 minutes of irradiation. This was because the cells were left in treatment slurry that does not contain nutrients and thereby the cells suffered from an additional exposure to inanition conditions. Moreover, it has been shown that the peroxidation of lipids, under photocatalysis, might lead to a chain reaction involving organic radicals that can in principle oxidize other molecules even in the absence of UV-light and produce a residual bactericide effect as a function of irradiation concentration [12].

3.3.2 Estimation of the nutrient assimilation by ^{1}H-NMR

To shed light on how transport and other metabolic pathways are affected by the treatment, 1H-NMR was used to determine the evolution of the concentration

of nutrient inside and outside the cells as treatment occurs. Two 1 mL samples (after 18 and 72 hours of culture) were collected and 1H-NMR spectra were acquired; the second sample was centrifuged prior to analysis to separate the cells from the slurry. Re-suspension of the cells in water allows determining the concentration of the nutrient up taken by the cells. After 18 hours of culture, no sugars were observed in the supernatant for irradiated samples (Fig. 4a), even in samples in which growth was not observed. This agrees with the spectra of centrifuged samples (Fig. 4b) indicating that the nutrients were transported inside the cells. This unregulated transport could be caused by a damaged lipid bi-layer or damaged permeases, a hypothesis that agrees with the cell growth experiments mentioned previously. Such modifications of the permeability (K+, proteins, and ARN release) were also reported by Saito and co-workers [15]. Moreover, UV-treated cells have been shown to retain residual metabolic activity and showed several levels of metabolic imbalance [18].

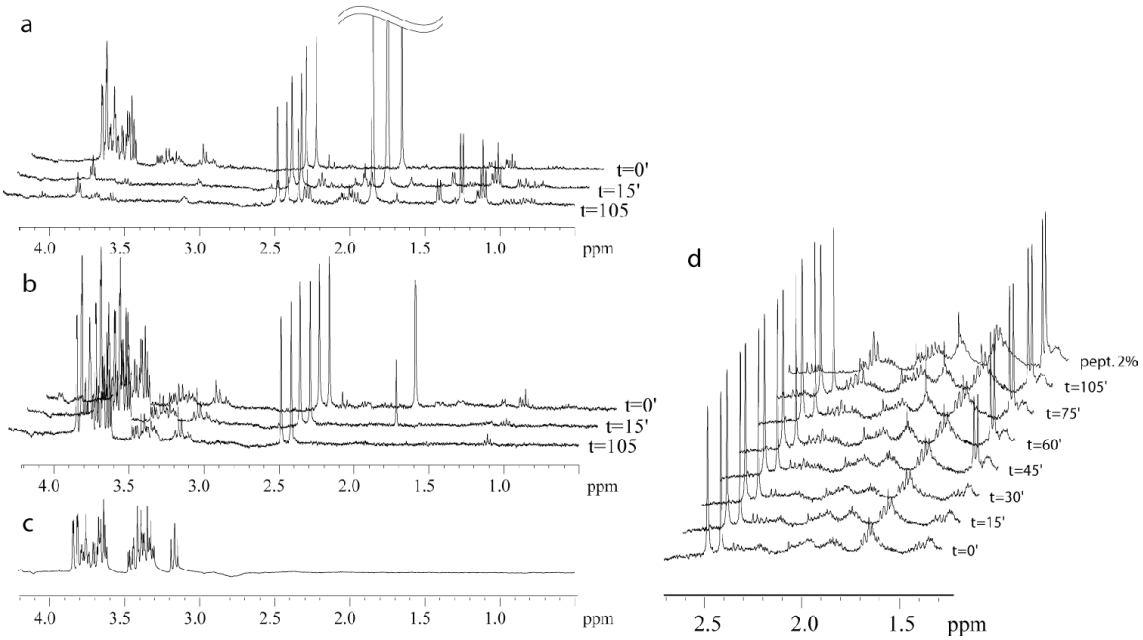

Figure 4. 1H-NMR spectra of supernatant and re-suspended pellets. (a) supernatant and (b) re-suspended pellets of samples transferred to glucose enriched media, (c) spectra of pure glucose, (d) supernatant of samples transferred to peptone media

The concentration of peptone in the supernatant (Fig. 4d) was found inversely correlated with cell growth, measured by optical density: the longer the irradiation, the higher concentration remained in the supernatant. Alter 105 minutes of illumination, the doublet observed at 1.35 ppm almost reached the initial concentration of peptone, a behavior that nicely correlates with the results obtained by FISH-DVC analysis and confirming that the processes of assimilation of peptone is more stable with respect to the disruptions caused to the cell membrane.

4. CONCLUSIONS

We demonstrated that the stress caused by photocatalytic treatment system could induce to a viable but non cultivable *E. coli* cells. This is a transitory state reached when the damage did not affect certain metabolic pathways, in particular the assimilation of peptone. It is presumable that this phenomenon could also be observed for other microorganisms, when treated by photocatalysis. For real-scale treatments using natural effluent or residual waters, the ability of a cell to grow again once the treatment is over represents an elevated risk for human health. This work clearly shows that the plate count method systematically underestimates the effective time required to completely remove the bacterial threat, since the loss of cultivability occurs before the loss of viability. Therefore, a better suited procedure, FISH-DVC, is proposed instead. Since this method becomes expensive, especially in undeveloped countries, experiments were carried out in a liquid nutrient media and were shown to nicely agree with the FISH-DVC results. This allows us to recommend the use of MPN

(Must Probable Number) methods, using specific nutrient broths when natural consortia are involved.

5. ABBREVIATIONS

DVC, direct viable count; FISH, Fluorescence in situ hybridisation; SPC, standard place count; VBNC, viable but not cultivable; LB, Luria broth; TSB, triptone soy broth; DAPI, 4',6'-diamidino-2-phenylindole; TAC , thricarboxylic acid cycle; NMR, Nuclear Magnetic Resonance.

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