The ability of electrochemical oxidation with a BDD anode to inactivate Gram-negative and Gram-positive bacteria in low conductivity sulfate medium

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Abstract

The disinfection of 100 mL of synthetic water containing 7 mM Na_2SO_4 with 10^6 CFU mL^{-1} of either Gram-negative or Gram-positive bacteria has been studied by electrochemical oxidation. The electrolytic cell was a stirred tank reactor equipped with a boron-doped diamond (BDD) anode and a stainless steel cathode and the trials were performed at acidic and neutral pH, at 33 mA cm^{-2} and 25 ºC. Reactive oxygen species, pre-eminently hydroxyl radicals, were efficiently produced in both media from water oxidation at the BDD anode and the bacteria concentration was reduced by $\geq$ 5 log units after 60 min of electrolysis, thus constituting a good chlorine-free disinfection treatment. All the inactivation kinetics were described by a logistic model, with no significant statistical differences between acidic and neutral suspensions. The electrochemical disinfection with BDD was very effective for Gram-negative bacilli like *E. coli* and *P. aeruginosa* and Gram-positive ones like *B. atrophaeus,* whereas the Gram-positive cocci *S. aureus* and *E. hirae* were more resistant. Thus, the latter organisms are a better choice than *E. coli* as process indicators. Scanning electron microscopy highlighted a transition from initial cells with standard morphology supported on clean filters to inactivated cells with a highly altered morphology lying on dirty filters with plenty of cellular debris. Larger damage was observed for Gram-negative cells compared to Gram-positive ones. The inactivation effect could then be related to the chemical composition of the outer layers of the cell structure along with the modification of the transmembrane potentials upon current passage.

Keywords: Bacillus atrophaeus; Electrochemical disinfection; *Enterococcus hirae*; *Escherichia coli*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

The production of safe water in areas with increasing number of people and scarcity of water is a need. Disinfection is used to reduce the number of pathogenic microorganisms to a low enough level to ensure healthy conditions (WHO, 2015), and must prioritize environmentally friendly methods. Chlorination is the most commonly used disinfection procedure, but it entails several drawbacks such as Cl_2 accumulation and formation of hazardous chloroderivatives. To solve these problems, other alternative methods including ozonation, UV light irradiation and electrochemical disinfection have been developed (Ghernaout and Ghernaout, 2010). To apply electrochemical disinfection as a green technology, its effect on different types of microorganisms under controlled conditions has to be tested.

Electrochemical advanced oxidation processes (EAOPs) have recently received increasing attention for both, removal of organic pollutants from wastewater (Ciríaco et al., 2009; Panizza and Cerisola, 2009; Sirés and Brillas, 2012; El-Ghenymy et al., 2014; Martínez-Huitle et al. 2015) and disinfection of urban and industrial water (Kraft, 2008; Cañizares et al., 2009; Rodrigo et al., 2010; Rajab et al.,2015; Werschkun et al., 2012), including swimming pools (Nakajima et al., 2004). Electrochemical oxidation (EO) is the most common EAOP utilized for electrochemical disinfection. It is characterized by easy and mild operation conditions (Sirés et al., 2014) and possesses environmental compatibility due to the in situ production of hydroxyl radical ($^{\bullet}OH$) from water discharge at the anode surface, without requiring the addition of noxious chemicals (Martínez-Huitle and Brillas, 2008; Oturan et al., 2012; Thiam et al., 2015a, b). The most important parameters affecting the disinfection process are the water composition, the hydrodinamics of the system, the kind of anode material and the applied current density (j), since they determine the distribution of oxidants and by-products (Mascia et al., 2012, 2013; Long et al., 2015). Metals like Pt

(Nakajima et al., 2004; Kerwick et al., 2005; Jeong et al., 2007; Delaedt et al., 2008), carbon electrodes like graphite and activated carbon fiber (Shang et al., 2013), mixed metal oxides of IrO_2, PbO_2, SnO_2, and/or TiO_2 (Martínez-Huitle and Brillas 2008; Panizza and Cerisola, 2008), and conductive boron-doped-diamond (BDD) (Furuta et al., 2004) have been used as anodes. The BDD electrode has excellent properties including large resistance to corrosion in very harsh media, large potential window, low adsorption of $^{\bullet}OH$ and organics and higher O_2 overpotential than other anodes (Martínez-Huitle, 2007; Anglada et al., 2009; Sirés et al., 2014). As a result, the BDD anode is considered the best one for EO, being able to mineralize most organic molecules in sulfate medium (Hamza et al., 2009; Rodrigo et al., 2010; Pipi et al., 2014; Scialdione et al., 2014).

It has been found that the direct electrolysis with conventional anodes such as Pt and RuO_2 only yields a large disinfection when the treated liquid contains chloride ions (Polcaro et al., 2007). This is due to the oxidation of Cl^- with formation of active chlorine species (Cl_2, HClO and/or ClO^-) that attack the bacterial cell. For example, Nakajima et al. (2004) reported the inactivation of all tested bacteria in 5 min upon generation of 30 mg L^{-1} active chlorine using a Pt-Ir anode at 30 mA. In contrast, the BDD anode produces a more active $^{\bullet}OH$, highly suitable for environmental application. The BDD anode is then very effective in the absence of Cl^- ion, thus preventing the accumulation of active chlorine and the possible formation of toxic organochlorinated products, chloramines, ClO_3^- and ClO_4^- (Vacca et al., 2013; Sirés et al., 2014; Martínez-Huitle et al., 2015). In electrochemical disinfection, $^{\bullet}OH$ is expected to exhibit a superior inactivation performance compared to active chlorine because of its much stronger oxidation ability (Cong et al., 2008). The most common supporting electrolyte to investigate the role of $^{\bullet}OH$ produced at BDD is Na_2SO_4, which presents enough conductivity, maintains a correct osmotic potential for the bacterial cells and is chlorine-free.

Some bactericidal mechanisms have been proposed to explain the action of EO with various electrodes under different experimental conditions. However, most of these studies have been focused on *Escherichia coli* as process indicator and use chlorine-containing media for the mediated oxidation by active chlorine (Drees et al., 2003; Kerwick et al., 2005; Jeong et al. 2009). *E. coli* is an indicator microorganism for sanitary water quality and has been widely used as a model organism due to its easy growth under laboratory conditions. Using a BDD electrode in non-chloride media, Polcaro et al. (2007) reported a reduction of three orders of magnitude of this bacterium in 60 s and Jeong et al. (2009) described an inactivation of 2.4 log units in 3 min. In 0.10% Cl^- suspensions, Yao et al. (2011) found that the use of the BDD anode was able to yield overall inactivation in a longer time of 30 min.

Other bacteria, with a different morphology and wall structure compared to *E. coli*, should be comparatively tested in order to determine the actual disinfection ability of electrochemical processes, as well as to validate *E. coli* as the right process indicator. To do this, we have undertaken a study on the EO treatment of different Gram-positive and Gram-negative strains that are the benchmark for the bactericidal tests according to the AENOR standard.

This paper reports the results obtained for the electrochemical inactivation of five bacteria with different cell walls and morphology, representatives of the bacterial pathogens found in the aquatic environment. Two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and three Gram-positive ones, *Bacillus atrophaeus*, *Staphylococcus aureus* and *Enterococcus hirae*, were treated by EO with a BDD anode in Na_2SO_4 medium under acidic and neutral conditions. The inactivation results of the five organisms were modelled and compared to establish the most appropriate indicators for the EO process. The changes in the outer structure of the cell walls were examined by scanning electron spectroscopy (SEM).

2. Materials and methods

2.1. Tested bacteria and culture preparation

Strains of two rod-shaped Gram-negative bacteria, *Pseudomonas aeruginosa* ATTC 15442 and *Escherichia coli* ATTC 10536, one rod-shaped Gram-positive bacterium, *Bacillus atrophaeus* ATCC 9372 (deposited as *B. subtilis var. niger*), and two Gram-positive cocci, *Staphylococcus aureus* ATCC 6538 and *Enterococcus hirae* ATCC 10541, were used. The bacteria were cultured in Trypticasein Soy Agar (TSA) plates, supplied by Laboratorio Conda, at 37 ºC for 24 h. Further, the cells were spiked in 2 mL of 7 mM Na_2SO_4 (analytical grade from Panreac) and centrifuged at 14,000 rpm for 2 min. After washing twice with 1 mL of 7 mM Na_2SO_4, the resulting pellet was resuspended in 1 mL of the same electrolyte giving rise to an optical density at 600 nm (O.D. 600) of 0.7±0.1, corresponding to about 10^8 colony-forming units per mL (CFU mL^{-1}).

2.2. Electrolytic system

All the EO trials were performed in a one-compartment, two-electrode cylindric glass tank reactor of 150 mL capacity. The cell was surrounded with a jacket to keep the treated suspension at 25 ºC under circulation of external thermostated water. The anode was a BDD thin-film electrode purchased from NeoCoat and the cathode was a stainless steel (AISI 304) sheet, both of 3 cm^2 area. The interelectrode gap was near 1 cm. Electrolytic trials were carried out at a low constant current density of 33.3 mA cm^{-2} to show the disinfection power of EO with a BDD anode and under vigorous stirring with a magnetic bar at 800 rpm for ensuring mixing and the transport of bacteria and cytoplasmic residues toward the anode. Before the assays, the anode surface was cleaned via polarization in a 50 mM Na_2SO_4 solution at 100 mA cm^{-2} for 180 min.

2.3. EO assays

136 For each disinfection experiment, 100 mL of aqueous solutions with 7 mM Na_2SO_4 were
137 prepared with ultrapure water of resistivity > 18 MΩ cm obtained from a Millipore Milli-Q
138 system. The solution was then spiked with a single bacterial strain (10^8 CFU mL^{-1}) to obtain a
139 suspension with 10^6 CFU mL^{-1}. The influence of pH on bacterial inactivation was studied at
140 acidic pH~3 by adding analytical grade H_2SO_4 from Panreac and at neutral pH~7 by adding
141 NaOH. The results were compared using the Kolmogorov-Smirnov statistical test.
142 After each treatment, the tank reactor was cleaned with 150 mL of a mixture composed of
143 100 mL of 33% (w/v) H_2O_2 (analytical grade from Panreac Química) and 100 ml of 96%
144 H_2SO_4 (analytical grade from Panreac Química) in 1 L of Milli-Q water for 10 min under
145 vigorous stirring. Afterwards, it was rinsed with ultrapure water and dried in an oven at 80 ºC.
146 The electrodes were cleaned by immersion in ultrapure water at 100 °C for 10 min and,
147 subsequently, they were dried using an air stream.

148 *2.4. Instruments and analytical methods*

149 The O.D. 600 was measured with a Camspec M108 spectrophotometer. The pH and
150 electrical conductance of bacterial suspensions were determined with a Crison GLP 22 pH-
151 meter and a Metrohm 644 conductimeter, respectively. For electrolysis, the constant current
152 was provided by an Amel 2053 potentiostat-galvanostat, using a Demestres 601BR digital
153 multimeter for the instantaneous measurement of cell voltage. TOC analysis of samples
154 withdrawn from electrolyzed suspensions was carried out using a Shimadzu TOC-VCNS
155 analyzer. Reproducible TOC values with an accuracy of ±1% were found by injecting 50 μL
156 aliquots into the analyzer.
157 Aliquots of 1 mL were withdrawn at regular times for 60-90 min of electrolysis. These
158 samples were diluted and cultured in duplicate on TSA plates and incubated at 37 ºC for 24 h.
159 Inactivation was determined from the reduction of culturability as log units reduction, i.e., log
160 (N_t/N_0), where N_t is the CFU value at given time and N_0 is the initial CFU value. The

theoretical detection limit was 1 bacterium per mL. All the EO trials were made thrice (independent experiments).

The surface structure and morphological changes of each bacterium during EO disinfection were analyzed by SEM (Gu et al., 2001; Diao et al., 2004). In each case, two samples were collected at initial time and after 45 min of electrolysis. Each bacterial suspension was filtered through a 0.2 μm polycarbonate membrane filter from Millipore. The filter was then immersed for 30 min in a 2.5% glutaraldehyde solution buffered with 0.1 M cacodylate at pH 7.4. Further, it was preserved at 4 ºC before being processed as follows: The filter was post-fixed in 1% OsO_4, washed with 0.2 M sodium cacodylate and dehydrated with a graded series of ethanol solutions from 30 to 100%, with 10% increments up to 80% and 5% up to 100%. After dehydration, samples were dried with critical point drying and coated with gold before observation. SEM images were obtained with a JEOL JSM-7001F equipment at 15 kV.

3. Results and discussion

3.1. Operation conditions during EO disinfection

The change in TOC, conductivity, cell voltage and pH for 100 mL of suspensions containing 7 mM Na_2SO_4 and the single bacteria strains at a concentration of 10^6 CFU mL^{-1} was determined after 60-90 min of EO treatment at 33.3 mA cm^{-2} to know the characteristics of the disinfection process. TOC analysis is an alternative way to monitor the degradation of the organic matter generated during the lysis of cells. An average TOC value of 2.55±1.50 mg C L^{-1} was found for the initial bacterial suspensions, which arouse from the bacteria content plus compounds remaining upon culture preparation. TOC was only reduced to 2.25±0.90 mg C L^{-1} in average at the end of EO. These findings point to a very small mineralization of the organic matter, either the culture compounds or the spread cell wall and cytoplasm, during the

185 disinfection process. This suggests that the main action of the electric field and the •OH
186 formed at the BDD surface during the electrolysis is the inactivation of the bacterial strains.
187 Similarly, the conductivity of 1.59±0.1 mS cm^{-1} of the untreated suspensions with 7 mM
188 Na_2SO_4 only increased slightly up to 1.70±0.1 mS cm^{-1}, as expected if only small amounts of
189 cytoplasmic salts were released during the process. Regarding the average cell voltage of the
190 BDD/stainless steel tank reactor, it also underwent a small decay from 16.5±1.6 V to 15.8±1.5
191 V during electrolysis, which can be related to the small increase in conductivity. In the case of
192 solution pH, no significant variation was observed during the EO treatments. Thus, the pH of
193 acidic solutions varied from 3.2±0.3 to 3.4±0.5, whereas the pH of neutral solutions changed
194 from 7.1±0.2 to 7.6±0.6. This indicates that, under our experimental conditions with an
195 undivided tank reactor, the H^+ formation from water oxidation at the BDD anode was
196 counterbalanced either by its reduction at the cathode under acidic conditions or,
197 preferentially, by OH^- production at the cathode in neutral medium (Cho et al., 2004; Sirés et
198 al., 2014).

199 All the aforementioned results bring to consider that the EO treatments of all bacteria
200 suspensions occurred under quasi-steady conditions and thus, the main effects were at cellular
201 and subcellular level but did not alter the macroscopic properties of the suspensions.

202 *3.2. Effect of pH on bacteria inactivation*

203 A first series of experiments was carried out to assess the stability of the different bacteria
204 in suspensions at pH near 3 and 7 before EO treatment. The acidic medium was chosen to
205 further check in future the viability of other EAOPs like electro-Fenton and photoelectro-
206 Fenton that operate at optimum pH close to 3 (Sirés and Brillas, 2012; Thiam et al., 2015c).
207 The two Gram-negative microorganisms, *P. aeruginosa* and *E. coli*, as well as the Gram-
208 positive cocci *S. aureus* and *E. hirae*, did not undergo any kind of inactivation in the two
209 tested media. In contrast, the content of the Gram-positive bacterium *B. atrophaeus* was not

stable in none of the sulfate media since it was reduced by two log units at pH~3 and half log unit at pH~7 after 60 min of stirring. Note that Geveke and Kozempel (2003) have also reported that acidification of *E.coli* suspensions did not cause any inactivation of this bacterium.

According to the above results, the suspensions of all the bacteria in 7 mM Na_2SO_4 were prepared just before their EO treatment at 33.3 mA cm^{-2} to obtain comparable inactivation data. Fig. 1 depicts the gradual reduction of log (N_t/N_0) with electrolysis time for the five bacteria in the two tested media. As can be seen, the content of all strains diminished more than 5 log units upon electrolysis, although with different inactivation kinetics.

Fig. 1 shows that the inactivation was already quantitative at 45 min, since at pH~3 the log (N_t/N_0) values of all the bacteria diminished about 6 units, except in the case of *E. hirae* since it decayed near 5 log units. Similarly, the results at pH~7 showed that the rod-shape bacteria decreased 6 log units or more, whereas the cocci dropped about 4.5 log units. At the end of the EO treatment, Fig. 1 evidences that the two Gram-negative as well as the Gram-positive bacilli were totally inactivated, whereas in the case of Gram-positive cocci, *S. aureus* and *E. hirae*, some few cells still survived, except in the case of *S. aureus* at pH~3. Despite the differences observed for the electrochemical disinfection at pH near 3 and 7, it can be concluded that the effect of pH was not statistically significant according to the Kolmogorov-Smirnov test, as will be discussed below.

The five strains suspended in synthetic water with 7 mM Na_2SO_4 at both pH values tested then showed a reduction of $\geq$ 5 log (N_t/N_0) units in 60 min by EO with a BDD anode at a current density of 33.3 mA cm^{-2}. This significant bacterial inactivation, greater than 99.999%, was mainly achieved by the action of the physisorbed BDD($^{\bullet}OH$) radicals formed at the BDD surface from the anodic oxidation of water (Martínez-Huitle and Brillas, 2008; Panizza and Cerisola, 2009). Under comparable conditions, our results highlight that the most resistant

235 bacteria were the cocci *S. aureus* and *E. hirae*, whereas the most fragile microorganism was
236 the bacillus *B. atrophaeus*, being all Gram-positive. Based on the electrochemical inactivation
237 found, one can divide the tested bacteria into three groups:

238 (i) The two Gram-negative bacilli, *E. coli and P. aeruginosa*, with a very similar
239 inactivation rate.

240 (ii) The two Gram-positive cocci, *S. aureus* and *E. hirae*, which were the most resistant
241 microorganisms to this kind of treatment, and

242 (iii) finally, the Gram-positive bacillum, *B. atrophaeus*, which was the most fragile,
243 which can be at least partly linked to its sensitivity to pH variations during EO.

244 It should be noted that *B. atrophaeus* has been called *B. subtilis var. niger* in previously
245 published literature and, consequently, much information about its inactivation is given
246 elsewhere, especially when it is in its sporulated form that largely increases its resistance
247 (Yoon et al., 2007). However, when survival studies were performed with vegetative cells, as
248 in our case (disinfection is only related to vegetative forms), it has simply been reported that
249 *B. subtilis* declined more rapidly than other Gram-positive bacteria like *P. fluorescens* in soils
250 (Van Elsas et al., 1986).

251 Other authors have also compared the inactivation of several bacteria by EO with a BDD
252 anode, showing similar trends to those found by us. Thus, Polcaro et al. (2007) reported a
253 reduction of the content of *E. coli*, coliforms and enterococci from 10^3 CFU mL^{-1} to their
254 detection limit after 60, 100 and 300 s of electrolysis, respectively, using 1 mM Na_2SO_4 at 10
255 mA cm^{-2}. On the other hand, Heim et al. (2015) described fast bacterial reduction rates, close
256 to 5 log units, for *E. coli*, *P. aeruginosa* and *E. faecium* up to a specific charge consumption
257 of 75 mAh L^{-1}, followed by a continuous but much slower inactivation.

258 It must be mentioned that, apart from the strong physisorbed oxidant BDD($^{\bullet}$OH)
259 generated at the BDD surface, this anode can also form other weaker reactive oxygen species

(ROS) from water oxidation such as atomic oxygen ($^{\bullet}O$), H_2O_2 and O_3 (Polcaro et al., 2007; Martínez-Huitle and Brillas, 2008). Furthermore, other weaker oxidants can be produced from the oxidation of the supporting electrolyte, like persulfate ($S_2O_8^{2-}$) ion from the oxidation of SO_4^{2-} ion (Sirés et al., 2014). All these oxidizing species are helpful for disinfection because they can damage the cell membranes, therefore altering their permeability and finally leading to their rupture (Diao et al., 2004). This point will be discussed below from SEM analysis of the untreated and inactivated bacteria.

3.3. Modelling inactivation kinetics

Numerous models have been proposed to describe bacterial survival curves, some of them including terms that account for shoulder and tailing phenomena. The logarithmic inactivation data for each bacteria shown in Fig. 1 were adjusted to a modified logistic model based on Kamau et al. (1990), expressed as follows:

$$\log (N_t/N_0) = \frac{I}{1 + a \exp (i\, t)} \qquad (1)$$

where I denotes the theoretical maximum log reduction achieved upon the EO treatment, a is a parameter of adjustment related to the shape of the first shoulder, i is the inactivation rate (in min^{-1}) and t is the electrolysis time (in min). It should be mentioned that ideal mixing is assumed here, thus ensuring the maximum mass transport toward/from the electrodes. Therefore, the existence of poor hydrodynamic conditions in our laboratory cell can be discarded. In contrast, Mascia et al. (2012) reported the significant effect of flow pattern inside the disinfection unit when treating larger volumes using a filter-press cell, owing to the dispersion phenomena, stagnant zones and bypass flows. When Eq. (1) was applied, it was observed that the second shoulder or tail was highly influenced by the detection limit of the processed volume and the initial concentration of the studied bacteria. Since the fittings were very similar at pH close to 3 and 7, a unique pH-independent plot has been represented from

284 the independent trials made for each bacterium. Fig. 2a-e depicts the graphs thus obtained for
285 the five bacteria, along with the corresponding curves (upper and lower dashed lines) related
286 to 95% confidence intervals on these fits. Table 1 summarizes the fitting parameters of Eq. (1)
287 found in each case, along with the square of their regression coefficients (R^2). The latter
288 values corroborate the goodness of Eq. (1) to describe the inactivation trends of all the tested
289 bacteria.

290 As expected, a first look to Fig. 2 confirms that *S. aureus* was the most resistant strain,
291 whereas *B. atrophaeus* was the most sensitive one. No significant differences can be observed
292 between the intermediate inactivation values of the other bacteria. For example, *S. aureus*
293 reached a 4 log reduction after 38 min of EO treatment, whereas 30 min were required for *E.*
294 *coli*, 28 min for *P. aeruginosa*, 23 min for *E. hirae* and only 14 min for *B. atrophaeus*. Total
295 inactivation of the latter one with a decrease of 6 log units was already reached in 30 min. A
296 similar drop of more than 6 log units for *E. coli* and *P. aeruginosa* was found after 60 min of
297 electrolysis, whereas *S. aureus* and *E. hirae* required longer time to attain their total
298 inactivation. For *E. coli*, *P. aeruginosa*, *S. aureus* and *E. hirae*, a first shoulder at short time
299 can be seen in Fig. 2a, b, d and e, respectively, whereupon the log (N_t/N_0) values decayed up
300 to reach overall disinfection, although for *E. hirae*, the inactivation rate seemed to become
301 drastically reduced once reached a 5 log reduction. The presence of the initial shoulder could
302 be related with the existence of more resistant cells within the whole bacteria populations.
303 This trend was not valid for *B. atrophaeus*, which underwent a much quicker inactivation
304 from the beginning of the electrolysis. Therefore, the classification mentioned in Section 3.2
305 is now verified:

306 (i) The two Gram-negative bacilli, *E. coli and P. aeruginosa*, presented a first shoulder in
307 the log (N_t/N_0)-t plot, followed by a rapid steep decay to end in their total inactivation.

(ii) The two Gram-positive cocci, *S. aureus* and *E. hirae*, presented a first shoulder in the log (N_t/N_0)-*t* plot as well, followed by a less pronounced drop than in case (*i*) to end in a tail. This behavior evidences the need of longer time to reach their total inactivation, and

(iii) finally, the Gram-positive bacillum, *B. atrophaeus*, was rapidly inactivated with no shoulder appearing during the treatment.

To better compare the EO disinfection of bacterial suspensions, the times for 1 log reduction or 90% of inactivation ratio (T_{90}), 2 log or 99% (T_{99}), 3 log or 99.9% ($T_{99.9}$), 4 log or 99.99% ($T_{99.99}$) and 5 log or 99.999% ($T_{99.999}$) were determined considering the kinetic relationship given by Eq. (1). The data obtained are summarized in Table 2. As can be seen, *B. atrophaeus*, a rod-shaped Gram-positive bacterium, required shorter times for inactivation compared to the others, regardless of the considered inactivation ratio. For both rod-shaped Gram-negative bacteria, *P. aeruginosa* and *E. coli*, $T_{99.99}$ was between 28 and 30 min and $T_{99.999}$ between 32 and 35 min. In contrast, the Gram-positive cocci needed longer times to reach a given inactivation ratio, in agreement with their higher resistance. While in the case of *E. hirae*, $T_{99.99}$ was similar to that found for Gram-negative bacteria, *S. aureus* presented a higher value. As for $T_{99.999}$ values, the difference was larger than 11 and 27 min compared to the Gram-negative and Gram-positive bacilli, respectively. For example, the differences between *E. coli* and *S. aureus* were of 7 and 11 min for $T_{99.99}$ and $T_{99.999}$, respectively, and up to 23 and 27 min in the case of *B. atrophaeus* vs. *S. aureus* to reach those inactivation ratios.

Interestingly, once reached an inactivation ratio of 90%, the three bacilli, *E. coli*, *P. aeruginosa* and *B. atrophaeus*, needed similar time intervals to ensure an additional log unit reduction, ranging between 3.1 and 4.8 min, whereas *S. aureus* required longer intervals (between 5.8 and 8.2 min). In the case of *E. hirae*, the time intervals up to $T_{99.9}$ were similar to those of bacilli (3.1 - 4.8 min), but from $T_{99.99}$ to $T_{99.999}$ the intervals became longer, being analogous to those of *S. aureus*. For most bacteria, the time intervals between T_{99} and $T_{99.9}$ and

between $T_{99.9}$ and $T_{99.99}$ were then shorter than the initial interval from T_{90} to T_{99} and the last interval to $T_{99.999}$. This behavior was also verified for *E. hirae* but only up to $T_{99.9}$.

Our results agree with those from other authors that pointed out that, in general, big cells tend to be more susceptible to an electric field than small and oval ones, which may justify the significantly slower inactivation of cocci compared to bacilli (Machado et al., 2010; Guillemes Peira, 2014). On the other hand, it has been reported that the Gram-negative bacteria are more sensitive than the Gram-positive ones to pulsed electric fields (Barsotti and Cheftel, 1999; Jeyamkondan et al., 1999). This has also been found in the present study, except in the case of *B. atrophaeus.*

3.4. SEM analysis during disinfection trials

SEM micrographs of the cells of the different bacterial strains were obtained before electrolysis and after 45 min of their EO treatments with a BDD anode at 33.3 mA cm^{-2}, as depicted in Fig. 3a-e. Before treatment, the cells showed their standard morphology, three bacillary forms of similar size and two coccoid forms, and the filters were clean. In contrast, their morphology was largely altered upon EO disinfection, becoming the cell surface of all the bacteria much rougher. Moreover, the filters became dirty with a great deal of cellular debris, probably because large amounts of cellular material were released from the inactivated cells. *E. coli* (see Fig. 3a) and *P. aeruginosa* (see Fig. 3b), both with a Gram-negative cell wall, underwent the most significant surface modification, which was less evident in the case of the bacteria with a Gram-positive wall, like the bacillus *B. atrophaeus* (see Fig. 3c) and the cocci *S. aureus* (see Fig. 3d) and *E. hirae* (see Fig. 3e). However, it seems that the cell appearance of the latter two organisms pointed to some shrinkage.

The inactivation kinetics of the tested strains and their morphological changes can then be related to the attack of ROS, like BDD($^{\bullet}OH$) and O_3, produced in situ by EO on their cellular walls having different structure. The effect of these oxidants can be explained from other

358 disinfection techniques. It has been described that they diffuse toward the outer layers of the
359 bacterial cells and then infiltrate into the membrane and cytoplasm, reacting with proteins and
360 unsaturated lipids. Consequently, the cell walls may be broken by lysis, causing the leackage
361 of inner compounds to the reaction medium and, simultaneously, the radicals can penetrate
362 into the cytoplasm and affect the enzymes and DNA molecules (Hunt and Mariñas, 1999).
363 Accordingly, our SEM results show that the ROS generated by BDD caused changes in the
364 cell envelope, which became rougher, especially in the Gram-negative bacilli. The vast
365 majority of the cells, after 45 min of electrolysis, lost their growth ability, despite the
366 apparently unaffected morphology of most of them, as also claimed by other authors
367 (Machado et al., 2010). Hunt and Mariñas (1999) explained that chemical reactions between
368 O_3 and biomolecules continue after loss of inactivation until the disinfectant is exhausted or
369 the biomolecules are completely oxidized. Thus, the generated ROS in EO directly affect the
370 cell walls causing their membrane cleavage (Diao et al., 2004). Other authors have also
371 reported that the EO treatment of *E. coli* in sulfate medium induces damage to the cell
372 membrane. The generated chemicals attack the membrane proteins and modify the K^+
373 balances, which affects the cell division and the synthesis of cellular ATP until causing the
374 bacterial inactivation (Polcaro et al, 2007, Jeong et al., 2009). Long et al. (2015) also
375 observed lipid peroxidation during electrochemical disinfection with a BDD anode.

376 According to our SEM observations, bacteria with Gram-positive wall preserve their cell
377 structure better than the Gram-positive ones. This could be explained by the molecular
378 composition of the outer layers, since the Gram-positive cells have a thick peptidoglycan
379 layer, whereas the Gram-negative ones only have a phospholipid bilayer with
380 lipopolysaccharide molecules and proteins under which there is a much thinner peptidoglycan
381 layer. Some studies have shown that the phospholipid membrane is hardly oxidizable,
382 whereas the proteins are easier to destroy under the direct effect of the electric current (Linley

et al., 2012). There is less information about the relationship between ROS and the outer membrane of Gram-positive cells, but our results suggest that their outer layer was quite resistant during inactivation, maintaining the initial structure despite the slight size reduction observed in the case of both cocci. It is also noteworthy that electrogenerated H_2O_2 and O_3 can go through the membranes and reach the vital centre of the cells (Drogui et al., 2001)

An additional significant difference observed upon EO treatment was the appearance of cellular debris, also described elsewhere. For example, Diao et al. (2004) observed substantial intracellular materials leaked out from the cells after electrochemical disinfection of *E. coli* suspensions using dimensionally stable anodes, which was ascribed to the oxidation of the membranes by electrogenerated ROS such as peroxides, $^{\bullet}OH$ and ozone. Finally, note that in the EO assays the cell membranes might undergo large modifications of their transmembrane potentials due to the concomitant electric field of ca. 16.5 V cm^{-1} in the BDD/stainless steel tank reactor. It has been reported that, if the resulting transmembrane potential value ranges between 0.2 and 1 V, reversible pore formation in the membrane (electroporation or electropermeabilization) may occur. Greater values lead to the cell death (Weaver and Chizmadzhev, 1996: Machado et al., 2010), therefore contributing to their inactivation. Since critical electric fields in the kV cm^{-1} range would be necessary to promote irreversible electroporation (García et al., 2016), its contribution in the present study seems rather insignificant.

4. Conclusions

It has been shown that the five tested bacterial strains, two Gram-negative and three Gram-positive, suspended at 10^6 CFU mL^{-1} in synthetic water with 7 mM Na_2SO_4, experienced a significant reduction of at least 5 log units within 60 min of EO with a BDD anode at 33.3 mA cm^{-2}. This method can then be considered a suitable chlorine-free

disinfection treatment. ROS, pre-eminently hydroxyl radicals, generated at the BDD surface were very efficient under acidic and neutral conditions. Although apparently the inactivation seemed more effective at pH~3, no relevant statistical differences were fond at pH~7. A modified logistic model has been used to describe the inactivation kinetics in all cases. The electrochemical disinfection with BDD was very effective for the bacilli *E. coli*, *P. aeruginosa* and *B. atrophaeus*, being the latter one much more sensitive. In contrast, the Gram-positive cocci *S. aureus* and *E. hirae* were more resistant and, consequently, they should be chosen as more appropriate indicators than *E. coli* for the EO treatment. The SEM micrographs of all bacteria showed a transition from cells with standard morphology supported on clean filters to cells with a highly altered morphology lying on dirty filters with plenty of cellular debris due to their lysis. These observations revealed a greater damage in the case of the Gram-negative organisms, due to their particular cell wall structure. The overall inactivation effect can then be explained not only on the basis of oxidizing electrogenerated ROS but also from the different chemical composition of the outer cell layers and the large modifications of the transmembrane potentials upon application of the electric current.

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580

Figure captions

Fig. 1. Logarithmic reduction of bacterium content with electrolysis time for the electrochemical oxidation (EO) treatment of 100 mL of aqueous suspensions with 7 mM Na_2SO_4 and 10^6 CFU mL^{-1} of a given bacterium using a BDD/stainless steel cell at 33.3 mA cm^{-2} and 25 ºC. Bacterium: *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Bacillus atrophaeus* (BA), *Staphylococcus aureus* (SA) and *Enterococcus hirae* (EH). The number 3 or 7 in each acronym accounts for the initial solution pH, i.e., 3.0 or 7.0, respectively.

Fig. 2. Logistic model applied to the electrochemical inactivation kinetics of: (a) *E. coli* (b), *P. aeruginosa*, (c) *B. atrophaeus*, (d) *S. aureus* and (e) *E. hirae* during the EO trials shown in Fig. 1. The dashed lines represent the 95% confidence intervals on these fits.

Fig. 3. SEM images for: (a) *E. coli* (b), *P. aeruginosa*, (c) *B. atrophaeus*, (d) *S. aureus* and (e) *E. hirae* supported on polycarbonate membrane filters. Samples correspond to bacteria suspensions in 7 mM Na_2SO_4 at pH 7.0, before (left) and after (right) 45 min of EO treatment with a BDD/stainless steel cell at 33.3 mA cm^{-2} and 25 ºC.

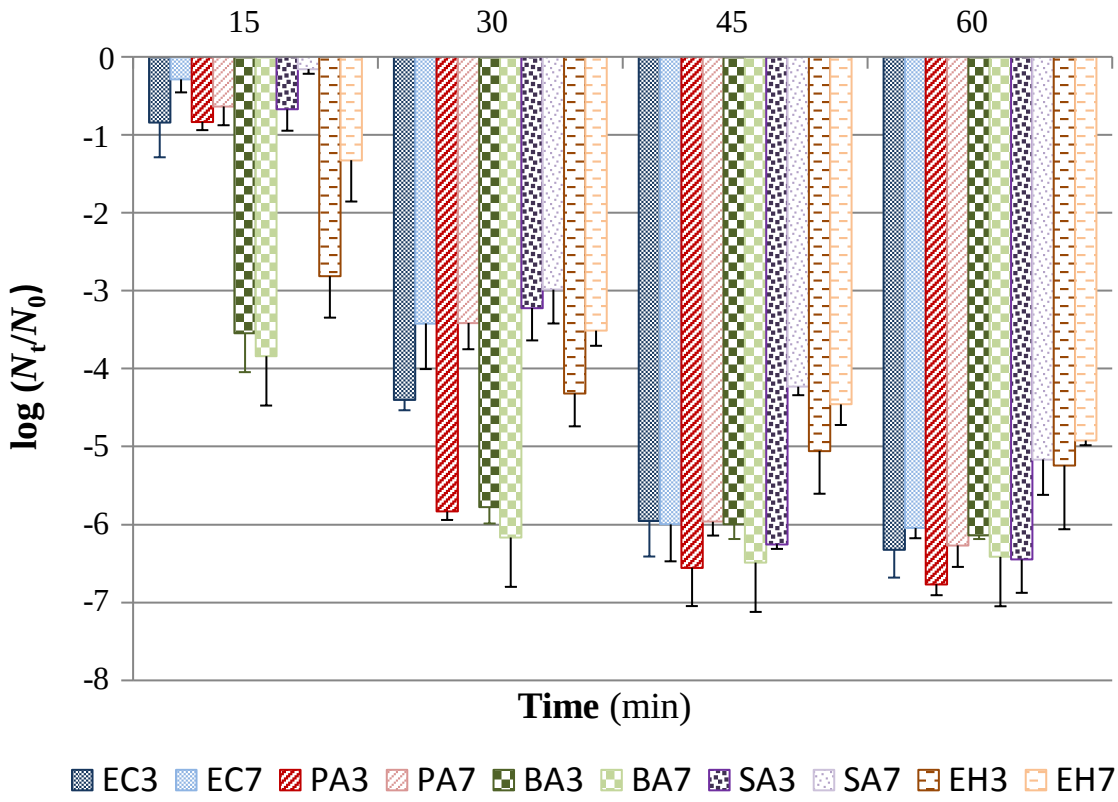

15
30
45
60
0
-1
-2
-3
-4
-5
-6
-7
-8
log (N_t/N_0)
Time (min)
EC3
EC7
PA3
PA7
BA3
BA7
SA3
SA7
EH3
EH7

Fig. 1

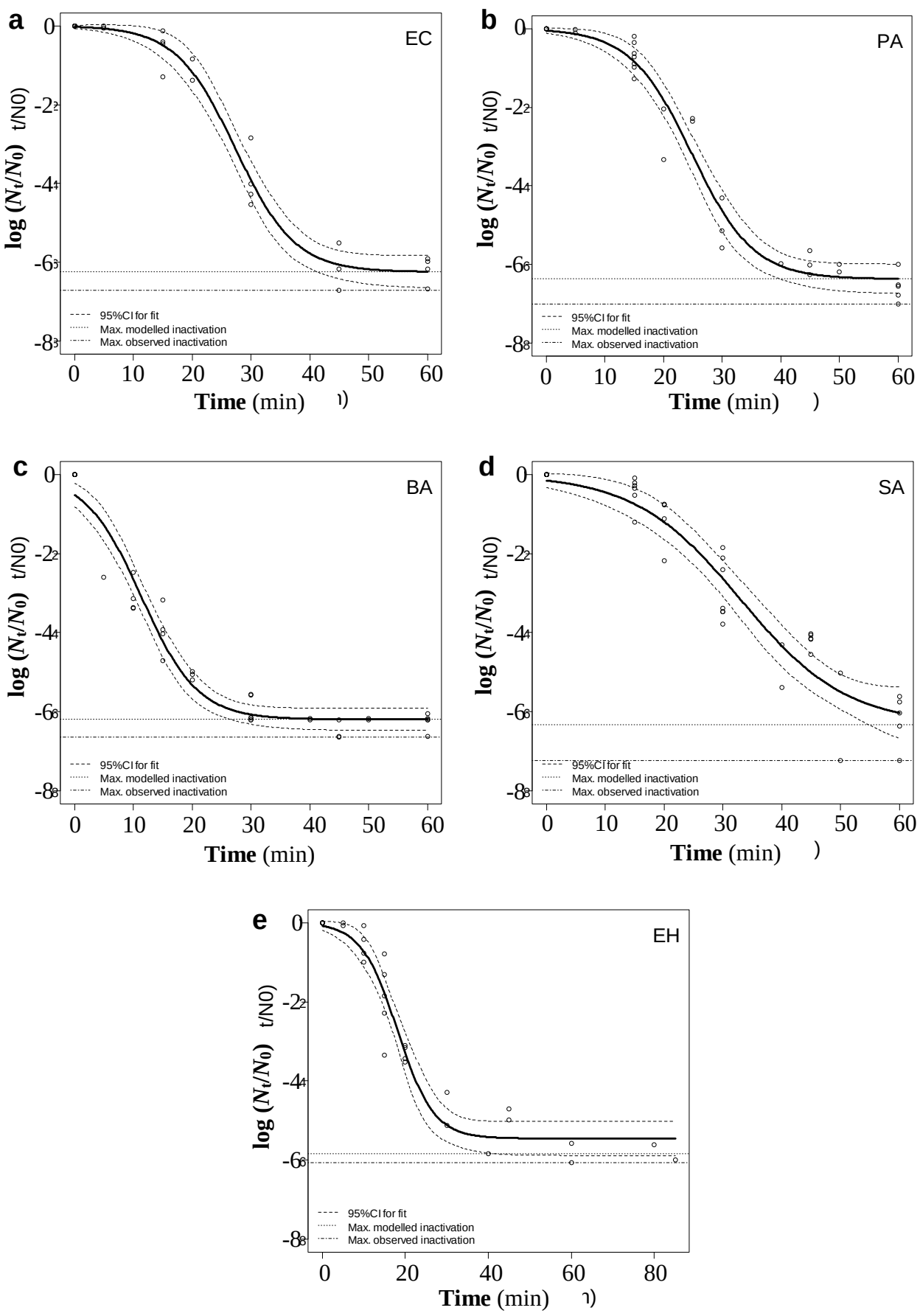
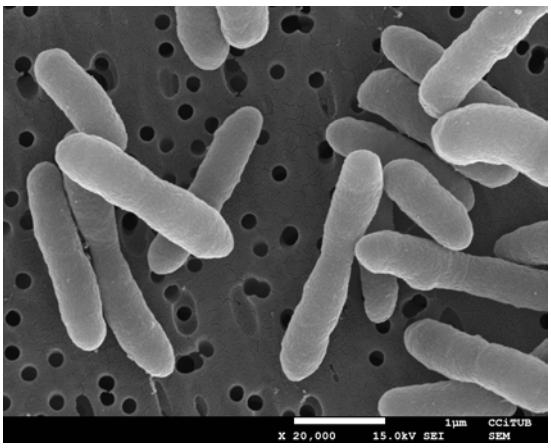
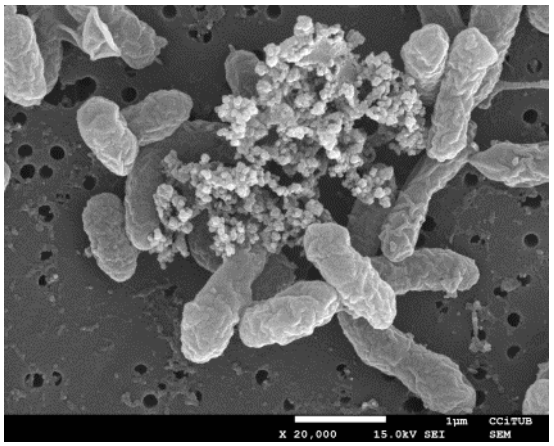

a
EC
b
PA
c
BA
d
SA
e
EH
log (Nt/N0) t/N0)
Time (min)
95%CI for fit
Max. modelled inactivation
Max. observed inactivation

Fig. 2

a


1µm CCiTUB
X 20,000 15.0kV SEI SEM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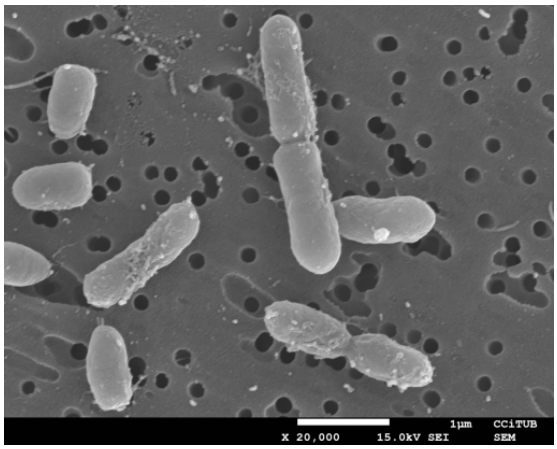
b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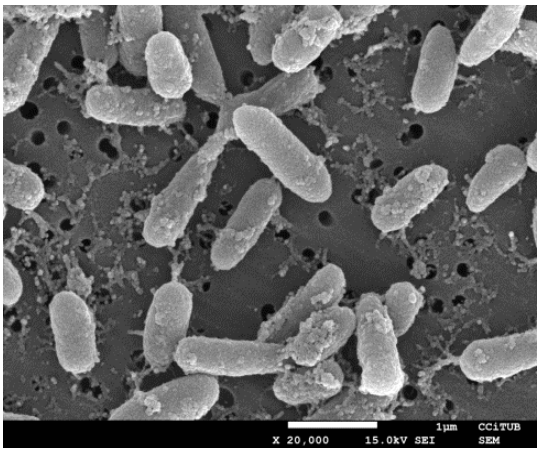
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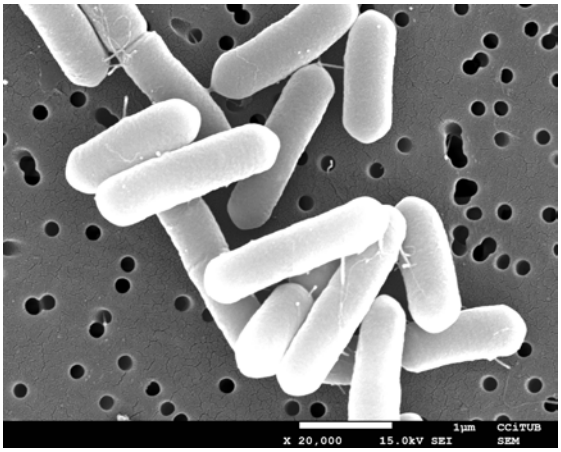
c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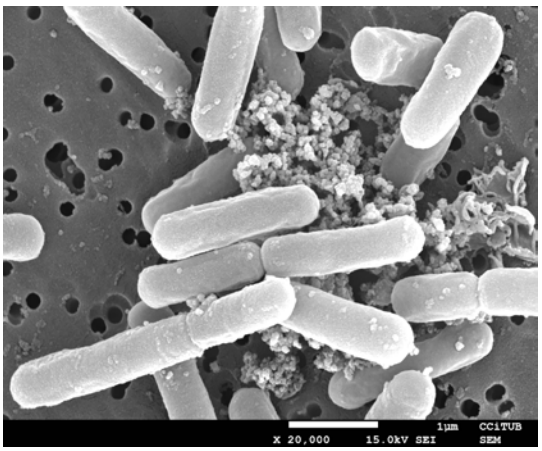
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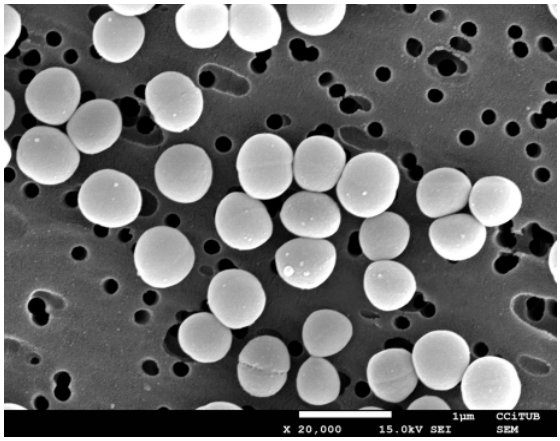
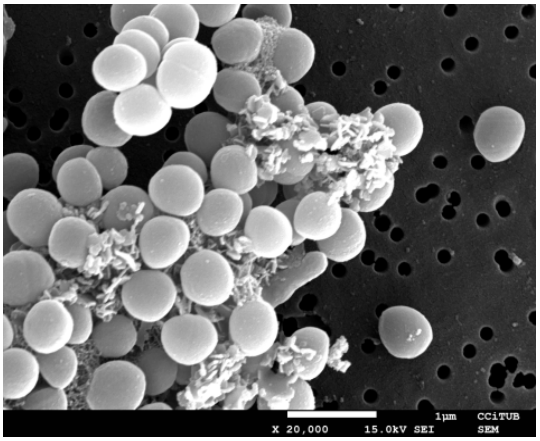
X 20,000 15.0kV SEI SEM

d


X 20,000 15.0kV SEI 1µm CCiTUB SEM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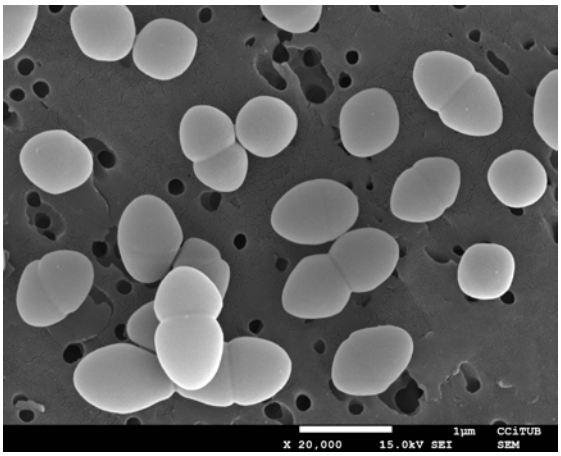
e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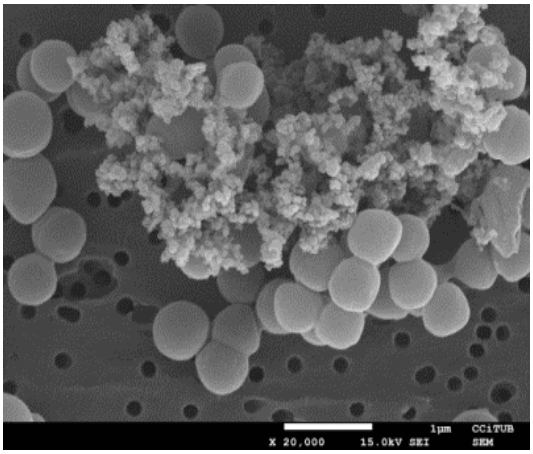
X 20,000 15.0kV SEI 1µm CCiTUB SEM

Fig. 3

693 Table 1

694 Parameters and goodness of fit for the logistic model curves of Fig. 2 for each bacterium.

695

Parameter	*E. coli*	*P. aeruginosa*	*B. atrophaeus*	*S. aureus*	*E. hirae*
I	-6.243	-6.369	-6.192	-6.327	-5.453
a	228.958	115.509	10.810	39.166	63.981
i	-0.198	-0.192	-0.2092	-0.111	-0.229
R^2	0.979	0.971	0.964	0.942	0.955

696

697

698

699 Table 2

700 Time required for selected inactivation ratios expressed in percentage for each bacterium
701 using the logistic model of Eq. (1).

702

Bacterium	Time for inactivation ratios (min)				
	T_{90}	T_{99}	$T_{99.9}$	$T_{99.99}$	$T_{99.999}$
E. coli	19.1	23.6	27.1	30.4	34.5
P. aeruginosa	15.9	20.7	24.1	27.5	31.5
B. atrophaeus	3.5	7.8	11.1	14.2	18.2
S. aureus	17.9	26.1	32.1	37.9	45.0
E. hirae	11.6	15.7	19.0	22.6	28.6

703

704