

Toluene biodegradation in an algal-bacterial airlift photobioreactor: influence of the biomass concentration and the presence of an organic phase

Raquel Lebrero*, Roxana Ángeles, Rebeca Pérez, Raúl Muñoz

Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina, s/n, 47011, Valladolid, Spain. Tel. +34 983186424, Fax: +34983423013.

*Corresponding author: raquel.lebrero@iq.uva.es

Keywords: Airlift bioreactor, algal-bacterial photobioreactor, toluene biodegradation, two-phase partitioning bioreactor

Abstract

The potential of algal-bacterial symbiosis for off-gas abatement was investigated for the first time by comparatively evaluating the performance of a bacterial (CB) and an algal-bacterial (PB) airlift bioreactors during the treatment of a 6 g m⁻³ toluene laden air emission. The influence of biomass concentration and of the addition of a non-aqueous phase was also investigated. A poor and fluctuating performance was recorded during the initial stages of the experiment, which was attributed to the low biomass concentration present in both reactors and to the accumulation of toxic metabolites. In this sense, an increase in the dilution rate from 0.23 to 0.45 d⁻¹ and in biomass concentration from ~1 to ~5 g L⁻¹ resulted in elimination capacities (ECs) of 300 g m⁻³ h⁻¹ (corresponding to removal efficiencies ~ 90 %). Microalgae activity allowed for a reduction in the emitted CO₂ and an increase in dissolved O₂ concentration in the PB. However, excess biomass growth over 11 g L⁻¹ hindered light penetration and severely decreased photosynthetic activity. The addition of silicone oil at 20 % (on a volume basis) stabilized system performance, leading to dissolved O₂ concentrations of 7 mg L⁻¹ and steady ECs of 320 g m⁻³ h⁻¹ in the PB. The ECs here recorded were considerably higher than those previously reported in toluene-degrading bioreactors. Finally, microbial population analysis by DGGE-sequencing demonstrated the differential specialization of the microbial community in both reactors, likely resulting in different toluene degradation pathways and metabolite production.

33 **1. Introduction**

34 Due to its valuable psychoactive properties, the amount of toluene used in the chemical
35 industry has noticeably increased over the last decades, leading to a concomitant
36 increase in the atmospheric emissions of this aromatic pollutant (WHO 2000, EURAR-
37 T 2003). Toluene causes toxic effects to both human health and the environment, and is
38 considered a priority pollutant by the US-Environmental Protection Agency. Physical-
39 chemical technologies for toluene removal, such as adsorption in activated carbon,
40 thermal incineration or UV oxidation, generate hazardous by-products and/or wastes
41 and entail high investment and operating costs. In this context, bioremediation based on
42 bacterial or fungal activity has arisen as a cost-effective and environmentally
43 sustainable alternative to conventional physical-chemical methods (Harding et al. 2003).
44 In particular, pneumatically agitated suspended-growth systems such as airlift
45 bioreactors have demonstrated a cost-effective toluene abatement performance while
46 avoiding typical operational problems encountered in packed-bed bioreactors: excessive
47 biomass growth, flooding, channeling, pressure drop build-up or formation of anaerobic
48 or dry zones (Vergara-Fdez et al. 2008). Nevertheless, airlift bioreactors for the
49 treatment of toluene still face severe limitations such as (i) a limited pollutant mass
50 transfer from the gas to the liquid phase due to its high Henry law, which results in a
51 low pollutant bioavailability, (ii) microbial inhibition at high toluene inlet
52 concentrations, and (iii) O₂ limitation when treating high toluene loads. An enhanced
53 oxygen supply to the microbial community in the reactor by increasing the turbulence of
54 the culture broth might be problematic since intensive mechanical aeration in
55 bioreactors is highly costly and might cause volatilization and re-dispersion of the
56 toluene present in the aqueous phase (Muñoz et al. 2005).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77

Photosynthetic oxygenation in algal-bacterial photobioreactors constitutes an efficient alternative to conventional aeration methods. In this process, the oxygen photosynthetically produced by microalgae in the presence of light and CO₂ is used by heterotrophic bacteria to *in situ* oxidize the organic pollutant, producing in return the CO₂ needed for microalgae photosynthesis. Unlike bacterial processes where the mineralization of organic pollutants releases CO₂ and H₂O as main end-products, microalgal processes are able to fix and recover carbon and other nutrients as valuable biomass besides furnishing O₂ to the heterotrophic community. Moreover, some microalgae are capable of biotransforming xenobiotic organic contaminants (Semple et al. 1999; Muñoz and Guieysse 2006). Therefore, microalgae may boost the biodegradation of toluene either by directly biotransforming the pollutant or by enhancing the degradation potential of the microbial community present in the bioreactor. Besides, additional O₂ supply by photosynthetic activity might prevent oxygen limitation and accelerate the bacterial degradation of toluene (Semple et al. 1999). Up to date, most studies on toluene biodegradation were based on the activity of bacteria and fungi. On the contrary, microalgae-supported biodegradation of aromatic contaminants has been scarcely investigated, and the catabolic pathways of biodegradation of aromatic compounds in microalgae are still largely unknown. Thus, despite the potential benefits of microalgae-based off-gas treatment, there is no single study comparatively evaluating the performance of algal-bacterial and bacterial bioreactors for the treatment of volatile organic compounds (VOCs).

78
79
80
81

On the other hand, the limited mass transfer of toluene to the liquid phase might be overcome by the addition to the bioreactor of a non-aqueous phase (NAP) with high affinity for this pollutant, resulting in a so-called two-liquid phase partitioning bioreactor (TLPPB). TLPPBs have been successfully applied to treat both high and low

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

82 VOC-laden gas emissions, improving the performance of biological processes (Muñoz
83 et al. 2012, Lebrero et al. 2013). The NAP not only supports an enhanced mass transfer
84 of the target pollutant and O₂ from the gas phase to the microorganisms, but also acts as
85 a buffer against surges in pollutant or metabolite concentrations that might be
86 potentially toxic to the microbial community (Lebrero et al. 2015).

87 This work was devised to comparatively evaluate a bacterial and an algal-bacterial
88 airlift bioreactors treating toluene at high loading rates. The influence of biomass
89 concentration and of the addition of a non-aqueous phase in order to overcome mass
90 transfer limitations and the inhibition resulting from high toluene loads were also
91 investigated. Moreover, the differential specialization of the microbial communities in
92 both bioreactors was also assessed by molecular techniques.

93

94 **2. Materials and Methods**

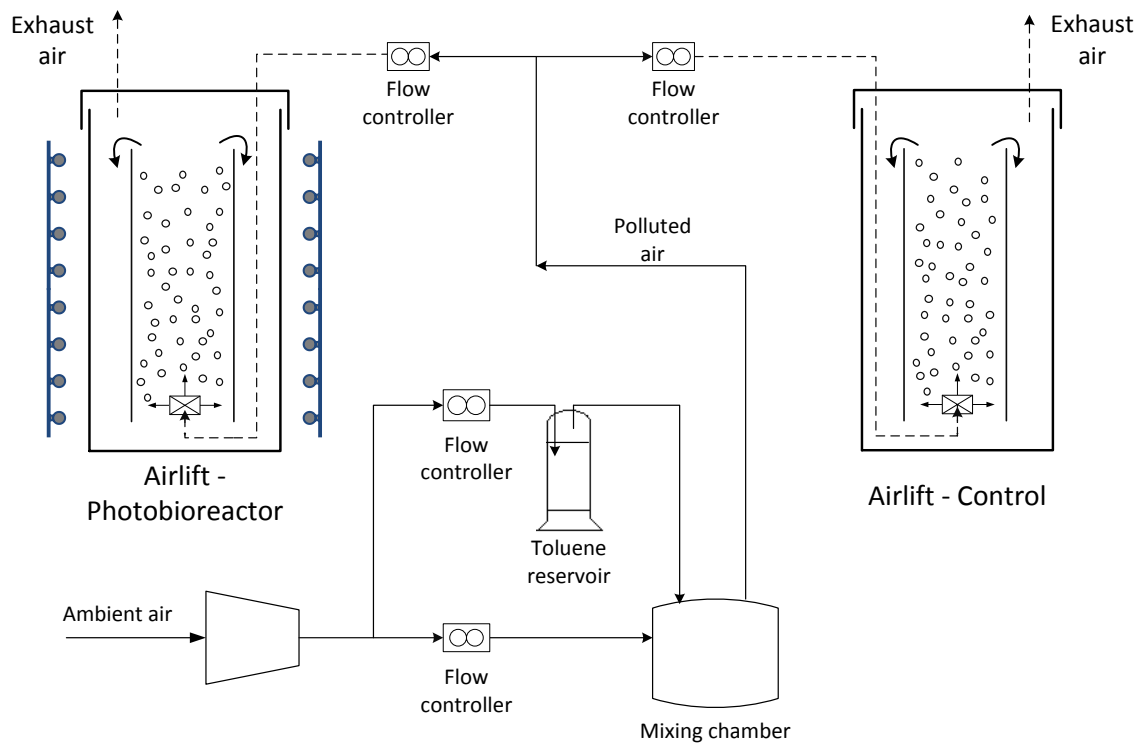
95 **2.1 Microorganisms and culture conditions**

96 Both bioreactors were inoculated with a mixture of *Chlorella sorokiniana* (0.5 L),
97 activated sludge from Valladolid wastewater treatment plant (0.1 L) and a toluene
98 acclimated *Pseudomonas putida* DSM 6899 culture (0.35 L) to an initial total suspended
99 solids concentration (TSS) of 0.79 g L⁻¹. The mineral salt medium (MSM) used
100 throughout the entire experiment was composed of (g L⁻¹): KNO₃ 1.25, CaCl₂·H₂O
101 0.1105, H₃BO₃ 0.1142, FeSO₄·7H₂O 0.0498, ZnSO₄·7H₂O 0.0882, MnCl₂·4H₂O
102 0.0144, MoO₃ 0.0071, CuCl₂·2H₂O 0.0157, CoCl₂·2H₂O 0.0049, EDTA (C₁₀H₁₆N₂O₈)
103 0.5, KH₂PO₄ 0.6247 and K₂HPO₄ 1.3251.

104

105 **2.2 Experimental set-up**

106 The experimental plant consisted of two identical internal-loop airlift glass bioreactors
 107 with a total volume of 2.5 L and a working volume of 2.2 L, operated in parallel in an
 108 air-conditioned room at a constant temperature of 25 °C (Figure 1). The inner tube had a
 109 diameter of 0.055 m and a height of 0.295 m, while the external cylinder diameter and
 110 height were 0.09 m and 0.42 m, respectively. A porous metallic diffuser, with an
 111 average pore diameter of 2 μm, was placed at the bottom of the inner tube to promote
 112 gas distribution. The air-lift photobioreactor (PB) was continuously illuminated with
 113 LED lamps arranged concentrically at an average intensity of 330.5 μmol m⁻² s⁻¹ (within
 114 the optimum range for the photosynthetic apparatus of microalgae, Muñoz and Guieysse
 115 2006), while the control air-lift (CB) was covered with an opaque structure to prevent
 116 diffuse light penetration.



117
 118 **Figure 1.** Schematic representation of the experimental set-up
 119

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

120 The toluene-contaminated stream was obtained by sparging ambient air (flow controller,
121 Aalborg, USA) in a reservoir containing liquid toluene kept at a constant temperature of
122 25 ± 2 °C. The toluene-laden stream was then diluted with ambient air in a mixing
123 chamber and subsequently divided into two identical streams (flow controller, Aalborg,
124 USA) to feed both air-lift reactors. The toluene inlet concentration was maintained at
125 6.2 ± 0.7 g m⁻³ and the reactors were operated at an empty bed residence time of ~1.1
126 min (corresponding to an inlet load of 369 ± 45 g m⁻³ h⁻¹).

128 **2.3 Operational procedure**

129 During the first 6 days of operation, 0.5 L of the culture broth were daily removed from
130 each bioreactor and replaced by fresh MSM. From day 6 to 26, the exchange of culture
131 medium was increased to 1 L in order to avoid metabolite accumulation, which resulted
132 in low TSS concentrations in both reactors. Therefore, 0.5 L of the 1L culture broth
133 daily replaced were centrifuged and the biomass returned to the corresponding reactor
134 from day 26 on. This strategy allowed controlling the biomass concentration and
135 investigating the effect of this parameter on toluene removal performance. From day 46
136 onwards, the biomass recovered by centrifugation was increased to that corresponding
137 to 0.9 L of the retrieved culture broth. Weekly cleanings were performed from day 14 to
138 remove any biomass attached to the reactor walls. Finally, 20% of the culture broth was
139 substituted by silicone oil 200 cSt (Sigma Aldrich, USA) at day 82 in order to improve
140 process robustness.

141 Inlet and outlet gas concentrations of toluene and CO₂ were daily measured in both
142 bioreactors. The pH, temperature and dissolved oxygen (DO) were daily recorded from
143 the culture broths. Three times per week, samples from the culture broth were also taken
144 to determine the concentration of TSS, total organic carbon (TOC) and total nitrogen

145 (TN) in both bioreactors. By day 68, samples from the liquid phase of each bioreactor
146 were taken and analyzed in duplicate by SPME-GC-MS in order to assess the presence
147 of any secondary metabolite accumulated in the culture broth.

148 Finally, six samples for microbial analysis were collected and stored immediately at
149 -20°C . Samples 1 and 2 corresponded to the CB and the PB inoculum, respectively.
150 The rest of the samples were retrieved at the end of the experiment (day 106) from the
151 suspended biomass in the CB (sample 3) and the PB (sample 4), and from the biomass
152 attached to the reactor walls in the CB (sample 5) and the PB (sample 6).

153

154 **2.4 Analytical methods**

155 Toluene gas concentration was measured using a Varian 3900 gas chromatograph (Palo
156 Alto, USA) equipped with a flame ionization detector and a SupelcoWax capillary
157 column ($15\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$). The injector, detector and oven temperatures
158 were set at 210, 250 and $140\text{ }^{\circ}\text{C}$, respectively. CO_2 concentration was determined in a
159 Bruker 430 gas chromatograph (Palo Alto, USA) coupled with a thermal conductivity
160 detector and equipped with a CP-Molsieve 5A ($15\text{ m} \times 0.53\text{ }\mu\text{m} \times 15\text{ }\mu\text{m}$) and a CP-
161 Pora-BOND Q ($25\text{ m} \times 0.53\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$) columns. The oven, injector and detector
162 temperatures were maintained at 45, 150 and 175°C , respectively.

163 Temperature and DO concentration in the culture broth of both reactors were monitored
164 by an OXI 330i oximeter (WTW, Germany). The pH of the liquid samples was
165 determined by a pH meter Crison 50 12T (Crison Instruments, Spain), while biomass
166 concentration was measured as TSS according to Standard Methods (American Water
167 Works Association, 2012). Samples for the determination of TOC and TN
168 concentrations were filtered through $0.22\text{ }\mu\text{m}$ filters (Merck Millipore, USA) prior to

169 analysis in a TOC-VCSH analyzer (Shimadzu, Japan) coupled with a
170 chemiluminescence detection TN module (TNM-1) (Shimadzu, Japan).

171 The presence of secondary metabolites in the culture broth was analyzed as follows:

172 1.7-mL glass vials were filled with 1.5 mL of the culture broth sample and closed with

173 Teflon/rubber caps. Samples were pre-concentrated by SPME by immersing an 85- μ m

174 Carboxen/PDMS fibre (Supelco, Bellefonte, USA) into the leachate for 5 min. The

175 SPME fibre was then injected and allowed to desorb for 1 min in an Agilent 6890N

176 GC-MS equipped with a DB-WAX column (30 m \times 0.250 mm \times 0.25 μ m) (J&W

177 Scientific[®], CA, USA). The injector temperature was set at 200 °C while the oven

178 temperature was initially maintained at 40 °C for 4 min and then increased at

179 10 °C min⁻¹ up to 200 °C. Source and MS quadrupole temperatures were set at 230 and

180 150 °C, respectively. Only compounds with a match quality \geq 90 % were considered in

181 the discussion.

182

183 **2.5 Microbiological procedures**

184 Genomic DNA was extracted using the protocol described in the Fast[®] DNA Spin Kit

185 for Soil (MP Biomedicals, LLC) handbook. The V6-V8 region of the bacterial 16S

186 rRNA genes was amplified by polymerase chain reaction (PCR) using the universal

187 bacterial primers 968-F-GC and 1401-R (Sigma-Aldrich, St. Louis, MO, USA). The

188 PCR mixture contained 1 μ L of each primer (10 ng μ L⁻¹ each primer), 25 μ L of

189 BIOMIX ready-to-use 2 reaction mix (Bioline, Ecogen), 2 μ L of the extracted DNA,

190 and Milli-Q water up to a final volume of 50 μ L. The PCR thermo-cycling program

191 consisted of 2 min of pre-denaturation at 95°C, 35 cycles of denaturation at 95°C for 30

192 s, annealing at 56°C for 45 s, and elongation at 72°C for 1 min, with a final 5-min
193 elongation at 72°C.

194 The DGGE analysis of the amplicons was performed with a D-Code Universal Mutation
195 Detection System (Bio Rad Laboratories) using 8 % (w/v) polyacrylamide gels with a
196 urea/formamide denaturing gradient of 45 to 65 %. DGGE running conditions were
197 applied according to Roest et al. (2005). The gels were stained with GelRed Nucleic
198 Acid Gel Stain (biotium) for 1 h. The most relevant bands were excised from the DGGE
199 gel in order to identify the bacteria present in the samples, resuspended in 50 µL of
200 ultrapure water and maintained at 60 °C for 1 h to allow DNA extraction from the gel.
201 A volume of 5 µL of the supernatant was used for reamplification with the original
202 primer set. Before sequencing, PCR products were purified with the GenElute PCR
203 DNA Purification Kit (Sigma-Aldrich, St. Louis, MO, USA).
204 DGGE profiles were compared using the GelCompar IITM software (Applied Maths
205 BVBA, Sint-Martens-Latem, Belgium). After image normalization, bands were defined
206 for each sample using the bands search algorithm within the program. The peak heights
207 in the densitometric curves were also used to determine the diversity indices based on
208 the Shannon–Wiener diversity index (H), calculated as follows:

$$209 \quad H = -\sum [P_i \ln(P_i)]$$

210 where H is the diversity index and P_i is the importance probability of the bands in a lane
211 ($P_i = n_i/n$, where n_i is the height of an individual peak and n is the sum of all peak
212 heights in the densitometric curves). Therefore, this index reflects both the sample
213 richness (relative number of DGGE bands) and evenness (relative intensity of every
214 band).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

215 Similarity indices of the compared samples were calculated from the densitometric
216 curves of the scanned DGGE profiles by using the Pearson product–moment correlation
217 coefficient (Häne et al. 1993). The taxonomic position of the sequenced DGGE bands
218 was obtained using the RDP classifier tool (50% confidence level) (Wang et al. 2007).
219 The closest cultured and uncultured relatives to each band were obtained using the
220 BLAST search tool at the NCBI (National Centre for Biotechnology Information)
221 (McGinnis and Madden 2004). The sequences generated from this work are deposited in
222 GenBank under accession numbers KT200332-KT200347.

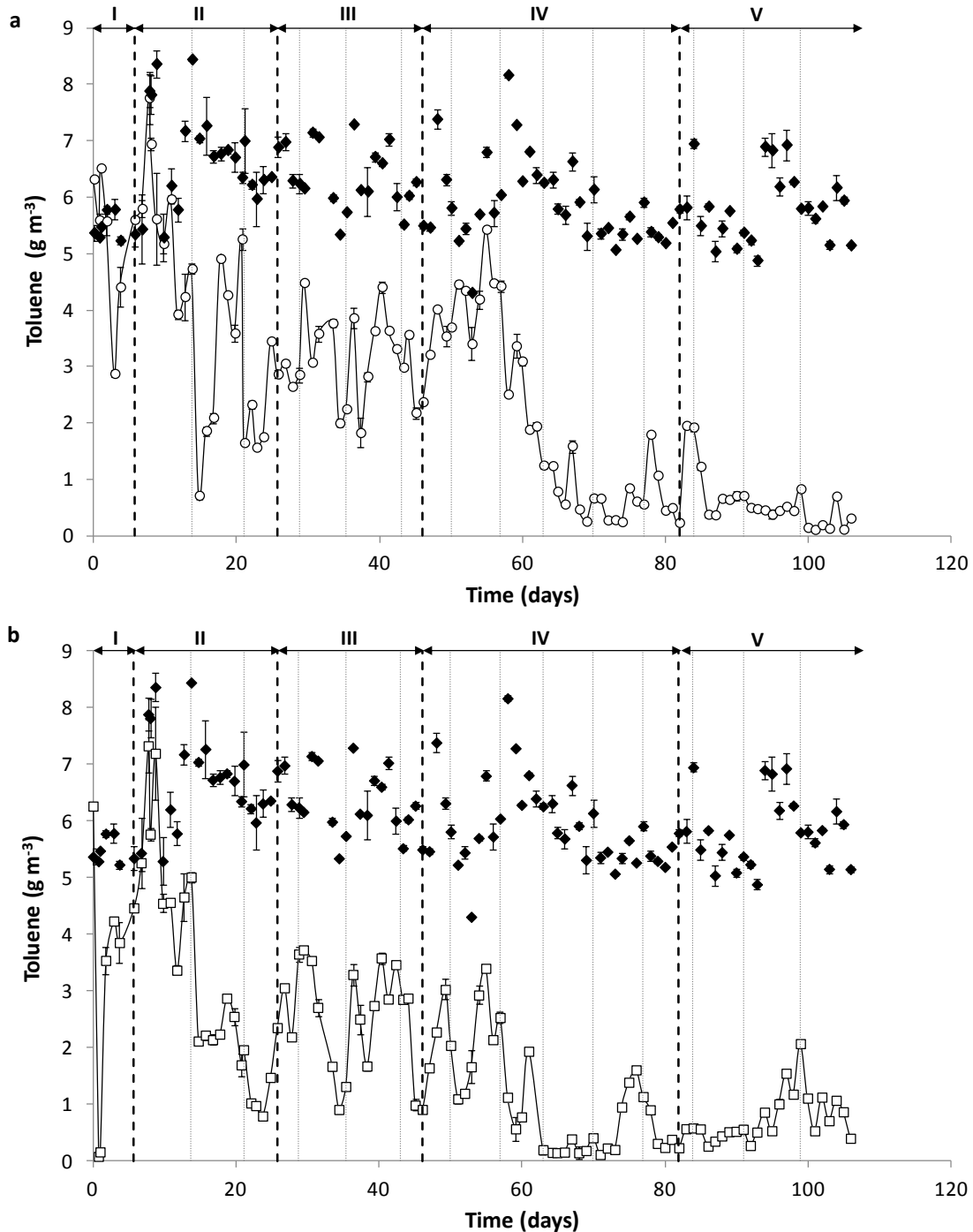
223

224 **3. Results and Discussion**

225 **3.1. Bacterial and algal-bacterial airlifts performance**

226 A different performance was observed during the start-up of both bioreactors despite
227 following the same inoculation procedure. Whereas toluene removal efficiency (RE)
228 peaked at 99 % immediately after start-up in the control bioreactor, decreasing
229 afterwards to ~16 % by day 5, a maximum RE of 50 % was recorded by day 3 of
230 operation in the photobioreactor, gradually decreasing to zero by day 5 (Figures 2a and
231 2b). The MSM renewal rate was then doubled by day 6 to 1 L day⁻¹ (corresponding to a
232 dilution rate of 0.45 d⁻¹) and maintained during the second operational stage. However,
233 a consistent low toluene biodegradation performance was recorded in both bioreactors
234 in spite of the higher MSM replacement rate until periodic reactor walls cleanings were
235 initiated. Biomass detachment allowed for a slight increase in the suspended biomass
236 concentration and consequently in the abatement performance recorded in stage II. In
237 this sense, toluene REs fluctuated from 17 to 90 % in the PB and from 58 to 87 % in the
238 CB between days 15 and 26, the maximum elimination capacities (ECs) being recorded
239 immediately after biomass detachment from the reactor walls in both systems (Figures 2

240 and 3). During these first and second operational stages, biomass concentration
 241 increased from 0.8 g L⁻¹ to 4 g L⁻¹ in the PB and to 2.2 g L⁻¹ in the CB as a result of an
 242 enhanced toluene degradation (Figure 4).



243

244 **Figure 2.** Time course of toluene inlet (◆) and outlet concentration in (a) the photobioreactor
 245 (○) and (b) control reactor (□) operated at an empty bed residence time of 1.1 min. Dotted lines
 246 represent the reactor cleanings and dashed lines the different operating stages as indicated in the
 247 upper part of each graph.

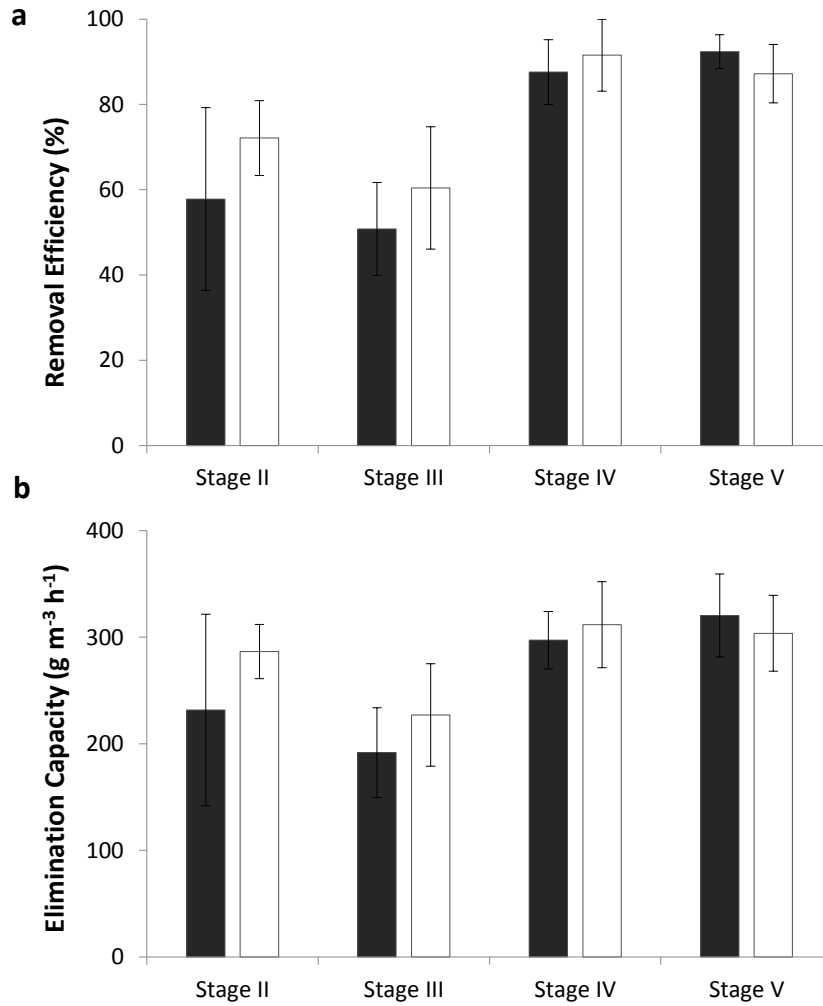
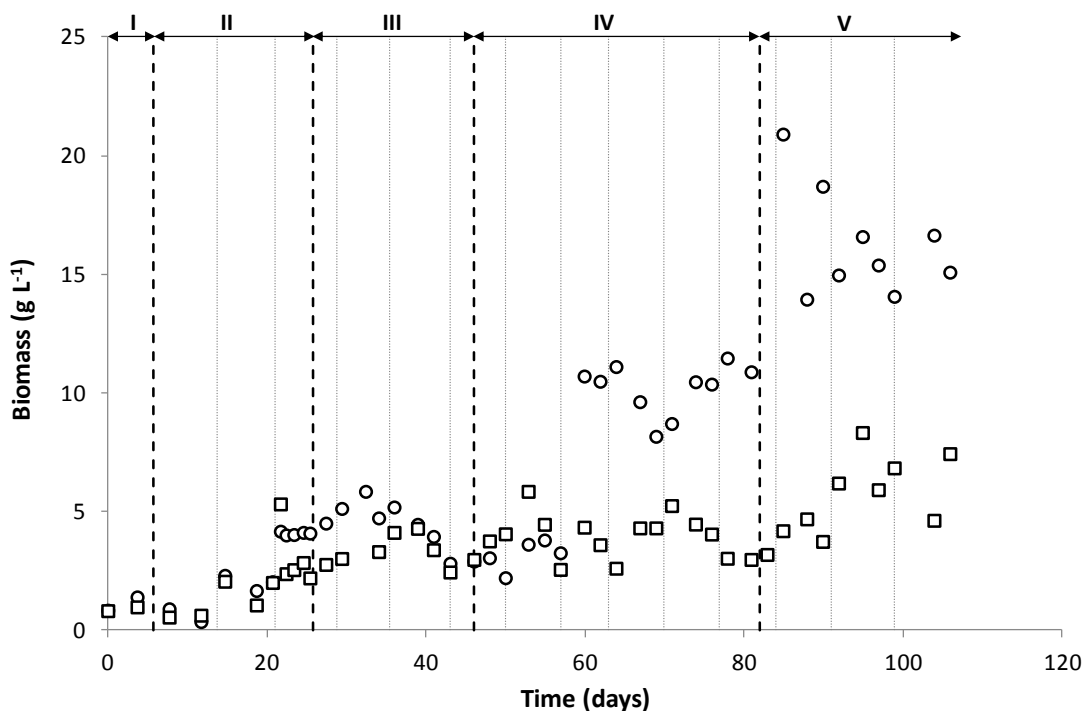


Figure 3. Average removal efficiency (a) and elimination capacity (b) recorded in the photobioreactor (black columns) and control airlift reactor (white columns) in the different operating stages. Vertical bars represent standard deviations.

The concentration of CO_2 also increased concomitantly with toluene removal, with average values (days 20-25) of $14.0 \pm 0.6 \text{ g m}^{-3}$ in the CB and of $3.1 \pm 0.8 \text{ g m}^{-3}$ in the PB. These lower CO_2 emissions in the PB confirmed the intense photosynthetic activity (Figure 5a). The dissolved O_2 concentration recorded during stage II was also higher in the PB ($5.9 \pm 0.8 \text{ mg L}^{-1}$) than in the CB ($0.4 \pm 0.1 \text{ mg L}^{-1}$) as a result of microalgal photosynthesis (Figure 5b). The unstable performance observed in both reactors during stage II (days 5-26) was attributed to the inhibition of microbial activity mediated by the accumulation of toxic metabolites and to the progressive accumulation of biomass in the

261 reactor walls, resulting in a low suspended biomass concentration. In this sense,
 262 biomass detachment from the reactor walls led to a higher suspended biomass
 263 concentration, which entailed a one-off increase in the removal efficiency. This increase
 264 in toluene removal resulted in higher metabolites concentrations and therefore in a
 265 subsequent decrease in the reactor performance, which was again restored after the
 266 following cleaning. Nevertheless, along this second stage, biomass accumulation
 267 globally resulted in higher toluene REs. Several authors have previously highlighted the
 268 key role of biomass concentration and metabolites accumulation on microbial instability
 269 in bioprocesses treating toluene (Bordel et al. 2007, Díaz et al. 2008). In this context,
 270 Díaz et al. (2008) observed that restoration of system performance after the
 271 accumulation of inhibitory metabolites in a suspended growth reactor treating toluene
 272 was not immediate likely due to the deterioration of the toluene degrading community.



273
 274 **Figure 4.** Time course of the biomass concentration as total suspended solids concentration in
 275 the photobioreactor (○) and control reactor (□). Dotted lines represent the reactor cleanings and
 276 dashed lines the different operating stages as indicated in the upper part of each graph.

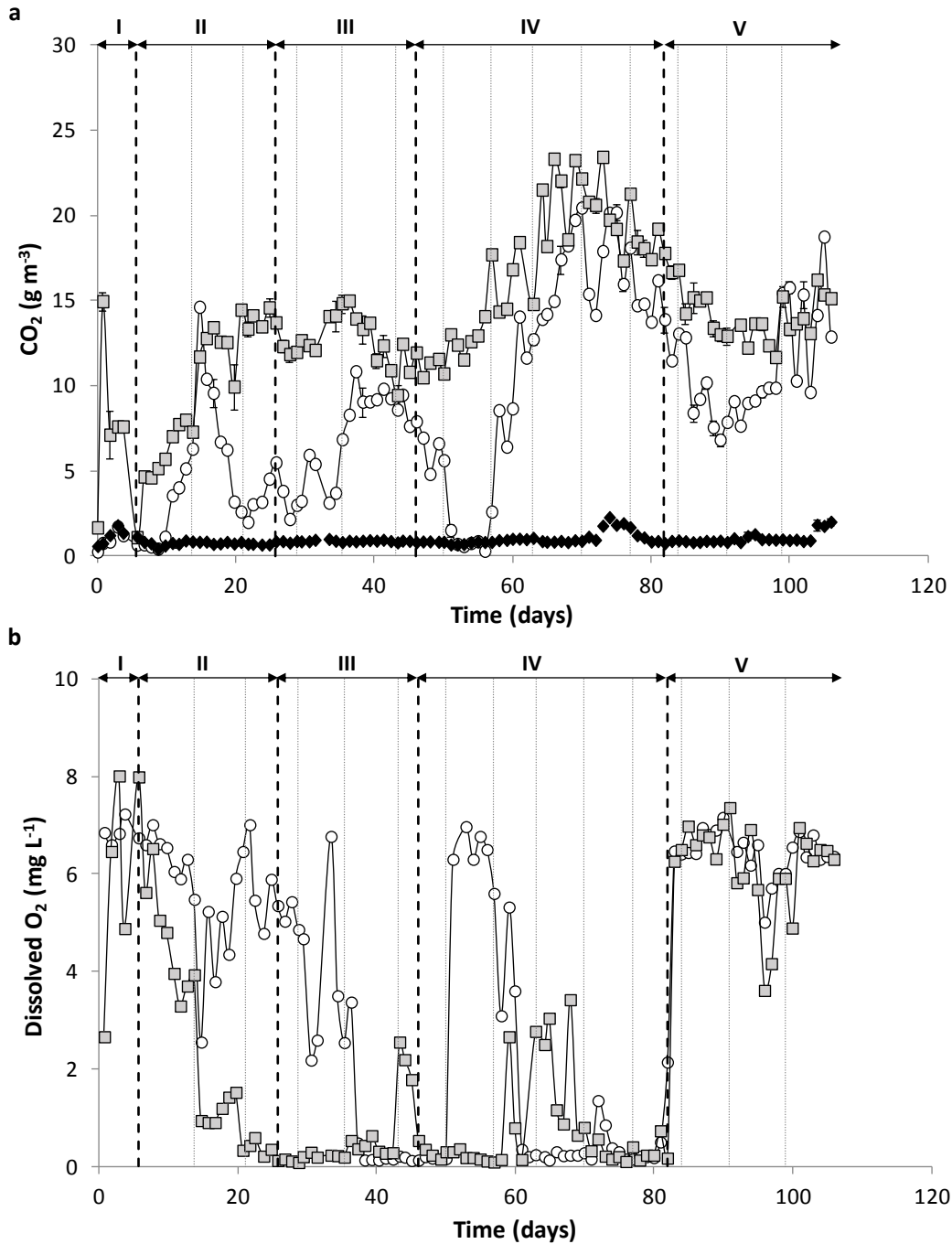
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

277 At this point, it was evident that microbial activity was limiting toluene abatement in
278 both bioreactor configurations. Thus, half of the exchanged media was daily centrifuged
279 and the biomass returned to the corresponding reactor during stage III in order to further
280 increase the suspended solids concentration in the reactors. This operational strategy
281 initially resulted in a TSS increase up to 5.8 and 4.3 g L⁻¹ in the PB and CB,
282 respectively, both concentrations stabilizing at ~2.9 g L⁻¹ by day 46 (Figure 4). During
283 this stage, average toluene removals of 51 ± 11% in the PB and 60 ± 14% in the CB
284 were recorded. Despite the enhancement in toluene abatement mediated by the increase
285 in biomass concentration, both bioreactors still exhibited a highly fluctuating
286 performance (Figures 2 and 3). A lower CO₂ concentration was also recorded along
287 stage III in the PB compared to that of the CB, although a gradual decrease in the
288 photosynthetic activity was observed from day 37. This deterioration in microalgae
289 activity resulted in higher CO₂ outlet concentrations and lower dissolved oxygen values
290 in the PB (9.1 ± 0.9 g m⁻³ and 0.2 ± 0.1 mg L⁻¹, respectively) (Figure 5). Unfortunately,
291 this behavior could not be attributed to any macroscopic change in the photobioreactor.
292 From day 46 onwards (stage IV), 90 % of the retrieved culture broth was centrifuged in
293 order to recover and return the biomass to both bioreactors. While this procedure
294 resulted in a significant biomass accumulation in the photobioreactor (10.2 ± 1.1 g L⁻¹
295 by day 60), the TSS value remained at 3.9 ± 0.8 g L⁻¹ in the control airlift (Figure 4).
296 Stabilization in the abatement performance at REs of 87.6 ± 7.6 % and 91.6 ± 8.4 %
297 was observed from day 63 onwards in the PB and the CB, respectively, corresponding
298 to ECs of 297.1 ± 26.9 and 311.7 ± 40.4 g m⁻³ h⁻¹. These high toluene removals resulted
299 in a decrease in the DO values in both reactors. Under these operational conditions,
300 photosynthetic activity in the PB was severely deteriorated as shown by the outlet CO₂
301 concentrations in the PB (16.5 ± 2.4 g m⁻³) compared to those recorded in the CB (20.1

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

302 $\pm 2.1 \text{ g m}^{-3}$) under comparable REs. At this point, it is important to stress that biomass
303 concentration in the PB is not only a key parameter governing pollutant removal rate,
304 but also determining the light utilization efficiency by microalgae. In this sense, no
305 further increase in photosynthetic oxygenation rate occurs above a critical biomass
306 concentration due to mutual shading and algal dark respiration as demonstrated by
307 Muñoz et al. (2004). This deterioration in photosynthetic activity was correlated to the
308 increase in biomass concentration in the PB by day 60, which likely hindered light
309 penetration with the concomitant reduction in microalgae CO₂-fixation capacity.

310 In order to identify the different metabolites present in the culture broth, samples from
311 the liquid phase of each bioreactor were taken at day 68 and analyzed in duplicate by
312 SPME-GC-MS. Despite the limitation of this technique due to the hydrophobic nature
313 of the PDMS fibre (which only allowed the detection of non-water soluble compounds),
314 significant differences were observed between both culture broths. For instance, among
315 the aromatic and alcohol derivative compounds detected, oxyme- methoxy- phenyl
316 benzene was retrieved from both samples, while 1,2 benzene dicarboxylic acid was only
317 found in the CB and 2- (2-ethoxyethoxy) ethanol was the most significant metabolite in
318 the PB. α -dimethylamino 4-ethoxy o-cresol was only detected in the photobioreactor
319 airlift. These results suggested the occurrence of different toluene degradation pathways
320 depending on the microbial community, thus resulting in the accumulation of different
321 extracellular metabolites.



323 **Figure 5.** Time course of (a) CO₂ inlet (◆) and outlet concentration in the photobioreactor (○)
 324 and control reactor (□); (b) dissolved oxygen concentration in the photobioreactor (○) and
 325 control reactor (□). Dotted lines represent the reactor cleanings and dashed lines the different
 326 operating stages as indicated in the upper part of each graph.

327
 328 Finally, the addition of silicone oil supported an increase in the DO concentration in
 329 both reactors (Figure 5b). The removal performance of the photobioreactor experienced
 330 both a slight increase and a stabilization at 92.4 ± 4.0 % ($EC = 320.4 \pm 38.9$ g m⁻³ h⁻¹),

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
331 while biomass accumulated up to $\sim 15 \text{ g L}^{-1}$. Steady REs (except for a sudden decrease
332 by day 99) of $87.2 \pm 6.9 \%$ were recorded in the control airlift during this last stage
333 (corresponding to an $\text{EC} = 303.6 \pm 35.6 \text{ g m}^{-3} \text{ h}^{-1}$), together with an increase in biomass
334 concentration up to $5.6 \pm 2.7 \text{ g L}^{-1}$. Several authors have demonstrated a mass transfer
335 enhancement of poorly water soluble compounds (as is the case of toluene and oxygen,
336 with Henry constants $H=Ca/Cg$ of 3.7 and 0.03, respectively (Sander 2014)) when a
337 non-aqueous phase such as silicone oil is added to the bioreactor. This enhanced toluene
338 mass transfer was not only related with the higher solubility of the toluene in the
339 silicone oil, but also with an enhanced gas/water interfacial area (Quijano et al. 2010,
340 Lebrero et al. 2013). Moreover, the addition of this non-aqueous phase to the
341 bioreactors acted as a metabolite reservoir, thus avoiding inhibitory concentrations in
342 the culture broth.

29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
343 The ECs here recorded were considerably higher than those reported in toluene-
344 degrading biofilters, ranging from 90 up to $165 \text{ g m}^{-3} \text{ h}^{-1}$ at toluene inlet concentrations
345 of $0.4\text{-}8 \text{ g m}^{-3}$ (Jorio et al. 1998, Singh et al. 2010, Gallastegui et al. 2013, Cheng et al.
346 2016). These ECs were also higher than previous experiments performed in airlift
347 bioreactors. Vergara-Fernandez et al. (2008) reported maximum ECs of $230 \text{ g m}^{-3} \text{ h}^{-1}$ at
348 $\sim 8 \text{ g m}^{-3}$ of toluene, while Harding et al. (2003) only achieved $35 \text{ g m}^{-3} \text{ h}^{-1}$ in an
349 external loop airlift fed with 15 g m^{-3} of toluene. Airlift bioreactors are a cost-effective
350 alternative for the implementation of aerobic off-gas treatment processes compared to
351 packed-bed bioreactors, besides providing a better process control. The high toluene
352 removal performance here achieved, together with its inherently low energy
353 consumption, support the applicability of this pneumatically aerated configuration.
354 Moreover, the presence of a microalgae community in the PB showed a beneficial effect
355 during stage II by reducing the amount of CO_2 emitted to the atmosphere compared to

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

356 that of the CB concomitantly with an increase in the DO concentration in the cultivation
357 media. Finally, the operation of the airlift as a two-phase partitioning reactor with the
358 addition of silicone oil further enhanced not only toluene but also oxygen mass transfer,
359 thus preventing O₂ limiting scenarios.

360

361 **3.2 Microbial analysis**

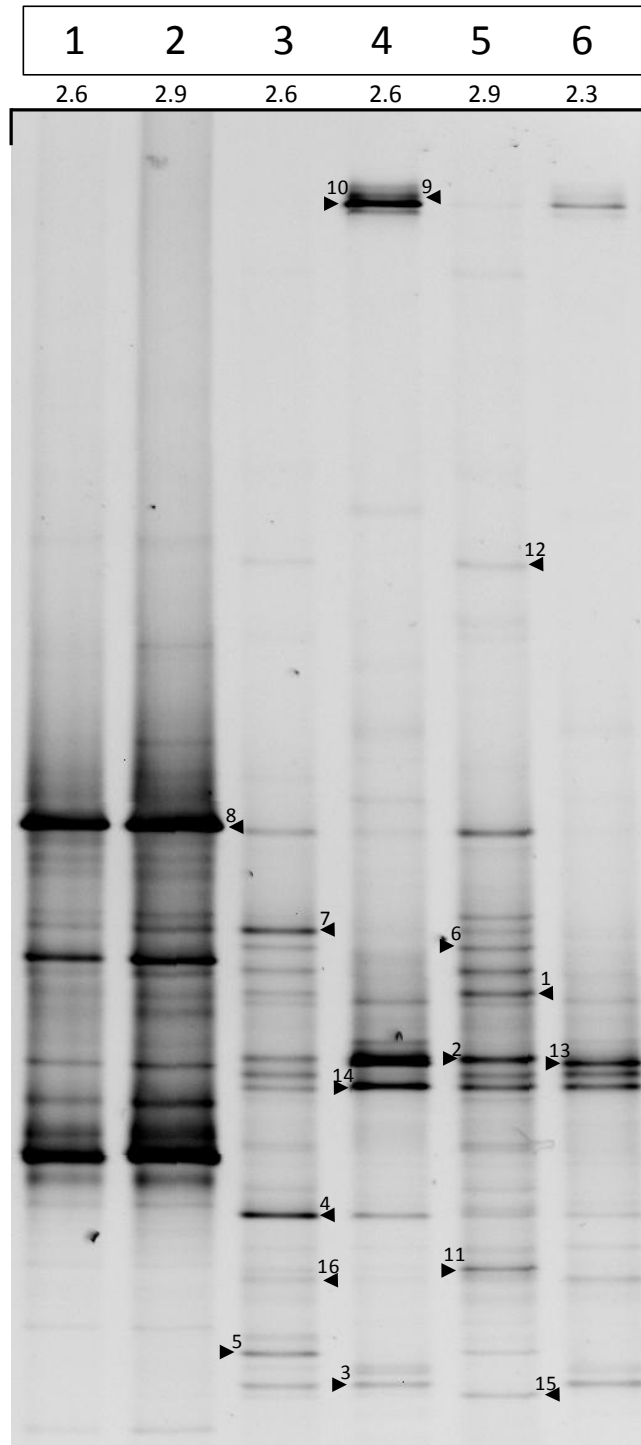
362 The Shannon-Wiener diversity indices obtained showed that the high diversity of the
363 inoculum was maintained throughout the experiment despite the selective pressure of
364 toluene and its metabolites (Figure 6). This diversity index, with typical values ranging
365 from 1.5 to 3.5 (low and high species evenness and richness, respectively) (McDonald
366 2003) was similar for all the samples retrieved, varying from 2.33 (biomass attached
367 onto the PB) to a highest value of 2.94 corresponding to the inoculum and the biomass
368 attached in the CB. As expected, a low Pearson similarity value of 32.4 % was recorded
369 between the suspended biomass samples of both bioreactors, although the attached
370 biomass retrieved from the CB and the PB showed a similarity of 74.9 %.

371 From the DGGE gel, 16 bands were sequenced (Figure 6, Table 1), rendering six
372 different phyla: *Proteobacteria* (eight bands), *Acidobacteria* (four bands),
373 *Cyanobacteria/Chloroplast* (one band), *Ignavibacteriae* (one band), *Actinobacteria* (one
374 band) and *Chloroflexi* (one band). The closest cultured and uncultured relatives of each
375 band were determined by NCBI BLAST analysis and summarized in Table 1 along with
376 the environment from which the closest organisms were retrieved. The VOC
377 biodegradation capacity of some of the microorganisms here identified has been already
378 reported in the literature. For instance, from the bands retrieved in both bioreactors,
379 bacteria from the genus *Xanthobacter flavus* (band 7) and *Bryobacter* (band 11) within
380 the phylum *Proteobacteria* are able to degrade phenol (Nagamani et al. 2009), while

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

381 members of the family *Enterobacteriaceae* (band 8) have been found to transform
382 aromatics under both aerobic and anaerobic conditions, tolerating and removing
383 different polycyclic aromatic hydrocarbons (Toledo et al. 2006). Microorganisms from
384 the genus *Ignavibacterium* (phylum *Ignavibacteriae*) (band 14) and from the phylum
385 *Chloroflexi* (band 16) have been also retrieved from toluene treatment systems (Lebrero
386 et al. 2012, Kuppardt et al. 2014). Six of the DGGE sequenced fragments were only
387 found in the CB (1, 3, 5, 6, 12 and 15), from which bands 6 (order *Rhizospirales*,
388 phylum *Proteobacteria*) and 15 (genus *Mycobacterium*, phylum *Actinobacteria*) were
389 also identified as PAH and BTEX degrading microorganisms, respectively (Martin et al.
390 2012, Zhang et al. 2013). Finally, DGGE fragments 9, 10 and 13 were only retrieved
391 from the PB. In particular, band 13 was identified as *Chlorella Sorokiniana*, with a
392 higher relative abundance in the suspended biomass sample. *Blastocatella*-like bacteria
393 (bands 9 and 10), commonly found in biological systems treating petrochemical
394 effluents, likely played a key role in toluene degradation in the photobioreactor based on
395 the intensity of the DGGE bands (Yang et al. 2015, Fu et al. 2016).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65



396

397 **Figure 6.** DGGE profile of the main bacterial communities present in the CB inoculum (1), PB
398 inoculum (2), suspended biomass at day 106 in the CB (3) and the PB (4), and attached biomass
399 at day 106 in the CB (5) and PB (6). The Shannon-Wiener diversity indexes are indicated in the
400 upper part of the gel. The sequenced bands are indicated by “▶” and the corresponding number
401 of each band.

Table 1. RDP classification of the DGGE bands sequenced and corresponding matches (BLASTN) using the NCBI database with indication of the similarity percentages and sources of origin. The presence/absence of each band in each sample tested together with its intensity are also shown.

| Taxonomic placement (50% confidence level) | Band n° | 1 | 2 | 3 | 4 | 5 | 6 | Closest relatives in Blast Name (accession number) | Similarity (%) | Source of origin |
|--|---------|-----|-----|-----|-----|-----|----|--|----------------|--|
| Phylum Proteobacteria | 1 | x | xx | xx | | xx | | Uncultured <i>delta proteobacterium</i> (JN038629) | 89 | Soil |
| Class Alphaproteobacteria | 2 | | | xx | xxx | xxx | xx | Uncultured bacterium (JQ072580) | 90 | Brewery wastewater pilot reactor |
| Order Rhodospirillales | | | | | | | | | | |
| Family Rhodospirillaceae | 3 | | | x | x | xx | | Bacterium (AB208736) | 96 | Culture collection |
| Genus Azospirillum | 4 | | | xxx | xx | x | x | <i>Azospirillum fermentarium</i> (NR_118484) | 99 | Nitrogen-fixing species |
| | | | | | | | | Uncultured bacterium (JX174657) | 97 | Microbial fuel cell fed with olive mill wastewater as sole carbon source |
| | 5 | | | xx | | x | | <i>Azospirillum</i> sp.(HQ694759) | 99 | Autotrophic enrichment culture En_UW_28 with Na ₂ S ₂ O ₃ as electron donor |
| | | | | | | | | <i>Azospirillum brasiliense</i> (FR667883) | 99 | Culture collection |
| | | | | | | | | <i>Azospirillum</i> sp. (AY061963) | 99 | Culture collection |
| Order Rhizospirales | 6 | | | xx | | xx | | Uncultured bacterium (FQ659151) | 90 | PAH degrading bacterial community in contaminated soil |
| Family Xanthobacteraceae | | | | | | | | | | |
| Genus Xanthobacter | 7 | x | xx | xxx | x | xx | x | <i>Xanthobacter</i> sp.(HF566355) | 99 | Vineyard soil |
| | | | | | | | | <i>Xanthobacter flavus</i> (AB512110) | 99 | Polyvinyl alcohol-degrading bacteria from activated sludge |
| Class Gammaproteobacteria | | | | | | | | | | |
| Order Enterobacteriales | | | | | | | | | | |
| Family Enterobacteriaceae | 8 | xxx | xxx | xx | x | xx | | <i>Erwinia</i> sp. (KF999718) | 99 | Extraction of Luffa cylindrica fruits |
| Phylum Acidobacteria | | | | | | | | | | |
| Class Acidobacteria_Gp4 | | | | | | | | | | |
| Genus Blastocatella | 9 | | | | xxx | | x | Uncultured bacterium (JX394456) | 98 | Aerosol microbiology in a metropolitan subway system |
| | | | | | | | | Uncultured bacterium (DQ984629) | 98 | Oil-contaminated soil |
| | 10 | | | | xxx | x | xx | Uncultured bacterium (JN603801) | 98 | Rhizosphere soil from field grown riceplants |
| | | | | | | | | Uncultured bacterium (DQ984629) | 98 | Oil-contaminated soil |
| Class Acidobacteria_Gp3 | | | | | | | | | | |
| Genus Bryobacter | 11 | x | x | x | x | xx | | Uncultured bacterium (FQ659827) | 97 | PAH degrading bacterial community of a contaminated soil |
| | | | | | | | | Uncultured bacterium (EF393024) | 97 | Anaerobic polychlorinated biphenyl dechlorinating consortia |
| Class Holophagae | | | | | | | | | | |
| Order Holophagales | | | | | | | | | | |
| Family Halophagaceae | | | | | | | | | | |
| Genus Geothrix | 12 | | | x | | x | | Uncultured bacterium (KC758898) | 98 | Sulfate-reducing MTBE and TBA plume |

404 **4. Conclusions**

1
2 405 This study confirmed the superior performance of bacterial and algal-bacterial airlift
3
4
5 406 reactors over conventional biofilters for the removal of toluene, which achieved ECs up
6
7 407 to $320 \text{ g m}^{-3} \text{ h}^{-1}$ at empty bed residence times of 1.1 min. Biomass concentration and
8
9 408 medium renewal rate played a key role on toluene abatement performance, with
10
11 409 optimum values of $\sim 5 \text{ g L}^{-1}$ and 0.45 d^{-1} (with a 90% biomass recycling), respectively.
12
13
14 410 Moreover, photosynthetic activity in the photobioreactor resulted in lower emissions of
15
16
17 411 CO_2 and higher dissolved oxygen concentrations when suspended biomass was
18
19 412 maintained below 5 g L^{-1} , with higher values leading to mutual shading and therefore a
20
21
22 413 low photosynthetic efficiency. The results also showed the positive impact of silicone
23
24 414 oil addition on the stabilization of toluene biodegradation due to an enhanced O_2 mass
25
26
27 415 transfer and buffering against toxic metabolites. Finally, the presence of microalgae in
28
29 416 the PB induced the enrichment of a microbial community different than that established
30
31
32 417 in the CB, as suggested by the low similarity observed between the microbial
33
34 418 communities identified by the end of the operation and between the metabolites detected
35
36
37 419 in the culture broth of both bioreactors.

38
39 420

421 **Acknowledgments**

42 422 This research was supported by MINECO (CTM2015-70442-R and Red Novedar) and the
43
44
45 423 Regional Government of Castilla y León (Project VA024U14 and UIC 71). CONACyT-México
46
47
48 424 is also gratefully acknowledged for the Master grant of Roxana Angeles.

49
50
51
52 425

55 426 **References**

56
57 427 *American Water Works Association* (2012) Standard methods for the examination of water and
58
59 428 wastewater. American Water Works Association 1469.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- 429 Bordel S., Muñoz R., Díaz L.F. and Villaverde S. (2007) Predicting the accumulation of
430 harmful metabolic byproducts during the treatment of VOC emissions in suspended growth
431 bioreactors. *Environmental Science and Technology* 41, 5875-5881.
- 432 Cheng Z., Lu L., Kennes C., Yu J. and Chen J. (2016) Treatment of gaseous toluene in three
433 biofilters inoculated with fungi/bacteria: Microbial analysis, performance and starvation
434 response. *Journal of Hazardous Materials* 303, 83-93.
- 435 Díaz L.F., Muñoz R., Bordel S. and Villaverde S. (2008) Toluene biodegradation by
436 *Pseudomonas putida* F1: targeting culture stability in long-term operation. *Biodegradation*
437 19, 197-208.
- 438 EURAR-T, 2003. European Union Risk Assessment Report –Toluene (EINECS No. 203-625-
439 9), vol. 9, PL2.
- 440 Fu L.Y., Wu C.Y., Zhou Y.X., Zuo J.E. and Ding Y. (2016) Treatment of petrochemical
441 secondary effluent by an up-flow biological aerated filter (BAF). *Water Science and*
442 *Technology*, in press.
- 443 Gallastegui G., Barona A., Rojo N., Gurtubay L. and Elías A. (2013) Comparative response of
444 two organic biofilters treating ethylbenzene and toluene after prolonged exposure. *Process*
445 *Safety and Environmental Protection* 91, 112-122.
- 446 Häne B.G., Jäger K. and Drexler H.G. (1993) The Pearson product-moment correlation
447 coefficient is better suited for identification of DNA fingerprint profiles than band matching
448 algorithms. *Electrophoresis* 14, 967–972.
- 449 Harding R.C., Hill G.A. and Lin Y.H. (2003) Bioremediation of toluene-contaminated air using
450 an external loop airlift bioreactor. *Journal of Chemical Technology and Biotechnology* 78,
451 406-411.
- 452 Jorio H., Kiared K., Brzezinski R., Leroux A., Viel G. and Heitz M. (1998) Treatment of air
453 polluted with high concentrations of toluene and xylene in a pilot-scale biofilter. *Journal of*
454 *Chemical Technology and Biotechnology* 73, 183-196.
- 455 Kuppardt A., Kleinstauber S., Vogt C., Lüders T., Harms H. and Chatzinotas A. (2014)
456 Phylogenetic and functional diversity within toluene degrading, sulphate reducing consortia
457 enriched from a contaminated aquifer. *Microbial Ecology* 68, 222-234.
- 458 Lebrero R., Rodríguez E., Estrada J.M., García-Encina P.A. and Muñoz R. (2012) Odor
459 abatement in biotrickling filters: Effect of the EBRT on methyl mercaptan and hydrophobic
460 VOCs removal. *Bioresource Technology* 109, 38-45.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- 461 Lebrero R., Rodríguez E., Pérez R., García-Encina P.A. and Muñoz R. (2013) Abatement of
462 odorant compounds in one- and two-phase biotrickling filters under steady and transient
463 conditions. *Applied Microbiology and Biotechnology* 97, 4627-4638.
- 464 Lebrero R., Hernández R., Pérez R., Estrada J.M. and Muñoz R. (2015) Two-liquid phase
465 partitioning biotrickling filters for methane abatement: Exploring the potential of
466 hydrophobic methanotrophs. *Journal of Environmental Management* 151, 124-131.
- 467 McDonald G (2003) *Biogeography: space, time and life*. Wiley, New York, p 409
- 468 McGinnis S. and Madden T.L. (2004) BLAST: at the core of a powerful and diverse set of
469 sequence analysis tools. *Nucleic Acids Research* 32, W20–25.
- 470 Martin F., Torelli S., Le Paslier D., Barbance A., Martin-Laurent F., Bru D., Geremia R., Blake
471 G. and Jouanneau Y. (2012) Betaproteobacteria dominance and diversity shifts in the
472 bacterial community of a PAH-contaminated soil exposed to phenanthrene. *Environmental
473 Pollution* 162, 345-353.
- 474 Muñoz R., Köllner C., Guieysse B. and Mattiasson B. (2004) Photosynthetically oxygenated
475 salicylate biodegradation in a continuous stirred tank photobioreactor. *Biotechnology
476 Bioengineering* 87, 797–803.
- 477 Muñoz R., Rolvering C., Guieysse B. and Mattiasson B. (2005) Photosynthetically oxygenated
478 acetonitrile biodegradation by an algal-bacterial microcosm: a pilot-scale study. *Water
479 Science and Technology* 51, 261-265.
- 480 Muñoz R. and Guieysse B. (2006) Algal-bacterial processes for the treatment of hazardous
481 contaminants: A review. *Water Research* 40, 2799-2815.
- 482 Muñoz R., Daugulis A.J., Hernández M. and Quijano G. (2012) Recent advances in two phase
483 partitioning bioreactors for the treatment of volatile organic compounds, *Biotechnology
484 Advances* 30, 1707–1720.
- 485 Nagamani A., Soligalla R. and Lowry M. (2009) Isolation and characterization of phenol
486 degrading *Xanthobacter flavus*. *Bioremediation Journal* 13, 1-6.
- 487 Quijano G., Rocha-Rios J., Hernández M., Villaverde S., Revah S., Muñoz R. and Thalasso F.
488 (2010). Determining the effect of solid and liquid vectors on the gaseous interfacial area and
489 oxygen transfer rates in two-phase partitioning bioreactors. *Journal of Hazardous Materials*
490 175, 1085-1089.
- 491 Roest K, Heilig H.G., Smidt H., de Vos W.M., Stams A.J.M. and Akkermans A.D.L. (2005)
492 Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater.
493 *Systematic and Applied Microbiology* 28, 175– 185.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

494 Sander R. (2014) Compilation of Henry's law constants, version 3.99. Atmospheric Chemistry
495 and Physics Discussions 14, 29615-30521.

496 Semple K.T., Cain R.B. and Schmidt S. (1999) Biodegradation of aromatic compounds by
497 microalgae. FEMS Microbiology Letters 170, 291-300.

498 Singh R.S., Rai B.N. and Upadhyay S.N. (2010) Removal of toluene vapour from air stream
499 using a biofilter packed with polyurethane foam. Process Safety and Environmental
500 Protection 88, 366-371.

501 Toledo F.L., Calvo C., Rodelas B. and González-López J. (2006) Selection and identification of
502 bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal
503 capacities. Systematic and Applied Microbiology 29, 244-252.

504 Vergara-Fernández A.O., Quiroz E.F., Aroca G.E. and Pulido N.A. (2008) Biological treatment
505 of contaminated air with toluene in an airlift reactor. Electronic Journal of Biotechnology 11,
506 1-7.

507 Wang Q., Garrity G.M., Tiedje J.M. and Cole J.R. (2007) Naive Bayesian classifier for rapid
508 assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental
509 Microbiology 73, 5261-5267.

510 WHO (2000) Air Quality Guidelines, Regional Office for Europe, Denmark. Second Edition,
511 Chapter 5.14

512 Yang Q., Xiong P., Ding P., Chu L. and Wang J. (2015) Treatment of petrochemical wastewater
513 by microaerobic hydrolysis and anoxic/oxic processes and analysis of bacterial diversity.
514 Bioresource Technology 196, 169-175.

515 Zhang L., Zhang C., Cheng Z., Yao Y. and Chen J. (2013) Biodegradation of benzene, toluene,
516 ethylbenzene, and o-xylene by the bacterium *Mycobacterium cosmeticum* byf-4.
517 Chemosphere 90, 1340-1347.