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Experimental and Modeling of Tetracycline degradation in water in a Flow-Through Enzymatic monolithic reactor

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1 **Abstract**

2 In this work, the laccase from *Trametes versicolor* was immobilized in highly porous silica monoliths (0.6
3 cm diameter, 0.5 cm length). These monoliths feature a unique homogeneous network of interconnected
4 macropores (20 μm) with mesopores (20 nm) in the skeleton and a high specific surface area (330 m^2/g). The
5 enzymatic monoliths were applied to degrade tetracycline (TC) in model aqueous solutions (20 ppm). For
6 this purpose, a tubular Flow-Through-Reactor (FTR) configuration with recycling was built. The TC
7 degradation was improved with oxygen saturation, presence of degradation products and recirculation rate.
8 The TC depletion reaches 50% in the FTR and 90% in a stirred tank reactor (CSTR) using crushed monoliths.
9 These results indicate the importance of maintaining a high co-substrate concentration near active sites. A
10 model coupling mass transfers with a Michaelis-Menten kinetics was applied to simulate the TC degradation
11 in real wastewaters at actual TC concentration ($2.8 \cdot 10^{-4}$ ppm). Simulation results show that industrial scale
12 FTR reactor should be suitable to degrade 90% of TC in 5 h at a flow rate of 1 mL/min in a single passage
13 flow configuration. Nevertheless, the process could certainly be further optimized in terms of laccase activity,
14 oxygen supply near active sites and contact time.

15

16

17 **Key words.** Water treatment, Enzymatic silica monoliths, pharmaceuticals degradation, tetracycline
18 degradation, Flow-Through-Reactor, Modelling, Scale-up.

19

20

21 1 Introduction

22 Pharmaceutical products (PPs) are found more and more frequently in the environment due to an increase in
23 their consumption, because humans have a better access to medicines in conjunction with the aging of the
24 population. Indeed, they can be found at very low concentrations ($\mu\text{g L}^{-1}$ - mg L^{-1}) in wastewaters (Halling-
25 Sørensen et al., 1998), rivers (Burns et al., 2018), sediments (Kerrigan et al., 2018) and sea (Björlenius et al.,
26 2018). Moreover, traditional wastewater treatment plants (WWTP) are inefficient to completely remove PPs,
27 (Alvarino et al., 2018; Thiebault et al., 2017; Verlicchi et al., 2012). Therefore, PPs remain in WWTP
28 effluents and as a consequence they can be transferred to underground or surface waters, which are among
29 the main sources of drinking water (Bruce et al., 2010; de Jongh et al., 2012). Several tertiary treatments have
30 been proposed to improve the removal of PPs from wastewaters, they include advanced oxidation treatments
31 (Kanakaraju et al., 2018; Kıdık and Doğan, 2018), physical adsorption (Rajapaksha et al., 2019 ; Rocha et
32 al., 2020) or even enzymatic degradation.

33 Enzymatic degradation of PPs can be an alternative option among the other tertiary treatments named
34 previously, since enzymes mediate biochemical reactions at a rapid rate under mild operating conditions (pH,
35 temperature, solvents, and ionic strength). In particular, oxidoreductase enzymes such as laccases, tyrosinases
36 and peroxidases have the ability to oxidize large variety of PPs like phenols, drugs, and hormones (Singh
37 Arora and Kumar Sharma, 2010; Demarche et al., 2012; De Cazes et al., 2014). Nevertheless, enzymatic
38 process can be expensive and unsustainable if enzymes are not recycled within the system.

39 Immobilization generally increases the stability of enzymes under reaction conditions while allowing their
40 reuse and then reducing costs. (Zhang et al., 2015 Ji et al., 2017). Several immobilization techniques like
41 adsorption, entrapment and encapsulation have been applied for enzyme immobilization on solid supports
42 however it is observed that covalent immobilization enhances enzymes stability and long-term process
43 sustainability (Zdarta et al., 2018). Along with nature of enzymes and immobilization techniques, choosing
44 suitable solid support is also very essential in enzymatic process. Inorganic support materials like silica,
45 zirconia, active carbons have high mechanical strength and temperature stability therefore are explored for
46 immobilization of enzymes (Sadeghzadeh et al., 2020; Bebić et al., 2020; Zdarta et al., 2020).

47

48 The application of enzymatic reactors for PPs degradation has been explored with different configurations
49 like packed bed reactors (Nguyen et al., 2016; Bilal and Iqbal, 2019), fluidized bed reactors (Lloret et al.,
50 2012; Piao et al., 2019) as well as enzymatic membrane reactors (EMR) (de Cazes et al., 2014; Barrios-
51 Estrada et al., 2018). EMRs have been widely studied for PPs degradation because they combine two
52 functions in a single unit: filtration and enzymatic reaction (Sanchez Marcano and Tostsis, 2002; Sanchez
53 Marcano, 2019). Nevertheless, EMRs can also present some drawbacks like membrane clogging or low
54 reactivity because the amount of biocatalyst grafted on the separative layer of membranes is relatively low.

55 Ji et al., (2016) observed less than 10 % of carbamazepine degradation with laccase from *Trametes versicolor*
56 in an EMR without using mediators. De Cazes et al., (2014) found only 56 % of tetracycline (TC) degradation
57 in 24 h with laccase from *Trametes versicolor* immobilized on ceramic membranes. Similarly, Barrios-
58 Estrada et al., (2018) found that only 33% of bisphenol-A was degraded in 24 h with an immobilized laccase
59 on the same type of membranes. To overcome some of these disadvantages, meso-/macroporous monoliths
60 have been recently applied for enzyme immobilization, the objective is to provide a big surface area to
61 immobilize a large amount of enzymes, together with a macroporosity which allows low pressure drop while
62 avoiding clogging (Ahmad et al., 2021; Biggelaar et al., 2019; Sebai et al., 2022). Moreover, as far as
63 substrates are forced to flow through the monolith porosity the probability of contact with the biocatalyst is
64 enhanced while allowing a precise control of the contact time (Sanchez Marcano, 2019). This configuration
65 is called Flow-Trough-Reactor (FTR). Silica monoliths prepared by emulsion templating and presenting a
66 large distribution of macropores (50 nm to 6 μm) have been recently used as supports in biocatalysis
67 (Biggelaar et al., 2017, 2019). Another type of silica monoliths have been prepared by spinodal
68 decomposition. They featured a narrow distribution of macropores and presented the advantage of an
69 independent control of macropores (in the range 1 to 50 μm) and mesopores diameters (in the range 4 to 30
70 nm, with a corresponding to surface area of 800 to 200 $\text{m}^2 \text{g}^{-1}$, respectively) (Fajula and Galarneau, 2019;
71 Galarneau et al., 2016b, 2016a).

72 There are few reported models of mass transport through monoliths. They consider the macroscopic structure
73 and developed approaches for simulation of velocity fields, diffusion, and dispersion of chemical species
74 within the porous structure (Jungreuthmayer et al., 2015; Meyers and Liapis, 1999; Tallarek et al., 2002).
75 These models, which are based on morphology and real structure of porous monoliths, require high
76 computational techniques like image processing techniques as well as relatively large computing times and
77 costs (Jungreuthmayer et al., 2015). This assertion is especially true for large-scale geometries required for
78 practical engineering problems. Furthermore, apart from the recent work of Ahmad et al., (2021), no work
79 has been reported in the literature coupling a reaction kinetics with transport of species within macroporous
80 silica monoliths for scale-up purposes.

81 The objective of this research work was to develop an original FTR configuration using enzymatic monoliths
82 for PPs degradation and studying by modeling and simulation the possibility of scale-up of the FTR for this
83 application. For this purpose the degradation of tetracycline (TC) was chosen as model molecule of PPs.
84 Firstly, a lab scale set up was used for studying experimentally the enzymatic degradation of TC by laccase
85 from *Trametes versicolor* covalently immobilized on silica monoliths with large homogeneous macropores
86 ($\sim 20 \mu\text{m}$) to assess low pressure drop and mesopores of 20 nm providing a large surface area ($330 \text{m}^2 \text{g}^{-1}$) to
87 improve enzymes immobilization. TC degradation tests were carried out in a tubular FTR configuration with
88 recycling for the degradation of TC in aqueous solutions. Oxygen effect on TC degradation as well as the
89 effect of TC degradation products on degradation rate were also studied. Moreover, a previous reported

90 model (Ahmad et al., 2021) coupling mass transfers with an apparent Michaelis-Menten kinetics, determined
91 under oxygen saturation conditions, was applied to simulate the TC degradation in real wastewaters having
92 a TC concentration six orders of magnitude lower than oxygen concentration at saturation conditions at 25
93 °C. The developed model was employed to simulate the geometrical scale up of monoliths for the complete
94 TC degradation at usual real wastewater concentrations at different flows. To our knowledge, it is the first
95 time that such a study of TC degradation in a FTR has been carried out considering the influence of oxygen
96 as co-substrate and the degradation products and performing the scale-up for an actual concentration of this
97 antibiotic in wastewaters.

98

99 2 Materials and Methods

100 Silica monoliths with hierarchical porosity were synthesized and functionalized by amino groups for being
101 used as solid support for covalent immobilization of laccases. The laccase-monoliths were then used for the
102 removal of tetracycline (20 ppm) contained in water in batch and in flow through configuration. conditions
103 using a recycling configuration. The effects of recirculation flow rate, of oxygen and presence of by-products
104 were analyzed. Simulation was performed to evaluate the performance of laccase-monoliths for the removal
105 of tetracycline in concentration ($2.8 \cdot 10^{-4}$ ppm) found in real wastewaters and to determine the size of
106 monoliths necessary to treat efficiently such effluents in flow condition using a single passage configuration.

107 2.1 Materials

108 Powdered commercial laccase (activity $\geq 0.5 \text{ U mg}^{-1}$ according to the provider), Tetracycline (TC) ($\geq 98.0\%$),
109 Glutaraldehyde (GLU) (25% v/v) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were
110 all purchased from Sigma-Aldrich, Germany.

111 Silica monoliths with macropores of 20 μm and mesopores of 20 nm diameters (specific surface area of 330
112 $\text{m}^2 \text{ g}^{-1}$) were prepared by a controlled sol-gel process with tetraethylorthosilicate (TEOS) and polyethylene
113 glycol (PEO) 100 kDa in acidic solution (HNO_3) followed by a basic treatment (NH_4OH 0.1 M) at 100 °C
114 for 24 h and then calcined at 550 °C for 8 h, according to a method previously reported (Galarneau et al.,
115 2016a). The monoliths were then activated at 250 °C under vacuum for 4 h to remove water prior to be
116 grafted with 3-aminopropylamine triethoxysilane (APTES) in ethanol under reflux (80 °C) overnight with an
117 excess of 10 amino groups per nm^2 . The resulting NH_2 -monoliths presented a grafting density of $1.5 \text{ NH}_2/\text{nm}^2$
118 corresponding to an amount of 0.8 mmol NH_2/g and leading to a specific surface area of $197 \text{ m}^2 \text{ g}^{-1}$. The
119 NH_2 -monoliths were then clad with a Teflon heat shrinkable gain at 180 °C for 2 h connected to stainless-
120 steel tubing (Ahmad et al., 2021).

121 **2.2 Activation of monoliths and laboratory scale setup for TC degradation in water** Cladded NH₂-
 122 silica monoliths were activated with a GLU solution (4% v/v) prepared in citrate phosphate buffer (pH 7, 0.1
 123 M). Then, monoliths were filled with 0.5 mL of laccase solution (5±1 U mL⁻¹) prepared by solubilizing the
 124 necessary amount of commercial powder of enzyme in the same citrate phosphate buffer. After
 125 immobilization, the resulting laccase-monoliths were stored in the buffer solution (pH 7) at 4 °C. The
 126 measurement of the enzymatic activity of laccase immobilized in monoliths, was carried out through the
 127 oxidation of 1 mM solution of 2,2-azino-bis(3-ethylbenzothiazoline-6 sulfonic acid (ABTS) at pH = 4. For
 128 this purpose, the laccase-monoliths were crushed, and experiments were carried out in a stirred tank reactor
 129 (25-50 mL) under vigorous magnetic agitation at 200 rpm. The same methodology was applied for the
 130 determination of kinetic constants of TC degradation. Immobilization methods and activity determination of
 131 crushed laccase-monoliths were described in detail in a previous publication (Ahmad et al., 2021).

132 A laboratory scale set up for TC degradation (Figure 1) was built by connecting in series three cladded
 133 enzymatic monoliths (0.6 cm diameter, 0.5 cm length, ~50 mg, each). A HPLC pump (Gibson model: 321,
 134 France) allowed recycling a TC solution in between the monoliths and a reservoir. A pressure transducer
 135 (Keller, 0-2 bar) was connected before the inlet of the monoliths to monitor the pressure changes during the
 136 process. The temperature of the feed tank was controlled placing the feed tank in a thermostatically controlled
 137 water bath. All the TC degradation tests were carried out in closed loop (complete recycling of the TC
 138 solution (20 ppm)) because of low TC degradation in a single pass. A TC solution (20 ppm, 30 mL) was
 139 flowed through the monoliths at three different flow rates (0.5, 1 and 5 mL min⁻¹). In additional experiments,
 140 air was bubbled in the feed tank solution at the air flow rate of 30-40 mL min⁻¹ to keep feed TC solution at
 141 oxygen saturation (measured with a DO meter VisiFerm RS-485 from Himlton (Switzerland)). Based on the
 142 macropore volumes of the monolith (3.41 mL/g), we can estimate the number of times (cycles) the solution
 143 passes through the FTR built with three enzymatic monoliths in series (~150 mg) (Table 1):

144

145 **Table 1.** Number of cycles through the laccase-monoliths reactor as a function of flow rate

Flow rate (mL/min)	Number of cycles in 24h (V _{macro} : 3.41 mL/g)
0.5	1409
1	2818
5	14090

146

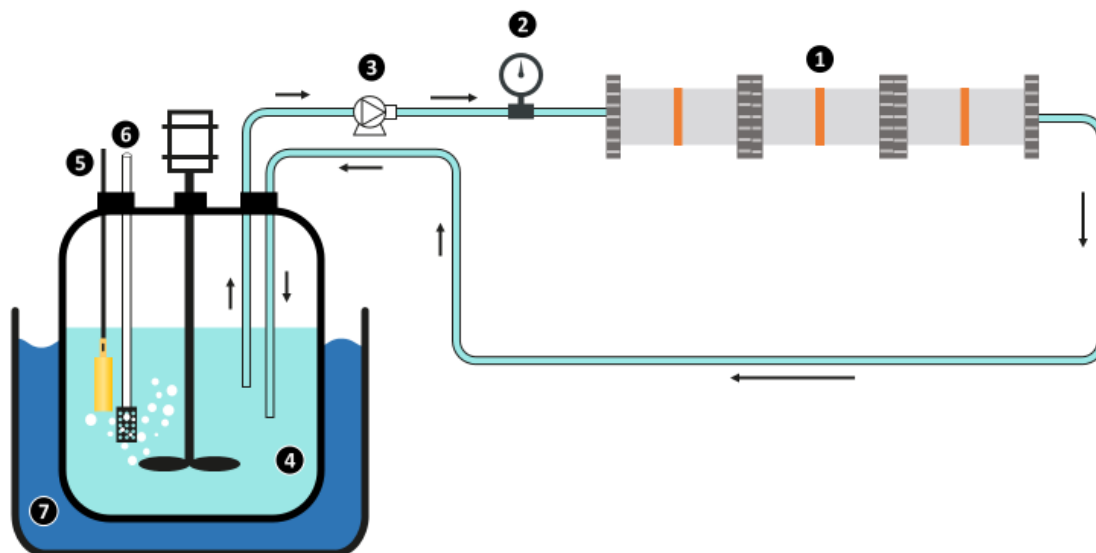
147 The Reynolds number (Re) (Bird, et al, 1960) was calculated from the well known following equation:

148 $Re = \frac{\rho u d_{macro}}{\mu}$ (1)

149 with ρ (0.997047 kg m⁻³) the density of water at 25 °C, μ (0.000891 kg m⁻¹ s⁻¹) the dynamic viscosity of water
150 at 25 °C, d_{macro} (2 10⁻⁵ m) the macropores mean diameter and u (m s⁻¹) the velocity of water within
151 macropores, $u = V/A$ with V the flow rate (5 10⁻⁴ to 5 10⁻³ L min⁻¹) and A the section of the monolith (6 mm
152 diameter).

153 For comparison batch experiments were carried out with crushed laccase-monomoliths (~150 mg) in a stirred
154 tank reactor (CSTR 50 mL) containing 30 mL of TC solution (20 ppm) under magnetic stirring (200 rpm).
155 In this case also additional experiments were carried out by bubbling air in the solution at the air flow rate of
156 30-40 mL min⁻¹ to maintain oxygen saturation conditions. Samples of 100 μ L were taken from feed tank
157 every 30 min to measure the TC concentration evolution during 24 h by HPLC-MS analysis (Waters 2695
158 separation module with micromass detector of Wythenshawe, Manchester, UK). Degradation products of TC
159 were separated and identified by means of liquid chromatography coupled to high resolution mass
160 spectrometer QExactive (LC-HRMS) equipped with a HESI ionization source operating in negative or
161 positive ionization mode, in separate injections. Data acquisition was performed in data dependent scan
162 where the 10 most intense ions from full scan (m/z 50-600) were further fragmented with an isolation of 1.0
163 Da at a collision energy of 30 a.u. Data results were processed manually with Xcalibur 3.1 software (IDAEA-
164 CSIC laboratory, Spain).

165 Control experiments to study the effect of TC self-degradation or adsorption on the evolution of TC
166 concentration were carried out with whole or crushed inactivated enzymatic monoliths. For this purpose,
167 enzymatic monoliths were deactivated by heating in oven at 100 °C for 2 h.



168

169 **Figure 1.** Schematic draw of the laboratory scale set up for TC degradation with recycling. 1: reactor
170 composed of three monoliths in series, 2: pressure transducer, 3: HPLC pump, 4: Stirred tank with TC
171 solution, 5: air bubbling system, 6: thermocouple, 7: Thermostatic water bath.

172 3 Results and discussions

173 3.1 Effect of recirculation rate on TC degradation

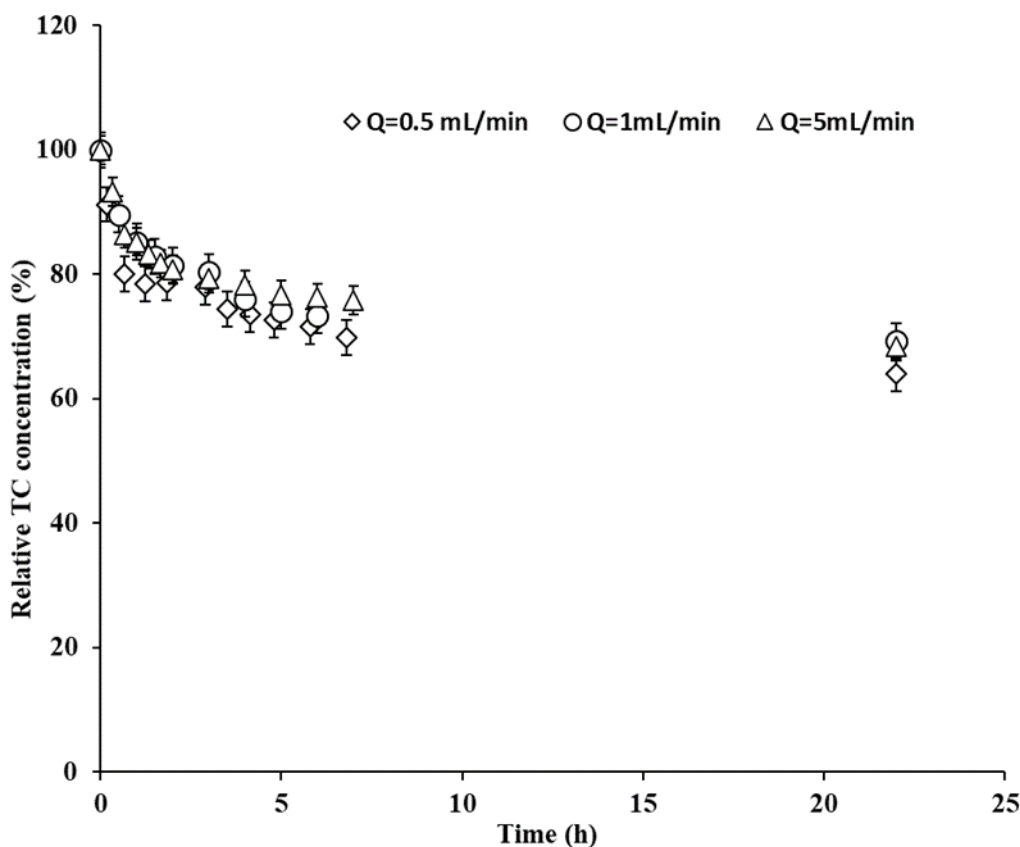
174 Recirculation rate in enzymatic reactor is an important parameter for continuous flow. When the recirculation
175 rate is slow, the reaction can behave a similarly as non-continuous processes (Hamam and Budge, 2010) with
176 a long contact time with the enzymes. When the recirculation rate is fast, more oxygen is is transported close
177 to the enzymes, but the contact time is shorter. Therefore, it was important to determine the optimum flow
178 rate for the TC degradation.

179 To study the effect of recirculation flow, TC degradation tests were carried out using the set up shown in
180 Figure 1 at different recirculation flow rates from 0.5 to 5 mL min⁻¹ at 25 °C. For each test, fresh laccase-
181 monoliths were used with initial TC solution concentration of 20 ppm and total volume of TC solution of 30
182 mL. The decrease in relative TC concentration was similar for all flow-rates (Figure 2): 30% of the TC was
183 degraded in the first five hours. For longer time reaction the degradation rate was extremely low (about 10%
184 of additional degradation over the next 20 hours). This effect was observed with all tested recirculation flow

185 rates, which means that there was no effect of flow rate on the TC degradation. This indicates that the
186 transport of substrates inside the reactors containing the enzymes immobilized on silica monoliths is
187 governed by the self-diffusion in the mesopores and not by external mass transfer in the macropores.

188 The increase of the flow rate did not allow modifying the hydrodynamic flow regime. Indeed, the Reynolds
189 number (Re) calculated from Eq (1) for the recirculation flow rates of 0.5 to 5 mL min⁻¹ was ranged between
190 0.01 - 0.1 and corresponded for all flow rates to a laminar flow regime (limit of the laminar flow regime (Re
191 < 1) (Bird et al, 1960). It can be concluded that the increase in flow rate was not high enough to further
192 decrease possible external mass transfer resistances (decrease of boundary layers). In previous studies,
193 Galarneau et al., (2016b) studied the dependence of flow rate on external mass transfer layer with silica
194 monoliths for Diels-Alder reaction. They observed that productivity was increased by increasing the flow
195 rate from 0.01 to 0.03 mL min⁻¹. In another study, Jatoi et al., (2021) studied the effect of flow rate on
196 external mass transfer limitations with silica monoliths loaded with Pt in the continuous-flow liquid-phase
197 hydrogenation of *p*-Nitrophenol. They observed that for the range of flow rate from 1 to 8 mL min⁻¹ there
198 was no effect on mass transfer limitations in macropores and attributed this behavior to the relative “high”
199 flow rate applied. From these results it can be concluded that boundary layer reduction and optimum flow
200 depends on several other factors like reaction rate, substrate concentration, pore size and porosity, which
201 needs to be optimized as well.

202



203

204 **Figure 2.** TC degradation at different flow rates (0.5-5 mL min⁻¹). Temperature: 25°C; TC initial
 205 concentration of 20 ppm; total volume of TC solution of 30 mL, 15 U of enzymes in laccase-monoliths.

206

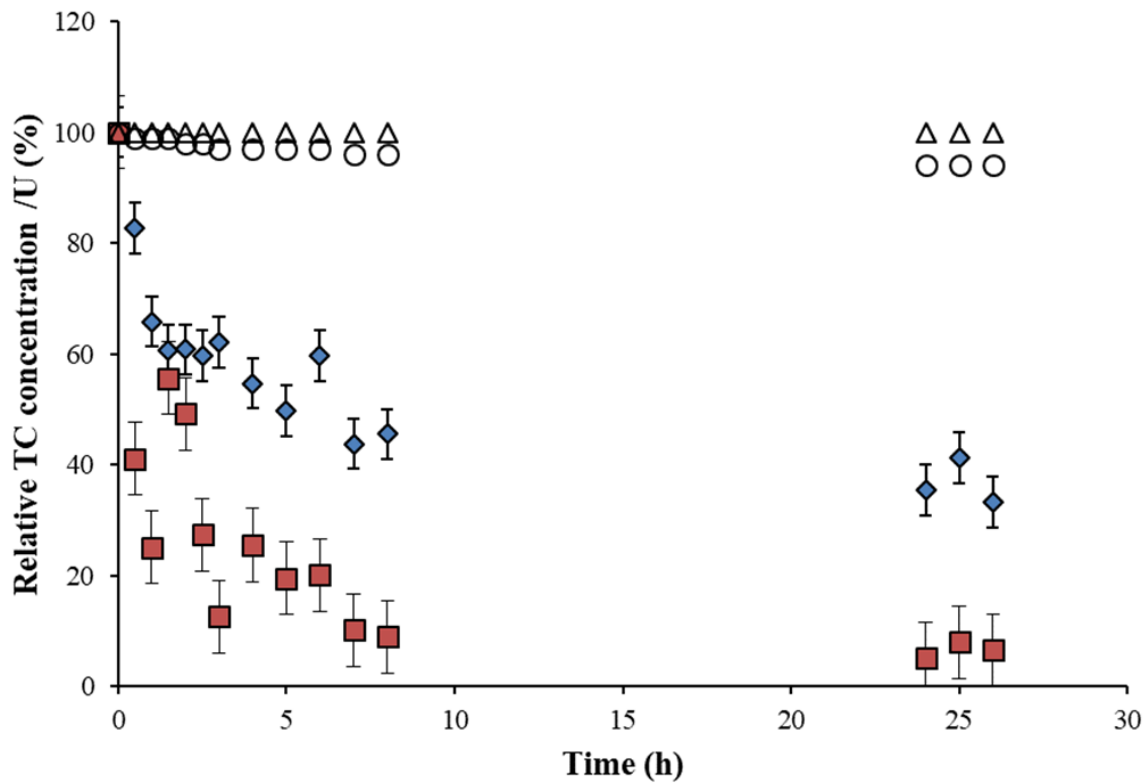
207 There are other possible explanations to the low experimental TC degradation (Figure 2). For example, the
 208 process can be controlled by internal mass transfer, i. e. diffusion inside the mesopores, which can be
 209 considered as stagnant regions in monoliths. In fact, silica monoliths feature skeletons with a thickness in the
 210 range 5-15 μm, which contain an interconnected mesoporous structure where the major part of enzyme
 211 molecules is immobilized. Therefore, the most part of reactive sites inside the mesoporous structure are only
 212 accessible through the diffusion of substrates.

213

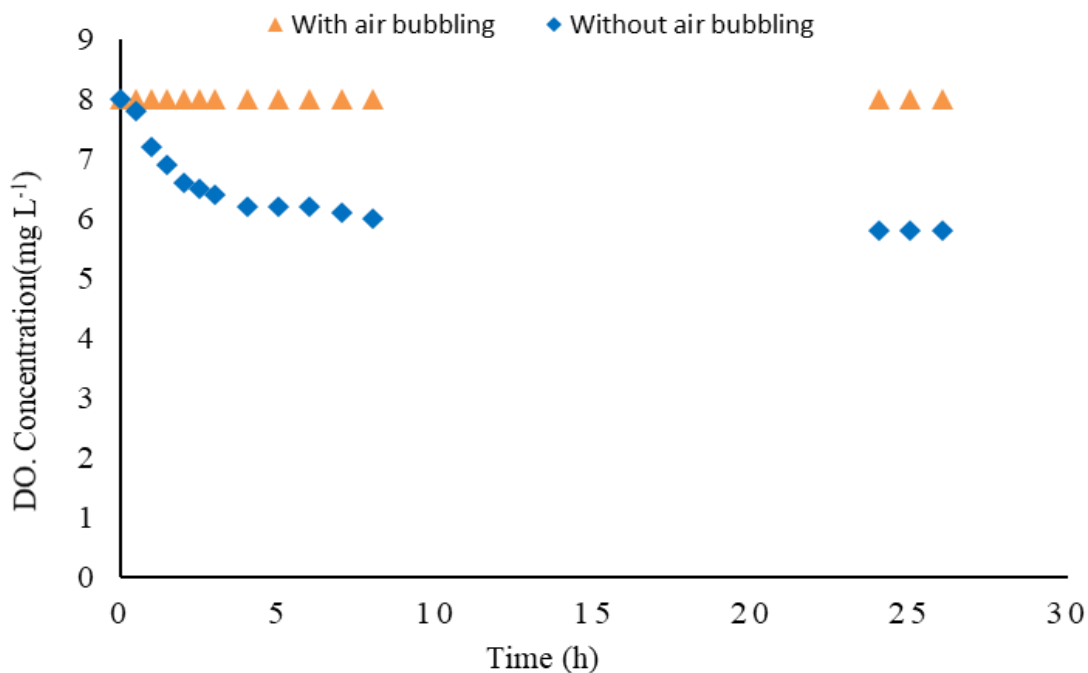
214 3.2 Effect of dissolved oxygen on TC degradation

215 Laccases₇ are multicopper oxidases that catalyze the oxidation of different aromatic compounds with
 216 molecular oxygen, which is concomitantly reduced to water (Nyanhongo et al., 2012). In order to study the
 217 importance of the amount of oxygen available for ~~on~~ TC degradation, the effect of the addition of air bubbling
 218 at a flow rate of 30-40 mL min⁻¹ was first studied in a stirred tank reactor with crushed monoliths as discussed

219 in section 2.2 (Figure 3). The concentration of the dissolved oxygen in the tank was measured during the
220 experiments with and without air-bubbling (Figure 4).



221
222 **Figure 3.** Evolution of TC concentration with crushed laccase-monoliths in a stirred tank reactor, without
223 (diamonds) and with (squares) air-bubbling. Control experiments with inactivated laccase-monoliths with
224 (circles) and without (triangles) air bubbling. Initial TC concentration of 20 ppm in osmosed water (pH 6).
225 Enzyme concentration of 18 U in the laccase-monoliths. Total volume 30 mL, temperature 25 °C.



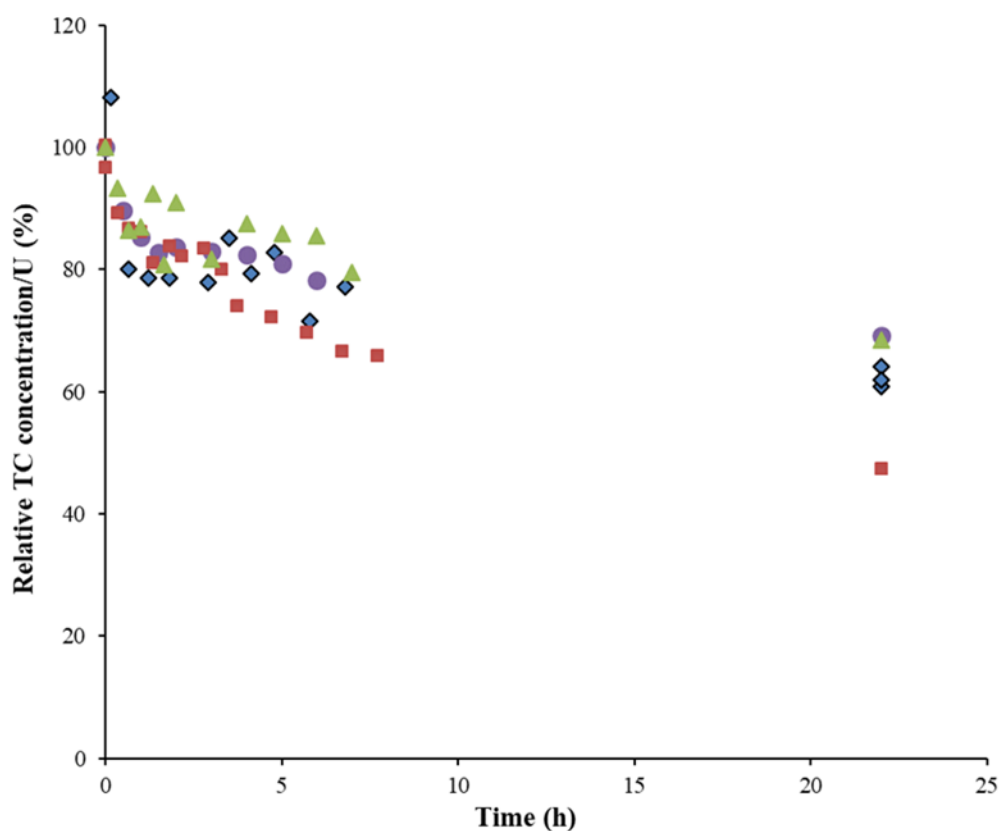
226

227 **Figure 4.** Evolution of dissolved oxygen (DO) concentration verse time in case of TC degradation test with
 228 crushed laccase-monoliths in a stirred tank reactor with (triangles) or without (diamonds) air bubbling. Initial
 229 TC concentration of 20 ppm in osmosed water (pH 6). Enzyme concentration of 18 U in the laccase-
 230 monoliths. Total volume 30 mL, temperature 25 °C.

231

232 From Figure 3 it can be observed that for control tests (with inactivated laccase-monoliths) TC self-
 233 degradation or adsorption (with or without air bubbling) was extremely low (less than 5%). Furthermore, For
 234 the crushed active monoliths without air bubbling the TC degradation was 55% in the first 6 h, while with
 235 air bubbling, the TC degradation reached more than 90% in the same period. These results show that oxygen
 236 seems to be a limiting substrate for TC degradation. The same conclusion was found in literature (Ortner et
 237 al., 2015) for the oxidation of lignins using laccase from *Trametes villosa*. The authors observed a rapid
 238 decrease of oxygen concentration at the beginning of the reaction, which limited the oxidation of phenolic
 239 compounds. They concluded that a sufficient oxygen concentration is necessary for an efficient laccase
 240 oxidation process. In the present work, when the solution was bubbled with air, the dissolved oxygen (DO)
 241 was always at saturation value ($\sim 8.3 \text{ mg L}^{-1}$ at 25 °C) (Figure 4), which corresponded to an excess of 5.6
 242 moles of oxygen/moles of TC. Without air bubbling, the DO concentration decreased of 20% in the first 6 h
 243 concomitantly with the decrease of TC concentration (55%) to reach a steady-state of DO concentration of 6
 244 mg L^{-1} . The calculation of the number of moles of TC and oxygen ($5.6 \cdot 10^{-6}$ and $1.35 \cdot 10^{-6}$, respectively)
 245 indicates that a final molar ratio O_2/TC of 4, is therefore not enough to proceed to further oxidation. An
 246 excess of at least 5.6 O_2 molecules per TC seems to be necessary for total TC oxidation.

247 The effect of air bubbling in the FTR was studied at two different flow rates (1 and 5 mL min⁻¹) by bubbling
248 air inside the feed tank (30-40 mL min⁻¹) (Figure 5). Initial TC concentration for each experiment was 20
249 ppm, the total volume of solution in the tank was 30 mL and the total activity of the 3 cladded laccase-
250 monoliths was 12 U. For the flow rate of 5 mL min⁻¹, the degradation of TC reached 50 % with air bubbling,
251 while without air bubbling the TC degradation was only 30%. Similarly, for the flow rate of 1 mL min⁻¹, the
252 TC degradation was 40 and 30% with and without air bubbling, respectively. With air bubbling, TC
253 degradation was slightly higher in the case of the larger flow rate (5 mL min⁻¹) due to the larger amount of
254 oxygen provided to the reactor. Nevertheless, this TC degradation ratio remains low compared to 90% TC
255 degradation reached in a stirred tank reactor with air bubbling (Figure 5).



256

257 **Figure 5.** Evolution of TC concentration with laccase-monoliths under recycling flow at different flow rates
258 without air bubbling (TC solution flow rates: circle, 1 mL min⁻¹; triangle, 5 mL min⁻¹) and with air bubbling
259 (TC solution flow rates: diamonds, 1 mL min⁻¹; squares, 5 mL min⁻¹) at 25°C. Conditions: initial TC
260 concentration of 20 ppm in osmosed water; total volume of TC solution of 30 mL; total activity of the laccase-
261 monoliths of 12 U.

262

263 These results suggest the decrease of oxygen concentration near the bio-catalytic sites (which are located
264 everywhere in the surface of monoliths, but mainly inside the mesoporosity), which limits the reaction rate

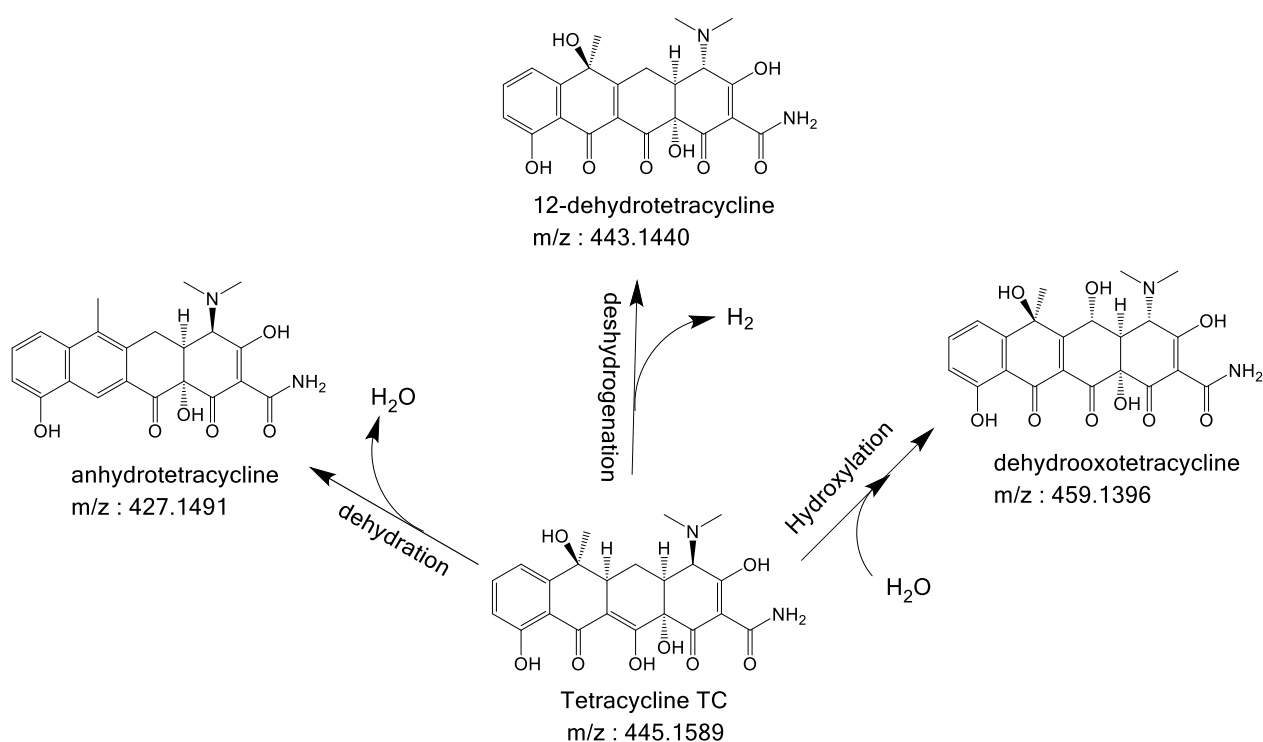
265 and the conversion in flow. Furthermore, maintaining a high oxygen concentration or even an excess of
266 oxygen in the solution of the reservoir is not enough to enhance the conversion; this emphasizes that the
267 decrease of co-substrate (O_2) concentration near catalytic sites certainly controls the process.

268

269 3.3 Effects of TC degradation products

270 Many different TC degradation products were identified using the same laccase immobilized in ceramic
271 membranes (De Cazes., et al., 2014). These products present a TC based structure like anhydrotetracycline,
272 4-epi-anhydrotetracycline, dehydrooxotetracycline and 12-dehydrotetracycline (Llorca et al., 2015). In this
273 study the main molecules identified by LC-HRMS (IDAEA-CSIC laboratory, Spain) during the degradation
274 of TC are presented in Figure 6. One of the major products identified was dehydroxytetracycline ($m/z =$
275 459.1396) obtained after hydroxylation of the tetracycline. Some other products were identified coming from
276 the loss of functional groups ($-OH$, $-NH_2$, etc.) of the tetracycline molecule. However the gap in the mass
277 balance between initial tetracycline and the amount of identified by-products indicates that the majority of
278 tetracycline was polymerized (as is usually the case for phenolic compounds), or completely oxydized into
279 CO_2 or transformed in other by-products, which were not detectable by LC-HRMS.

280



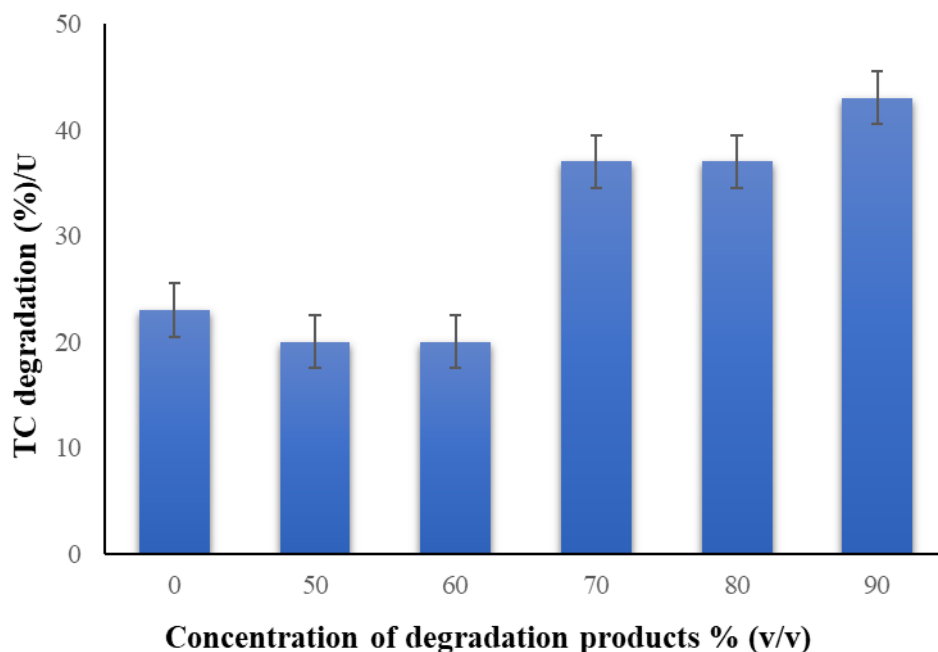
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282 **Figure 6.** Degradation products of tetracycline and possible reaction pathways using laccase from *Trametes*
283 *versicolor* immobilized on silica monolith.

284

285 As enzymes can be inhibited by the products of the enzymatic conversion (Jing et al., 2009; Barth et al.,
286 2015), the effect of degradation products on TC degradation rate was studied with crushed monoliths and
287 under vigorous stirring to avoid any influence of mass transport limitations. For this purpose, an initial TC
288 degradation test was carried out for 24 h, with 50 mL of fresh TC solution (20 ppm) and a total enzymatic
289 activity of crushed monoliths of 6 U. After 24 h of reaction (without air bubbling), the reaction mixture
290 (unconverted TC + degradation products) was separated from the immobilized enzymes by filtration.
291 Afterward, a series of TC degradation experiments were carried out at different concentrations of degradation
292 products by adding a volumetric fraction (50-90%) of the reaction mixture obtained from the initial
293 experiment to a fresh TC solution (up to reach an initial TC concentration of 20 ppm) mixed with a fresh
294 crushed enzymatic monolith (6 U). The results obtained are shown in Figure 7. It is important to notice that
295 a lower degree of TC degradation (20%) was observed in comparison to the results (60% degradation)
296 reported in Figure 3. This is due to the use of a lower enzymatic monolith activity (6 U instead of 18 U).

297



298

299 **Figure 7.** Effect of the amount of TC degradation products on TC degradation in batch. Initial TC
300 concentration of 20 ppm; crushed laccase-monolith (6 U); temperature 25 °C; reaction time of 24 h.

301

302 The main result of this series of experiments is the enhancement of TC degradation with the increase of
303 concentration of degradation products. Without degradation products and until a concentration of 60% of
304 degradation products, TC degradation is around 20% in 24 hours, whereas for more than 70% of degradation
305 products present in the solution, TC degradation reaches 40% after 24 hours. These results show that the TC
306 degradation products formed did not inhibit the TC degradation, but in opposite they enhanced the conversion

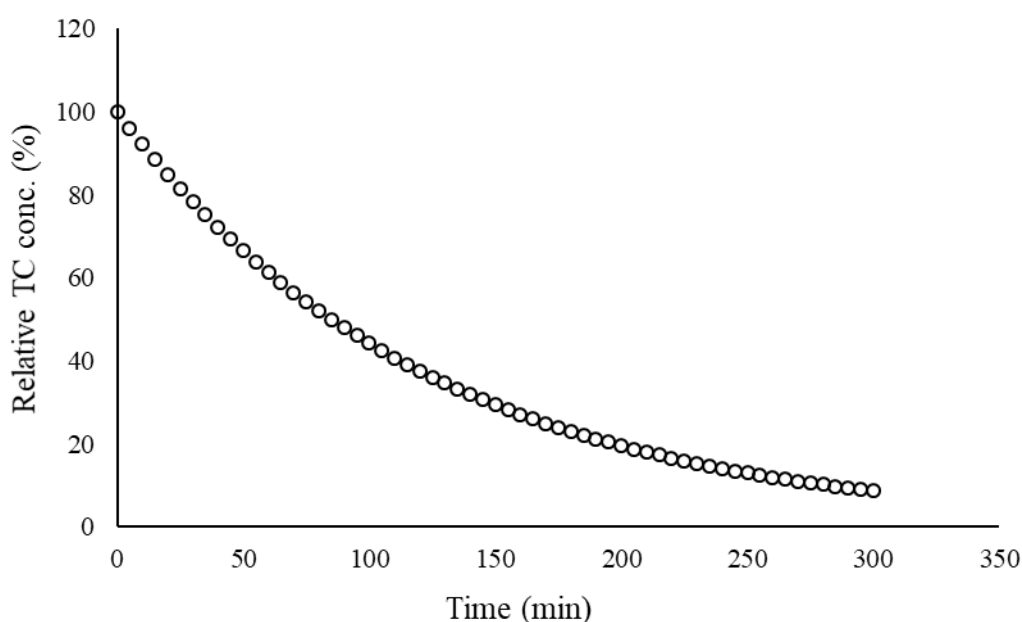
307 when their concentration reached a minimum level. A similar phenomenon has been recently observed by
308 Parra Guardado et al., (2019) and has been explained by the fact that some products of the reaction can act
309 as redox mediators enhancing the enzyme reactivity and the conversion. This could also explain the higher
310 degradation rate of TC (by a factor 2) obtained in the stirred tank reactor in comparison to the FTR, where
311 degradation products do not spend enough time in the enzyme vicinity.

312

313 **3.4 Application of a model for simulation of TC degradation in actual wastewater concentrations** 314 **and proposed scale up of monolith system**

315 In a previous work (Ahmad et al., 2021) reported a computational fluid dynamics (CFD) model allowing
316 computing TC degradation. The model was built coupling an apparent Michaelis-Menten reaction kinetics
317 of immobilized enzymes, flow hydrodynamics within the FTR and a dynamic mass balance on feed tank. As
318 far as this model was built in conditions of oxygen saturation conditions and with a reaction kinetics, which
319 only takes into account TC and not oxygen, it should not be applied for TC concentrations as high as used in
320 this work (20 ppm). Indeed it was demonstrated here that oxygen becomes rapidly a limiting substrate.
321 However, in some municipal wastewaters the TC concentration currently encountered is very low ($2.8 \cdot 10^{-4}$
322 ppm) (Abejón et al., 2015b; Danner et al., 2019). Thus, in this case and considering the saturation
323 concentration of oxygen at 25 °C (8 mg L^{-1}), the concentration of O_2 ($\text{O}_2/\text{TC} = 4 \cdot 10^5$) would be five orders
324 of magnitude larger than the requirement previously founded ($\text{O}_2/\text{TC} = 5.6$). Under such conditions, we can
325 consider that oxygen is no longer a limiting substrate and then the model can be reasonably applied for
326 simulation purposes.

327



328

329 **Figure 8.** Simulated degradation of TC at actual initial TC concentration (2.8×10^{-4} ppm) found in wastewater
 330 in a FTR. Simulation conditions: flow rate (1 mL min^{-1}), continuous recycled mode, Temperature $25 \text{ }^{\circ}\text{C}$, 15
 331 U of enzymatic activity in the monoliths.

332

333 The simulation of the TC degradation carried out with the FTR (3 monoliths in series of 0.6 cm diameter and
 334 0.5 cm length, each) shows that for real TC concentration (2.8×10^{-4} ppm) a removal of 90% of TC was reached
 335 in 5 h at a flow rate of 1 mL min^{-1} with a recycling configuration (see Figure 8). Taking account of these
 336 results, we then proposed to determine the minimum size of a monolith that allows a complete degradation
 337 of the TC in only one passage (without recycling) through the FTR. For this purpose, monoliths with different
 338 size (length and diameter ranged in between 5-50 cm and 1-20 cm, respectively) were considered for
 339 simulations (Table 2). When the size of monoliths is increased the conversion is enhanced. For example, for
 340 a single pass, when the monolith geometry was set at 5 cm length and 1 cm diameter only 0.5% of initial TC
 341 was converted. However, with monoliths of 50 cm length and 20 cm diameter it was possible to completely
 342 degrade TC at a flow rate of 1 mL min^{-1} . These theoretical “large-scale monoliths” were then used for further
 343 simulations.

344

345 **Table 2.** Effect of the scale up of the silica monoliths (by increasing the length and diameter of the monoliths)
 346 towards TC degradation.

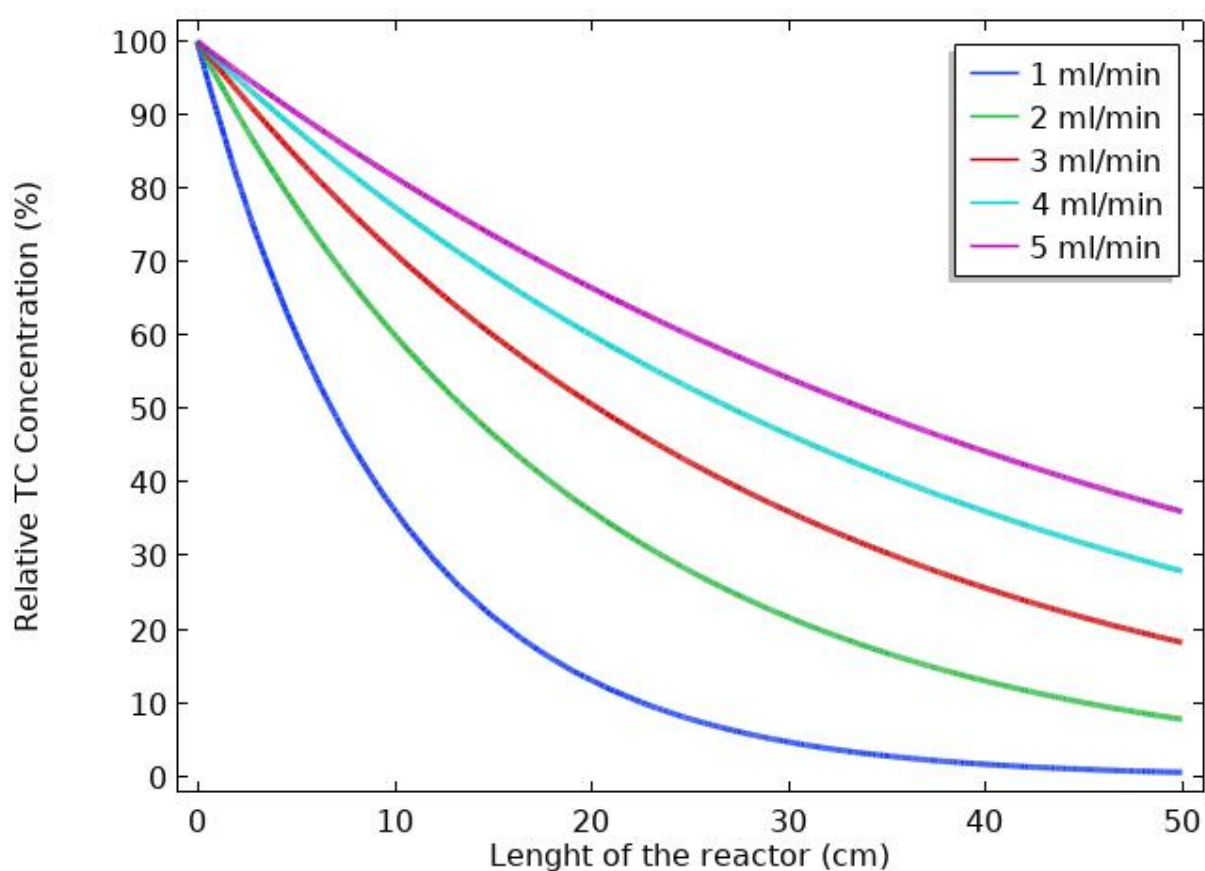
Length (cm)	Diameter (cm)	Inlet concentration (ppm) $\times 10^{-4}$	TC Outlet concentration (ppm) $\times 10^{-4}$	TC conversion in a single pass (%)
0.15	0.06	2.8	2.79	0.3
5	2	2.8	2.78	0.7
10	4	2.8	2.68	4
15	6	2.8	2.43	13
20	8	2.8	2.01	28
25	10	2.8	1.34	52
50	20	2.8	0	100

347

348 The TC conversion along the length of the optimum reactor (20 cm diameter, 50 cm length) was then studied
 349 at different flow rates (Figure 9). Complete TC degradation was achieved at the flow rate of 1 mL min^{-1} .

350 However, it can be observed that the rate of TC depletion in the last 15 cm was relatively low; this last section
351 of the monolith is not enough efficient. For flow rates higher than 1 mL min⁻¹, TC conversion decreases up
352 to 50% at a flow rate of 5 mL min⁻¹ (Figure 9). In fact, the global process is controlled by reaction kinetics
353 and at high flow rates, the residence time is too low to reach a complete TC depletion. However, as real
354 wastewater fluxes are extremely high (600-70,000 m³/day) a compromise must be found between the level
355 of TC depletion, the contact time and process effectiveness. For this, an optimization has to be carried out
356 with multi-objective programming like Pareto optimality, this analysis has to be coupled with the cost of the
357 process (Abejón et al., 2015b).

358



359

360 **Figure 9.** Relative tetracycline concentration along the length of the reactor from inlet to outlet at different
361 TC flow rates from 1 to 5 mL min⁻¹. Reactor length: 50 cm, diameter: 20 cm; Inlet TC concentration: 2.8 10⁻⁴
362 ppm; Reaction rate: 4.4 10⁻⁵ μmol L⁻¹ min⁻¹.

363

364 Conclusions

365 Biocatalytic reactors were built by grafting laccase into the mesopores of macroporous silica monoliths. The
366 depletion of tetracycline (TC) (20 ppm) in aqueous solutions was carried out in a tubular flow through reactor

367 (FTR) configuration (3 monoliths in series of 0.6 cm diameter and 0.5 cm length, each) with recycling in a
368 reservoir tank. Only 30-40% of TC was degraded during the first 5 h and then the conversion slowed down
369 to a threshold. The increase of flow rate from 0.5 to 5 mL min⁻¹ did not change TC depletion showing that
370 the phenomenon was not due to external mass transfer limitation, but rather to diffusion limitation of
371 substrates inside the mesopores. Experiments performed in stirred tank reactors (CSTR) with crushed
372 monoliths showed a higher TC depletion of 55% in 6 h, which was increased to 90% when the solution was
373 bubbled with air. This showed that O₂ is a limiting substrate for this reaction and that a molar excess of at
374 least 5.6 O₂/TC is necessary for a complete TC depletion. In FTR configuration, the addition of air-bubbling
375 in the reservoir lead to an increase of TC depletion of only 10%, with a maximum of 50% at flow rate of 5
376 mL min⁻¹, this result indicates that oxygen limitations are enhanced in FTR configuration due to the mass
377 transport limitations inside the mesopores. It was observed that the presence of by-products of degradation
378 increases the TC depletion, this result could be explained by a possible the role of redox mediators of such
379 by-products. In real municipal wastewater TC concentration is very low (2.8 10⁻⁴ ppm) and O₂ will be no
380 more a limiting substrate as its excess of 5 orders of magnitude higher than TC. The modeling of FTR showed
381 that in such conditions TC depletion should reach 90% in 5 h at a flow rate of 1 mL min⁻¹ in a recirculating
382 flow mode. The model allowed to predict that a monolith of 20 cm diameter and 50 cm length could be used
383 for a total depletion of TC in a single pass (without recirculation) at a flow rate of 1 mL/min. However for
384 higher flow rates (as the one encountered in real wastewater treatments) the decrease of the TC depletion was
385 noticed. This is due by the fact that the depletion is governed by the reaction kinetic of the laccase. Further
386 improvement on laccase activity could contribute to optimize the process. New monolithic configurations
387 and processes have to be designed in order to feed oxygen continuously inside the porosity.

388

389 **Declarations**

390

391 **Ethics approval and consent to participate**

392 Not applicable

393

394 **Consent for publication**

395 Not applicable

396

397 **Availability of data and materials**

398 The datasets used and/or analyzed during the current study are available from the corresponding author on
399 reasonable request, additional information and results are given in supplementary material.

400

401 **Competing interests**

402 The authors declare that they have no competing interests

403

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408

409 **Authors' contributions**

410 Sher Ahmad and Wassim Sebai carried out the experimental work, synthesis of monoliths and enzymatic
411 degradation of tetracycline, Marie-Pierre Belleville designed degradation experiments and contributed to
412 the scientific discussion, Nicolas Brun, designed monoliths synthesis and contributed to the scientific
413 discussion, Anne Galarneau and Jose Sanchez Marcano supervised all of the work, contributed with the
414 discussions and wrote the whole manuscript. All of the authors revised the manuscript.

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