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**Intensification of sequencing batch reactors by cometabolism and bioaugmentation with *Pseudomonas putida* for the biodegradation of 4-chlorophenol**

**Cometabolic biodegradation of 4-chlorophenol in bioaugmented SBR with *Pseudomonas putida***

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**ABSTRACT**

**BACKGROUND:** This study is focused on the application of intensified biological systems for the degradation of halogenated organic compounds. The single and combined effects of cometabolism and bioaugmentation with *Pseudomonas putida* on the aerobic degradation of 4-chlorophenol (4-CP) in sequencing batch reactors was studied. Phenol was added as growth substrate to enhance 4-CP biodegradation through cometabolic transformation.

**RESULTS:** Adaptation of activated sludge by increasing 4-CP loads addressed to a progressive acclimation to that compound, which could be successfully degraded at loading rates below 55 mg g<sup>-1</sup> VSS d<sup>-1</sup>. Using phenol as cosubstrate allowed almost a threefold decrease of the

time required for the exhaustion of 4-CP. The addition of phenol also reduced the toxic effect of 4-CP over *P. putida*. The bioaugmentation of the SBR with *P. putida* frankly enhanced the 4-CP removal rate, allowing to deal with 4-CP loads up to 120 mg g<sup>-1</sup> VSS d<sup>-1</sup>.

**CONCLUSION:** Bioaugmentation of SBR with *P. putida* improves the capacity of this system to withstand high toxic shocks. Cometabolic degradation of 4-CP with phenol improves the removal rates achieved by the SBR at similar 4-CP loads. Both strategies are more convenient intensification techniques than acclimation for the biological treatment of 4-CP.

**Keywords:** Sequencing batch reactor (SBR); chlorophenols; biodegradation; bioaugmentation; cometabolism; *Pseudomonas putida*

# INTRODUCTION

Chlorophenols constitute an important group of hazardous compounds widely used in pesticides, herbicides and dyes manufacture. Among the monochlorophenols, 4-chlorophenol (4-CP) has been reported to be the less biodegradable<sup>1</sup> and more toxic compound ( $EC_{50} = 1.9 \text{ mg L}^{-1}$ )<sup>2</sup> under aerobic conditions, and it has showed refractory in anaerobic systems.<sup>3</sup> 4-CP has been widely used as target toxic compound to evaluate the treatability of hardly biodegradable compounds by different biological systems like conventional activated sludge,<sup>4, 5</sup> fluidized bed reactors,<sup>6</sup> packed-bed reactor,<sup>7, 8</sup> SBR,<sup>9-11</sup> rotating tubes biofilm reactors,<sup>12</sup> rotating brush biofilm reactors,<sup>13</sup> moving bed biofilm reactors,<sup>14</sup> and membrane sequencing batch reactor.<sup>15, 16</sup>

Biological treatment of wastewater containing chlorophenols by conventional activated sludge (AS) often fail to achieve high removal efficiencies due to microbial inhibition<sup>17</sup> by interfering with energy transduction of cells.<sup>18</sup> Therefore, several strategies could be used in order to enhance the removal efficiency of hardly-biodegradable compounds. These methods include adaptation of the microbial population to the pollutant<sup>19, 20</sup> and/or the addition of alternative carbon sources like phenol, acetate or sugars. These cosubstrates can act as inducing agents of oxidizing enzymes or by providing reducing capability for the degradation of recalcitrant compounds under aerobic conditions. Works found in the literature have concluded that cometabolism is a powerful way of enhancing chlorophenols biodegradation, which allows dealing with higher concentrations of those compounds.<sup>9, 21</sup> Several authors have shown that phenol is a better growth cosubstrate than biogenic compounds because of its similitude to chlorophenols.<sup>22</sup>

The introduction of specialist degrading bacteria in biological systems is a promising cost-effective intensification technique for improving both the removal efficiency and the start-up of bioreactors treating xenobiotics-bearing wastewaters.<sup>7</sup> The so-called bioaugmentation has been mostly applied for the remediation of soils contaminated with hazardous pollutants and also for treating wastewaters bearing hardly biodegradable compounds in small-scale AS systems.<sup>23</sup> However, its application to other biological systems has been scarcely studied so far. Nevertheless, 4-CP can be partial or completely removed by different aerobic strains, being *Azotobacter*, *Alcaligenes*, *Rhodococcus*, *Phragmites* and specially

*Pseudomonas* the most studied genus. The bioaugmentation of aerobic AS with *P. putida* has been successfully applied to improve the biological removal of chlorophenols batchwise.<sup>24</sup> It is noteworthy that the simple addition of allochthonous bacteria possessing metabolic capabilities does not guarantee enhanced transformation in a mixed culture.<sup>25</sup> So far, very few examples of successful bioaugmentation of AS units using either naturally isolated or genetically engineered microorganisms have been reported.<sup>26</sup> Among the different biological systems used for the treatment of xenobiotics-bearing wastewaters, sequencing batch reactors (SBR) have received increasing attention due to some of advantages of this technology such as easier control of the process, high nutrients removal efficiency and low energy requirements. In addition the operating conditions can be periodically modified in that system allowing to control the abundance and activity of allochthonous strains in multi-species microbial communities<sup>27</sup> and facilitating the survival and growth of the bacteria introduced.<sup>28, 29</sup> In this work, the effect of using phenol as cosubstrate and bioaugmentation of AS with *P. putida* for the biodegradation of 4-CP from synthetic wastewater by SBR was investigated.

## EXPERIMENTAL

### SBR description and operation

The experiments were carried out in three SBR of 2.1 L inoculated with AS (control SBR), *P. putida* or bioaugmented AS with *P. putida* (0.3%, w:w). The runs were conducted in a series of sequential stages of 12 h consisting of anoxic filling (1 h), aerated reaction (9.5 h), settling (1 h) and discharging (0.5 h). The reactors were operated at each 4-CP concentration investigated for at least three weeks, in order to ensure the steady performance before collection of the corresponding data. The operating conditions were 30 ± 1 °C, neutral pH, 120 rpm, 5% of volume exchange ratio, and 10.5 d hydraulic residence time (HRT).<sup>9</sup>

### Microorganisms and growth conditions

*P. putida* strain CECT 4064 was purchased from the Spanish Type Culture Collection (Valencia). Stock cultures were maintained at -40 °C in a nutrient medium supplemented with 15% (v/v) glycerol. *P. putida*

was transferred to a nutrient medium containing 1 g beef extract, 2 g yeast extract, 5 g peptone and 5 g NaCl per litre of deionised water. The cell suspension resulting from the late exponential growth phase was subcultured in a mineral salts medium<sup>30</sup> with phenol (25 mg L<sup>-1</sup>) as sole carbon source and stirred in a thermostated orbital shaker at 120 rpm, 30 °C and neutral pH for 10 - 12 h. The resulting culture was inoculated into the corresponding SBR.

The AS used as inoculum was collected from a cosmetics wastewater treatment plant. Biomass concentration in the reactors was maintained at 2,500 ± 200 mg VSS L<sup>-1</sup>. Organic load rates of 2.4 and 3.8 mg g<sup>-1</sup> VSS d<sup>-1</sup> of 4-CP and phenol, respectively, were used during the acclimation period which extended for fifteen weeks.

#### Wastewater composition

A wide range of 4-CP loading rates was tested when this compound was the sole carbon source (210 - 3,150 mg L<sup>-1</sup>) and also in the studies of cometabolism (525 - 3,150 mg L<sup>-1</sup>) in the presence of a wide range of phenol loads, as can be seen in Table 1. Ammonium sulphate and phosphoric acid were used as nitrogen and phosphorous sources, respectively. A COD:N:P:micronutrients ratio of 100:0.5:0.1:0.05 (w:w) was fixed. The mineral solution added as micronutrients supply consisted on FeCl<sub>3</sub>, CaCl<sub>2</sub>, KCl and MgSO<sub>4</sub>.

#### Analytical methods

4-CP and phenol were analyzed by HPLC/UV (Prostar, Varian) using a C18 column as stationary phase (Microsorb MW-100-5) and a mixture of acetonitrile and H<sub>2</sub>O (40:60, vol.) as mobile phase. The flow rate was maintained at 1.0 mL/min and a wavelength of 280 nm was used. Total organic carbon (TOC) was measured by an OI Analytical Model 1010 TOC apparatus. Total and volatile suspended solids (TSS and VSS) were determined according to the APHA Standard Methods.<sup>31</sup> SEM micrographs were obtained with a Hitachi S-3000N apparatus. Contribution of abiotic processes such as volatilisation and adsorption onto biomass was evaluated. Adsorption of 4-CP was measured on biomass samples after extraction with Soxhelt following the US-EPA 8041 method. Tests of volatilisation were performed under identical operating conditions to those employed in the biodegradation experiments but in the absence of biomass.

The results reported were the average values from duplicate runs. In all the cases, the standard errors were lower than 10%.

## RESULTS AND DISCUSSION

### Effects of acclimation and bioaugmentation during the start-up

Acclimation and bioaugmentation during the start-up period of SBR treating a 4-CP load of  $2.4 \text{ mg g}^{-1} \text{ VSS d}^{-1}$  with the addition of phenol as cosubstrate ( $3.8 \text{ mg g}^{-1} \text{ VSS d}^{-1}$ ) were evaluated. Runs were carried out using non-adapted and acclimated biomass. The time-evolution of phenol and 4-CP concentrations along the aerobic phase of the start-up period of SBRs inoculated with different biomass is shown in Figure 1. Whereas non-adapted AS did not show any biodegrading activity, both compounds were depleted after 8 h upon adaptation. Owing to the high tolerance of *P. putida* to the presence of toxic compounds, removal efficiencies higher than 35% were obtained for both phenol and 4-CP with non-adapted *P. putida*. However, those removal efficiencies were greatly increased after acclimation, reaching values of 82 and 100% for 4-CP and phenol, respectively.

Regarding the effect of bioaugmentation, the use of non-adapted *P. putida* did not enhance the removal rates observed with adapted AS. This fact recommends the use of specialist degrading bacteria previously adapted to the toxic compounds for the bioaugmentation of bioreactors. The enhancement of the removal capacity of *P. putida* upon acclimation would improve the viability of the strain introduced.<sup>32</sup> The bioaugmented SBR showed the highest removal rates indicating the existence of synergic effects between the activated sludge and *P. putida*.

### Cometabolic removal of 4-CP in the presence of phenol

Figure 2 shows the time-evolution of phenol and 4-CP concentration along the aerobic reaction stage in a SBR inoculated with several adapted biomass. It is worthy to note that phenol concentrations detected after filling in the control and bioaugmented SBR were lower than expected, which indicates that this

1 compound was partially removed upon the anoxic filling phase (Fig. 2a). With respect to 4-CP degradation,  
2 a long lag stage was observed in the SBR-*P. putida*, since 4-CP removal starts once phenol is almost  
3 depleted. The 4-CP removal rate increases sharply once phenol was nearly exhausted. This diauxic  
4 consumption of both compounds is commonly found in monoculture biological systems as well as negative  
5 interactions between chemically similar compounds in binary systems.<sup>33</sup> In this particular case, the initial  
6 step in the transformation of 4-CP is hydroxylation promoted by an NADPH-dependent monooxygenase  
7 giving rise to 4-chlorocatechol. The NADPH consumed in the monooxygenase reaction can be regenerated  
8 by the oxidation of phenol or phenol-induced biomass.<sup>34</sup> Similarly, the oxidation of phenol is also initiated  
9 by a NADPH-dependent hydroxylase leading to catechol.

10 On the opposite, simultaneous degradation of both substrates occurred in control and bioaugmented SBR.  
11 It can also be seen that the bioaugmentation of AS with acclimated *P. putida* led to a significant increase of  
12 the removal rates. The bacterial pull present in these systems minimizes the competitive inhibition  
13 between 4-CP and phenol since different microorganisms can be involved in the degradation pathway of  
14 those compounds and the corresponding intermediates. Additionally, increasing phenol load up to 120 mg  
15 g<sup>-1</sup> VSS d<sup>-1</sup> allowed treating 4-CP loads up to 60 and 120 mg g<sup>-1</sup> VSS d<sup>-1</sup> in control and bioaugmented SBR,  
16 respectively. A possible explanation could be that phenol supplies the electrons required for the initial  
17 monooxygenase step of 4-CP biodegradation.<sup>35</sup> The high performance showed by the bioaugmented system  
18 can be caused by the exchange of genetic elements via cell-to-cell contact which is thought to be significant  
19 for bacteria residing in aggregates representing high cell density environments.<sup>36</sup>

20 In all the experiments a residual fraction of TOC was measured in the resulting effluents, which was similar  
21 (20 mg L<sup>-1</sup>) for control and bioaugmented SBR but almost triple in the case of SBR-*P. putida*. The remaining  
22 TOC has been related to the presence of soluble microbial products, whose concentration was significantly  
23 decreased by using AS as inoculum. Discounting that fraction, TOC removal was complete in all the systems,  
24 reaching higher removal rates in bioaugmented SBR.



#### 4-CP removal as sole substrate

The treatment of water with 4-CP as sole carbon and energy source by SBR inoculated with *P. putida* led to the inactivation of the strain. Thus, the application of that single culture for the treatment of 4-CP in absence of a cosubstrate is unlikely. Figure 3 shows the time-evolution of 4-CP concentration during the aerated stage in both non-bioaugmented (Fig. 3a) and bioaugmented (Fig. 3b) SBR. Although 4-CP disappearance rates were significantly lower than those achieved by the cometabolic systems, similar loads of 4-CP could be treated. The maximum 4-CP load ( $120 \text{ mg 4-CP g}^{-1} \text{ VSS d}^{-1}$ ) treated efficiently by the bioaugmented SBR was considerably higher than for the SBR inoculated with AS. In this last system loads of 4-CP above  $55 \text{ mg g}^{-1} \text{ VSS d}^{-1}$  caused a dramatic decrease of the removal efficiency, indicating that *P. putida* provides a greater resistance to toxicity. Although the decrease of indigenous AS concentration in the control SBR was negligible after the acclimation to the toxic compound, it should not be ruled out that bioaugmentation of AS can exert a protective effect against the toxicity derived from the presence of 4-CP up to certain concentration.

The time-evolution of 4-CP concentration in both control and bioaugmented SBR showed inhibition profiles which were accurately described by using Haldane inhibition model (Fig. 3). The experimental data were fitted to the Haldane model. Fitting to the experimental results was accomplished by using a non-linear least squares minimization of the error using a simplex algorithm followed by a Powell minimization algorithm (Micromath® Scientist 3.0) at the 95% probability level to obtain the values of the fitting parameters. The optimal-fitting values of the parameters (maximum specific 4-CP consumption rate,  $V_{\max}$ , saturation,  $K_s$ , and inhibition,  $K_i$ , constants) for both systems are shown in Table 2. 4-CP loading rates above  $30 \text{ mg 4-CP g}^{-1} \text{ VSS d}^{-1}$  led to a decrease of the maximum removal rate in the case of the control SBR, whereas in the bioaugmented SBR the value of this parameter continued increasing up to a load of 4-CP 2.5 times higher. The erratic trend of  $K_s$  and  $K_i$  values in the control SBR suggests the occurrence of changes in the microbial population. However, the saturation and inhibition constants increased when increasing the 4-CP concentration in the bioaugmented SBR. The increase of the  $K_i$  value could indicate a progressive acclimation of the microbial population to the presence of 4-CP. From this result it can be concluded that

bioaugmentation is a more convenient intensification technique than acclimation for treating high toxic loads.

The bioindication analysis of the bioaugmented SBR after three months working showed the formation of 1.0 - 2.0 mm aggregates of free living bacteria (*P. putida*) caused by the presence of 4-CP in the medium (Fig. 4a). The toxic shock of 4-CP modified the cell morphology from bacillus to coccus, which led to spherical colonies in the bioaugmented SBR (Fig. 4b). The structural and morphological modifications seem to evolve towards a coccoid stress-resistant form, responsible of the residual viability of the microbial population.<sup>37</sup> In certain instances, not only the cell shape changes but also the volume of the cell decreases.<sup>38</sup>

The colonization process started with the surface adhesion of both *P. putida* and the bacteria of the AS. In a second step *P. putida* was covered with exopolysaccharides from the AS. The autoaggregation capacity and the inclusion of *P. putida* into the flocs may help to a successful bioaugmentation since it has been reported that non-flocculant strains did not survive in the AS system and required a higher inoculum size to accomplish substrate removal.<sup>17</sup>

## CONCLUSIONS

The removal of 4-CP as representative of hardly biodegradable compounds by SBR was enhanced using cometabolism or bioaugmentation as intensification techniques. Acclimation of biomass improves significantly the extent and rate of 4-CP biodegradation during the start-up of the SBR. Furthermore, this period can be accelerated by bioaugmentation with specialist degrading bacteria. Bioaugmentation with *P. putida* enhanced the ability of this system to degrade 4-CP. Specialist degrading bacteria also must be adapted to the pollutant before being introduced in the SBR.

The addition of phenol as cosubstrate greatly enhanced the 4-CP removal rates at similar 4-CP loads. On the other hand, bioaugmentation with *P. putida* enhanced the ability of this system to degrade higher 4-CP loads. The biodegradation kinetics was satisfactorily described by the Haldane model. The morphology of *P. putida* changed from bacillus to coccus when 4-CP was added, giving rise to the formation of spherical

colonies. These aggregates were integrated in the AS flocs, which favoured the retention of *P. putida* into the system. The high survival of this strain and a possible genetic transference with the microorganisms contained in the AS make the SBR a more suitable system for the biodegradation of recalcitrant pollutants.

## ACKNOWLEDGEMENTS

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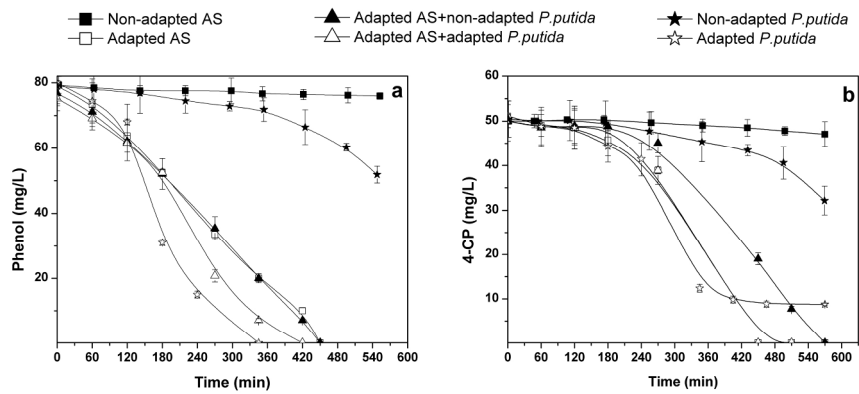


Figure 1. Time-evolution of phenol (a) and 4-CP (b) along the aerobic reaction stage of the start-up of SBR inoculated with different biomass.  
119x55mm (600 x 600 DPI)



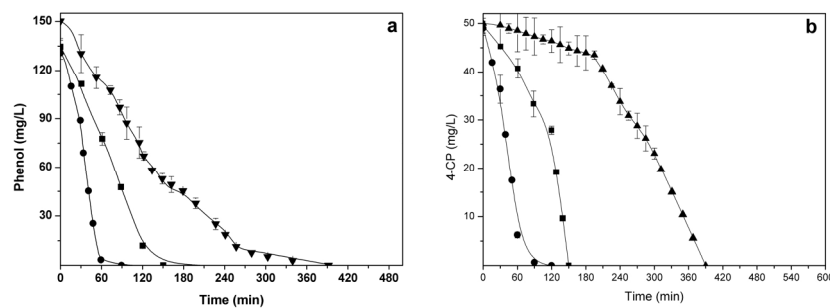


Figure 2. Time evolution of phenol (a) and 4 CP (b) along the aerobic reaction stage in SBR inoculated with adapted AS (■), *P. putida* (▲) and AS+*P. putida* (●) treating  $120 \text{ mg g}^{-1} \text{ VSS d}^{-1}$  of phenol and  $40 \text{ mg g}^{-1} \text{ VSS d}^{-1}$  of 4 CP.

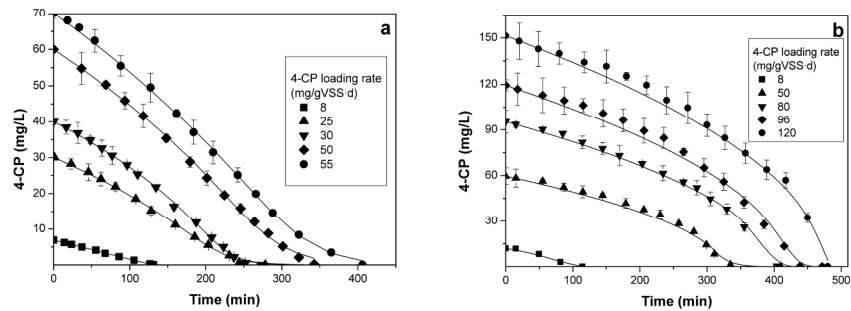


Figure 3. Time-evolution of 4-CP during the aerobic reaction stage of control (a) and bioaugmented (b) SBR treating 4-CP as sole substrate. Fittings to Haldane model (solid lines).  
128x64mm (600 x 600 DPI)

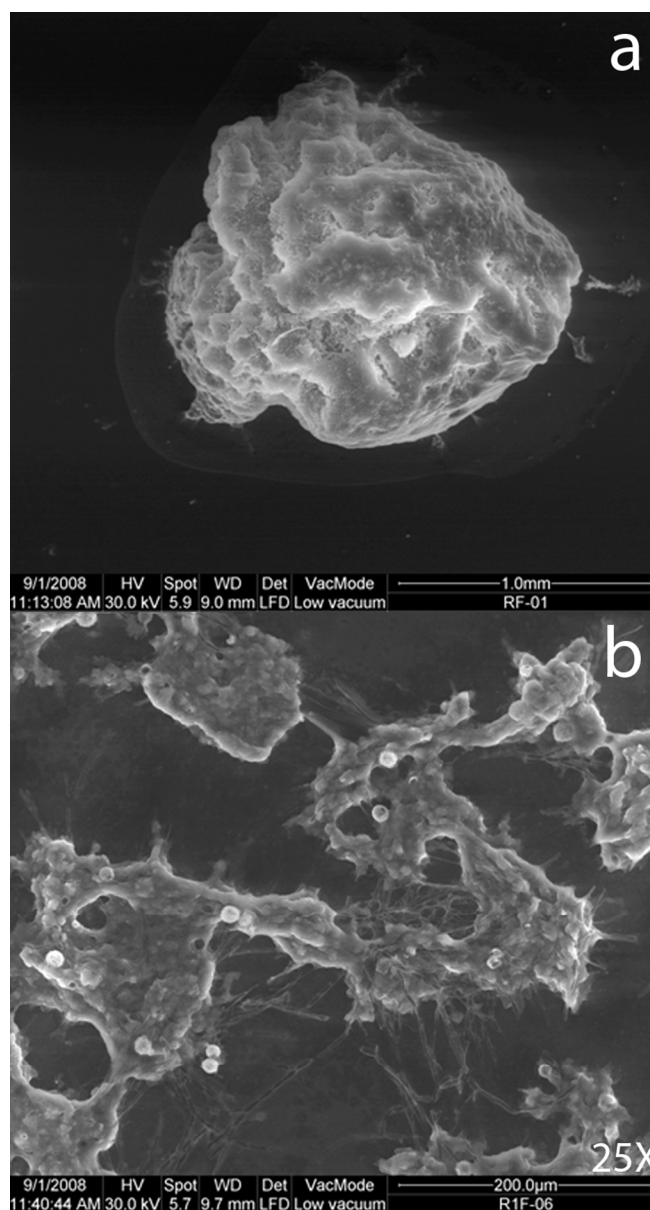


Figure 4. SEM micrographs of one aggregate of *P. putida* during the treatment of 4-CP in the SBR-*P. putida* (a) and bioaugmented activated sludge with *P. putida* (b).  
76x139mm (300 x 300 DPI)

TABLES

**Table 1.** Treatable 4-CP and phenol loading rates (mg g<sup>-1</sup> VSS d<sup>-1</sup>) in different SBR arrangements.

Reactor	Biomass	4-CP	Phenol
Cometabolic tests			
SBR-control	AS	20 - 60	20 - 120
SBR- <i>P. putida</i>	<i>P. putida</i>	20 - 60	120
SBR-bioaugmented	Biougmented AS	120	20 - 120
4-CP as sole carbon source			
SBR-control	AS	8 - 55	-
SBR-bioaugmented	Biougmented AS	8 - 120	-

**Table 2.** Fitting values of the parameters\* of Haldane model for 4-CP biodegradation as sole carbon source in control and bioaugmented SBR.

Control SBR					Bioaugmented SBR				
4-CP loading rates (mg g <sup>-1</sup> VSS d <sup>-1</sup> )	$V_{max}$ (mg 4-CP g <sup>-1</sup> VSS min <sup>-1</sup> )	$K_S$ (mg L <sup>-1</sup> )	$K_I$ (mg L <sup>-1</sup> )	$r^2$	4-CP loading rates (mg g <sup>-1</sup> VSS d <sup>-1</sup> )	$V_{max}$ (mg 4-CP g <sup>-1</sup> VSS min <sup>-1</sup> )	$K_S$ (mg L <sup>-1</sup> )	$K_I$ (mg L <sup>-1</sup> )	$r^2$
8	0.14±0.01	1.28±0.41	84.84±41.22	0.999	8	0.60±0.52	1.78±0.74	1.86±1.08	0.997
25	0.34±0.06	5.57±2.04	12.81±3.59	0.999	50	1.26±0.15	1.99±1.37	2.31±0.70	0.997
30	1.42±0.41	29.48±10.08	3.68±1.16	0.999	80	4.35±1.09	2.94±1.09	2.81±0.74	0.999
50	1.14±0.24	39.45±10.36	9.40±2.27	0.999	100	2.37±0.38	2.42±1.03	7.96±1.44	0.998
55	0.79±0.11	27.97±5.45	18.22±3.13	0.999	120	5.14±0.97	2.24±0.72	5.02±0.99	0.999

\* Individual confidence limits at the 95% probability level.