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Sabrine Ben Ouada, Rihab Ben Ali, Nicolas Cimetiere, Christophe  
Leboulanger, Hatem Ben Ouada, Sami Sayadi

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1 **Biodegradation of Diclofenac by two green microalgae: *Picocystis* sp. and**  
2 ***Graesiella* sp.**

3 Sabrina BEN OUADA\*<sup>123</sup>, Rihab BEN ALI<sup>2</sup>, Nicolas CIMETIERE<sup>4</sup>, Christophe  
4 LEBOULANGER<sup>3</sup>, Hatem BEN OUADA<sup>2</sup>, Sami SAYADI<sup>15</sup>

5 <sup>1</sup> Laboratory of Environmental Bioprocesses, Center of Biotechnology of Sfax, BP 1177, 3018 Sfax,  
6 Tunisia

7 <sup>2</sup> Laboratory of Blue Biotechnology and Aquatic Bioproducts, National Institute of Marine Sciences  
8 and Technologies, 5000 Monastir, Tunisia

9 <sup>3</sup> UMR 9190 MARBEC (IRD - Université de Montpellier – CNRS - IFREMER), CS30171, 34203  
10 Sète, France.

11 <sup>4</sup> Ecole Nationale Supérieure de Chimie de Rennes, CNRS, UMR 6226, 11 Allée de Beaulieu, CS  
12 50837, 35708 Rennes Cedex 7, France

13 <sup>5</sup> Center for Sustainable Development, College of Arts and Sciences, Qatar University, Doha 2713,  
14 Qatar

15 Corresponding author: Sabrina BEN OUADA

16 Center of Biotechnology of Sfax (CBS)  
17 Route Sidi Mansour km 6, B.P. 3038, Sfax, Tunisia  
18 Tel: +216-52907 460

19 [sabrinebenouada@gmail.com](mailto:sabrinebenouada@gmail.com)

## Abstract

The aim of the present study was to provide an integrated view of algal removal of diclofenac (DCF). Two isolated microalgal strains *Picocystis* sp. and *Graesiella* sp. were cultivated under different DCF concentrations and their growth, photosynthetic activity and diclofenac removal efficiency were monitored.

Results showed that DCF had slight inhibitory effects on the microalgal growth which did not exceed 21% for *Picocystis* and 36% for *Graesiella* after 5 days. Both species showed different patterns in terms of removal efficiency. In presence of *Picocystis* sp., the amounts of removed DCF were up to 73%, 43% and 25% of 25, 50 and 100 mg L<sup>-1</sup> respectively; whereas only 52%, 28% and 24% were removed in the presence of *Graesiella* at same DCF tested concentrations. DCF removal was insured mainly by biodegradation. To better reveal the mechanism involved, metabolites analyses were performed. Two DCF biodegradation/biotransformation products were detected in presence of *Picocystis*.

This study indicated that *Picocystis* performed a satisfactory growth capacity and DCF removal efficiency and thus could be used for treatment of DCF contaminated aqueous systems.

## Keywords

Microalgae; Extremophiles; Diclofenac; Removal; Biodegradation; Biotransformation.

## 1. Introduction

The presence of emerging contaminants as pharmaceutical and personal care products (PPCPs) in the aquatic environment continues to give rise to concern due to their environmental risk and toxicological properties. Indeed, these chemical compounds can cause multiple changes

in the physiological state of organisms, and their occurrence in the environment may affect non-target species (Valavanidis et al., 2014).

Among the most used PPCPs, Diclofenac (DCF) is a non-steroidal anti-inflammatory drug (NSAID) widely prescribed as an antipyretic analgesic. It is often found as a persistent toxic waste and one of the most widely available drugs in the world. Recent studies based on the Intercontinental Marketing Services (IMS) health data (which serves 82% of the global population) from 86 countries estimated that about  $1443 \pm 58$  tons of DCF are consumed globally on an annual basis (Acuña et al., 2015). However, this is only an indication of the DCF consumption for human health-related applications and does not include DCF's veterinary uses.

A large part of the consumed DCF is excreted in urine and feces in original form so entering municipal wastewater. DCF is ineffectively removed by conventional wastewater treatment plants (WWTPs) (Langenhoff et al., 2013; Sophia and Lima, 2018). Thus, it can be discharged into the environment with treated wastewater effluent, recycled water, and wastewater plant sludge. Actually, DCF is detected in several aquatic environments all over the world at concentration ranges from few hundreds to thousands of ng/L (Lonappan et al., 2016). It may be transported through food chains (Cuellar-Bermudez et al., 2017) causing toxic adverse effects on many aquatic organisms, even at environmentally low concentrations (Xu et al., 2019).

Therefore, it is mandatory to investigate alternative treatments for DCF removal from wastewaters. Among them, those based on the use of microalgae are emerging as a sustainable and economical solution (Escapa et al., 2017). Recent studies reported that many microalgae species could remove several pharmaceutical contaminants, including DCF (Xiong et al., 2018). Therefore, using microalgae to remove DCF from wastewater could be prospective.

To date, only very few studies have been carried out on the assessment of DCF removal using microalgae (Escapa et al., 2017; Villar-Navarro et al., 2019). Furthermore, the ability of microalgae to accumulate DCF as well as its biodegradation and/or biotransformation products were not yet investigated. Thus, information regarding DCF effects, removal and biotransformation products in the presence of microalgae are required.

Among microalgae, a particular interest is given to the use of extremophilic species in bioremediation systems (Varshney et al., 2014). Such organisms are assumed to have specific qualities allowing them to tolerate high concentration of several pollutants and further could be more efficient in the fast contaminant removal from wastewaters (Peeples, 2014).

The aim of the present work is to evaluate and compare, under laboratory culture conditions, the effects and the removal efficiency of DCF by two extremophilic microalgae strains *Picocystis* sp. and *Graesiella* sp. isolated from two polluted ponds in Tunisia for the purpose of selecting resistant species for their bioremediation use. To fulfill those purposes, the effect of DCF on growth and photosynthesis of both species was assessed together with their abilities to remove and accumulate DCF. Then, the resulting biotransformation and biodegradation products were addressed.

## **2. Materials and methods**

### **2.1 Chemicals and reagents**

Diclofenac sodium salt ( $\geq 98\%$ ) was purchased from Sigma Aldrich. Ultra-pure water (UPW) was delivered by Elga Pure Lab System (resistivity 18.2 M $\Omega$ .cm, COT <50  $\mu$ g C/L). Acetonitrile (ACN) with 0.1% formic acid (FA) was purchased from JT Baker (LC-MS grade) and used in association with UPW with 0.1% formic acid.

Salts and reagents for medium preparation suppliers are specified in Supplementary material 1. All chemicals used were of analytical grade.

## 2.2 Algal strains and culture conditions

Two extremophilic chlorophyta species were investigated in this study: an alkaliphilic *Picocystis* sp. CINS 23 and a thermophilic *Graesiella* sp. CINS 60. The strains have been deposited in the National Institute of Marine Sciences and Technologies Collection. *Picocystis* sp. was isolated from a household sewage “Essed valley” located in Center East of Tunisia (35° 59' 23" N, 10° 30' 10" E) and *Graesiella* sp. from a hot water catchment basin emerging from “Ain Echfa,” a hot spring located in northern Tunisia (36°49' N, 10°34' E). Preliminary laboratory experiments were conducted to define the optimal autotrophic growth conditions: culture media, temperature and light intensity (Ben Ali et al., 2017; Ben Ouada et al., 2018; Mezhoud et al., 2014). The strains were cultivated separately in batch culture under sterile conditions: *Graesiella* in Bold's Basal Medium (BBM) (Bischoff and Bold, 1963) modified according to the Elser concept for freshwater microalgae with C:N:P ratio equal to 166:20:1 (Elser et al., 2000), and *Picocystis* in Zarrouk medium (Zarrouk, 1966) (Supplementary material 1). The initial pH was adjusted to 6.8 and 8.4 for BBM and Zarrouk media, respectively. Cultures were maintained in optimal growth conditions at temperature of 30°C and light intensity of 75  $\mu\text{mol m}^{-2} \text{s}^{-1}$  under 8/16-h illumination cycle as established by preliminary laboratory experiments. Experimental cultures were conducted at these optimal growth conditions for all the designed experiences.

Exponentially growing cultures from *Picocystis* and *Graesiella* were separately inoculated in 500 mL Erlenmeyer flasks containing respectively 300 mL of Zarrouk and BBM media, with an initial cell density of  $10^6$  cells  $\text{mL}^{-1}$ . Cultures were exposed separately for 5 days to DCF at 0 (control), 25, 50, 100 and 200  $\text{mg L}^{-1}$  initial concentrations. For each experimental DCF concentration and separately for each tested strain, a series of six Erlenmeyer flask cultures were used in triplicate. Daily, 3 ml of each culture served for the determination of the optical density and the photosynthetic activity. The determination of the residual, adsorbed,

accumulated, and biodegraded DCF amounts of 25, 50 and 100 mg L<sup>-1</sup> initial tested concentrations, was carried out at the end of the experiment (day 5). Blank controls (without algae cells) served also for the determination of the DCF abiotic removal (Supplementary material 2).

The stock solution of diclofenac with the concentration of 1 g L<sup>-1</sup> was prepared by dissolving an appropriate amount of the compound in distilled water and it was stored below the temperature of 5 °C. The testing solutions were diluted to the desired concentrations during the experiments.

## 2.2 Growth inhibition test

Growth was followed spectrophotometrically in each culture condition by measuring the daily changes in OD<sub>680 nm</sub> during five days of DCF exposure.

Growth curves of microalgae population were obtained by plotting OD<sub>680 nm</sub> values and incubation time. The areas under each growth curve were evaluated using Origin 8.5 software (OriginLab, Northampton, USA). Inhibition of algal activity was expressed as the percentage inhibition (PI%) defined according to **De Orte et al. (2013)** by the following equation:

$$PI\% = \left[ \frac{\text{Control area} - \text{Treated area}}{\text{Control area}} \right] \times 100 \quad (\text{Eq. A1})$$

Where, Control area and Treated area are the integrated areas under the growth curve of control and treated culture, respectively.

## 2.3 Photosynthetic parameters

Chlorophyll fluorescence for each culture condition was measured every day using the Aquapen-CAP-C 100 fluorometer (Photon Systems Instruments, Brno, Czech Republic). A 3 mL sample was collected daily from each experimental culture and diluted with fresh medium

to a final optical density at 680 nm of 0.3. Before the measurement, each micro-algal sample was dark-adapted for 15 min at room temperature to allow the complete re-oxidation of PSII reaction centers. The chlorophyll fluorescence transients (OJIP) were induced by light pulses at a fixed excitation wavelength of 650 nm and intensity of 3000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and recorded for up to 1 s on a logarithmic time scale. The OJIP-test parameters were determined from transient analysis according to **Strasser et al. (2000)** and the maximal PSII photochemical efficiency ( $F_v/F_m$ ) was derived, wherein  $F_v = F_m - F_o$  is the variable fluorescence, and  $F_o$  and  $F_m$  are minimal and maximal fluorescence yields in dark-adapted state, respectively.

The non-photochemical quenching (NPQ) was measured according to the manufacturer's instructions (protocol NPQ1, Photon Systems Instrument). The intensities of actinic light (450 nm) and pulse-saturating light were 300 and 3000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , respectively. The NPQ was calculated as  $\left( \left( F_m - F'_m \right) / F'_m \right)$  (**Campbell et al., 1998**), wherein  $F'_m$  is the maximal fluorescence in a light-adapted state.

## **2.4 Determination of DCF removal and degradation intermediates**

After 5 days of DCF exposure, 150 mL of each algae culture were centrifuged at 10,000 $\times$ g for 10 minutes. The supernatant was used for the determination of the residual DCF in the medium. The cell pellets were washed three times with de-ionized water and then centrifuged. The supernatant was used for the determination of DCF adsorbed on the microalgae surface. The cell pellets were mixed with 2 mL of dichloromethane-methanol (1:2 v/v) and digested by sonication for 45 min (**Ji et al., 2014**). After centrifugation for 10 min at 4500 $\times$ g, the resulting supernatant was conserved to determine the accumulated DCF within the microalgae cells.



The same extraction procedure was applied for media without algae cells at all tested DCF concentrations exposed to the same culture conditions during five days to determine the abiotic removal of DCF.

Total DCF removal percentage was calculated according to the following equation:

$$\text{DCF removal (\%)} = \frac{(\text{initial DCF concentration} - \text{final DCF concentration})}{\text{initial DCF concentration}} \times 100 \quad (\text{Eq. A2})$$

Biotransformation/biodegradation percentage (Bp) of DCF by microalgae was calculated according to the following equation (Li et al., 2009):

$$\text{Bp(\%)} = (\text{CI} - \text{Ar} - \text{Aa} - \text{Ad} - \text{Ac}) \times 100 / \text{CI} \quad (\text{Eq. A3})$$

Where CI is the initial amount of DCF, Ar is the residual amount in the medium, Aa is the amount of abiotic removal, Ad is the amount adsorbed to the algal cells and Ac is the amount accumulated on algal cells. Specific removal of DCF (RS) was calculated according to the following equation:

$$\text{RS (mg g}^{-1}\text{)} = \frac{(\text{Ad} + \text{Ac} + \text{Bp})}{\text{dw}} \quad (\text{Eq. A4})$$

Where Ad is the DCF amount adsorbed to the algal cells, Ac is the amount accumulated on algal cells, Bp is the biodegraded amount and dw the dry weight of microalgae cells (g L<sup>-1</sup>).

## DCF Analysis

DCF analysis was carried out using a Waters (Aquity UPLC) liquid chromatographic system coupled to a mass spectrometer detector (Quattro Premier; Micromass) equipped with an electrospray ionization source. Chromatographic separation was performed on a BEH-C18 chromatographic column (100 mm × 2.1 mm ID; 1.7 μm). LC elution was performed with 100% acetonitrile as mobile phase A and an ultrapure water 9:1 acetonitrile (v/v) as mobile

phase B. The pH of the eluent was adjusted to 2 with 0.1 % formic acid. Gradient elution at a flow rate of 0.4 mL min<sup>-1</sup> consisted in 0% A (100% B) from 0 to 1 min, followed by a linear increase to 90% A (10% B) in 4 min and held for one minute; finally, from 6 to 7 min, mobile phase A decreased back to 0%. Nitrogen was used as the collision and nebulizing gas. The injection volume was 5 µL. The analysis was performed using ESI positive mode under the following conditions: capillary, 23 V; collision, 29 eV; column temperature, 45°C; sample temperature 5°C. The multiple-reaction monitoring (MRM) mode was applied for the DCF detection and quantification. The retention time is 4.47 min and MRM parameters: parent and daughter ions are 296.35 and 214.21 Da, respectively.

Complete calibration curves were performed at the beginning and at the end of the sample set, the mean slope value of these curves was used for DCF quantification. The limit of detection (LOD) and the limit of quantification (LOQ) were 0.180 and 0.547 mg L<sup>-1</sup>, respectively.

For the detection of DCF degradation intermediates, the full scan (FS) mode was performed, in a scan range of 60 to 500 m/z. Spectra were recorded with an average of 20-25 scans under identical experimental conditions. The reference full scan spectrum is presented in Fig. 1. The structural identity of each biodegradation product was performed with the LC-MS/MS fragmentation analysis. The analytical device was controlled by Micromass MassLynx 4.1 software.

## **2.5 Statistical analysis**

Mean and standard deviation values of the three biological replicates were calculated for each treatment and for the control. In all cases, data are given as mean value with standard deviation. Two-way ANOVA was used to analyze the effect of DCF tested concentrations and culture duration on growth and photosynthetic activities and one-way ANOVA was performed to determine the significant differences between removal efficiencies of studied

species among DCF tested concentrations, using IBM SPSS Statistic 21.0.0 software. A P-value  $< 0.05$  was considered statistically significant. When significant differences were observed, means were compared using the Post Hoc test for Two-way ANOVA analysis at a level of significance of 0.05 ( $P < 0.05$ ). For the DCF removal efficiencies, the significance of differences between the control and treated samples was analyzed by the one-way ANOVA analysis using the multiple-range Duncan test at the same level of significance ( $P < 0.05$ ).

### 3. Results

#### 3.1 Effect of DCF on microalgae growth

The effect of DCF concentrations on *Picocystis* sp. and *Graesiella* sp. growth during 5 days of exposure was evaluated by the growth inhibition percentage (PI%) (Table 1). Results showed that the DCF has slight effect on both species. The PI of *Picocystis* sp. and *Graesiella* sp. did not reach 40 % for both species during the whole period of exposure and even at the highest DCF tested concentration ( $200 \text{ mg L}^{-1}$ ). The maximum PI recorded was 21% for *Picocystis* and 36% for *Graesiella* implying that *Picocystis* was relatively more tolerant to DCF. Furthermore, a negative PI was observed when *Picocystis* was exposed to 25 and 50 mg/L DCF, which reflects a stimulation of the microalgae growth by 4 and 21 %, respectively.

#### 3.2 Effect of DCF on microalgae photosynthetic activity

The effect of DCF on the photochemical efficiency of photosystem II ( $F_v/F_m$ ) and the non-photochemical quenching (NPQ), an indicator of the excess-radiant energy dissipation to heat in PSII antenna complexes during light-adapted state, of *Picocystis* and *Graesiella* were determined during 5 days of DCF exposure at 25, 50, 100 and  $200 \text{ mg L}^{-1}$  initial DCF concentrations. Compared to the controls, the  $F_v/F_m$  ratios of *Picocystis* treated cultures were not inhibited during the first 3 days of DCF exposure (Fig. 2). A slight decrease was recorded

only at the end of the exposure duration (4th and 5th day) in *Picocystis* cultures exposed to high DCF concentrations (50 - 200 mg L<sup>-1</sup>). For *Graesiella*, no inhibitory effect on Fv/Fm ratios was recorded during the whole experiment period regardless the DCF tested concentrations. Contrariwise, an increase in the NPQ values of both species was observed when exposed to high DCF tested concentrations (50 - 200 mg L<sup>-1</sup>) as compared to the controls especially since the third day of DCF exposure (Fig. 3). After 5 days' exposure to 200 mg L<sup>-1</sup> DCF, the NPQ values of *Picocystis* and *Graesiella* treated cells were about two folds of the control groups.

### 3.3 DCF removal from the culture medium

The DCF removal by *Picocystis* and *Graesiella* was investigated at 25, 50 and 100 mg L<sup>-1</sup> DCF after 5 days of treatment (Table 2). Blank controls (without algae cells) were also investigated. Both species showed different patterns in terms of removal efficiency. In presence of *Picocystis* sp., the amounts of removed DCF were up to 73%, 42% and 25% of 25, 50 and 100 mg L<sup>-1</sup>, respectively; whereas only 52%, 28% and 24% were removed in the presence of *Graesiella* at the same DCF concentrations.

DCF removal in blank controls did not exceed 8% regardless of initial concentrations indicating that abiotic losses were negligible, and the observed DCF decrease was mainly due to the removal by microalgae.

The quantity of DCF adsorbed and accumulated within the microalgae cells did not exceed also 0.5% in all tested concentrations for both studied species (Table 2) that implies that the DCF removal by microalgae was insured mainly by biotransformation/biodegradation.

As illustrated in table 2, the DCF Biotransformation/biodegradation percentage (Bp) in the presence of *Picocystis* were about 69%, 36% and 21% of 25, 50 and 100 mg L<sup>-1</sup> DCF,

respectively. For *Graesiella*, the Bp were 44%, 21% and 18% of DCF at the same tested concentrations.

The specific removal (RS) of DCF by *Picocystis* was between 12.39 and 18.96 mg g<sup>-1</sup> for 25-100 mg L<sup>-1</sup> initial DCF concentrations. In *Graesiella*, the maximal RS was 12.23 mg g<sup>-1</sup> observed for 100 mg L<sup>-1</sup> initial DCF concentration.

### 3.4 Characterization of biodegradation products

DCF degradation products were separated by HPLC and characterized by on-line electrospray ionization–ion trap mass spectrometry (ESI–MS). In the presence of *Picocystis*, the comparison of chromatograms obtained (c) with and (a) without microalgae (Fig. 4) showed the appearance of two additional peaks at retention times of 1.21 and 3.82 min, respectively. The first one, labeled DP1 in Fig. 5a, yielded a molecular ion at m/z 312 Da and two additional ion fragments at m/z 266 and 230. The MS2 fragmentation spectrum of m/z 312 revealed a fragment ion at m/z 266 corresponding to the loss of one CO<sub>2</sub> molecule, and the MS3 fragmentation spectrum of m/z 266 showed a fragment ion at m/z 230 corresponding to the DCF dechlorination. The difference in mass between the molecular ion of the product DP1 (312 Da) and DCF (296 Da) was 16 Da, which might be related to the hydroxylation of the compound. The addition of an hydroxyl to the molecule supported the lower retention time observed for the compound by increasing the molecule's polarity (Rigobello et al., 2013). Although information provided by LC-MS instruments is not enough to establish the precise position of the OH groups in the molecule, knowledge of the reactivity of the different species and the cited literature allowed us to propose probable structure as seen in Fig. 5a.

The second peak, labeled DP2 in Fig. 5b, showed a molecular ion at m/z 152 Da and two additional ion fragments at m/z 134 and m/z 106. The MS2 fragmentation of m/z 152 revealed a fragment ion at m/z 134 corresponding to the dehydration by an intramolecular cyclization,

and the MS3 fragmentation spectrum of  $m/z$  134 showed a fragment ion at  $m/z$  106 corresponding to the loss of one CO molecule. The difference of 144 Da between diclofenac and DP2 is consistent with a loss of the dechlorinated benzene ring as proposed in Fig. 5b. In the presence of *Graesiella*, no difference was recorded between the EIS-MS chromatograms obtained with and without microalgae.

#### 4. Discussion

Microbial removal of pollutants relies on a trade-off between tolerance of the organisms to the toxicity of the targeted compounds, and their efficiency to effectively sequester or degrade them. To date only few studies on DCF toxicity to microalgae were reported, mostly on freshwater cyanobacteria and chlorophyta species (Supplementary material 3). The measured sensitivities to diclofenac spread from 84.5% growth inhibition in the freshwater cyanobacterium *Cylindrospermopsis raciborskii* exposed to 0.1 mg L<sup>-1</sup> (Bácsi et al., 2016) to 50% growth inhibition (EC50) in the marine chlorophyceae *Dunaliella tertiolecta* (DeLorenzo and Fleming, 2008) exposed to 185.7 mg L<sup>-1</sup> DCF. Comparatively, *Picocystis* and *Graesiella* were characterized in the present study by a high tolerance to DCF, since an exposure up to 200 mg L<sup>-1</sup> did not reach 40% inhibition for both strains. This behavior could at least partly attributed to the origin of the model organisms, considering that extremophile algae have already reported to be tolerant to chemical contamination (Ben Ali et al., 2017; Ben Ouada et al., 2018; Rehman and Shakoori, 2004). Furthermore, growth stimulation up to 21% was observed in *Picocystis* exposed to 50 mg L<sup>-1</sup> DCF, as reported for other chlorophyta strains exposed to 25 mg L<sup>-1</sup> DCF (Escapa et al., 2017). This behavior is consistent with potential use of DCF as organic carbon source, since chlorophyta species were shown able to photo-heterotrophic metabolism when culture medium was complemented by organic substrates (Ratha et al., 2013; Zili et al., 2015).

300 Toxic chemicals often target the photosynthetic apparatus in microalgae (**Choi et al., 2012**),  
301 but raw *Picocystis* and *Graesiella* PSII potentials (Fv/Fm) were not altered by DCF exposure  
302 in the present study. To date, no toxicity of DCF to PSII integrity was reported for  
303 microalgae, whereas higher plants were found sensitive (**Kummerová et al., 2016**).  
304 Comparatively, the non-photochemical quenching NPQ, an indicator of the excess-radiant  
305 energy dissipation during light-adapted state, increased for both species after DCF exposure.  
306 The same trend was reported in river biofilms and in plant species (**Kummerová et al., 2016**),  
307 and suggests an adaptive mechanism in *Picocystis* and *Graesiella* to protect PSII antenna and  
308 dissipate the excess of absorbed energy (**Müller et al., 2001**).  
309 The high tolerance of *Picocystis* and *Graesiella* to DCF could be related to their high DCF  
310 removal abilities. Among many studies about chemical remediation by microalgae, little  
311 attention was paid to NSAIDs including DCF. **Matamoros et al. (2015a; 2015b)**, **Xiong et al.**  
312 **(2018)** and **Zhou et al. (2014)** demonstrated that microalgal inoculation in wastewaters  
313 enhanced pharmaceutical removal efficiencies of treatment systems up to 80%. In laboratory  
314 monocultures, the amount of DCF removed by green microalgae were about 20 to 80 %, at  
315 DCF initial concentration  $\leq 25 \text{ mg L}^{-1}$  (**Escapa et al., 2017**). These values are comparable to  
316 DCF removal efficiencies of both *Graesiella* (52%) and *Picocystis* (73%) at  $25 \text{ mg L}^{-1}$  initial  
317 DCF concentration. Even at higher initial concentrations, reaching  $100 \text{ mg L}^{-1}$ , *Picocystis* and  
318 *Graesiella* were able to remove about 25-42% and 24-28%, respectively; after 5days of  
319 exposure, which demonstrated their high DCF removal abilities; even when compared to other  
320 biological treatments (**Langenhoff et al., 2013**).  
321 In the present work, a decrease of DCF initial concentrations was observed in the controls  
322 without algae suggesting abiotic degradation, most likely by photooxidation (**Michael et al.,**  
323 **2014**). However, abiotic loss of DCF did not exceed 8% at all tested concentrations, and

biotic removal is the most probable process in accordance with literature data (**Escapa et al., 2017**).

Biotic removal can be passive, by hydrophobic binding on microalgal cell walls or to the extracellular polysaccharides (**Xiong et al., 2018**), and active when accumulation and biotransformation/biodegradation occur within cells. In the present results, less than 1% of DCF was removed by adsorption and in equal amount by intracellular accumulation. Since EPS can be released into the medium (**Mezhoud et al., 2014**), it is possible to hypothesize that free EPS contributed significantly to DCF removal. Taking into account the method used in this study, the proportion of adsorbed DCF could be higher than that estimated. Nevertheless, **Bácsi et al. (2016)** demonstrated in several microalgae that DCF was not permanently bound to the membranes, and that no intracellular accumulation occurred, in accordance to the data presented here. As reduction of DCF concentration in the cultures cannot be balanced by adsorbed and/or accumulated DCF in the cells, these results suggest that biodegradation processes occurred in both *Picocystis* and *Graesiella* cultures. So far, little is known about microalgae that degrade diclofenac and the involved biodegradation pathways. In this study, two degradation products of DCF were detected in *Picocystis* cultures, a hydroxylated compound and a mono-aromatic product. Hydroxy diclofenac has already been reported as a major product of DCF metabolism in bacteria, fungi and microalgae, with a toxicity reduction compare to the parent product (**Domaradzka et al., 2015; Escapa et al., 2018**). The mono-aromatic derivative results from the loss of the benzene dechlorinated ring, as detected by **Cooper and Song (2012)** in advanced oxidation processes of DCF.

However, no DCF metabolites were detected in *Graesiella* cultures despite the high decrease in initial DCF concentrations observed. This can be either due to analytical issues (metabolites below detection limits, procedures not suitable for unforeseen compounds, ...) or kinetic factors, as the hypothesis of total mineralization cannot be excluded.



There is no evidence that the biodegradation is intracellular and known biodegradation processes involve the production of reactive oxygen species (ROS) in the medium as consequence of photosynthesis, and resulting photo-oxidation or hydroxylation of contaminants (Oral and Kantar, 2019). Extracellular enzymes are also identified in extracellular matrix of microalgae involved in chemicals hydrolysis (Otto et al., 2015). Indeed, EPS can form a hydrated biofilm matrix acting as an external digestive system where extracellular enzymes close to cells, allowing them to metabolize organic compounds (Flemming and Wingender, 2010; Xiong et al., 2018). In this context, DCF byproducts formed could be physically trapped or included in EPS matrix and escape analysis, which need further investigation.

## 5. Conclusion

Based on IC<sub>50</sub> values, *Picocystis* and *Graesiella* were, relatively, tolerant to DCF compared to other chlorophyta species as reported in the literature. The growth of both species was inhibited by less than 40% even at 200 mg L<sup>-1</sup> DCF. Interestingly, *Picocystis* and *Graesiella* exhibited high DCF removal efficiencies reaching 73% and 52% of 25 mg L<sup>-1</sup> as initial concentration, respectively.

The main proportion of DCF removal (69% and 44% of 25 mg L<sup>-1</sup> initial DCF concentration) was insured by biodegradation processes. Two biodegradation products of DCF were identified in the presence of *Picocystis*: hydroxy-diclofenac and a mono-aromatic derivative of DCF. In *Graesiella* culture, no metabolites were detected despite the high DCF biodegradation percentage, this may be associated either to low metabolites concentrations below the detection limit of the analytical method used or to the total mineralization of diclofenac. Moreover, the role of EPS in removal processes may be investigated. Further research on the biodegradation pathway and the toxicity assessment of the bioproducts still

required. The obtained results open up promising prospects for the application of the microalgae here considered, mainly *Picocystis*, in DCF bioremediation systems. The high tolerance of studied species allows their use in continuous flow culture systems particularly in the case of bioremediation of wastewaters, characterized by lower DCF concentrations. Therefore, we can concentrate or degrade the pollutant faster and with low culture volume. Although, confirmation of these results in pilot-scale at environmental conditions still needed.

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## Figures caption

**Fig. 1.** Full scan spectrum of the DCF reference solution (DCF in ultra-pure water) in a scan range of 60 to 500 m/z

**Fig. 2.** Variation in the PSII photochemical efficiency (Fv/Fm) of (a) *Picocystis* sp. and (b) *Graesiella* sp. during 5 days of DCF exposure. Data are represented as mean  $\pm$  SD (n=3). Same letters indicate no significant difference according to Post Hoc test ( $P \geq 0.05$ ) (uppercase letters for culture duration and lowercase letters for tested concentrations).

**Fig. 3.** Variation in the non-photochemical quenching (NPQ) of (a) *Picocystis* sp. and (b) *Graesiella* sp. under exposure to 0, 25, 50, 100 and 200 mg L<sup>-1</sup> DCF for 5 days. Data are represented as mean  $\pm$  SD (n=3). Same letters indicate no significant difference according to Post Hoc test ( $P \geq 0.05$ ) (uppercase letters for culture duration and lowercase letters for tested concentrations).

**Fig. 4.** LC-MS/MS ion chromatograms showing the degradation of 100 mg L<sup>-1</sup> initial DCF concentration by *Picocystis* sp.: (a) DCF without microalgae, (b) microalgae without DCF and (c) DCF in presence of *Picocystis* sp.

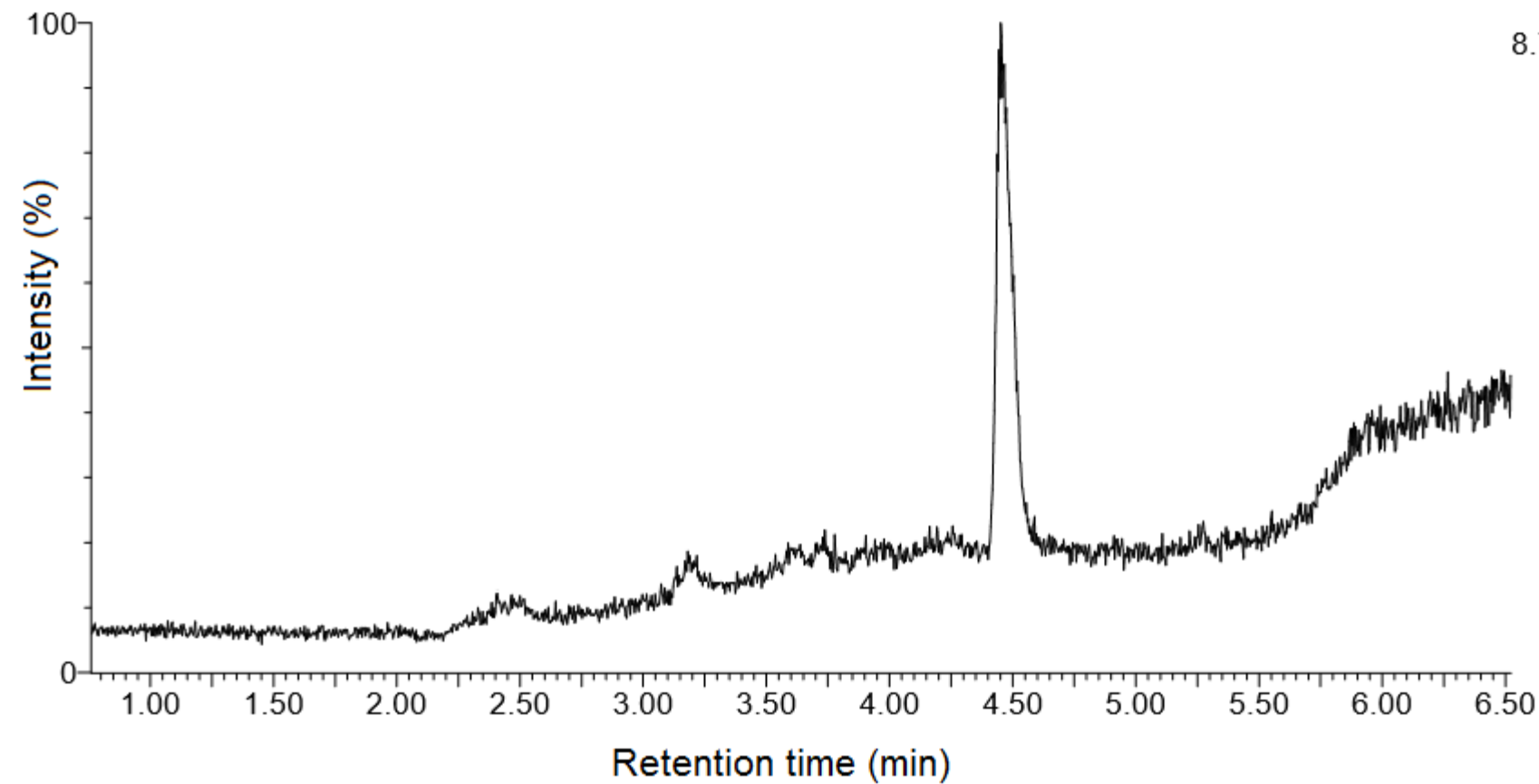
**Fig. 5.** ECI-MS2 spectra of DCF degradation products obtained in presence of *Picocystis* sp.: (a) DP1 and (b) DP2.

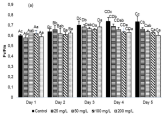
## Tables caption

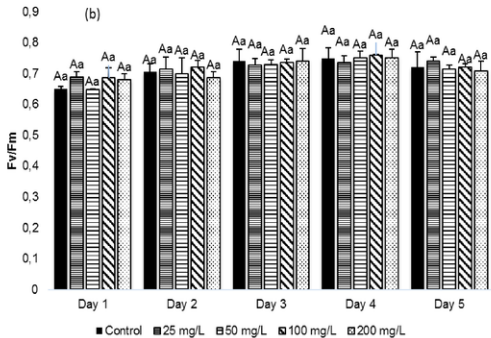
**Table 1.** Growth inhibition percentage of *Picocystis* sp. and *Graesiella* sp., calculated from the area under the growth curves, as a function of DCF concentrations after 1, 2, 3, 4 and 5 days of exposure. Data are represented as mean  $\pm$  SD (n=3). Same letters indicate no significant difference according to Post Hoc test ( $P \geq 0.05$ ) (uppercase letters for culture duration and lowercase letters for tested concentrations).

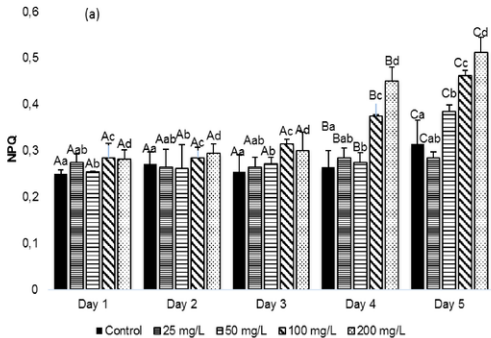
**Table 2.** Total (Tr) and abiotic removal (Ab), accumulated/adsorbed amount (Ac/Ad), biodegradation percentage (Bp) and specific removal (RS) of DCF by *Picocystis* sp. and *Graesiella* sp. cells exposed during five days to initial concentrations of 25, 50 and 100 mg L<sup>-1</sup> DCF. Data are represented as mean  $\pm$  SD (n=3). Different letters within the same column indicate significant difference according to Duncan test ( $P \geq 0.05$ ).

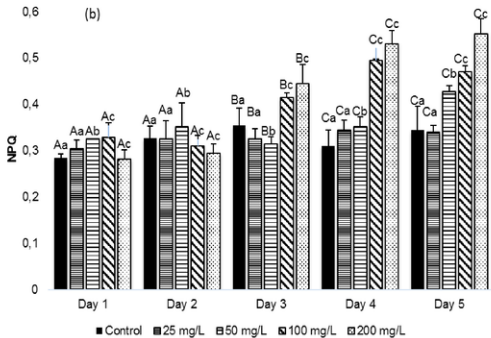
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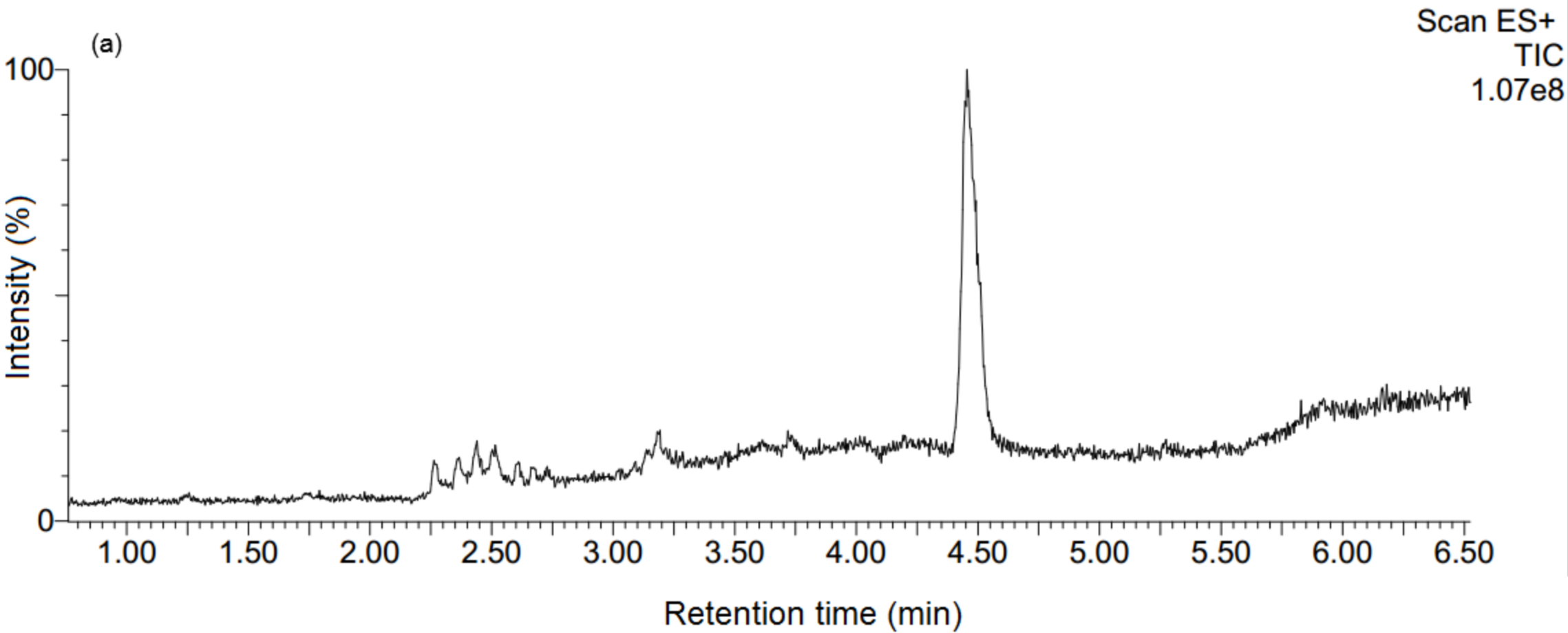




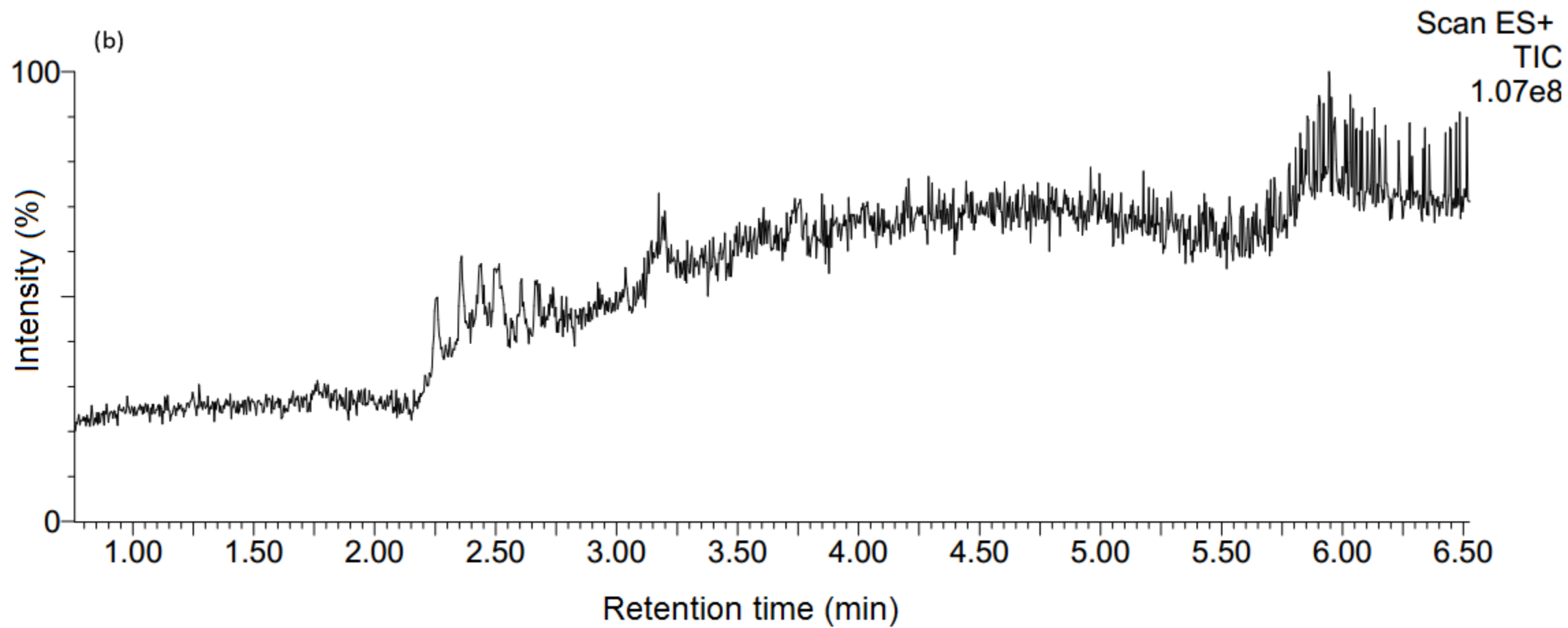


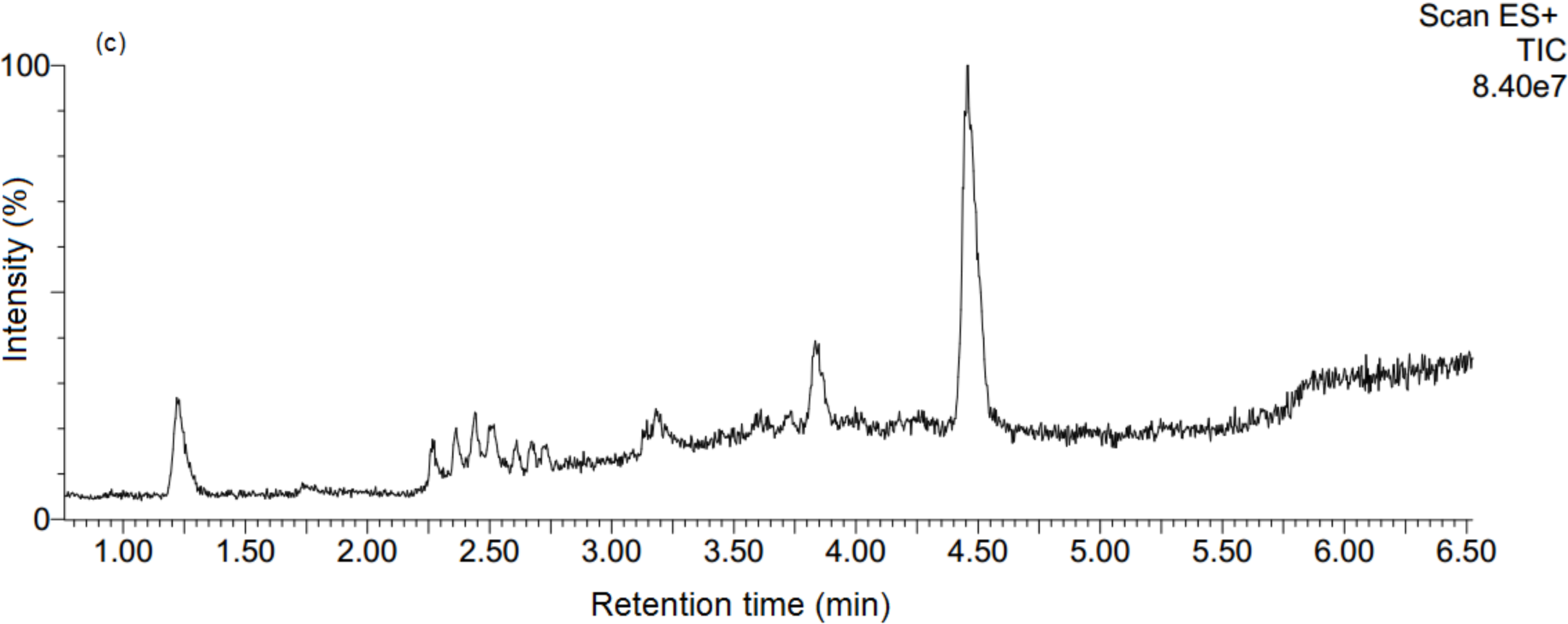


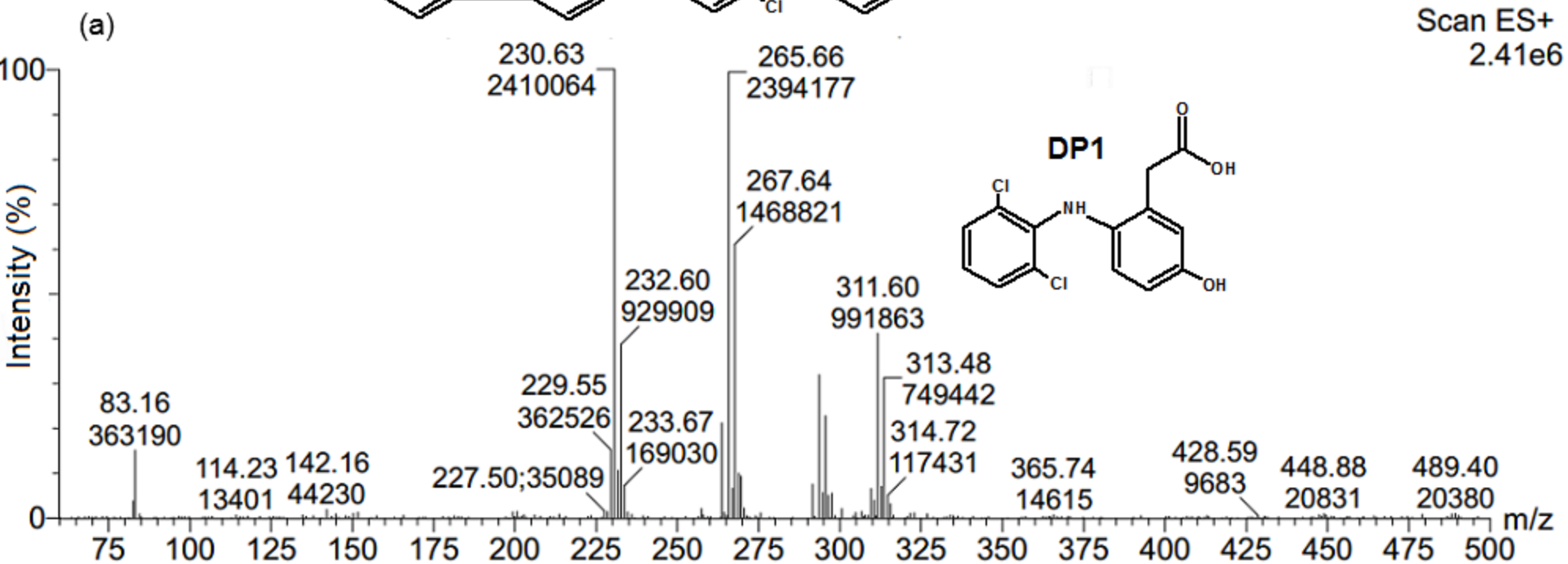




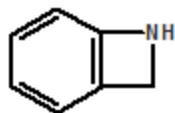
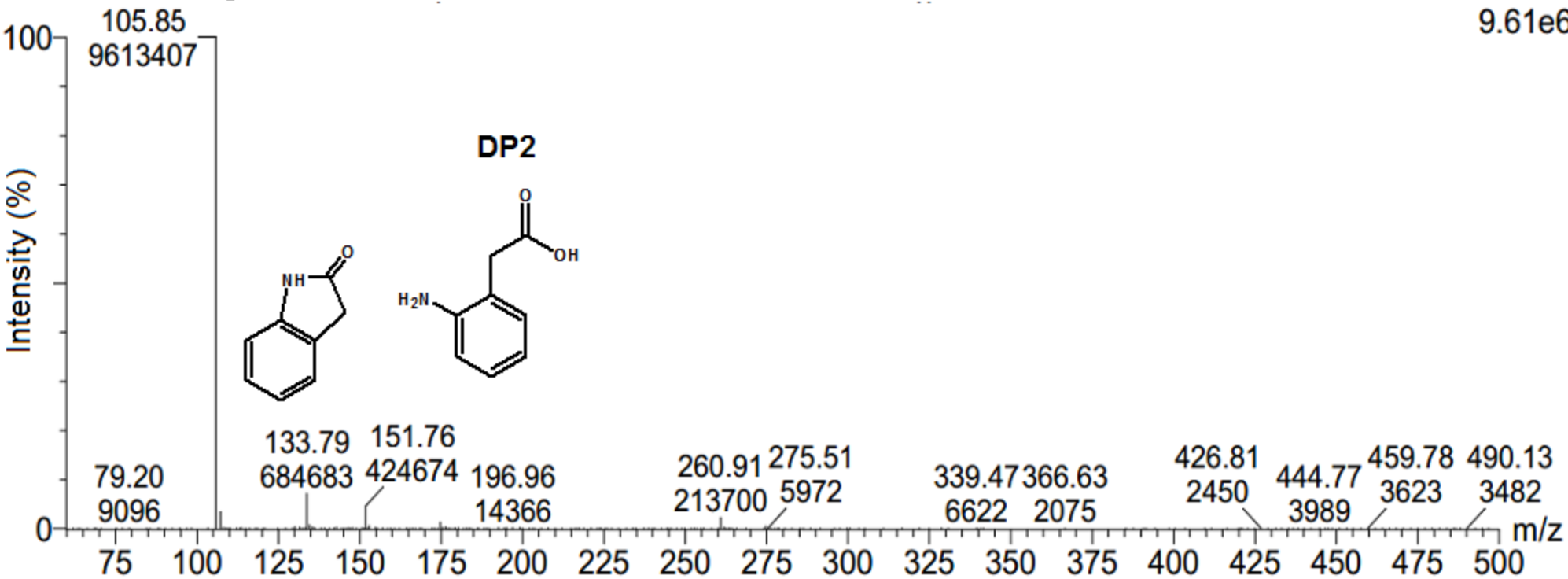






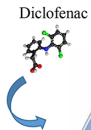


(b)

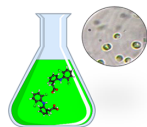
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Growth inhibition percentage (%)					
	Day 1	Day 2	Day 3	Day 4	Day 5
<i>Picocystis</i>					
<b>25 mg L<sup>-1</sup></b>	-4.37 ± 0.85Cb	-6.55 ± 0.91Ab	-8.54 ± 0.82ABb	-8.18 ± 0.62BCb	-14.27 ± 2.62Ab
<b>50 mg L<sup>-1</sup></b>	-6.32 ± 1.02Ca	-10.48 ± 1.32Aa	-12.95 ± 0.41Aba	-14.51 ± 1.03BCa	-21.36 ± 2.03Aa
<b>100 mg L<sup>-1</sup></b>	8.41 ± 3.24Cc	7.61 ± 1.69Ac	11.77 ± 0.61Abc	13.42 ± 1.06BCc	16.20 ± 1.06Ac
<b>200 mg L<sup>-1</sup></b>	12.67 ± 1.53Cd	11.05 ± 0.96Ad	14.24 ± 0.52Abd	16.68 ± 0.56BCd	20.56 ± 3.56Ad
<i>Graesiella</i>					
<b>25 mg L<sup>-1</sup></b>	7.10 ± 0.24Aa	7.65 ± 0.50Ba	11.41 ± 1.24Ca	13.67 ± 1.53Da	16.48 ± 1.38
<b>50 mg L<sup>-1</sup></b>	7.95 ± 0.90Ab	9.31 ± 1.76Bb	12.61 ± 1.69Cb	16.05 ± 0.96Db	19.27 ± 2.02
<b>100 mg L<sup>-1</sup></b>	9.30 ± 1.15Ac	12.76 ± 0.26Bc	16.77 ± 0.61Cc	20.24 ± 1.52Dc	27.48 ± 0.99
<b>200 mg L<sup>-1</sup></b>	16.93 ± 0.32Ad	20.74 ± 1.49Bd	26.42 ± 1.06Cd	28.68 ± 0.56Db	36.33 ± 2.18

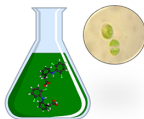
	<b>Tr (%)</b>	<b>Ab (%)</b>	<b>Ac/Ad (%)</b>	<b>Bp (%)</b>	<b>SR (mg g<sup>-1</sup>)</b>
<i>Picocystis</i>					
<b>25 mg L<sup>-1</sup></b>	73.04 ± 3.82a	3.83± 1.05a	0.04± 0.01a	69.17± 4.88a	13.22 ± 1.27a
<b>50 mg L<sup>-1</sup></b>	42.46 ± 2.08b	6.81± 2.22b	0.12± 0.02b	35.53± 4.32b	12.39 ± 1.66a
<b>100 mg L<sup>-1</sup></b>	24.81± 1.58c	4.28 ± 1.32ab	0.17± 0.04b	20.36± 2.94c	18.96±3.55a
<i>Graesiella</i>					
<b>25 mg L<sup>-1</sup></b>	52.21 ± 3.22a	7.53± 1.14a	0.21± 0.06a	44.47± 4.42a	6.65± 0.95a
<b>50 mg L<sup>-1</sup></b>	28.34± 1.17b	6.52± 1.27a	0.44± 0.04a	21.38± 2.48b	7.31± 1.19a
<b>100 mg L<sup>-1</sup></b>	23.5± 1.82c	5.15± 1.53a	0.31± 0.03a	18.04± 3.38b	12.23± 3.24b



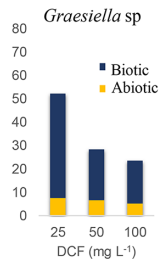
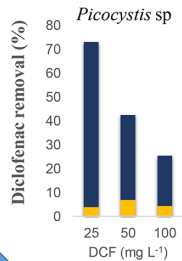
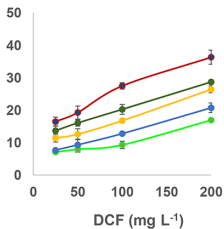
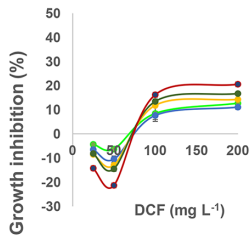
Abiotic control



*Picocystis sp.*



*Graesiella sp.*



Biodegradation products

