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Research Article

Biological Removal and Fate Assessment of Diclofenac Using *Bacillus subtilis* and *Brevibacillus laterosporus* Strains and Ecotoxicological Effects of Diclofenac and 4′-Hydroxy-diclofenac

Camille Grandclément,[1,2,3] Anne Piram,[1] Marie-Eléonore Petit,[4] Isabelle Seyssiecq,[2] Isabelle Laffont-Schwob,[4,5] Guillaume Vanot,[3] Nicolas Tiliacos,[3] Nicolas Roche,[6] and Pierre Doumenq[1]

[1]*Aix-Marseille University, CNRS, LCE, Marseille, France*
[2]*Aix-Marseille University, CNRS, Cent Marseille, M2P2, Marseille, France*
[3]*Société Prodibio SAS, Groupe Ovalee, Technopôle de Château-Gombert, Héliopolis, Marseille, France*
[4]*Aix-Marseille University, Univ Avignon, CNRS, IRD, IMBE, Marseille, France*
[5]*Aix-Marseille University, IRD, LPED, Marseille, France*
[6]*Aix-Marseille University, CNRS, IRD, INRAE, Coll France, CEREGE, Aix-en-Provence, France*

Correspondence should be addressed to Anne Piram; anne.piram@univ-amu.fr

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Since bacterial consortia involved in conventional wastewater treatment processes are not efficient in removing diclofenac (DCF), an emerging pollutant frequently detected in water bodies, the identification of microorganisms able to metabolise this pharmaceutical compound is relevant. Thus, DCF removal was investigated using bacteria isolated from aqueous stock solutions of this micropollutant and identified as *Bacillus* and *Brevibacillus* species using 16S rRNA gene sequencing. A 100% DCF removal was achieved after 17 hours of experiment at 20°C in a nutrient medium; the biodegradation kinetic followed a pseudo-first order ($k_{biol} = 11\ L{\cdot}g_{SS}^{-1}{\cdot}d^{-1}$). Quantitative assessment of DCF removal showed that its main route was biotic degradation. The main degradation product of DCF, 4′-hydroxy-diclofenac (4′-OH-DCF), was identified using liquid chromatography-electrospray ionisation high-resolution mass spectrometry. Since the ecotoxicological impact of 4′-hydroxy-diclofenac was not reported in the literature, the ecotoxicity of DCF and its metabolite were tentatively evaluated using *Vibrio fischeri* bioassays. Results from these tests showed that this metabolite is not more toxic than its parent compound and may hopefully be an intermediate product in the DCF transformation. Indeed, no significant difference in ecotoxicity was observed after 30 min between DCF (50 should be writtten in subscript all along the manuscript in $EC_{50} = 23 \pm 4\ mg{\cdot}L^{-1}$) and 4′-hydroxy-diclofenac ($EC_{50} = 19 \pm 2\ mg{\cdot}L^{-1}$). Besides, the study highlighted a limit of the Microtox® bioassay, which is largely used to assess ecotoxicity. The bioluminescence of *Vibrio fischeri* was impacted due to the production of microbial activity and the occurrence of some carbon source in the studied medium.

1. Introduction

The widespread occurrence of organic micropollutants has become a growing concern during the past few years because of their potentially adverse impacts on aqueous ecosystems and human health. Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) (DCF) is a nonsteroidal anti-inflammatory prescription drug (NSAID) used as analgesic, antiarthritic, and antirheumatic. Based on International Marketing Services health data from 86 countries, it has been estimated that an average of 1443 ± 58 tons of DCF are annually consumed: 39.5% in Asia and 28.7% in Europe [1]. The high consumption level of this compound leads to an increasing probability to find it as a

persistent micropollutant into the environment. Indeed, due to its extensive use and low removal by sewage treatment plants [2], DCF is one of the most frequently detected pharmaceuticals in effluents, at various concentration levels. DCF has not only been found in groundwater in the concentration range of hundreds of $ng{\cdot}L^{-1}$ [3] and in marine waters in the range of $\mu g{\cdot}L^{-1}$ [4] but also in drinking waters in the range of a few $ng{\cdot}L^{-1}$ [5]. According to literature data, the maximal measured concentrations of DCF in municipal wastewater range between 0.44 and 7.10 $\mu g{\cdot}L^{-1}$, and the mean concentrations are between 0.11 and 2.30 $\mu g{\cdot}L^{-1}$ [6]. Known to cause severe damage to various environmental species at concentration levels as low as 1 $\mu g{\cdot}L^{-1}$, DCF is one of the three pharmaceuticals included in the first "watch list" of the European Directive 2013/39/EU (EU 2013), requiring its environmental monitoring in the member states. According to this proposed Evaluation of Quality Standards document, the maximum allowable concentrations are of 0.10 $\mu g{\cdot}L^{-1}$ in fresh waters and 0.01 $\mu g{\cdot}L^{-1}$ in marine waters.

According to Nguyen et al. [7], biodegradation and adsorption were the main removal pathways of DCF in activated sludge, and biodegradation (less than 50% in the study) occurred via cometabolic degradation rather than direct metabolism. An efficient biodegradation path for DCF could lead to the development of low-cost biological removal processes. However, using conventional processes such as activated sludge (AS), membrane bioreactors (MBRs), or sequencing batch reactors (SBRs), DCF is only moderately biodegradable with an average removal rate of 20 to 40% [8]. Using these processes, DCF biodegradation rate is very low, which can be explained by its chemical structure and its properties. Indeed, Tadkaew et al. [9] brought into focus the relationship between some molecular features, such as the occurrence of electron-withdrawing or electron-donating functional groups and the removal of trace organic contaminants by a laboratory-scale MBR system. In their work, the removal efficiencies of all hydrophilic and moderately hydrophobic (Log $D < 3.2$) micropollutants possessing strong electron-withdrawing functional groups were very low (below 20%). Consequently, DCF, which is a chlorinated compound containing amines (strong electron donor) and carboxylic functional groups (strong electron acceptor), and has a log $D < 3.2$ at pH = 8.0, also showed a poor removal rate (17%) [9]. Furthermore, there are still gaps in the literature devoted to the biodegradation process of DCF. The main microorganisms used in biotransformation of micropollutants are fungi. For instance, *P. chrysosporium* is well known for its biosorption ability regarding xenobiotics and heavy metals, but its use in wastewater treatment processes is limited. That is why recent studies investigated the removal of environmental contaminants by *P. chrysosporium*, combined with another process such as silver nanoparticles [10], or a polyvinyl alcohol support material [11]. White rot fungi such as *Bjerkandera* sp. R1, *Bjerkandera adusta*, *Trametes versicolor*, *Phanerochaete sordida*, and *Phanerochaete chrysosporium* were identified as potential microorganisms responsible for the degradation of DCF [12–14]. More recently, an *Ascomycota* fungus, *Penicillium oxalicum*, was used to biodegrade DCF at flask and bench bioreactor scales [15]. In these studies, the hydroxylation of DCF was catalysed by cytochrome P-450 monooxygenases [16]. However, much less is known about the biodegradation of DCF by bacteria. Research on bacterial biodegradation of micropollutants has intensified in the last decade, but the bacterial strains responsible for the DCF degradation were not always mentioned. For instance, 75% of DCF, initially added at 300 $mg{\cdot}L^{-1}$, was degraded after 3 weeks by an enriched bacterial culture, but the microorganisms responsible for the DCF removal were not identified [17]. Thus, identified bacterial strains isolated from the appropriate environment, and with the ability to biologically remove DCF, could be used to seed bioreactors and enhance the removal of this drug. To the best of our knowledge, the degradation of DCF by *Bacillus subtilis* and *Brevibacillus laterosporus* strains was not monitored. Few authors have studied the degradation of micropollutants by *Bacillus subtilis*. For instance, *Bacillus subtilis*, isolated from column reactors, degrades sulfamethoxazole and trimethoprim into NH_4^+, and then into NO_3^- in a continuous process [18]. After a four-day incubation period at 30°C, *B. subtilis* was able to transform approximately 40% of pyrene and 50% of benzo[a]pyrene, initially added at 20 $\mu g{\cdot}mL^{-1}$ [19].

DCF is one of the most relevant compounds regarding ecotoxicity and environmental persistence. Most of the studies on the toxic effects of DCF have been focused on its adverse effects on aquatic animals [20]. The toxicity of DCF in freshwater environment has been studied in laboratories with the help of a standard organism for toxicological studies ([21–23]). At acute concentrations ($mg{\cdot}L^{-1}$), DCF induced high mortality rates in *Daphnia magna* sp. after 48 h of exposure. The EC_{50} values were reported to be 22.4 $mg{\cdot}L^{-1}$ and 39.9 $mg{\cdot}L^{-1}$ ([21, 24]). Cleuvers [25] investigated the toxicity of a mixture of pharmaceutical compounds using acute *Daphnia* and algal tests. He revealed that DCF was potentially harmful to aquatic organisms. Regarding the *Vibrio fischeri* assay, studies concluded that DCF has a relatively acute toxicity on the tested bacteria. However, studies regarding the toxicity of DCF metabolites are scarce.

Besides, since the toxicity of transformation products was not evaluated in previous studies, and because some of them may potentially be more toxic than the parent compound itself, it seems necessary to assess their toxicity in order to draw objective conclusions regarding the removal efficiency of the studied process.

Considering all the facts mentioned above regarding the removal of DCF, it seems necessary to add an efficient wastewater treatment process to classical wastewater treatment plants (WWTP), to avoid releasing such a compound into the environment. The aim of the present study was to select microorganisms able to biologically remove DCF. After a first screening to select promising strains, the reaction kinetic of the selected microorganism consortium was monitored in order to evaluate degradation time scales, with the aim of further implementing these strains in a bioreactor. In a second part, the degradation products were tentatively identified by LC-MS/MS, and a degradation pathway for DCF was proposed. Finally, since the Microtox® test needs a small test sample, and is a standard easily comparable to

literature data, this method was used to assess the potential changes in ecotoxicity during the degradation process. To the best of our knowledge, this work is the first report giving an ecotoxicological value for the identified main degradation product: 4′-hydroxy-diclofenac (4′-OH-DCF). However, there seems to be a high variability between the values found in the literature for the ecotoxicological effects of DCF, and this work questions the use of a test for acute ecotoxicity.

2. Materials and Methods

2.1. Chemicals. Diclofenac was purchased from the Cayman Chemical Company, with a purity of 99%. Stock solutions of DCF were prepared at a concentration of 50 mg·L^{-1} in deionised water (Milli-Q), stored at 4°C, and used within one month. The following chemicals were used in the analyses for quantification of the molecules: CH_3CN (hipersolv chromanorm, VWR Chemicals), CH_3OH (Emsure®, Merck), CH_3CO_2H (100%, v/v) reagent grade (VWR Chemicals), and ultrapure water (Direct-Q 5UV, Milipore).

Glucose, K_2HPO_4, $MgSO_4$, and glycerol, also used for culture media, were purchased from Sigma. All other chemicals, such as tryptone/peptone from casein, casein hydrolysate standard, yeast extract, and agar used for the preparation of culture media were of the highest available purity and were purchased from Roth.

2.2. Media and Culture Conditions. The bacteria were cultivated in an appropriate liquid nutrient medium. The nutrient media for *Bacillus* spp. (pH 7.2 ± 0.2) was used for bacillus communities, whereas the nutrient media for *Pseudomonas* spp. (pH 7.0 ± 0.2) was used for *Pseudomonas* communities [26]. The pH was adjusted if needed. All media were sterilised by autoclaving at 121°C for 20 min, according to the standard NF EN 14885. The strains were incubated at 20°C for 3 days under shaken conditions (125 rpm).

2.3. Microorganisms. Commercial strain consortia BHB, OBXL, CB2, G1B, OMP, and *Pseudomonas putida* were kindly provided by the Prodibio company (Marseille, France).

Strains DCF1, DCF2, and DIU2 were isolated from aqueous stock solutions, including a DCF stock solution at 50 mg·L^{-1}. Serial 10-fold dilutions were dispensed on agar medium, and the plates were aerobically incubated at 30°C for 24 hours. Different morphologies of colonies were chosen and isolated by repeated streak culturing. The selected strains were identified by the MIO laboratory (Mediterranean Institute of Oceanography, UMR 7294), using Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE). The partial 16s sequence of DCF1, DCF2, and DIU2 showed the highest similarity with *Brevibacillus laterosporus* strain BC2, *Brevibacillus laterosporus* strain TK3, and *Bacillus subtilis* strain mammoth-10, respectively. Strain DCF2 (*Brevibacillus laterosporus* strain TK3) was deposited in the BCCM/LMG Bacteria Collection (Belgium) under accession number LMG S-30273 (DCF2).

2.4. Experimental Procedures

2.4.1. Screening Test. Different strains kindly provided by the Prodibio company from their water-treating activity were tested individually in order to evaluate their efficiency to remove DCF. Degradation experiments were carried out in 100 mL Erlenmeyer flasks containing 10 mL of the studied strains in a total volume of 50 mL of an appropriate nutrient medium following Atlas' [26] recommendations. Degradation experiments included a stability control containing 1.0 mg·L^{-1} of DCF in the defined medium (pH = 7.0) and uninoculated controls containing 35 mL of the defined medium (pH = 7.0). Uninoculated controls, as well as abiotic controls in which life forms were deactivated using 1.0 g·L^{-1} of sodium azide (Sigma) added to the medium, were also performed under identical conditions in order to assess contamination or sorption effects, respectively. DCF was added into the flasks to give the desired final concentration (approximately 1.0 mg·L^{-1}, concentration chosen following the performance of analytical detection) from a stock solution in ultrapure water. After target molecule addition, flasks were incubated under shaken conditions (at 125 rpm) at 20°C.

After 3 days of experiment, 2.0 mL samples were withdrawn and were filtered through a regenerated cellulose 0.2 μm filter. Then, 0.75 mL of acetonitrile and 0.48 g of ammonium sulphate were added to 0.75 mL of each sample, under magnetic stirring. Finally, solutions were centrifuged at 4°C, 18,900g for 15 min, and supernatants were filtered at 0.2 μm on PTFE filters. Extraction efficiency of DCF, evaluated by recovery experiments, was 90%.

Then, the concentration of the target molecule was analysed by HPLC analysis. Degradation after 3 days of experiment was calculated by comparing target molecule concentration in the stability controls with target molecule concentration in the experimental flasks (see equation (1)). All the biodegradation values were corrected for the sorption values determined in sodium azide control flasks. Degradation due to abiotic pathway is described in equation (2), given as follows:

$$\text{biotic degradation}\,(\%) = \left(1 - \frac{\left[X_{\text{abiotic control}}\right]_t - \left[X_{\text{sample}}\right]_t}{\left[X_{\text{stability control}}\right]_t}\right) \times 100, \quad (1)$$

$$\text{abiotic degradation}\,(\%) = \left(1 - \frac{\left[X_{\text{abiotic control}}\right]_t}{\left[X_{\text{stability control}}\right]_t}\right) \times 100. \quad (2)$$

2.4.2. Batch Test Protocol Using Isolated Strains. The efficiency of isolated strains was evaluated individually, as well as a bacterial consortium. For the bacterial consortium, 10 mL of each selected strain was added in 20 mL of liquid nutrient medium for *Bacillus* sp. and the mix of isolated

strains was incubated at 20°C for 3 days in shaken conditions (at 125 rpm).

Degradation experiments of DCF, initially added at 1.0 mg·L^{-1}, were performed in 100 mL Erlenmeyer flasks containing 10 mL of isolated strains or the bacterial consortium in a total volume of 50 mL of a chemically defined medium, which contained 1.0 g of glucose, 6.0 g of tryptone/peptone from casein, 3.0 g of casein hydrolysate standard, and 3.0 g of yeast extract per litre. Degradation experiments including stability, uninoculated, and abiotic controls as described in Section 2.4.1 were incubated under shaken conditions (at 125 rpm) at 20°C.

After 3 days of experiment, the extraction and analytical procedures described in Section 2.4.1 were applied, and DCF biotic and abiotic degradations were calculated, thanks to the equations (1) and (2).

2.4.3. Kinetic Tests. Kinetic experiments were performed in 100 mL Erlenmeyer flasks containing 10 mL of a mix of isolated strains in a total volume of 50 mL of a chemically defined medium. The same nutrient medium was used as previously described. As for the batch test protocol, kinetic experiments including uninoculated controls, as well as controls, killed using sodium azide performed under identical conditions. DCF was added into the flasks to give the desired final concentration (1.0 mg·L^{-1}) from a stock solution in deionised water. After DCF addition, flasks were incubated under shaken conditions (at 125 rpm) at 20°C.

A 2.0-mL sample was taken once an hour and was filtered through a regenerated cellulose 0.2 μm filter. The target compound in the liquid nutrient medium was extracted by SPE with Oasis hydrophilic-lipophilic balance (HLB) cartridges (1 cc; 30 mg adsorbents, 30 μm particle size, Waters), after filtration through a 0.2 μm regenerated cellulose filter. 1.0 mL of each sample was acidified with 0.1% of acetic acid and spiked with an internal standard, naproxen, at 1.0 mg·L^{-1}. The SPE cartridges were installed on a vacuum manifold and preconditioned with 1.0 mL of acetonitrile, and then 1.0 mL of 0.1% of acetic acid in water (Milli-Q). 1.0 mL of the sample was loaded under vacuum at a flow rate of 300 to 400 μL·min^{-1}. Then, the Oasis HLB cartridges were washed with 1.0 mL of a solution containing 20% of acetonitrile and 80% of 0.1% of acetic acid in Milli-Q water, and dried under vacuum for 30 minutes. Finally, elution was performed with 1.0 mL of a solution containing 80% of acetonitrile and 20% of 0.1% of acetic acid in Milli-Q water at a flow rate of 200 to 300 μL·min^{-1}. The final extract was spiked with an internal standard, gemfibrozil, added up to a concentration of 5.0 mg·L^{-1}. Concentration of target molecule was analysed by LC-MS, and degradation was calculated as described previously.

2.5. Analytical Procedures

2.5.1. HPLC Analysis. Analysis of DCF was performed using a PerkinElmer Flexar FX10 UHPLC equipped with a Flexar PDA (Photodiode Array Detector) Plus Detector, a Flexar FX-10 UHPLC (ultrahigh-performance liquid chromatography) pump, a Flexar Column Oven, a Flexar FX UHPLC Autosampler Cool only, and a Flexar Fluorescence Detector. The column was disposed in a column oven, temperature was maintained at 35°C, and a sample volume of 10 μL was injected. The chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (150 mm × 2.1 mm, particle size 3.5 μm) equipped with a guard column Zorbax Eclipse Plus C18 (50 mm × 2.1 mm, particle size 5.0 μm). The mobile phase consisted of acetonitrile : methanol (1 : 1, v/v) (solvent A) and 0.1% of acetic acid in Milli-Q water (v/v) (solvent B). It was delivered at 0.3 mL·min^{-1}. DCF detection was performed at 276 nm, the maximum wavelength obtained with a UV spectrum of DCF. The extraction recovery of DCF in UV-detection of quality control samples was up to 99%. The calibration curve was linear within the concentration range of 0.1–20 mg·L^{-1}, with the correlation coefficient (R^2) above 0.9999. The method was specific and sensitive with a quantification limit of 0.1 mg·L^{-1}.

2.5.2. LC/ESI-MS Analysis. LC/ESI-MS analyses were conducted using an Agilent 1290 Infinity system coupled to an Agilent 6530 Q-TOF tandem mass spectrometer equipped with an Agilent jet stream (AJS) electrospray (ESI) ion source. MassHunter Workstation software B4.00 was used for instrument control, data analysis, and processing.

10 μL of the sample was injected after filtration on a 0.2-μm cellulosic filter, of 15 mm inner diameter, provided by Agilent Technologies. Separation was performed using a Zorbax Eclipse Plus C_{18} column (150 mm × 2.1 mm, particle size 3.5 μm) equipped with a guard column Zorbax Eclipse Plus C_{18} (50 mm × 2.1 mm, particle size 5.0 μm) supplied by Agilent Technologies. The column was kept at 35°C in the column oven. The mobile phase was composed of water acidified with 0.1% acetic acid (solvent A) and acetonitrile (solvent B) at 0.3 mL·min^{-1}. To ensure a better separation, a gradient program was used: 0–2.7 min 80% A, 2.7–4.7 min 38% A, 4.7–9.7 min 15% A, 9.7–9.8 min 80% A, and 9.8–11 min 80% A.

Mass calibration was carried out following the method developed by Meribout et al. [27]. Source parameters were defined as follows: fragmentor (130 V), capillary (2500 V), skimmer (65 V), and nitrogen was used as the drying agent (350°C, 10 L·min^{-1}), nebuliser (30 psi), and sheath gas (350°C, 8 L·min^{-1}). Scanning was realised from m/z 100 to 1000 amu with 10 000 transients per spectrum. Analytes were ionised by ESI in positive ion mode inducing to the formation of the $[M + H]^+$ ions of several products.

2.5.3. Identification of Degradation Products by LC-MS/MS QqToF. Screening of transformation products was carried out following the recommendations of Meribout [27]. Specific collision energies of 10, 20, and 40 eV were specified, and C, H, N, O, S, and Cl were selected as possible elements present.

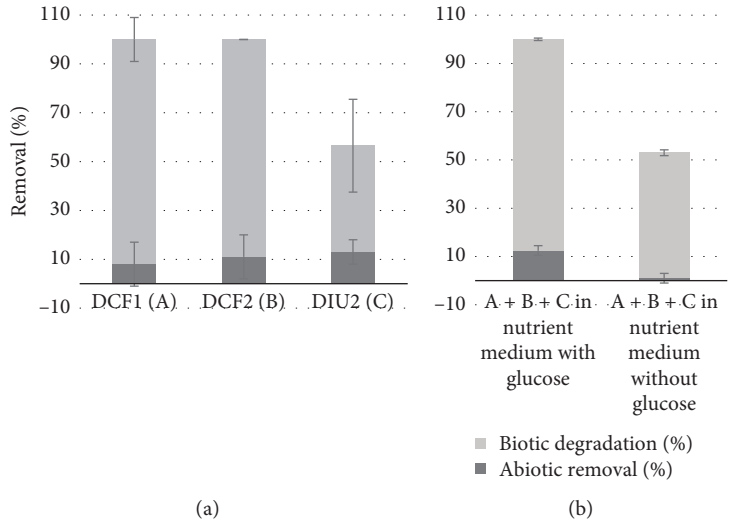


Figure 1: Removal of diclofenac using DCF1, DCF2, and DIU2 (A, B, and C, respectively), and conditions at 20°C after 3 days of degradation. The experiments were performed in triplicate in nutrient medium with or without glucose. Values are means ± standard error of the mean (SEM) ($n = 3$).

2.6. Vibrio fischeri Bioluminescence Test (Microtox® Test). Ecotoxicity of the samples was evaluated through an in vitro bioassay, the Microtox® toxicity test. This test is based on the evaluation of the bioluminescence inhibition of the marine bacteria *Vibrio fischeri*. In attendance of contaminants, the natural bioluminescence of *Vibrio fischeri* is reduced. The inhibitory effect of water samples on the light emission of *Vibrio fischeri* (NRRL B-11177) was evaluated following the recommendations of the standard ISO 11348–3: 2007. Microtox® toxicity test is generally performed with the standard Acute Toxicity Test Basic 81.9% protocol, using a Microtox® M500 analyser (R-Biopharm, France). Reagent well was maintained at 5.5 ± 1°C, and both the incubator part and the read wells were maintained at 15 ± 1°C. All the materials for analysis, such as freeze-dried *Vibrio fischeri* cells, diluents, osmotic adjusting solution, and reconstitution solution, were distributed by R-Biopharm.

Samples were collected and centrifuged at 4,000g for 10 min at 5°C. Then, the resulting supernatants were filtered on a 0.2 μm regenerated cellulose membrane to remove turbidity, avoiding interferences with particulate matter. Regarding the pH of the samples, this parameter should be between 6.0 and 8.0. Since pH of tested samples was globally above 8.0, this parameter was adjusted using few drops of 1 N HCl solution. To induce a suitable osmotic pressure (above 2%), Osmotic Adjusting Solution (a nontoxic 22% sodium chloride solution) was added to each sample. Then, a dilution series of each sample was prepared in sodium chloride diluent solution (2%). Depending on the expected EC_{50} values, various concentration intervals of the target compounds were selected. Bioluminescence was measured after 5, 15, and 30 min of exposure, and compared with the measured value of a bacterial control solution, which contains a diluent without the tested compound. Each sample was tested in triplicate.

2.7. Statistical Analysis. Prior to statistical analyses, normality and homoscedasticity of data were tested and did not enable the use of parametric tests. Thus, EC_{50} was compared using Wilcoxon sum-rank test at $\alpha = 0.05$, followed by the Kruskal–Wallis post hoc test when necessary. Data were in triplicates, and statistical analyses were performed using the JMP Pro 12 software.

3. Results and Discussion

3.1. Removal Efficiency of the Studied Strains. BHB, OBXL, CB2, G1B, OMP, and *Pseudomonas putida* used in the screening tests did not induce a significant removal of DCF.

On the contrary, significant removal was observed using strains isolated from stock solutions. Results of 3-day degradation experiments, with DCF initially added at 1.0 mg·L^{-1}, and a nutrient media containing glucose used as an easily degradable carbon source are shown in Figure 1, in terms of biotic (grey part), abiotic (black part), and total (biotic + abiotic) degradations. On Figure 1(a), DCF degradation (biotic + abiotic) can firstly be observed to nondetectable levels after 3 days of incubation with either the strain DCF1 (A) or DCF2 (B), while DIU2 (C) strain leads to the lowest percentage of degradation with around 55% of total degradation after 3 days. Palyzová et al. [28] observed 92% of biotransformation of DCF at 1.0 g·L^{-1} after 72 h at 28°C with a strain isolated from polluted soils, *Raoultella* sp. KDF8. *Brevibacterium* D4, a strain isolated from domestic wastewater treatment plant (WWTP) biodegraded 35% of 10 mg·L^{-1} of DCF used as a sole carbon source [29]. Biotransformation of 70% of DCF supplied as the sole carbon source was also biodegraded by *Labrys portucalensis* F11 after 30 days [30]. Finally, a study of Nguyen et al. [7] revealed the functional capacity of *Nitratireductor*, *Asticcacaulis*, and *Pseudoxanthomonas* in the cometabolism of DCF but required further experimental investigations to confirm it.

Compared with the contribution of adsorption on the surface of the fungal cells observed in previous studies, the results obtained with the strains DCF1, DCF2, and DIU2 revealed that adsorption, calculated as the difference in DCF concentration between the azide-killed controls and the uninoculated ones, was lower than 13%. Indeed, a complete removal of DCF, initially added at relatively high (10 mg·L^{-1}) and environmentally relevant low concentration (45 μg·L^{-1}), was observed using *Trametes versicolor*, mainly due to sorption (47% and 80% of the removal, respectively) [13]. In a recent study, a strain isolated from an activated sludge, and identified as *Enterobacter hormaechei*, metabolised DCF with a removal percentage of 53%, due to biodegradation pathways since no decrease regarding DCF concentration was noticed in abiotic and adsorption controls [31]. According to Nikolaou et al. [32], the acidic pharmaceutical compounds with pKa value from 4.1 to 4.9, like DCF, occur as ions at a neutral pH and remain in the aqueous media. Indeed, such compounds are not easily sorbed onto sludge, even if a slight adsorption may be observed at a low pH.

Other experiments were carried out in order to assess the efficiency of a mixture of the three strains to remove DCF. As shown in Figure 1(b) (A + B + C in nutrient media with glucose histogram), no inhibition due to a competition between the selected strains was observed. DCF was still completely removed (biotic + abiotic) after 3 days using the bacterial consortium under identical growth conditions compared with the previously described results obtained with the isolated bacterial strains.

Since organic micropollutants such as DCF are present at very low concentration in wastewater treatment plants, they cannot support any significant microbial growth. This is why it is usually considered that biodegradation of such compounds occurs, thanks to co-metabolic pathways rather than metabolic ones [33]. However, the number of studies regarding co-metabolic biodegradation of pharmaceuticals is limited. According to Barra Caracciolo et al. [34], DCF could be biodegraded co-metabolically, since its degradation potential is very low and no enrichment DCF-depleting microbial activity was observed. More studies using co-metabolic conditions are needed in order to have a better understanding of the micropollutant degradation mechanisms, and regarding applications for *in situ* bioremediation. Since two of the selected strains used in the present study were isolated from the DCF stock solution (DCF1 and DCF2), these strains are probably able to use DCF as a sole source of carbon and energy. Thus, removal efficiency of the selected microorganisms was investigated in the presence or in the absence of an easily degradable external carbon source (glucose). By comparing the removal efficiencies obtained from the consortium with and without glucose addition (Figure 1(b)), results displayed that the efficiency of the consortium is lower using a nutrient medium without glucose. Indeed, only 52% of degradation was observed after 3 days of experiment without glucose, while it reached 100% using the consortium with glucose added as an easily degradable external carbon source. It is likely that the occurrence of this co-substrate could serve as a growth substrate, improving the bacterial growth rate, and, thus, the DCF biodegradation rate. Other authors [30] also observed that the bacterial growth was enhanced when the acetate supply was increased, which in turn improved DCF biodegradation efficiency by *L. portucalensis* F11. The authors also noticed a higher reaction rate with acetate used as a complementary carbon and energy source. Indeed, a complete degradation of DCF was observed after 6 and 25 days for 1.7 μM and 34 μM of DCF, respectively [30]. Periodic feeding with acetate as an additional carbon source also improved the removal of DCF by *Brevibacterium* D4 strain which achieved 90% of removal after 30 days of experiment [29]. Aissaoui et al. [31] investigated the DCF biodegradation using the strain D15 of *Enterobacter hormaechei* and observed a metabolization of DCF by this strain, achieving 53% of elimination after 48 h, with a maximal growth rate observed at the end of the experiment (OD ≈ 0.4). In the presence of glucose, the DCF elimination rate by *Enterobacter hormaechei* increased and 82% of the initial DCF concentration (10 mg·L^{-1}) was removed with a simultaneous increase of bacterial growth (OD ≈ 1.2) due to glucose assimilation [31]. Finally, a bacterial strain, Klebsiella sp. KSC, was recently identified and studied for DCF removal. This is the first study that points out an environmental isolated strain capable of DCF biodegradation at high concentration (70 mg·L^{-1}) and in less than 72 h [35].

It is also important to notice that, in the frame of the present study, the DCF concentrations tested (1.0 mg·L^{-1}) are several orders of magnitude higher than those observed in the influents of wastewater treatment plants, which are commonly in the range of μg·L^{-1}. However, concentrations of pharmaceuticals in the environment are not always very low. Indeed, health care industries can release very high concentrations of pharmaceuticals in the environment (in the mg·L^{-1} range) [36]. The aim of the present study was merely to ascertain whether the selected strains are able to degrade DCF. Further work will concentrate on wastewater under real-world operating conditions, in order to assess the field efficiency of these strains. Nonetheless, since the degrading capability may be affected by the DCF concentration in the culture medium [30], a compromise needs to be found between an appropriate amount of DCF and the microorganisms used to remove it.

3.2. Removal Kinetics of Diclofenac. In order to evaluate the degradation rate over 20 hours of DCF initially spiked at 1.0 mg·L^{-1} using the selected bacterial consortium, a degradation kinetic was measured by a sampling every hour, followed by a DCF concentration measurement. During the first hours, no biodegradation was observed (see Figure 2); this period is a biodegradation lag phase.

Indeed, the bacterial consortium under consideration needed to adapt to the disturbance caused by the addition of DCF. Besides, bacteria needed a certain amount of time to produce the specific degradation enzymes required for their growth and for the metabolization of the medium. The duration of this adaptation period is dependent on the pollutant concentration and should be reduced in the presence of lower environmental concentrations [37]. It is

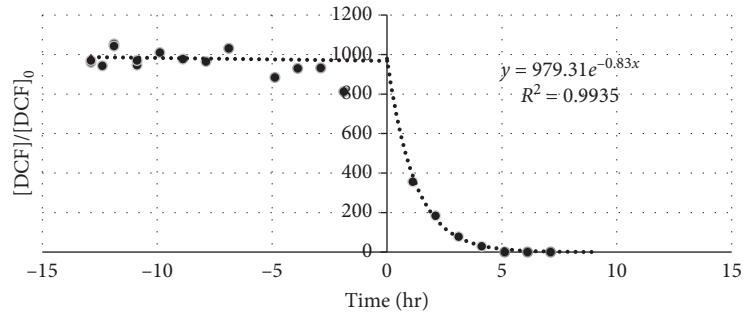


FIGURE 2: Pseudo-first-order kinetic plot for the degradation of diclofenac initially spiked at 1.0 mg·L^{-1} over time at 20°C. The dotted line (time > 0 h) represents the exponential regression of the measured data using equation (3) and the corresponding coefficient of determination (R^2).

worth noting that only concentrations corresponding to the current degradation phase (e.g., significantly different from the initial concentration) were taken into account to evaluate the DCF removal kinetic. Thus, measured concentrations that were not statistically different from the initial concentration were considered to be part of the lag phase and were not used for the calculation of the removal kinetic and thus of k_{biol}. Thereafter, the duration of the lag phase observed in Figure 2 (artificially linear dotted line corresponding to time < 0) is about 13 hours. Then, the degradation phase of DCF begins (at $t = 0$) and is recorded during 7 hours (exponential trend dotted line).

According to previous studies, the biodegradation of DCF should obey a pseudo-first-order kinetic, where the degradation rate of DCF only depends on the concentration of the target analyte itself [38], as follows:

$$\frac{\mathrm{d}[C]}{\mathrm{d}t} = k_{\text{biol}} \times X_{\text{SS}} \times S, \tag{3}$$

where $[C]$ is the DCF concentration (dissolved and sorbed onto suspended solids) in μg·L^{-1}, S the soluble compound concentration (μg·L^{-1}), k_{biol} the reaction rate constant in L·g$_{\text{SS}}$·d^{-1}, t time in days, and X_{SS} the suspended solids (SS) concentration in g$_{\text{SS}}$·L^{-1}, which can be assumed to be a constant for short-term batch observations ($X_{\text{SS}} = 1.8$ g$_{\text{SS}}$·L^{-1} in the present study).

Plotting [DCF]/[DCF$_0$] versus time, an exponential relationship of the form $[\text{DCF}] = [\text{DCF}]_0 \times e^{-kt}$ may represent the decrease of DCF concentration after the lag phase (see Figure 2). From this equation, we can deduce the value of $k = k_{\text{biol}} \times X_{\text{SS}} = 0.83\ \text{h}^{-1} = 19.9\ \text{d}^{-1}$. Thus, $k_{\text{biol}} = 11$ L·g$_{\text{SS}}^{-1}$·d^{-1}. This result highlights that DCF is highly biodegradable by the selected consortium of bacterial strains.

Previous studies have successfully used an identical kinetic model for the biodegradation of organic micropollutants in batch experiments using activated sludge [38]. However, DCF was classified in the group of low biodegradability compounds ($k_{\text{biol}} < 0.1$ L·g$_{\text{SS}}^{-1}$·d^{-1}) considering a first-order degradation constant in WWTPs, suggesting no substantial removal by biodegradation using a classical WWTP activated sludge bioreactor [38]. Consequently, the consortium of selected strains used in the present study is much more efficient than activated sludge to remove DCF, leading to an increase of k_{biol} by a factor of 100.

Since conventional WWTPs usually exhibit removal efficiencies of DCF mainly in the 21–40% range, the removal efficiency of the consortium of strains tested in this work is very promising for future studies. At the moment, DCF is not sufficiently removed using conventional wastewater treatment processes. The use of the strains selected in the present study could allow significant removal of DCF, which is a persistent drug very frequently detected in water bodies.

3.3. Identification of Transformation Products. The identification of DCF metabolites produced by the studied strains in the liquid medium, after 3-day degradation experiments, was performed by high-resolution mass spectrometry coupled with liquid chromatography (LC-MS/MS). Both positive and negative ionisation modes were assessed. Since biological degradation samples were complex, a distinction had to be made in order to identify the chromatographic peaks resulting from DCF biodegradation and those resulting from other activities such as nutrient metabolism and bacterial lysis. The mass chromatograms of the samples from the experiments inoculated with active bacterial strains and DCF were compared with those of the control experiments containing bacterial strains without DCF. At the beginning of the experiments, the compounds not detected in the controls were considered as potential biodegradation products of DCF. Furthermore, the identification of biodegradation intermediates was facilitated by a targeted extraction of the main transformation products reported in the literature, performed using theoretical m/z of each potential metabolite. Table 1 collects the main transformation products identified in the literature, which have been investigated in the degradation samples.

Different collision energies were selected and tested in order to obtain more information regarding the hypothetical and unknown structure of the transformation products. After isolation and fragmentation of the precursor ion, one unique metabolite was observed. Main information related to the possible DCF transformation products is gathered in Table 2.

The (+)-ESI product ion profile, shown in Figure 3, clearly indicates a monohydroxylated product with a mass of m/z 312 $[\text{M} + 1]^+$, confirming the molecular composition of $C_{14}H_{11}C_{l2}NO_3$. The fragmentation pattern of the monohydroxylated product was identical to that of the

Table 1: Diclofenac transformation products reported in the literature.

Transformation products	Formula	$[M+H]^+$ (m/z theoretical)	Reference
2,6-Dichloro-N-(phenyl) aniline	$C_{12}H_9Cl_2N$	238.0185	[39]
1-(2,6-Dichlorophenyl)-1,3-dihydro-2H-indol-2-one	$C_{14}H_9Cl_2NO$	278.0134	[39]
5-Hydroxydiclofenac quinone imine	$C_{14}H_9Cl_2NO_3$	310.0032	[40]
4′-Hydroxy-diclofenac	$C_{14}H_{11}Cl_2NO_3$	312.0189	[41]
5-Hydroxy-diclofenac	$C_{14}H_{11}Cl_2NO_3$	312.0189	[41]
1-O-[2-[(2′,6′-dichlorophenyl)amino]phenyl acetyl]-b-D-glucopyranuronic acid	$C_{20}H_{19}Cl_2NO_8$	472.0560	[6]

Table 2: Diclofenac and its biotransformation product defined by their retention times, measured and theoretical protonated molecules (fragment ions), and formula.

Compound	t_{ret} (min)	$[M+H]^+$ (m/z measured)	$[M+H]^+$ (m/z theoretical)	Mass error (mDa)	Formula	Mass difference
Diclofenac	5.0	296.0243	296.0240	0.3	$C_{14}H_{11}C_{l2}NO_2$	0.00000
4′-Hydroxy-diclofenac	4.0	312.0209	312.0189	2.0	$C_{14}H_{11}Cl_2NO_3$	15.9966

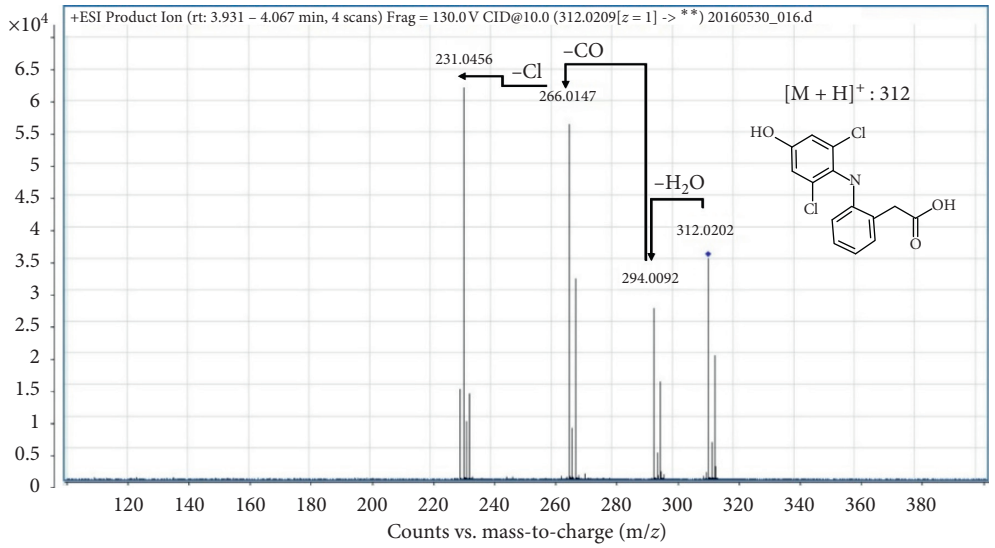


Figure 3: (+) ESI-QqToF-product ion spectra of 4′-OH-DCF, $[M+H]^+ = m/z$ 312; CE = 10 eV.

transformation product 4′-OH-DCF. The loss of 18 Da (-H_2O) from the molecular ion resulted in the formation of a fragment ion at m/z 294.0092. Then, the loss of 28 Da (-CO) resulted in the formation of a fragment ion at m/z 266.0147, and a less intense fragment ion at m/z 231.0456 corresponded to a loss of -Cl from m/z 266.0147.

Only one metabolite of DCF was identified, due to the hydroxylation of DCF by the selected strains, which is a bottleneck in DCF degradation [42]. 4′- OH-DCF is one of the major metabolites also formed during the human metabolization of DCF, catalysed by different types of cytochromes in the hepatocytes [43]. The 4′-OH-DCF is formed, thanks to a hydroxylation of the phenyl group containing molecules of chlorine. According to Bouju et al. [42], both human and microbial activities may be the source of production of 4′-OH-DCF in municipal sewage plant effluents. Indeed, some fungal and bacterial organisms are known to mimic the mammalian metabolism of micropollutants such as DCF. Some bacteria are able to metabolise DCF in aerobic conditions due to a redox reaction with the cytochrome P450 (CYP450) [44]. For instance, DCF was hydroxylated by a strain of the bacterium *Actinoplanes* sp. by means of a cytochrome P450 [44]. Besides, 4′-OH-DCF was also identified as a major metabolite after biodegradation by *Raoultella* sp. KDF8 [28] and *L. portucalensis* [30]. This hydroxylated metabolite is generated by CYP450 2C9 enzymes ([45, 46]).

3.4. Ecotoxicity Evaluation. As it may happen in the case of other organic micropollutants, such as pesticides [47], it can be assumed that DCF transformation products might be more toxic than DCF itself. Thus, the ecotoxicity of both 4′-OH-DCF and DCF using samples of batch experiments at $t = 0$ and $t = 3$ d have been evaluated.

3.4.1. DCF and Its Metabolite Ecotoxicity in Simple Medium. The ecotoxicity given by EC_{50} values obtained with DCF and its hydroxylated metabolite are shown in Table 3. To the best of our knowledge, this is the first report of an ecotoxicological value for this metabolite. The values of EC_{50} obtained

at 5, 15, and 30 min were almost constant; thus, we only reported results after 30 min.

Previous studies evaluated the toxic effect of DCF on *Vibrio fischeri* and concluded that DCF has a relatively limited acute toxicity on the tested bacteria. However, it has been recognised that there are high discrepancies between EC_{50} values for a same pharmaceutical in the literature [48]. For example, results vary depending on the exposure time. After 15 min of exposure, some authors observed an EC_{50} between 9.7 and 14.31 $mg{\cdot}L^{-1}$ ([23, 49, 50]). Ferrari et al. [51] evaluated the toxic effect of DCF on *Vibrio fischeri* after 30 min of exposure and noticed an $EC_{50} = 11.45\ mg{\cdot}L^{-1}$, which is below half of the EC_{50} given in Table 3. Moreover, as far as we know, the ecotoxicity of 4′-hydroxy-diclofenac has never been evaluated on *Vibrio fischeri*. Recently, Stylianou et al. [35] investigated the acute toxicity of DCF and the biotransformation products created by *Klebssiella* sp. KSC with *Vibrio fischeri*. However, the results are expressed as the inhibition percentage of the bioluminescence of the bacteria and did not differentiate each by-product. According to the authors, the DCF biotransformation products by the isolated strain were not able to cause toxic effects in contrast to DCF [35].

It is worth noting that, based on the EC_{50} values reported in Table 3, no significant difference in ecotoxicity was found between DCF and its metabolite (4′-OH-DCF), using a Wilcoxon/Kruskal-Wallis test ($n = 3$, $\alpha = 0.05$). This result is quite reassuring because such concentration levels (about 20 $mg{\cdot}L^{-1}$) are not observed in the aquatic environment. Indeed, in untreated sewage, 4′-OH-DCF is usually found at a much lower concentration level (average concentration of 237 $\mu g{\cdot}L^{-1}$), corresponding to concentration levels of the same order of magnitude as its parent compound (DCF average concentration of 338 $\mu g{\cdot}L^{-1}$) [40]. Even though the interest of degrading DCF into 4′-OH-DCF may be discussed, since no significant difference of ecotoxicity has been found, it has been demonstrated that this metabolite may hopefully be an intermediate product in the DCF transformation. Indeed, 4′-OH-DCF could not be detected anymore after 9 days of batch degradation experiments using activated sludge and was transformed into two other by-products, including OH-DCF-lactam [42].

Finally, it seems important to keep in mind that the Microtox® ecotoxicological test evaluates the acute toxicity of a given compound, but it does not mean that the occurrence of lower concentrations of this compound are harmless. One of the widely used and highly standardised methods for measuring toxicity is the acute immobilisation test (using *Daphnia magna*). According to Cleuvers [25], DCF was potentially harmful to aquatic organisms. Indeed, he investigated the toxicity of a mixture of pharmaceuticals using acute toxicity tests with *Daphnia* and algae, and noticed an EC_{50} value of 68 $mg{\cdot}L^{-1}$. Generally, EC_{50} values using acute toxicity tests with *Daphnia magna* varied between 22 and 80 $mg{\cdot}L^{-1}$, which are far higher values than the environment concentrations usually reported for DCF ([21, 24]). Schwaiger et al. [52] investigated the toxicity of DCF on *Daphnia magna* reproduction. The no-observed effect concentration (NOEC) was reported to be 1.0 $mg{\cdot}L^{-1}$ and the lowest-observed-effect concentration (LOEC) to be 0.2 $mg{\cdot}L^{-1}$.

Table 3: Ecotoxicity values of diclofenac and its metabolite after 30 min of exposure (triplicate).

Compound	Formula	EC_{50} after 30 min ($mg{\cdot}L^{-1}$)
Diclofenac	C14H11Cl2NO2	23 ± 4
4′-Hydroxy-diclofenac	$C_{14}H_{11}Cl_2NO_3$	19 ± 2

Likewise, other acute toxicity tests have been performed, notably using one of the most commonly used organisms in toxicity testing, the fish *Danio rerio*. For instance, the acute toxicity of DCF has been investigated on different stages of development of *Danio rerio*. Praskova et al. [53] determined the 96 h LC_{50} for juvenile and 144 h LC_{50} for embryonic stages: median lethal concentration (LC) and 50% mortality after a 96 h or 144 h interval, respectively. The toxicity of DCF to the juvenile and embryonic stages of this fish were compared. The study revealed a statistically higher sensitivity to DCF ($P < 0.05$) in the embryonic stages (mean 144 h $LC_{50} = 6.11 \pm 2.48\ mg{\cdot}L^{-1}$) compared with the juvenile fish (mean 96 h $LC_{50} = 166.6 \pm 9.8\ mg{\cdot}L^{-1}$) [53]. Regarding chronic toxicity, Ferrari et al. [24] tested the viability of *Danio rerio* embryos exposed 10 days to DCF. In their study, NOEC and LOEC were reported to be 4,000 and 8,000 $\mu g{\cdot}L^{-1}$, respectively.

Regarding the low environmental concentrations of DCF detected in surface waters, the acute toxicity risk of this pharmaceutical seems to be low on *Vibrio fisheri*, *Daphnia magna*, and *Danio rerio*. In spite of this, further investigations are needed to evaluate the potential negative effects of chronic exposure to DCF. Indeed, the exposure to DCF at environmental concentrations can cause acute lethal and chronic sublethal toxicity in higher plant development (fern spore bioassay) [54].

3.4.2. Limits of the Microtox® Test on a Complex Medium. Different preliminary experiments were carried out in order to evaluate the ecotoxicity of the 0.2-μm filtered nutrient medium after 3 days of incubation in the presence of the studied bacterial consortium and DCF. However, this complex medium gave inconsistent results. Indeed, the release of metabolism by-products during bacterial growth, or the occurrence of some carbon source in the medium used for the degradation experiments, showed negative effects on the luminescence of the bacteria. Thus, the influence of the nutrient media itself on *Vibrio fischeri* bioluminescence was tested. All EC_{50} values obtained during these different experiments are collected in Table 4.

First, the nutrient media alone was tested. The EC_{50} of this media showed a toxic effect on *V. fischeri* (EC = 31.9% with a Basic Test 45%). It has been assumed that the toxicity of glucose was responsible for the toxicity of the nutrient media. Indeed, it has been demonstrated that the presence of glucose in growth medium represses *V. fischeri* luminescence [55]. Screening test 81.9% revealed an EC_{50} at 8.14% for a solution of glucose at 1.0 $g{\cdot}L^{-1}$ (e.g.,

Table 4: EC_{50} values obtained during the evaluation of the ecotoxicity of the medium used for degradation experiments.

Compounds (initial concentration)	Test protocol	EC_{50} after 30 min of exposure Mean [% min–% max]
Tryptone/peptone from casein (6.0 g·L^{-1}) Casein hydrolysate standard (3.0 g·L^{-1}) Yeast extract (3.0 g·L^{-1}) Glucose (1.0 g·L^{-1})	Basic test 45% ($n = 3$)	31.9% [20.3–52.9]
Glucose (1.0 g·L^{-1})	Screening test 81.9% ($n = 2$)	8.14% [4.81–11.5]
Tryptone/peptone from casein (6.0 g·L^{-1}) Casein hydrolysate standard (3. 0 g·L^{-1}) Yeast extract (3.0 g·L^{-1})	Screening test 81.9% ($n = 2$)	20.7% [4.36–37.0]
Tryptone/peptone from casein (6.0 g·L^{-1}) Casein hydrolysate standard (3. 0 g·L^{-1}) Yeast extract (3.0 g·L^{-1}) $NaHCO_3$ (1.0 g·L^{-1}) + glycerol (3.0 g·L^{-1})	Screening test 81.9% ($n = 2$)	17.0% [0.39–33.7]
Tryptone/peptone from casein (6.0 g·L^{-1})	Screening test 81.9% ($n = 2$)	40.3% [34.5–46.1]
Casein hydrolysate standard (3. 0 g·L^{-1})	Screening test 81.9% ($n = 2$)	26.6% [19.8–33.3]
Yeast extract (3.0 g·L^{-1})	Screening test 81.9% ($n = 2$)	18.8% [33.1–45.5]

EC_{50} = 81.4 mg·L^{-1}). However, glucose may not be the only component of the nutrient media to have a negative effect on *V. fischeri*. Even when removing glucose or using another carbon source such as $NaHCO_3$ with glycerol, EC_{50} values given by the screening test 81.9% were still close to 20%. EC_{50} values of the other components of the nutrient media were 2.4 g·L^{-1} (e.g., EC_{50} = 40.3% for a concentration at 6.0 g·L^{-1}), 0.80 g·L^{-1} (e.g., EC_{50} = 26.6% for a concentration at 3.0 g·L^{-1}), and 0.56 g·L^{-1} (e.g., EC_{50} = 18.8% for a concentration at 3.0 g·L^{-1}), for tryptone/peptone from casein, casein hydrolysate standard, and yeast extract, respectively. Each component of this nutrient media seemed to have a negative effect on *V. fischeri*.

Furthermore, the ecotoxicity of compounds released by the selected microorganisms to remove micropollutants was also evaluated. Thus, a sample from the growth control was used to evaluate its toxicity. The EC_{50} values were 100%, which means that the complex medium generated by the development of the selected bacteria and the nutrients were very toxic for *V. fischeri* and unusable to evaluate the potential change of ecotoxicity of the pollutant directly in the culture media.

The Microtox® bioassay integrates an overall effect of the composition of the sample tested and cannot be used to evaluate the effect of a single pollutant in a complex medium rich in nutrients. However, the Microtox® test is a useful technique for the determination and the comparison of the acute toxicity of single contaminants and a mixture of them directly in the reactional medium of this bioassay [56].

4. Conclusions

The DCF biodegradation by *Bacillus subtilis* and *Brevibacillus laterosporus* strains achieved 100% after 17 hours of experiment and followed a pseudo-first-order kinetics. The degradation rate constant was 11 L·g_{SS}^{-1}·d^{-1} after a lag phase during which the selected strains produced the specific degradation enzymes necessary for the metabolization of the medium and their growth. A screening of transformation products by high-resolution mass spectrometry coupled with liquid chromatography (LC-MS/MS) in a positive mode was carried out in order to find out the degradation pathway of this drug. The biodegradation of DCF led to the formation of one metabolite, 4′-hydroxy-diclofenac, after hydroxylation of the parent compound. No significant difference of ecotoxicity was noticed between both compounds. However, the degradation of DCF into 4′-hydroxy-diclofenac could lead to the transformation of this metabolite in another by-product, potentially decreasing the toxic effect in the studied medium. Further experiments to evaluate the acute toxicity should be required to confirm results from the Microtox® test, and other experiments on the evaluation of potential chronic effects are required to complete this preliminary study.

Data Availability

The data used to support the findings of this study are included within the article.

Additional Points

Biodegradation of diclofenac using selected microorganisms was performed. 4′-OH-DCF was identified as the major degradation product by LC-MS analysis. DCF kinetic biodegradation followed a pseudo-first order (k_{biol} = 11 L·g_{SS}^{-1}·d^{-1}). The ecotoxicity of DCF and 4′-OH-DCF was 23 and 19 mg·L^{-1}, respectively. Compounds of the complex medium showed an apparent toxicity to *Vibrio fisheri*.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Table A1: composition of nutrient media, Table A2: information regarding different strains provided by Prodibio company, Table A3: removal efficiency of the different studied strains tested after 3 experiments, Figure A1: (+) ESI-QqToF-product ion spectra of 4′-OH-DCF, $[M + H]^+ = m/z$ 312; CE = 10 eV. (*Supplementary Materials*)

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