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Biodegradation of Diclofenac by the bacterial strain *Labrys portucalensis* F11

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ABSTRACT

Diclofenac (DCF) is a widely used non-steroidal anti-inflammatory pharmaceutical which is detected in the environment at concentrations which can pose a threat to living organisms. In this study, biodegradation of DCF was assessed using the bacterial strain *Labrys portucalensis* F11. Biotransformation of 70% of DCF (1.7–34 μM), supplied as the sole carbon source, was achieved in 30 days. Complete degradation was reached via co-metabolism with acetate, over a period of 6 days for 1.7 μM and 25 days for 34 μM of DCF. The detection and identification of biodegradation intermediates was performed by UPLC-QTOF/MS/MS. The chemical structure of 12 metabolites is proposed. DCF degradation by strain F11 proceeds mainly by hydroxylation reactions; the formation of benzoquinone imine species seems to be a central step in the degradation pathway. Moreover, this is the first report that identified conjugated metabolites, resulting from sulfation reactions of DCF by bacteria. Stoichiometric liberation of chlorine and no detection of metabolites at the end of the experiments are strong indications of complete degradation of DCF by strain F11. To the best of our knowledge this is the first report that points to complete degradation of DCF by a single bacterial strain isolated from the environment.

1. Introduction

Pharmaceuticals have emerged as environmental contaminants for which concern is increasing (Kümmerer, 2009), as their bio-accumulation potential and ecotoxicity could pose a risk for living organisms. After consumption, unmetabolized pharmaceuticals and their metabolites are excreted and enter wastewater as biologically active substances (Zhang et al., 2008). Conventional wastewater treatment plants (WWTPs) have a limited capability to remove these compounds, resulting in their release into the environment. Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) (DCF) is a widely prescribed nonsteroidal anti-inflammatory drug, with an estimated global consumption of 940 t per year (Zhang et al., 2008). Nowadays, this drug is ubiquitously present in the aquatic environment (Patrolecco et al., 2015) due to its continuous release from WWTPs, being considered a pseudo-persistent pollutant (Bu et al., 2016). The maximum DCF concentrations in municipal wastewaters vary between 0.44 and 7.1 μg/L (mean concentrations 0.11 − 2.3 μg/L) (Vieno and Sillanpää, 2014). The removal efficiencies by WWTPs ranges from 0% to 80%, but lies mainly in the 21–40% range (Zhang et al., 2008). DCF has also been detected in surface water (Fernández et al., 2010; Patrolecco et al., 2015), groundwater (Lapworth et al., 2012), and drinking water (Simazaki et al., 2015). The ecotoxicity of this pharmaceutical has been pointed out by several studies. Exposure to 0.1 μg/L of DCF affects biochemical processes of duckweed plants (Kummerová et al., 2015) and at a concentration of 0.2 μg/Kg has a negative impact in freshwater fish (Guiloski et al., 2015). DCF poisoning was also identified as the major, and possibly the only, cause of rapid population declines of Gyps vultures across the Indian subcontinent (Green et al., 2004). DCF is one of the substances on the watch list for European Union-wide monitoring in the Decision 2015/495/EU.

Despite the progress in advanced technologies for wastewater treatment, such as advanced oxidation and membrane-based technologies, the latter systems have as a drawback their high operational costs (Barbosa et al., 2016; Schröder et al., 2016) whereas the advanced oxidation methods usually result in the formation of transformation products, which might be more toxic and/or persistent than DCF (Fischer et al., 2015; Márquez Brazón et al., 2016; Rizzo et al., 2009).

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Methods which can either lead to complete degradation of micropollutants or may turn them into stable harmless compounds are needed.

DCF is generally considered to be poorly biodegradable, which results in incomplete elimination from wastewater in conventional WWTP (Patrolecco et al., 2015; Pereira et al., 2015; Vieno and Sillanpää, 2014). Despite the existing reports on the degradation of DCF by microorganisms, there are still gaps on the knowledge related with the degradation process. Of the few reports claiming the biodegradation of DCF, in the majority of previously published studies the bacteria responsible for the degradation are unknown. DCF was found to be biodegradable in agricultural soils, but the organisms involved were not identified (Al-Rajab et al., 2010). Langenhoff et al. (2013) reported degradation of 75% of 300 mg L^{-1} of DCF during three weeks by enriched bacterial culture. However, the microorganisms responsible for degradation were not identified and the persistence of the formed metabolites was not assessed. Bouju et al. (2016) reported that MBR biomass was able to transform 40% of DCF during 25 days; two metabolites were identified as end products of the biotransformation. Palyzová et al. (2017) reported the biotransformation of DCF (1.0 g L^{-1}) by *Raoultella* sp. KDF8, but this capability was only achieved after the strain was modified by chemical mutagenesis. An isolate retrieved from domestic WWTP has shown capacity to degrade DCF: strain *Brevibacterium* D4 was able to biodegrade 35% of 10 mg L^{-1} of diclofenac as a sole carbon source and 90% of the same amount when periodic feeding with acetate as a supplementary carbon source was present; metabolites were not assessed (Bessa et al., 2017). On the other hand, degradation of DCF was achieved by white rot fungus *Phanerochaete sordida* YK-624, supplied at a concentration of 100 μM (Hata et al., 2010). Marco-Urrea et al. (2010) reported complete removal of DCF by *Trametes versicolor*, at a concentration of 10 mg L^{-1} and 45 μg L^{-1}, but the adsorption on the surface of the fungus cells contributed to 47% and 80% of the removal, respectively (Marco-Urrea et al., 2010). Rodarte-Morales et al. (2011) also reported removal of DCF from a mixture of pharmaceuticals (1 mg L^{-1}) by white rot fungus, *Bjerkandera* sp. R1, *Bjerkandera adusta* and *Phanerochaete chrysosporium* but formation of stable intermediates was not assessed.

The first step of the transformation is usually hydroxylation catalyzed by cytochrome P-450 monooxygenases, similar to the mammalian metabolism, or oxygenation by laccases and peroxidases (Domaradzka et al., 2015). However, in the cited reports is not known what happens with the formed hydroxylated compounds, which seems to be end-products of DCF transformation. *Actinoplanes* sp. hydroxylated DCF (50 μM) with 100% turnover in less than 5 h (Osorio-Lozada et al., 2008). The cytochrome P450 enzyme from this strain was identified and expressed in *Escherichia coli* for the production of 4′-hydroxydiclofenac metabolite (Prior et al., 2010). Gröning et al. (2007) observed a rapid removal of DCF in a concentration of 3–35 μM in river sediment, with transient accumulation of a major metabolite, the p-benzoquinone imine derivative of 5-hydroxydiclofenac, which is further abiotically adsorbed on the biofilm, revealing a lack of biodegradation potential for this intermediate compound. Microbial transformations products in activated sludge bioreactors were detected and identified, showing the formation of stable metabolites (Kosjek et al., 2009, 2008). Another study reported a decrease of 42% of DCF during 57 days (70 ± 14 days half-life) due to biodegradation; however, the three transformation products observed were persistent until the end of the experiment (Poirier-Larabie et al., 2016). These investigations revealed that degradation processes often do not lead to complete degradation, but result in persistent transformation products, which may still pose a threat to the environment.

The main aim of this study was to investigate the degradation of DCF by a previously isolated and fully characterized bacterial strain - *Labrys portucalensis* F11, able degrade other pharmaceuticals, like fluoxetine and fluoroquinolones (Amorim et al., 2014; Moreira et al., 2014). Kinetics of DCF biodegradation was studied as a sole carbon source and in co-metabolism with acetate. Intermediate compounds formed during the biodegradation process were also identified. To the best of our knowledge, this is the first report that points to complete biodegradation of DCF by a single bacterium isolated from the environment.

2. Materials and methods

2.1. Chemicals and materials

Methanol and ammonium acetate employed for the instrumental analyses as well as for standard solutions preparation were HPLC grade (Riedel-de Haën, Baker). A Milli-Q Gradient A-10 (Millipore) system was used for delivering ultrapure water (18.2 MΩcm, organic carbon ≤ 4 μg/L) to be used for both ultra-high pressure liquid chromatography (UPLC) and standard solutions preparation. Acetonitrile was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid 99% (TFA), trimethylamine (≥99%) (TEA) and DCF sodium salt were obtained from Sigma-Aldrich (Steinheim, Germany). HPLC grade solvents were filtered with 0.45 μm Glass microfiber filters (Whatman™). Minimal salts medium (MM) (Moreira et al., 2013) was prepared with analytical-grade chemicals (Sigma–Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany). Sodium acetate was purchased from Merck (Darmstadt, Germany).

2.2. Cultivation conditions

L. portucalensis F11 (GenBank/EMBL/DDBJ accession number AY362040) is a bacterial strain previously isolated (Carvalho et al., 2005). *L. portucalensis* F11 was deposited at BCCM/ LMG Bacteria Collection, Ghent, Belgium, under accession number LMG 23412 and at DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, under the accession number DSM 17916. This organisms was selected to evaluate the biodegradation of DCF due to its biodegradation abilities (Amorim et al., 2014, 2013a, b; Carvalho et al., 2016; Moreira et al., 2014, 2012). The microorganism was routinely cultivated on nutrient agar (NA) plates incubated for 2 d at 25 °C to prepare the inoculum for the degradation experiments.

2.3. Biodegradation of DCF

As a first test, the degradation of DCF was tested at a concentration of 34 μM with the addition of a supplementary carbon source, sodium acetate, at 5.9 mM, this was done to simulate the carbon content of an influent of a WWTP (de Kreuk et al., 2005). Moreover, assays with a periodic feeding with the same concentration of sodium acetate (at days 6, 10, 15, 20 and 24), were established. Cells of *L. portucalensis* strain F11 were inoculated to an OD600 of ca. 0.05 into 250 mL flasks containing 75 mL MM supplemented with DCF. The experiment was monitored during 30 d. Further degradation experiments were then performed testing DCF in a concentration range between 1.7 and 34.0 μM, to establish the effect of the DCF concentration on the degradation process.

All the cultures were incubated at 25 °C on a rotary shaker (130 rpm). Experiments were performed in triplicate under sterile conditions. Control assays without inoculation and controls inoculated with autoclaved (i.e. non-viable) *L. portucalensis* F11 cells were performed to evaluate abiotic degradation and adsorption. A control for cell growth was established with the same concentration of acetate without DCF addition. In order to prevent photolytic degradation, all the flasks were completely wrapped to protect the cultures from any source of light. Samples were taken at regular intervals to assess the cellular growth and the degradation of DCF. The purity of the cultures was evaluated through regular plating on NA plates. DCF concentration was determined by a validated HPLC method.

Degradation rate constants were calculated, assuming first-order