Chemosphere 103 (2014) 336–342

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Use of stable isotope probing to assess the fate of emerging contaminants degraded by white-rot fungus

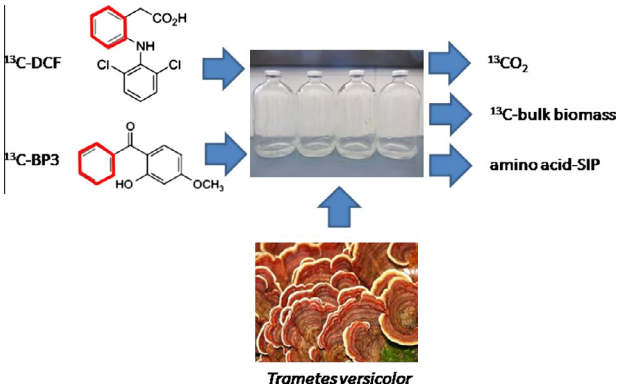
Marina Badia-Fabregat [a], Mònica Rosell [b], Glòria Caminal [c], Teresa Vicent [a], Ernest Marco-Urrea [a,*]

[a] Departament d'Enginyeria Química, Escola d'Enginyeria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Grup de Mineralogia Aplicada i Medi Ambient, Departament de Cristal·lografia, Mineralogia i Dipòsits Minerals, Facultat de Geologia, Universitat de Barcelona, Martí Franquès s/n, 08028 Barcelona, Spain
[c] Institut de Química Avançada de Catalunya, IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

HIGHLIGHTS

- Use of carbon stable isotope tracer experiments to assess fungal degradation.
- We observed 11% mineralization for DCF and 45% for BP3.
- BP3 and DCF carbon accumulated intracellularly according to bulk biomass EA-IRMS.
- Amino acid-SIP proved the use of BP3 as carbon source by *Trametes versicolor*.
- Mass balances for ^{13}C labelling fate were calculated.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 October 2013
Received in revised form 27 November 2013
Accepted 11 December 2013
Available online 3 January 2014

Keywords:
Fungi
Stable isotope probing (SIP)
Diclofenac
Benzophenone-3
Mineralization
Amino acids

ABSTRACT

The widespread of emerging contaminants in the environment and their potential impact on humans is a matter of concern. White-rot fungi are cosmopolitan organisms able to remove a wide range of pharmaceuticals and personal care products (PPCP) through cometabolism (i.e. laccases and peroxidases) or detoxification mechanisms (i.e. cytochrome P450 system). However, the use of PPCP as carbon source for these organisms is largely unexplored. Here, we used carbon stable isotope tracer experiments to assess the fate of anti-inflammatory diclofenac (DCF) and UV filter benzophenone-3 (BP3) during degradation by *Trametes versicolor*. The comparison between carbon isotopic composition of emitted carbon dioxide from ^{13}C-labelled DCF ([acetophenyl ring-$^{13}C_6$]-DCF) and ^{13}C-BP3 ([phenyl-$^{13}C_6$]-BP3) versus their ^{12}C-homologue compounds showed mineralization of about 45% and 10% of the ^{13}C contained in their respective molecules after 9 days of incubation. The carbon isotopic composition of the bulk biomass and the application of amino acid-stable isotope probing (SIP) allowed distinguishing between incorporation of ^{13}C from BP3 into amino acids, which implies the use of this emerging contaminant as carbon source, and major intracellular accumulation of ^{13}C from DCF without implying the transformation of its labelled phenyl ring into anabolic products. A mass balance of ^{13}C in different compartments over time provided a comprehensive picture of the fate of DCF and BP3 across their different transformation processes. This is the first report assessing biodegradation of PPCP by SIP techniques and the use of emerging contaminants as carbon source for amino acid biosynthesis.

* Corresponding author. Tel: +34 93 581 2694.
E-mail address: Ernest.Marco@uab.cat (E. Marco-Urrea).

0045-6535/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.chemosphere.2013.12.029

1. Introduction

The presence of emerging contaminants in the environment raises concerns about its potential to harm human or environmental health (Murray et al., 2010; Brausch and Rand, 2011). The anti-inflammatory diclofenac (DCF) and the UV filter benzophenone-3 (BP3) have high levels of consumption, widespread presence in the environment (Fent et al., 2010; Liu et al., 2011; Duan et al., 2013; Barbara Morasch, 2013) and significant associated environmental risk (Hernando et al., 2006). DCF was recently proposed to be included as priority substance in the Water Framework Directive 2000/60/EC (European Commission, 2012). Regarding BP3, it is regulated by the 2002/72/EC Directive, relating to compounds in contact with food, and was listed as substance with potential evidence of endocrine disrupting effects (category 2) (European Commission, 2007).

In order to avoid the release of xenobiotics, some alternative strategies are under study as conventional wastewater treatment plants (WWTP) are not effective in totally degrading these compounds (Jelic et al., 2011). The use of ligninolytic fungi is one of these alternatives and their application as decontaminating agent has been intensively studied during the last years (Golan-Rozen et al., 2011; Harms et al., 2011). Both DCF and BP3, together with many other PPCP, were previously shown to be biodegradable by white-rot fungi (Marco-Urrea et al., 2009, 2010; Gago-Ferrero et al., 2012). However the mechanistic of PPCP degradation is still not fully understood and has stated to proceed either cometabolically by means of extracellular enzymes such as laccases and peroxidases or via detoxification reactions such as cytochrom P450 and conjugations (Yang et al., 2013). For bioremediation purposes, metabolic or growth-linked reactions are preferred over cometabolic or detoxification mechanisms since microorganisms can derive their carbon and energy directly from the pollutant. However, evidence of the use of xenobiotics as a carbon source is limited in white-rot fungi.

To accomplish the aim of identifying biodegradation strategies that entail mineralization, the use of contaminants labelled with stable carbon isotopes and further determination of carbon isotopic signatures of CO_2 has been widely applied. With the advent of stable-isotope probing (SIP) analyses, the range of applications increased including tracking the carbon flow through microbial communities (Bastida et al., 2010) and identifying unannotated pathways in certain microorganisms (Marco-Urrea et al., 2012), among others. The basis of this technique is labelling certain type of microbial biomarkers with stable isotopes (usually ^{13}C) and then, using chromatography coupled to mass spectrometry (MS) or to isotope ratio mass spectrometry (IRMS) for higher sensibility, determine the increase in the ^{13}C atom percentage (at.%) of the labelled biomarker pools. Thus, protein-SIP (Bastida et al., 2010), total lipid fatty acids (TLFA)-SIP (Jakobs-Schönwandt et al., 2010; Bastida et al., 2011), DNA-SIP (Lu and Chandran, 2010) or RNA-SIP (Bastida et al., 2011) analyses can be performed.

The application of isotope techniques to fungi can shed light on the role of these widespread organisms in decontamination processes and also predict contaminant fate in the environment (Harms et al., 2011). The use of SIP-techniques in fungi is scarce and limited to a recent study demonstrating the incorporation of the carbon-based nanomaterial C_{60} fullerol into the lipid biomass of two white-rot fungi (*Trametes versicolor* and *Phlebia tremellosa*) (Schreiner et al., 2009).

In the present study, we combine the analysis of carbon isotopic composition of CO_2, bulk biomass and individual amino acids (by amino acid stable isotope probing [aa-SIP]) during the degradation of ^{13}C-DCF and ^{13}C-BP3 by the white-rot fungus *T. versicolor* to track the ^{13}C fate of these emerging contaminants and the degradation mechanism used by the fungus. This is the first work to demonstrate assimilation of xenobiotics into fungal amino acids using SIP techniques.

2. Materials and methods

2.1. Reagents and fungal strains

The nonlabelled BP3 (^{12}C-BP3) was kindly provided by Merck (Darmstadt, Germany). [Phenyl-$^{13}C_6$]-oxybenzone (^{13}C-BP3) was obtained from Cambridge isotopes (Cambridge, UK) with a chemical purity >99% and an isotope purity 99 at.%. The nonlabelled DCF (^{12}C-DCF) was purchased from Sigma–Aldrich (Saint Louis, USA). [Acetophenylring-$^{13}C_6$]-diclofenac (^{13}C-DCF) was obtained from Alsachim (Strasbourg, France) with a chemical purity >99% and an isotope purity 99 at.%. All other chemicals used were of analytical grade.

T. versicolor (ATCC #42530) was obtained from the American Type Culture Collection and was maintained by subculturing on petri dishes in malt extract (2%) and agar (1.5%) medium at 25 °C.

2.2. Media and cultures for fungal production

Pellets production was done as previously described by Font et al. (2003). The blended mycelia suspension used for the experiments was obtained by grinding the pellets in 8% NaCl solution with a X10/20 homogenizer (Ystral GmbH, Dottingen, Germany). For the experiments, a defined medium was used (Blánquez et al., 2004), with a glucose initial concentration of 0.5 g L^{-1} and 2 g L^{-1} of KH_2PO_4 instead of dimethyl succinic acid to minimize other possible carbon sources than glucose and contaminant.

2.3. Experimental design

Each experiment included, apart from the experimental bottles, uninoculated and sodium azide killed controls for abiotic degradation and biotic sorption determination respectively. DCF experiment also included heat killed controls. All experiments were conducted in duplicate in 125-mL serum bottles (Wheaton, Mealville, NJ). Cultures were incubated at 25 °C and 130 rpm orbital agitation.

^{12}C- or ^{13}C-BP3/DCF were added from 100 mg L^{-1} stock solution in acetonitrile (BP3) or ethanol (DCF) to a final concentration of 1 mg L^{-1}, in a total volume of 10 mL of medium. Acetonitrile and ethanol were totally evaporated with nitrogen before the addition of medium in order to avoid their possible use as carbon source by the fungus. Finally, one millilitre of blended mycelia was added to obtain a concentration of 0.5 g d.w. L^{-1} in the bottles. In the sodium azide killed controls, 100 µL of sodium azide at 100 g L^{-1} were additionally added the day before and left shacking overnight with the media and the fungus to ensure the total inactivation of the fungus prior to pollutant addition. Heat killed controls were previously autoclaved 30 min at 121 °C. The existing air inside the bottles was replaced by a higher oxygen content air by means of displacing the air with pure oxygen in order to avoid a potential oxygen limitation as *T. versicolor* is an aerobic organism (Marco-Urrea et al., 2008). Bottles were then closed with Teflon-coated butyl-stoppers (Wheaton, Millville, NJ) and aluminium crimps (Baxter Scientific Products, McGaw Park, IL).

At each sampling point (initially, at 3, 6 and 9 days), the procedure was the same: for the non sacrificed bottles at that time, the air was replaced by blowing pure oxygen inside again and, for the sacrificed bottles, the procedure performed was as follows. CO_2 was sampled with a gas-tight syringe from the headspace of the bottle and directly injected to a gas chromatograph coupled to an