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

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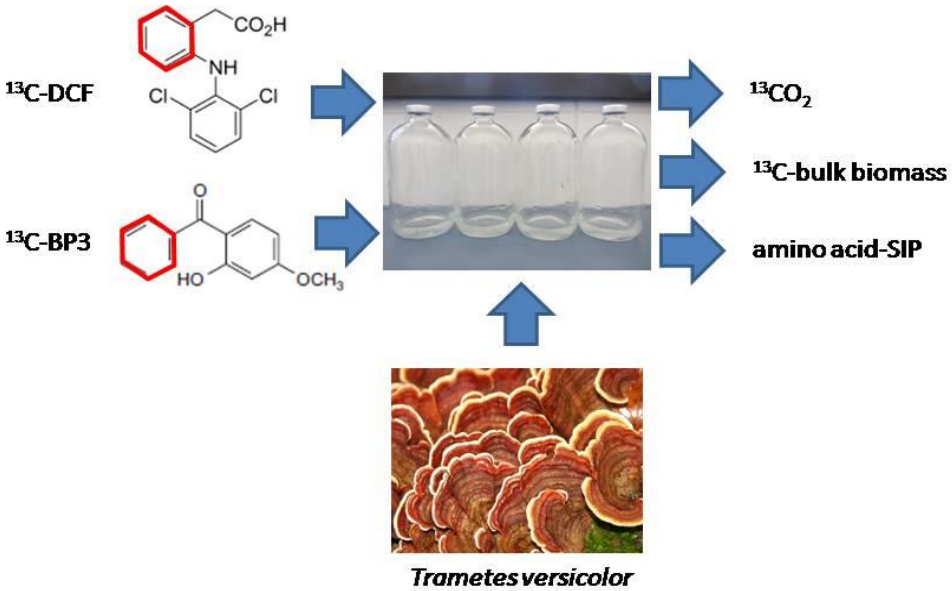
Graphical abstract/TOC



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.

Keywords

Fungi, Stable Isotope Probing (SIP), Diclofenac, Benzophenone-3, Mineralization, Amino acids.

1. Introduction

The presence of emerging contaminants in the environment raises concerns about its potential to harm human or environmental health (Brausch and Rand, 2011; Murray et al., 2010). The anti-inflammatory diclofenac (DCF) and the UV filter benzophenone-3 (BP3) have high levels of consumption, widespread presence in the environment (Duan et al., 2013; Fent et al., 2010; Liu et al., 2011; 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 (Gago-Ferrero et al., 2012; Marco-Urrea et al., 2010; Marco-Urrea et al., 2009). 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 (Bastida et al., 2011; Jakobs-Schönwandt et al., 2010), 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 25ºC.

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 gL^{-1} and 2 gL^{-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 25ºC and 130 rpm orbital agitation.

^{12}C- or ^{13}C-BP3/DCF were added from 100 mg/L stock solution in acetonitrile (BP3) or ethanol (DCF) to a final concentration of 1 mg/L, 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 121ºC. 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 isotope ratio mass spectrometer through a combustion interface (GC-C-IRMS). Then, for BP3 experiment, the bottles were opened and 1 mL was sampled and filtered through 0.22 µm PVDF syringe filter (Millipore, US) for glucose and laccase activity analyses. Afterwards, BP3 was solubilised by adding 6 mL of ethanol and the mixture was centrifuged for 10 min at 4ºC and 13000 *g*. In the case of DCF, ethanol addition was not needed due to its higher solubility in water. The supernatant was filtered by 0.45 µm nylon filter (Millipore,

US) (BP3) or 0.22µm PVDF syringe filter (Millipore, US) (DCF) and analysed by HPLC-UV for contaminant quantification. The pellet was further processed for elemental analysis of biomass and amino acid-SIP (aa-SIP) analysis as described below.

2.4. Protein extraction, purification and amino acids derivatization

Protocol used for protein extraction, purification and amino acids derivatization was adapted from Bastida et al. (2011). Detailed information can be found at Supplementary Material.

2.5. Analytical methods

2.5.1. HPLC-UV analysis

Filtered samples of the supernatant were placed in amber HPLC vials to avoid natural photodegradation during the analysis. A Dionex 3000 Ultimate HPLC equipped with UV detector and autosampler Dionex were used. The chromatographic separation was achieved on a LiChrosphere RP-18 (125 mm x 4 mm, 5 µm) LC column (Merck, Barcelona, Spain). The method used for BP3 analysis is extensively described in Gago-Ferrero et al. (2012) and the method for DCF was modified from Marco-Urrea et al. (2010), changing the isocratic mobile phase for a gradient elution. Acetonitrile changes from 35% to 55% in 20 min, then, from 55% to 100% in 5 min, remaining at 100% during 5 minutes and decreasing rapidly to 35% where it is maintained 5 more minutes.

2.5.2. Stable isotope analysis and calculations

Isotope ratios were reported in δ-notation (‰) relative to the Vienna Pee Dee Belemnite standard (V-PDB, IAEA-Vienna). The $\delta^{13}C$ value is defined as

$\delta^{13}C=(Rs/Rr-1)\times1000$, where Rs and Rr are the $^{13}C/^{12}C$ ratios in the sample and V-PDB standard, respectively. To convert $\delta^{13}C$ to atom% ^{13}C, the equation atom% $^{13}C=100/(1/(\ (\delta/1000+1)R_{PDB})+1)$ was used, where δ is the measured $\delta^{13}C$ (‰) of the sample and R_{PDB} is the isotope ratio of V-PDB (R_{PDB}=0.0112372).

2.5.2.1. CO_2 analysis by GC-C-IRMS

$^{13}C/^{12}C$ ratios of headspace CO_2 were determined by a GC–C–IRMS system consisted of an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a split/splitless injector, coupled to a Delta Plus isotope ratio mass spectrometer through a GC-Combustion III interface (ThermoFinnigan, Bremen, Germany). Column specifications and settings are explained in detail in the Supplementary Material.

2.5.2.2. Bulk biomass analysis by EA-IRMS

The carbon isotopic composition of the bulk freeze dried biomass was determined using a Flash EA1112 elemental analyser (EA) coupled to a Delta C isotope ratio mass spectrometer through a Conflo III interface (ThermoFinnigan, Bremen, Germany). Delta values ($\delta^{13}C$) of the samples were corrected using the linear regression derived from three international reference materials (USGS 24, IAEA-CH-6 and IAEA-CH-7) and with respect to the Vienna Pee Dee Belemnite (VPDB) standard according to Coplen et al. (2006).

2.5.2.3. Amino acids SIP analysis by GC-C-IRMS

Carbon isotopic compositions of individual amino acids were determined with the same GC–C–IRMS system described for CO_2. Column, temperature program and other specifications can be found in the Supplementary Material.

2.5.3. Other analyses

Glucose concentration was measured with a biochemical analyser YSI 2700 SELECT (Yellow Spring Instruments) in the concentration range 0-20 ± 0.04 g L^{-1}. Laccase

activity was measured using a modified version of the method for the determination of manganese peroxidase (Kaal et al., 1993) as described elsewhere (Gago-Ferrero et al., 2012). Biomass amount was determined as the constant weight at 100 ºC.

3. Results and discussion

3.1. Mineralization

Under the described experimental conditions, DCF was totally removed from the solution within 3 d (Fig. 1A). As Figure 1B shows, also BP3 concentration decreased quite fast from the liquid the first 3 d, but afterwards a plateau was reached achieving a final removal of 81.2±5.6%. In both cases, negligible removal of DCF and BP3 was observed in the inactivated controls, indicating that removal was not due to sorption. Glucose was completely consumed during the first 3 days and laccase maximum activity was around 20-30 U L^{-1} (Fig. S1).

As shown in Fig. 2A, CO_2 production rate in the experimental bottles reached a peak within the first 3 d of incubation, corresponding with the period of glucose consumption. No significant differences in total CO_2 production were detected between samples containing labelled and unlabelled compounds. The percentage of $^{13}CO_2$ released from the labelled phenyl moieties of ^{13}C-DCF and ^{13}C-BP3 was calculated taking into account the amount of CO_2 produced and the $\delta^{13}C$ values at each time. As shown in Fig. 2B, mineralization of ^{13}C from DCF reached a final percentage of only 11% the ninth day with respect to the initial ^{13}C, while 30% of ^{13}C from BP3 was already detected as CO_2 on day 3 and 45% on day 9. At 6 and 9 d the increase in the labelled ring mineralization was still considerable because CO_2 was much more enriched in ^{13}C ($\delta^{13}C$ is higher) although the production was lower.

The $\delta^{13}C$ values of controls containing sodium azide plus ^{13}C-BP3 were identical to those of controls containing sodium azide with ^{12}C-BP3 indicating that $^{13}CO_2$ production was biotic. However, an unexpected ^{13}C isotopic enrichment of CO_2 was observed in controls inactivated with sodium azide plus ^{13}C-DCF that reached $\delta^{13}C$ values up to 25.9 ± 2.1 at 9 d. However, the percentage of initial ^{13}C-DCF mineralized in these controls was negligible when referred to the low CO_2 emitted in these microcosms. This enrichment was probably produced by the oxidative action of extracellular laccase, not fully deactivated by sodium azide, which was able to remove rapidly DCF from the medium in previous *in vitro* experiments (Marco-urrea et al., 2010). To confirm this aspect, thermically inactivated controls with ^{13}C-DCF were also included and resulted in $\delta^{13}C$ values comparable to inactivated controls containing ^{12}C-DCF.

This is the first study reporting biological mineralization of BP3. Mineralization of other xenobiotics had already been reported for *T. versicolor*: [$^{13}C_2$]-trichloroethylene (Marco-Urrea et al., 2008), [U-^{14}C]-pentachlorophenol (Tuomela et al., 1999) and 2,4',5-[U-^{14}C]-trichlorobiphenyl (Beaudette et al., 1998). However, mineralization percentages in those cases were lower (11% on day 22 for TCB, 29% in 42 d for PCP). On the other hand, other labelled PPCP compounds such as [$^{13}C_6$-phenyl]-sulfamethazine and [$^{13}C_3$-propionic]-ibuprofen, did not show $^{13}CO_2$ production after being degraded by white-rot fungi (García-Galán et al., 2011; Marco-Urrea et al., 2009).

Regarding DCF mineralization, we expected higher mineralization values since degradation rate and removal of identified transformation products occurred more rapidly than in the case of BP3 (Gago-Ferrero et al., 2012; Marco-urrea et al., 2010). Biological mineralization of [$^{14}C_1$-carboxyl]-DCF was previously shown (Al-Rajab et al., 2010) in soils by the indigenous microbiota although it should be noted that the ^{14}C labelled carbon was in the carboxyl group, probably easier to convert to CO_2 than the aromatic ring carbons. In the present work, although only one aromatic ring of each

target compound was labelled, the fact that both aromatic rings are equally oxidized leads to hypothesize that ring cleavage would occur similarly to the whole molecule.

Summarizing, mineralization rate and yield can not be inferred from the removal rate of the parent compound and the main metabolites and experiments on CO_2 isotopic signature are needed to unequivocally confirm mineralization.

3.2. ^{13}C incorporation into fungal biomass by EA-IRMS and amino acid-SIP

A key aspect that remains vaguely explored on the use of fungi in bioremediation is their ability to use xenobiotics as carbon source. Indeed, it is thought that bacteria can outcompete fungus in contaminated sites because the latter cannot obtain energy for growth from contaminants (Harms et al., 2011). The first step, thus, is to determine whether *T. versicolor* can use the xenobiotics as carbon source or not. Afterwards, further experiments should be done to confirm if fungi can grow on them as a sole carbon source. Some experiments have already been done with withe-rot fungi growing on xenobiotics as sole carbon source (Prenafeta-Boldú et al., 2001), but they lacked the information about incorporation of compounds in the anabolic pathways.

The bulk analysis of the $\delta^{13}C$ values of fungal biomass by EA-IRMS showed $\delta^{13}C$ enrichment in both cultures spiked with ^{13}C-DCF and ^{13}C-BP3 in comparison with the $\delta^{13}C$ values observed in the unlabeled controls (^{12}C-BP3 and ^{12}C-DCF) which remained constant over time. A marked shift in the $\delta^{13}C$ enrichment was observed the first 3 d of incubation in cultures spiked with ^{13}C-BP3 (from -21.9 ± 0.3 to -6.7 ± 2.5) and afterwards remained constant. On the other hand, a continuous incorporation of ^{13}C into biomass was observed for cultures containing ^{13}C-DCF, with a $\delta^{13}C$ increasing from -6.4 ± 1.2 at time zero to +28.4 ± 3.9 at 9 days. The carbon isotopic signature in heat killed and sodium azide killed controls spiked with either labelled or unlabelled BP3 and DCF resulted in $\delta^{13}C$ values statistically identical, indicating that ^{13}C-

incorporation in live cultures was not due to passive uptake associated to physico chemical processes such as sorption. The time-course of ^{13}C enrichment into biomass is depicted in Fig. 3 as percentage of ^{13}C atom (at%).

Previous indirect evidences using specific inhibitors suggested a role to cytochrome P450 system in the primary oxidation of DCF and BP3 (Gago-Ferrero et al., 2012; Marco-urrea et al., 2010). This mechanism would imply an active uptake of contaminants through the fungal cell wall and membranes and their further intracellular transformation. In addition, the role of cytochrome P450 in DCF and BP3 degradation by *T. versicolor* was underpinned by the previous identification of hydroxylated and demethylated transformation products typically catalyzed by this intracellular enzymatic system (Gago-Ferrero et al., 2012; Marco-urrea et al., 2010). On the one hand, DCF was rapidly transformed to 4'-hydroxydiclofenac and 5-hydroxydiclofenac by *T. versicolor* and these intermediates disappeared after 24 h from the medium (Marco-urrea et al., 2010) (Fig. S2). Other white-rot fungi, such as *Phanerochaete chrysosporium*, also degrade effectively DCF (Rodarte-Morales et al., 2012) and the metabolites produced by *Phanerochaete sordida* are hydroxylations as well (Hata et al., 2010). On the other hand, fungal BP3 byproducts are 4-hidroxybenzophenone, 2,4- and 4,4'-dihydroxybenzophenone together with glucose and pentose conjugates (Fig. S3). Both hydroxylated and conjugated byproducts of BP3 disappeared from the medium after two weeks of incubation (Gago-Ferrero et al., 2012). Therefore, the observed biomass labelling could be attributed to the intracellular presence of labelled untransformed DCF and BP3 or associated byproducts without implying a final incorporation into anabolic products.

Amino acids-SIP was used to unequivocally demonstrate the transformation of DCF and/or BP3 into anabolic products. In Fig. 4, the amino acid enrichment (as atom % ^{13}C) in cultures of *T. versicolor* spiked with ^{13}C-BP3 compared with controls spiked with ^{12}C-BP3 are presented. Incorporation of ^{13}C was observed in all the target amino acids

since the third day of incubation, except for serine that did not show significant enrichment (0.016 at%) until the sixth day of incubation. Fluctuations in the isotope content over time were attributed to the continuous turnover of amino acids to precursor intermediates that was reflected in the labelling patterns. The extent of enrichment varies between amino acids. The highest ^{13}C incorporation (>0.05%) was observed for alanine, glutamate and aspartate, which can be biosynthesized by transamination of pyruvate and tricarboxylic acid (TCA) cycle intermediates α-ketoglutarate and oxaloacetate respectively. A common degradation pathway of aromatic compounds by white-rot fungi involves the ring cleavage to produce β-ketoadipate and finally succinate and acetyl-CoA, essential compounds of TCA cycle (Wells and Ragauskas, 2012). Therefore, an hypothetical metabolic pathway of BP3 would include i) the ring oxidation by the action of cytochrome P450 system (as previously reported in Gago-Ferrero et al. (2012)), ii) catechol formation and ring cleavage by ortho-fission generating an intradiol, and iii) anabolism precursors formation via β-ketoadipate pathway (Fig. S3). However, these intermediates were not detected by nuclear magnetic resonance analyses during the experiment, probably due to their low concentration in the medium and rapid transformation (data not shown). Interestingly, ^{13}C incorporation of amino acids are in the same range than the global incorporation observed in bulk biomass (0.02 at%), except for glutamate, aspartate, alanine, threonine and proline that showed higher values.

Regarding DCF, no significant ^{13}C incorporation was detected in the amino acids. These results may suggest that DCF (or byproducts) can be accumulated into the cell for being further transformed by the cytochrome P450 system, as previous inhibitory experiments indicated (Marco-urrea et al., 2010) and thus contributing to the high isotopic enrichment observed in the bulk biomass. Therefore, our results indicate that DCF would be mineralized mainly by cometabolic pathways instead of being used as carbon source by *T. versicolor*.

3.3. Calculation of mass balances

A ^{13}C-mass balance of labelled DCF and BP3 was performed across four different sections over time i) ^{13}C remaining in the liquid media as non-transformed parent compound, ii) ^{13}C newly formed byproducts in the media, iii) ^{13}C mineralized as carbon dioxide, iv) ^{13}C incorporated into fungal biomass (Fig. 5). Estimation of byproducts was calculated by subtracting to the initial amount of ^{13}C added in form of parent compound, the remaining non-transformed parent compound, the amount accumulated into biomass and mineralization. With regards to the ^{13}C calculated as incorporated into fungal biomass, it is not possible to distinguish between the ^{13}C assimilated into biomass and ^{13}C deriving from the accumulation of labelled parent compounds and/or byproducts into the fungus cell. However, amino acid-SIP allows us to confirm that BP3 was used as carbon source whereas DCF degradation might proceed via cometabolism or detoxification mechanisms. As observed in Fig. 5B, DCF could be rapidly transformed to intermediates that would be slightly removed from the media with a gradual accumulation into the fungal biomass. At the end of the incubation, ~ 90% of DCF remained either as byproduct or accumulated into the cells. As observed in Fig. 5A, mineralization of BP3 did not reach a plateau, suggesting that higher mineralization rates could be reached if cultures were incubated longer. The mass balance fits well with previous evidences of BP3 degradation by *T. versicolor*, since 30% of the initial BP3 was reported to be in the medium as glycoconjugate the ninth day of incubation (Gago-Ferrero et al., 2012).

3.4. Conclusions

The use of amino acid-SIP and EA-IRMS techniques allowed us determining whether the fungus used the tested emerging contaminants as carbon source for e.g. amino

acid biosynthesis or, on the contrary, the elimination process occurred via alternative mechanisms. The use of these techniques are relevant for white-rot fungi due to the complexity of the enzymatic system involved in the transformation of contaminants that include i) cometabolism (ligninolytic enzymes, i.e. laccases and peroxidises), ii) detoxification mechanism (i.ecytochorme P450), and iii) metabolism (i.e. via lipid or amino acid synthesis). By using amino acid-SIP, unequivocal use of xenobiotics in metabolism can be proved, and it is more reliable than indirect evidences such as the removal of the target compound when added as sole carbon source (Prenafeta-Boldú et al., 2001), mineralization of xenobiotics by measuring isotopic signature of emitted CO_2 (Tuomela et al., 1999), or disappearance of both parent compounds and byproducts (Marco-Urrea et al., 2010). In all, the use of amino acid-SIP can shed light on the fate of xenobiotics during white-rot fungi degradation and help to improve bioremediation strategies using these organisms.

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Figure 1. Time course degradation of DCF (A) and BP3 (B). Symbols: uninoculated controls with ^{12}C-DCF/^{12}C-BP3 (●), heat-killed controls with ^{13}C-DCF (■), controls containing sodium azide with ^{13}C-DCF/^{13}C-BP3 (▼), experimental cultures with ^{12}C-DCF/^{12}C-BP3 (○) and experimental cultures with ^{13}C-DCF/^{13}C-BP3 (Δ).

Figure 2. A) Carbon dioxide produced every three days in cultures of *T. versicolor* spiked with DCF. Symbols: controls containing sodium azide with ^{12}C-DCF (●) and ^{13}C-DCF (○); experimental cultures with ^{12}C-DCF (▼) and ^{13}C-DCF (Δ). B) Cumulative $^{13}CO_2$ production in cultures of *T. versicolor* spiked with either ^{13}C-BP3 (●) or ^{13}C-DCF (○).

Figure 3. Carbon stable isotopic composition (atom%) of the fungal biomass spiked with either DCF or BP3. Symbols: experimental cultures spiked with ^{12}C-BP3 (●), ^{12}C-DCF (▼), ^{13}C-BP3 (○), and ^{13}C-DCF (Δ).

Figure 4. Carbon stable isotopic composition (atom%) of the amino acids in cultures spiked with ^{12}C-BP3 and ^{13}C-BP3 at different experimental times. Values were corrected for the carbon introduced during derivatization.

Figure 5. Mass balance of ^{13}C in BP3 (A) and DCF (B) experiments. In black non-transformed ^{13}C-BP3/^{13}C-DCF remaining in the media, in dark grey estimated ^{13}C remaining in the media in form of byproducts to complete the mass balance, in light grey ^{13}C into fungal biomass, and in white ^{13}C mineralized ($^{13}CO_2$).