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Biodegradation of carbamazepine and clarithromycin by *Trichoderma harzianum* and *Pleurotus ostreatus* investigated by liquid chromatography – high-resolution tandem mass spectrometry (FTICR MS-IRMPD)

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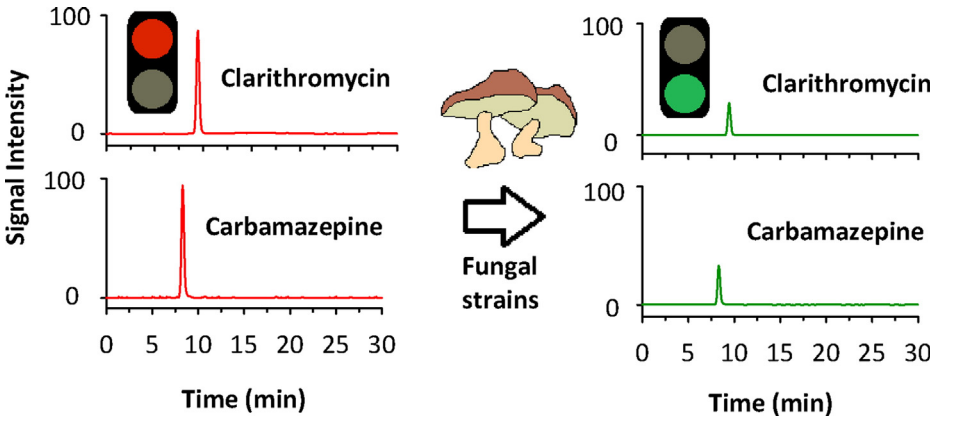
HIGHLIGHTS

- Pharmaceuticals biodegradation of fungus Trichoderma harzianum was ascertained.
- T. harzianum degraded carbamazepine and clarithromicyn in aqueous-aerobic conditions
- LC-ESI/FT-ICR MS allowed to identify some unknown pharmaceutical metabolites

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the capability of pharmaceutical biodegradation of fungus *Trichoderma harzianum* was evaluated through the comparison with the well-known biodegradation capability of white-rot fungus *Pleurotus ostreatus*. The study was performed in aqueous phase under aerobic conditions, using two of the most frequently detected drugs in water bodies: carbamazepine and clarithromycin, with concentrations commonly found in treated wastewater (4 μg/l and 0.03 μg/l respectively). For the first time, we demonstrated that *T. harzianum* is able to remove carbamazepine and clarithromycin. The analyses were performed by reversed-phase liquid chromatography/mass spectrometry, using high-resolution Fourier-transform ion cyclotron resonance mass spectrometry upon electrospray ionization in positive ion mode. The high selectivity and mass accuracy provided by high-resolution mass spectrometry, allowed us to identify some unknown metabolites. On the basis of our study, the major metabolites detected in liquid culture treated by *T. harzianum* were: 14-hydroxy-descladinosyl- and

Abbreviations: RPLC-ESI-FTICRMS, reversed phase liquid chromatography-electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry; XIC, extracted ion chromatogram; SPE, solid phase extraction; WWTP, wastewater treatment plant.

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Pleurotus ostreatus
Trichoderma harzianum
HPLC-HRMS

descladinosyl-clarithromycin, which are pharmacologically inactive products not dangerous for the environment.

1. Introduction

Nowadays the consumption of pharmacologically active substances to treat illness and prevent diseases is steadily increasing, up to thousands of tons per year (Kosjeka et al., 2005) and their occurrence as unmetabolized substances or as active metabolites, is more and more frequent in wastewater, rivers, lakes, and hence in drinking water (Zuccato et al., 2000). The uncontrolled presence of biologically active substances in the environment is worrying and dangerous. All the species target and non-target organisms will face the same bioactive properties that are used in therapeutic applications. For these reasons, the pharmaceutical investigation is attracting attention and interest from the scientific community, in order to define and assess the real environmental risks (Kümmerer, 2009). Although the pharmaceutical compounds and their metabolites are present in trace concentrations in water bodies (ng/l or μg/l), which are at least three to four orders of magnitude lower than those required to produce pharmacological effects (Halling-Sorensen et al., 1998), bioaccumulation and biomagnification processes lead to an increase in detectable biologically active substances, with toxic effects for both fauna and flora (Christensen, 1998).

Among the pharmaceuticals most frequently detected in the aquatic environment are carbamazepine, an antiepileptic drug, and clarithromycin, an antibiotic, selected as the target molecules in this study (Zhang and Geißen, 2010; Calza et al., 2012). Carbamazepine, 5H-dibenzazepine-5-carboxamide, is an iminostilbene derivate with a tricyclic structure largely used for the treatment of epilepsy (Fertig and Mattson, 2008). Again, clarithromycin (6-*O*-methyl erythromycin) belongs to the group of macrolide antibiotics and it is one of the most prescribed drugs in human medicine (Kummerer and Henninger, 2003). Carbamazepine and clarithromycin have always aroused great interest as environmental pollutants for their high persistence in various environmental matrices (Zhang et al., 2008; McArdell et al., 2003) and their partial or no degradation during wastewater treatment (Clara et al., 2005; Joss et al., 2005). The incomplete removal by processes of mechanical and chemical treatments (Gros et al., 2007; Miao et al., 2004) results in quite high concentration levels of these contaminants, ranging from ng/l up to μg/l in surface water or wastewater (Jelic et al., 2011; Calamari et al., 2003) and at low ng/l in drinking water (Benotti et al., 2009; Ye et al., 2007). Moreover, bioassays performed on bacteria, algae and micro-crustaceans proved that carbamazepine and clarithromycin are among the most hazardous compounds for the aquatic environment (Ferrari et al., 2003; Isidori et al., 2005). Once in the receiving water bodies, possible adverse effects of carbamazepine on aquatic organisms such as the blockade of pupation were reported (Oetken et al., 2005). The removal of clarithromycin in WWTPs varies considerably ranging from its almost total disappearance (Gracia-Lor et al., 2012) to lower degradation levels (0–80%) (Verlicchi et al., 2012). Carbamazepine is even more persistent than clarithromycin, and its removal efficiencies by the WWTPs are mostly below 10% (Zhang et al., 2008). The highest removal efficiency for carbamazepine in a WWTP was observed by Paxéus (2004), equal to 53%.

The removal inefficiency of pharmaceuticals by conventional water treatments has encouraged an interest to develop efficient techniques for achieving the total destruction of contaminants, by using biodegradation of microorganisms or physical-chemical treatments such as ozonation and phototransformation (Poyatos et al., 2010) with high degradation percentages (>90%) (Esplugasa et al., 2007) but with a limitation of the formation of undesirable and toxic by-products.

Several groups of microorganisms, such as white-rot fungi, have the potential to be useful biocatalysts due to their high specificity to attack substrates, through the action of intracellular and extracellular enzymes. This versatility in degrading a wide variety of xenobiotics makes these microorganisms potentially useful in bioremediation applications (Marco-Urrea et al., 2009; Marco-Urrea et al., 2010). The ability of white-rot fungi to degrade carbamazepine and clarithromycin has already been demonstrated in several experiments. Jelic et al. performed degradation experiments in Erlenmeyer flasks and evaluated the percentage of degradation of carbamazepine by *Trametes versicolor* after 6 days (Jelic et al., 2012). Golan-Rozen et al. studied the degradation capability of three *Pleurotus ostreatus* strains and concluded that it is more efficient at removing carbamazepine than other fungi (Golan-Rozen et al., 2011). In addition, numerous studies have proved that white-rot fungi are useful as potential agents for the degradation of a large number of pharmaceuticals, including clarithromycin, at environmentally relevant concentrations in sewage sludge (Rodríguez-Rodríguez et al., 2011). The use of white-rot fungi, such as *Pleurotus ostreatus* or *Trametes versicolor*, for removing pharmaceuticals also presents some advantages not associated with other bioremediation systems. Indeed, white-rot fungi can simultaneously degrade complex mixtures of contaminants because a part of the enzymes useful in the degradation is produced constitutively while others are produced after induction by nutrient privation (Muñoz et al., 1997). Studies concerning the use of *T. harzianum* for the biodegradation of pharmaceuticals has not been reported. *T. harzianum* is one of the most abundant culturable fungi and it is found in many soil types thanks to its ability to colonize the roots of most plant species (Harman et al., 2004).

In the present study, the degradation of carbamazepine and clarithromycin by *T. harzianum* was assessed in aqueous phase under aerobic conditions through the comparison with the well-known biodegradation capability of *P. ostreatus*. Moreover, the formation of several

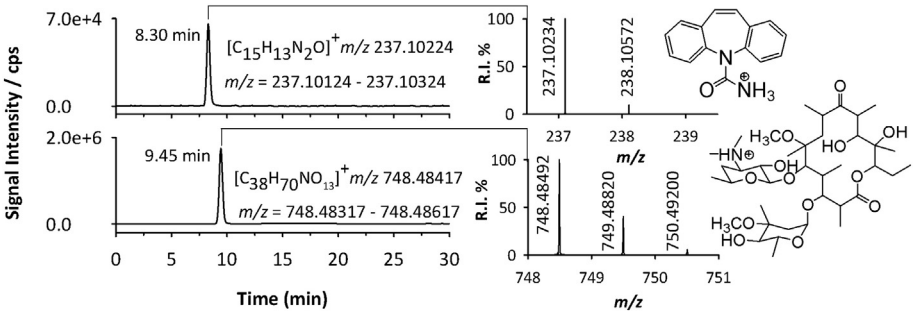


Fig. 1. XICs by LC/ESI-FTICR acquired in positive mode of standard solution containing carbamazepine and clarithromycin using a restricted windows of ±0.0010 *m/z* unit centered around exact mass value of both pharmaceuticals. In the insets high-resolution ESI-FTICR mass spectrum and structures of protonated molecules are showed.

potential transformation products was investigated. To accomplish this, we used liquid chromatography coupled with high-resolution mass spectrometry.

2. Experimental

2.1. Chemicals

Methanol (MeOH), acetonitrile (ACN) and formic acid used for sample pretreatment and chromatography separations had an analytical or LC-MS grade and were purchased from Sigma Aldrich (Schnelldorf, Germany). Analytical standards of carbamazepine and clarithromycin were purchased from Sigma Aldrich (Schnelldorf, Germany). Ultrapure water was produced using a Milli-Q RG system from Millipore (Bedford, MA, USA). Stock solutions of carbamazepine and clarithromycin were prepared by dissolving the substances in methanol at a concentration of 1 mg/l and stored at −20 °C. Standard solutions for LC-MS analyses were prepared by diluting the stock solution to the desired concentration with ACN/H_2O (70/30, v/v). Five calibration solutions over the range (0.005 μg/l–5 μg/l) of desired concentrations were prepared by appropriate dilutions of the calibration stock solution. Pure nitrogen (99.996%) was delivered to the LC-MS system as sheath gas. The ion-trap pressure was maintained with helium 99.999%, which was used for trapping and for collisional activation of the trapped ions.

2.2. Fungal strains, growth conditions and sample preparation

Samples of 40-day-old pure *P. ostreatus* and *T. harzianum* strain T-22 (T22) were separately cultured in Petri dishes filled with solid Potato Dextrose Agar (PDA; Oxoid Ltd., Cambridge, UK). The same culture medium was used for growing both fungal strains to avoid any matrix effect and to guarantee a true comparison between the two species. To prevent bacterial growth, the samples were implemented with 0.03 mg/ml streptomycin (insufficient amount for antifungal activity) and 0.02 mg/ml tetracycline and incubated for 9 days at 25 °C. Both the fungal samples were separately transferred in liquid Potato Dextrose Broth (PDB; Oxoid Ltd.), pH 7.6, and implemented with 0.03 mg/ml streptomycin and 0.02 mg/ml tetracycline. At the end, fungal samples were incubated for 7 days at 25 °C on a rotary shaker at 100 rpm. After incubation, the liquid medium was filtered through two layers of Whatman No. 1 filter paper (Whatman Ltd., Maidstone, UK) to remove hyphal fragments. Ten-fold serial dilutions of the two liquid cultures were prepared in a sterile 1/4 strength Ringer solution (NaCl 2.25 g/l, KCl 0.105 g/l, $CaCl_2$ 0.045 g/l, $NaHCO_3$ 0.05 g/l, and citric acid 0.034 g/l) to avoid osmotic shock, and sonicated for 2 min to disperse fungal hyphae and cells. Aliquots were inoculated in triplicate on 1/10 strength solid Malt Extract Agar (MEA; PDB; Oxoid Ltd.), containing 0.03 mg/ml streptomycin and 0.02 mg/ml tetracycline, for fungal counting. Fungal counting took place after 72 h at 25 °C (Lorch et al., 1998). At the same time, aliquots (100 ml) of liquid broth (PDB) were added to 900 ml of 1/10 strength Murashige and Skoog liquid medium without vitamins (Sigma-Aldrich®, St. Louis, MO, USA) implemented with 5 g/l glucose. Aliquots (50 ml) of this second liquid medium, with and without fungi, were poured in 125 ml glass sealed containers and divided in three groups (12 containers with *P. ostreatus*, 12 with *T. harzianum*, and 12 uninoculated controls). Each group of 125 ml glass containers were divided in three sub-groups in duplicates, containing respectively: a) 4.0 μg/l carbamazepine, b) 0.03 μg/l for clarithromycin, c) 4.0 μg/l carbamazepine + 0.03 μg/l for clarithromycin, d) 10 mg/l carbamazepine, e) 10 mg/l clarithromycin, and f) 10 mg/l carbamazepine + 10 mg/l clarithromycin. The 125 ml glass containers were oxygenated for 1 min sealed and incubated at 25 °C on a rotary shaker at 100 rpm in the dark for 15 days.

Degradation, at a specified interval (at 7 and 15 days), was evaluated by comparing the concentrations in the uninoculated controls with that in the experimental flasks. Carbamazepine and clarithromycin were extracted from water samples by solid-phase extraction (SPE) (OASIS HLB cartridges, 6 cm^3, 0.2 g, Waters, Milford, MA, USA) (Gros et al., 2009). In detail, each SPE cartridge was conditioned with 6 ml of

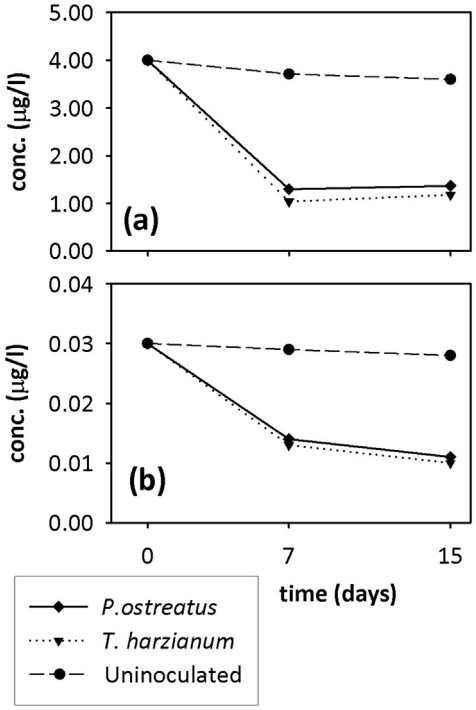


Fig. 2. Comparison of (a) carbamazepine concentrations of water samples inoculated with *P. ostreatus* and T. *harzianum*, and (b) clarithromycin concentrations of water samples inoculated with *P. ostreatus* and T. *harzianum*. Values plotted are means for duplicate cultures.

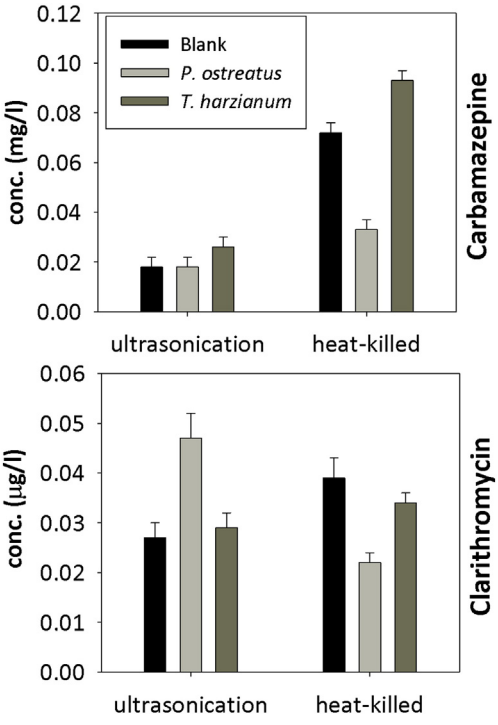


Fig. 3. Comparison of the concentrations of carbamazepine and clarithromycin after ultrasonication and heat-killed. Cell disruption treatments were performed on SPE cartridges employed for the extraction of both pharmaceuticals from the cultures with initial concentration of 10 mg/l after 7 days of incubation. Values plotted are means for duplicate cultures.

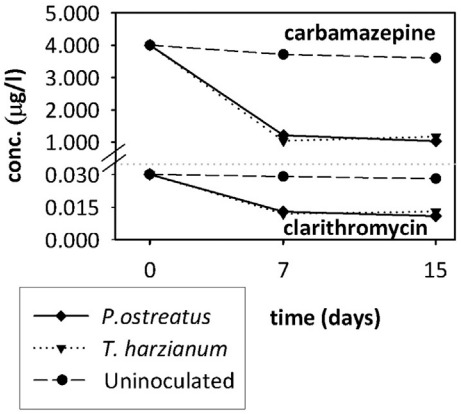


Fig. 4. Comparison of both pharmaceutical concentrations of water samples inoculated with *P. ostreatus* and *T. harzianum*.

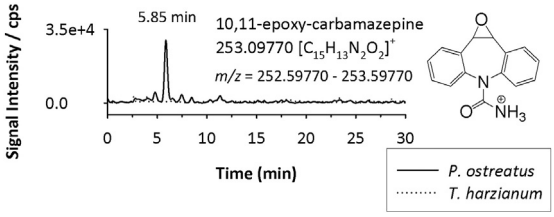


Fig. 6. Extracted ion chromatogram (XIC) using LC/ESI-FTICR of 10,11-epoxy-carbamazepine in 10 mg of carbamazepine water samples inoculated for 7 days with *P. ostreatus* or *T. harzianum*.

MeOH and equilibrated with 5 ml of ultra-pure water (Milli-Q RG system). The water sample (20 ml) was loaded on the SPE cartridge to allow absorption of carbamazepine and clarithromycin on the sorbent phase. After washing the SPE cartridge with 5 ml of ultra-pure water, the analytes were eluted from the cartridge with 5 ml (×2 times) of MeOH, while fungal cells remained absorbed in the cartridge. The methanol eluate was evaporated to full dryness under a gentle nitrogen flow, re-dissolved in 1 ml of MeOH and filtered by 0.2 μm PTFE membrane (Whatman, Maidstone, UK) before LC-MS analysis. All the SPE extractions were performed in duplicate.

The two sets of SPE cartridges employed for the extraction of each fungal water sample at 10 mg/l of carbamazepine and clarithromycin, after elution with MeOH, underwent two comparative methods of fungal cell disruption, i.e. ultrasonication and heat-killed, in order to evaluate the amount of pharmaceutical adsorbed by fungi (Klimek-Ochab et al., 2011).

When the ultrasonication method was employed, the SPE sorbent phase was removed from the cartridge and sonicated in 10 ml of pure MeOH for 30 s. Suspended cells were cooled in an ice bath for 2 min and an additional sonication cycle was performed. The total time of sonication was 5 min. The extract obtained was centrifuged at 6500 rpm (3900 g) at 4 °C for 15 min (Kontron A8.24 rotor centrifuge); the supernatant was filtered by 0.2 μm PTFE membrane, evaporated to full dryness under a gentle nitrogen flow and re-dissolved in methanol (1 ml) before LC-MS analysis. When the heat-killed cell-free disruption procedure was employed, the second set of SPE cartridge was autoclaved at 121 °C for 30 min. The sorbent phase was washed with 5 ml (×2) of methanol, while the eluate was concentrated to full dryness under a gentle nitrogen flow and the cell free-extract was re-dissolved in methanol, filtered and analyzed.

2.3. ESI-LTQ-FTICR MS instrumentation and separation conditions

Chromatographic experiments were performed using a Surveyor LC system coupled to hybrid linear trap/7-T FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LC separation was performed at ambient temperature on an Accucore-150-C18 column (150 × 4.6 mm i.d., 2.6 μm particle size), equipped with Accucore-150-C18 Defender Guard Cartridges (10 × 4.6 mm i.d., particle size 2.6 μm, Thermo Scientific, USA). Both LC-MS water and acetonitrile (ACN), A and B solvents, respectively, were added with 0.1% formic acid. Initial conditions of the gradient profile were 70%:30% (A:B, v/v) and were brought to 25%:75% (A:B, v/v) in the first 14 min. The gradient profile was then increased to 5% of solvent A and 95% of solvent B in the next 2 min and held constant for 4 min. It was finally changed to 0%:100% (A:B, v/v) in the next minute. Afterwards, the starting conditions were restored in 4 min and the column was reconditioned for 5 min. The flow rates were 0.5 ml/min in the column and 125 μl/min in the ESI source (split 4:1). 20 μl of each sample was injected into the chromatographic column. Positive ion ESI-MS was chosen for the detection of the analytes. The source voltage was set at +4.5 kV, temperature of the heated capillary was set at 350 °C. While, capillary voltage and tube lens were set at +5 V and +10 V respectively. The sheath gas (N_2) flow rate was 5 arbitrary units (a.u.). Full-scan MS experiments were performed in the ICR cell in the range *m/z* 50–1500. The laser was mounted vertically on the back of the magnet, downstream of the FTICR cell. In the upper part of the electronics cabinet at the rear side of the instrument, a mirror unit deflects the laser beam into the magnet bore. The ion trap and FT-ICR calibration were carried out using a solution of Ultramark mix (*m/z* 1122–1822), MRFA (*m/z* 524) and caffeine (*m/z* 195). Mass spectra were acquired as profile data at a resolution

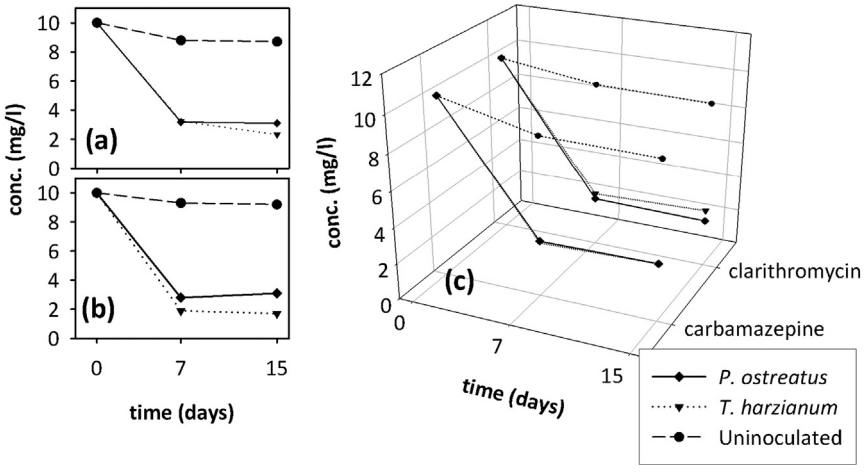


Fig. 5. Comparison of (a) carbamazepine, (b) clarithromycin and (c) both pharmaceutical concentrations of water samples inoculated with *P. ostreatus* and T. *harzianum*.

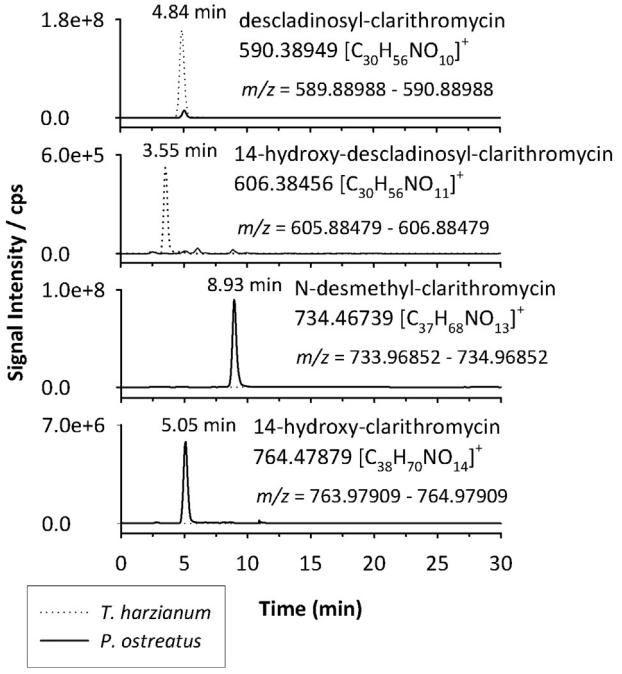


Fig. 7. Extracted ion chromatograms using LC/ESI-FTICR of major metabolites in 10 mg of clarithromycin water samples inoculated for 7 days with *P. ostreatus* or *T. harzianum*.

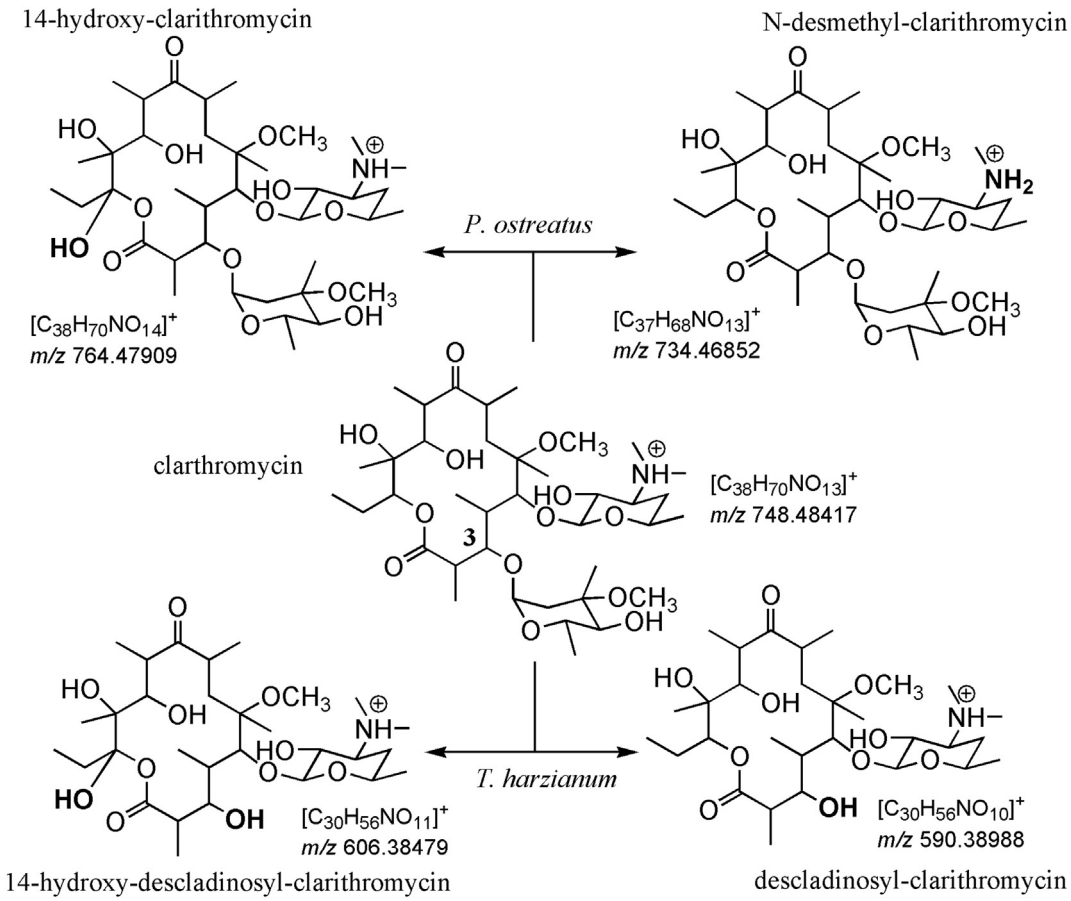
of 100,000 (FWHM) at *m/z* 400. The automatic gain control (AGC) ion population target in full scan MS was 5,000,000 for FTICR MS. The maximum ion injection time was 1000 ms for FTICR. Averages of *m/z* measurements of five LC runs and mass measurement accuracy were reported in terms of root-mean-square (RMS) for each ion (i.e., both precursor and product ions). Data acquisition and analyses were accomplished using the Xcalibur software package (version 2.0 SR1 Thermo Electron). The chromatographic raw data were imported, elaborated and plotted by SigmaPlot 11.0 (Systat Software, Inc., London, UK).

3. Results and discussion

3.1. RPLC-ESI-FTICRMS of carbamazepine and clarithromycin

LC-ESI-(+)-FT-ICR-MS separation of carbamazepine (CBZ) and clarithromycin (CLT) is shown in Fig. 1. Thanks to the high mass accuracy capability of the FT-ICR trapping cell, very selective XICs were generated with a tight mass-to-charge ratio window of ±0.0010 units around each targeted molecule (i.e., $[M + H]^+ \pm 1.0$ mDa).

ESI-FTICR mass spectra of both pharmaceuticals, collected at the apex of the chromatographic peak, exhibit $[M + H]^+$ as the most abundant ion, at *m/z* 237.10234 ($[C_{15}H_{12}N_2O + H]^+$ (A + 0), exact *m/z* value 237.10224, error 0.45 ppm) for carbamazepine and at *m/z* 748.48492 ($[C_{38}H_{69}NO_{13} + H]^+$ (A + 0), exact *m/z* value 748.48417, error 1.22 ppm) for clarithromycin, along with isotopic peaks (Fig. 1). Thanks to accurate values of *m/z*, it was possible to assign to each isotopic peak the chemical composition, with an error lower than 2.0 ppm (see Supplementary Material, Fig. S1). To evaluate the formation of undesired byproducts and/or potential metabolites, fragmentation behavior of carbamazepine and clarithromycin was investigated by high resolution FT-ICR tandem mass spectrometry performed by infrared multiphoton dissociation (IRMPD) (see Supplementary Material, Fig. S2). The figures of merit of the proposed method were evaluated: the values of the detection limit were in the range between 0.003 μg/l and 0.03 μg/l, a



Scheme 1. Degradation by-products of clarithromycin by *P. ostreatus* and *T. harzianum*.

good linearity (r^2 not lower than 0.9996 for both drugs) and a broad linear range (0.05–5 μg/l and 0.001–10 mg/l for carbamazepine and 0.005–0.5 μg/l and 0.001–10 mg/l for clarithromycin, respectively) were obtained.

Intraday RSD values (1.8% for carbamazepine and 2.8% for clarithromycin) and inter-day values (3.3% and 6.3%, respectively) indicated satisfactory results.

3.2. Degradation of carbamazepine and clarithromycin by Tricoderma harzianum

As reported in the introduction section, the degradation of carbamazepine and clarithromycin by *T. harzianum* was assessed through the comparison with the well-known biodegradation capability of *P. ostreatus* (Golan-Rozen et al., 2011). The results of the experiments at the lower initial concentrations (4 μg/l and 0.03 μg/l for carbamazepine and clarithromycin, respectively) are reported in Fig. 2. An overlapping of concentration trends for both carbamazepine and clarithromycin samples, treated with *P. ostreatus* and *T. harzianum*, was observed. The removal efficiencies, calculated on the seven day water samples, are equal to 68 and 72% for carbamazepine, treated with *P. ostreatus* and *T. harzianum*, respectively, and 55 and 57% for clarithromycin.

It is possible to observe (Fig. 2) that both fungi completed almost all their biodegrading activity within the first seven days. Indeed, after 15 days, no further decrease of concentration of the target molecules could be observed. For the first time, biodegradability performance of *T. harzianum* was evaluated and it has proved to be comparable with that of white-rot fungi (*T. versicolor* showed a percentage of degradation of around 57% for carbamazepine (Marco-Urrea et al., 2009)). In order to demonstrate that the removal capacity already shown in Fig. 2 is not due to sorption only, but reflects the actual amount of pharmaceuticals degraded, we compared the concentrations obtained from both methods of fungal cell disruption (ultrasonication and heat-killed) with the corresponding blanks. Fig. 3 shows that each pharmaceutical concentration does not significantly differ ($\alpha = 0.05$) from the corresponding blank.

These experimental results are very promising concerning the degradation ability of *T. harzianum* and its employment in remediation of wastewater and their reuse. In fact, our attention has been focused on this species of fungus for its features, very useful in order to potentially reuse contaminated water. Indeed, the concentrations tested in this study are approximately those present in the effluents of municipal wastewater treatment plants (Zuccato et al., 2000). In particular, *T. harzianum* is a filamentous fungus whose presence has been ascertained in nearly all soils, where it also exerts beneficial plant growth promoting activity. This could suggest, in the best case, a possible wastewater reuse for irrigation of soils containing this fungus, without a previous decontamination treatment. As already mentioned, white-rot fungi are able to simultaneously degrade more pollutants (Muñoz et al., 1997). In order to ascertain this behavior also for *T. harzianum*, we performed an additional experiment on a water sample containing both pharmaceuticals. Again, *T. harzianum* showed a similar degradation ability compared to *P. ostreatus*, as shown in Fig. 4. The initial amount of pharmaceuticals was degraded after 7 days and no further decrease of the concentration was observed.

3.3. Identification of degradation products of carbamazepine and clarithromycin

LC-ESI(+)-FTICR MS allowed the identification of putative metabolites of carbamazepine and clarithromycin produced by both fungi under study. To identify the molecular ions of the by-products of the two pharmaceuticals, extracted ion chromatograms (XICs) were used, starting from the exact values of mass to charge ratio (m/z) of all putative metabolites. We show the results of the metabolic study carried out by employing water samples with high pharmaceutical concentrations (10 mg/l), conditions in which the fungi exhibited the highest removal ability (Golan-Rozen et al., 2011), as reported in Fig. 5.

The metabolite identification is attracting increasing interest since many of the degradation products are still bioactive substances and hazardous for the environment. Therefore, often not only the parent compound should be the subject of a risk assessment but also the active metabolites.

Several studies demonstrated that the CYT P450 is the enzymatic mechanism employed in the degradation of carbamazepine and other pharmaceuticals by white-rot fungi. The oxidative action of extracellular fungal enzymes, in liquid medium, was demonstrated by comparing the degradation rates obtained with and without CYT P450 inhibitors (Marco-Urrea et al., 2009; Golan-Rozen et al., 2011). The major metabolite, among the oxidation products, detected in all treatments of carbamazepine with white-rot fungi is 10,11-epoxy-carbamazepine. It is worthwhile to note that 10,11-epoxy-carbamazepine is a pharmacologically active drug (Tomson and Bertilsson, 1984) and its presence in water bodies is undesirable (Miao et al., 2005). Usually, epoxides obtained by oxidation of polycyclic aromatic hydrocarbons are unstable and rapidly transformed to hydroxy derivates or dihydrodiols (Cajthaml et al., 2002), produced also by human liver (Larraya et al., 1999). Fortunately, it is already reported that *P. ostreatus* is able not only to oxidize carbamazepine to epoxide, but also to continue its metabolism up to diols (Golan-Rozen et al., 2011). Otherwise, in samples with high carbamazepine concentrations (i.e. 10 mg/l) the accumulation of 10,11-epoxy-carbamazepine can be observed (Fig. 6).

In this study, 10,11-epoxycarbamazepine was never detected in water samples inoculated with *T. harzianum*. Fig. 7 shows the major putative metabolites of clarithromycin produced by *P. ostreatus* and *T. harzianum* biodegradation. The degradation processes of the white-rot fungus overlaps the oxidative pathway proposed for human liver (Rodrigues et al., 1997). Both oxidative metabolisms employ the CYT P450 and give 14-hydroxy-clarithromycin and *N*-desmethyl-clarithromycin as the major metabolites (Scheme 1). Among these oxidative products, 14-hydroxy-clarithromycin is still a pharmacologically active molecule, whereas *N*-desmethyl-clarithromycin is described to be inactive (Baumann et al., 2015). However, the metabolites generated by *T. harzianum* degradation are both inactive pharmacologically and characterized by the cleavage of the glycosidic bond and the loss of L-cladinose moiety at position 3 (Scheme 1).

The formation of descladinosyl and 14-hydroxy-descladinosyl-clarithromycin could be considered a confirmation of the involvement of hydrolytic enzymes produced by *T. harzianum*. In conclusion, these results have shown that the use of *T. harzianum* is more suitable for clarithromycin degradation compared to *P. ostreatus*. Indeed, *T. harzianum* ensures the formation of degradation products pharmacologically inactive and not dangerous for the environment, in addition to a degradation capability comparable to that of *P. ostreatus*.

4. Conclusions

This is one of the first studies reporting the ability of *T. harzianum* to degrade carbamazepine and clarithromycin under concentrations commonly found in treated wastewater reused for irrigation (4 μg/l and 0.03 μg/l respectively). Results suggest that the *T. harzianum* could be useful for the degradation of both pharmaceuticals. Thanks to the high selectivity and mass accuracy provided by reversed-phase liquid chromatography coupled with high-resolution Fourier-transform ion cyclotron resonance (FTICR) MS, we were able to identify some unknown metabolites produced by the degradation processes of the two fungi under study.

Our experimental data suggest that the degradation of carbamazepine and clarithromycin occurred intracellularly and no sorption processes were involved.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.03.119.

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Electronic Supplementary Material

Biodegradation of carbamazepine and clarithromycin by *Trichoderma harzianum* and *Pleurotus ostreatus* investigated by liquid chromatography-high resolution tandem mass spectrometry (FTICR MS-IRMPD)

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Number of Figures:2;

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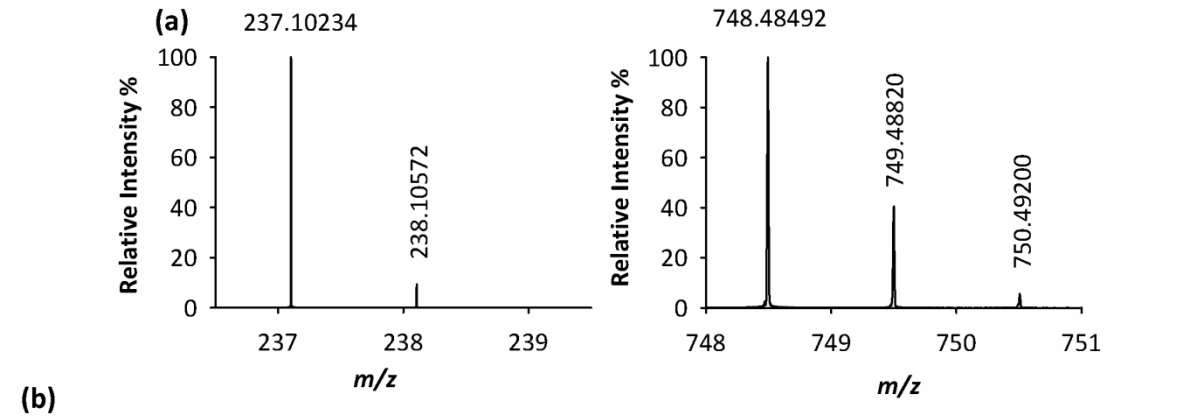

(b)

m/z	Relative Intensity %	Composition
237.10224	100	$C_{15}H_{13}N_2O^+$
238.09927	0.74	$C_{15}H_{13}N^{15}NO^+$
238.10559	16.22	$C_{14}{}^{13}CH_{13}N_2O^+$
238.10852	0.15	$C_{15}H_{12}{}^2HN_2O^+$
239.10263	0.12	$C_{14}{}^{13}CH_{13}N^{15}NO^+$
239.10649	0.21	$C_{15}H_{13}N_2{}^{18}O^+$
239.10895	1.23	$C_{13}{}^{13}C_2H_{13}N_2O^+$

m/z	Relative Intensity %	Composition
748.48417	100.00	$C_{38}H_{70}NO_{13}{}^+$
749.48120	0.37	$C_{38}H_{70}{}^{15}NO_{13}{}^+$
749.48752	41.10	$C_{37}{}^{13}CH_{70}NO_{13}{}^+$
749.48838	0.50	$C_{38}H_{70}NO_{12}{}^{17}O^+$
749.49044	0.81	$C_{38}H_{69}{}^2HNO_{13}{}^+$
750.48456	0.15	$C_{37}{}^{13}CH_{70}{}^{15}NO_{13}{}^+$
750.48841	2.67	$C_{38}H_{70}NO_{12}{}^{18}O^+$
750.49088	8.22	$C_{36}{}^{13}C_2H_{70}NO_{13}{}^+$
750.49174	0.20	$C_{37}{}^{13}CH_{70}NO_{12}{}^{17}O^+$
750.49380	0.33	$C_{37}{}^{13}CH_{69}{}^2HNO_{13}{}^+$

Figure S1.

The carbamazepine isotopic peak at *m/z* 238.10572 was assigned to $[C_{14}{}^{13}CH_{12}N_2O+H]^+$ (A+1, exact m/z value 238.10559, error 0.57 ppm) and the clarithromycin isotopic peaks at *m/z* 749.48820 and at *m/z* 750.49200 were assigned to $[C_{37}{}^{13}CH_{69}NO_{13}+H]^+$, (A+1, exact m/z value 749.58752, error 1.90 ppm) and $[C_{36}{}^{13}C_2H_{69}NO_{13}+H]^+$ (A+2, exact m/z value 750.49088, error 1.63 ppm), respectively. The relative intensities (%) evaluated within the signal-intensity precision of the mass spectrometer which was typically up to about ±10 % were in good agreement with reported those of the simulated spectra (**Figure S1**).

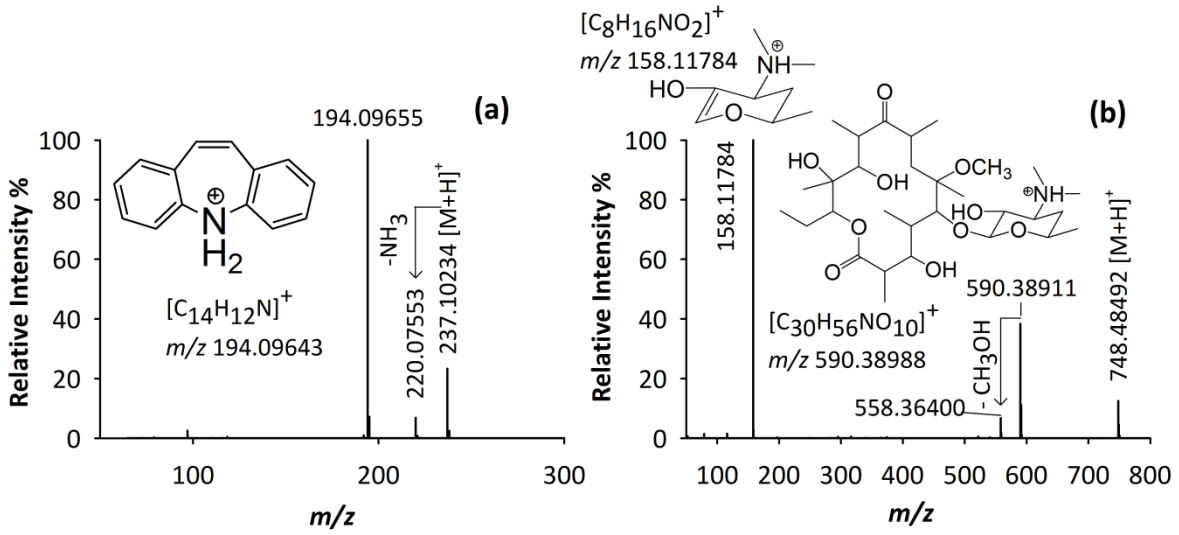

Figure S2.

Precursor ion isolation of mono-protonated adducts, at *m/z* 237.1 and at *m/z* 748.5, were performed with a relatively wide window of 6 m/z, which included all main isotopologues ions. For each target molecule, the IRMPD conditions, in terms of laser power and duration pulse, were optimized in order to obtain the best improving S/N ratio. In particular, 100% of power by a 20 W CO_2 laser source and 150 ms of duration pulse for carbamazepine and 50 ms for clarithromycin, were applied to obtain effective gas-phase fragmentation.

The product ion spectra after photon irradiation of protonated adducts are shown in Figure 2. The high resolution MS and mass accuracy allowed the identification of the molecular formula of fragments along with the determination of the chemical composition of closely related species. The fragmentation conditions employed generate only product ions at *m/z* 220.07553 and at *m/z* 194.09655 for carbamazepine. The identity and the formation of all product ions was proposed. Specifically, the product ion at *m/z* 220.07553 is presumably originated by the loss of ammonia (17.02655 Da) and the base peak at *m/z* 194.09655 may be due to the neutral loss of formic acid (73.94410 Da) and subsequent formation of

iminostilbene residue. The electron delocalization on the iminostilbene moiety confers high stability to the molecule and prevents it from being extensively fragmentated.

Also as regards the fragmentation pathway of clarithromycin, it not involve the large macrocyclic lactone ring. The IRMPD FT-ICR tandem mass spectrum revealed three fragment ions at *m/z* 590.38911, 558.36400 and 158.11784. The fragment ion at *m/z* 590.38911 is generated to the loss of L-cladinose moiety at position 3. This fragment ion could undergo loss of a small neutral molecule of methanol (CH_3OH 32.02621 Da) to form the ion at *m/z* 558.36400. The clarithromycin can undergo also the loss of the second sugar moiety. During the cleavage of glycosidic bond in position 5, the charge is retained on the desosamine sugar moiety revealed as protonated adduct at *m/z* 158.11784.

FIGURE CAPTIONS

Figure S1. (a) Experimental high-resolution ESI-FTICR mass spectrum of protonated clarithromycin and carbamazepine and (b) theoretical relative isotope intensities (%)

Figure S2. IRMPD FT-ICR MS/MS of protonated adduct of (a) carbamazepine and (b) clarithromycin fragmented by photon irradiation for 150 ms and 150 ms respectively at 100% CO_2 laser power