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Removal of micropollutants by fungal laccases in model solution and municipal wastewater: evaluation of estrogenic activity and ecotoxicity

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ABSTRACT

This study describes a multidisciplinary approach that investigates the breakdown potential of a laccase mediated system from *Trametes pubescens* MUT 2400 against several micropollutants including already recognized endocrine disrupting chemicals at their natural residual concentrations (from μg/L up to ng/L).

In model solution, the chemical speciation focused on a mixture of 18 analytes and adopted stir bar sorptive extraction with directed in-situ derivatization followed by gas chromatography and mass spectroscopy analysis. The method's key performance parameters were evaluated in consideration of the chemical peculiarities and complexity of real wastewaters: precision, accuracy, estimated working range extended to a wide residual concentration interval (10 ng/L to100 μg/L) indicated its fitness for purpose.

Laccases were extremely active towards all the target compounds, both in term of removal yields and rate. The maximal percentage of removal was obtained for 4-*t*-butylphenol, 2-hydroxybiphenyl, 4-*n*-octylphenol, salicylic acid and estrone (percentage of removal above 90%). Enzymes concentration played a central role and in most of the cases, the catalyzed reactions were very fast: the initial concentration of 9 compounds was halved within the first 3 h.

The laccase-mediated treatment was then applied to a municipal wastewater collected in a real wastewater treatment plant, containing at least 9 xenobiotics as drugs, pesticides, plasticizers and personal care products. Although the harsh chemical and biological conditions of the effluent influenced enzyme stability, the reaction took place, and above 70% transformation was obtained for most analytes during the 24 h experiment. Bioassays were carried out to estimate the estrogenic activity (the *E-screen* test and the MELN gene-reporter luciferase assay) and the ecotoxicity (*Lepidium sativum*, *Pseudokirchneriella subcapitata* and *Vibrio fischeri*), demonstrating the capability of laccases to mediate an effective detoxification of the wastewater and a decrease of the estrogenic activity.

Abbreviation: EDCs, endocrine disrupting chemicals; PCPs, personal care products; ER, estrogen receptor; SBSE, multi-shot stir bar sorptive extraction; TDU, thermal desorption unit; GC, gas chromatography; MS, mass spectrometry; WWTP, wastewater treatment plant; U/L, enzymatic activity expressed as International Unit per liter; PR, percentage of removal; MEC, minimal effective concentration; $t_{1/2}$, analyte half-life; Ti, Target Ions; RSD%, relative standard deviation percentage; LOQ, limit of quantification; QCx, quality control samples; Rel.Err %, relative error percentage; COD, chemical oxygen demand; GI%, germination index; I%, inhibition percentage of the algal growth or of the bacterial bioluminescence; EEQ, 17-β-estradiol equivalent quantity; RPE %, relative proliferative effect; TRANS%, rate of luciferase gene expression.

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1. Introduction

Endocrine disrupting chemicals (EDCs) are defined as "exogenous substances that cause adverse health effects in an organism, or its progeny, consequent to changes in endocrine functions" and have become a major issue due to their ability to interact with human estrogenic receptors. In this context, European Union has prioritized the reduction of surface-water pollution by municipal and industrial wastewaters, so as to limit the presence of EDCs and other harmful chemicals in the water cycle (Directive 2000/60/EC).

EDCs include biologically active compounds (pesticides, herbicides, and pharmaceuticals), heat stabilizers, plasticizers, personal care products (PCPs), etc. but a complete list is not available yet. One of the main limitations to detect their presence in water bodies is the very low concentration level (from μg/L up to pg/L), prioritizing the development of high-performance analytical techniques (Fatta-Kassinos et al., 2011).

Since several EDCs are designed to be chemically and biologically stable over a wide range of environmental conditions, wastewater treatment plant (WWTP) often proves ineffective causing accumulation phenomena, even into drinking waters (Chong et al., 2012; Mompelat et al., 2009; Snyder et al., 2003). Novel environmentally-sustainable biological processes are an attractive option to the costly and energy-consuming chemical and physical approaches, which often cause undesired by-product formation (Husain and Qayyum, 2012; Ikehata et al., 2006). Many fungi have already been recognized capable to degrade several xenobiotics, including some EDCs as pesticides, plasticizers, pharmaceuticals, etc. by means of their extracellular enzymes (Corvini et al., 2006; Kabiersch et al., 2011; Karas et al., 2011).

Laccases are glycosilated multicopper oxidases, able to catalyze the electron transfer from a substrate to a molecule of oxygen, which is thereby reduced to water. Their activity mainly targets phenolic moieties, but very often thanks to natural or synthetic mediators oxidation of non-phenolic compounds is also possible through an indirect electron transfer (Strong and Claus, 2011). On the whole, laccase-mediated system triggers oxidative reaction cascades, showing low substrate specificity (Strong and Claus, 2011). Owing to their biochemical and catalytic properties, laccase are considered as good green biocatalysts, potentially exploitable for in-field uses: they indeed mediate versatile reactions, being thermostable with a long shelf-life even at room temperature (Youshuang et al., 2011; Liu et al., 2013). Treatments based on these enzymes have been successfully applied towards many xenobiotics including EDCs (Cabana et al., 2007a; Manda et al., 2014). However mostly model single-component solutions have been tested (Catapane et al., 2013; Hommes et al., 2012; Murugesan et al., 2010).

Furthermore because of the heterogeneous and often unknown composition and the mixture interactions of municipal wastewaters, their chemical characterization is not sufficient to examine the intrinsic toxicity. In detail, synergic behavior has been associated to many compounds: for example, the effects of 17-β-estradiol, bisphenol A and DDT were additive (Rajapakse et al., 2001). In this context, bioassays represent effective monitoring tools to estimate the estrogenic activity and the ecotoxicity (Leusch et al., 2010). Moreover, considering the intrinsic sensitivity of each model assay, a battery of tests is generally recommended, using organisms belonging to different trophic levels and different end points as lethality, growth ability, respiration rate, etc. (Leusch et al., 2010; Soupilas et al., 2008; Tigini et al., 2011). Several *in vitro* bioassays have recently been suggested as a screening tool for suspected estrogenic chemicals. The most common ecotoxicological tests involve terrestrial plants (*Cucumis sativus*, *Lepidium sativum*, *Sorghum bicolour*, *Triticum aestivum*, etc.), aquatic plants (*Lemna minor*), algae (*Pseudokirchneriella subcapitata*, *Selenastrum capricornutum*, etc.), crustacea (*Daphnia magna*, *Artemia franciscana*) and bacteria (*Vibrio fischeri*) (Lundstrom et al., 2010; Tigini et al., 2011) whereas the endocrine interference is generally evaluated by means of human cell lines or yeasts (Jobling, 1998). Assays include estrogen receptor (ER) binding, ER-dependent transcription system, and proliferation of estrogen dependent cell lines such as MCF-7 cells; they can determine the total estrogenic EDCs activity of environmental samples (Berckmans et al., 2007; Nelson et al., 2007; Schilirò et al., 2009).

Because of the large number of micropollutants potentially present in WWTP samples, the present multidisciplinary study investigated the effectiveness of laccases of *T. pubescens* MUT 2400 against a complex mixture of EDCs, pharmaceuticals, pesticides and PCPs. A multi-residue screening analytical approach, based on multi-shot stir bar sorptive extraction (SBSE) with directed in-situ derivatization followed by gas chromatography and mass spectrometry (GC–MS) was identified as the most suitable method to contemporarily quantify 18 target compounds on model solution and a real wastewater sample in order to evaluate the full potential of the enzymatic treatment. In this intriguing context, the present research covered some scientific areas in order to critically discuss the potentials of a biological oxidation method for the treatment of civil wastewaters. The risk assessment was carried out combining advanced multi-residue analytical technique and biological tests to globally evaluate the effectiveness of fungal laccases.

This study was then focused to describe the capability of fungal laccases: i) to remove micropollutants including EDCs, PCPs, etc. in model and real solutions through the definition of the maximal analyte removal and half-life, and the minimal effective concentration of laccases; ii) to reduce the ecotoxicological hazard and the estrogenic activity of a real municipal wastewater.

2. Materials and methods

Certified EDCs, pharmaceuticals and PCPs with known or suspected estrogenic activity were used: 2,4-dichlorophenol, 4-t-butylphenol, diethyl phthalate, 2-hydroxybiphenyl, 4-n-octylphenol, salicylic acid, alachlor, 4-n-nonylphenol, oxybenzone naproxen, diclofenac, triclosan, ketoprofen, bisphenol A, bis(2-ethylhexyl) phthalate, estrone, 17-α-ethynyl estradiol, 17-β-estradiol. Standard stock solutions of each compound were prepared in acetone at a concentration of 1 g/L, and stored at −18 °C. All the chemicals (purity 97–99%) were purchased from Sigma–Aldrich (Milan, Italy).

2.1. Municipal wastewater

Sample was collected from a real municipal WWTP in northwestern Italy (Turin), treating more than 260 million m^3 of sewage from an area of about 450 km^2. The mean treated wastewater flow is approximately 615,000 m^3/day mainly released by 1.5 million inhabitants and 1800 industries, amounting to a total equivalent population of 3 million.

Twenty-four hour composite sample (4 L) was collected after primary sedimentation and stored in brown glass bottles at 4 °C. The effluent parameters are listed in Table 1.

2.2. Enzymatic treatment

The strain selected for the study and its enzymatic pathway is the result of screening more than 300 different Basidiomycetes with interesting key-features for this specific application: high chemical stability in complex environmental samples, wide range of operative pH, high production yield in optimized cultural

Table 1
Physico-chemical parameters of the wastewater collected in the real municipal WWTP. N tot, total content of organic and inorganic nitrogen; P tot, total content of phosphorous; TSS, total suspended solid.

COD mg/L	Solid COD mg/L	Sediment mL/L	N tot mg/L	NH_4 mg/L	P Tot mg/L	TSS mg/L	pH
205	60	0.5	26.5	27	2.76	84.6	6.7

conditions and high elimination efficiency of xenobiotics (Anastasi et al., 2010, 2012).

T. pubescens MUT 2400 (Mycotheca Universitatis Taurinensis, Turin, Italy) was grown in a rich nutrient medium in agitated conditions (120 rpm), as described by Nair et al. (2013). After the 0.2 μm filtration, the laccase crude extract was stored at 4 °C until the experiments.

The enzymes were used avoiding any further purification step, in order to preserve compounds secreted by the fungus, which might positively influence laccases catalytic activity and stability over time. As a crude enzymatic extract was used, kinetic models were not reliable.

The enzymatic stock solution contains more than 300 U/L, where one International Unit (U) corresponds to the amount of enzyme that oxidizes 1 μmol of model substrate per minute under standard condition, as already described by Liu et al. (2013). The model assay is described below in paragraph 2.4. Laccases constituted the major protein fraction secreted by the fungus and no other oxidative enzymes (i.e. peroxidases) were detected (data not shown).

2.2.1. Tests on model spiked solution

The model solution was prepared by spiking ultrapure water with 1 μg/L of 18 analytes, listed in Table 2. The initial pH (6.9 ± 0.2) was not modified, because it was representative of the values usually registered in the wastewaters of WWTP Turin (data from WWTP management, personal communication).

Different concentrations of laccase (10, 25, 50, 100, 250 and 500 U/L) were tested towards the mixture solution. The experiment was conducted in 500 mL flask (300 mL final volume). Three biological replicates were set and maintained under constant stirring (100 rpm) at room temperature. A control was set by inoculating laccases in ultrapure water to estimate the enzymatic stability in a model system. In addition, an abiotic control without enzyme inoculum was prepared to detect unexpected variability due to experimental conditions and experiments run in parallel to monitor possible changes in analytes concentration profiles.

Table 2
Cumulative RSD% calculating considering all the calibration samples during the entire validation period and LOQ of each compound (ng/L).

	RSD%	LOQ
2,4-Dichlorophenol	17.1	0.43
4-t-butylphenol	16.4	0.34
Diethyl phthalate	9.0	0.11
2-Hydroxybiphenyl	11.7	0.14
4-n-ocylphenol	16.0	0.14
Salycilic acid	19.8	2.03
Alachlor	21.7	0.69
4-n-nonylphenol	12.9	0.61
Oxybenzone	10.0	0.21
Naproxen	14.1	3.01
Diclofenac	18.9	1.51
Triclosan	16.0	0.49
Ketoprofen	19.3	7.50
Bisphenol A	19.1	0.19
Bis(2-ethylhexyl) phthalate	11.5	0.23
Estrone	19.2	0.45
17-α-ethynyl estradiol	15.0	8.74
17-β-estradiol	17.7	9.16

At the end of the incubation experiment (24 h), three aliquots were collected and immediately submitted to SBSE targeted sampling with ad hoc derivatization (i.e., phenols, acids and amines and apolars).

The percentage of removal (PR) of each pollutant was calculated by quantifying the residual concentration after the enzymatic treatment. The minimal effective concentration (MEC) able to reduce at least at 30% of the initial concentration of the target analyte was estimated. The 30% of removal was arbitrarily fixed as minimal yield also in consideration of the method precision and uncertainty with analytes at ultra-trace level. When indicated, the conversion profile over time (sampling at 1-3-6-24 h) was followed, identifying the time required to lose half of the initial concentration ($t_{1/2}$, European Environment Agency http://www.eea.europa.eu) by extrapolation from the model functions that best fit the experimental data (curve fit was considered acceptable for R^2 values above 0.8).

2.2.2. Tests on real municipal wastewater

According to previous results, 100 U/L of laccases were used to treat a real municipal wastewater sample. An abiotic control without enzyme inoculum was also set and run in parallel for 24 h as the enzymatic treatment. The experiment was carried out as described for model spiked solution.

Residual laccase activity was measured during the experiment, in order to monitor possible inactivation effects due to the chemical and physical conditions of the real wastewater.

2.3. Analytes extraction and quantification

A reference multi-target SBSE procedure was optimized to extend its sampling effectiveness to the wide range of chemicals tested in the present study. Chemicals are reported in Supplementary Table 1 together with CAS registry numbers, in-situ derivatizing agent adopted, absolute retention time of the main derivative (min) and, Target Ions (Ti) and Qualifiers adopted for quantitation. Based on the specific functionalities and polarities, analytes were submitted to a directed in-situ derivatization, which enhances absolute recoveries and improves method sensitivity. SBSE was run simultaneously for the different chemical classes (phenols, acids/amines and a polar compounds) and was followed by a multi-shot thermal desorption unit (TDU)-GC-MS analysis as previously described (Bicchi et al., 2009; Van Hoeck et al., 2009).

Gas chromatography was run with a 30 m long, 0.25 mm ID and 0.25 μm df Mega 5 FSOT column (5% diphenyl, 95% dimethylsiloxane) (Mega, Legnano, Italy). The oven temperature was programmed from 70 °C (2 min) to 150 °C at 25 °C/min, then to 200 °C at 3 °C/min and to 280 °C at 8 °C/min (10 min), using helium in constant pressure mode as carrier gas. The mass spectrometer was equipped with transfer line, ion source and quadrupole analyzer temperatures which were maintained at 280 °C, 230 °C and 150 °C, respectively; a solvent delay of 4 min was used. Electron impact ionization mass spectra were recorded at 70 eV with an ionization current of 34.6 μA. A dwell time of 10 ms was used in SIM mode, and one target ion and two qualifiers for each analyte were chosen. The simultaneous SIM/SCAN acquisition option was adopted for data acquisition which were then analysed by MSD ChemStation

software (G1701CA; version D.03.00 SP1; Agilent Technologies, Little Falls, DE, USA).

The method performance was studied by evaluating precision, sensitivity, accuracy and matrix effect, a three-weeks long protocol was applied and detailed results are reported as supplementary material (Supplementary Table 2). The precision of the analytical method was verified over the working range of expected residual concentrations (i.e., 10 ng/L to 100 μg/L) to assess the robustness of data expressed as quantified analyte concentration. It was estimated according to ISO definition (1998) and expressed through relative standard deviation percentage (RSD%) calculated over three replicates samplings at each concentration level within three weeks (W1–W3) over a three months period. Cumulative RSD% represents *intermediate precision* and was calculated by considering all calibration samples analyzed over the entire validation period.

Limit of Quantification (LOQ) was experimentally measured and was considered as the lowest analyte concentration at which target ion peak area precision (over three replicates) fell below an arbitrarily fixed value of 35% (limit that is double the acceptable reproducibility limit fixed by the Horwitz function (Horwitz, 2003) at the ppt level). Combined standard uncertainty (expressed as Relative Uncertainty %) is applicable to quantitative results within the method working range. Accuracy was determined on Quality Control samples (QC1 and QC2) obtained by spiking real wastewaters with known amounts of chemicals and is reported as Relative Error % (Rel.Err %).

In order to evaluate the occurrence of a matrix effect and due to the limitations posed by in-situ derivatization process that excludes the possibility to apply the direct quantitation of analytes thus evaluating absolute recovery %, spiked ultrapure water samples were compared with spiked thermally-inactivated laccase crude extracts (100 U/L), at 10 ng/L, 100 ng/L, 1 μg/L, 10 μg/L and 100 μg/L of the target compounds.

Calibration curve slopes were used to evaluate the magnitude of the matrix effect.

2.4. Laccase activity and COD determination

Laccase activity was assayed at 25 °C following oxidation at 420 nm of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid, ABTS), in 0.1 M sodium citrate buffer pH 3 (Niku-Paavola et al., 1988) and expressed as U/L.

Chemical oxygen demand (COD) was measured before and after each experiment on wastewater samples following the dichromate method (Lange kit, LCK 614).

2.5. Ecotoxicological assays

The tests were carried out before and after each experiment, using the following model organisms and standard methods: *L. sativum* (UNICHIM 1651: 2003 method), *P. subcapitata* (UNI EN ISO 8692: 2005 method) and *V. fischeri* (UNI EN ISO 11348-3). To evaluate whether the experimental procedures perturbed the response of the bioassays, the analyses were carried out also on the abiotic control (wastewater without enzymatic inoculum).

Phytotoxicity on *L. sativum* was expressed as the germination index (GI%), calculated from the following equation:

$$GI\% = (Gs \times Ls)/(Gc \times Lc) \times 100 \quad (1)$$

where Gs is the mean number of germinated seeds in the sample, Ls is the mean root length of the sample, Gc is the mean number of germinated seeds in the control (i.e. ultrapure water), and Lc is the mean root length of the control.

As regard *P. subcapitata* assay, the results were expressed as percentage of inhibition of the algal growth (I%) comparing the treated samples with a control made up by nutrient solution only.

Luminescent bacteria test was performed using the Microtox® toxicity system (Microtox Model 500, Microbics Corp., USA) and following the procedure described in the Microtox® manual (1995). The principle of this system is based on evaluation of the decrease of the luminous energy naturally emitted by *V. fischeri* bacteria (Azur Environmental, Carlsbad, CA, USA). After a screening test, the luminescence was measured at time zero and after 5, 15 and 30 min and compared to the control. When the relative inhibition percentage (I%) was above 20%, the EC50 was calculated and subsequently converted in toxic units (TU):

$$TU = (1/EC50) \times 100 \quad (2)$$

The GI% combines the toxicity effect on seed germination and on root elongation in respect to the control (ultrapure water). Thus, toxicity is associated to values below 100, whereas a stimulation effect is outlined by GI% > 100. Conversely, toxicity against algae and bacteria is expressed as the inhibition (I%) of the growth and the bioluminescence, respectively; therefore no effect is indicated as zero, inhibition gives I% > 0, while stimulation gives I% < 0.

2.6. Estrogenic activity

The extraction of the untreated and treated effluent was evaluated using a previously described method (Schilirò et al., 2009); 200 mL aliquots of the sample were extracted. The effluent was evaluated before and after the laccase treatment to assess the impact of the enzymatic treatment on estrogenic activity; the abiotic control was also analyzed.

After solid phase extraction, the real municipal WWTP primary effluent was subjected to two *in vitro* tests: the human breast cancer cell line (MCF-7 BUS) proliferation test (*E-screen* test) and the luciferase-transfected human breast cancer cell line gene-reporter assay (MELN assay).

The estrogenic activity was evaluated by the methods reported by Schilirò et al. (2012). The 17-β-estradiol equivalent quantity (EEQ) was measured in both tests. The results were presented by relative proliferative effect (RPE %) and rate of luciferase gene expression (TRANS%) as regards *E-screen* test and MELN assay, respectively.

3. Results and discussion

This section is organized in three sub sections: the first one deals with the methodological approach and the strategy adopted to evaluate the laccase activity against target analytes selected; the second part reports analytical data on real wastewaters while the last section is devoted to the effects on ecotoxicity and estrogenic activity.

3.1. Methodological aspects: SBSE-GC-MS method validation

One of the main issues for the detection of potentially active compounds in water bodies is their presence at very low concentration level (from μg/L up to pg/L): hence toxicological assessment and chemical speciation appear strongly conditioned by the key performance of analytical techniques adopted in screening studies (Mompelat et al., 2009; Touraud et al., 2011). Precision, sensitivity and accuracy should be indeed maximized to comply for trace and ultra-trace level detection of chemicals, which indeed are still estrogenically active at pg/L residual concentration (Flint et al., 2012).

Hence, the performance of the method used in this study was verified and data are reported in Table 2 (and Supplementary Table 2). Relative standard deviation percentage (RSD %) is an expression of the precision of the method to calculate the actual concentration of each analyte. For most of the analytes, this value was close to or below 15% (i.e. 10% for oxybenzone) but the quantification was less precise (19–20%) for 6 compounds, as salicylic acid, alachlor, diclofenac, ketoprofen, bisphenol A and estrone.

Limit of Quantification (LOQ) of the method was in a ng/L range but substantial differences were observed among analytes. The maximal precision was reached for at least 13 analytes being LOQ lower than 0.7 ng/L: as regards diethyl phthalate, 2-hydroxybiphenyl, oxybenzone and bis(2-ethylhexyl)phthalate, the extreme accuracy in the quantification was also associated to the method highest precision (RSD% approximately 10%). The quantification range was approximately 1.5–3.0 ng/L for salicylic acid, naproxen and diclofenac, and reached higher values (7–9 ng/L) for ketoprofen, 17-α-ethynyl estradiol and 17-β-estradiol.

Practical recovery depends in the first instance on sampling temperature, analyte solubility and ionic strength, and may be strongly influenced by the matrix effect, which was indeed evaluated in the present study. Results indicated a minimal matrix effect only for 4-n-octylphenol and estrone: Fig. 1a and c histograms show the different resulting instrumental response (i.e, Normalized Area) as a function of the laccase matrix effect; Fig. 1b and d shows the matrix effect on calibration curves. Estrone and 17-α-ethynyl estradiol are considered because of their divergent behavior, that of estrone being affected by a moderate matrix effect. Even though the method accuracy was rarely and barely influenced, these data suggested that analyte quantification would be more reliable if modified media (i.e. thermally-inactivated laccase extracts diluted in ultrapure water) were used to produce the calibration curves.

3.2. Results on model spiked solutions treatment

The occurrence of micropollutants in water bodies has been deeply discussed and reported, highlighting the ineffectiveness of traditional techniques adopted in WWTP: surface and even drinking waters contain traces of several harmful compounds, showing a clear estrogenic activity (Bicchi et al., 2009; Mompelat et al., 2009; Vulliet et al., 2011). The presence of small amounts of EDCs should not mislead the attention, and a proper exposure assessment should evaluate the outcome of the combination of several compounds: a complex and hardly predictable mixture effect may be generated (Kortenkamp et al., 2007). Hence, the enzymatic treatment based on laccases of *T. pubescens* MUT 2400 was tested towards a model solution spiked with several analytes representative of the actual complexity of surface waters (Bicchi et al., 2009). The target analytes are indicated in Table 3.

3.2.1. Minimal effective concentration (MEC)

Experimental results outlined that an initial laccase concentration of up to 50 U/L induced a very high PR values, in particular for 4-t-butylphenol, 2-hydroxybiphenyl, 4-n-octylphenol, salicylic acid, 4-n-nonylphenol, bisphenol A, estrone, 17-α-ethynyl estradiol and 17-β-estradiol. Similar enzymes concentration has been successfully applied also by other authors (Cabana et al., 2007b; Torres-Duarte et al., 2009), but in few cases, very high enzymatic loads were necessary. Noteworthy, 5000-800 U/L of laccases were required to treat naproxen, diclofenac, hormones, bisphenol A and triclosan (Lloret et al., 2010; Murugesan et al., 2010; Nakamura and Mtui, 2003), towards which laccases of *T. pubescens* MUT 2400 reduced up to 60% of the initial concentration already at 100 U/L. However, the comparison of these data with literature ones remains rough because of the huge difference of the treated compounds concentration. Indeed, also due to the limits of the applied

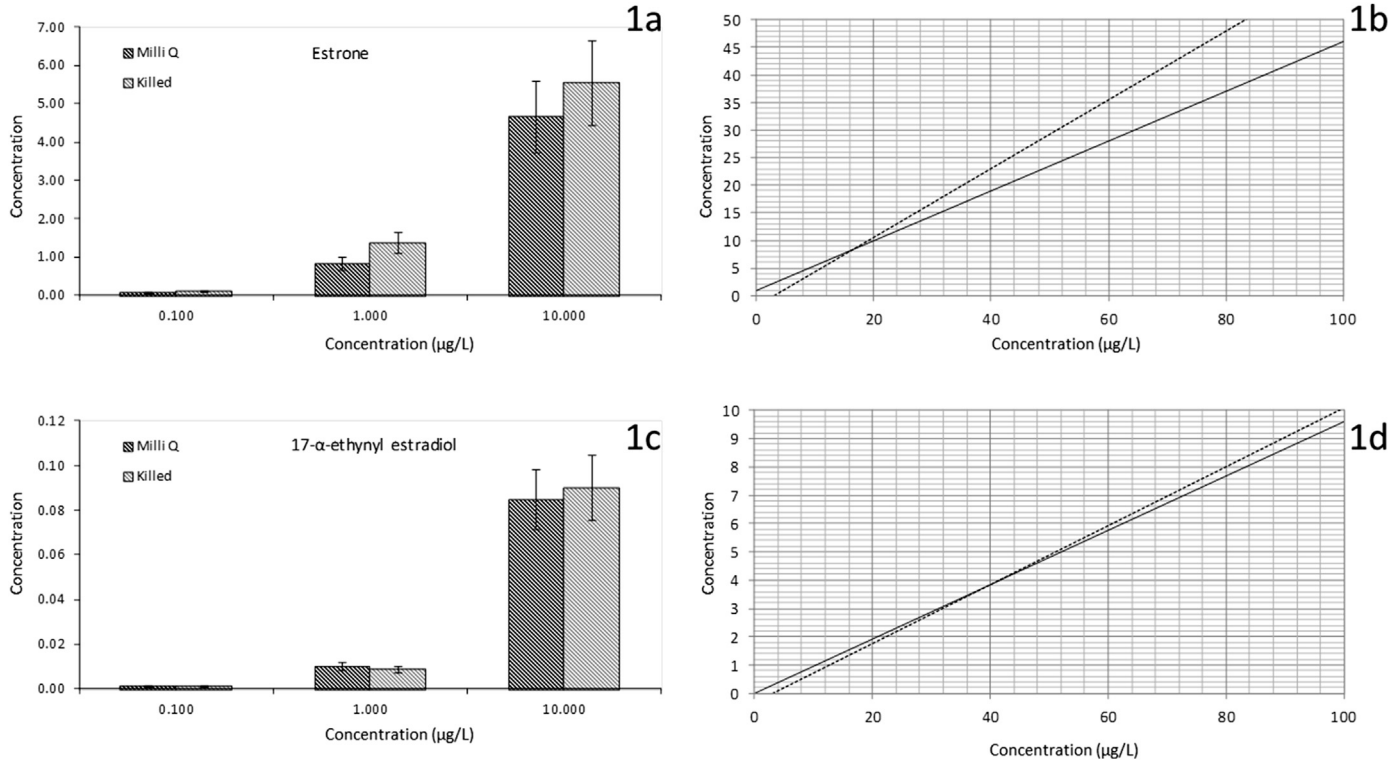

Fig. 1. Matrix effect of estrone and 17-α-ethynyl estradiol: comparison of instrumental response (1a, 1c) and calibration curves (1b, 1d) in ultrapure water (dotted lines) or in presence of thermally inhibited crude extract (solid lines).

Table 3
Degradation groups defined in accordance to the MEC, PR and RSD% calculated on model solutions treated with different laccases concentrations and analytes $t_{1/2}$ corresponding to the time (h) needed to halve the initial concentration of pollutant. Group: A MEC <10 U/L; B 10 < MEC <25 U/L; C 25 < MEC <50 U/L; D MEC >100 U/L. nc: not calculated, elimination profile not coherently represented by a model function.

Compound name	Group	500 U/L		250 U/L		100 U/L		50 U/L		25 U/L		10 U/L		100 U/L	500 U/L
		PR	RSD%	PR	RSD%	PR	RSD%	PR	RSD%	PR	RSD%	PR	RSD%	$t_{1/2}$	
2,4-Dichlorophenol	B	26.7	44.3	17.8	50.1	21.2	37.5	30.8	25.7	35.4	7.7	14.5	17.7	4.4	2.4
4-t-butylphenol	B	92.7	2.0	91.0	3.1	91.8	1.7	87.5	7.0	69.3	3.3	11.5	19.8	1.1	0.7
Diethyl phthalate	D	39.0	33.0	23.1	10.3	48.8	10.3	24.8	38.9	0	–	0	–	nc	nc
2-Hydroxybiphenyl	B	96.3	0.7	91.2	4.0	90.9	4.3	90.1	4.2	68.4	4.4	0	–	2.2	1.1
4-n-octylphenol	A	95.5	1.4	95.8	0.8	91.3	4.5	96.1	1.2	65.8	10.0	64.3	8.5	3.1	10
Salicylic acid	A	94.3	1.9	91.7	2.8	92.5	1.8	98.3	1.0	99.4	12.9	99.0	12.7	0.7	1.6
Alachlor	D	28.1	29.7	30.9	11.3	37.3	11.5	21.1	36.5	0	–	0	–	150	150
4-n-nonylphenol	C	59.1	39.6	54.0	14.1	41.8	33.8	73.8	16.8	17.3	39.5	19.6	6.2	2.4	2.6
Oxybenzone	A	54.0	23.0	56.3	5.9	63.3	12.7	58.7	26.2	53.9	20.7	33.9	20.5	6.6	6.0
Naproxen	B	68.9	9.7	52.2	17.8	62.2	28.6	48.2	20.0	56.9	6.3	0	–	126	114
Diclofenac	C	52.3	22.2	43.1	29.6	56.0	25.2	50.0	21.3	0	–	0	–	32	14
Triclosan	C	57.2	21.3	49.7	11.2	61.7	18.9	41.0	26.9	25.3	19.2	0	–	144	144
Ketoprofen	D	41.5	24.1	32.2	19.4	17.5	27.0	15.2	16.7	0	–	0	–	19	9.0
Bisphenol A	A	83.4	10.9	77.2	10.7	79.4	5.6	74.3	14.1	69.4	16.0	45.7	3.3	1.4	0.6
Bis(2-ethylhexyl)phthalate	B	46.2	24.1	56.6	11.3	60.2	4.9	35.5	9.0	31.5	21.3	6.3	8.8	nc	nc
Estrone	B	98.8	0.5	96.2	1.0	96.2	0.7	95.8	1.7	42.8	20.8	24.4	26.5	0.9	0.6
17-α-ethynyl estradiol	A	100	< LOQ	100	< LOQ	91.5	16.1	93.6	11.8	100	< LOQ	100	< LOQ	0.1	0.1
17-β-estradiol	A	100	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	100	< LOQ	0.1	0.1

analytical methodology, compounds concentration was often higher (above 5 mg/L) (Murugesan et al., 2010; Nakamura and Mtui, 2003) than in the present study (1 μg/L).

Synthetic mediators were not added, even though they were found to be fundamental for the conversion of persistent micropollutants (Lloret et al., 2010; Murugesan et al., 2010). However, this should not mislead the attention being aware that the enzymatic attack rarely leads to a complete mineralization of the compounds, forming instead many active radicals, which may themselves trigger oxidative cascades (Corvini et al., 2006). Indeed in presence of a mixture of micropollutants, the oxidation reaction may proceed following a multi-step oxidation scenario: direct laccase activity is hence coupled with indirect oxidations mediated by sub-products of the former reactions.

Target analytes can be grouped on account of the laccase MEC able to reduce at least 30% of their initial concentration and their removal rates. The first group (Group A), characterized by a MEC below 10 U/L, consists of: 4-n-octylphenol, salicylic acid, oxybenzone, bisphenol A, 17-α-ethynyl estradiol and 17-β-estradiol; the second (Group B) with MEC values between 25 and 10 U/L, includes 2,4-dichlorophenol, 4-t-butylphenol, 2-hydroxybiphenyl, naproxen, bis(2-ethylhexyl) phthalate and estrone; Group C, characterized by MEC values between 50 and 25 U/L, includes 4-n-nonylphenol, diclofenac and triclosan. Alachlor and ketoprofen showed a MEC higher than 100 U/L (Group D).

On the basis of the maximal removal yields, target analytes were grouped, identifying those most easily oxidized by laccases: 4-t-butylphenol, 2-hydroxybiphenyl, 4-n-octylphenol, salicylic acid and estrone were almost completely removed (PR > 90%), even if this result was obtained with relatively high enzymatic concentration (100 U/L). It is noteworthy that for 17-α-ethynyl estradiol and 17-β-estradiol, the residual concentration after the enzymatic treatment was always below the method LOQ, giving an apparent removal equal to 100%.

Noteworthy fungal laccases were effective against the majority of the compounds: 60% of conversion was measured for 13 up to 18 analytes, among which 7 (4-t-butylphenol, 2-hydroxybiphenyl, 4-n-octylphenol, salicylic acid, estrone, 17-α-ethynyl estradiol and 17-β-estradiol) were almost completely eliminated (PR > 90%). On the opposite, alachlor and ketoprofen confirmed to be very recalcitrant to laccase oxidation, even at high enzymatic concentrations, the PR did not exceed 20–30%.

The advantages of using crude extracts instead of purified enzymes are emphasized by the results obtained for triclosan and oxybenzone. In the absence of specific natural or synthetic mediators, purified fungal laccases are ineffective on triclosan (Cabana et al., 2007b; Murugesan et al., 2010), oxybenzone and several organohalogenated pesticides (Torres-Duarte et al., 2009). Since synthetic mediators were not added in this study, mediators produced by *T. pubescens* MUT 2400 still present in the crude extract may be responsible of the high removal yields (above 50%) obtained for these compounds.

Even though the MEC of 15 up to 19 analytes was equal or lower than 50 U/L, higher enzyme concentration is more suitable for a further application, due to the expected reduction when crude extract is added to a complex matrix (i.e. wastewaters) with variable pH and active microflora. Hence, 100 U/L were adopted for the treatment of a real wastewater sample.

The comparison of PR and enzyme concentration did not show a linear profile; sometimes above a certain enzymatic concentration no significant differences transformation could be observed (i.e. oxybenzone). This phenomenon could be due to the limited enzymatic affinity for the substrate as well as problems of substrate diffusion. Indeed at ppb concentration, the bioavailability of the substrate is a crucial and limiting factor.

3.2.2. Analytes half-life and laccase stability over time

Table 3 lists the calculated half-life ($t_{1/2}$) values, with the only exception of phthalates for which the data were often inconsistent and probably sensibly altered by the technical procedure. Laccase treatment was very rapid and most of the compounds were halved within the first 24 h. In particular, $t_{1/2}$ of 2,4-dichlorophenol, 4-t-butylphenol, 2-hydroxybiphenyl, 4-n-octylphenol, salicylic acid, 4-n-nonylphenol and bisphenol A was lower than 4 h. On the opposite, alachlor, naproxen, triclosan and ketoprofen were the most recalcitrant analytes to the enzymatic oxidation, showing $t_{1/2}$ values higher than 5 days.

In general, the highest initial concentration (500 U/L) of laccase was more effective, resulting in a lower $t_{1/2}$. Fig. 2 reports the removal profiles of 4-n-octylphenol, salicylic acid, bisphenol A and estrone.

Laccase activity was stable at least for the first 8 h; at the end of the experiment, a minimal loss (10%) was observed, and the pH remained unvaried around 7. Previous experiments at controlled

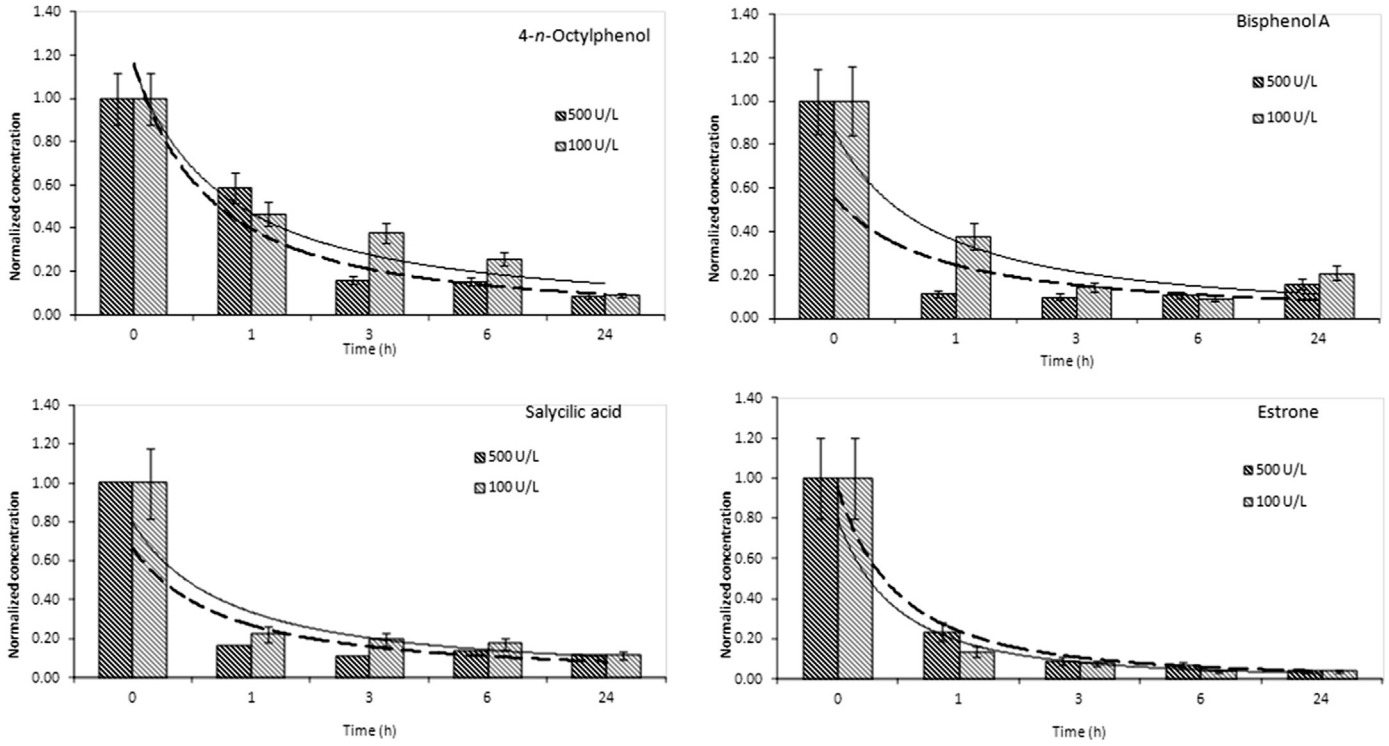

Fig. 2. Graphical representation of normalized chromatographic area as a function of the incubation time for 4-n-octylphenol, salicylic acid, bisphenol A and estrone by means of 100 and 500 U/L of laccases. The curves that better fit with data are also drawn: (—) for 100 U/L and (——) for 500 U/L.

conditions showed that laccase activity of *T. pubescens* MUT 2400 crude extract was stable over time between pH 6 and 9 (Spina, 2013). The minimal loss of active enzymes can therefore be ascribed to the micropollutants and aromatic compounds present in the tested samples.

3.3. Real municipal wastewater treatment

Since laccases proved to be very effective to reduce the micropollutants concentration in model solution, they have been assessed towards a municipal wastewater whose chemical and toxicological characterization has been carried out before and after the laccase treatment.

3.3.1. Occurrence and removal of detected analytes

The detected compounds included quite ubiquitous biologically active molecules as drug, pesticides and herbicides, and plasticizers, routinely found in surface and drinking waters (Benotti et al., 2009; Mompelat et al., 2009). On the whole, 8 out 9 of the detected molecules exceeded the correspondent concentration previously used in the model solution (1 μg/L) (Table 4). The most abundant compounds were diethyl phthalate, oxybenzone, naproxen and bisphenol A, being detected at concentration above 15 μg/L. The detected concentrations (μg/L) are in the same order of magnitude of the average chemical composition of surface waters and municipal effluents; meanwhile it usually tends to decrease at ng/L level in drinking water (Mompelat et al., 2009).

Besides, the occurrence of these molecules is strongly dependent on time, season and point of collection, limiting a proper comparison of these finding with other available data. For example, Lundstrom et al. (2010) found a minor content of anti-inflammatory drug as naproxen, ketoprofen and diclofenac (0.3–0.4 μg/L) but the presence of high amounts of bisphenol A (1.7 mg/L) increased the potential hazard of the sewage effluents. Moreover worldwide, deep variations have been associated to bisphenol A concentration in water cycle, ranging from few ng/L to mg/L (Flint et al., 2012).

The effectiveness of fungal crude extracts in transforming target analytes was evaluated in the real municipal wastewater, where the presence of suspended particles, colloids, solvents and xenobiotics as well as autochthonous microorganisms may interfere with enzymatic activity. Table 4 reports the target analytes identified and quantified in the sample, together with PR values measured by inoculating 100 U/L of laccases.

Laccases were able to actively convert all the compounds present in the effluent and, with the only exception of 2,4-dichlorophenol and bisphenol A, the final yields were always above 72%. Noteworthy 2-hydroxybiphenyl, a fungicide mainly used as a post harvest treatment on fruit and vegetables, was almost completely removed by laccases (PR 92%), with a residual concentration of approximately 0.4 μg/L. The most persistent compound was instead bisphenol A, for which only 35% removal could be detected.

Table 4
Uses and quantified concentration (μg/L) of the detected compounds in the real wastewater sample, and PR values obtained by the enzymatic treatment for each analyte.

	Use	Initial concentration	PR
2,4-Dichlorophenol	herbicide	0.13	51.0
Diethyl phthalate	plasticizer	67.6	79.7
2-Hydroxybiphenyl	fungicide	5.0	91.9
Salicylic acid	drug	9.5	75.0
Oxybenzone	PCP	43.7	77.3
Naproxen	drug	15.3	79.3
Diclofenac	drug	3.5	76.8
Ketoprofen	drug	2.8	72.5
Bisphenol A	plasticizer	27.4	35.0

Since the chemico-physical conditions of the wastewater may disturb the proper folding of enzymes as well as their catalytic activity, the stability of the enzymatic inoculum was followed during the experiment. A significant inactivation of the laccase occurred: the activity was stable during the first 8 h (100% recovery), then slightly decreased, with an irreversible loss of 54% at the end of the experiment (residual 45 U/L). Probably the interaction with some components of the real effluent inhibited or destabilized enzymes. Reducing anions, organic solvents, heavy metals, cyanide, salts and suspended particles, phenolic secondary products of EDCs oxidation pathway as well as the autochthonous microflora may actually interfere with laccase catalytic activity and stability (Cabana et al., 2007a; Garcia et al., 2011; Murugesan et al., 2010).

Besides, COD was determined before and after laccase treatment on real sample, highlighting a consistent reduction, close to 36%. The results were consistent with residual analytes concentration determined by SBSE-TDU-GC-MS and in line with the measured PR. It must be stressed that, although residual COD is still relatively high, the partial reduction of this parameter mediated by laccases could improve the final efficiency of the process, since the metabolites produced are usually more efficiently biodegraded by subsequent treatments (Nakamura and Mtui, 2003).

3.3.2. Ecotoxicity and estrogenic activity evaluation

Knowing the actual amount of compounds that potentially come into contact with aquatic organisms and human beings is important but not sufficient to predict a reasonable risk assessment and bioassays are useful tools to integrate the analytical data measuring the effects of complex mixtures. Besides, it should be also considered that synergistic or antagonistic interactions among chemical substances may occur. Actually, a mixture of compounds is often more toxic than expected by summing the effects of each single component (Kortenkamp et al., 2007). Thus, the risk associated to real wastewaters could be deeply underestimated without additional toxicological information obtained by means of bioassays, able to describe the ecotoxicity and the estrogenic activity ascribable to a certain sample.

In the present study, the ecotoxicity of the effluent after the laccase treatment was assessed applying standardized methods: three model organisms were used as the alga *P. subcapitata*, the plant *L. sativum* and the luminescent bacterium *V. fischeri*.

As shown in Table 5, the methods had different sensitivities to the intrinsic toxicity of the effluents. *L. sativum* was not disturbed by the wastewater (GI% 100%) and at the end of the enzymatic treatment, the sample induced even a biostimulation of the plant (GI% > 100%). As regard the *P. subcapitata* test, untreated effluent was mildly toxic (I% 33%) but laccases of *T. pubescens* MUT 2400 detoxified it, significantly decreasing I% to 12%. On the whole, both the tests described a significant detoxification of the sample, due to the laccase treatment.

Noteworthy Microtox® test was not able to record any toxic effect. Indeed during the screening test the inhibition of *V. fischeri* luminescence was always below or close to 20%, which is ascribable to the physiological variability of the bacterium itself. On the sample after the treatment (I% 22.7 ± 3.1) the definitive test was performed, nevertheless it has not been possible to calculate the EC50 (and then TU) and the sample could be considered non-toxic. Actually even though this test is one of the most commonly used test for water risk assessment, contrasting results are available about its suitability to follow the EDCs toxicity. In particular, *V. fischeri* was able to evidence negative effects of many compounds as triclosan, chlorophenol, before and after the treatment with fungal laccases (Gaitan et al., 2011; Kim and Nicell, 2006) but in contrast it proved not sensitive to a complex mixture of pharmaceutical, hormones and EDCs (Lundstrom et al., 2010). Interestingly, only at 10 mg/L, toxic effects were associated to naproxen (Marco-Urrea et al., 2010), explaining the insensitiveness of the test to the naproxen presence in the presently studied effluent (Table 4, 15.3 μg/L). These data suggested that the analyte typology able to trigger a response into the bacterium is very specific and deeply influenced by the actual dose.

Table 5
Ecotoxicological evaluation (germination index-GI%, algal growth inhibition-I% and bacterial luminescence inhibition-I%) before and after the laccase treatment. Data referred to the abiotic control are also reported.

	L. sativum	*P. subcapitata*	*V. fischeri*
	GI%	I%	I%
Before	97.0 ± 2.4[a]	33.2 ± 9.1[a]	9.0 ± 1.7[a]
After	122.4 ± 2.4[b]	12.1 ± 0.8[b]	22.7 ± 3.1[b]
Control	94.4 ± 4.7[a]	39.5 ± 16.9[a]	13.7 ± 1.5[a]

a/b: letters indicate significant differences ($p \leq 0.05$) of values.

The ecotoxicological analyses did not give a unique description of the hazard associated to the municipal wastewater. This evidence could be associated to the different interaction and response that organisms have towards such a heterogeneous sample. For example, the untreated sample was almost not toxic for the plant and the bacteria, but interfered with the proper growth of the algae. These results suggested that *P. subcapitata* is the most adequate organism to give information about the ecotoxicological risk ascribable to this wastewater sample.

Estrogenic activity, expressed as EEQ (ng/L), was evaluated by means of the *E-screen* test and the MELN gene-reporter luciferase assay. Both of them gave a common response towards the untreated municipal wastewater, displaying its moderate capability to interfere with the function of the endocrine system (EEQ 16–18 ng/L) (Table 6). The detected estrogenic activity is in agreement with other reported findings in literature (Fernandez et al., 2007; Kralchevska et al., 2013), even though few data fall even outside this range. For example, Yang et al. (2011) measured a higher activity in an influent from a WWTP, 47.7–80.1 ng/L.

Besides, the estrogenic activity of the studied effluent seemed to be strongly reduced by laccase treatment. However, because of the strong biology variability of the sample it was not possible to assess significant differences using the *E-screen* test. The MELN assay instead showed that laccases were able to almost nullify the estrogenic activity of the municipal wastewater (Table 6).

4. Conclusions

The research reported here emphasizes the advantages of multidisciplinary studies that combine advanced and multi-target analytical approaches enlarging the spectrum of testable analytes even at low residual concentrations (ng/L), so as to comprehensively evaluate the performance of biocatalyzed reactions in complex systems. Micropollutants confirmed to be an actual reality in

Table 6
Estrogenic activity of the wastewater sample before and after the treatment in *E-screen* as represented by RPE% and EEQ, and in MELN gene-reporter luciferase cells as represented by increased TRANS% and EEQ. bts: below the test sensitivity. Data refereed to the abiotic control are also reported.

	E screen		MELN luciferase assay	
	RPE %	EEQ (ng/L)	TRANS %	EEQ (ng/L)
Before	99 ± 17	15.7 ± 15.5[a]	95 ± 13	18.0 ± 9.1[a]
After	64 ± 20	1.7 ± 0.4[a]	bts	bts[b]
Control	83 ± 13	5.1 ± 1.6[a]	62 ± 11	6.6 ± 3.7[a]

a/b: letters indicate significant differences ($p \leq 0.05$) of values.

superficial waters of the district area of Turin (Italy) able to cause consistent effects towards the aquatic ecosystem and human beings, even interfering with the endocrine system. These data confirmed that the continuous and constant release of plasticizers, surfactants, pesticides, drugs, etc. from industrial processes and daily routine life of the modern society directly cause the accumulation of harmful concentration of micropollutants. The general concern about municipal wastewaters discharge into the environment is then confirmed, justifying the growing social and institutional attention for their effective treatment.

Laccases of *T. pubescens* MUT 2400 demonstrated their great potential to remove xenobiotics both in model and real samples. For several analytes (salicylic acid, alachlor and ketoprofen) this study provides a novel perspective, since they are for the first time the targets of an enzymatic treatment. Practical parameters obtained on model solutions (i.e. PR, MEC and $t_{1/2}$) would be useful to guide experimental design of an enzymatic treatment at larger scale.

Even though a consistent enzymatic fraction was inactivated due to the harsh chemical and biological conditions of the real effluent, laccases were able to trigger efficient oxidative reactions cascade. In the real municipal wastewater, decrease of up to 70% of the initial concentration of most the target analytes occurred, leading to a direct effect on the ecotoxicity and the estrogenic activity. After the enzymatic treatment, the endocrine interference on cells has been almost completely eliminated.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jclepro.2015.03.047.

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