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Removal of endocrine disrupting chemicals by fungal laccases in model solutions and real municipal wastewater: effect on the estrogenic activity and the 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 solutions, the chemical speciation focused on 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 to 100 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 degradation were obtained for 4-*t*-butylphenol, 2-hydroxybiphenyl, 4-*n*-octylphenol, salicylic acid and estrone (percentage of degradation above 90%). Enzymes concentration played a central role but in most of the cases, the minimal effective concentration was below 50 U/l. Moreover, laccases catalyzed very fast reactions: at 100 U/l, the initial concentration of 9 compounds was already halved within the first three hours.

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, degradation took place, and above 70% reduction 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 (*L. sativum*, *P. subcapitata* and *V. fischeri*), demonstrating the capability of laccases to mediate an effective detoxification of the wastewater and a decrease of the estrogenic activity.

Keywords:

endocrine disrupting chemicals; estrogenic activity; fungal laccases; municipal wastewater; ecotoxicity; stir-bar sorptive extraction.

Abbreviation:

EDCs: endocrine disrupting chemicals

PCPs: personal care products

LMS: laccase mediated system

ER: estrogen receptor

SBSE: multi-shot stir bar sorptive extraction

WWTP: wastewater treatment plant

PD: percentage of degradation

MEC: minimum effective concentration

$t_{1/2}$: analytes half-life

RSD%: relative standard deviation percentage

LOQ: limit of quantification

COD: chemical oxygen demand

GI%: germination index

I%: algal inhibition percentage

EEQ: 17- β -oestradiol equivalent quantity

RPE %: relative proliferative effect

TRANS%: rate of luciferase gene expression

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 limitation is the possibility to detect their presence in water bodies at very low concentration level (from $\mu\text{g/l}$ up to pg/l), prioritizing the development of high performances 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 results ineffective causing accumulation phenomena, even into drinking waters (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 glycosylated 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 oxidation of non-phenolic compounds is also possible thanks to natural or synthetic mediators that catalyze indirect electron transfer reactions (Baldrian, 2006; Strong and Claus, 2011). On the whole, laccase mediated system (LMS) triggers oxidative reaction cascades, showing a low substrate specificity (Strong and Claus, 2011). The biochemical and catalytic skills candidate them 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), even though 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 resulted additive (Rajapakse et al., 2001). In this context, bio-assays 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 has recently been suggested as a screening tool for suspected estrogenic chemicals. The most common ecotoxicological tests involve terrestrial plants (*Cucumis sativus*, *Lepidium sativum*, *Sorghum bicolor*, *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 *Trametes 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 solutions and a real wastewater sample in order to evaluate the full potential of the enzymatic treatment.

The decontamination effectiveness by fungal laccases was followed both in term of analytes concentration reduction (i.e. maximal degradation, minimal effective enzymatic concentration, analyte half-life) and detoxification of the wastewater evaluating both the estrogenic activity and the ecotoxicological hazard.

2. Materials and Methods

2.1. Chemicals

Certified EDCs, pharmaceuticals and PCPs with known or suspected estrogenic activity were used: all the chemicals (purity 97-99%) were purchased from Sigma-Aldrich (Milan, Italy). Standard stock solutions of each compound were prepared in acetone at a concentration of 1 g/l, and stored at -18 °C.

2.2 Municipal wastewater

Sample was collected from a real municipal WWTP in north-western Italy (Turin), treating more than 260 million m³ of sewage from an area of about 450 km². The mean treated wastewater flow is approximately 615,000 m³/day mainly released by 1.5 million inhabitants and 1,800 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.

2.3 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 conditions and high degradation efficiency of xenobiotics (Anastasi et al., 2010; Anastasi et al., 2012).

Trametes 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. Water treatment was carried out avoiding further purification steps, in order to preserve secreted compounds by the fungus, which might positively influence the LMS catalytic activity and stability over time.

2.3.1 Model spiked solutions

Different concentrations of laccase (10, 25, 50, 100, 250 and 500 U/l) were tested towards the model solution spiked with 1 µg/l of 18 analytes, listed in Table 2. Three biological replicates were set and maintained under constant stirring (100 rpm) at room temperature. In addition, an abiotic control without enzyme inoculum, was prepared to detect unexpected variability due to experimental conditions. After 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 degradation (PD) of each pollutant was calculated by quantifying the residual concentration after the enzymatic treatment. The minimum effective concentration (MEC) able to

reduce at least at 30% of the initial concentration of the target analyte was estimated. The 30% of degradation was arbitrarily fixed as minimal degradation yield also in consideration of the method precision and uncertainty with analytes at ultra-trace level. When indicated, the degradation profile over time (5 sampling points during the experiment) 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).

2.3.2 Real municipal wastewater

With respect with the previous results, 100 U/l of laccases were used to treat a real municipal wastewater sample. The experiment was carried out as described for model spiked solutions.

2.4 Analysis

2.4.1 Extraction and GC-MS

A reference multi-target SBSE procedure was optimized to extend its sampling effectiveness to the wide range of chemicals tested in the present study. 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 apolar compounds) and was followed by a multi-shot thermal desorption-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).

2.4.2 Method performances

The method performance was studied by evaluation the precision, the sensitivity and the matrix effect. 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 considering all calibration samples analysed during 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 Horowitz function (Horwitz, 2003) at the ppt level).

In order to evaluate the occurrence of a matrix effect, the analytes quantification carried out in ultrapure water was compared with samples containing thermally-inactivated laccase crude extracts (100 U/l), spiked at 10 ng/l, 100 ng/l, 1 µg/l, 10 µg/l and 100 µg/l of the target compounds.

2.5 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). Enzymatic activity was expressed as International Units (U), where 1 unit corresponds to the amount of enzyme that oxidizes 1 µmol of substrate per minute.

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

2.6 Ecotoxicological assays

The tests were carried out before and after each experiment, using the following model organisms and standard methods: *Lepidium sativum* (UNICHIM 1651: 2003 method), *Pseudokirchneriella subcapitata* (UNI EN ISO 8692: 2005 method) and *Vibrio fischeri* (UNI EN ISO 11348-3).

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

$$\text{(Eq. 1) GI\%} = (G_s * L_s) / (G_c * L_c) * 100$$

where G_s is the mean number of germinated seeds in the sample, L_s is the mean root length of the sample, G_c is the mean number of germinated seeds in the control (i.e. ultrapure water), and L_c is the mean root length of the control.

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 *Vibrio fischeri* bacteria (Azur Environmental, Carlsbad, CA, USA). After a screening test then the luminescence was measured at time zero and after 5, 15 and 30 min and compared to the control. The EC50 values were subsequently converted in toxic units (TU):

$$\text{(Eq. 2) TU} = (1/\text{EC50}) \times 100$$

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 bacterium 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.

2.7 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.

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 -oestradiol 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

3.1 Method validation

One of the main issue for the detection of potentially active compounds in water bodies is their presence at very low concentration level (from $\mu\text{g/l}$ 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. 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 closed 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 effect only for 4-n-octylphenol and estrone: Figure 1 evidences the effect of enzyme extract on calibration curve of estrone, together with not affected case (i.e. 17- α -ethynyl estradiol). 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 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 *Trametes 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 PD 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. This observation may be also explained considering that enzymatic attack rarely leads to a complete mineralization of the compounds, forming instead many active radicals, which may then trigger oxidative cascades (Corvini et al., 2006). Indeed, the chemicals oxidation of a complex wastewater 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 thus be grouped on account of the laccase MEC able to reduce their initial concentration to a residual 30% and their degradation rates. The first group (Group A), characterized by a MEC below 10 U/l, consists of: 4-n-octylphenol, salicylic acid, oxybenzone, 17- α -ethynyl estradiol and 17- β -estradiol; the second (Group B) with MEC values between 20 and 10 U/l, includes 2,4-dichlorophenol, 4-t-butylphenol, 2-hydroxybiphenyl, naproxen, bisphenol A, bis(2-ethylhexyl) phthalate and estrone; Group C, characterized by MEC values between 50 and 20 U/l, includes 4-n-nonylphenol, diclofenac and triclosan. Alachlor and ketoprofen showed a MEC higher than 100 U/l.

On the basis of the maximal degradation 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 (PD > 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 degradation equal to 100%.

Two other groups of compounds can be drawn. The first one presented a high degradation, above 60%, and included oxybenzone, naproxen, diclofenac, triclosan, bisphenol A and bis(2-ethylhexyl) phthalate. Few compounds instead were moderately degraded (PD < 50%): 2,4-dichlorophenol, diethyl phthalate, alachlor, 4-n-nonylphenol and ketoprofen. Alachlor and ketoprofen confirmed to

be very recalcitrant to laccase oxidation, even at high enzymes concentrations (Group D, MEC > 100 U/l), the PD did not exceeded 20-30%.

The advantages of using crude extracts instead of purified enzymes are emphasized by the results obtained for triclosan and oxybenzone. As has been reported, 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 degradation 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 are 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.

3.2.2 Analytes $t_{1/2}$ 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 inconstant 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 $t_{1/2}$. Figure 2 reports the degradation 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 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

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 of 9 of the detected molecules exceeded the correspondent concentration previously used in the model solution (1 µg/l). The most abundant compounds were 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 by the 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 drugs 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 degrading 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 PD values measured by inoculating 100 U/l of laccases.

Laccases were able to actively degrade all the compounds present in the effluent and, with the only exception of 2,4-dichlorophenol and bisphenol A, the final yields of degradation 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 (PD 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.

A significant inactivation of the laccase occurred: the activity was stable during the first 8 h, then slightly decreased, with an irreversibly lost of 60% at the end of the experiment. 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, closed to 36%. The results were consistent with residual analyte concentrations determined by SBSE-TDU-GC-MS and in line with the measured PD. It must be stressed that, although residual COD is still relatively high, the partial degradation mediated by laccases could improve the final efficiency of the process, since the metabolites produced are in general more completely biodegraded by subsequent treatment (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 algae *Pseudokirchneriella subcapitata*, the plant *Lepidium sativum* and the luminescent bacterium *Vibrio fischeri*.

As shown in Table 5, the methods had different sensitivities to the intrinsic toxicity of the effluents. As regard the *P. subcapitata* and *L. sativum* bioassays, untreated effluent was mildly toxic but laccases of *T. pubescens* MUT 2400 detoxified it, significantly increasing the GI% and decreasing the I%, respectively. Actually, at the end of the enzymatic treatment, the sample induced even an important biostimulation of the plant (GI%> 100%).

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 closed to 20%, which is ascribable to the physiological variability of the bacterium itself. On the sample after the treatment ($\Delta I\% 22.7 \pm 3.1$) the definitive test was performed, nevertheless it has not been possible to calculate the EC50 and the sample could be considered non-toxic. Actually even though this test is one of the most commonly use 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 resulted 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.

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 a influent from a WWTP, 47.7-80.1 ng/l.

Besides, the estrogenic activity of the studied effluent was almost completely eliminated by the laccase treatment. According to *E-screen* test, the EEQ was reduced up to 89%, whereas MELN assay was not able to record any effect at all after the enzymatic treatment (Table 6).

4. Conclusion

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 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 *Trametes 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 by the LMS on model solutions (i.e. PD, 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, degradation 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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Figure captions

Figure 1. Matrix effect of estrone and 17- α -ethynyl estradiol: comparison of calibration curves in ultrapure water (dotted lines) or in presence of thermally inhibited crude extract (solid lines).

Figure 2. Timing of the degradation of 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.

Table captions

Table 1. Physico-chemical parameters of the wastewater collected in the real municipal WWTP. TSS, total suspended solid.

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

Table 3. Degradation group defined in accordance to the MEC, PD and RSD% calculated on model solutions treated with different laccases concentrations and analytes $t_{1/2}$ correspond to the time (h) needed to half the initial concentration of pollutant.

Table 4. Uses and quantified concentration ($\mu\text{g/l}$) of the detected compounds in the real wastewater sample, and PD values obtained by the enzymatic treatment for each analyte.

Table 5. Ecotoxicological (germination index-GI%, algal growth inhibition-I% and bacterial luminescence inhibition- $\Delta\text{I}\%$) and estrogenic activity (estradiol equivalent concentrations, EEQ, ng/l) evaluation before and after the laccase treatment.

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.