CHEMICAL ENGINEERING TRANSACTIONS
VOL. 32, 2013
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-23-5; **ISSN** 1974-9791
DOI: 10.3303/CET1332066

Endocrine Disrupting Chemicals (EDCs) in Municipal Wastewaters: Effective Degradation and Detoxification by Fungal Laccases

Federica Spina[a], Chiara Cordero[b], Barbara Sgorbini[c], Tiziana Schilirò[c], Giorgio Gilli[c], Carlo Bicchi[b], Giovanna C. Varese[a]

[a]Department of Life Sciences and Systems Biology, University of Torino, viale P.A. Mattioli 25, 10125 Torino, Italy
[b]Department of Scienza e Tecnologia del Farmaco, University of Torino, via P. Giuria 9, 10125 Torino, Italy
[c]Department of of Public Health and Microbiology, via Santena 5bis, University of Torino, 10126 Torino, Italy
federica.spina@hotmail.it

The present study investigated the real potential of a crude extract produced by *T. pubescens* MUT 2400 with high laccases concentration, to degrade pharmaceuticals and recognized EDCs. The enzymatic treatment was applied toward a model solution and real municipal wastewater samples, containing some pharmaceutically active compounds. Laccases were able to extensively degrade all the tested compounds and an important detoxification of real wastewaters was also observed.

1. Introduction

Pharmaceuticals are part of the daily routine life, being used in human and animal medicine as therapeutics. After excretion as metabolites modified during Phase I and Phase II metabolism, more than 4,000 pharmaceutically active compounds (PhACs) may even reach the aquatic system (Mompelat et al., 2009). Landfill leachates, hospital and pharmaceutical effluents and, above all, municipal wastewaters result contaminated (Fatta-Kassinos et al., 2011).
In the past decades, several PhACs (i.e. acetylsalicylic acid, ibuprofen, paracetamol, ketoprofen, naproxen, diclofenac, β-blockers) have been detected in river, lakes, and even drinking waters with the average concentration ranging between µg/L and ng/L (Christen et al., 2010; Vulliet et al., 2011).
The knowledge about the fate of these contaminants in waters is barely known as well as their putative impact on aquatic organisms and human beings (Christen et al., 2010). One of the main concern is due to the fact that actually, some PhACs interfere with the endocrine system, mimicking natural hormones activity by selectively interact with estrogens receptors active site (Fent et al., 2006a). Hence, they have been classified as Endocrine Disrupting Chemicals (EDCs). In particular, an estrogenic activity has been already associated to estradiol derivates, genistein, paracetamol, antibiotics, etc. (Christen et al., 2010; Frye et al., 2011). They cause adverse effects on reproduction and fetal development, but they have been also associated to the rising of antibiotic resistance and allergies reactions (Christen et al., 2010).
According to these results, PhACs, including EDCs, have to be degraded before their release in the environment. Wastewater treatment plants (WWTPs) are the first and the main barrier that have to face the presence of these pollutants. Unfortunately, the conventional methods are not design for this purpose and they result usually inefficient. As a consequence, PhCAs are released in the receiving bodies, contaminating surface and ground waters (Snyder et al., 2007).
In order to solve this problematic, novel cost-effective and eco-friendly processes based on white-rot fungi are an attractive option (Cabana et al., 2007). Many strains have already recognized capable to degrade several xenobiotics, by means of their extracellular enzymatic pattern (Rodríguez Couto and Toca Herrera, 2006). From an applicative point of view, the use of whole fungal cells poses serious drawbacks, both in terms of economical and operative sustainability. A promising alternative could be given by enzymatic treatments, which are eco-friendly and display low energy requirements and easy process control (Torres

Please cite this article as: Spina F., Cordero C., Sgorbini B., Schiliro T., Gilli G., Bicchi C., Varese G.C., 2013, Endocrine disrupting chemicals (edcs) in municipal wastewaters: effective degradation and detoxification by fungal laccases, Chemical Engineering Transactions, 32, 391-396 DOI: 10.3303/CET1332066

et al., 2003). In particular, laccases are oxidoreductive enzymes with a low substrates specificity, which allow them to degrade many xenobiotics, including EDCs and pharmaceuticals (Cabana et al., 2007; Lloret et al., 2012).
Considering available data, PhACs and EDCs are an actual issue of superficial waters in the Turin district (Schilirò et al., 2004). Hence, aim of this study was the development of a competitive treatment, active toward these pollutants: the crude extract of *Trametes pubescens* MUT 2400 with high laccases concentration was then used to treat model and real solutions, containing high amount of PhACs.

2. Materials and Methods

2.1 Chemicals

All the chemicals, including several certified EDCs standards and pharmaceuticals, were purchased from Sigma-Aldrich (Milan, Italy): salicylic acid (99 %), naproxen (98 %), diclofenac (99 %), ketoprofen (99 %), estrone (99 %) and ethynyl estradiol (98 %) (Figure 1).

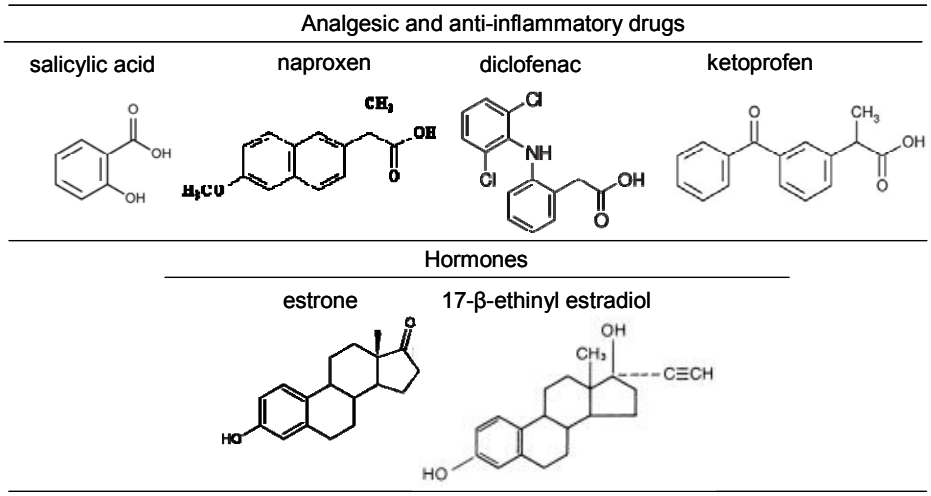

Figure 1: Chemical structures of the anti-inflammatory drugs salicylic acid, naproxen, diclofenac, ketoprofen) and hormones (estrone, 17-β-ethinyl estradiol) used in this study.

2.2 Municipal wastewaters

Water samples were collected from Turin municipal WWTP which treats around 42,000 m^3/day and serves four towns of the metropolitan area and almost 250,000 inhabitants. The plant has several treatments including primary sedimentation, nitrification/denitrification, activated sludge oxidation, filtration, etc. Two municipal water samples were taken after the primary sedimentation (MW1 and MW2): they were representative of a 24 h cycle and were collected in different periods of the year (MW1 in April and MW2 in July 2012). Each sample (3 L) was stored in brown-glass flasks at 4 °C until experiments were run.

2.3 Analytical analysis

The analytical procedure combined Stir Bar Sorptive Extraction (SBSE) with in situ derivatization followed by Gas Chromatography and Mass Spectrometric Detection (GC-MS) (Bicchi et al., 2009). The analyses were carried out in the Department of Scienza e Tecnologia del Farmaco (University of Torino, Italy).
The analytical method was validated according to international guidelines and by evaluating, its accuracy and the actual limit of quantification (LOQ) for each compounds. In particular, the accuracy was verified by analyzing water samples spiked with pure standards of suitable purity and stability at 10 µg/L. The accordance between the actual and the estimated concentration was compared in term of relative error percentage. The percentage of degradation (PD) of each pollutant was calculated by quantifying the residual concentration after the enzymatic treatment, in comparison with the data measured at the beginning of the experiment (untreated sample). Moreover, the half-life of the compounds ($t_{1/2}$), was determined by extrapolation from the function that better fits with experimental values.

2.4 Enzymatic treatment

The culture filtrate of *T. pubescens* MUT 2400 was used at the final laccase concentration of 100 U/L. The experiment was carried out in 500 mL flask, using approximately 450 mL of each sample. The flasks were

maintained in a mild agitation (100 rpm) in order to keep a proper aeration. After 24 h, three aliquots of 10 mL each were collected and immediately submitted to SBSE targeted sampling. The percentage of degradation (PD) and the half life ($t_{1/2}$) of each pollutant were calculated by quantifying the residual concentration after the enzymatic treatment.
Laccases were used towards a model solution and real municipal water samples. For the model solution, ultrapure water was spiked with 1 µg/L of selected compounds, indicated as potentially present in water courses by literature data and previous studies (Bicchi et al., 2009).
During the entire experiment, in order to assess enzymes stability, the laccase activity was followed by means of a standardized method (Anastasi et al., 2012).

2.5 Ecotoxicological analysis

The ecotoxicity of real water samples was evaluated both before and after the enzymatic treatment. Since, little is known about the sensitivity of bioassays to the chemical complexity of superficial waters, in the present study, *Lepidium sativum* (plant) and *Pseudokirchneriella subcapitata* (alga) were selected because they have previously showed to be sensitive to contaminated industrial wastewaters (Anastasi et al., 2012).

3. Results and Discussion

3.1 Laccases treatment of model solution

To evaluate the potentials and the effectiveness of any treatment against xenobiotics, it is necessary to monitor the fate of the highest number of toxicants (Snyder et al., 2007) and, hence, a powerful and versatile validated procedure has to be used. Methodology performance parameters confirmed the fitness for purpose of the candidate method, and several figures of merit are reported in Table 1 as regard non steroidal anti-inflammatory drugs (salicylic acid, naproxen, diclofenac, ketoprofen) and hormones (estrone, 17-β-ethinyl estradiol). Experimental data demonstrated that the method resulted to be highly sensitive to the presence of these compounds. With the only exception of ketoprofen, the Limit of Quantitation (LOQ) was always below 10 ng/L. This represents a key technical advantage, because the combination of GC-MS allowed to surely identify and quantify several compounds, environmentally and toxicologically relevant, at very low concentration. In fact, many compounds have been recognized noxious for aquatic organisms, even at ng/L level. For example, hormones, as estrone or 17-β-ethynyl estradiol, are responsible of alterations in the normal reproduction system of fish and mudsnail already at 0.1 - 10 ng/L: they may cause sex differentiation, reduction of fertility or increase of eggs production (Christen et al., 2010).
The effectiveness of the laccase crude extract of *T. pubescens* MUT 2400 towards PhACs was first investigated on a model solution and the results are reported in Table 1. Salicylic acid, estrone and 17-β-ethynyl estradiol were almost completely removed (PD > 90 %), lowering their concentration below 100 ng/L. Naproxen and diclofenac were also extensively degraded, whereas ketoprofen was very recalcitrant (PD < 18 %) to the enzymatic oxidation.
In general, laccase treatment was very rapid and, for most of the PhACs, their initial concentration halved within the first 24 h (Table 1). Interestingly, salicylic acid and hormones were degraded very rapidly, showing a $t_{1/2}$ below 1 h. For the most recalcitrant compound (ketoprofen) the calculated $t_{1/2}$ was higher than 5 days.
Laccase activity remained stable at least for the first 8 h; at the end of the experiment, a minimal loss, around 10 %, was observed. The pH of the solution (7.3) did not have a destabilizing effect towards the enzymes, confirming that, in neutral solutions (pH 6 - 8), laccases result quite stable during time (Baldrian, 2006).

Table 1: Evaluation of parameters after the laccase treatment of the model solution.

	LOQ	laccases at 100 U/L		
		PD	residual conc	$t_{1/2}$
salicylic acid	2.03	92.5	75	0.7
naproxen	3.01	62.2	378	< 24.0
diclofenac	1.51	56.0	440	32.0
ketoprofen	12.5	17.5	825	144.0
estrone	0.45	96.2	38	0.9
17-β-ethinyl estradiol	8.74	91.5	85	0.1

LOQ: limit of quantification in ng/L of the pharmaceuticals used to spike the model solutions; PD: percentage of degradation; residual concentration in ng/L; $t_{1/2}$: half life in hours of each compound after the laccases treatment of the model solution.

3.2 Laccases treatment of municipal wastewaters

Real water samples were collected in the Turin municipal WWTP in different periods of the year, in order also to get a first indication on the seasonal variability of the PhCAs present in the effluents.

Working with complex and heterogeneous waters, the capability of the method to precisely quantify the presence of target compounds should be first evaluated. In fact, the heterogeneous chemical composition, suspended solid materials, etc. may eventually interfere with the analytical procedure. The results showed a good degree of accuracy, always below 20 % (data not shown).

Thanks to this previous validation, it was possible to correctly estimate the concentration of several target PhACs in real wastewaters (Table 2). The chemical composition of the wastewaters differed both from a quantitative and qualitative point of view: MW1 showed a more various and consistent presence of PhACs in comparison with MW2. The main concern was ascribable to naproxen in MW1 and to ketoprofen in MW2, being present at quite high concentration (14 - 15 µg/L). Also for the other PhACs, the actual amount always exceeded the concentrations used to spiked the model solution.

Even though hormones derivates were not detected in real municipal wastewaters, the hazard of these samples could be still considerable. In fact, some PhACs can bind the estrogen receptor as well as mediate other disturbing effects. For example, in rainbow trout, alterations of kidney and gills already happened at 5 µg/L (Fent et al., 2006a). Hence, as already pointed out by other authors, real samples confirmed to be highly contaminated (Christen et al., 2010).

Knowing that the real water samples were actually contaminated, the enzymatic treatment was applied and the final yields are reported in Figure 2. Laccases of *T. pubescens* MUT 2400 were able to degrade all target compounds up to 70 %.

It is important to underline that even though real samples were obviously more toxic and had a higher pollutants load than model solution, laccases still maintained their degradation capability. In fact, in a real water sample, many factors may inhibit or interfere with laccase activity, reducing the actual capability of the enzymes to mediate fruitful oxidation processes. In agreement with this assumption, a consistent inactivation of laccases was seen in both samples: after 24 h, only less than 40 % of the initial activity was left. Similar data were reported by other authors, highlighting the considerable destabilizing effect of reducing anions, organic solvents, heavy metals, cyanide, salts and suspended particles as well as phenolic secondary products of EDCs oxidation pathway (Cabana et al., 2007; Garcia et al., 2011).

According to these evidences, the maintenance of the same process efficiency would have been already a good result. Interestingly, except for salicylic acid, the treatment of MWs highlighted a consistent enhancement of the PD values, in comparison with degradation yields obtained towards the model solution (Figure 2 and Table 1). A possible explanation of this unexpected result could be the presence, in the real water samples, of natural mediators, enhancing the enzymatic activity against pollutants (Cabana et al., 2007; Garcia et al., 2011).

After the enzymatic treatment, the residual concentration of the investigated compounds was comparable to the lowest concentration reported in the output effluents of the WWTPs (Figure 2), which ranges from few ng/L to even 12.5 µg/L for naproxen (Fent et al., 2006a). As already mentioned, the presence of small amounts of PhACs and EDCs should not mislead the attention, because toxic effects on the aquatic ecosystem have already been observed even at very low concentration of PhACs (Fent et al., 2006a).

Table 2: Estimated concentration (µg/L) of the target compounds in the two municipal wastewater samples. nd = not detected.

	estimated concentration	
	MW1	**MW2**
salicylic acid	9.5	nd
naproxen	15.3	2.2
diclofenac	3.5	0.8
ketoprofen	2.8	14.3
estrone	nd	nd
17-β-ethinyl estradiol	nd	nd
total	31.1	17.3

MW1 and MW2: two municipal water samples taken after the primary sedimentation.

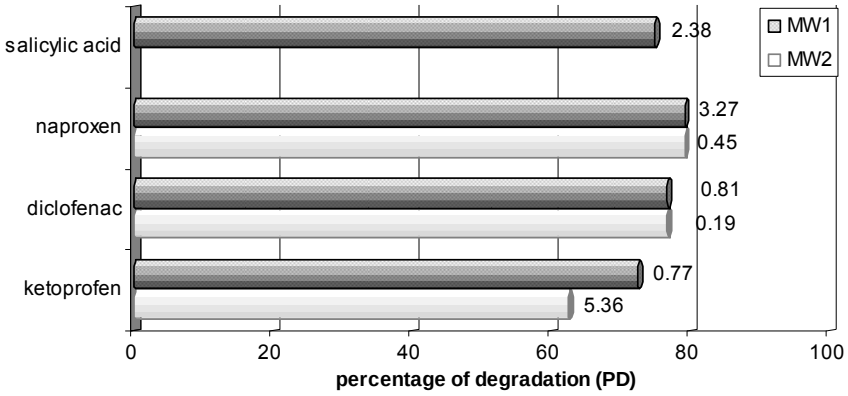

Figure 2: Percentage of degradation (PD) of the compounds detected in MW1 and MW2, after the enzymatic treatment. The final residual concentration (µg/L) of each compound is written closed to the bar.

Moreover, as required by the European Medicines Evaluation Agency (EMEA), the ecotoxicological evaluation of PhACs compounds is necessary (Fent et al., 2006b). In the present study, both untreated samples were mildly toxic, inhibiting almost 20 % of the algal growth and of the seed germination index (IG %) (Figure 3).
According to *P. subcapitata* test, the enzymatic treatment did not significantly change the hazard of the real samples: the modification of the chemical components mediated by laccases did not influence the algal development. Otherwise, considering the *L. sativum* test, laccase activity reduced the initial toxicity of MW1 and MW2. Actually, the enzymatic treatment even induced an important biostimulation of the seed germination, showing an IG > 100 % (Figure 3).
Hence, the two model organisms did not respond in the same way to the MWs, confirming the need of a battery of ecotoxicological tests, able to give a complete picture of the potential hazard of a water sample (Tigini et al., 2011).

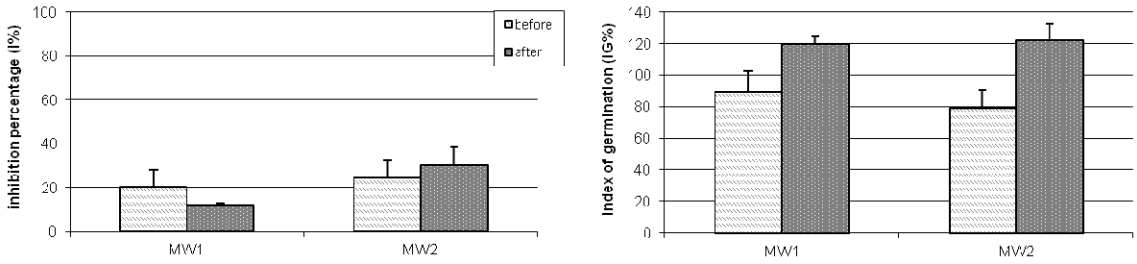

Figure 3: Inhibition of the algal growth (I%) (left) and the seed index germination (IG %) (right) in presence of the untreated (before) and treated (after) MW1 and MW2.

4. Conclusions

The optimized analytical methodology applied in the present study, allowed to reliably identify and quantify many PhACs in real municipal water samples. Hence, PhACs are confirmed to be an actual issue in superficial waters, reaching even harmful levels. However, at least in Italy, the complete absence of any law regulation and restriction have not stimulated the development of suitable techniques, applicable in WWTPs and effective in degrading PhACs, including EDCs.
Laccases of *T. pubescens* MUT 2400 highlighted a great potential to degrade several PhACs which can be found in municipal wastewaters. To date, for several compounds (i.e. salicylic acid, ketoprofen), this is the first time that an enzymatic treatment was successfully applied for their degradation, not only of model spiked solutions but also of real municipal wastewaters. Even though a consistent fraction of the enzymes was inactivated in real effluents, laccases were able to trigger efficient oxidative reactions cascade: the concentration of most of the molecules was reduced up to 70 % with a direct effect on samples ecotoxicity.
In conclusion, despite the very promising results, the process has still to be optimized, improving its stability and efficiency. Several solutions are under investigation, including the immobilization of enzymes,

the addition of mediators and stabilizers and the selection of the most suitable location for the enzymatic treatment in WWTPs (Cabana et al., 2007).

Acknowledgement

Authors are grateful to SMAT, Società Metropolitana Acque Torino S.p.A., by supplying the wastewaters samples used during experiments.

References

Anastasi A., Spina F., Romagnolo A., Tigini V., Prigione V., Varese G.C, 2012, Integrated fungal biomass and activated sludge treatment for textile wastewaters bioremediation, Bioresour. Technol. 123, 106-111.

Baldrian P., 2006, Fungal laccases occurrence and properties, FEMS Microbiol. Rev. 30, 215-242.

Bicchi C., Schilirò T., Pignata C., Fea E., Cordero C., Canale F., Gilli G., 2009, Analysis of environmental endocrine disrupting chemicals using the E-screen method and stir bar sorptive extraction in wastewater treatment plant effluents, Sci. Total Environ. 407, 1842-1851.

Cabana H., Jones J.P., Agathos S.N., 2007, Elimination of Endocrine Disrupting Chemicals using White Rot Fungi and their Lignin Modifying Enzymes: A Review, Eng. Life Sci. 7, 429-456.

Christen V., Hickmann S., Rechenberg B., Fent K., 2010, Highly active human pharmaceuticals in aquatic systems: a concept for their identification based on their mode of action, Aquat. Toxicol. 96, 167-181.

Fatta-Kassinos D., Meric S., Nikolaou A., 2011, Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research, Anal. Bioanal. Chem. 399, 251-275.

Fent K., Weston A.A., Caminada D., 2006a, Ecotoxicology of human pharmaceuticals, Aquat. Toxicol. 76, 122-159.

Fent K., Escher C., Caminada D., 2006b. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system, Reprod. Toxicol. 22, 175-185.

Frye C.A., Bo E., Calamandrei G., Calza L., Dessı`-Fulgheri F., Fernandez M., Fusani L., Kah O., Kajta M., Le Page Y., Patisaul H.B., Venerosi A., Wojtowicz A.K., Panzica G.C.., 2011, Endocrine disrupters: a review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems, J. Neuroendocrinol. 24, 144-159.

Garcia H.A., Hoffman C.M., Kinney K.A., Lawler D.F., 2011, Laccase-catalyzed oxidation of oxybenzone in municipal wastewater primary effluent, Water res. 45, 1921-1932.

Lloret L., Eibes L., Feijoo G., Moreira M.T., Lema J.M., Continuous biotransformation of estrogens by laccase in an enzymatic membrane reactor. Chemical engineering transactions 27, 31-36. DOI:10.3303/CET1227006

Mompelat S., Le Bot B., Thomas O., 2009, Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water, Environ. Int. 35, 803-814.

Rodríguez-Couto S., Toca Herrera J.L., 2006, Industrial and biotechnological applications of laccases: a review, Biotechnol. Adv. 24, 500-513.

Tigini V., Giansanti P., Mangiavillano A., Pannocchia A., Varese G.C., 2011, Evaluation of toxicity, genotoxicity and environmental risk of simulated textile and tannery wastewaters with a battery of biotests, Ecotoxicol. Environ. Saf. 74, 866-873.

Torres E., Bustos-Jaimes I., Le Borgne S., 2003, Potential use of oxidative enzymes for the detoxification of organic pollutant, Appl. Catal. B 46, 1-15.

Schiliró T., Pignata C., Fea E., Gilli G., 2004, Toxicity and estrogenic activity of a wastewater treatment plant in Northern Italy, Arch. Environ. Contam. Toxicol. 47, 456-462.

Snyder S.A., Wert E.C., Lei H., Westerhoff P., Yoon Y., 2007, Removal of EDCs and pharmaceuticals in drinking and reuse treatment processes, Awwa Research Foundation, Denver, USA.

Vulliet E., Cren-Olive C., Grenier-Loustalot M., 2011, Occurrence of pharmaceuticals and hormones in drinking water treated from surface waters, Environ. Chem. Lett. 9, 103-114.