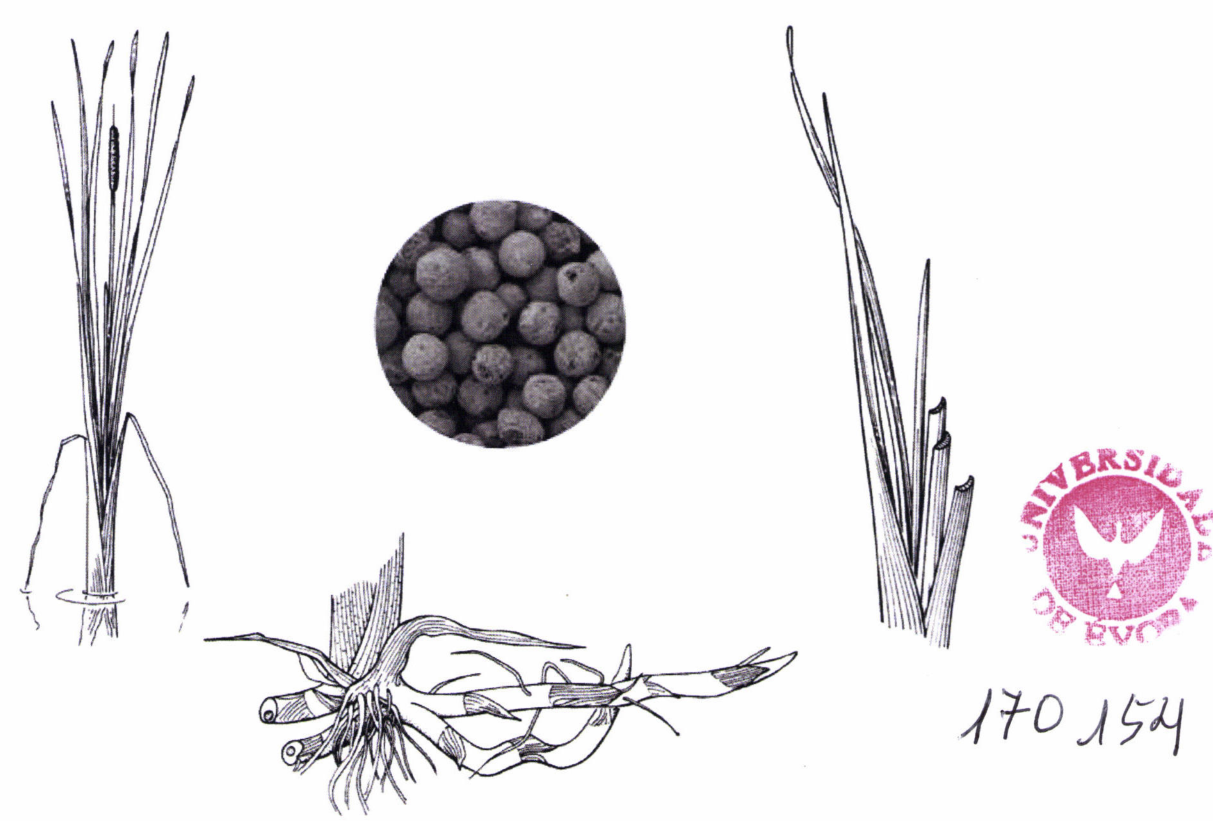
Studies on Pharmaceuticals Removal from Water
Potential use of Constructed Wetlands Systems

Ana Vitória Martins Neves Barrocas Dordio



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Supervisors: Doctor Cristina Maria Barrocas Dias Teixeira da Costa
Doctor Ana Paula Honrado Pinto

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2009

To the loving memory

of my Mom

Thank you for all

U.E
Serviços
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Abstract

Pharmaceutical residues in the environment and their potential toxic effects have been recognized as one of the emerging research areas in environmental chemistry. Many reports are available in the literature about the detection of several of the most consumed pharmaceuticals, their metabolites and transformation products in effluents of domestic wastewater treatment plants (WWTPs) as well as surface and ground waters and even in drinking waters worldwide. This situation can be attributed to the general inadequacy of conventional treatment processes used in WWTPs in dealing with trace pollutants.

An option for removal of organic xenobiotics from WWTPs effluents is the implementation of constructed wetlands systems (CWS). In comparison with other tertiary or advanced treatment technologies, CWS present the advantage of being an essentially low-cost and low-maintenance technology. CWS have been already applied with success for the treatment of other organic xenobiotics, but their use for pharmaceuticals removal has been only scarcely tested. Moreover, these systems have been approached primarily as a "black-box", without a thorough understanding of the processes involved. The efficiency of CWS in the removal of pollutants can be significantly enhanced by using adequate support matrices with a greater capacity to retain contaminants by sorption phenomena, ionic exchange or other physico-chemical processes and plant species with high capacity to tolerate and remove pollutants from contaminated waters.

The main goal of this work was to evaluate the ability of a microcosm CWS to remove selected pharmaceuticals from domestic wastewater that has received secondary treatment.

The selection of the pharmaceuticals to be studied was based on the following criteria: data on consumption and presence in the environment, behavior in WWTPs, and characteristics such as biodegradability, acid-base character, hydrophobicity and water solubility. Four pharmaceuticals were selected, in particular two acidic substances (ibuprofen, IB, and clofibric acid, CA), a neutral one (carbamazepine, CB) and another with an alkaline character (atenolol, AT). These are moderately lipophilic compounds, with the exception of AT, which is a somewhat hydrophilic substance. Among these, one of the compounds is biodegradable (IB), another one is only moderately biodegradable (AT), whereas the other two (CA and CB) are hardly biodegradable.

For the quantification of the studied pharmaceuticals in liquid and solid samples, analytical methodologies were developed and optimized for each sample type. For liquid samples (pharmaceuticals contaminated water and wastewater), the methodology comprised, whenever necessary, an optimized step of pre-concentration with solid phase extraction (SPE), chromatographic separation with high performance liquid chromatography (HPLC) and detection by UV/Vis spectrometry. For solid samples (plant tissues), a methodology was developed consisting of the extraction of CB from tissues by the sea sand disruption method (SSDM), separation by liquid chromatography and selective detection and quantification by quadrupole ion trap mass spectrometry with an electrospray interface (LC-ESI-MS/MS).

In order to select a suitable support matrix to be used in the microcosm CWS, assays were performed with several different materials. A preliminary screening of common materials (LECA 2/4, LECA 3/8, sand and expanded perlite) evidenced the high sorption capacity of LECA (the 2/4 grade in particular) for phenoxyacetic acids, a family of chemicals which CA is part of. Given the sorption qualities shown, this material was tested with the pharmaceuticals CB and IB as well. For several different conditions (aqueous solutions of the single compounds; aqueous solutions of CA, CB and IB mixtures; and treated wastewater spiked with the three compounds) LECA removed the pharmaceuticals at initial concentrations of 1 mg L^{-1} in large extent (50-95%). The following decreasing order was observed for the removed amounts of each pharmaceutical: CB > IB > CA. The kinetics of the sorption process was characterized by an initial fast step, with most pharmaceuticals being removed within the first 24 h. Equilibrium was attained, in general, after approximately 72–96 h, and the kinetic behavior seemed similar in all tested conditions. In subsequent studies, two other clay materials – sepiolite and exfoliated vermiculite – were also tested for the removal of the most recalcitrant compound, CA. In this study, vermiculite exhibited higher removal efficiency than LECA, but the low density of exfoliated vermiculite and its mechanical properties make it a less appropriate material for using in CWS.

In respect to the vegetation component, studies were conducted in hydroponic conditions under a controlled environment (temperature and humidity) to assess the ability of *Typha* spp. to withstand and remove, from water, the pharmaceuticals CA, IB and CB. In such assays, after 21 days of exposure to a solution spiked with 20 µg L^{-1} of each pharmaceutical, *Typha* spp. was able to remove, for all pharmaceuticals, over 80% of their initial amounts. Moreover, just within the initial period of 24–48 h, over 50% of

the compounds were removed, and even when the plants were subjected to concentrations several orders of magnitude higher (up to 2000 μg L^{-1}), still high removal efficiencies were observed.

In order to study the fate and metabolism which may affect some pharmaceuticals as they are removed from water by *Typha* spp., one of the studied compounds, CB, was quantified in leaf tissues of plants exposed to this xenobiotic in hydroponic assays. Non-conjugate CB could be detected in tissue samples of plants exposed to any of the tested pharmaceutical concentrations (500 – 2000 μg L^{-1}) which shows that CB is taken up by their roots and translocated to their leaves. Additionally, the metabolite 10,11-dihydro-10,11-epoxycarbamazepine was tentatively identified in *Typha* leaf extracts which is indicative of the metabolization of CB.

Some biochemical and physiological parameters were also determined in these assays in order to evaluate the ability shown by *Typha* spp. to cope with the presence of the studied xenobiotics. Exposure to high concentrations of CA, CB and IB did affect *Typha*'s growth in every case, but by the end of the assays, the plants' growth as well as photosynthetic pigments had, in general, approached normal values. An observed alteration in antioxidant enzymes' activities provided an indication that both roots and leaves were affected by the xenobiotics presence but, in general, *Typha* spp. seemed able to resist the pharmaceuticals' induced oxidative burst except when plants are subjected to the highest concentrations (largely above normal environmental concentration levels) of some of the pharmaceuticals, where the inhibition of some antioxidant enzymes might be regarded as an early sign of toxicity.

Taking in consideration the results obtained, a microcosm CWS was assembled using LECA and *Typha* spp. plants which was then evaluated for its capacity to remove the pharmaceuticals AT, CA, CB and IB from spiked wastewaters. Removal efficiencies of the studied pharmaceuticals were generally high (75-97%) after a maximum retention time of 7 days, with an earlier trend towards stabilization of the removal efficiencies being observed after 96 h. It was observed that the solid matrix composed by LECA 2/4 was responsible for most of the pharmaceuticals removal from wastewater, but the presence of the *Typha* spp. plants made an additional contribution to the removal efficiency of 6-32% in the summer (although of only 2-8% in the winter) and they also significantly increased the rate of the pharmaceuticals removal. Seasonal variability of this system's performance showed that there is some loss of efficiency during the winter season when compared to the removal efficiencies in the summer. The difference is

highest for the compounds where biological processes play a more important role (either through plant uptake, as in the case of CA, or biodegradation, as for IB). Temperature variations were observed to have a much lower impact on sorption, as this remained a relevant removal process even during the winter. In fact, the removal efficiencies of those pharmaceuticals for which sorption is the most important removal process (as was the case for CB) were minimally affected by seasonal variability. As expected, the removal during the summer occurred at a much faster rate than during the winter.

The results obtained in this work show the potential of a CWS using LECA 2/4 and *Typha* spp. plants for removing the studied pharmaceuticals from contaminated wastewaters.

Estudos para a remoção de fármacos de águas. Potencial utilização de Leitos Construídos de Macrófitas

Resumo

A qualidade da água é uma das grandes preocupações actuais em química analítica ambiental. Em particular, a ocorrência e destino de fármacos no ambiente aquático tem vindo a ser reconhecido como um dos problemas emergentes nesta área.

De facto, a frequente detecção de fármacos utilizados em medicina humana, seus metabolitos e produtos de transformação em efluentes de estações de tratamento de águas residuais urbanas (ETARs) bem como em águas naturais, superficiais e subterrâneas, e até mesmo em águas para consumo, tem demonstrado a existência de um problema real devido à crescente quantidade e diversidade de substâncias deste tipo que são excretadas e libertadas nos sistemas de recolha de efluentes líquidos urbanos. Como consequência da geralmente baixa eficiência de remoção destes contaminantes pelos processos de tratamento convencionais utilizados na maioria das ETARs, resulta que muitas destas substâncias acabam por ser descarregadas com os efluentes nos meios receptores hídricos e disseminadas pelos meios aquáticos.

As implicações resultantes desta descarga de fármacos no ambiente aquático sugerem a urgente necessidade de encontrar processos complementares ou alternativos para a sua remoção dos efluentes antes de alcançarem os cursos de água. A utilização de processos de tratamento terciário ou de afinação nas ETARs, como por exemplo a utilização de processos de membranas ou oxidação química, podem permitir o aumento das eficiências de remoção obtidas. No entanto, a implementação e manutenção deste tipo de processos têm custos elevados e são difíceis de adaptar para uma utilização em larga escala em ETARs.

Os leitos construídos de macrófitas (LCMs) têm surgido ultimamente como sistemas complementares ou, nalguns casos, alternativos de tratamento de águas residuais urbanas. Os LCMs, ou zonas húmidas construídas, são sistemas artificiais projectados e construídos para tirar partido do mesmo tipo de processos que ocorrem nas zonas húmidas naturais, utilizando vegetação, solos e colónias de microorganismos típicas destes sistemas, mas actuando de uma forma controlada e optimizada com o propósito de aplicação para tratamento de efluentes.

Os mecanismos de tratamento nos LCMs são extremamente diversificados, caracterizados por uma interacção complexa de processos físicos, químicos e biológicos,

que ocorrem ao nível das plantas, da matriz de suporte e dos microrganismos adaptados à toxicidade do efluente.

Os LCMs (geralmente usados numa fase secundária ou terciária do tratamento dos efluentes) têm apresentado bons resultados na remoção de vários xenobióticos orgânicos. Contudo, actualmente existem ainda poucos estudos relativos à remoção de resíduos farmacêuticos de águas contaminadas utilizando LCMs. Para além disso, os estudos existentes tratam o sistema como uma "caixa negra" sendo apenas avaliada a eficiência global de remoção, não tendo sido ainda estudada a contribuição de cada uma das componentes para os processos de remoção dos poluentes que ocorrem nestes sistemas. A eficiência de remoção dos poluentes nos LCM pode, no entanto, ser significativamente melhorada através de uma selecção criteriosa de matrizes de suporte que possuam uma elevada capacidade para reter contaminantes por sorpção, permuta iónica ou outros processos fisico-químicos, e de plantas que revelem uma boa tolerância aos poluentes e capacidade de os removerem de águas contaminadas.

Este trabalho teve, assim, como objectivo principal, avaliar a eficiência de um microcosmos de leitos construídos de macrófitas optimizado, por selecção prévia da matriz de suporte e avaliação das características da vegetação, para a remoção de quatro fármacos de um efluente recolhido após tratamento secundário.

A selecção dos fármacos que foram estudados teve como critérios principais o seu consumo em Portugal e em outros países, a sua presença no ambiente, o seu comportamento em ETARs convencionais, a sua biodegradabilidade, e a variedade de propriedades fisico-químicas apresentadas, nomeadamente características ácido-base, hidrofobicidade (K_{ow}) e solubilidade em água. Assim, para este estudo foram seleccionados o anti-inflamatório ibuprofeno (IB), o anti-epiléptico carbamazepina (CB), o beta-bloqueador atenolol (AT) e o metabolito de alguns reguladores lipídicos ácido clofíbrico (CA). Destes compostos o IB e o CA apresentam um carácter ácido, enquanto a CB é essencialmente neutra e o AT apresenta características básicas. Estes compostos são moderadamente lipofílicos com excepção do AT que é razoavelmente hidrofílico. Dois destes compostos são dificilmente biodegradáveis (CA e CB), o AT é considerado moderadamente biodegradável e o IB é um composto facilmente biodegradável.

Para a quantificação dos fármacos em dois tipos diversos de amostras, líquidas (soluções aquosas, soluções nutritivas e efluentes) e sólidas (tecidos de plantas), foram desenvolvidas e optimizadas metodologias analíticas adequadas. A metodologia desenvolvida para amostras líquidas incluiu, sempre que necessário, um passo de pré-concentração por SPE (solid phase extraction), seguida de separação por cromatografia

líquida de alta performance (HPLC – high performance liquid chromatography) e detecção por espectrometria UV/Vis. Para amostras sólidas, a metodologia desenvolvida para permitir a quantificação de CB em tecidos de folhas de planta consistiu na extracção deste composto pelo método de disrupção da amostra por areia (SSDM – sea sand disruption method), separação por cromatografia líquida e detecção e quantificação por espectrometria de massa (quadrupole ion trap).

No âmbito deste trabalho foram testados vários materiais com vista a uma possível utilização como matriz de suporte em LCMs para a remoção dos fármacos em estudo de efluentes. Num estudo preliminar foram avaliados alguns materiais vulgarmente utilizados (areia, LECA 2/4, LECA 3/8 e perlite expandida) tendo sido determinada a sua eficiência para a remoção de compostos da família dos fenoxiácidos, entre os quais se encontra o CA. Dos resultados obtidos destacou-se a LECA como o material com maior capacidade de sorpção para estes compostos, em particular em granulometrias mais pequenas (2/4). Dadas as qualidades demonstradas como sorbente, este material foi também testado com os fármacos CB e IB. Para várias condições (soluções aquosas dos compostos isolados; soluções aquosas de misturas de CA, CB e IB; e efluente dopado com os três fármacos) a LECA 2/4 apresentou elevadas remoções desses fármacos para concentrações iniciais de 1 mg L^{-1} (50-95%). Nestes ensaios observou-se a seguinte relação para as quantidades removidas de cada composto, por ordem decrescente: CB > IB > CA. A cinética do processo de sorpção caracterizou-se por uma fase inicial rápida, em que a maior parte da quantidade inicial dos fármacos foi removida durante as primeiras 24 h. Em geral, o equilíbrio estabeleceu-se aproximadamente no período de 72-96 h, tendo sido o comportamento cinético bastante similar em todas as condições testadas.

Para o CA, que foi o fármaco que se revelou mais recalcitrante de entre os estudados, foram ainda testados os materiais vermiculite esfoliada e sepiolite. Destes ensaios verificou-se que a vermiculite tem uma capacidade de sorpção bastante elevada para este composto. Este material tem, no entanto, uma baixa densidade e características mecânicas que tornam menos adequada a sua utilização em leitos.

No que concerne à componente vegetal, foram realizados estudos com *Typha* spp. em condições hidropónicas e num ambiente de humidade e temperatura controlados, de modo a avaliar a capacidade desta espécie de macrófitas para a remoção de CA, CB e IB bem como a resposta da planta à toxicidade induzida pela presença destes xenobióticos no seu meio nutritivo. Dos resultados obtidos verificou-se que a *Typha* spp. apresenta uma

elevada capacidade de remoção dos 3 fármacos estudados (>80%) após 21 dias de exposição a soluções nutritivas dopadas com 20 μg L^{-1}. Verificou-se também, em todos os casos, que mais de 50% dos compostos foram removidos em apenas 24-48 h, e que mesmo quando as plantas foram expostas a concentrações até cem vezes superiores (2000 μg L^{-1}) continuaram a observar-se elevadas eficiências de remoção sem sinais visuais de toxicidade.

De modo a estudar o destino e possível metabolização que alguns fármacos poderão ter na *Typha* spp. ao serem removidos por esta, efectuaram-se ensaios para quantificar um dos fármacos (CB) nos tecidos de folhas de plantas previamente expostas a este xenobiótico. Para toda a gama de concentrações iniciais de CB testadas em solução nutritiva (500 – 2000 μg L^{-1}) conseguiu-se detectar CB não-conjugada em amostras desses tecidos o que mostra que este fármaco foi efectivamente absorvido pelas plantas e translocado para a parte aérea, nomeadamente as folhas. Adicionalmente, foi possivelmente identificado o metabolito 10,11-dihydro-10,11-epoxycarbamazepine em extractos de folhas de *Typha* o que indicará a metabolização deste fármaco pela planta.

De modo a avaliar a capacidade da *Typha* spp. para tolerar a presença dos compostos xenobióticos estudados, foram determinados alguns parâmetros bioquímicos e fisiológicos, nomeadamente a actividade de enzimas antioxidantes como catalase, superóxido dismutase, ascorbato peroxidase e guaiacol peroxidase, bem como a variação do teor de pigmentos fotossintéticos (clorofila total, *a* e *b* e carotenoides) e ainda a taxa relativa de crescimento das plantas. Foi observado que o crescimento das plantas expostas às concentrações mais elevadas de CA, CB e IB foi, de facto, afectado. No entanto, verificou-se que ao fim dos 21 dias de duração destes ensaios os valores de crescimento bem como os teores em pigmentos fotossintéticos tinham, de um modo geral, regressado a níveis próximos do controlo. Observaram-se ainda alterações nas actividades das enzimas antioxidantes estudadas, tanto em tecidos de folhas como de raízes, dando uma indicação clara de as plantas terem sido afectadas pela presença dos xenobióticos. No entanto, no final dos ensaios, as plantas demonstraram terem sido capazes de reagir ao stress oxidativo induzido pela exposição aos fármacos, observando-se a reposição dos níveis de actividade enzimática para valores próximos aos dos ensaios controlo, excepto para alguns casos que correspondiam a plantas sujeitas às concentrações iniciais de fármacos mais elevadas (correspondentes, no entanto, a níveis de concentração muito acima dos normalmente encontrados em amostras ambientais).

Após a obtenção dos resultados relativos aos componentes do sistema estudados, procedeu-se à montagem de um microcosmos de LCM usando LECA 2/4 como matriz de suporte e

plantado com a espécie *Typha* spp. e avaliou-se a eficiência deste sistema na remoção dos fármacos AT, CA, CB e IB de um efluente dopado com estes compostos. As eficiências de remoção obtidas para os fármacos estudados foram elevadas (75-97%) após um período máximo de retenção de 7 dias, verificando-se contudo uma tendência para a estabilização da remoção dos compostos logo a partir das 96 h. Constatou-se que a matriz de suporte constituída por LECA foi a responsável pela remoção da maior parte da quantidade inicial dos fármacos presente no efluente. A presença da *Typha* spp. contribuiu para um aumento de remoção de 6 a 32% no Verão (mas apenas de 2 a 8% durante o Inverno) tendo também sido responsável por um aumento significativo da velocidade de todo o processo. Avaliou-se também o efeito sazonal sobre a performance deste sistema, tendo a comparação entre as eficiências de remoção de CA, CB e IB pelo microcosmos durante um período de Inverno e outro de Verão mostrado que, de facto, se observa uma perda de eficiência do sistema durante o Inverno. A diferença entre épocas é maior para os compostos em cuja remoção os processos biológicos desempenham um papel mais importante (seja a absorção pelas plantas, como é o caso do CA, seja a biodegradação, no caso do IB). Observou-se que nos processos de sorpção as alterações de temperatura têm uma menor influência, tendo estes permanecido os mais importantes processos de remoção mesmo durante o Inverno. Em consequência, os fármacos removidos essencialmente através de processos de sorpção, como foi o caso da CB, tiveram uma menor variação sazonal da eficiência de remoção. Tal como esperado, verificou-se ainda que a remoção dos fármacos ocorreu mais rapidamente durante o Verão do que durante o Inverno.

Os resultados obtidos durante a realização deste trabalho, e apresentados nesta dissertação, apontam para a potencial utilização dos LCM tendo LECA como matriz de suporte e plantados com a espécie *Typha* spp. para a remoção dos fármacos estudados de efluentes contaminados.

List of original publications and the author's contribution

Paper I: Dordio, A. V., Teimão, J., Ramalho, I., Carvalho, A. J. P., Candeias, A. J. E., 2007. Selection of a support matrix for the removal of some phenoxyacetic compounds in constructed wetlands systems .Sci. Total Environ. 380, 237-246.

Ana Dordio planned and performed the experimental work with the exception of the parts relating to the mineralogical characterization of the media by X-ray diffraction and the analysis of the media particles' morphology and macroporous structure by optical observation of polished surfaces and thin sections. She interpreted the results and wrote the paper.

Paper II: Dordio, A. V., Candeias, A. J. E., Pinto, A. P., Teixeira da Costa, C., Carvalho A. J. P., 2009. Preliminary media screening for application in the removal of clofibric acid, carbamazepine and ibuprofen by SSF-constructed wetlands. Ecol. Eng. 35, 290-302.

Ana Dordio planned and performed the experimental work with the exception of the parts relating to the media's mineralogical characterization by X-ray diffraction and the determination of their microstructural properties by SEM-EDX and optical microscopy. She interpreted the results and wrote the paper.

Paper III: Dordio, A. V., Duarte, C., Barreiros, M., Carvalho, A. J. P., Pinto, A. P., Teixeira da Costa, C., 2009. Toxicity and removal efficiency of pharmaceutical metabolite clofibric acid by *Typha* spp. – Potential use for phytoremediation? Biores. Technol. 100, 1156-1161.

Ana Dordio planned and performed the experimental work, interpreted the results and collaborated in the writing of the paper.

Paper IV: Dordio, A., Ferro, R., Teixeira, D., Carvalho, A. J. P., Pinto, A. P., Barrocas Dias, C., 2009. Potential of *Typha* spp. for the phytotreatment of water

contaminated with Ibuprofen. In preparation, to be submitted to Ecological Engineering (WETPOL 2009 special issue).

Ana Dordio planned and performed the experimental work, interpreted the results and wrote the paper.

Paper V: Dordio, A., Carvalho, A. J. P., Teixeira, D. M., Barrocas Dias, C., Pinto, A. P., 2009. Removal of pharmaceuticals in microcosm constructed wetlands using *Typha* spp. and LECA. Submitted to Bioresource Technology (BITE-S-09-02355).

Ana Dordio planned and performed the experimental work, interpreted the results and wrote the paper.

Paper VI: Dordio, A., Pinto, J., Barrocas Dias, C., Pinto, A. P., Carvalho, A. J. P., Teixeira, D. M., 2009. Atenolol removal in microcosm constructed wetlands. Accepted for publication by International Journal of Environmental Analytical Chemistry.

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List of abbreviations and symbols

ACN	Acetonitrile
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Interfaces
APX	Ascorbate Peroxidase
Aq	Aqueous
AsA	Ascorbic acid
AT	Atenolol
BTEX	Benzene, Toluene, Ethylbenzene, and Xylenes
CA	Clofibric Acid
CAT	Catalase
CB	Carbamazepine
CWS	Constructed Wetlands System
d_{10}	Effective grain size – maximum diameter of the grains that correspond to 10% of the sample mass
d_{60}	Maximum diameter of the grains that correspond to 60% of the sample mass
DAD	Diode Array Detector
DCM	Dichloromethane
DHA	Dehydroascorbate
DHAR	Dehydroascorbate Reductase
DNA	Deoxyribonucleic Acid
EC_{50}	Median Effect Concentration – the concentration of a toxicant inducing a response halfway between baseline and maximum effect
ESI	Electrospray Ionization
EtOAc	Ethyl acetate
FA	Formic acid
FTICR	Fourier transform ion cyclotron resonance
FWS	Free Water Surface
GC	Gas Chromatography
GPX	Glutathione Peroxidase
GR	Glutathione Reductase

GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
GTN	Glycerol Trinitrate
HAc	Acetic acid
HFBA	Heptafluorobutyric acid
HLB	Hydrophilic Lipophilic Balanced
HPLC	High Performance Liquid Chromatography
HSSF	Horizontal Subsurface Flow
IB	Ibuprofen
INFARMED	Autoridade Nacional do Medicamento e Produtos de Saúde, I. P. (formerly denominated, Instituto Nacional da Farmácia e do Medicamento)
K_d	Sorption distribution coefficient
K_F	Freundlich constant
K_H	Henry's law constant
K_L	Langmuir isotherm's constant
K_{ow}	Octanol-Water partition coefficient
LC	Liquid Chromatography
LECA	Light Expanded Clay Aggregates
LOD	Limit of Detection
LOQ	Limit of Quantification
MAX	Mixed-mode Anion Exchange
MCX	Mixed-mode Cation Exchange
MDA	Monodehydroascorbate
MDAR	MDA Reductase
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix Solid Phase Dispersion
MTBE	Methyl *tert*-Butyl Ether
NHS	National Health System
NOEC	No observed effects concentration
NSAID	Non-Steroidal Anti-Inflammatory Drug
OC	Organic Carbon

PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	Polychlorinated Biphenyls
pKa	Acid dissociation constant
PS	Primary Sludge
PZC	Point of Zero Charge
QIT	Quadrupole ion trap
QTOF	Quadrupole time-of-flight
RDX	Cyclotrimethylenetrinitramine
RGR	Relative Growth Rate
ROS	Reactive Oxygen Species
RP	Reversed Phase
S/N	Signal-to-noise
SF	Surface flow
SIM	Single Ion Monitoring
SOD	Superoxide Dismutase
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
SRT	Solids Retention Time or Sludge Age
SS	Secondary Sludge
SSDM	Sea Sand Disruption Method
SSF	Subsurface Flow
S_w	Water solubility
TCE	Trichloroethylene
TEA	Triethylamine
TNT	Trinitrotoluene
tQ	Triple Quadrupole
TSCF	Transpiration Stream Concentration Factor
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV/Vis	Ultraviolet-Visible
VSB	Vegetated Submerged Bed
VSSF	Vertical Subsurface Flow

WWTPs	**Wastewater Treatment Plants**

Chapter 1

1. Introduction

Increasing amounts of pharmacologically active substances are consumed yearly in human medicine for diagnosis, treatment, or prevention of diseases. Due to the variety of functions that these substances must perform, pharmaceuticals present very different physicochemical properties but most notably they can be characterized by their usual polar or ionic nature as they commonly must take effect in aqueous environments.

Through excretion or disposal of unused or expired products, pharmaceuticals and their metabolites are continuously introduced into the sewage system. As many of these compounds receive inefficient treatment in wastewater treatment plants (WWTPs) (which were not designed to deal with this type of pollutants) they eventually are released in the environment. This is considered to be the main route for contamination of the aquatic environment by pharmaceuticals (Nikolaou et al., 2007; Aga, 2008; Kasprzyk-Hordern et al., 2009; Kümmerer, 2009b). Over the last years, in numerous monitoring studies, residues of lipid regulating drugs, analgesics and anti-inflammatory drugs, antibiotics, hormones, antidiabetics, neuroactive compounds and beta-blocker drugs have all been detected worldwide in wastewaters, surface waters, ground waters and even drinking waters (Fent et al., 2006; Aga, 2008; Barceló and Petrovic, 2008; Miège et al., 2009; Kümmerer, 2009b).

The foreseeable environmental consequences of high environmental loads of pharmaceuticals points out to the urgent need of finding ways to retain and remove these pollutants before they reach the receiving water bodies. Optimization of the WWTP processes has been tried by increasing hydraulic and solid retention times. In addition, some advanced technologies have been evaluated to decrease their discharge into water bodies. However, despite the sometimes high removal efficiencies attained, these processes are generally not cost-effective on a large scale (Fent et al., 2006). In fact, there is still a great need for finding applicable technologies for removing pharmaceuticals from wastewater with higher efficiencies at reasonable cost of operation and maintenance.

A phytoremediation technology for wastewater treatment that is gaining increasing popularity is the constructed wetlands systems (CWS). Nowadays, CWS are becoming an alternative to conventional wastewater treatment processes or are being integrated in WWTPs as a secondary or tertiary treatment step. Low cost and low maintenance are some of its most attractive characteristics (Kadlec and Wallace, 2009).

These systems are now becoming a mature technology for the removal of bulk pollutants such as suspended solids, organic matter, pathogens and nutrients. Focus is now moving into the removal of more specific and recalcitrant compounds for which the conventional treatment systems are not effective. CWS are nowadays being increasingly used for the cleanup of specific pollutant types such as organic xenobiotics and new challenges have been emerging such as the removal of pharmaceuticals and other micropollutants which present new environmental problems to be solved.

Often CWS have been studied under a "black box" approach where only influent and effluent pollutants concentrations were measured and no more in-depth investigations were run. However, in order to use CWS as a more efficient response to these new challenges, a thorough understanding of the processes involved in pollutants removal in CWS is direly needed, as well as some knowledge of the ways the several CWS components may interact with each other synergistically. This has, in fact, been an effort which increasingly has been undertaken in the most recent years in the area of CWS research, not only in field studies but also in numerous lab studies as well.

1.1. Objectives of this study

This thesis addresses the problem of pharmaceuticals removal from wastewaters by studying a solution based on the constructed wetlands technology. Specific aims of this study were to:

- develop analytical methodologies for quantification of selected pharmaceuticals in aqueous (water and wastewater) and solid samples (plant tissues) (Chapters **2**, **3**, **4**)
- screen materials for selection of a suitable CWS's support matrix (Chapter **2**)
- study the potential of *Typha* spp. to tolerate and remove selected pharmaceuticals from aqueous solutions (Chapter **3**)
- evaluate the ability of a microcosm CWS to remove the selected pharmaceuticals from a spiked wastewater (Chapter **4**)
- characterize the role of the CWS components, in particular the solid matrix and the vegetation, in the performance of those systems (Chapter **4**)
- study the influence of seasonality on the removal of the selected pharmaceuticals in a microcosm CWS (Chapter **4**)

1.2. Human pharmaceuticals consumption and use

Human pharmaceuticals comprise a wide ranging class of bioactive compounds with substantial variability in chemical structures, functions, behavior and activity. Despite being consumed worldwide in increasing quantities, there are not enough data available on the total use of pharmaceuticals.

The consumption and application of human pharmaceuticals may vary considerably from country to country due to differences in the prevalence of diseases, treatment habits and options, or simply for market reasons. For instance, some pharmaceuticals are, in some countries, sold over the counter without prescription, while in others they are only available by prescription.

In the European Union over 3000 different pharmaceutical active substances are used in human medicine such as analgesics and anti-inflammatory drugs, beta-blockers, lipid regulators, neuroactive compounds, antibiotics among others (Fent et al., 2006; Hummel et al., 2006; Ternes et al., 2007).

In Portugal, the national regulatory institute (INFARMED) publishes annually statistics on the quantities of pharmaceuticals and active compounds sold nationwide. Detailed data, as published by INFARMED, on pharmaceuticals consumption (number of packages) in Portugal in the last 5 years, grouped by pharmacotherapeutic groups, is presented in Appendix A (Table A-4). As can be seen from these data, drugs consumption has increased in Portugal by 8% over the last five years, corresponding to gross sales of about 130 million packages in 2007. Increase in overall pharmaceuticals sales is expected to continue in the coming years due to ageing of the population, longer life expectancy, improving healthcare system and increasing availability of cheaper generic drugs due to patents expiration.

In Portugal, the top selling pharmaceutical classes are cardiovascular system drugs and central nervous system drugs, which together account for nearly 50% of the total packages sold in the National Healthcare System (NHS). A second group with significant sales is composed by drugs in the classes of the locomotor system, endocrine system and anti-infectious products (INFARMED, 2008).

Focusing on individual active substances, those with the highest number of packages sold in the Portuguese NHS in 2007 were the analgesic paracetamol and the psychodrug alprozolam (Xanax®). Anti-inflammatory drugs such as ibuprofen, diclofenac and

acetylsalicylic acid (Aspirin®) were also highly consumed and amoxicillin was the most sold antibiotic (INFARMED, 2008).

Among the top 100 active substances with greatest number of packages sold in the Portuguese NHS in 2007 (INFARMED, 2008) one can find many compounds commonly detected as surface and ground waters contaminants worldwide, including those already mentioned above and other substances such as carbamazepine, diazepam, bisoprolol or fenofibrate (Fent et al., 2006; Petrovic and Barceló, 2007; Aga, 2008; Miège et al., 2009). Figure 1.1 presents, for comparison, the sales of a few selected top 100 active substances, chosen mainly based on the criteria of their national consumption, representation of the main pharmacotherapeutic groups, occurrence in the environment (worldwide detection frequency), behavior in WWTPs, and their ecotoxicity.

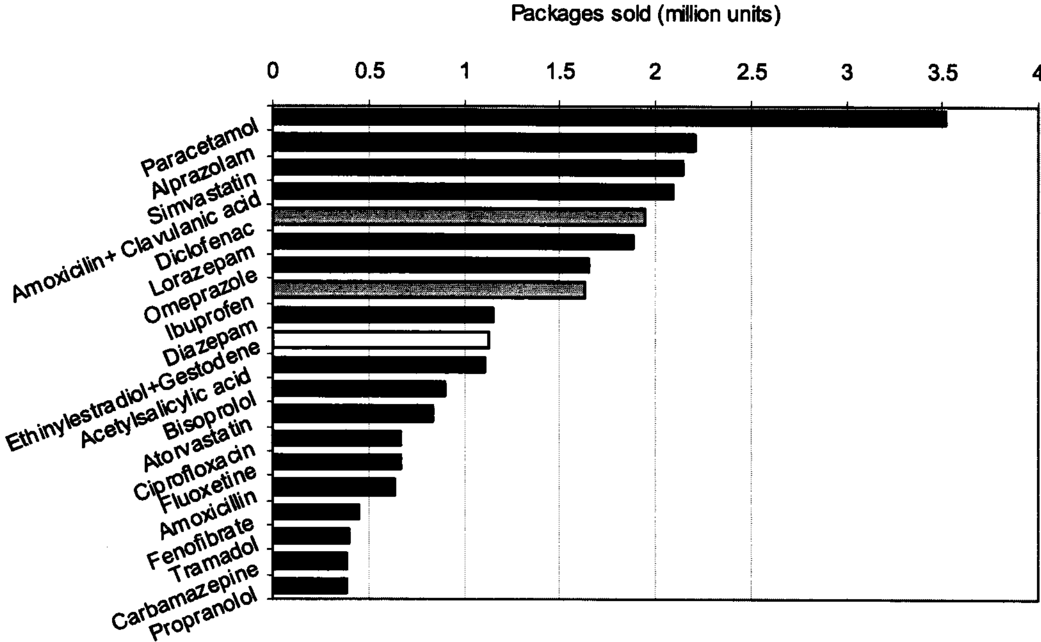

Figure 1.1. Consumption (million packages) of some specific pharmaceuticals in the Portuguese NHS in 2007 (source: INFARMED (2008)).

The Portuguese pharmaceuticals consumption profile is not easily comparable with those typical of other European countries, because the only global sales statistics available from the INFARMED are presented in terms of the number of packages sold whereas the statistics for other countries are usually presented in a weight basis (Table 1.1). However, it is clear from those lists that a lot of the most popular active substances in Portugal are also top selling abroad.

Table 1.1. Consumption of active substances (tons/year) in different countries

Compound name	USA[a] 2000	UK[b] 2000	Italy[c] 2001	Switzerland[d] 2002	Spain[e] 2003	Finland[f] 2005	Wales[g] 2006
Paracetamol	-	390.9	-	-	-	-	140.68
Aspirin	-	18.11	-	-	-	-	6.308
Ibuprofen	2300	162.2	1.90	17.98	276	-	11.00
Carbamazepine	-	40.35	-	-	20.0	4.6	2.515
Ketoprofen	-	-	-	0.254	-	-	0.07
Diclofenac	-	26.12	-	3.883	32.3	-	2.200
Naproxen	-	35.07	-	-	42.6	-	2.198
Atenolol	-	28.98	22.07	-	-	0.86	2.328
Bezafibrate	-	-	7.60	-	4.0	-	0.504
Amoxicillin	-	71.47	209.6	-	-	-	-
Sulfamethoxazole	309	-	12.7	-	-	-	0.115
Erythromycin	-	26.48	3.92	-	-	-	-
Mefenamic acid	-	14.52	-	17.257	-	-	3.145

[a] (Schwab et al., 2005); [b] (Jones et al., 2002); [c] (Zuccato et al., 2005); [d] (Tauxe-Wuersch et al., 2005); [e] (Carballa et al., 2008); [f] (Vieno et al., 2007a); [g] (Kasprzyk-Hordern et al., 2009)

1.3. Human pharmaceutical compounds in the environment

Pharmaceuticals have become one of the most important emerging classes of pollutants that have been detected in raw and treated wastewater, biosolids and sediments, receiving waters, groundwaters and drinking water (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Heberer, 2002a; Fent et al., 2006; Petrovic and Barceló, 2007; Nikolaou et al., 2007; Aga, 2008; Barceló and Petrovic, 2008; Miège et al., 2009; Kasprzyk-Hordern et al., 2009; Kümmerer, 2009b). Despite an only recent public awareness, pharmaceuticals, their metabolites and transformation products have, nevertheless, been entering the environment for many years. However, it was the recent advances in analytical methodologies and instrumentation (significantly lowering the detection and quantification limits for analyses of organic compounds in complex environmental matrices) that have allowed in-depth monitoring studies, which have called a public attention to this problem.

The widespread detection of a large range of pharmaceuticals in the environment has raised concern about the potential impact of these bioactive substances. The significance of pharmaceuticals as trace environmental pollutants deserves special attention, owing to several facts (Petrovic and Barceló, 2007; Enick and Moore, 2007):

- pharmaceuticals are continuously introduced in the aquatic environment via effluents from WWTPs, and for this reason they are referred to as "pseudo-persistent" contaminants (i.e. high removal rates are overcome by their continuous introduction into the environment);
- they are originally developed with the intention of performing a biological effect;
- they often have the same type of physico-chemical behavior as other harmful xenobiotics (persistence in order to avoid the substance to be inactivated before having a curing effect, and lipophilicity in order to be able to pass cell membranes); and
- they are present at minute concentrations, thus requiring more sophisticated and laborious analytical tools for their separation and accurate quantification.

1.3.1. Human pharmaceuticals sources, fate and effects in the environment

The distinction between source, origin, and fate of pharmaceuticals in the environment is often vaguely/not clearly made. In particular, the terms "source" and "origin" are often used interchangeably and the meanings are confused. The concepts are perhaps made clearer by distinguishing "origin" as the point at which the chemicals first begin to exist in any form, from "source" as being the point from which the chemicals are derived or obtained (Daughton, 2007).

Along a pollutant's environmental transport chain, a diversity of different exposure situations and effects can occur and, at some points in the chain, additional sources for pollutants may be considered (but not necessarily other origins). An actual "source" is often difficult to define precisely as a pharmaceutical leaves the manufacturer and goes through the supply-consumption cycle until it is released to the environment, where it can reside in several environmental compartments and exchange among them.

Identification/consideration of sources tends to focus on parent, unaltered pharmaceuticals. However, many of the sources of parent pharmaceuticals also serve as sources not only of excreted metabolites but also of environmental transformation products such as those resulting from microbial metabolism or photodecomposition, and this is a fact which is important to not disregard.

Over the last decade, scientists have established a large, diverse, and sometimes unexpected variety of routes through which human pharmaceuticals cross (and are distributed to) various environmental compartments. Figure 1.2 presents a representation of possible pathways for human pharmaceuticals in the environment.

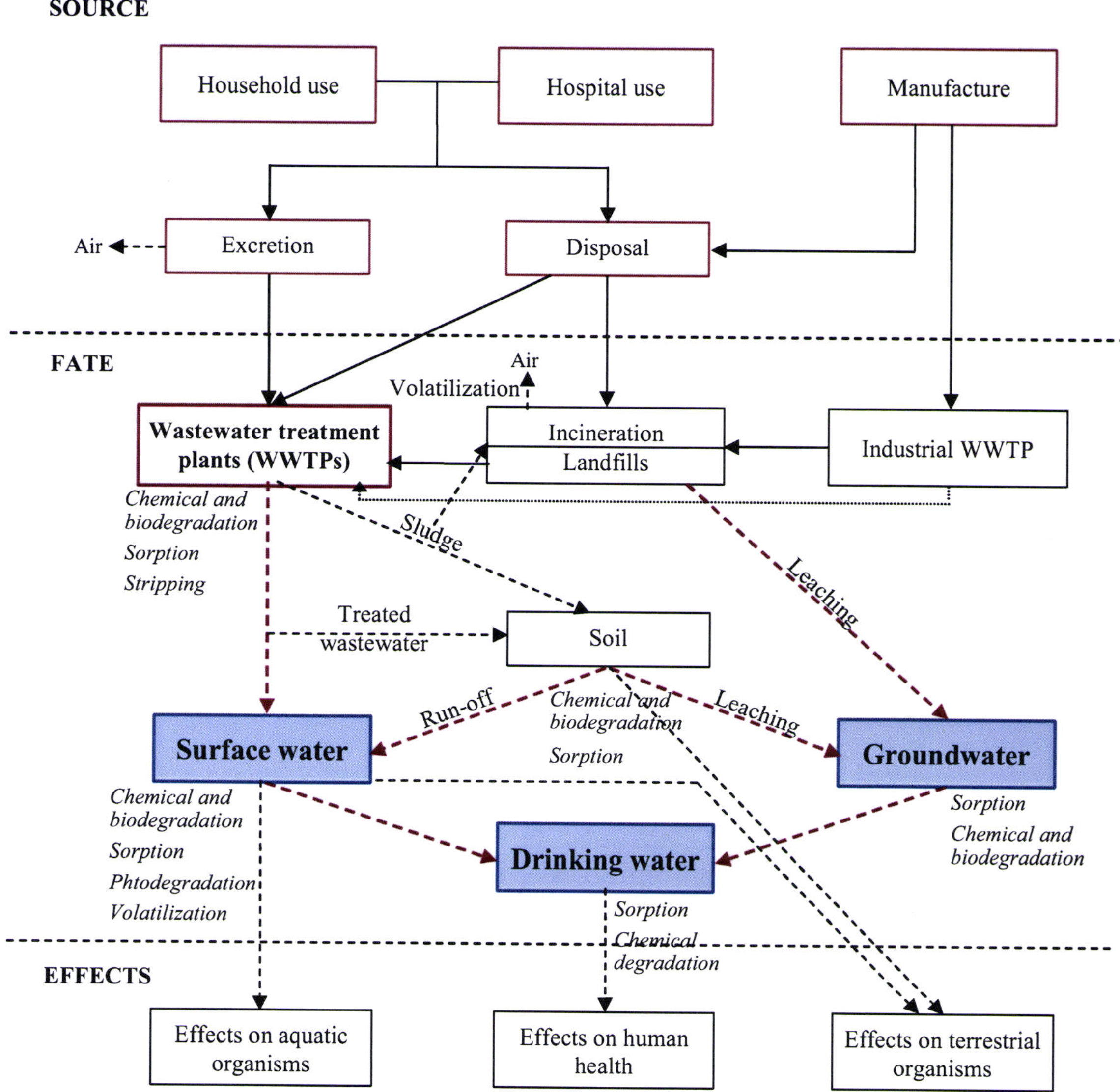

Figure 1.2. Pathways of human pharmaceuticals in the environment (adapted from Heberer (2002a)).

The primary route of entry for the human pharmaceuticals, their metabolites and transformation products into the environment is through wastewater point sources (Nikolaou et al., 2007; Aga, 2008). Pharmaceuticals enter the sewage system either through excretion of unmetabolized products and their metabolites or through the use of the sewage system to dispose of excess medications. In the WWTPs these compounds generally evade efficient removal by the conventional wastewater treatment processes.

Once released into the environment via the discharge of treated wastewater, pharmaceuticals are subjected to the same potential type of transport, transfer and transformation/degradation processes as other organic contaminants. Thus, the interaction of pharmaceuticals with soil, surface and ground water is similarly complex.

Aquatic transport and transformation processes of pharmaceuticals in the environment may include sorption, hydrolysis, biological transformation/degradation, redox reactions, photodegradation, volatilization and precipitation/dissolution (Petrovic and Barceló, 2007; Aga, 2008; Kümmerer, 2008; Farré et al., 2008; Kümmerer, 2009b). These processes occur continuously in the environment and influence the presence and mobility of pharmaceuticals in aquatic ecosystems. Response of drugs to any of these pathways for partitioning, degradation or transformation in the environment may reduce their concentrations in the environment or remove them entirely and thereby reduce their potential to impact human health and aquatic life. Pharmaceutical compounds that are marketed in large quantities and are water soluble or slightly soluble, yet resistant to degradation through biological or chemical processes, have the greatest potential to reach steady-state levels in the environment and to be detected in surface and ground waters and in drinking water supplies (Jjemba, 2006; Petrovic and Barceló, 2007; Aga, 2008).

A major difference between pharmaceuticals and other "traditional" environmental organic pollutants (e.g. solvents, pesticides, PCBs, PAHs) is that pharmaceutical compounds, in general, have passed through the human digestive tract and possibly through a conventional WWTP. Two consequences of this pre-exposure to biochemical metabolism are that many drugs will enter the aquatic environment in a modified form and those that are unaltered, consequently, share a resistance to biotic transformation. This allows certain inferences to be made regarding the importance of various abiotic transformation processes for pharmaceutical compounds in the aquatic environment (Arnold and McNeill, 2007).

Given the water solubility of many pharmaceuticals, the abiotic processes most likely to transform them and to more permanently remove them from the aquatic environment include hydrolysis and photodegradation (Petrovic and Barceló, 2007; Aga, 2008; Kümmerer, 2008). However, considering the passage of pharmaceuticals through the digestive tract and their relatively long-residence time in aqueous environments within the WWTPs, hydrolysis reactions likely play a less important role in the aquatic fate of many pharmaceuticals that reach the environment (Arnold and McNeill, 2007). On the other hand, direct photodegradation by sunlight may be an important elimination process for pharmaceuticals with absorbances in the 290-800 nm region (Velagaleti, 1997; Andreozzi et al., 2003).

In any case, the extent of the several abiotic and biotic processes that may potentially have an influence on the short-term behavior and long-term fate of a pharmaceutical in the environment are controlled by many factors related both with the pharmaceutical properties and with the environmental conditions.

Some of the most important pharmaceuticals properties that affect their fate in the environment are:

- ***Molecular Structure:*** several factors related to the pharmaceutical's structure are relevant for the removal processes and pharmaceutical distribution between the different compartments in the environment. Structural factors of major importance are pharmaceutical's molecular size, hydrophobicity, molecular charge, ability for hydrogen bonding, arrangement and interactions of molecular fragments and its coordination (Dragun, 1998). In general, pharmaceuticals are large and complex molecules with several functional groups. In this respect they form a very heterogeneous group (in comparison with other classes of pollutants such as PAHs, PCBs or dioxins) varying widely in their molecular structures, functionalities, molecular weights, etc (Cunningham, 2008).
- ***Polarity:*** the varied heteroatom content and multifunctional composition of many pharmaceuticals makes them compounds that are polar or ionizable, and this fact plays a major role in their solubility and mobility in the environment (Cunningham, 2008). A polar pharmaceutical will be very water soluble and tend to not be adsorbed onto organic matter. On the other hand, a non-polar compound will have a higher tendency to leave water and be adsorbed onto the solid matrix (e.g. soil, sediments or sludge) especially if it contains a substantial amount of organic matter (Cunningham, 2008). However, a fact that can not be ignored is that the ionization of some pharmaceuticals may lead to more complex ionic, ion pairing or complexation mechanisms of sorption which are not accounted for in the conventional simple mechanism of non-polar partitioning to organic matter in the solid matrix (Cunningham, 2008).
- ***Ionization constant (pKa):*** whether an acid or basic pharmaceutical exists as the neutral molecule or as its conjugate ionic species will have a profound influence on its behavior, availability to biological organisms and, ultimately, its environmental fate (Cunningham, 2008). Firstly, the ionic species will be much more water soluble than its neutral counterpart. Consequently, if the acid or basic pharmaceutical is easily ionized, it will not distribute as readily into a hydrophobic

(lipid-like) compartment, neither will it have a tendency to volatilize. Secondly, the pharmaceutical's charge can directly affect its affinity with the solid phase and determine the type of sorption mechanism (hydrophobic or ionic). It is not uncommon of pharmaceuticals to have more than one ionizable functional group which results, in such cases, to have several degrees of ionization for the molecules, controlled by the pH of the medium (Cunningham, 2008).

- ***Water solubility (S_w):*** the tendency of a compound to be in the aqueous medium is an important factor in determining its mobility and distribution. Solubility in water is mainly determined by the compound's polarity or tendency to ionize (as indicated by its pKa). However, other factors may strongly influence a compound's solubility such as temperature, pH and ionic strength of the solution, and dissolved organic matter content (Tinsley, 2004).
- ***Octanol-water partition coefficient (K_{ow}):*** is a measure of the hydrophobicity of an organic compound. An inverse relation between the K_{ow} and aqueous solubility is intuitive since compounds that are soluble in water are usually less soluble in non-polar organic solvents like octanol (playing the role of a surrogate for lipids) and vice versa. Therefore, the more hydrophobic a compound is, the less water soluble it becomes and the more likely it will adsorb to inorganic as well as organic solid particles (Cunningham, 2008).
- ***Sorption or solid-water distribution coefficient (K_d):*** is commonly used as a quantitative measure of the sorption of compounds from the aqueous compartment onto a solid phase (e.g. sludge, sediments or soil). The K_d value is simply a ratio of the sorbed phase concentration to the solution phase concentration at equilibrium (Cunningham, 2008). The K_d value is often estimated from the log K_{ow} of the contaminant and the organic matter content of the media. However, this approach is based on the assumption that the partitioning will be essentially into the organic fraction of the solid which is valid only for neutral and hydrophobic compounds. In fact, many pharmaceuticals are multifunctional ionizable compounds with a much more complex partitioning behavior which may include ionic, ion pairing and/or complexation interactions with solid particles. In this case, K_d should be experimentally determined at different pH values and solution ionic strengths in order to assess the role of ionic interactions.
- ***Volatility:*** this depends on the Henry's law constant (K_H) of the compound. Alternatively, for practical applications, it is most convenient to use a

"dimensionless" air-water partition constant or Henry's coefficient (K_{aw}) which is defined as the ratio between concentrations in the vapor and in the water phase (Schwarzenbach et al., 2003). Pharmaceuticals generally have low volatilities ($K_{aw} < 10^{-5}$ (POSEIDON, 2006)), since these are mostly compounds meant to take effect in an aqueous environment and are therefore rather hydrophilic. The volatility of a compound depends on temperature and may also be diminished if the compound is not available in water, e.g. when it is sorbed.

- ***Persistence*:** the ability of a pharmaceutical to remain active in the environment is measured by its half-life. Half-life is not an absolute characteristic of a substance but depends on environmental conditions, which accounts for the usual variety of reported values of this property for a given pharmaceutical. Persistence is a function of the chemical and biological degradation processes, which break down the pharmaceuticals into simpler compounds.

In addition to the compound's properties, the fate of pharmaceuticals is also determined by the environmental conditions. Some of those factors include the temperature, sunlight, pH, content of organic matter in soils and sediments and redox conditions.

Temperature strongly affects some physical properties of the pharmaceuticals such as vapor pressure, water solubility and, thereby, also the Henry's law constant. Furthermore, warmer temperatures tend to accelerate some physical, chemical, and biological processes such as volatility, chemical and microbial degradation.

Sunlight intensity also influences the rates of photodegradation processes which are responsible for some transformation of less photostable compounds.

The acidity or alkalinity of an aqueous environment can as well affect the fate and removal of pharmaceuticals by influencing some of their properties such as the degree of ionization, solubility and, in some cases, even the chemical stability of the compound. Additionally, the physiology of microorganisms and the activity of extra-cellular enzymes is strongly influenced by the medium pH as biological processes are usually only effective within a narrow range of pH values.

Organic matter content influences the sorption affinity of the solid particles for compounds that are more hydrophobic (such as the non-polar pharmaceuticals) as well as the level of biological activity. Organic matter also serves as an energy source for microorganisms that is essential for the breakdown of some biodegradable pharmaceuticals.

The abundance of dissolved oxygen is an environmental condition that determines the extent and type of redox phenomena present, both in chemical and in biological processes. In general, it is observed that pharmaceuticals are more efficiently degraded in aerobic conditions than in anoxic or anaerobic conditions (Zwiener et al., 2000; Zwiener et al., 2002; Jones et al., 2005).

Evidence being accumulated over the latest years supports the case that, under ordinary conditions, pharmaceuticals have such physico-chemical properties which makes them in many cases refractory to degradation and transformation and, consequently, do indeed have the potential to reach the environment (Halling-Sørensen et al., 1998; Fent et al., 2006; Petrovic and Barceló, 2007; Nikolaou et al., 2007; Aga, 2008; Kümmerer, 2009b). However, little is known about the impending human or ecological hazards that can arise from the cumulative exposure to the "cocktail" of pharmaceuticals and metabolites present in the different environmental compartments (notwithstanding the low concentrations at which they are observed to occur).

Human pharmaceuticals are designed to target specific metabolic and molecular pathways and, as side-effect, when introduced in the environment they may affect analogous pathways in animals having identical or similar target organs, tissues, cells or biomolecules. Even in animals lacking or having different receptors for drugs, dissimilar modes of action may occur. It is important to recognize that, for many drugs, their specific modes of action are not well known and often not only one but many different modes of actions occur. Therefore, the ecotoxicity of most pharmaceuticals is difficult to assess (Fent et al., 2006).

The current literature about ecotoxicological effects of human pharmaceuticals deals mainly with the short-term exposure acute toxicity evaluated in standardized tests and generally focused on aquatic organisms. Acute toxicity values are in the mg L^{-1} dose range for most of the pharmaceuticals detected in the environment (Halling-Sørensen et al., 1998), but reported levels in surface water are at least three orders of magnitude below (Fent et al., 2006; Nikolaou et al., 2007; Aga, 2008). It is, however, more difficult to assess (but more relevant) whether there is any environmental significance with regard to long-term chronic effects as these toxicity data is generally lacking (Fent, 2008).

Nonetheless, some main effects can be identified which derive from the presence of pharmaceuticals and related substances in the environment:

- ***Cumulative impacts:*** pharmaceuticals in the environment are often present as complex mixtures which may result in additive, synergistic, or antagonistic effects even when individual components might be present at concentrations too low to raise concern. The probabilities of these effects depend on the type of compound and its mode of action, although even mixtures of compounds with different modes of action may cause non-negligible effects. Some mixtures have been shown to have additive effects, such as concentration addition, so that above a certain threshold even very small amounts of a compound will contribute to the overall toxicity. It is also possible for the mode of action of one compound to have an effect on others. This effect may be antagonistic, thus reducing the overall toxicity of the mixtures, or synergistic and therefore increasing the toxicity beyond what would be expected for concentration addition. In fact, additive or even synergistic effects can render some pharmaceuticals mixtures dangerously potent (Daughton and Ternes, 1999; Hernando et al., 2004; Bendz et al., 2005; Fent et al., 2006; Fent, 2008).
- ***Endocrine disruption:*** chemicals which can disturb the normal function of hormones, cause environmental damages even if they are found in very low concentrations. Endocrine disruption studies have focused mainly on the sexual/reproductive hormone system. Knowledge about the disruption of other hormone systems is scarce (Jørgensen and Halling-Sørensen, 2000; Bendz et al., 2005; Fent, 2008; Bolong et al., 2009).
- ***Development of antibiotic-resistant bacteria:*** antibiotics have effects that are different from those of common xenobiotics, because bacteria are the target organisms of antibiotics. The increasing consumption of antibiotics during the last decades has caused a genetic selection of more resistant bacteria which are, therefore, more difficult to eliminate. Moreover, it seems that development of antibiotics resistance is favored, not only by widespread consumption, but also by pollution or concentrations of antibiotics in waters and/or sediments (Halling-Sørensen et al., 1998; Kim and Aga, 2007; Cooper et al., 2008; Kümmerer, 2009a).
- **Genotoxic effects:** genotoxicity is a measure of the ability of a substance to damage the DNA and chromosomes of cells. Several pharmaceuticals are genotoxic (e.g. cytostatic substances, antibiotics and antineoplastic drugs) (Isidori et al., 2007; Zounkova et al., 2007; Farré et al., 2008; Sarikaya and Yüksel, 2008).

Overall, the ecotoxicity of pharmaceuticals can be characterized as a game of risk. The possibility of negative impacts is present, and a number of researchers are trying to quantify the risk posed by various pharmaceuticals (Crane et al., 2006; Emblidge and DeLorenzo, 2006; Hernando et al., 2006b; Enick and Moore, 2007; Cooper et al., 2008; Cunningham et al., 2009). Risk assessments rely on models that predict the physical, chemical, and biological properties and the corresponding ecotoxicity potential of non-assessed compounds by comparing them to assessed compounds. Sanderson et al. (2004) have prioritized drug classes in terms of their predicted toxicity, ranking sedatives and anti-psychotics as high priority, while anti-epileptics were ranked lower on the priority list. For specific pharmaceutical compounds Hernando et al. (2006b) and Cooper et al. (2008) calculated risk quotients from known toxicology data, and identified a set of high risk pharmaceuticals among which are ibuprofen, carbamazepine, naproxen, diclofenac and ketoprofen.

1.3.2. Human pharmaceuticals occurrence in the environment

The environmental occurrence of pharmaceuticals was first reported in 1976 by Garrison et al. in the USA, who detected clofibric acid in treated wastewater at concentrations from 0.8 to 2 $\mu g\ L^{-1}$. In Europe, the first comprehensive studies of the occurrence of pharmaceuticals in rivers and streams were reported in the mid 1980s by Watts et al. (1983), Waggott (1981), and Richardson and Bowron (1985). In Canada, ibuprofen and naproxen were also detected in wastewaters in 1986 (Rogers et al., 1986; Nikolaou et al., 2007; Hao et al., 2007). After these findings, the occurrence of pharmaceuticals in environmental samples has been investigated in several countries: Brazil (Stumpf et al., 1999), Canada (Lishman et al., 2006; Hao et al., 2006; Comeau et al., 2008), UK (Ashton et al., 2004; Zhang and Zhou, 2007; Kasprzyk-Hordern et al., 2008), France (Andreozzi et al., 2003; Rabiet et al., 2006; Leclercq et al., 2009), Germany (Ternes, 1998; Heberer, 2002a; Weigel et al., 2004; Hernando et al., 2006a; Osenbrük et al., 2007), Greece (Koutsouba et al., 2003; Andreozzi et al., 2003), Italy (Andreozzi et al., 2003; Zuccato et al., 2005), Spain (Hernando et al., 2006a; Carballa et al., 2008; Kuster et al., 2008), Sweden (Andreozzi et al., 2003; Bendz et al., 2005; Zorita et al., 2009), USA (Stackelberg et al., 2004; Benotti and Brownawell, 2007; Palmer et al., 2008; Benotti et al., 2009), among others. Where pharmaceuticals have been detected in WWTPs effluents or water resources, their concentration levels are between ng L^{-1} and $\mu g\ L^{-1}$, as is shown in Table 1.2.

Table 1.2. Occurrence of pharmaceuticals residues in environmental samples (ng L^{-1})

Compound	Surface water	Ground water	Drinking water	WWTPs effluent	Reference
Analgesics and anti-inflammatory drugs					
Ibuprofen	< 2	< 2	< 2	30 [a]	(Lin et al., 2005)
	152 [b]	-	<12	6900 [b]	(Hernando et al., 2006b)
	5850	-	510-1350	-	(Loraine and Pettigrove, 2006)
	0.3-4.5	0.2-0.6	-	20-220	(Rabiet et al., 2006)
	(28) 38 [c]	-	-	(65) 137 [c]	(Kim et al., 2007)
	-	-	-	(0.8) 24.6 [d]	(Miège et al., 2009)
	(22) 48 [c]	-	-	(236) 424 [c]	(Kasprzyk-Hordern et al., 2009)
Ketoprofen	30 [a]	< 2	< 2	< 2	(Lin et al., 2005)
	< 26	-	< 26	< 75	(Hernando et al., 2006a)
	2.8-15	2.8-15	-	20-1080	(Rabiet et al., 2006)
	-	-	-	(0.21) 1.62 [d]	(Miège et al., 2009)
Naproxen	30 [a]	< 1	< 1	170 [a]	(Lin et al., 2005)
	70 [b]	-	-	630 [b]	(Hernando et al., 2006a)
	(11) 18 [c]	-	-	(128) 483 [c]	(Kim et al., 2007)
Diclofenac	-	< 2	< 2	< 2	(Lin et al., 2005)
	72 [b]	< 7	-	1420 [b]	(Hernando et al., 2006a)
	1.4-33	-	1.4-2.5	210-490	(Rabiet et al., 2006)
	(3.0) 6.8 [c]	-	-	(40) 127 [c]	(Kim et al., 2007)
	-	-	-	(0.42) 1.95 [d]	(Miège et al., 2009)
Blood lipid regulators					
Clofibric acid	(66) 550 [c]	-	-	(360) 1600 [c]	(Ternes, 1998)
	-	< 18	-	-	(Sacher et al., 2001)
	35 [b]	-	< 17	107 [b]	(Hernando et al., 2006a)
	(14.7) 118.5 [b]	-	-	-	(Moder et al., 2007)
	-	-	-	(0.15) 0.23 [d]	(Miège et al., 2009)
Gemfibrozil	(6.6) 9.1 [c]	-	-	(11.2) 17 [c]	(Kim et al., 2007)
Bezafibrate	-	-	-	(2200) 4600 [c]	(Ternes, 1998)
	< 2	-	-	-	(Hao et al., 2006)
Beta-blockers					
Atenolol	-	< 8.2	-	-	(Sacher et al., 2001)
	10-60	-	-	160	(Bendz et al., 2005)
	-	-	-	(0.15) 0.38 [d]	(Miège et al., 2009)
	(63) 258 [c]	-	-	(2870) 7602 [c]	(Kasprzyk-Hordern et al., 2009)
Metoprolol	-	-	-	10-390	(Andreozzi et al., 2003)
	30-70	-	-	190	(Bendz et al., 2005)
Sotalol	-	560 [b]	-	-	(Sacher et al., 2001)
Neuroactive compounds					
Fluoxetine	1.7	-	-	NA	(Kim et al., 2007)

Values with "<" were below the limit of quantification or limit of detection; NA= not applicable
[a]mean; [b]maximum; [c](mean) maximum; [d](median) maximum; [e]median; [fig]= estimated from a figure
(Note: wherever the type of data is not specified (i.e. whether mean, median, etc.) it is because that is not clearly stated in the bibliographic source)

Table 1.2. Occurrence of pharmaceuticals residues in environmental samples (ng L^{-1}) (cont.)

Compound	Surface water	Ground water	Drinking water	WWTPs effluent	Reference
Carbamazepine	(250) 1100 [d]	-	-	(2100) 6300 [c]	(Ternes, 1998)
	60-1500 [fig]	-	258 [b]	-	(Stackelberg et al., 2004)
	< 8	< 6	< 6	420 [c]	(Lin et al., 2005)
	24.56	14-43	-	160-290	(Rabiet et al., 2006)
	(25) 61 [c]	-	-	(226) 729 [c]	(Kim et al., 2007)
	-	-	-	(0.52) 2.30 [d]	(Miège et al., 2009)
	(11) 27 [c]	-	-	(2499) 4596 [c]	(Kasprzyk-Hordern et al., 2009)
Antibiotics					
Sulfamethoxazole	(20) 36 [c]	< 50	-	-	(Stackelberg et al., 2004)
	10 [b]	-	-	70	(Bendz et al., 2005)
	70 [b]	-	-	-	(Weigel et al., 2004)
	-	-	-	(0.07) 0.32 [d]	(Miège et al., 2009)
Ofloxacin	-	-	-	120-580	(Andreozzi et al., 2003)
Ciprofloxacin	-	-	-	250 [e]	(Zuccato et al., 2005)

Values with "<" were below the limit of quantification or limit of detection; NA= not applicable
[a]mean; [b]maximum; [c](mean) maximum; [d](median) maximum; [e]median; [fig]= estimated from a figure

The occurrence of pharmaceuticals in different environmental compartments, especially waters, has been already reviewed by several authors (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Kümmerer, 2001; Jones et al., 2001; Heberer, 2002a; Fent et al., 2006; Petrovic and Barceló, 2007; Nikolaou et al., 2007; Khetan and Collins, 2007; Aga, 2008; Kümmerer, 2008; Kümmerer, 2009b). Many of the compounds that have become ubiquitous in surface waters and treated wastewater are mostly from the classes of the anti-inflammatories, antibiotics, blood lipid regulators, beta-blockers or neuroactive drugs (Nikolaou et al., 2007; Aga, 2008; Miège et al., 2009). The anti-inflamatories ibuprofen, naproxen, diclofenac are widely consumed drugs that are also very often detected in WWTPs effluents, ground and surface waters and even in drinking waters (Table 1.2). Among the blood lipid regulators, the highly prescribed fibrates drugs also appear frequently in wastewaters as well as natural water bodies, but particularly in the form of their bioactive metabolite clofibric acid, one of the earliest detected and most notorious water contaminants of pharmaceutical origin (Table 1.2). Several beta-blockers (atenolol, metoprolol, propanolol and sotalol) have also been detected in effluents and in surface waters in many studies, of which atenolol seems to be, in this class, the most frequently found worldwide (Table 1.2). Within neuroactive drugs, carbamazepine, fluoxetine, and diazepam are the most studied and frequently

detected substances, with carbamazepine having an especially common presence in the aquatic environment due to a long history of clinical usage and very recalcitrant behavior. In addition, the high ubiquity of several antibiotics (e.g. ofloxacin, roxithromycin, ciprofloxacin and sulfamethoxazole) in WWTPs effluents is also confirmed by several studies (Table 1.2). In all countries with developed medical care systems, some other compounds such as X-ray contrast media can also be expected to be present at appreciable concentrations in wastewaters (Heberer, 2002a; Putschew and Jekel, 2007).

Among the most consumed drugs, those harder to biodegrade typically tend to be more frequently present in treated wastewaters and environmental samples. Nevertheless, the presence of some of the easily biodegradable pharmaceuticals may also still occur in effluents of WWTPs, even though conventional wastewater treatment processes may achieve high removal efficiencies for some of these compounds. However, the heavy influent loads of some of the most consumed pharmaceuticals will result, even after efficient treatment, in the discharge of these compounds into the receiving water bodies.

In addition to the parent pharmaceutical compounds, a series of ensuing metabolites and transformation products are also of great environmental importance as in some cases their ecotoxicity may even exceed that of the original drugs. However, the occurrence of metabolites or transformation products has not yet been studied in much detail apart from some specific compounds (e. g. clofibric acid, 10,11-dihydro-10,11-epoxycarbamazepine, 3 – hydroxycarbamazepine, salicylic acid, hydroxyl-ibuprofen, carboxy-ibuprofen) (Farré et al., 2008; Miège et al., 2009; Leclercq et al., 2009).

The existing published data show (Table 1.2) that the majority of the pharmaceuticals detected worldwide are also widely prescribed drugs in Portugal according to INFARMED, in particular drugs used to treat cardiovascular diseases (e.g. beta-blockers, blood lipid regulators). Therefore many of such drugs can be expected to be present in Portuguese water samples as well. Remarkably, however, there is no reported study on their prevalence in Portugal to date and the characterization of the Portuguese scenario concerning water contamination with pharmaceuticals is yet to be done.

1.3.3. Studied pharmaceuticals – selection of pertinent pharmaceuticals

Given the large number and variety of pharmaceutical compounds detected in the aquatic environment, a pre-selection of the pharmaceuticals to be studied in this work was necessary. The criteria taken into account were: i) portuguese yearly consumption;

ii) worldwide reported occurrence in the environment; iii) biodegradability; iv) behavior in conventional WWTPs; v) suitability for the available analytical methods; and vi) physical-chemical properties of the compounds. A variety of characteristics, namely the acid-base characteristics, hydrophobicity (K_{ow}), water solubility and volatility were considered.

This work began by initially focusing on two acidic and one neutral pharmaceuticals: ibuprofen, clofibric acid and carbamazepine respectively. In order to complement the studies with a pharmaceutical with basic character, at a later stage, tests were extended to include atenolol. A summary of the most important physico-chemical properties of these substances is presented in Table 1.3.

Table 1.3. Physico-chemical properties of studied pharmaceuticals

Compound	Henry's law constant ($atm\ m^3\ mol^{-1}$) [a]	Water solubility ($mg\ L^{-1}$) [a]	pKa	log K_{ow}	log K_d	
					Sludge [d,e]	Soil and sediment [f,g,h]
Clofibric acid	2.19×10^{-8}	583	3.18 [b]	2.57 [a]	0.68 [SS]	-0.52 [Sed] 0.49 – 0.73 [Soil]
Ibuprofen	1.50×10^{-7}	21	4.91 [a]	2.48 [c]	< 1.3 [PS] 0.85 [SS]	-0.76 – 0.23 [Sed]
Carbamazepine	1.08×10^{-10}	17.7	13.9 [a]	2.45 [a]	< 1.3 [PS] 0.09 [SS]	-0.68 – 0.72 [Sed] 1.57 [Soil]
Atenolol	1.37×10^{-18}	13300	9.6 [a]	0.16 [a]	-1.42	-

[a](SRC, 2009); [b](Packer et al., 2003); [c](Scheytt et al., 2005a); [d](Ternes et al., 2004); [e](Maurer et al., 2007); [f](Beausse, 2004); [g](Drillia et al., 2005); [h](Scheytt et al., 2005b)
[PS] = primary sludge; [SS] = secondary sludge; [Sed] = sediment; - = data not found

These pharmaceuticals are widely consumed in Portugal (and worldwide) and, in several studies, traces of all these substances have been detected in treated wastewaters, surface and ground waters and drinking waters worldwide, thus indicating their incomplete removal in conventional WWTPs (Heberer, 2002a; Tixier et al., 2003; Paxeus, 2004; Fent et al., 2006; Nikolaou et al., 2007; Zhang et al., 2008; Miège et al., 2009; Kasprzyk-Hordern et al., 2009).

Acidic pharmaceuticals: Clofibric acid and Ibuprofen

Clofibric acid stands as a representative for a chemically stable, recalcitrant metabolic product of a pharmaceutical. This non-biodegradable active metabolite of the some blood lipid regulators drugs, the fibrates (clofibrate, etofyllin clofibrate and etofibrate), is well-known for its persistence and widespread occurrence in water resources (Winkler et al., 2001; Tixier et al., 2003; Khetan and Collins, 2007; Evangelista et al.,

2008). This substance was detected early in the 1970s in domestic wastewaters and, since then, it is one of the most frequently reported pharmaceuticals in monitoring studies (Garrison et al., 1976; Heberer, 2002a; Khetan and Collins, 2007; Miège et al., 2009; Kasprzyk-Hordern et al., 2009). Clofibric acid is also classified as a plant growth regulator (antiauxin) pesticide (Wood, 2009). Its structure is suggestive of chlorophenoxy acid herbicides being, in fact, an isomer of one such herbicide, mecoprop (Emblidge and DeLorenzo, 2006).
Ibuprofen, a non-prescription drug that is among the most consumed pharmaceuticals all over the world, is a non-steroidal anti-inflammatory drug (NSAID) used in the treatment of rheumatic disorders, pain and fever. Ibuprofen environmental contamination is a result of the very high amounts of this drug entering the WWTPs which, despite the also high removal rates (up to 90%) (Paxeus, 2004; Fent et al., 2006; Petrovic and Barceló, 2007; Aga, 2008; Kasprzyk-Hordern et al., 2009), still results in the discharge of contaminated effluent, thus making it a frequently detected pharmaceutical in receiving water bodies downstream the WWTPs (Tixier et al., 2003; Weigel et al., 2004; Petrovic and Barceló, 2007; Nikolaou et al., 2007; Khetan and Collins, 2007).

Neutral pharmaceutical: Carbamazepine

The antiepileptic drug carbamazepine is widely used and is also a well-known ubiquitous contaminant of municipal WWTPs effluents and natural water bodies (Heberer, 2002a; Petrovic and Barceló, 2007; Khetan and Collins, 2007; Aga, 2008; Zhang et al., 2008). The frequent presence of carbamazepine in treated wastewaters is due to the general inefficiency of conventional wastewater treatment processes to remove this recalcitrant contaminant from wastewaters (Paxeus, 2004; Kasprzyk-Hordern et al., 2009; Leclercq et al., 2009; Wick et al., 2009). In fact several studies confirmed that carbamazepine is a compound persistent to biodegradation (Stamatelatou et al., 2003; Zhang et al., 2008).

Basic pharmaceutical: Atenolol

The beta-blocker atenolol is used in the treatment of high blood pressure as well as in the recovery from heart attacks. In several studies, trace concentrations of this compound was detected in natural water bodies and in domestic wastewaters, thus indicating its incomplete degradability in WWTPs (Paxeus, 2004; Gros et al., 2006b; Maurer et al., 2007; Hernando et al., 2007b; Palmer et al., 2008; Snyder, 2008; Wick et

al., 2009). Those studies also showed that, despite the low concentrations detected, some damaging effects were caused by atenolol on the aquatic ecosystems (Maurer et al., 2007; Snyder, 2008).

A more detailed information on the pharmaceuticals used in this study is provided in Appendix A.

1.4. Why are pharmaceuticals not efficiently removed in conventional WWTPs?

Recent studies have clearly shown that the removal of pharmaceutical compounds in municipal WWTPs is often incomplete. In fact, up to 90% of the initial amounts of pharmaceuticals entering the WWTPs may remain in effluent after treatment (Fent et al., 2006; Aga, 2008; Cooper et al., 2008). As a consequence, a significant fraction of the pharmaceuticals and their metabolites entering the WWTPs are discharged with the final effluent into the aquatic environment.

The treatment processes in municipal WWTPs are designed to remove bulk constituents of wastewater such as suspended solids, dissolved biodegradable organic matter, pathogens and nutrients by physical, chemical and biological processes available along the consecutive stages of a conventional treatment (Figure 1.3).

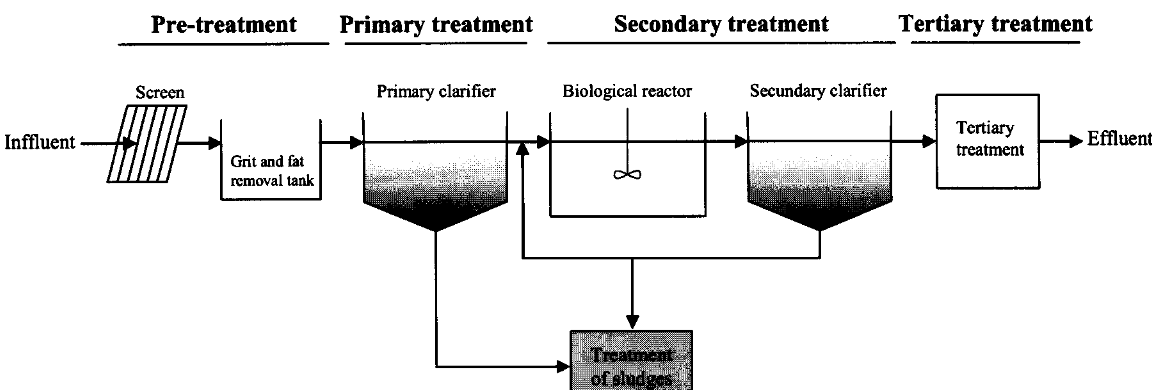

Figure 1.3. Diagram of a conventional wastewater treatment plant (adapted from Tchobanoglous et al. (2003)).

Conventional WWTPs were not designed to deal with pharmaceuticals or trace pollutants in general. Due to the highly variable physical and chemical properties of these compounds, the efficiencies by which they are removed may vary substantially. It

is also mostly unknown whether WWTPs could be cost-effectively modified to reduce pharmaceutical discharges. Typically, there is very little elimination of organic micropollutants from the preliminary treatment of wastewater, and it is also unlikely that many pharmaceuticals will be removed during screening or primary sedimentation (Jones et al., 2005). As there is little biological activity, any pollutant removal at this stage will rely on both the tendency of the individual drugs to adsorb to solids and the degree of suspended solids removal in the primary sedimentation tanks (Zhang et al., 2008). Usually, there is little change in dissolved polar organics (such as pharmaceuticals) at this point, so little to no loss of polar drugs may be expected here. In general, elimination of any compound by sorption to sludge is considered relevant only when the log K_d for that compound is higher than 2.48 (i.e. $K_d > 300$ L kg^{-1}) (Joss et al., 2005). That is far from being the case for the drugs clofibric acid, ibuprofen, carbamazepine and atenolol, which all have log K_d in the range of 0.9-1.6 (Table 1.3).

Activated sludge and trickling filters are the more common types of secondary biological treatment used in conventional WWTPs. Losses of drugs in both treatments may occur by the same mechanisms as other organic micropollutants, which include sorption to and removal in sludge and/or chemical degradation/transformation (such as hydrolysis) and biotransformation/biodegradation (aerobic, anoxic and anaerobic). In activated sludge processes, little loss by volatilization during aeration (stripping) is expected due to the low volatility of most pharmaceuticals (Larsen et al., 2004; Miège et al., 2009), including the ones studied here (Table 1.3). In fact, it is found that Henry coefficients of over ~ 10^{-3} are required for significant stripping in a bioreactor with fine bubble aeration (Larsen et al., 2004).

According to the literature ibuprofen has been found to be readily biodegraded and, consequently, highly removed during secondary (biological) treatment (usually > 90%). However, little removal is usually obtained at this stage for recalcitrant pharmaceuticals such as carbamazepine (< 20%) or clofibric acid (< 40%) which are harder to biodegrade (Table 1.4). Atenolol has been found to be biodegradable in WWTPs (Table 1.4). However the degradation rates are too low for the complete biodegradation of the compound and therefore, elimination in WWTPs has been found to be incomplete (Table 1.4).

Drugs remaining in the wastewater after primary and secondary treatment may be eliminated by tertiary or advanced treatments. However, in most countries only a small number of WWTPs include these adaptations. Advanced treatment techniques such as

chemical oxidation (e.g. ozonation) and membrane treatment (e.g. ultrafiltration) have been shown to remove pharmaceuticals in some cases to levels below detection limits in drinking water treatment works, (Ikehata et al., 2006; Guil et al., 2007; Esplugas et al., 2007) but how effectively they do so varies with the treatment conditions employed.

Typical pharmaceutical loads in wastewaters and their removal by conventional treatment are presented in Table 1.4, which is illustrative of the inadequacy of conventional WWTPs to deal with pharmaceutical contamination.

Table 1.4. Influent and effluent concentrations and removal efficiency in WWTPs

Compound	Influent concentration (μgL^{-1})	Effluent concentration (μgL^{-1})	Removal (%)	Reference
Clofibric acid	1 [fig]	(0.66-0.85) [fig]	15-34	(Stumpf et al., 1999)
	0.46	0.48	< 0	(Heberer, 2002b)
	0.15-0.25	0.15-0.25	0	(Tauxe-Wuersch et al., 2005)
	(0.25) 0.56 [a]	(0.15) 0.23 [a]	20-40	(Miège et al., 2009)
Ibuprofen	2.64-5.70	0.91-2.10	60-70	(Carballa et al., 2004)
	1.2-3.68	2.4 [b]	80-100	(Clara et al., 2005)
	(0.67) 1.13 [a]	(0.02) 0.07 [a]	> 90	(Nakada et al., 2006)
	28	3	98	(Roberts and Thomas, 2006)
	(84) 168 [c]	(7.1) 28 [c]	95	(Gómez et al., 2007)
	(59-156) 167-373 [c]	(6.2-12.6) 26.5-48.2 [c]	88-93	(Santos et al., 2007)
	(3.2) 83.5 [a]	(0.80) 24.6 [a]	80	(Miège et al., 2009)
	6.9	0.048	99	(Zorita et al., 2009)
Carbamazepine	(1.78) 3.80 [c]	(1.63) 5.00 [c]	8	(Heberer, 2002b)
	0.33-1.85	0.47-1.62	< 20	(Clara et al., 2005)
	(0.05) 0.27 [a]	(0.05) 0.16 [a]	< 45	(Nakada et al., 2006)
	(0.15) 0.3 [c]	(0.13) 0.23 [c]	20	(Gómez et al., 2007)
	(0.28-0.36) 0.94-2.15 [c]	(0.29-0.50) 0.47-1.29 [c]	0-25	(Santos et al., 2007)
	(0.29) 0.82 [a]	(0.50) 2.44 [a]	-121	(Vieno et al., 2007a)
	(0.73) 1.9 [a]	(0.52) 2.3 [a]	< 20	(Miège et al., 2009)
Atenolol	n.r.	n.r.	< 10 (0-10)	(Andreozzi et al., 2003)
	0.03	0.16	< 0	(Bendz et al., 2005)
	(1.65) 2.21 [a]	(0.99)1.68 [a]	40	(Lee et al., 2007)
	2.23 [d]	0.54 [d]	76	(Maurer et al., 2007)
	(0.73)1.71 [a]	(0.29) 1.18 [a]	58	(Vieno et al., 2007a)
	0.03 [d]	(0.154) 0.38 [c]	< 20	(Miège et al., 2009)

[fig]=estimated from a figure; [a](median) maximum; [b]maximum; [c](mean) maximum; [d]mean; n.r: not reported; (**Note:** wherever the type of data is not specified (i.e. whether mean, median, etc.) it is because that is not clearly stated in the bibliographic source)

1.4.1. Factors affecting the removal of pharmaceuticals in WWTPs

The elimination of pharmaceuticals in WWTPs is affected by some of the same factors that affect the fate of pharmaceuticals in the environment, namely the physico-chemical properties of the pharmaceuticals and environmental conditions (section 1.3.1). Other factors which are specific to WWTPs, such as wastewater characteristics (e.g. pH, temperature, dissolved oxygen concentration, total suspended solids, organic matter, presence of inhibitors such as antibiotics, pharmaceuticals concentrations) and operation parameters (e.g. hydraulic and solid retention time, biomass concentration, agitation conditions), may also affect pharmaceuticals removal (Aga, 2008; Miège et al., 2009).

In the case of biodegradable pharmaceuticals, wastewater temperature is one of the factors to have a major influence in the removal efficiency in WWTPs. Comparing the removal of ibuprofen and atenolol in WWTPs during the winter and summer seasons, Castiglioni et al. (2006) observed significantly higher removals during the latter period. This increased efficiency may be attributed to the enhanced microbial activity at higher wastewater temperatures which leads to improved biodegradation. On the other hand, poor biodegradable pharmaceuticals, which may be removed primarily by sorption processes, are affected by temperature in a less significant extent.

High rates of rainfall can also significantly influence the operation of WWTPs, not only by a dilution effect, but also by reducing the hydraulic retention times and disturbing the microbial populations, with a possible washout of important microorganisms species, as suggested by some authors (Ternes, 1998; Drewes, 2007), which contribute to a reduced performance of the systems.

Biodegradation of pharmaceuticals may occur under different redox conditions, namely aerobic, anaerobic or anoxic conditions. For the former processes, the concentration of dissolved oxygen will be determinant for the efficiency of biodegradation. For example, biodegradation of ibuprofen has been reported to be faster under aerobic than anoxic conditions (Zwiener et al., 2002). Consequently, adequate aeration will have a major influence in the removal of this pharmaceutical (and other similar cases) in WWTPs.

Biodegradation being, in general, one of the slowest processes involved in the elimination of pharmaceuticals, it can benefit from an increased residence time of the wastewater in the system, which provides for a longer and more efficient contact of it with microorganisms as well as enough time for the slow metabolic processes to take effect. Therefore, longer hydraulic retention times leads to higher removal efficiencies,

especially for biodegradable pharmaceuticals, as was observed for ibuprofen, ketoprofen, atenolol, among other drugs (Tauxe-Wuersch et al., 2005; Maurer et al., 2007; Miège et al., 2009).

Several studies report that better removal efficiencies for some pharmaceutical compounds, e.g. bezafibrate and ibuprofen (Clara et al., 2005), are achieved in biological wastewater treatment processes with longer (>10 days) solid retention time or sludge age (SRT). This has been attributed to the enrichment of slowly growing microbial communities in activated sludge that are able to break down a large number of pharmaceuticals (Zhang et al., 2008; Cirja et al., 2008). On the other hand, poor biodegradable pharmaceuticals such as carbamazepine are mostly unaffected by changes in SRT (Clara et al., 2005; Bernhard et al., 2006; Zhang et al., 2008).

Aiming to improve the efficiency of WWTPs in removing pharmaceuticals, optimization of conventional wastewater treatment processes and WWTPs operation parameters has been attempted in addition to the evaluation of some advanced technologies. However, despite the sometimes higher removal efficiencies attained, most of these improvements have still been considered insufficient or not economically viable to be widely adopted (Fent et al., 2006).

Nonetheless, the issue of emergent pollutants such as pharmaceuticals and the need for regulating water quality parameters for this type of contamination have been raised several times by specialists (Robinson et al., 2007; Bolong et al., 2009). In fact, environmental agencies worldwide are evolving towards a greater awareness to this problem, electing these new substances as priority pollutants and requiring new environmental risk assessments to be carried out as part of the process of approving new substances for public use (Kot-Wasik et al., 2007). In this context, it is foreseeable that wastewater treatment requirements become more stringent in the coming years in terms of the limiting concentrations of many of these substances in the WWTPs effluents. To meet these new requirements, many of the existing conventional WWTPs will have to be adapted or reformed in the coming years. Consequently, there is a growing need for alternative wastewater treatment processes for removing pharmaceuticals from waters that have higher efficiencies at reasonable costs of operation/maintenance.

An alternative low-cost wastewater treatment option for removal of pharmaceuticals from wastewater may be the use of constructed wetlands systems which have already shown high efficiencies in removing some other organic recalcitrant compounds (e.g. pesticides) from contaminated waters (Haberl et al., 2003; Imfeld et al., 2009).

1.5. Constructed wetlands systems – can they be an option?

Constructed wetlands systems (CWS) are engineered systems designed and constructed to make use of the natural processes involving wetland vegetation, soils and their associated microbial assemblages to assist in wastewater treatment. They take advantage of many of the same processes that occur in natural wetlands, but do so within a more controlled environment. CWS have been used to treat a variety of wastewaters including urban runoff, municipal, industrial and agricultural (USEPA and USDA-NRCS, 1995; Cooper et al., 1996; Vymazal et al., 1998; Sundaravadivel and Vigneswaran, 2001; Stottmeister et al., 2003; Haberl et al., 2003; Scholz and Lee, 2005; Kadlec and Wallace, 2009; Vymazal, 2009).

In the past, CWS have been used mainly as wastewater treatment alternatives or complementary to the conventional treatment for domestic wastewaters of small communities. Thus, CWS have been mostly applied in the removal of bulk wastewater pollutants such as suspended solids, organic matter, excess of nutrients and pathogens.

More recently, CWS applications for dealing with more specific pollutants, such as organic xenobiotics, have been meeting a larger interest and have been the subject of an increasing number of studies. In many of such studies, CWS have been proving to be efficient and cost-effective solutions for the removal of some organic xenobiotics such as pesticides, azo dyes, explosives and petroleum hydrocarbons (Williams, 2002; Haberl et al., 2003; Braskerud and Haarstad, 2003; Low et al., 2008; Davies et al., 2008; Imfeld et al., 2009; Vymazal, 2009; Moore et al., 2009; Tang et al., 2009). However, CWS have not yet been fully evaluated for the removal of pharmaceuticals, their metabolites and transformation products. Such studies are, in fact, scarce and the subject is still largely unexplored (Gross et al., 2004; Matamoros and Bayona, 2006; Matamoros et al., 2007a; Matamoros et al., 2007b; Conkle et al., 2008; Matamoros et al., 2008a; Matamoros et al., 2008b; Matamoros et al., 2009; Park et al., 2009).

Ultimately, the optimization of CWS for the removal of more specific target compounds requires a basic knowledge of the processes involved in the removal of the pollutants and the interactions between those and the CWS components. New trends in CWS research are moving towards the study of such processes and interactions and focus on the selection and optimization of the CWS components for more specific applications.

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1.5.1. Types of Constructed Wetlands Systems

The CWS classification is based on the water flow regime. Depending on the level of the water column with respect to the solid matrix bed, in practice two general types of CWS are used (Vymazal et al., 1998; Kadlec and Wallace, 2009):

- free water surface (FWS) constructed wetlands systems (also called surface flow (SF) wetlands or aerobic wetlands), and
- subsurface flow (SSF) constructed wetlands systems (also known as vegetated submerged bed (VSB) systems).

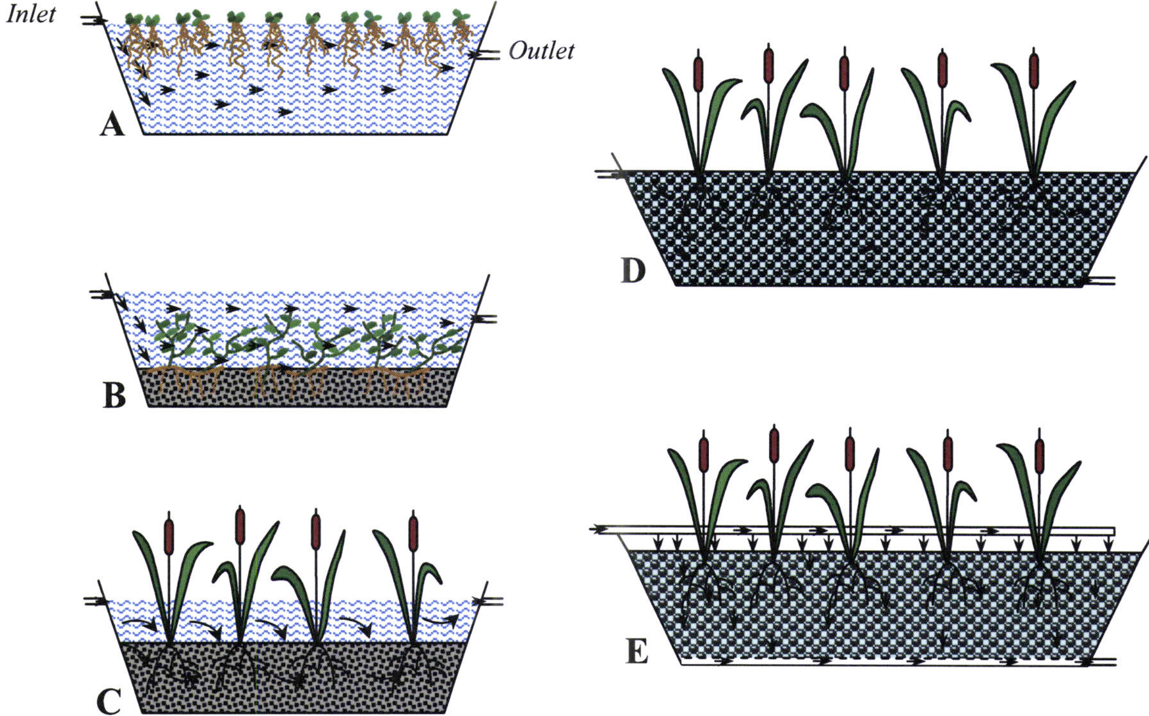

Figure 1.4. Different types of CWS (A, FWS with free-floating plants; B, FWS with submerged plants; C, FWS with emergent plants; D, Horizontal SSF; E, Vertical SSF) (adapted from Dordio et al. (2008)).

FWS-CWS more closely resemble natural wetlands in appearance as they contain aquatic plants that are free floating or rooted in a solid matrix layer on the bottom of the wetland (USEPA, 2000a; Haberl et al., 2003; Kadlec and Wallace, 2009). In this type of CWS the water flows horizontally through the leaves and stems of the plants, above the support matrix from the inlet to the outlet (Figure 1.4-A, B and C). The near-surface layer of water is aerobic while the deeper waters and the matrix are usually anaerobic regions (USEPA and USDA-NRCS, 1995; USEPA, 2000a; Kadlec and Wallace, 2009).

FWS-CWS can further be sub-classified according to the dominant type of wetland vegetation growing in the system. This wetland systems can be: a floating macrophyte system (Figure 1.4-A); a submerged macrophyte system (Figure 1.4-B) and a rooted emergent macrophyte system (Figure 1.4-C) (USEPA and USDA-NRCS, 1995; Vymazal et al., 1998; USEPA, 2000a; Kadlec and Wallace, 2009).

On the contrary, SSF-CWS do not resemble natural wetlands because they do not show a standing water layer; they contain a solid matrix bed (usually consisting of small rocks, gravel, sand or soil) which has been planted with aquatic plants. The wastewater flows beneath the surface of the support matrix, in contact with the plants roots. In the SSF-CWS water can flow horizontally (HSSF) or vertically (VSSF) through the permeable support matrix or substrate (Figure 1.4-D and E, respectively).

SSF-CWS are thought to have several advantages over FWS-CWS (USEPA and USDA-NRCS, 1995; USEPA, 2000a). The former systems show, in general, higher contaminant removal efficiencies than the latter per unit area of land occupation (USEPA and USDA-NRCS, 1995; USEPA, 2000a). In SSF-CWS the support matrix provides more surface area for the occurrence of sorption processes and for the development of microorganisms, and this fact is in part responsible for its increased efficiency. Consequently, SSF-CWS usually require the occupation of a smaller area in comparison with FWS-CWS, in order to achieve a particular level of treatment. As the water surface in a SSF-CWS is below the support matrix surface (Figure 1.4-D and E) there is also little risk of odors and insect vectors which are associated with the standing wastewaters of the FWS-CWS as well as a minimal risk of public or animal exposure. The accumulation of plant debris in the surface of the SSF-CWS can additionally provide some thermal protection in colder climates. Percolation of the wastewater through the support matrix in the SSF-CWS also provides faster depuration with the respect to the organic pollutants and nutrients (USEPA and USDA-NRCS, 1995; USEPA, 2000a).

Given the advantages presented by the SSF-CWS, they are, in general, more frequently constructed in Europe as secondary or tertiary treatment systems. Additionally, most cases of success attained by using CWS for removing organic xenobiotics from wastewaters, as mentioned above, have employed SSF-CWS. Therefore, a preference was given in this study to a SSF-CW type of system for evaluating its ability to deal with pharmaceuticals contamination in waster.

1.5.2. Components of SSF-CWS

A SSF-CWS consists of a properly-designed basin that contains soil (or other selected support matrix), water column and wetland vegetation as the main elements. Other important components that assist in the treatment of the wastewater, such as the communities of microorganisms, develop naturally. The concerted action of all these components (matrix, vegetation and microbial population), through a variety of chemical, physical and biological processes, is responsible for the depuration of wastewaters achieved in a SSF-CWS (Figure 1.5).

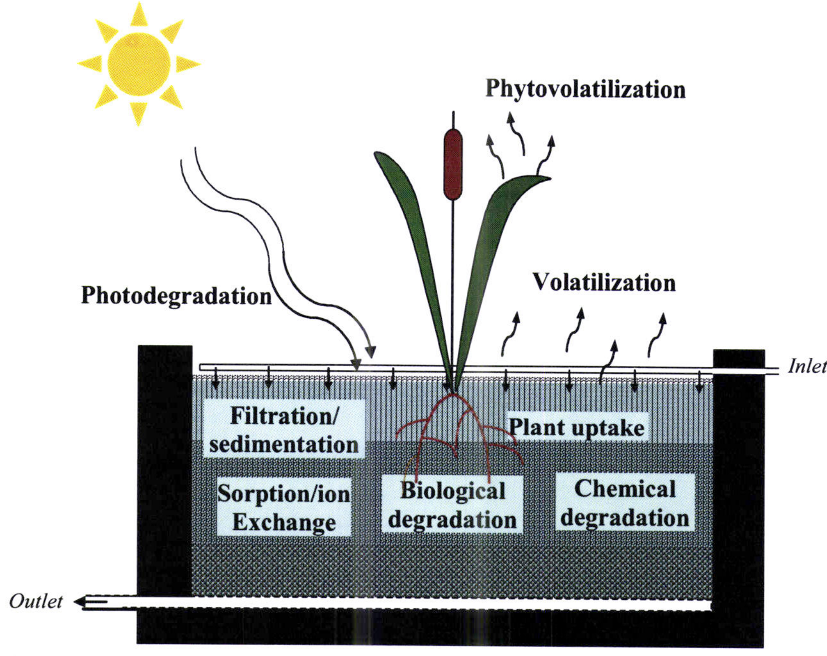

Figure 1.5. Summary of the major physical, chemical and biological processes controlling pollutant removal in a SSF-CWS (adapted from Dordio et al. (2008)).

The plants growing in natural wetlands (often called wetland plants or macrophytes), are also typically the plant species used in constructed wetlands as these are well adapted to the water saturated conditions found in these systems (Brix, 1994; Brix, 1997; Kadlec and Wallace, 2009). Major roles of macrophytes in CWS include the filtration of large debris; provide surface area for microorganisms development and release exudates by roots (normally including organic acids, sugars, amino acids, vitamins and enzymes (Macek et al., 2000; Alkorta and Garbisu, 2001)) that stimulate their growth; promote hydraulic conductivity of the matrix (roots and rhizomes growth help to prevent clogging in the matrix); transport and release oxygen through the roots (which increases aerobic degradation and nitrification); and diminish the wastewater pollutant load (nutrient and xenobiotics uptake) (Brix, 1994; Sundaravadivel and Vigneswaran, 2001; Haberl et al., 2003; Kadlec and Wallace, 2009).

In SSF-CWS, an increasingly important role is being attributed to plants in removing poorly or non-biodegradable organic xenobiotics through their capacity to sorb them in their roots and even uptake them and sequester/transform them in their tissues (Macek et al., 2000; Korte et al., 2000; Dietz and Schnoor, 2001; Pilon-Smits, 2005; Collins et al., 2006). In addition, even in the case of biodegradable compounds, where the major role in the removal of such pollutants is played primarily by microorganisms and their metabolism, some enzymes produced by plants and released in roots exudates can assist in the availability of faster compound removal processes (Macek et al., 2000; USEPA, 2000b; Pilon-Smits, 2005).

The primary function of the support matrix in a SSF-CWS, is to provide a physical support where the plant's root systems are anchored and some surface area for the attachment and growth of the microorganisms (Sundaravadivel and Vigneswaran, 2001). In addition, this component can provide some moderation of environmental conditions (e.g. temperature or pH) which affect the development of the biotic components. The matrix should also allow an even infiltration and movement of the wastewater.

Beyond this supportive role, the matrix acts as a filter medium which will trap particulate pollutants and sorb organic matter. Some mineral materials also have ion exchange capacity which may provide for some ability to retain polar or ionic compounds, either inorganic (including heavy metals) or organic (e.g. some pesticides).

In practice, every SSF-CWS should be constructed with adequate support matrix materials, according to their local availability and the usefulness for the aims of the treatment. A careful selection of the type of materials used as the solid matrix may prove an important step in the optimization of the constructed wetland's performance.

The solid matrix and vegetation components were studied in more depth in this work. The fact that most of the pharmaceuticals to be removed in these systems are poorly biodegradable compounds (e.g. carbamazepine, clofibric acid) and are, therefore, refractory to the biological treatment in WWTPs, it is expected that the microbial populations of SSF-CWS will have a limited role in the removal efficiency of these systems. On the other hand, it could be expected that a major role should be played by the solid matrix and vegetation in the removal of these recalcitrant compounds and since the microbial component always develops naturally in SSF-CWS, it is worthwhile to focus in a more thorough study of the other two components.

1.5.3. Selection of support matrix

The support matrix (or substrate) is vital in a SSF-CWS as it provides the link between all the components and the main treatment processes occurring within the system.
The choice of the support matrix materials should be determined both in terms of their physical and chemical characteristics. Chemical properties (e.g. point of zero charge, PZC) and composition as well as physical parameters such as grain-size distributions, porosity, effective grain size and the hydraulic conductivity are all important factors that influence the performance of the system (Vymazal et al., 1998; Brix et al., 2001; Akratos and Tsihrintzis, 2007; Kadlec and Wallace, 2009).
The hydraulic conductivity is a fundamental characteristic of the support matrix as it should allow for an even distribution of the inlet flow and collection of the outlet flow. As a general rule, successful operation requires hydraulic conductivities of approximately 10^{-3} to 10^{-4} m s^{-1} (USEPA, 1993a; Cooper et al., 1996; Vymazal et al., 1998; Sundaravadivel and Vigneswaran, 2001).
Particle size fractions in the range of 0-30 mm are normally used depending on the type of material (Vymazal et al., 1998). Typically, gravel sizes lie between 0 and 12 mm (Vymazal et al., 1998; Sundaravadivel and Vigneswaran, 2001). Often additional criteria have to be complied with, like d_{10} (effective grain size) being greater than 0.3 mm or d_{60}/d_{10} (uniformity coefficient) larger than 4 (Vymazal et al., 1998). In the uniformity coefficient expression, d_{60} and d_{10} represent the grain diameter, in mm, for which 60% and 10% of the sample mass, respectively, are finer than a sieve mesh of that size.
Typically the porosity of a sand or gravel media is in the range 30-45% (Kadlec and Wallace, 2009).
A wide variety of materials has been used as support matrix to fill SSF-CW beds. The most frequent choices have been gravel, crushed rock, sand (usually river sand), gravel/sand mixtures and local soil (USEPA, 1993b; USEPA and USDA-NRCS, 1995; Cooper et al., 1996; Vymazal et al., 1998; USEPA, 2000a; Kadlec and Wallace, 2009). However, alternatives to these more common materials have also been investigated, e.g. light expanded clay aggregates (LECA), rice husk, vermiculite, anthracite and ceramic filter (Brix et al., 2001; Zhang et al., 2007; Calheiros et al., 2008; Tee et al., 2009). As complement, the additions of straw, compost, LECA and wood chips have been used to improve the performance of these systems (Billeter et al., 1998). Straw, compost and

wood chips in particular represent an additional carbon source for the microorganisms while clay materials like LECA can increase the surface area for sorption of different types of pollutants.

The selection of support matrices with appropriate physical and chemical characteristics for removal of specific pollutants can be guided by the numerous studies of sorption properties of several types of materials: common natural minerals (e.g. gravel, sand, soil), industrially processed natural materials (expanded clay, exfoliated vermiculite, expanded perlite) or modified natural materials (e.g. organoclays), and newly developed artificial ones (e.g. zeolites) (Carrizosa et al., 2001; Li et al., 2004; Abate and Masini, 2005; Sanchez-Martin et al., 2006; Dogan et al., 2007; Apreutesei et al., 2008).

In the present work four different industrially processed natural materials were studied: LECA with granulometric grades of 2/4 and 3/8; sepiolite; exfoliated vermiculite and expanded perlite. In addition a natural unprocessed material, sand, which is frequently used in filter beds, was also tested.

Most of the tested materials are clays since, in numerous studies, a variety of clay minerals (Gonzalez-Pradas et al., 2003; Gianotti et al., 2008; Peng et al., 2009) and soils containing clay constituents (Gennari et al., 2007; Liu et al., 2008; Ahangar et al., 2008) have been found to sorb substantial amounts of organic pollutants such as pesticides and other xenobiotics. Commercially available clay materials such as sepiolite, vermiculite and LECA are thus being increasingly considered as alternative low-cost adsorbents for organic pollutants.

Light expanded clay aggregates (LECA) is a processed natural material that is produced by subjecting clay materials to a high temperature treatment, causing injected CO_2 to expand within the clay aggregates and thus creating a highly porous, lightweight material. LECA is mainly used for construction purposes (such as in building blocks and as insulation material), but over the last years it is also being increasingly used for different types of treatment processes for water and wastewater such as filtering and as support matrix in CWS (Zhu et al., 1997; Brix et al., 2001; Scholz and Xu, 2002; Heistad et al., 2006; Calheiros et al., 2008; Albuquerque and Labrincha, 2008).

Figure 1.6. Aspect of LECA grains.

Sepiolite is an abundant clay mineral which consists of a natural hydrated magnesium silicate. Structurally, it is formed by blocks and channels extending in a fibrous texture containing a significant number of silanol groups on the surface (Gonzalez-Pradas et al., 1999; Tekin et al., 2006; Alkan et al., 2007). These groups can play an important role in providing an ionic type of sorption mechanism. Several studies were done using sepiolite as a catalyst support, in wastewater and solid wastes treatment, and in reducing the toxic effect of some pollutants (Rytwo et al., 2002; Rajakovic et al., 2007; Dogan et al., 2007; Ugurlu, 2008; Donat, 2009).

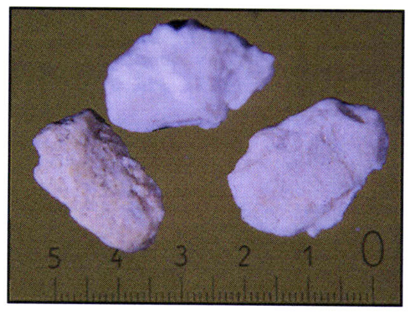

Figure 1.7. Aspect of sepiolite grains.

Vermiculite is a group of clay minerals formed by alteration of micas. They consist of porous and flaky particles which expand to about 20 times their volume when heated, producing exfoliated vermiculite which is a low-density, thermally insulating, and inert material used in plaster, insulation, packing material and as medium for raising plants from seeds. Recently, several studies have been carried out on the use of vermiculite, usually after chemical modification, as an adsorbent of organic compounds from water (da Silva Jr. et al., 2003; Abate and Masini, 2005; Mysore et al., 2005).

Figure 1.8. Aspect of vermiculite grains.

Perlite is a glassy volcanic rock which is essentially a metastable amorphous aluminum silicate. As perlite contains a high silica content, it is chemically inert in many environments and hence is also an excellent filter aid and filler in various industrial processes. In addition, when mixed with soil, expanded perlite increases the soil porosity which improves its capacity to retain air and water and consequently the growing conditions for plants. Therefore, another important use for expanded perlite is found in horticultural applications. Recently, the properties of perlite for adsorption of water

Figure 1.9. Aspect of perlite grains.

contaminants have been investigated in a number of papers (Chakir et al., 2002; Alkan et al., 2005; Roulia and Vassiliadis, 2008).

Further information about the characteristics of the tested materials can be found in Appendix B.

1.5.4. Selection of the wetland vegetation

Several different species of wetland vegetation (e.g. macrophytes) can be used in SSF-CWS but, the selection of the more suitable ones to be used in each case should be carefully made, because plants play an important role, directly and indirectly, in the global system efficiency.

Overall, plants to be used in SSF-CWS should have the following characteristics (Sundaravadivel and Vigneswaran, 2001; Scholz and Lee, 2005):

- ecological adaptability – the chosen plants should pose no risk to the natural ecosystem surrounding;
- tolerance to local conditions in terms of climate, pests and diseases;
- tolerance to the hypertrophic water-logged conditions of wetlands;
- ready propagation, rapid establishment and growth, and good root system development;
- perennial duration rather than annual;
- tolerance towards the pollutants toxicity;
- high pollutant removal capacity.

Specific requirements of plants characteristics will vary depending on the functional role of wetland plants in the treatment systems. This will be related to the type of SSF-CWS design and its mode of operation (continuous or batch), loading rate, and wastewater characteristics. Other additional objectives (such as ecological, aesthetic, recreational, and economic) of wetland developments may also affect the choice of the plants (Sundaravadivel and Vigneswaran, 2001; Scholz and Lee, 2005; Kadlec and Wallace, 2009).

Despite the fact that the dominant species of macrophyte varies locally, *Phragmites* spp. and *Typha* spp. are the most commonly used in SSF-CWS in temperate zones (Cooper et al., 1996; Vymazal et al., 1998; Scholz and Lee, 2005; Kadlec and Wallace, 2009).

In this work the species *Typha* spp. (cattail) was selected because it is a local emergent hydrophytic plant well adapted to the Alentejo climate conditions with a rapid growth rate and a good root system development, and it is a species that has been frequently used in SSF-CWS. In addition, *Typha* spp. seems to be a species that tolerates a great variety of organic xenobiotics in concentrations normally found in typical wastewater compositions. For these reasons, one important application of *Typha* in CWS has been the removal of various organic xenobiotics in wastewaters, namely petroleum hydrocarbons, explosives, pesticides, etc. (Table 1.5).

Figure 1.10. *Typha* spp.

Table 1.5. Classes of organic xenobiotics removed by *Typha* spp. in CWS

Type of pollutants	Type of system	Reference
Petroleum hydrocarbons/ PAHs:		
BTEX	FWS	(Haberl et al., 2003)
PAHs	Hybrid system	(Machate et al., 1997)
Petroleum hydrocarbons	FWS	(Gessner et al., 2005)
Phenanthrene	CW pilot	(Machate et al., 1997)
Chorineted solvents		
TCE	Wetland microcosms	(Bankston et al., 2002)
Explosives		
RDX	SSF mesocosms	(Low et al., 2008)
Pesticides		
Atrazine	CW cells	(Runes et al., 2003)
	VSSF	(McKinlay and Kasperek, 1999)
Azinphos-methyl	CW	(Schulz et al., 2003)
Endosulfan	CW	(Schulz and Peall, 2001)
Others		
PCBs	FWS	(Haberl et al., 2003)
Phenols	HSSF	(Calheiros et al., 2008; Tee et al., 2009)
Estrogenic hormones	SSF	(Gray and Sedlak, 2005)

In these systems, *Typha* spp. may contribute to the overall removal of xenobiotics by direct uptake of some of these compounds. In fact, in hydroponic studies, the ability of *Typha* spp. to uptake some organic xenobiotics from the nutrient solutions has been observed (Wilson et al., 2000; Amaya-Chavez et al., 2006; Ma and Havelka, 2009).

In the few available studies reporting the use of CWS for pharmaceuticals removal, the *Phragmites* species has mostly been used (Matamoros et al., 2005; Matamoros and Bayona, 2006; Matamoros et al., 2007a; Matamoros et al., 2007b; Matamoros et al., 2008a). Although *Typha* spp. has been rarely tested with pharmaceuticals in CWS, recent work indicates that it is capable of uptaking several pharmaceuticals (Park et al., 2009) and suggests that this species should be more thoroughly studied as well.

In addition to the individual plant characteristics, the efficiency of pollutant removal in SSF-CWS is also affected by the plants' density on the beds as well as its maturity stage. In the literature, a wide range of values can be found for the reported choice of plants densities. In the case of *Typha* spp., systems with such low plants densities as around 4 plants m^{-2} and as densely planted as 80 plants m^{-2} have all been used in assays as well as in fully operating CWS (Sawaittayothin and Polprasert, 2007; Gebremariam and Beutel, 2008; Kadlec and Wallace, 2009).

Plants maturity is also a relevant aspect influencing the efficiency of a CWS. Only a well established vegetation can achieve top performance, which normally requires a acclimation period of 1 – 2 full year cycles, depending on the plant species. Furthermore, the seasonal year cycle (vegetative stage), which determines the plants activity, with periods of full activity intercalated with periods of dormancy, gives rise to seasonal variations in the planted beds performance (Kadlec and Wallace, 2009).

More detailed information about *Typha* spp. is presented in Appendix C.

1.6. Organic xenobiotics removal in SSF-CWS

A specialized use of SSF-CWS for the removal of specific organic compounds or classes of compounds has been developing as a growing type of CWS application in comparison to the treatment of bulk pollutants. Significant work exists with wastewater contaminated with other organic xenobiotics from the petroleum industry, food processing industry, pesticide contaminated agricultural runoff, landfill leachates, and waters containing surfactants, solvents or mineral oils (Vymazal et al., 1998; Williams, 2002; Haberl et al., 2003; Imfeld et al., 2009; Vymazal, 2009).

Very little is commonly known, about the exact pathways of the organic xenobiotics removal in SSF-CWS. Given the diversity of chemical characteristics of these

compounds, which despite being classified under a common designation of xenobiotics are in fact formed by widely unrelated families of chemical substances, it is of no surprise that several very diverse mechanisms are responsible for their removal. The same observation can be made specifically for pharmaceuticals as this subset of xenobiotics is equally varied in terms of their chemical properties. A comprehensive description of organic xenobiotics removal in SSF-CWS is, thus, not an easy task to accomplish and these systems have been and still are largely operated as a "black box".
Although much of the design of SSF-CWS in the past has been done with little knowledge of the roles played by each component and how its function could be optimized, nowadays the knowledge that has been accumulating is beginning to be applied. A much greater variety of plant species, matrix materials and constructed wetlands designs is now, being introduced. The goals of the target contaminants to remove in CWS are also becoming progressively more ambitious.

1.6.1. Main removal processes in SSF-CWS

Organic xenobiotics removal by SSF-CWS involves several interdependent processes which may be classified as abiotic (physical or chemical) or biotic (carried out by living organisms such as plants and microorganisms). These processes are basically the same occurring in natural wetlands and also identical to those responsible for the fate of pharmaceuticals in the environment as described in section 1.3.1. The way in which CWS differ from natural wetlands is that CWS are engineered systems where these processes occur in a controlled environment and conditions are optimized in order to maximize pollutants removal.
The primary abiotic and biotic processes that participate in removing organic xenobiotics from contaminated water in a SSF-CWS are described in Table 1.6.

Removal of pollutants from water may be accomplished through storage in the wetland solid matrix and in the vegetation, or through losses to the atmosphere. A basic understanding of how these processes operate in wetlands is extremely helpful for assessing the potential applications, benefits and limitations of SSF-CWS.

Table 1.6. Abiotic and biotic processes involved in xenobiotics removal in SSF-CWS (Pilon-Smits, 2005; Reddy and DeLaune, 2008a)

Processes	Description
Abiotic	
Sorption	Including adsorption and absorption, the chemical processes occurring at the surface of plants roots and solid matrix that result in a short-term retention or long-term immobilization of xenobiotics.
Hydrolysis	The chemical breakdown of organics by the action of water, a process which is pH-dependent.
Photodegradation	Degradation of organic xenobiotics by the action of sunlight
Redox reactions	Modification, which sometimes may be quite substantial, of xenobiotics due to the action of oxidizing or reducing agents. Redox reactions are also frequently brought about by biotic agents (e.g. bacteria), or enzymatically catalyzed.
Precipitation	For those compounds which can exist in several forms with different water solubilities, the conversion into the most insoluble forms.
Filtration	Removal of particulate matter and suspended solids.
Volatilization	Release of some organic xenobiotics as vapors, which occurs when these compounds have significant vapor pressures.
Biotic	
Aerobic/anaerobic biodegradation	Metabolic processes of microorganisms, which play a significant role in organic xenobiotics removal in CWS.
Phytodegradation	Breakdown of organic xenobiotics having first been taken up by plants.
Rhizodegradation	Enhancement of microbial degradation of some organic xenobiotics by the stimulus provided by substances released in roots exudates.
Phytovolatilization	Uptake and transpiration of volatile organics through the aerial plant parts.

1.6.1.1. Abiotic processes

A wide range of physical and chemical processes are involved in the abiotic removal of contaminants in SSF-CWS. The most important abiotic removal process occurring in SSF-CWS, at the surface of plants roots and solid media, is sorption, resulting in a short-term retention or long-term immobilization of the contaminants (Vymazal et al., 1998; Reddy and DeLaune, 2008a; Kadlec and Wallace, 2009). Other common abiotic processes such as hydrolysis, photodegradation, redox reactions and volatilization (Table 1.6) can also contribute, in varied extents, to the removal of some particular classes of compounds, depending on their specific properties. In general, however, apart from sorption, they are not major removal processes for most organic compounds such as pharmaceuticals because either the conditions in SSF-CWS or the properties of the compounds are not suitable. For example, photodegradation in SSF-CWS does not occur in appreciable extent as the water level is below the solid matrix surface and, therefore, exposure of the pollutants to sunlight is very limited in this type of systems.

The process of volatilization is also of modest importance for substances with low volatility, as is the case of most pharmaceuticals including the compounds studied. Where SSF-CWS are used as a tertiary treatment stage after conventional secondary treatment in a WWTP, organic compounds do not suffer, in most cases, appreciable hydrolysis either, as they have been already subjected to such type of processes in secondary treatment stage (and, in case of pharmaceuticals, previously in the human digestive system as well). Therefore, the organic xenobiotics present in SSF-CWS influents are those that have resisted such hydrolysis processes or they are the transformation products of those substances that did not resist it.

1.6.1.2. Biotic processes

CWS are biological systems in which biological processes play a major role in the removal of pollutants. The two biotic components which are responsible by the biological action in organics removal are the wetland vegetation and the microbial populations.

Direct uptake by plants is a widely recognized process for inorganic pollutants removal. In the case of some organic substances, plant uptake has been observed to also play a significant role among biotic processes, especially for those compounds possessing a moderate hydrophobicity ($0.5 < \log K_{ow} < 3.5$) (Dietz and Schnoor, 2001; Schroder and Collins, 2002; Pilon-Smits, 2005). Nevertheless, action of microorganisms is generally accepted to be the major route for organic xenobiotics elimination in wetlands. However, even in these microbial processes, plants do play a relevant role, through the influence of exudates released in the rhizosphere which have the effect of stimulating the development and the activity of microorganisms (apart from contributing also, in some cases, to the catalytic degradation of pollutants) (Macek et al., 2000; Singer et al., 2003; Pilon-Smits, 2005).

Although microorganisms may also provide a measurable amount of contaminant uptake and storage, it is their metabolic processes that play the most significant role in the decomposition of organic compounds through the transformation of complex molecules into simpler ones (Reddy and DeLaune, 2008a). This provides an important biological mechanism for removal of a wide variety of organic compounds. However, the efficiency and rate of organic compounds degradation by microorganisms is highly variable for different compounds types.

1.6.2. Factors affecting organic xenobiotics removal efficiency in SSF-CWS

The degree to which each process will contribute to the overall removal of the organic xenobiotics from wastewaters in SSF-CWS will depend on many of the same factors affecting the fate and removal of pharmaceuticals in WWTPs (see section 1.4.1) and in the environment (see section 1.3.1), namely the physico-chemical properties of the compounds, environmental conditions and wastewater characteristics. A few additional factors are particular to SSF-CWS, namely the characteristics of the solid matrix and of the plants species.

The type of materials that compose the solid matrix will have a strong influence over the occurrence of certain removal processes, sorption in particular, as well as to the development of the vegetation and microbial populations. A good hydraulic conductivity, which avoids the occurrence of overland flows and preferential channeling, is crucial for a good contact of the wastewater with the CWS media and, thus, for the efficiency of the system (Vymazal et al., 1998; Kadlec and Wallace, 2009). The chemical characteristics of the solid matrix determines its capacity to sorb pollutants (Muller et al., 2007; Reddy and DeLaune, 2008b) but retention in abiotic components is also a function of several characteristics of the wastewater, such as its dissolved organic matter content, pH and electrolyte composition, as well as of the pollutant itself.

The characteristics of the biotic components (vegetation and microorganisms) obviously also have a tremendous influence on the SSF-CWS behavior. Microorganisms populations develop naturally in SSF-CWS and are affected by similar factors as those present in WWTPs. Important characteristics of both microorganisms and plants are their tolerance to the more toxic pollutants (at typical wastewater concentrations) and their capacity to, respectively, biodegrade or uptake them. In the case of the vegetation, other factors related with the SSF-CWS design such as plant density and layout of the specimens (e.g. the way specimens of different species may be intermixed when planted in the beds) all have to be considered and carefully planned as their influence may range from subtle differences in the system's behavior to more pronounced impacts in the overall efficiency (Kadlec and Wallace, 2009). In particular, the cycles of vegetative activity of some species in addition to variations of climate conditions may lead to significant seasonal changes in the system's efficiency, which in some cases may be mitigated by using polycultures of vegetation (Kadlec and Wallace, 2009).

1.7. Analysis of pharmaceuticals in environmental samples

Pharmaceutical residue analysis in environmental samples is an example of the improvement in analytical techniques in recent years. Following an appropriate sample preparation, the instrumentation and methodologies available nowadays allow the detection of such active compounds in waters and wastewaters at concentration levels as low as ng L^{-1}.

Analysis of pharmaceuticals in environmental samples poses a difficult challenge to the researchers for the following reasons (Kot-Wasik et al., 2007):

- the great variety of pharmaceutical compounds at typically low concentrations;
- the need to identify not only the parent pollutants but also their transformation products and metabolites;
- the diversity of matrices (e.g., benthic sediment, sludge, surface water, wastewater and biological samples) and large differences in pollution-load levels;
- the possibility of interference between sample components of similar physico-chemical characteristics which can be present in the same sample at different concentrations; and
- the lack of suitable standards and certified reference materials.

The detection of these compounds in environmental samples requires therefore highly sensitive analytical methods. Different approaches have been chosen, but the most commonly followed ones consist of chromatographic separations, either gas (GC) or liquid chromatography (LC), coupled with a variety of detectors among which the most popular ones are UV/Vis, fluorescence and mass spectrometry (MS) (Robinson et al., 2007; Fatta et al., 2007; Kot-Wasik et al., 2007). The decision to use GC or LC is usually based on the physicochemical characteristics of the target analyte. LC is commonly used for the analysis of polar compounds, and explores the differences in the compounds' partition between a mobile phase and a stationary phase with different polarities. The technique GC, where separation is obtained due to differences in analytes volatilities, can also be used for pharmaceuticals analysis, but many of those compounds are thermolabile. Thus, most GC methods must incorporate a derivatization step to overcome this limitation (Boyd et al., 2003; Radjenovic et al., 2007; Fatta et al., 2007; Kostopoulou and Nikolaou, 2008).

The MS detector is usually chosen due to its selectivity and sensitivity. Tandem MS is generally more sensitive than single MS in measuring the contents of analytes in

complex matrices, because it is associated with higher and more stable signal-to-noise (S/N) ratios, as a result of the specific fragmentations of isolated precursor ions and the elimination of background noise. The main disadvantage in the LC-MS/MS analyses is the matrix interference associated with the analytes ionization. This results either in enhancement or, more frequently, suppression of the analytes ionization, thus causing uncertainty in the detection and quantification of the target compounds (Radjenovic et al., 2007; Fatta et al., 2007).

Matrix interferences are normally most extensive during the analysis of extremely complex matrices as is usually the case with environmental samples (e.g. wastewaters) (Hernando et al., 2004; Petrovic et al., 2006; Radjenovic et al., 2007; Fatta et al., 2007). Moreover, the concentrations in which the pharmaceuticals are generally found in the samples requires an initial step of concentration and purification of the analytes prior to their analysis. Each analytical step is crucial in obtaining correct and meaningful results, but sample preparation is usually the key component of the entire analytical process (Pavlovic et al., 2007; Kostopoulou and Nikolaou, 2008).

1.7.1. Sample preparation

The basic concept of sample preparation methods is to convert a real matrix into a sample suitable for analysis. This process almost inevitably changes the interactions of compounds with their chemical environment. These interactions are determined by the physical and chemical properties of both analytes and matrices, and they affect the applicability of different sample preparation techniques and analytical methods as well as their efficiency and reproducibility. Hence, characterization of the initial physico-chemical state of a sample is a precondition of all further sample preparation steps (Reemtsma and Quintana, 2006; Pavlovic et al., 2007).

It is very important to have information on the physical and chemical properties of an analyte. Most pharmaceuticals have acidic and/or basic functionalities; their ionization rate depends on acidic dissociation constants (i.e. pKa values) and is controlled by solution pH. With this knowledge, one can choose the best option for the pharmaceuticals extraction (e.g., pKa value enables adjustment of the pH value of sample solution; log K_{ow} shows affinity of pharmaceuticals towards water or non-polar media) (Reemtsma and Quintana, 2006; Pavlovic et al., 2007).

Sample preparation can be achieved by employing a wide range of techniques, but all methods have the same major goals (Smith, 2003; Pavlovic et al., 2007):

- to remove potential interferences;
- to increase the concentration of an analyte;
- if necessary, to convert an analyte into a more suitable form for analysis;
- to provide a robust, reproducible method that is independent of variations in the sample matrix.

Sample preparation must also be tailored to the final analysis, considering the instrumentation to be used and the degree of accuracy required, whether quantitative or qualitative. In addition, different procedures must be followed depending on whether the analytes are to be extracted from a liquid or a solid matrix.

1.7.1.1. Extraction from aqueous samples

The extraction step of pharmaceuticals in liquid samples (water and wastewater) should be as selective as possible in order to minimize the co-extraction of matrix compounds that may interfere with the analyte detection. In terms of sample preparation methodology, a matrix effect can be defined as the influence of a property of the sample, independent of the presence of the analyte, on recovery efficiency and thereby on the quantity extracted (e.g., pharmaceuticals may sorb to organic matter in the samples, causing the concentrations of freely dissolved pharmaceuticals to be lower and therefore more difficult to detect).

Solid Phase Extraction

Solid phase extraction (SPE) has become the essential technique for pharmaceuticals extraction from aqueous environmental samples (Radjenovic et al., 2007; Fatta et al., 2007; Kostopoulou and Nikolaou, 2008). In SPE, most commonly the analytes are extracted from the initial matrix by partitioning between a solid phase and a liquid phase. When compared to the classic liquid-liquid extraction procedures, SPE minimizes the volumes of sample and toxic solvents used, and the time and effort needed for analysis, with higher recoveries, reproducibility and selectivity.

The effectiveness of SPE for each class of pharmaceutical compounds depends on the properties of the compounds as well as on the kind of stationary phase through which the sample is percolated and on the elution solvent. Ideally, the target compounds adhere to the stationary phase, while interferents in the sample are washed away. The type of stationary phase and elution solvent can both affect recoveries and LODs of individual target compounds, so they need to be tested in order to optimize the

extraction of the analytes. Such optimization is particularly difficult when there is a need to determine different classes of compounds simultaneously. Recently, there has been a great deal of research to assess the effectiveness of different SPE stationary phases for the recovery of pharmaceuticals from aqueous samples.

The compounds adsorbed on the stationary phase are normally eluted by a small volume of organic solvent, such as methanol, acetonitrile, ethyl acetate or acetone. Prior to elution, the sorbent material is sometimes washed with a few milliliters of water or with water containing a low portion of an organic solvent (Öllers et al., 2001; Stolker et al., 2004; Lin et al., 2005; Hao et al., 2006). This results in a cleaner extract by removing matrix constituents that can interfere with the analysis of the target compounds.

Choice of the sorbent is the key point in the SPE procedure because it can control parameters such as selectivity, affinity and capacity. This choice depends strongly on the compounds to be analyzed and on the interactions that the sorbent can establish with functional groups of the pharmaceuticals molecules. However, the overall SPE efficiency is also highly dependent on the kind of sample matrix and its interactions with both the sorbent and the analytes (Pavlovic et al., 2007).

A wide variety of silica or co-polymer base SPE materials are nowadays commercially available and have been used for the analysis of pharmaceutical compounds in environmental samples (Weigel et al., 2004; Tauxe-Wuersch et al., 2005; Castiglioni et al., 2005; Gros et al., 2006a; Gros et al., 2006b; Fatta et al., 2007; Kostopoulou and Nikolaou, 2008).

Traditionally, reversed phase sorbents (usually an alkyl bonded silica, such as C_{18} silica) have been used to retain hydrophobic compounds. However, co-polymer based sorbent materials (such as the Oasis™ HLB, MCX and MAX) have lately become increasingly popular in the environmental analysis of pharmaceuticals because they show a greater stability over a wider range of pH values, whereas bonded silica should only be used in a narrower pH window ($2 < pH < 8$). Especially popular are the SPE materials that allow the retention of wide variety of compounds. For example, the co-polymer poly(divinylbenzene-co-N-vinylpyrrolidone) used in Oasis™ HLB (hydrophilic-lipophilic balanced), has both hydrophilic and lipophilic retention characteristics and can be used to retain both polar and non-polar compounds. This material has excellent wetting properties, thus providing the advantage of no negative "running dry" effects on the analyte recovery (Öllers et al., 2001; Radjenovic et al., 2007). Neutral and acidic compounds are retained on the solid phase exhibiting van der Waals and H-donor-H-

acceptor interactions (Radjenovic et al., 2007). In the literature, pharmaceuticals such as clofibric acid, ibuprofen, carbamazepine and atenolol are reported as having been successfully retained on the Oasis™ HLB material (Table 1.6).
For more selective sample concentration, SPE materials that allow the retention of a group of compounds with similar physico-chemical properties can be used. For example, Oasis™ MCX (mixed-mode cation exchange) and MAX (mixed-mode anion exchange) materials have high selectivity for basic and acidic compounds respectively. The retention of the compounds on the SPE material can be controlled by adjusting the sample pH. In fact, control of the sample pH can also be a very important step to be performed before the sample is loaded in the SPE cartridge. This is particularly important for compounds with acid-base characteristics and, depending on the cartridge chemical nature, target analytes might be ideally neutral or charged. For instance, when C_{18} silica material is used, acidic pharmaceuticals (e.g., the anti-inflammatories and bezafibrate) are often extracted at acidic pH where the ionization of the compounds is suppressed (Löffler and Ternes, 2003; Stolker et al., 2004; Lin et al., 2005; Castiglioni et al., 2005). Due the same reasons, basic compounds (e.g., the beta-blockers like atenolol) are sometimes extracted at basic pH (Hernando et al., 2004; Vieno et al., 2006; Hernando et al., 2007b).

Different SPE cartridges and methodologies were used to concentrate the pharmaceuticals studied in this work. Details regarding the optimization of the SPE conditions and the obtained recoveries for the different analytes in the studied matrices (water and wastewater samples) are described in detail in the published manuscripts presented in the appropriate section of this thesis.

1.7.1.2. Pharmaceuticals extraction from solid samples

The classical extraction method, both for polar and non-polar analytes, was Soxhlet extraction, which consumes large amounts of solvent as well as of the sample, and which is relatively time consuming (Reemtsma and Quintana, 2006). Currently, a variety of other more recent methods are normally preferred for the extraction of pharmaceuticals in solid environmental samples, following the already referred trend towards cheaper, faster and more environmentally friendly methods (Reemtsma and Quintana, 2006; Kristenson et al., 2006).

Matrix solid phase dispersion

Matrix solid phase dispersion (MSPD) is a process that permits simultaneous disruption and extraction of solid, semi-solid or highly viscous samples (Barker, 2007). This technique is based on the blending of the sample with an abrasive solid support material with dispersive properties to sorb the analyte molecules, such as an appropriate bonded-phase (e.g. octadecylsilyl (C_{18}) silica has been traditionally used)(Barker, 2007). More recently, many applications of MSPD have involved the blending of samples with underivatized silica materials (silica gel, sand, etc.) (Morzycka, 2002; Perret et al., 2002; Michel and Buszewski, 2004; Bogialli et al., 2004; Teixeira and Costa, 2005) or other organic (graphitic fibers) (Blasco et al., 2004; Furusawa, 2005) or inorganic (Florisil, alumina, etc.) (Kishida and Furusawa, 2001; Gómez-Ariza et al., 2002; Furusawa, 2004) solids which cause sample disruption but do not, apparently, possess the same dispersive properties (Barker et al., 1993; Barker, 2000). However, some of these methods have the advantage of using an inexpensive disruptive material (e.g. sea sand as in the sea sand disruption method, SSDM) which may provide for an interesting balance between cost and efficiency (Teixeira and Costa, 2005).

MSPD has been applied mainly to the analysis of pesticides and other micropollutants from animal or plant tissues as well as other (mainly biological) matrices (Barker, 2007). However, the use of MSPD for pharmaceutical extraction has been reported in only a limited number of publications (Kishida, 2007).

The main factors to consider when performing a MSPD extraction include: the type of solid supports, the best ratio of sample to solid support material, the optimum choice of elution solvents and the sequence of their application to a column, the elution volume, and the effect of the sample matrix itself.

In this work, the sample disruption methodology was used to investigate the presence of the pharmaceutical compounds inside plant tissues. Different materials were used to disrupt and disperse the sample, which included octadecyl silica (as in MSPD) and sea sand (as in SSDM). The latter technique proved to have similar efficiencies as MSPD with the advantages of being of lower cost and providing more reproducible analyte recoveries. Detailed information on the analytical methodology optimization and the results obtained can be found in the "Complementary Results" section in Appendix D.

1.7.2. Detection methods

Most of the methods reported in the literature apply liquid chromatography in the environmental analysis of pharmaceuticals in environmental samples. This is due to the low volatility and high hydrophilicity of most of the pharmaceuticals which often precludes the use of gas chromatography (Fatta et al., 2007; Kostopoulou and Nikolaou, 2008; Chen et al., 2008).

In LC, reversed phase chromatographic columns (typically C_{18} or C_8-bonded silica) are most often used in the separation of pharmaceuticals (Table 1.7). Both acetonitrile and methanol are used as organic mobile phases for the LC separation. In order to obtain sufficient retention for acidic drugs and reproducible retention times, it is usually recommend the use of a buffer in the eluent or acidification of the mobile phase, although this sometimes reduces the signal intensities whenever mass detection is used as a result of the effects of suppression at the MS interface. Volatile compounds (e.g., ammonium acetate, ammonium formate or formic acid) are used as mobile phase additives whenever MS detection is used (Kot-Wasik et al., 2007).

LC complete separation of the analytes from the matrix components and from each other is especially important when detection methods such as UV or fluorescence are used. The MS detector is becoming more commonly used in pharmaceuticals analysis because of its high sensitivity and its ability to confirm the chemical nature of the compounds when compared with conventional ultraviolet (UV) or fluorescence detection. Moreover, when using MS, the analytes do not have to be fully separated from each other due to the selectivity of the detection method. However, better chromatographic separation generally improves detectability and reduces the ion-suppression effects.

The use of the mass detector requires that the eluting solvent coming out of the LC column be eliminated and the target analytes be ionized before they can be drawn into the mass spectrometer analyzer. The flow rates produced by an LC would overwhelm a high-vacuum mass spectrometer pinhole interface. The mass spectrometer analyzers are operated at 10^{-5} to 10^{-7} torr vacuum pressure to prevent collision with air molecules of the molecular ions produced in the interface, which could cause them to fragment before being detected. Modern LC/MS systems come equipped with nonevacuated interfaces in which solvent is being removed as the samples are ionized for introduction into the high-vacuum environment of the mass spectrometer analyzer. The two types of in-flow

interfaces commonly used in modern LC/MS systems are the electrospray (ESI) and atmospheric-pressure chemical ionization (APCI) also known as atmospheric pressure interfaces (API) (McMaster, 2005).

The ESI interface has been by far the most frequently applied in pharmaceutical residue determination, since it is particularly suitable for polar and moderately non-polar analytes and for thermally labile substances, although it is well known to be more prone to signal suppression than the APCI interface (Sørensen and Elbæk, 2004).

In the ESI interface, the eluent coming out of the LC column in which the analyte(s) of interest is dissolved, is passed through a capillary, at atmospheric pressure, maintained at high voltage. The liquid stream breaks up with the formation of highly charged droplets which are desolvated as they pass through the atmospheric-pressure region of the source towards a counter electrode. Desolvation is assisted by a stream of a drying gas, usually nitrogen, being continually passed into the spraying region. Analyte ions are obtained from these droplets which then pass through two differentially pumped regions into the source of the mass spectrometer. A schematic of an electrospray system is shown in Figure 1.11.

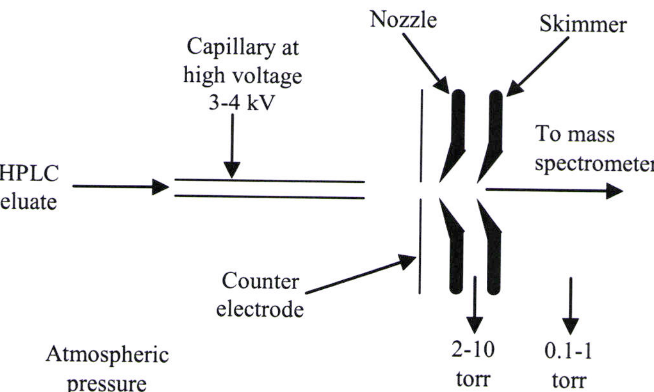

Figure 1.11. Schematic of an electrospray LC–MS interface (adapted from Ardrey (2003)).

The introduction of atmospheric pressure ionization interfaces (API) and MS equipments that enable tandem analysis has greatly improved the sensitivity and the selectivity of detection. Nevertheless, API-ionization interfaces are susceptible to matrix interferences, and, in the case of a very complex matrix (e.g., wastewater and plant extracts), even when using selected reaction monitoring (SRM) detection, both false-negative results (due to "ion suppression") and false-positive results (due to insufficient

selectivity, "ion enhancement") can be obtained (Ternes, 2001). In order to minimize problems of inaccurate quantification, several strategies have been adopted as standard practices (Benijts et al., 2004; Kloepfer et al., 2005). The most often applied approach involves suitable calibration (e.g., standard addition or internal standard calibration using structurally similar unlabeled pharmaceuticals or isotopically labeled standards).

LC systems coupled to different MS detectors like quadrupole ion trap mass spectrometry (QIT-MS), triple-quadrupole mass spectrometry (QqQ-MS) or quadrupole time-of-flight mass spectrometry (QTOF-MS) are, nowadays, the most extensively applied methods for analyzing pharmaceutical residues in complex environmental samples especially due to the possibility to perform tandem mass spectrometry (Lin et al., 2005; Castiglioni et al., 2005; Petrovic et al., 2006; Trenholm et al., 2006; Kim et al., 2007; Vieno et al., 2007b).

Tandem mass spectrometry (MS/MS) is a technique where structural information on sample molecules is obtained by using multiple stages of mass selection and mass separation. A prerequisite is that the sample molecules can be transferred into gas phase and ionized intact and that they can be induced to fall apart in some predictable and controllable fashion after the first mass selection step. Multistage MS/MS, or MS^n, can be performed by first selecting and isolating a precursor ion (MS^2), fragmenting it, isolating a primary fragment ion (MS^3), fragmenting it, isolating a secondary fragment (MS^4), and so on as long as you can obtain meaningful information or the fragment ion signal is detectable. A variety of imaginative modes of tandem MS are described in the literature, but the selected reaction monitoring (SRM) is the most often used in combination with online chromatographic separation to quantify a specific compound in a complex matrix. Frequently three degrees of separation: elution time, precursor m/z, and fragment m/z are necessary to obtain an unambiguous and quantifiable signal.

As mentioned before, tandem MS has been more or less successfully performed with a wide variety of analyzer combinations. What analyzers to combine for a certain application is determined by many different factors, such as sensitivity, selectivity, and speed, but also size, cost, and availability. The two major categories of tandem MS methods are tandem-in-space and tandem-in-time, but there are also hybrids where tandem-in-time analyzers are coupled in space or with tandem-in-space analyzers. A tandem-in-space mass spectrometer consists of an ion source, a precursor ion activation device, and at least two nontrapping mass analyzers (e.g., triple-quadrupole (QqQ) or quadrupole-time of flight (Qq-TOF)). In tandem-in-time MS systems, ions produced in

the ion source are trapped, isolated, fragmented, and m/z separated in the same physical device. This is only possible in trapping devices, such as the (quadrupole ion trap (QIT) or the Fourier transform ion cyclotron resonance (FTICR), where ions can reside in the trap for a long time.

In order to propose structures and to obtain information about unknown ions, exact mass analysis can be performed in tandem in-time instruments, which are, typically, ion-trap mass spectrometers (e.g., two-dimensional and three-dimensional quadrupole ion traps and, much less often due to their cost, Fourier transform ion cyclotron resonance (Hager, 2004; Díaz-Cruz and Barceló, 2005; Ekman et al., 2009)). These instruments are able to record a complete mass spectrum of each pulse of ions introduced into the trapping volume, so the sensitivity they achieve is extremely high (Hager, 2004).

LC-MS and LC- tandem MS operating in the SIM (single ion monitoring) and SRM mode, respectively, provide the most sensitive methodology for the analysis of the pharmaceutical compounds. LC-tandem MS is usually chosen because of its high sensitivity and its ability to confirm the chemical nature of the compounds having the same molecular mass but different product ions, even if they co-elute. MS^n detection is therefore preferred for increased analytical sensitivity and selectivity in complex matrices, such as wastewaters (Díaz-Cruz and Barceló, 2005).

Despite the enormous potential of the mass detector, its use is not devoid of problems, namely with hard to ionize compounds, matrix effects, and the high costs associated with the acquisition and maintenance of the equipments. Whenever the concentrations of the analytes are not too low (100-1000 $\mu g\ L^{-1}$) neither is the matrix too complex, their analysis and quantification can be done with sufficient accuracy in the much more accessible LC-UV or LC-DAD instrumentation. The use of an appropriate methodology for sample preparation can result in analytical methods with very LOD and LOQ, even when UV detectors are used.

In this work, the UV/Vis and DAD detectors were successfully used to determine the studied pharmaceuticals in some assays, especially when this analytical methodology was coupled with SPE with high recovery rates. Details on the methodology used can be found in the manuscripts presented in the appropriate section of this thesis.

However, the application of LC with tandem MS (in a LC-ESI-QIT-MS apparatus) was required to determine the minute amounts of pharmaceutical compounds in extracts

from plant tissues. Due to the complexity of the matrix, only the sensitivity and selectivity of the mass detector was able to provide an accurate detection and quantification of the studied pharmaceuticals in the plant extracts. Details of the analytical methodology used are described in the “Complementary Results” section in Appendix D.

Table 1.7. Analytical methods based on SPE, liquid chromatography and mass spectrometry for quantification of the pharmaceuticals in environmental samples

Analyzed drugs	SPE			LC separation		Detection method	LOQ (ng L^{-1})	References
	Material	pH	Elution solvent	Column	Mobile phase			
CB	RP-C_{18}	7.5	MeOH	C_{18}	Aq. NH_4Ac/ACN	ESI-tQ	5 (tap water) 50 (effluent)	(Ternes, 1998; Ternes, 2001)
AT	PPL Bond-Elut	7	MeOH	C_{18}	Aq. NH_4Ac/ACN+MeOH+NH_4Ac	ESI-tQ	8.2 (tap and surface water)	(Sacher et al., 2001)
IB	Strata X	3.0	MeOH	C_{18}	Aq. NH_4Ac/MeOH	ESI-tQ	20-50 (effluent and surface water)	(Hilton and Thomas, 2003)
CA, IB	Oasis MCX	2.0	Acetone	C_{18}	Aq. HAc/ACN	APCI-tQ	0.4 ng g^{-1} (solid sample)	(Löffler and Ternes, 2003)
CB	Oasis HLB	7.0	MeOH	C_8	Aq. NH_4Ac+Aq. FA/ACN+MeOH	ESI-tQ	nr	(Miao and Metcalfe, 2003)
CB, IB	Oasis HLB	2.0	MeOH+MTBE	C_{12}	Aq. FA/MeOH	APCI or ESI-tQ	1.0 (surface water)	(Vanderford et al., 2003)
AT, CA	RP-C_{18}	10.5	MeOH	C_{18}	H_2O/ACN	ESI-tQ	17-50 (effluent)	(Hernando et al., 2004)
CB	Oasis MCX	3.0	MeOH+Ammonia	C_{18}	Aq. NH_4Ac/MeOH+NH_4Ac	ESI-Q-TOF	5-10 (effluent and surface water)	(Stolker et al., 2004)
AT, CA, IB CB	Oasis MCX Lichrolut EN	2.0 7.0	MeOH+Ammonia/MeOH + NaOH/MeOH MeOH+EtOAc	C_8	ESI^+: Aq. FA/ACN ESI^-: Aq. TEA/ACN	ESI-tQ	0.36-1.38 (effluent)	(Castiglioni et al., 2005)
AT, CB, IB	Oasis HLB	2, 4, 7	MeOH	C_{18}	ESI^+: Aq. FA/ACN ESI^-: H_2O/ACN	ESI-tQ	19-86 (effluent)	(Gómez et al., 2006)
AT, CA, CB, IB	Isolut ENV+, Oasis HLB and MCX, RP-C_{18}	2, 7	MeOH	C_{18}	ESI^+: Aq.NH4Ac+HAc/ACN+MeOH ESI^-: H_2O/MeOH	ESI-tQ	3-42 (surface water) 6-40 (effluent)	(Gros et al., 2006b)
CA, CB, IB	Oasis HLB	8.2	MeOH	C_{18}	ESI^+: Aq. HFBA+Aq. NH_4Ac+MeOH/ACN ESI^-: Aq. NH_4Ac/ACN	ESI-Ion Trap	0.5-25 (surface water)	(Hao et al., 2006)
CB, IB, AT	Oasis HLB	nr	MeOH	C_{18}	ESI^+: Aq. NH_4Ac+Aq. HAc/ACN+MeOH ESI^-: H_2O/MeOH	ESI-Q-TOF	15-500 (effluent)	(Petrovic et al., 2006)
AT	Oasis MCX	3.0	DCM+2-propanol+NH_4OH	C_8	Aq. FA/ACN	ESI	6-9 (effluent)	(Lee et al., 2007)

Abreviations: ACN, acetonitrile; APCI, Atmospheric pressure chemical ionization; Aq., aqueous; AT, atenolol; CA, clofibric acid; CB, carbamazepine; DCM, dichloromethane; ESI, Electrospray ionization; EtOAc, Ethyl acetate; HAc, acetic acid; HFBA, heptafluorobutyric acid; IB, ibuprofen; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MTBE, Methyl tert-butyl ether; nr, not reported; RP, reverse phase; TEA, triethylamine; TOF, time of flight; tQ, triple quadrupole

Chapter 2

2. Studies for selection of the more suitable support matrix materials

Article 1

Title: Selection of a support matrix for the removal of some phenoxyacetic compounds in constructed wetlands systems

Authors: Ana V. Dordio, José Teimão, Idália Ramalho, A. J. Palace Carvalho, A. J. Estêvão Candeias

Published by: Science of The Total Environment (2007, Vol. 380, Issues 1-3, pp. 237-246)

Article 2

Title: Preliminary media screening for application in the removal of clofibric acid, carbamazepine and ibuprofen by SSF-constructed wetlands

Authors: Ana V. Dordio, A.J. Estêvão Candeias, Ana Paula Pinto, Cristina Teixeira da Costa, A.J. Palace Carvalho

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Motivations

The efficiency of a subsurface flow-constructed wetlands system (SSF-CWS) in the removal of pollutants such as pharmaceutical compounds can be significantly enhanced by a greater capacity of the support matrix to retain contaminants by sorption phenomena, ion exchange or other physico-chemical processes occurring in solid media. The hydraulic conductivity, grain size distribution and mechanical resistance are also important parameters to be considered in the selection of materials for SSF-CWS support matrices.

The present chapter discusses the selection of suitable materials to compose a support matrix to be used in the construction of a microcosm constructed wetlands system (CWS) for removal of pharmaceuticals from wastewaters. In this work different materials were evaluated. Light expanded clay aggregates (LECA) was studied with solutions of three pharmaceuticals (clofibric acid, carbamazepine and ibuprofen). For the most recalcitrant of the three compounds, clofibric acid, other materials were also tested, namely exfoliated vermiculite, expanded perlite, sepiolite and sand. These materials were chosen because they are either low cost industrial products or widely available natural materials which have varied characteristics and are, therefore, expected to present different sorption behaviors.

Several factors that influence the sorption phenomena were investigated. In particular, the dependence of the sorption extent on the pharmaceuticals concentrations was studied by changing the initial concentrations of the compounds. Effects due to the complexity of the liquid matrix were also examined by comparing the sorption capacity of the materials for simple aqueous solutions of the compounds and spiked wastewater. The behavior of the pharmaceuticals sorption in single-compound solutions was also compared with that of solutions where all three pharmaceuticals were present simultaneously (and where competition effects could be assessed).

Physical, chemical and mineralogical characterization of the materials (e.g. particle size distribution, point of zero charge, XRD analysis) was conducted in order to shed some light into how their differences influence the sorption capacities.

The selection of the support matrix to be used in a CWS is an opportunity to significantly enhance the solid matrix's role in the removal of the pollutants, which may contribute to an overall increase in the efficiency of the system.

For the quantifications of the studied pharmaceuticals in spiked water and wastewater samples, an analytical methodology was developed and optimized. The method used for quantification of studied pharmaceuticals in aqueous samples included the following steps: isolation and pre-concentration with solid phase extraction (whenever necessary), separation by liquid chromatography (HPLC) and detection/quantification by UV/Vis spectrometry.

2.1. What is the role of the support matrix in organic xenobiotics removal at SSF-constructed wetlands?

Many phenomena relevant for organic xenobiotics removal occur within the support matrix compartment of the SSF-CWS or is influenced by the solid media characteristics. However, through sorption, the solid matrix does play an active role which can be complimentary with other processes occurring within the other SSF-CWS components.

Sorption of organic contaminants to solid surfaces (e.g., suspended material in the water column or surface of the support matrix) is an important retention process because it lowers the concentrations of organic xenobiotics in the aqueous medium and transfers them into a phase that makes them less available (Reddy and DeLaune, 2008a). Sorption of non-polar organic compounds onto the solids suspended in water and onto the support matrix can be best described as a water-lipid partitioning process, whereby the organic xenobiotic is partitioned between the water and the organic matter fraction in the solid phase (Reddy and DeLaune, 2008a). Sorption of polar or ionic organic xenobiotics, on the other hand, is dominated by electrostatic interactions and ion exchange phenomena. Although the large majority of priority pollutants are non-polar, most pharmaceuticals are polar or ionizable (Reddy and DeLaune, 2008a).

In contact with the support matrix in a SSF-CWS pharmaceuticals are exposed to the same type of processes determining their fate in these wastewater treatment systems as they do in the environment in contact with soil. The type of interactions as well as the way pharmaceuticals' properties influence their fate is described in some detail in section 1.3.1.

Sorption isotherms can be useful tools for studying the interactions of organic chemicals with solid media composed by several types of materials and for getting details about surface phenomena occurring within this compartment. Isotherms data are generally described by using either the Langmuir or Freundlich equations (Reddy and DeLaune, 2008a).

Langmuir equation – this equation has a sound conceptual base and was originally developed for defining the adsorption of gases over solids. The following assumptions are made in the deduction of this expression (Tinsley, 2004):

- the energy of adsorption is constant and independent of the extent of surface coverage by the adsorbed molecules;

- adsorption occurs on localized sites and there is no interaction between the adsorbed molecules; and
- the maximum possible adsorption extent is that of a complete monolayer.

In the Langmuir equation the moles of solute adsorbed per gram of adsorbent (n_{sorb}) are expressed as a function of the equilibrium concentration of solute in solution (C_e) (Langmuir, 1918):

$$n_{sorb} = \frac{n_m \cdot K_L \cdot C_e}{1 + K_L \cdot C_e} \tag{2.1}$$

with n_m being the number of moles adsorbed per gram of adsorbent corresponding to a complete monolayer and where K_L is a constant related to the energy of adsorption (when C_e=1/K_L, then $n_{sorb} = n_m/2$).

At low concentrations of solute the relation is approximately linear ($n_{sorb} \approx n_m K_L C_e$), but the extent of adsorption is limited at high concentration due to the limited number of sites (Tinsley, 2004).

The Langmuir equation can be expressed in a linear form:

$$\frac{1}{n_{sorb}} = \frac{1}{n_m} + \frac{1}{K_L \cdot n_m} \cdot \frac{1}{C_e} \tag{2.2}$$

Freundlich equation – this equation expresses the empirical relation between the amount of compound sorbed (n_{sorb}) and the equilibrium concentration, C_e (Freundlich, 1926):

$$n_{sorb} = K_F \cdot C_e^{1/n} \tag{2.3}$$

A logarithmized form of this expression gives a linear relation from which values for the Freundlich constant, K_F, and the exponent, $1/n$, can be derived:

$$\log n_{sorb} = \log K_F + \frac{1}{n} \log C_e \tag{2.4}$$

Values of K_F provide an indication of the extent of sorption. The exponent n, simply indicates whether the relation between n_{sorb} and C_e is linear (n=1), concave down (n>1) or concave up (n<1) (Tinsley, 2004; Reddy and DeLaune, 2008a).

For nonpolar organic xenobiotics a distribution constant (K_d) can be calculated corresponding to a modified Freundlich equation where $1/n$ is approximately equal to 1:

$$K_d = \frac{n_{sorb}}{C_e} \tag{2.5}$$

where n_{sorb} is the organic xenobiotics adsorbed on the sediments and C_e the equilibrium concentration of organic xenobiotics.
Using the K_d and sediment's organic Carbon (OC) fraction, another constant can be determined that is independent of soil type and is specific for each sorbed organic xenobiotic being investigated (Reddy and DeLaune, 2008a):

$$K_{OC} = K_d \cdot f_{OC} \quad (2.6)$$

where f_{OC} is the fraction of soil OC. K_{OC} is, thus, a coefficient describing the distribution of the organic xenobiotic between the water and sediment organic matter phases.
Bioavailability of toxic organics to degradation is strongly influenced by sorption. For a given solid matrix, organic chemicals with smaller K_d values are sorbed to lesser extent and, therefore, more water soluble which makes them highly mobile and more available for degradation (Reddy and DeLaune, 2008a).
Both the physical and chemical characteristics of the materials used as support matrix in SSF-CWS can greatly influence the efficiency of the retaining processes such as sorption as described in section 1.5.2.
In most CWS used for the removal of organic xenobiotics, as described in the literature, the media materials used have been mainly gravel, sand or local soils (USEPA and USDA-NRCS, 1995; Cooper et al., 1996; Vymazal et al., 1998; Sundaravadivel and Vigneswaran, 2001; Kadlec and Wallace, 2009) and little importance has been given to the selection of more appropriate materials which can enhance the removal of this type of compounds by sorption. The few studies on CWS for pharmaceuticals removal have been following this general trend, with gravel beds being frequently used (Matamoros et al., 2005; Matamoros and Bayona, 2006; Matamoros et al., 2007a; Matamoros et al., 2008a).
However, there is a substantial number of studies on the sorption of organic xenobiotics, including pharmaceuticals, by a variety of different materials (Table 2.1). Some of these materials have appropriate mechanical and hydraulic properties which make them suitable to be used as support matrices in SSF-CWS.
Clay minerals and organic matter are generally identified as the two most important constituents of widely available, low-cost, natural materials with a good potential for retaining organic xenobiotics through sorption. Clay minerals, in particular, due to their

ionic nature, are especially suitable for the sorption of polar or ionic compounds, as many pharmaceuticals are. Activated carbon is also a well known good sorbent for non-polar compounds, but it has the disadvantage of being a more expensive material and better suited to be used as a filter rather than a CWS support matrix.

Table 2.1. Studies of organic compounds sorption by several synthetic, natural and modified natural materials

Material	Compound	Reference
Activated carbon	Halogenated organics	(Pavoni et al., 2006)
	Pesticides	(Ayranci and Hoda, 2005)
	Pharmaceuticals	(Yu et al., 2008; Putra et al., 2009)
Clays (bentonite)	Pharmaceuticals	(Putra et al., 2009)
	Several organic pollutants	(Zhu and Chen, 2009)
Clays (montmorillonite)	Pharmaceuticals	(Gao and Pedersen, 2005)
Clays (rectorite)	Pharmaceuticals	(Chang et al., 2009)
Clays (sepiolite)	Dyes	(Alkan et al., 2004; Alkan et al., 2007; Dogan et al., 2007)
Clays (smectite)	Pesticides	(Li et al., 2004; Li et al., 2006)
Clays (various)	Pesticides	(Laila et al., 2008; Baglieri et al., 2009)
	Pharmaceuticals	(Figueroa et al., 2004)
	Phenols	(Sagar and Shigh, 2007)
Diatomaceous earth	PAHs	(Mittal and Rockne, 2009)
Fly ash	Several organic pollutants	(Ahmaruzzaman, 2009)
Natural zeolite-clays	VOCs	(Breus et al., 2008)
Organoclays (organovermiculite)	Pesticides	(Abate and Masini, 2005)
Organoclays (various)	Pesticides	(Baglieri et al., 2009)
Organo-zeolites	PAHs	(Lemic et al., 2007)
Perlite	Dyes	(Dogan and Alkan, 2003; Acemioglu, 2005)
	Surfactants	(Alkan et al., 2005)
Soil	Pharmaceuticals	(Drillia et al., 2005; Uslu et al., 2008)

Other synthetic or modified natural materials that have been reasonably tested are organoclays and zeolites, but its use as support matrix is yet to be evaluated. Testing of new support matrix materials in experimental CWS is an attractive area of research which remains to be adequately explored for the removal of organic xenobiotics, in particular of pharmaceuticals.

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Selection of a support matrix for the removal of some phenoxyacetic compounds in constructed wetlands systems

Ana V. Dordio *, José Teimão, Idália Ramalho, A. J. Palace Carvalho, A. J. Estêvão Candeias

Department of Chemistry, University of Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal

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Abstract

The efficiency of constructed wetlands systems in the removal of pollutants can be significantly enhanced by using a support matrix with a greater capacity to retain contaminants by sorption phenomena, ionic exchange or other physico-chemical processes. The aim of this work was to evaluate the efficiencies of 3 different materials, Light Expanded Clay Aggregates [LECA] (in two different particle sizes), Expanded Perlite and Sand, for the removal from water of one pharmaceutical compound (clofibric acid) and one pesticide (MCPA). Both belong to the class of phenoxyacetic compounds. In addition, relationships were established between the compounds' removal efficiencies and the physico-chemical properties of each material. LECA exhibited a high sorption capacity for MCPA, while the capacity for clofibric acid was more modest, but still significant. In contrast, perlite had a very limited sorption capacity while sand did not exhibit any sorption capacity for any of the compounds. LECA with smaller particle sizes showed higher efficiencies than larger grade LECA and can achieve efficiencies near 100% for the lower concentrations in the order of 1 mg l^{-1}. However, the use of these smaller particle media at larger scales may present problems with hydraulic conductivities. The results show that expanded clay presents important advantages in laboratory studies and could be used as a filter medium or a support matrix in constructed wetlands systems.
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1. Introduction

Over the last decades, an ever-increasing number of xenobiotic compounds is being detected in water bodies. These emerging pollutants include pesticides, in particular herbicides such as MCPA, 2,4-D, Mecoprop and Atrazine (Azevedo et al., 2000; Lacorte et al., 2001; Laganá et al., 2002; Cerejeira et al., 2003), as well as pharmaceuticals and their metabolites such as Ibuprofen, Diclofenac and Clofibric acid (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Heberer, 2002a; Ferrari et al., 2003; Packer et al., 2003; Zuccato et al., 2005). This raised concerns about the contamination of the water resources and the impact on ecosystems and on drinking water supplies.

The threat presented by these types of compounds is due to their generally high mobility and persistence in the aqueous media and to their toxicity. For example, MCPA (4-chloro-2-methyphenoxyacetic acid) is a widely used systemic herbicide which belongs to the group of related synthetic herbicides, the chlorophenoxy herbicides. It is commonly used in combination with

* Corresponding author. Tel.: +351 266745343.
E-mail address: avbd@uevora.pt (A.V. Dordio).

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other herbicides for the post-emergence control of broad-leaved weeds (Tomlin, 1994). This herbicide is relatively mobile and is frequently detected in water bodies. It persists in the environment for several weeks (Santos et al., 2000; Lacorte et al., 2001; Laganá et al., 2002; Cerejeira et al., 2003). MCPA is classified, among other phenoxy acid herbicides, as a possible human carcinogen (class 2B-carcinogen) by the International Agency for Research on Cancer, and has recently been reported to have long-term reproductive effects such as mutagenicity. Clofibric acid [2-(4-chlorophenoxy)-2-methylpropanoic] acid is the bioactive metabolite of various blood lipid regulating pro-drugs such as clofibrate, etofibrate and etophyllinclofibrate (Daughton and Ternes, 1999; Stumpf et al., 1999; Winkler et al., 2001; Ferrari et al., 2003). Clofibric acid is also classified as a plant growth regulator (antiauxin) pesticide (Compendium of Pesticide Common Names). Its structure is suggestive of chlorophenoxy acid herbicides (it is, in fact, an isomer of one such herbicide, Mecoprop [2-(4-chloro-*o*-tolyloxy) propionic acid]). However, clofibric acid is resistant to degradation and appears to persist in the environment much longer than these herbicides (Daughton and Ternes, 1999). Its lifetime might be as long as 21 years (Zuccato et al., 2000). Because of this high persistence, clofibric acid is a common contaminant of wastewater and sewage treatment plants. It has frequently been detected in surface and ground waters (Daughton and Ternes, 1999; Heberer, 2002a; Ferrari et al., 2003; Emblidge and DeLorenzo, 2006) with a potential to contaminate drinking water supplies if not removed efficiently from effluents by the treatment plants (Heberer and Stan, 1997; Ternes, 1998; Heberer, 2002b; Ternes et al., 2002; Webb et al., 2003; Petrović et al., 2003; Stackelberg et al., 2004). The major sources of contamination by pharmaceuticals are primarily point sources such as effluents which did not receive an efficient treatment. For pesticides, also diffuse sources such as agricultural runoffs are important.

One of the reasons for the presence of these emerging compounds in the water bodies is that they frequently escape elimination during wastewater treatment in conventional sewage treatment plants. These were designed to deal with nutrients and other common contaminants such as organic matter, suspended solids and microorganisms, that arrive regularly and in large quantities. The conventional technology for wastewater depuration is not capable to completely remove these very specific chemicals which then enter the aquatic environment via the sewage effluents. Pharmaceuticals have been measured in the effluents of various European wastewater treatment plants (Daughton and Ternes, 1999; Ternes et al., 2002; Petrović et al., 2003; Carballa et al., 2004; Larsen et al., 2004; Stackelberg et al., 2004). New approaches to wastewater treatment are needed to sufficiently eliminate these xenobiotic compounds.

In addition to removal during biological wastewater treatment, pharmaceuticals can also be attenuated in engineered natural systems (i.e., subsurface flow constructed wetlands systems, riverbank infiltration) (Matamoros et al., 2005). Experiments with constructed wetlands have illustrated their ability to also remove several pesticides from contaminated waters (Moore et al., 2000; Kao et al., 2001; Braskerud and Haarstad, 2003; Sherrard et al., 2004). In subsurface flow constructed wetlands systems (SSF-CWS) the xenobiotic compounds are subjected to biological (biological degradation, plants and aquatic organisms' uptake), chemical (chemisorption, photo-decomposition and degradation) and physical (volatilization and sorption) processes. A concerted action between plant rhizomes, microorganisms and matrix components can decrease the concentration of the compounds to levels that are safe for the aquatic biota or production of drinking water.

Processed natural materials which are used as filter media in water and wastewater treatment systems can present additional functions which extend beyond the simple process of filtration. Their surface areas can constitute a support for microbial population growth in biofilters as well as for the development of macrophytes in CWS. The efficiency of these biological systems in the removal of pollutants can be significantly enhanced by a greater capability of the filter media to retain contaminants by sorption phenomena, ionic exchange or other physico-chemical processes (Yang et al., 1997). Consequently, the selection of a medium with a high sorption capacity can be an important step in the optimization of the CWS performances. These capabilities will be dependent upon the chemical and physical properties of the material chosen. Naturally, under field conditions, CWS performance will be far from ideal. The interaction among the several components of the CWS (matrix, plants and microorganisms) as well as the varying effluent compositions and environmental conditions will influence system performance. Yet, laboratory assays remain useful as a first step in designing the setup of a CWS as they allow to screen and test potential filtering materials in a fast and cost effective manner.

The aim of the present work was to evaluate the removal efficiencies of two phenoxyacetic compounds (MCPA and clofibric acid) by sand and two different processed natural materials (Light Expanded Clay Aggregates [LECA] and Expanded Perlite). These

materials are commonly used as filter media but are being increasingly introduced as support matrices in CWS as well (Johansson, 1997; Brix et al., 2001). This work constitutes a preliminary selection of materials that could make a significant contribution to the removal of the studied compounds from water.

2. Materials and methods

2.1. Media tested

In this study two different natural processed materials as well as sand were tested:

(i) Light Expanded Clay Aggregates (LECA) was supplied by MaxitGroup Portugal (two types of granular sizes were tested namely with grade 2/4 and grade 3/8). LECA consists of natural clay and is manufactured by running pelletised clay aggregates through rotary kilns at 1200 °C, producing lightweight (300–400 kg m^{-3}) ceramic pebbles. LECA is mainly used for construction purposes (such as in building blocks and insulating material), however over the last years it is being also used for different types of water and wastewater treatment processes such as filter processes and constructed wetlands systems (Johansson, 1997; Zhu et al., 1997; Brix et al., 2001).

(ii) Expanded Perlite was supplied by the Portuguese company Hubel. Perlite is a glassy volcanic rock which is essentially a metastable amorphous aluminum silicate.

Expanded Perlite is an excellent insulator, both thermal and acoustic, resists fire and is classified as an ultralight-weight material. Due to these properties, this material finds its major use in the construction industry. In addition, when mixed with soil, perlite increases the amount of air and water retained in the soil which obviously improves the growing conditions for plants and, therefore, another important use for perlite is found in horticultural applications which represent approximately 10% of the market (Mathialagan and Viraraghavan, 2002). As perlite contains a high silica content, it is chemically inert in many environments and hence is also an excellent filter aid and filler in various industrial processes.

Recently, the properties of perlite for adsorption of water contaminants have been investigated in a number of papers (Chakir et al., 2002; Mathialagan and Viraraghavan, 2002, Alkan et al., 2005).

(iii) Sea sand was also used in this study, which was collected in Faro Beach, Portugal.

A common use of sand is in water treatment plants as a medium of the filtering process and as a support matrix in constructed wetlands (Brix et al., 2001).

2.2. Chemicals

4-chloro-2-methyphenoxyacetic acid (MCPA) (97% purity) and clofibric acid (CA) (97% purity) were purchased from Aldrich. Some of the most relevant physico-chemical properties of these phenoxy compounds are listed in Table 1.

Table 1
Physico-chemical properties and fate characteristics of the MCPA herbicide and the clofibric acid pharmaceutical

Common name	MCPA	Clofibric acid (CA)
IUPAC name	(4-chloro-2-methylphenoxy) acetic acid	2-(4-chlorophenoxy)-2 methylpropionic acid
Chemical function	Pesticide (herbicide)	Active metabolite (lipid regulator)
Structure		
Molecular weight (g mol^{-1})	200.6	214.65
Melting point (°C)	(118–119)[a]	(120–123)[b]
Vapor pressure (20 °C) (mPa)	0.2[a]	–
Ionization constant, pK_a	3.07[c]	2.5[d]–3.18[e]
Water solubility (25 °C) (mg l^{-1})	825 (acid)[a]	582.5[b]
Log K_{ow}	(1.37–1.43)[f]	2.57[b]
Soil sorption coefficient, K_{oc}	100[a]	75.8[d]
Soil half-life (days)	7–60[a, c, g]	–

[a] Source: EXTOXNET (1996).
[b] Source: Tomlin (1994).
[c] Source: Ferrari et al. (2003).
[d] Source: Drillia et al. (2005).
[e] Source: Packer et al. (2003).
[f] Source: Montgomery (1993).
[g] In relation with microbial activity, moisture content and the concentration of organic matter in soil.

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2.3. Physico-chemical characterization of the media

Commercially available media contains considerable amounts of superficial mineral impurities and therefore, prior to use, it was required to always wash it several times with doubly distilled water until no further suspended materials were visible. This operation significantly reduced the amount of those impurities.

The particle-size distribution on a weight basis was analyzed in triplicate by the conventional dry-sieving technique (Day, 1965). Grain-size distribution plots were used to estimate d_{10} (effective grain size) and d_{60}, and the uniformity of the particle size distribution (the uniformity coefficient) was calculated as the ratio between d_{60} and d_{10}. The porosities of the media (or apparent porosity, or void space) were determined from the amount of water needed to saturate a known volume of the solid (replicate number $n=5$) (Brix et al., 2001; Del Bubba et al., 2003), and the bulk density was determined based on the ratio between the dry weight and the bulk volume of the media ($n=5$) (Brix et al., 2001). Hydraulic conductivity was measured as described in Cooper et al. (1996) ($n=5$).

The mineralogical composition of the media was studied by X-ray diffraction (XRD) using a Bruker AXS-D8 Advance diffractometer. Optical observation of polished surfaces and thin sections at the transmitted light microscope was used to analyze the morphology and macroporous structure of the particles. Finally, to evaluate the acidic properties of the media, the point of zero charge (PZC) was determined using the mass titration method (Noh and Schwarz, 1989; Žalac and Kallay, 1992).

2.4. Experimental design for sorption assays

Two series of independent batch assays, using clofibric acid and MCPA respectively, were conducted to study the removal efficiencies of these compounds by several sorbent media.

The assays were set up in 5 liter plastic containers filled with the tested media. The solid to liquid ratios used were 0.8, 0.65, 0.17 and 4.0 g ml^{-1} respectively for LECA 2/4, LECA 3/8, perlite and sand, both in the MCPA and in the clofibric acid assays. These ratios were chosen in each assay to correspond to a flooding rate near 100%. These liquid saturated conditions allow for a better contact between the media and the compounds' aqueous solutions and simulate the most usual flooding rates used in SSF-CWs.

The influence of some experimental conditions, such as the contact time, the initial concentrations

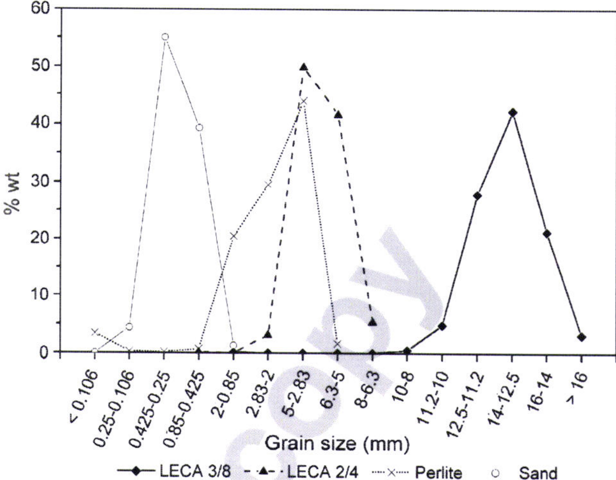

Fig. 1. Grain size distribution of LECA 3/8, LECA 2/4, perlite and sand.

(from 1 mg l^{-1} up to 70 mg l^{-1}) and LECA particle size, was investigated.

The compounds aqueous solutions were prepared using Millipore water. The pH of the solutions in contact with the media varied in the range of 5.5 (with sand) to near 7.5 (with LECA). Experiments were all done in triplicate and at the same controlled temperature of 20 °C. No stirring of the solutions was performed in order to provide a better simulation of the kinetic behavior in a SSF-CWS type of system, where low flow rates are normally used.

In these assays, the media were sterilized before use in order to minimize any microbial development and experiments were conducted in the dark to avoid any photocatalytic degradation reactions.

For the determination of the sorbed amounts of MCPA and clofibric acid, aliquots were collected every 24 h. After centrifugation, MCPA and clofibric acid concentrations were determined spectrophotometrically at $\lambda=228.6$ nm and $\lambda=227.6$ nm, respectively, using a Hitachi U-2000 spectrometer. The range of concentrations of MCPA and clofibric acid standards used to determine the calibration curve was within 0.3–70 mg l^{-1}. In this concentration range a linear behavior is observed ($R^2=0.999$ in both cases). For this analytical methodology the limits of detection (defined as LOD$=y_B+3$ S.D.$_B$) were 0.08 mg l^{-1} for MCPA determination and 0.09 mg l^{-1} for clofibric acid and the limits of quantification (defined as LOQ$=y_B+$ 10 S.D.$_B$) were 0.27 mg l^{-1} for MCPA and 0.30 mg l^{-1} for clofibric acid.

Sorbed amounts of MCPA and clofibric acid were determined by difference between the concentrations in the aliquots and the initial concentration of the respective compound.

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3. Results and discussion

3.1. Physical and chemical properties of the media

The materials tested exhibited a large variation in the particle size distribution (Fig. 1). LECA 3/8 contained, by far, the biggest proportion of large particles > 11.2 mm (94.6%) while sand had the largest proportion of small particles <0.85 mm (98.7%) (Fig. 1).

All the materials had similar uniformities with the exception of Perlite, which had a much higher uniformity coefficient.

Sand has a much lower porosity than the other materials studied (Table 2). The porosity of sand is exclusively due to the interparticle void space while the other materials absorb substantial amounts of water in the interior macroporous cavities of their particles.

The natural processed materials all have large porosities, which contribute to the high hydraulic conductivities of these materials and render them suited for use as filter media or as support matrices in CWS (Cooper et al., 1996; Brix et al., 2001). For the smaller particles however, preferred flow channels may develop (Bowmer, 1987; Fisher, 1990; Netter, 1994) that lead to less extensive contact between the solution and the solids. For sand of fine to medium particle size, Ks values reported vary between 10^{-4}–10^{-5} m s^{-1} (Cooper et al., 1996; Brix et al., 2001; Del Bubba et al., 2003). For fine and coarse perlite 3.13×10^{-4} and 4.65×10^{-4} m s^{-1}, respectively, have been measured (Heiskanen, 1995). Therefore, these characteristics alone would not preclude the use of perlite and sand as filter media as well (Brix et al., 2001).

As can be seen by the values of the points of zero charge, all the materials have a slightly basic character. The values for perlite and LECA are within the range that is commonly reported for these materials (Brix et al., 2001; Acemioğlu, 2005). The point of zero charge of the siliceous sand is slightly more basic than would be expected but can be attributed to the presence of calcitic materials such as mollusc shells.

Observation on a petrographic microscope showed that the sand is mainly composed by quartz. Rests of mollusc shells were also found along with some sandstone aggregates.

XRD analysis confirmed the mineralogical composition of this sand. The composition was mainly quartz with traces of orthoclase and calcite, which explains the high PZC found in this material (Fig. 2).

Thin section observation showed that perlite consists of agglomerated glass-like particles with an interparticle porous structure. XRD showed a very broad peak which indicates clearly its amorphous nature (Fig. 2).

Thin section observation revealed that LECA is an extensively porous material with a highly interconnected porous structure characterized by a wide meso-/macropore size distribution. It is mainly composed of quartz and in smaller amounts of hercynite (an iron alluminate) and anorthite (an alluminosillicate). These minerals are formed during thermal decomposition of the clay precursors. Traces of calcite were also found (Fig. 2).

3.2. Removal efficiency of the media

All the materials were tested for the removal of MCPA and clofibric acid at initial concentrations of 35 mg l^{-1} (Fig. 3). Sand did not adsorb any of these compounds which is mainly due to its relatively low surface area since it is mainly composed of dense quartz crystals. Expanded perlite exhibited a very low removal capacity for both phenoxy compounds. On the contrary, expanded clay presents high sorption capacities for both the MCPA and the clofibric acid. These results can also be explained partly by the higher PZC of LECA which leads to a more extensive protonation of its surface at working pH conditions (pH ≈ 7.5). At these pH values, the phenoxyacids are mostly in the anionic form ($pH > pK_a$) while the surfaces are positively charged (pH < PZC). It can be hypothesized that in such cases adsorption is mainly controlled by electrostatic interactions. Therefore, it is the more extensively charged surface of LECA that should

Table 2
Physical characteristics of the natural processed materials and sand

Filter media	d_{10} (mm)	d_{60} (mm)	d_{60}/d_{10}	Porosity (%)	Bulk density (kg m^{-3})	Ks (m $s^{-1} \times 10^{-3}$)	PZC
LECA 2/4	3.18	5.25	1.65	47±2	387±9	7.6±0.2	8.49±0.12
LECA 3/8	10.35	12.55	1.21	41±2	255±4	21.1±1.2	8.92±0.05
Perlite	1.20	3.32	2.77	61±2	115±6	nd	8.09±0.05
Sand	0.27	0.43	1.59	36±0	1525±23	nd	9.18±0.02

Values for d_{10}, d_{60} and uniformity coefficient (d_{60}/d_{10}) are means of triplicate analyses. Values for porosity, bulk density and hydraulic conductivity (Ks) are means±1 S.D. (n=5).
nd — not determined.

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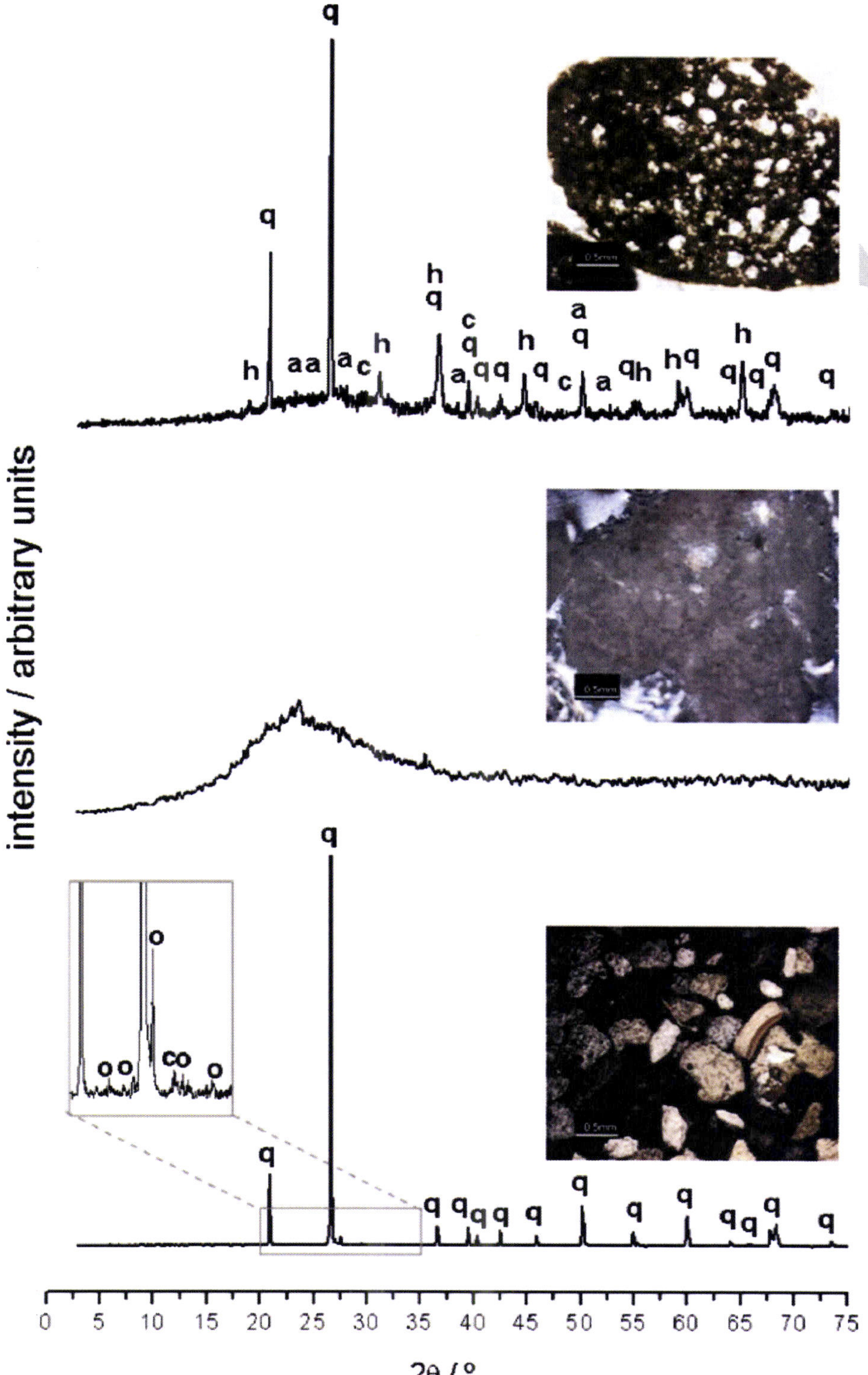

Fig. 2. X-ray diffraction and thin section micrographs of the media. From top to bottom: LECA, perlite and sand. (q — quartz, o — orthoclase, c — calcite, h — hercynite, a — anorthite).

have the strongest interaction with the anionic phenoxy compounds. These higher affinities of positively charged surfaces for anionic organic compounds have also been reported in other studies on the adsorption properties of various clay materials (Inacio et al., 2001; Alkan et al., 2004; Özcan et al., 2004) and shown to be dependent on the pH. On the other hand, studies of CWs using silica-based support matrices (Matamoros et al., 2005) and studies of pollutant fate in sandy soils with high silica contents (Thorstensen et al., 2001; Löffler et al., 2005)

have shown that anionic organic compounds have high mobility into water and low sorption affinity for the sediments, which is consistent with the present results and may be attributed to a poorer interaction with the neutral silica surface.

For all adsorbents, the removal efficiencies of the clofibric acid are somewhat smaller than those of MCPA. This may be due to a difference in the molecular conformations of these adsorbates and effective cross-sectional areas thus leading to a difference in the packing efficiency of the molecules on the adsorbent surface.

3.3. Kinetic studies

The effect of contact time and initial concentrations on the removal of MCPA and clofibric acid from the solution onto the LECA is shown in Fig. 4. Sorption of MCPA reached equilibrium in 144 h for all MCPA concentrations in the tested range (1–50 mg/l) with a maximum removal of 100% for the most diluted solutions. Solutions of clofibric acid with highest initial concentrations attained equilibrium slightly earlier while the most diluted ones require contact times similar to the MCPA's.

It is clear, especially in the most diluted solutions, that the kinetics of the process is governed by two different regimes. During the first hours, a rapid sorption was observed followed by a slower sorption over a longer period of time. This effect is less pronounced in the most concentrated solutions. The reasons for this dual-regime kinetics are not clear. Further investigation of this phenomenon would be interesting for the clarification of the mechanism responsible for this behavior.

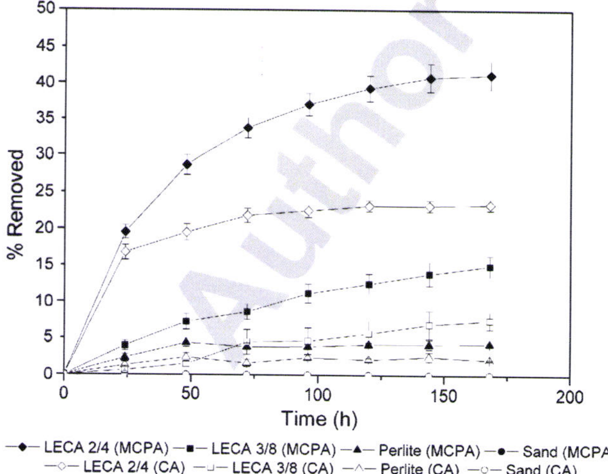

Fig. 3. Removal efficiencies of MCPA and CA, at initial concentrations of 35 mg/L, by each of the media. The curves represent the average of 3 replicates and error bars represent the range of ±1 S.D.

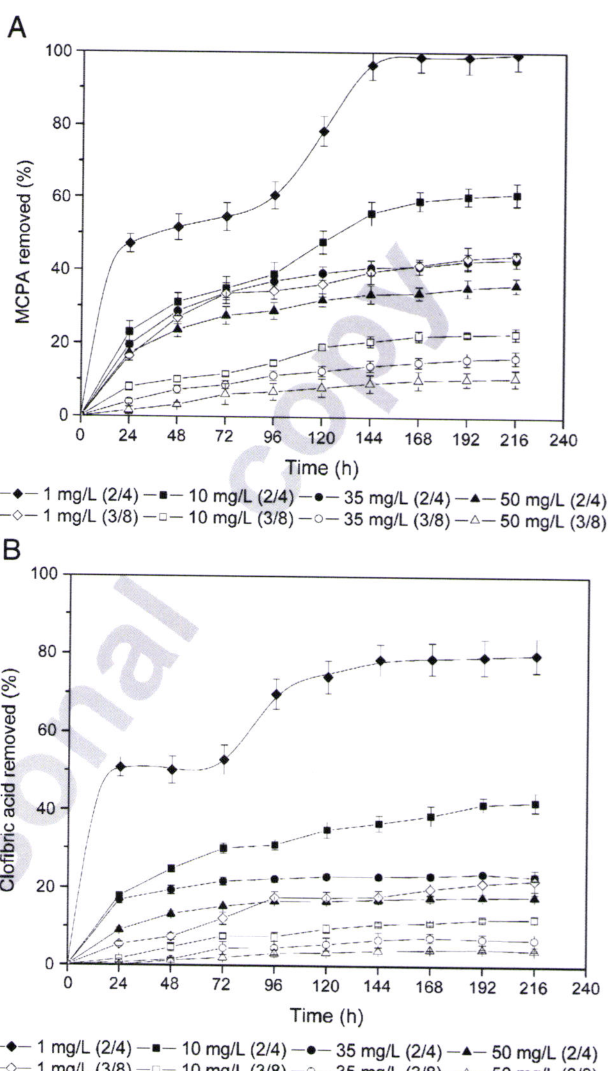

Fig. 4. On A, effect of contact time and initial concentrations on MCPA removal efficiencies by LECA. The curves represent the average of 3 replicates and error bars represent the range of ±1 S.D. On B, effect of contact time and initial concentrations on CA removal efficiencies by LECA. The curves represent the average of 3 replicates and error bars represent the range of ±1 S.D.

3.4. Effect of LECA particle size

LECA with grade 2/4 is more efficient in the removal of both phenoxy compounds (Fig. 5). This fact is not surprising as fine-grained materials have larger specific surface areas and therefore the potential to enhance the sorption capacity of the compounds. However, such materials often have lower hydraulic conductivities (Table 2). This may lead to the occurrence of overland flows and insufficient contact between the solutions and the overall media surfaces. Therefore, it should be assured that the material is permeable enough to prevent surface channeling when applied in filters or CWS.

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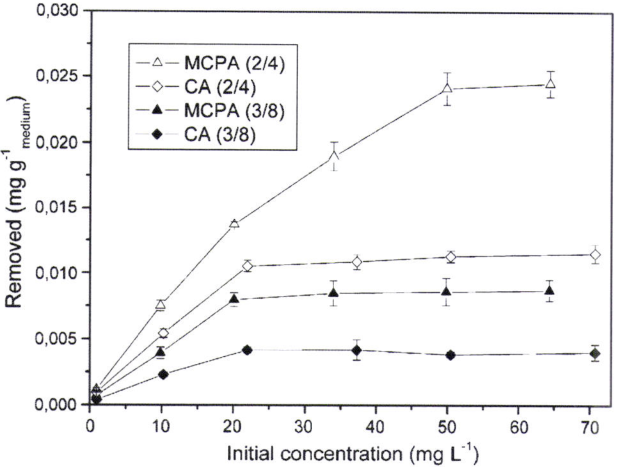

Fig. 5. Relation between the amount of MCPA and CA removed after 216 h versus the initial loading. The curves represent the average of 3 replicates and error bars represent the range of ±1 S.D.

The adsorption isotherms were reasonably well (with R^2 in the range of 0.983 to 0.993 — Table 3) described by the Langmuir equation for adsorption in monolayer:

$$\frac{C_e}{n} = \frac{1}{bn_m} + \frac{C_e}{n_m}.$$

Here, C_e is the solute concentration at equilibrium (in mg l^{-1}), n is the solute adsorbed per unit weight of adsorbent (in mg g^{-1}) and n_m is the monolayer capacity, which relates to the maximum solute adsorption. The latter parameter has an important practical application in the dimensioning of filters and CWS for attaining a particular maximum removal of these compounds. Additionally it can be divided by the solute's molar mass to express the number of active sites (n_s) on the surface for each adsorbent (Table 3).

The manufacturing process of the two grades of LECA is common to both and therefore they should have similar internal porous structures. The difference in the amounts of compound adsorbed should then be related to the difference of the two external surface areas. Considering the mean particle size of each LECA grade and assuming a spherical shape, the external area per unit volume of the LECA grains can be calculated in each case. The ratio of these two areas should be similar to the ratio of the amounts of solute adsorbed by each grade. In fact, this agreement was very close for both compounds (Table 4).

Table 3
Adsorption data of the Langmuir equation

	MCPA			CA		
	n_m/mg g^{-1}	n_s/mmol g^{-1}	R^2	n_m/mg g^{-1}	n_s/mmol g^{-1}	R^2
LECA 2/4	0.0278	0.138	0.953	0.0123	0.0573	0.993
LECA 3/8	0.0099	0.0494	0.985	0.0045	0.0210	0.985

Table 4
Relation between particle size and adsorption capacity

	Mean particle size (mm)	External specific surface area (m^2 m^{-3})	Ratio of external specific surface areas	Sorbed MCPA ratio	Sorbed CA ratio
LECA 2/4	5.00	1200	2.65	2.79	2.73
LECA 3/8	13.25	453			

4. Conclusions

LECA presented important advantages in the laboratory studies. It may be an appropriate choice to be used as a filter medium or support matrix in CWS. It has a high sorption capacity for phenoxy compounds (MCPA and clofibric acid), a pH buffering capability and a suited hydraulic permeability. The two other media tested, expanded perlite and sand, do not exhibit a significant sorption capacity for the studied phenoxy compounds. When different grades of LECA are compared, the kinetics of the sorption process seems to be independent of the particle size. The equilibrium state is attained in any case after approximately 144 h, suggesting that the mechanism of adsorption is similar in both cases. On the other hand, the sorption capacity is highly correlated to the average particle size of the materials. Smaller particle sizes correspond with a larger external surface area and a higher sorption capacity.

Finally, the adsorption of this type of compounds is highly dependent on the extent of surface protonation. Materials with high PZCs appear to interact strongly with these anionic molecules. Therefore, LECA or other materials with such properties should be a preferable choice for testing in filters and CWS. It should be noted, however, that the positive charge of some surfaces may be altered by the development of a biofilm under field conditions. It might cause a net negative charge to build up on the surfaces, which may lead to significantly less retention of acidic compounds such as MCPA and clofibric acid, as shown by Carlson and Silverstein (1998). This phenomenon has to be looked into when setting up a CWS for the removal of this type of pollutants.

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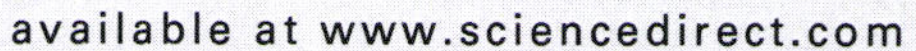

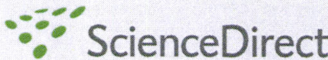

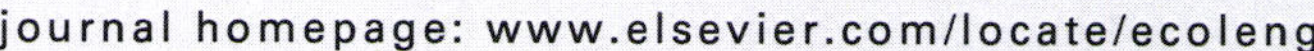

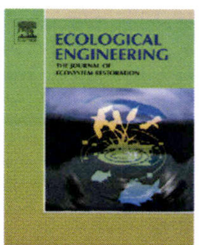

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Preliminary media screening for application in the removal of clofibric acid, carbamazepine and ibuprofen by SSF-constructed wetlands

Ana V. Dordio[a,*], *A.J. Estêvão Candeias*[a,b], *A.P. Pinto*[a,c], *C. Teixeira da Costa*[a,b], *A.J. Palace Carvalho*[a,b]

[a] *Department of Chemistry, University of Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal*
[b] *Centro de Química de Évora, University of Évora, Évora, Portugal*
[c] *ICAM, University of Évora, Évora, Portugal*

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ABSTRACT

The aim of the present work was to evaluate the sorption capacity of light expanded clay aggregates (LECA) to remove mixtures of ibuprofen, carbamazepine and clofibric acid in water and wastewater. High removal efficiencies were attained for carbamazepine and ibuprofen while a less satisfactory performance was observed for clofibric acid. In a mixture of the three compounds in water a slight decrease in the sorbed amounts is observed in comparison with solutions of the single compounds, indicating some competitive sorption. In wastewater, the pharmaceuticals mixture also undergoes a slight reduction in the sorbed amounts of carbamazepine and ibuprofen, probably due to the presence of dissolved organic matter which increases their solubility. These compounds were removed in the following order of efficiencies in all the tested conditions: carbamazepine > ibuprofen > clofibric acid. Two other clay materials – sepiolite and vermiculite – were tested for the removal of the more recalcitrant clofibric acid, and vermiculite exhibited higher removal efficiency than LECA. The sorption is characterized by an initial fast step, with most pharmaceuticals being removed within the first 24 h. The results of this study are a first step in the process of selecting an appropriate material or combination of materials to be used as media in SSF-CWs designed for the removal of pharmaceuticals from wastewaters.

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1. Introduction

Pollution by pharmaceuticals as well as other organic xenobiotic compounds presents a challenge which is not satisfactorily solved by conventional wastewater treatment technologies (Daughton and Ternes, 1999; Heberer, 2002; Jones et al., 2005; Fent et al., 2006). Many of these substances are not significantly degraded/removed in wastewater treatment plants (WWTP) and end up in the receiving water bodies, having already been detected in surface, ground and drinking waters worldwide (Heberer, 2002; Fent et al., 2006; Ellis, 2006). Despite the trace level concentrations of pharmaceutical residues detected ($\mu g\,L^{-1}$ to $ng\,L^{-1}$), potentially adverse effects can still occur due to the continuous exposure especially for the aquatic environment (Halling-Sørensen et al., 1998; Ferrari et al., 2003; Fent et al., 2006; Crane et al., 2006; Hernando et al., 2006; Enick and Moore, 2007).

* *Corresponding author*. Tel.: +351 266 745343; fax: +351 266 745303.
E-mail address: avbd@uevora.pt (A.V. Dordio).
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The widespread use of some drugs and their generally inefficient removal from wastewaters in WWTPs are the main reasons for the common detection of compounds such as clofibric acid (CA), carbamazepine (CB) and ibuprofen (IB) in many monitoring studies reported worldwide (Ternes, 1998; Heberer, 2002; Tixier et al., 2003; Fent et al., 2006). Clofibric acid (2-(4-chlorophenoxy)-2-methylpropanoic acid), a bioactive metabolite of the fibrates (drugs widely used as blood lipid regulators) was the first prescription drug metabolite reported in environmental studies (Garrison et al., 1976; Hignite and Azarnoff, 1977). CA is identified in several studies as a refractory contaminant of municipal WWTP influents and effluents (Tauxe-Wuersch et al., 2005; Fent et al., 2006). The anti-epileptic drug carbamazepine (benzo[*b*][1]benzazepine-11-carboxamide) is frequently detected in environmental samples due to the inefficiency of WWTP processes to remove this contaminant from wastewaters (Fent et al., 2006). Ibuprofen (2-[4-(2-methylpropyl)phenyl]propanoic acid), a non-prescription drug that is among the most consumed pharmaceuticals all over the world, is a well known non-steroidal anti-inflammatory drug. IB environmental contamination is a result of the very high amounts of this drug entering the WWTPs which, despite the high removal rates (up to 90%) (Heberer, 2002; Fent et al., 2006), still result in the discharge of contaminated effluent.

Some advanced technologies have been evaluated to decrease the xenobiotic discharge into water bodies, e.g. oxidative processes, activated carbon and membrane filtration (Andreozzi et al., 2002; Fent et al., 2006; Kim et al., 2007; Esplugas et al., 2007; Snyder et al., 2007), but these, despite the sometimes high removal efficiencies attained, are generally too costly to be considered as viable alternatives on a large scale (Fent et al., 2006).

Constructed wetlands (CWs) are a low-cost technology which has not been fully evaluated for the removal of pharmaceutical residues. When CWs have been used for wastewater treatment they have shown some capacity for removing several organic pollutants, but studies have mainly focused on pesticide removal from agricultural or urban runoff (Rodgers and Dunn, 1992; Moore et al., 2002; Schulz et al., 2003; Braskerud and Haarstad, 2003; Schulz, 2004; Matamoros et al., 2007), while fewer studies exist on pharmaceuticals depuration (Gross et al., 2004; Matamoros et al., 2005; Matamoros and Bayona, 2006; Matamoros et al., 2008).

Pollutant removal in CWs is achieved through the concerted action of its several components. Their performance can be optimized by a better understanding of the processes occurring in the system and the careful selection of the CWs components. The support medium in subsurface flow constructed wetlands (SSF-CWs) has a fundamental role in the growth and development of plants and microorganisms. In addition to providing physical support, the materials can directly interact with the pollutants mainly through sorption processes. The extent of these interactions may strongly influence the system's behavior and performance, and an appropriate selection of the materials to be used as medium is therefore an important step in CW optimization.

Some well-known good sorbents are relatively high priced, which limits their usage as CWs media. Many researchers have been investigating low-cost and locally available materials, produced from natural sources, which may have high surface areas and a high sorption capacity to remove organic pollutants from contaminated waters (Clausen et al., 2001; Abate and Masini, 2005; Sanchez-Martin et al., 2006).

Clays are soil components and therefore are widely available materials whose sorption qualities for organic compounds such as pesticides have already been studied to some extent (Fushiwaki and Urano, 2001; Davies and Jabeen, 2002; Li et al., 2003; Sanchez-Martin et al., 2006). Commercially available clay materials such as sepiolite, vermiculite and light expanded clay aggregates (LECA) are thus being increasingly considered as alternative low-cost adsorbents for organic pollutants (Abate and Masini, 2005; Sanchez-Martin et al., 2006; Dordio et al., 2007). Sepiolite, which forms an important group of clay minerals, is an abundant natural, hydrated magnesium silicate, with a fibrous texture, which is currently being used in many industrial, catalytic and environmental applications. Several studies were done using sepiolite as a catalyst support, in wastewater and solid wastes treatment, and in reducing the toxic effect of some heavy metals and pesticides (Gonzalez-Pradas et al., 1999; Rytwo et al., 2002; Sanchez-Martin et al., 2006). Vermiculite is a group of hydrated laminar minerals which are aluminium–iron–magnesium silicates resembling mica in appearance. Vermiculite has several different applications, but has been used mostly in agriculture and for insulation purposes. Recently, several studies have been carried out on the use of vermiculite, usually after chemical modification, as an adsorbent of organic compounds from water (da Silva et al., 2003; Abate and Masini, 2005; Mysore et al., 2005). LECA is an artificially modified natural material that is produced by subjecting clay materials to a high temperature treatment, causing injected CO_2 to expand within the clay aggregates and thus creating a highly porous, lightweight material. LECA is mainly used for construction purposes but over the last years it is also being used for different types of water and wastewater treatment processes such as filtering and in CWs (Zhu et al., 1997; Brix et al., 2001).

Previous studies on the removal of CA from aqueous solution have shown that LECA exhibits some sorption capacity for this compound (Dordio et al., 2007), while other more commonly used media materials such as siliceous sand (Dordio et al., 2007) and granitic gravel (Matamoros et al., 2005; Matamoros and Bayona, 2006; Matamoros et al., 2008) showed almost no capacity to sorb it.

The aim of the present work was to evaluate the sorption capacity of LECA to remove ibuprofen and carbamazepine as well as clofibric acid. In addition to LECA, two other clay materials, sepiolite and vermiculite, were tested for the removal of clofibric acid, which is reported as the most recalcitrant of the three compounds tested. The sorptive properties of LECA were also investigated using treated wastewater doped with a mixture of the three compounds at the same concentrations tested for the aqueous solutions. Physical, chemical and mineralogical characterization of the three clay materials was pursued in order to shed some light into their differences of sorption capacities. Due to the diverse chemical nature of the studied pharmaceuticals, the results of this study are a first step in screening for a material or combination of materials with a potential to be used as media in SSF-CWs designed for the removal of pharmaceuticals from wastewaters.

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2. Materials and methods

2.1. Chemicals

The assays were performed on aqueous solutions and doped wastewater solutions of the following pure pharmaceutical compounds: clofibric acid (CA) (Sigma–Aldrich, 97% purity), ibuprofen (IB) (Sigma–Aldrich, 99.8% purity) and carbamazepine (CB) (Sigma–Aldrich, > 99% purity). Some of the most relevant physical and chemical properties of these substances are listed in Table 1.

2.2. Media tested

In this study three different processed natural materials were tested: LECA with a granulometric grade of 2/4 (MaxitGroup Portugal); sepiolite (ActivPet, Portugal); and exfoliated vermiculite (Aguiar Mattos, Portugal).

2.3. Physical and chemical characterization of the media tested

The commercially available media contain considerable amounts of fine materials which were significantly reduced by washing them with Millipore water (Simplicity® UV, Millipore Corp., France) until no further suspended materials were visible. The washed media were then air dried and used throughout this study.

The particle-size distribution on a weight basis was analyzed in triplicate by the conventional dry-sieving technique (Day, 1965). Grain-size distribution plots were used to estimate d_{10} (effective grain size) and d_{60}, and the uniformity of the particle size distribution (the uniformity coefficient) was calculated as the ratio between d_{60} and d_{10}. The apparent porosity (void space) of the media was determined from the amount of water needed to saturate a known volume of the solid (number of replicates $n=5$) (Brix et al., 2001; del Bubba et al., 2003). Bulk density was determined based on the ratio between the dry weight and the bulk volume of the media ($n=5$) (Brix et al., 2001). Hydraulic conductivity was measured as described in Cooper et al. (1996) ($n=5$).

The microstructural properties of the media were investigated by scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectrometry (EDX) and by optical microscopy.

The optical microscopy observations and the corresponding photographic documentation were obtained by a stereomicroscope Leitz-Wetzeler equipped with a Leica DC500-2002 photo camera at different magnifications.

The SEM micrographs and EDX spectra were carried out using a FEG-SEM JEOL 7001F coupled with an OXFORD EDX spectrometer with Be window and Si(Li) detector. The samples were coated with a thin, conductive film of gold. The current used was 10–25 kV.

The mineralogical composition of the media was studied by X-ray diffraction (XRD) using a Bruker AXS-D8 Advance diffractometer with Cu Kα radiation and a speed of 0.05°/s, from 3 to 75°: 2θ, after grinding the samples so as to pass in a 106 μm sieve.

Finally, to evaluate the acidic properties of the media, the point of zero charge (PZC) was determined using the mass titration method (Noh and Schwarz, 1989; Zalac and Kallay, 1992).

Table 1 – Physical and chemical properties of clofibric acid, iburofen and carbamazepine

Common name	Clofibric acid (CA)	Ibuprofen (IB)	Carbamazepine (CB)
IUPAC name	2-(4-Chlorophenoxy)-2-methylpropanoic acid	2-[4-(2-Methylpropyl)phenyl] propanoic acid	Benzo[b][1]benzazepine-11-carboxamide
CAS number	882-09-7	15687-27-1, 31121-93-4 (sodium salt)	298-46-4
Chemical function	Active metabolite of clofibrates blood lipid regulators	Non-steroidal anti-inflammatory drug (NSAID)	Anticonvulsant
Structure			
Molecular weight ($g\,mol^{-1}$)	214.65	206.28, 228.26 (sodium salt)	236.27
Melting point (°C)	120–122	75–77	191–192
Ionization constant, pKa	2.5[a], 3.18[b]	4.42[c], 4.51[d], 4.9[e]	13.9[e], 14[f]
Water solubility (25 C) ($mg\,L^{-1}$)	582.5[g,h]	21[i], 49[c]	17.7 (estimated)[j], 112[h]
Soil sorption coefficient, $\log K_{oc}$	0.9–1.36[k], 1.88[a]	2.14–2.21[f], 2.19[k]	2.00–3.42[f], 2.24–2.27[k]

Sources: (a) Drillia et al. (2005), (b) Packer et al. (2003), (c) Avdeef et al. (2000), (d) Wan et al. (2002), (e) Jones et al. (2002), (f) Scheytt et al. (2005), (g) Tomlin (1994), (h) Ferrari et al. (2003), (i) Carballa et al. (2005), (j) Meylan et al. (1996), (k) Scheytt et al. (2004).

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2.4. *Pharmaceutical removal assays*

Sorption studies were performed on CA, CB and IB solutions prepared with Millipore water (Simplicity® UV, Millipore Corp., France) at concentrations of 1, 10, 35, and 50 mg L^{-1}. In addition to the studies using single-compound solutions, some studies were also carried out with solutions containing a mixture of the three compounds, which were prepared in order to have each compound at a concentration of 1, 10, 35, or 50 mg L^{-1}.

The sorptive media were previously sterilized and the experiments were conducted in the dark (to avoid photodegradation) and without stirring to provide a better simulation of the hydraulic behavior in a SSF-CW where low flow rates are normally used. Experiments were done in triplicate and at a controlled temperature of 20 °C.

For the determination of the amounts of pharmaceuticals sorbed by the different matrices, aliquots were collected at every 24 h from each batch replicate. The quantification was done using the high-performance liquid chromatography (HPLC) technique as described in Section 2.5.

2.4.1. *Sorption of CA, CB and IB in aqueous solution by LECA*

Eight series of batch assays (four of which used the aqueous solutions of the individual compounds separately, and another four carried out with a mixture of three compounds; each series corresponded to assays on one of the four tested concentrations, 1–50 mg L^{-1}) were set up in 5 L plastic containers filled with LECA 2/4. A solid to liquid ratio of 1.0 kg L^{-1} was used in each assay, corresponding to a flooding rate close to 100%.

The possible sorption of the compounds onto the containers walls was investigated for all the concentrations tested and using the same assay set up but without the sorptive medium. This effect was found to be negligible both for plastic as well as for glass vessels.

2.4.2. *Sorption of CA, CB, IB spiked in treated wastewater by LECA*

Four series of batch assays (each series, consisting of four assays corresponding to the mixture of the three compounds at the four studied concentrations) were conducted to study the efficiency of LECA in removing mixtures of CA, IB and CB spiked in treated wastewater at the different tested concentrations (1–50 mg L^{-1}). The wastewater used in these assays was collected at a secondary treatment stage in a wastewater treatment plant (WWTP) serving a small rural community population of *ca.* 400 inhabitants. The treatment processes used in this WWTP include screening, primary sedimentation and conventional activated sludge treatment.

The collected effluent was characterized by the determination of the following wastewater quality parameters, according to the APHA-AWWA-WPCF methods (Clescerl et al., 1998): total suspended solids (TSS), pH and total and soluble chemical oxygen demand (COD_t and COD_s) of samples filtered through 0.2 μm.

2.4.3. *Sorption of CA by sepiolite and vermiculite*

Two series of independent batch assays (each consisting of four assays testing each of the four concentrations studied) were set up in 5 L plastic vessels containing either sepiolite or vermiculite and filled with clofibric acid aqueous solution (1–50 mg L^{-1}) with a solid to liquid ratio of, respectively, 1.25 kg L^{-1} and 0.8 kg L^{-1} which correspond in both the cases to a flooding rate close to 100%.

2.5. *Quantification of CA, CB and IB by HPLC-DAD*

Samples were collected every 24 h for the full duration of the assays. These samples were filtered through 0.45 μm PTFE filters (Macherey-Nagel, Germany) and analyzed using HPLC (Elite LaChrom, Hitachi, Japan) with UV detection at 210, 222 and 227 nm for CB, IB and CA, respectively. The analytical column used was a reversed-phase Zorbax Eclipse XDB-C18 with 5 μm particle sizes (Agilent Technologies, Germany), and the mobile phase was a mixture of water:acetonitrile. For the single-pharmaceutical solutions the HPLC analyses were performed in isocratic mode whereas for the solutions containing the mixed three pharmaceuticals the separation was performed using a gradient program. In any case the water was acidified with 0.1% (v/v) phosphoric acid. The flow rate was 1.0 mL min^{-1} and the injection volume was 20 μL. Five replicate injections were made for each sample.

Calibration curves were derived for standard solutions of CB, IB and CA individually, as well as with solutions containing the three mixed compounds. Standard solutions of 100 mg L^{-1} were used to prepare standards between 0.5 and 60 mg L^{-1} of each compound. Three replicates were made for each standard solution and each solution was injected five times. The average areas of the compounds' peaks were plotted against the standards concentrations resulting in linear correlations with R^2 equal to or higher than 0.999 in every calibration curve.

Whenever the measured concentrations were below the limits of quantification of 0.27 mg L^{-1}, 0.39 mg L^{-1} and 0.13 mg L^{-1} for CB, IB and CA, respectively (LOQ, calculated as the blank signal plus 10 standard deviations of the blank, according to Miller and Miller, 2000), the samples were pre-concentrated on LiChrolut® RP-18 (40–63 μm) 3 mL (500 mg) standard PP-tubes (Merck, Germany). Recovery assays were done using an aqueous solution spiked with the individual analytes and their mixture at a concentration of 2 mg L^{-1} using the same procedure applied to the samples. Recoveries above 90% were obtained for all the individual compounds and their mixture.

2.6. *Statistical analysis*

Data were analyzed by the analysis of variance method (ANOVA, single factor) at different significance levels.

3. Results and discussion

3.1. *Physical and chemical properties of the media*

Relevant physical and chemical properties which were determined experimentally for the media used in this work are presented in Table 2.

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Table 2 – Physical and chemical characteristics of LECA 2/4, vermiculite and sepiolite

Media	LECA 2/4	Vermiculite	Sepiolite
d_{10} (mm)	3.00	1.22	1.40
d_{60} (mm)	3.95	2.88	3.10
Uniformity coefficient (U)	1.32	2.36	2.21
Apparent porosity/void space (%)	46 ± 1	69 ± 1	67 ± 1
Bulk density (kg m^{-3})	486 ± 7	132 ± 2	563 ± 9
Ks ($m^3\,m^{-2}\,s^{-1} \times 10^{-3}$)	7.7	nd	nd
pH (in water)	9.93 ± 0.02	7.31 ± 0.07	8.36 ± 0.02
PZC	10.54 ± 0.03	7.54 ± 0.09	8.35 ± 0.06
Electrical conductivity at 20 °C (mS cm^{-1})	537 ± 15	355 ± 4	369 ± 5

The values for d_{10}, d_{60} and uniformity coefficient (d_{60}/d_{10}) are means of triplicate analyses. The values for apparent porosity, bulk density and hydraulic conductivity (Ks) are means ± 1 S.D. (n = 5). nd – Not determined.

All the materials tested have comparable particle size distributions (Fig. 1). LECA 2/4 has the most uniform distribution (Table 2), with most of its particles (93.3%) having diameters within 2.83–5.00 mm. Vermiculite and sepiolite have slightly wider particle size distributions, extending especially towards the finer particles, with respectively 93.8% and 98.0% of the particles having diameters within the 0.85–5.00 mm range. Despite these differences, these materials can be considered quite uniform in terms of particle size.

The apparent porosities of all the materials are quite large (Table 2), which may contribute to high hydraulic conductivities. In comparison with the other two tested materials, LECA 2/4 has a somewhat lower porosity but it is, nevertheless, still significantly porous. These high porosities are due not only to interparticle void space but especially to the available space in the materials' internal structures which still are large enough to absorb and accommodate substantial amounts of water (Fig. 2).

The experimental values of the points of zero charge indicate that vermiculite has an essentially neutral surface whereas sepiolite and LECA have an alkaline character, which is more pronounced in the latter material. This may be attributed to the presence of alkaline components such as oxides and carbonates, especially in LECA, as was verified in the media mineralogical characterization. The pH values of water which had been in contact with the media closely follow the trend observed in the PZC of each material and are, in general, in accordance with the values reported in the literature (Mysore et al., 2005; Alkan et al., 2007; Ádám et al., 2007). Sepiolite and LECA surfaces are, therefore, likely to be protonated to some extent under ordinary pH conditions in water (pH = 6–7). However, characteristic pH values of a wastewater are slightly higher (pH = 7–8.5) and, under these conditions, only LECA should be significantly protonated. It should be noted that LECA is a mixture of several minerals, presenting a more complex mineralogical composition than the other two media, and the properties reported in the literature for this material, in particular its acid–base characteristics, are more variable (Drizo et al., 1999; Brix et al., 2001).

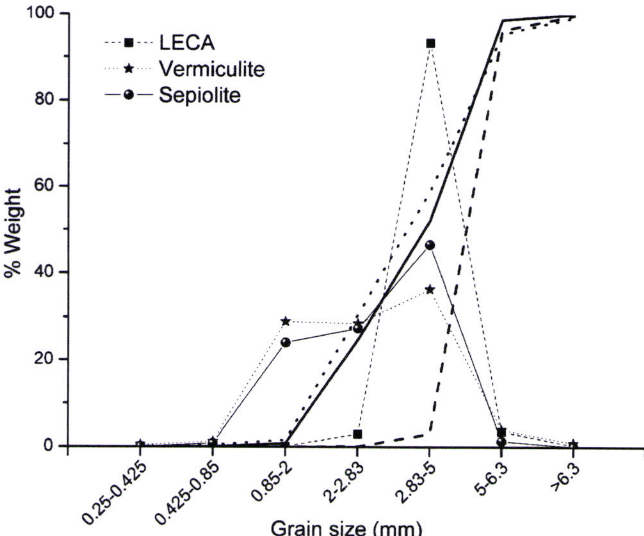

Fig. 1 – Grain size distributions and cumulative curves for LECA 2/4, vermiculite and sepiolite particles.

The micrographs (Fig. 2) show that LECA is an extensively porous material with a highly porous internal structure which extends over a wide range of pore sizes. The external surface exhibits lower roughness than the internal surface which results from the release of gas bubbles during the production of expanded clays.

Observation by SEM-EDX and optical microscopy of vermiculite shows that it is composed of large crystalline plates, which is typical of this type of phyllosilicates. It is also possible to see its exfoliated or expanded laminar structure produced during the industrial heating process of vermiculite (Fig. 2).

SEM observations also show the fibrous nature of sepiolite. It was also possible to see that the sepiolite used is composed of aggregate particles with the typical morphology of fiber bundles which are produced during the grinding operation of sepiolite industrial processing (Fig. 2).

EDX semi-quantitative relative elemental analysis of the support media is shown in Table 3. The results are consistent with the composition of the crystalline phases identified by XRD. The high values for oxygen are due to the fact that this element is present in the oxides and silicates that form these media. The results corroborate that sepiolite is composed mainly by magnesium silicates whereas in the case of vermiculite and LECA other major elements are present, namely Fe, Al and K, revealing their clayish nature. Results also show that LECA particles exhibit a high variability in composition.

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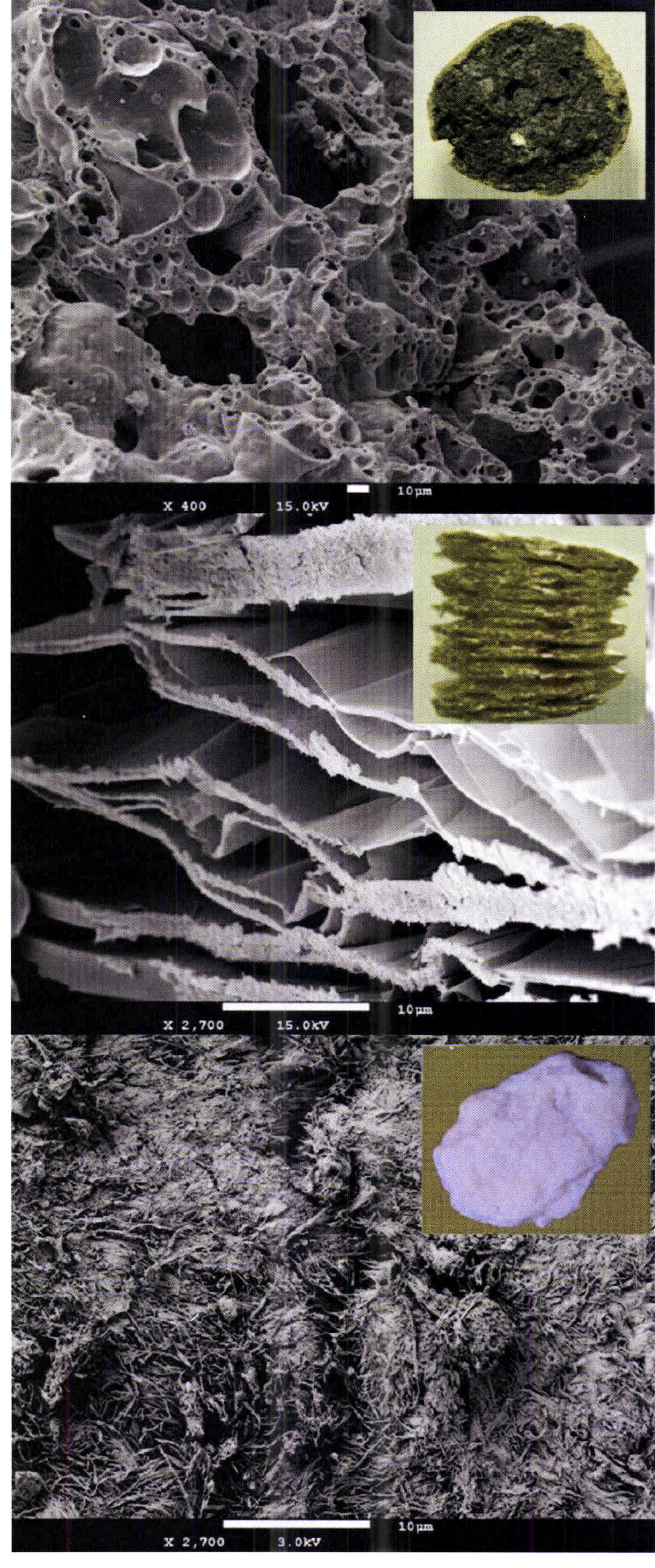

Fig. 2 – Scanning electron micrographs of the clay materials LECA, vermiculite and sepiolite (from top to bottom).

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Table 3 – Semi-quantitative relative elemental composition of the media in weight% estimated by energy dispersive X-ray spectrometry

Element	Vermiculite (weight%)	Sepiolite (weight%)	LECA (weight%)
O	24.5	77.7	53.9–39.2
Si	8.4	15.9	19.4–26.8
Mg	9.1	6.4	2.7–3.6
Fe	55.0	–	9.7–11.7
Al	3.0	–	9.8–13.0
K	–	–	4.5–5.7

The X-ray diffraction of LECA shows that the particles are mainly composed of quartz with a relative contribution of hercynite, a high temperature iron aluminate, and low quantities of albite and calcite. In addition, significant amounts of amorphous materials are observed in the diffractogram. This more heterogeneous mineralogical composition, resulting from the industrial processing of the material, is also reflected in the range of values observed in the elemental analysis by SEM-EDX (Table 3). This contrasts with the more precise values of the other two materials, which due to their crystalline nature, present a much more uniform elemental composition.

The sepiolite sample shows a typical XRD powder diffractogram with a characteristic peak at d_{001} = 12.2 Å, corresponding to the interlayer distance in the sepiolite structure. The sample also has quartz and traces of mica.

Vermiculite is a phyllosilicate composed of large crystalline plates. The XRD pattern shows that this sample is composed mainly of vermiculite with small quantities of biotite and phlogopite which is in agreement with the fact that vermiculite is formed by alteration of these minerals.

3.2. *Removal efficiency of pharmaceuticals by LECA*

The capacity of LECA to remove each of the three pharmaceuticals studied (CB, IB and CA) was tested initially in the ideal conditions of the three compounds dissolved individually in water and, subsequently, in more realistic assays using mixtures of the three compounds in water and in treated wastewater.

3.2.1. *Individual compounds in water*

In the assays using the single-compound aqueous solutions (Fig. 3a) LECA displays a great affinity for CB and IB, while more modest removals are attained for CA. CB is the compound sorbed in greater extent with removals of 60.1–95.1% for initial concentrations in the range of 1–50 mg L^{-1}. IB is also extensively sorbed (removals of 54.5–92.5%) whereas CA is the least sorbed of the three compounds (removals of 30.6–58.1%).

It is noticeable that % removal decreases with increasing load. Thus, higher % removals are obtained for lower initial concentrations. Despite the lower % removals at higher concentrations, the actual absolute amounts keep increasing with increasing initial concentrations. In fact, there seems to be a linear relationship between the sorbed amount and the initial concentration for the concentration range tested (Fig. 4). For the heavier initial loads of 50 mg L^{-1}, LECA sorption capacity is not yet exhausted for any of the compounds as the sorbed amounts continue to be in an increasing trend.

When comparing the amounts sorbed by LECA, the following order is consistently observed for all initial concentrations: CB > IB > CA.

Both IB and CA are carboxylic acids with pK_a values in the range of 3–5 and are therefore anionic under the pH conditions of the experiments (pH = 8–9). Electrostatic interactions with the surface of LECA, which is protonated under the same pH conditions, are expected to play a significant role in the sorptive process and favor the removal of these compounds from water. Higher affinities of positively charged surfaces for anionic organic compounds have also been reported in other

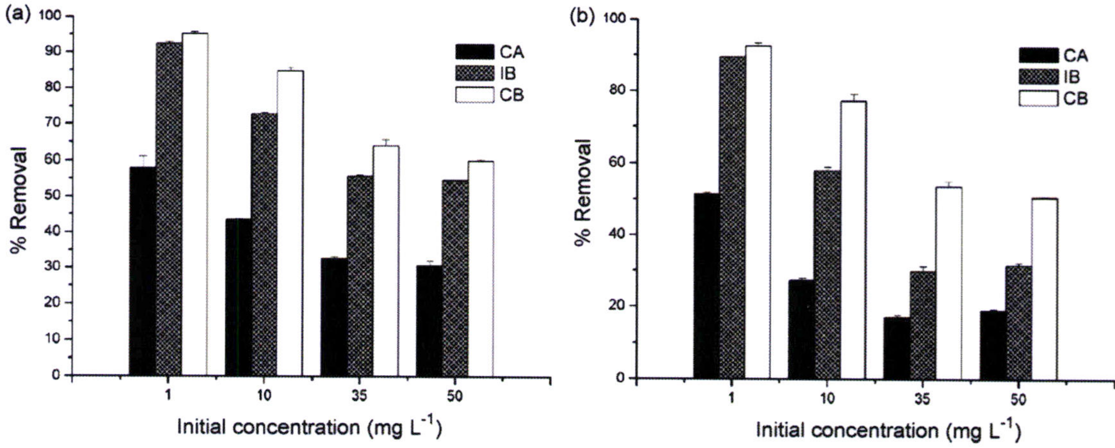

Fig. 3 – Removal efficiencies of CA, IB and CB by LECA for each initial concentration after 144 h of contact time in (a) assays of single-compounds in water and (b) assays of mixed-compounds in wastewater. Vertical bars represent the averages of three replicates and error bars represent the range of ±1 S.D. All removal efficiencies are ANOVA significantly different at P < 0.0001 within each initial concentration class.

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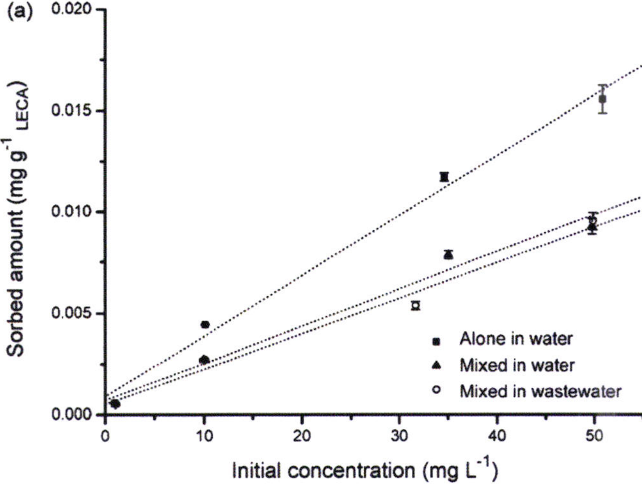

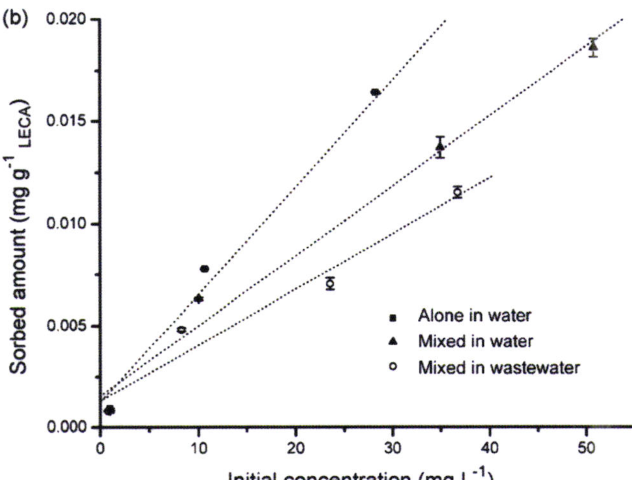

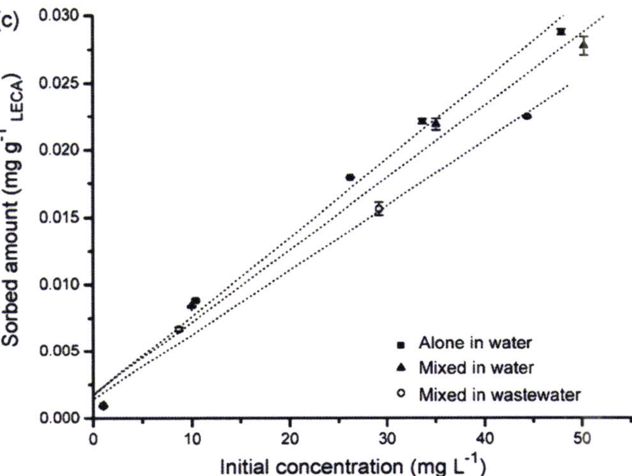

Fig. 4 – Total removed amounts of (a) CA, (b) IB and (c) CB after 144 h of contact time for each initial concentration in water and wastewater. Points represent the averages of three replicates and error bars represent the range of ±1 S.D. Lines are linear fits to the data (R^2 > 0.96).

studies on the adsorption properties of various clay materials (Inacio et al., 2001; Ozcan et al., 2004; Alkan et al., 2004; Sanchez-Martin et al., 2006) and, as expected, have shown to be pH-dependent. CA exhibits a lower affinity for LECA than IB, which may be due to the much stronger affinity of this compound for the aqueous medium (higher solubility) and consequently a decreased distribution into the solid matrix.

CB is a neutral compound and, consequently, any surface charge on LECA should not have a significant influence in the sorption of this compound. It is therefore expected that the strong sorption observed should correspond simply to a physisorption process driven by van der Waals interactions. The CB molecule has a quite large polarizability due to its extensively conjugated aromatic system of two benzene rings linked by an also conjugated 7-side ring. This characteristic should be responsible for very strong van der Waals interactions with the medium. In addition, the lower water solubility of CB should also favor the distribution of this compound into the solid medium.

The observed order in the sorption strengths of the three pharmaceuticals onto LECA follows the trend in soil sorption coefficients (Table 1) and has also been observed in other studies of sorption onto sediments (Scheytt et al., 2005).

3.2.2. *Mixtures of the three compounds in water and treated wastewater*

In order to assess possible efficiency losses arising under less than ideal conditions, the behavior of the isolated pharmaceuticals in solution was compared to that of the compounds mixed in an aqueous solution as well as in a more realistic and complex liquid matrix such as a treated wastewater, for which some of the characteristics are presented in Table 4.

A slight loss of efficiency of LECA is observed for the removal of the pharmaceuticals from wastewater (Fig. 3b) when compared with the ideal conditions of the individual solutions of each compound in water (Fig. 3a). Still significant removal efficiencies are observed for CB (50.6–92.6%) and IB (31.4–89.5%), especially for the lower concentrations, but for CA (19.1–51.4%), unfortunately, the observed removal efficiencies are somewhat poor.

The total amounts of pharmaceuticals removed are presented in Fig. 4 along with the single-compound solutions for comparison. A decrease in the sorbed amounts of CA and IB is observed when all compounds are present in the same aqueous solution which is probably due to a competitive sorption effect. This decrease is more pronounced for the 50 mg L^{-1} initial concentrations. In contrast with these two compounds, the sorbed amounts of CB are less affected probably because its greater affinity for LECA is less disturbed by the presence of the other two compounds.

Table 4 – Physical and chemical properties of the treated wastewater used in the assays (*n* = 4)

Parameters	Treated wastewater
pH	8.29 ± 0.05
TSS (mg L^{-1})	57 ± 3
COD_t (mg L^{-1})	133 ± 2
COD_s (mg L^{-1})	82 ± 2

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When the three compounds are all simultaneously present in a treated wastewater (Fig. 4), CA's sorption seems to be only marginally affected. However, a more significant decrease is observed in the sorbed amounts of CB and IB possibly because the dissolved organic matter of the wastewater slightly increases the compounds solubility in this medium, thus making the distribution into the solid compartment less favorable. The LECA's contents in calcite and its high PZC may favor the competitive sorption of inorganic ions present in the wastewater, such as phosphates (Johansson, 1997; Brix et al., 2001; Öövel et al., 2007) which, added to the sorption of other soluble organic compounds, may be an additional cause of the pharmaceuticals' decreased sorption. However, the less pronounced decrease in CA sorption in comparison with CB's and IB's suggests that, in the present study, differences of solubilities in the wastewater may be an essential cause for this behavior. Despite the decrease in efficiency, still substantial amounts of these compounds are sorbed in the medium, even when these more realistic conditions are used.

It should be noted that the positive charge of some surfaces may be altered over time by the development of biofilms after some time of operation of the beds. This might cause a net negative charge to build up on the media surfaces, which may lead to a lower retention of the anionic acidic compounds, as shown by Carlson and Silverstein (1998).

3.3. *Sorption models*

Some insight into the sorption processes can be gained by exploring how the equilibrium concentrations in solution, C_e, and corresponding sorbed amounts, n_{sorb}, fit some of the most popular model isotherms equations for sorption from liquid phase, namely the Langmuir equation (Langmuir, 1918):

$$\frac{C_e}{n_{sorb}} = \frac{1}{K_L n_m} + \frac{C_e}{n_m}$$

where K_L is the Langmuir constant, which relates to the energy of sorption, and n_m is the monolayer capacity, i.e. the maximum amount of sorbed compound, and the Freundlich equation (Freundlich, 1926):

$$n_{sorb} = K_F C_e^{1/n}$$

where K_F is the Freundlich constant, which relates to the extent of sorption, and $1/n$ is the Freundlich exponent, which determines the concavity of the isotherm and in the special case of $1/n = 1$ corresponds to a partition equilibrium in which case K_F is the Nernst partition coefficient K_d.

The results of the fits are presented in Table 5. In general, the Freundlich equation models the experimental data very well whereas the fit of the Langmuir equation is not so good.

The Langmuir model assumes an energetically homogenous surface and the absence of lateral interactions between sorbed molecules. The Freundlich equation has an empirical origin, but can be derived theoretically by considering an exponential variation of the sorption enthalpy with surface coverage. In fact, the Freundlich equation can be depicted as a summation of a distribution of Langmuir isotherms and hence it is able to account for the heterogeneity of the sorption process. In the present case, the best fit to the Freundlich equation reflects the heterogeneity of the sorbent material as well as possible lateral interactions between the sorbed molecules.

3.4. *Kinetic behavior*

The effect of contact time in the removal of CA, IB and CB from single-compound solutions onto LECA is shown in Fig. 5 for the lower and upper limit concentrations of the tests (1 and 50 mg L^{-1}). The qualitative features of the kinetic behavior of the pharmaceuticals removal by LECA in water do not seem to be much affected by the initial concentrations of the compounds. The kinetics are characterized by an initial fast step that should be mostly due to adsorption over the LECA's surface through which most of compound is removed within the first 24 h. Subsequently, a slower process is responsible for additional compound removal which stabilizes at equilibrium concentrations after around 72–96 h of contact time. Although the removal rates are obviously different for the three compounds, equilibrium is attained for all of them essentially within the same period. Additionally, it should be noted that

Table 5 – Fits of experimental sorption data to the Freundlich and Langmuir equations

Pharmaceutical	Freundlich			Langmuir		
	K_F	$1/n$	R^2	K_L	n_m	R^2
Alone in water						
CB	0.005393	0.5652	0.9999	0.2127	0.03276	0.9110
CA	0.001161	0.7398	0.9962	0.05297	0.02269	0.9599
IB	0.004129	0.5789	0.9994	0.1777	0.02883	0.8903
Mixed in water						
CB	0.005223	0.5681	0.9892	0.3013	0.03046	0.9787
CA	0.0007569	0.6845	0.9977	0.04776	0.01380	0.8946
IB	0.002312	0.5911	0.9997	0.1073	0.02230	0.9089
Mixed in wastewater						
CB	0.004223	0.5721	0.9912	0.2163	0.02680	0.8344
CA	0.0007997	0.6275	0.9910	0.05177	0.01185	0.7512
IB	0.002346	0.4636	0.9837	0.2455	0.01145	0.8589

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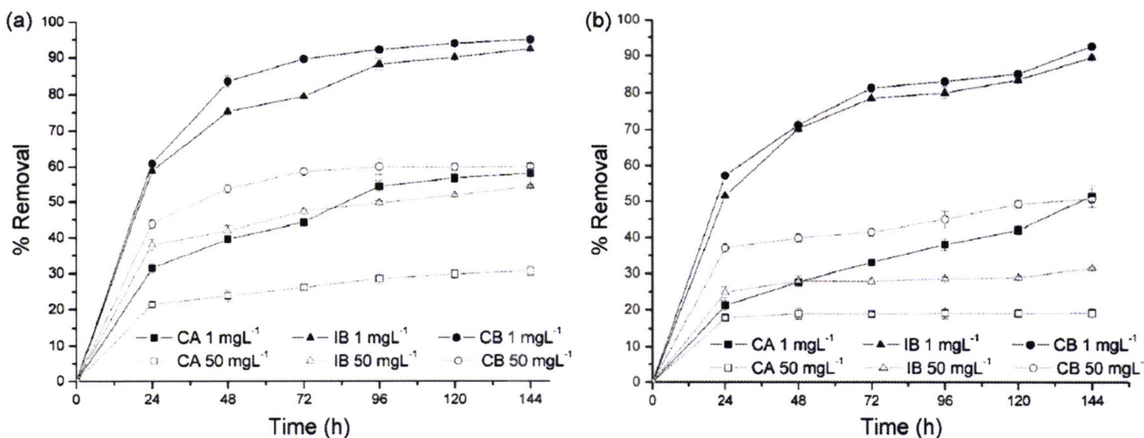

Fig. 5 – Effect of contact time and initial concentrations on CA, IB and CB removal efficiencies of (a) the single compounds in water and (b) the compounds mixed in the wastewater. The points represent the averages of 3 replicates and error bars represent the range of ±1 S.D.

the two anionic compounds, CA and IB, show a behavior which is qualitatively similar (especially evident in the 1 mg L^{-1} initial concentration assay) and differs from the behavior of the neutral compound CB. This observation suggests that the two classes of compounds are sorbed *via* two different mechanisms. The small step in the 72–96 h time window of the anionic compounds kinetics reveals a slower process contributing additionally to the sorption of these compounds, possibly an anion exchange process or molecule rearrangement over the LECA's surface.

Changing the conditions of the assays to the other two tested conditions, i.e. mixtures in water (data not show) and in wastewater, does not appreciably affect the kinetics in any of the cases. Still most of the compounds' removal occurs within the first 24 h, and equilibrium is attained within the same period of contact time (until 96 h).

The first 24 h-period seems, therefore, to be the most important stage of the whole process and, although the initial aim of the present work was to study the removal processes in a longer time frame, a better characterization of these first 24 h would probably prove useful.

3.5. *Removal of clofibric acid in water by the three different clay materials*

In face of the less satisfactory results obtained with LECA for the removal of CA, two other clay materials, namely vermiculite and sepiolite, were tested, attempting to increase the removal efficiency of this recalcitrant compound from water. Physical and chemical characterization of those materials was presented before (see Section 3.1).

As can be seen in Fig. 6, removal of CA by vermiculite is very significant. In addition to the large amounts removed, the kinetics is also very fast, with essentially all of the CA removal by vermiculite occurring only within the first 24 h. This may be indicative that sorption of CA on vermiculite is exclusively a surface effect, which is consistent with the laminar structure of this clay, making most of its large surface area readily available.

In contrast with vermiculite, sepiolite is slightly less efficient than LECA. This may be related with the differences in the chemical properties of these two materials. The greater acid–base activity of LECA's surface, which is evident by a

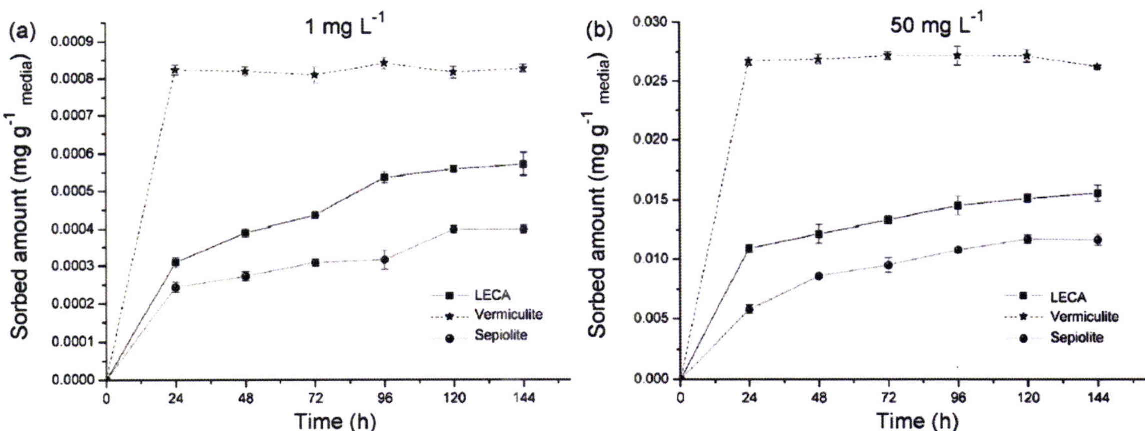

Fig. 6 – Removed amounts of CA by each of the media, for initial concentrations of (a) 1 mg L^{-1} and (b) 50 mg L^{-1}. The points represent the averages of 3 replicates and error bars represent the range of ±1 S.D. Removed amounts after 144 h of contact time are ANOVA significantly different at $P < 0.0001$.

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higher point of zero charge, makes it appropriate for the development of a positively charged surface at lower pH values under assay conditions and therefore leads to enhanced electrostatic interactions with the negatively charged CA anions.

While vermiculite did exhibit a much higher efficiency than LECA, its mechanical characteristics may present some operative problems. In fact, the flakes of exfoliated vermiculite easily fracture into smaller particles and adhere to other materials. In addition, due to its low density, vermiculite flakes tend to float over the water surface. LECA, on the other hand, is becoming an increasingly popular alternative as media of SSF-constructed wetland systems for the removal of other types of pollutants. All these issues may influence the final decision about the media selection.

4. Conclusion

The present laboratory studies illustrate the good sorption properties of LECA for the removal of three pharmaceuticals from aqueous solutions. In all the tested conditions (aqueous solutions of the single compounds, aqueous solutions of mixtures of the three compounds and treated wastewater doped with the three compounds) the amounts removed are ranked as follows: carbamazepine > ibuprofen > clofibric acid.

There is some loss of removal efficiency in the more complex conditions: the sorbed amounts of ibuprofen and clofibric acid are reduced when the three compounds are mixed in water, probably due to the competitive sorption of carbamazepine; in the wastewater, the sorbed amounts of carbamazepine and ibuprofen are also reduced, probably due to the increased solubility promoted by the wastewater's dissolved organic matter.

The first 24 h-period seems to be the most important stage of the whole sorption process. Equilibrium is attained, in general, after approximately 72–96 h, and the kinetic behavior seems to be similar in all tested conditions.

The assays of clofibric acid sorption by two other clay materials revealed that vermiculite presents higher removal efficiency than LECA whereas sepiolite is slightly less efficient.

In summary, in these laboratory studies LECA presented important advantages for the removal of certain pharmaceuticals from water. The results obtained with vermiculite show, however, that there is some room for improvement and other materials with sorption qualities similar to vermiculite but with better mechanical properties may be available either as a stand-alone alternative or to create composite media with LECA.

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Chapter 3

3. Studies for evaluation of phytotoxicity and removal capacity of selected pharmaceuticals by *Typha* spp.

Article 1

Title: Toxicity and removal efficiency of pharmaceutical metabolite clofibric acid by *Typha* spp. – Potential use for phytoremediation?

Authors: Ana V. Dordio, Cátia Duarte, Margarida Barreiros, A.J. Palace Carvalho, A.P. Pinto, Cristina Teixeira da Costa

Published by: Bioresource Technology (2009, Vol.100, Issue 3, pp.1156-1161)

Article 2

Title: Potential of *Typha* spp. for the phytotreatment of water contaminated with Ibuprofen

Authors: Ana Dordio, Raquel Ferro, Dora Teixeira, A.J. Palace Carvalho, A. P. Pinto, Cristina Barrocas Dias

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Motivations

Constructed wetlands systems (CWS) take advantage of the ability of plants to adsorb, uptake, translocate and detoxify or volatilize organic compounds which contaminate the aquatic environment, as well as to release root exudates that can either enhance compound transformation rates or stimulate microbial degradation processes in rhizosphere. However, the effectiveness of a plant species for phytoremediation technologies depends on its contaminant tolerance and on its removal/degradation capacity.

The main purpose of the work presented in this chapter is to evaluate the ability of *Typha* spp. to tolerate and remove some pharmaceuticals (clofibric acid, ibuprofen and carbamazepine). The assays were conducted exposing *Typha* specimens to different concentrations of the pharmaceuticals under hydroponic conditions in a plant growth chamber.

Physiological parameters monitored in the evaluation of the pharmaceuticals phytotoxicity included photosynthetic pigments contents (chlorophyll and carotenoids) and relative growth rates. In order to evaluate the enzymatic response to the oxidative stress conditions that may have been induced by the pharmaceuticals, the alterations of the following antioxidant enzymes activities were studied: superoxide dismutase, catalase, and two peroxidases, namely, guaiacol peroxidase and ascorbate peroxidase. These are key enzymes in the first line of defense against the oxidative burst induced by the presence of organic xenobiotics. In the case of clofibric acid, the non-enzymatic anti-oxidant response was also assessed by quantification of phenolic compounds contents in the plants.

The pharmaceuticals uptake capacity of the plants was assessed by determination of the decrease of pharmaceuticals concentrations in the spiked nutrient solution.

In order to shed some light into the fate and metabolism of one of the studied pharmaceuticals, carbamazepine, in plant tissues, this compound was quantified in leaf tissues of *Typha* spp. plants exposed to spiked nutrient solutions. In addition to the parent compound, carbamazepine, an attempt was made to also identify some of its metabolites and conjugation products (Appendix D).

Two analytical methods were developed and optimized to allow the quantification of studied pharmaceuticals in aqueous samples and one of them (carbamazepine) also in plant tissues. The method used for quantification of the studied pharmaceuticals (carbamazepine, clofibric acid and ibuprofen) in spiked nutrient solutions included the following steps: isolation and pre-concentration with solid phase extraction (whenever necessary), separation by liquid chromatography (HPLC) and detection with UV/Vis spectrometry.

The method used for quantification of carbamazepine in plant tissues (leaves) included the following steps: disruption by the sea sand disruption method, separation by liquid chromatography and detection with quadrupole ion trap mass spectrometry.

3.1. Uptake, translocation and detoxification of organic xenobiotics by plants

Plants play an important role in the biotic processes of organics removal in CWS, as described in section 1.5.2, involving numerous processes (see Table 1.5, page 36) of which many details still remain to be known or fully understood. Subjected to the direct or indirect action of plants, xenobiotics can be stabilized or degraded in the rhizosphere, adsorbed or accumulated in the roots and transported to the aerial parts, volatilized or degraded inside the plant tissues (Figure 3.1).

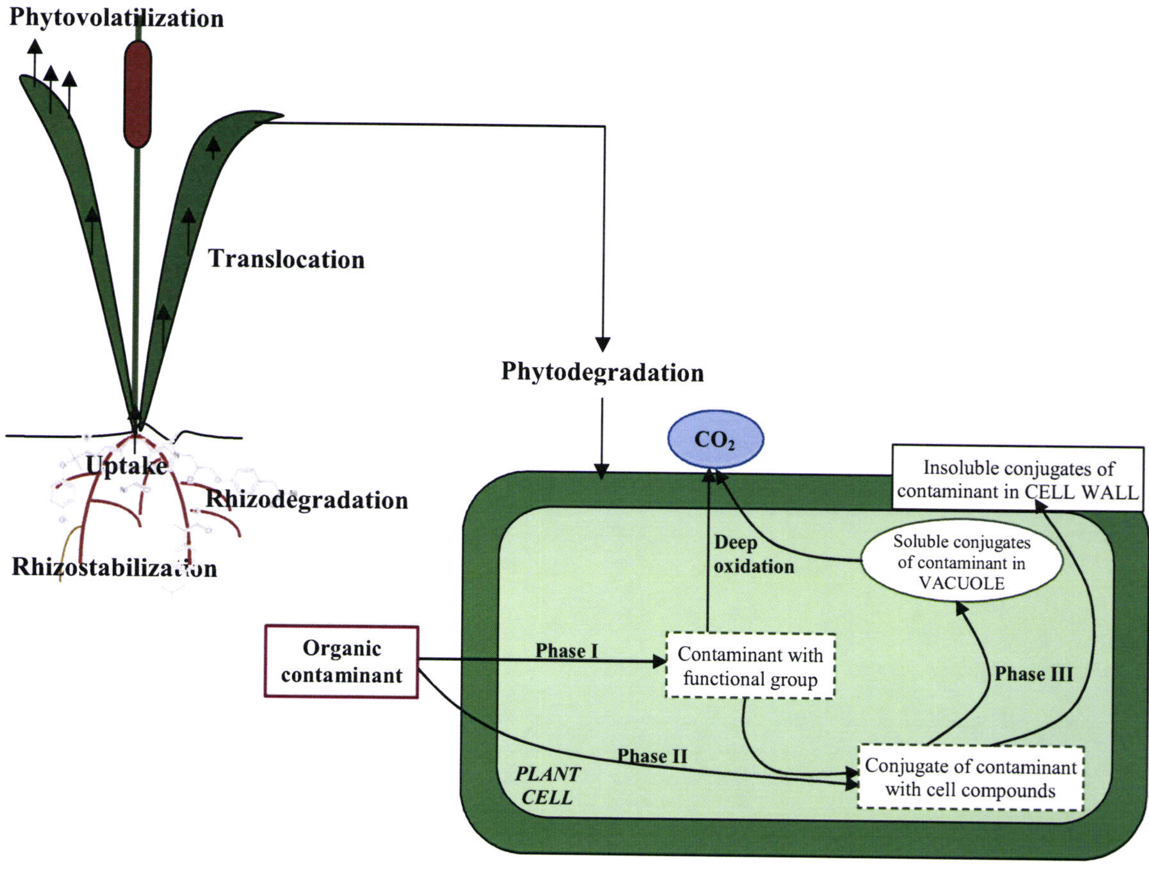

Figure 3.1. Major removal processes and transformation pathways of organic xenobiotics in plants (adapted from Kvesitadze et al. (2006) and Abhilash et al. (2009)).

Many organic pollutants can be readily taken up by plants but, as consequence of being xenobiotic, there are no specific transporters for these compounds in the plant membranes. Therefore, they move into and within plant tissues via diffusion (passive uptake) through the cell walls (Dietz and Schnoor, 2001; Pilon-Smits, 2005; Collins et al., 2006). The flux is driven by the water potential gradient created throughout the plant during transpiration, which depends on the plants characteristics and the CWS

during transpiration, which depends on the plants characteristics and the CWS environmental conditions. Translocation of the compounds is highly dependent on their concentrations and physico-chemical properties such as water solubility, log K_{ow} and pKa (Korte et al., 2000; USEPA, 2000b; Alkorta and Garbisu, 2001).

An optimal hydrophobicity may exist such that the organic compound has a tendency to bind to the lipid bilayer of the membrane but not too strongly so that transport can still occur. Direct uptake by roots is usually an efficient removal mechanism for moderately hydrophobic organic chemicals (log K_{ow} = 0.5 – 3.5) (USEPA, 2000b; Dietz and Schnoor, 2001; Pilon-Smits, 2005). In general, hydrophobic chemicals (log K_{ow} > 3.5) are bound so strongly to the lipophilic root solids and cell walls that they cannot easily enter and be translocated in the plant. On the other hand, chemicals that are quite water soluble (log K_{ow} < 0.5) are not sufficiently sorbed to roots nor effectively transported through the lipid layer of plant membranes. Some studies, however, indicate that the log K_{ow} value of a chemical may not be the sole factor determining its tendency to be taken up and some compounds have been shown to be able to penetrate plant membranes despite a low log K_{ow} (Renner, 2002). The capacity of a compound to be removed from water by a given plant, may also depend on other factors such as initial pollutant concentration, the anatomy and the root system of the plant (Chaudhry et al., 2002). In addition, very hydrophobic chemicals (log K_{ow} > 3.5) are also candidates for phytostabilization and/or rhizosphere bioremediation by virtue of their long residence times in the root zone (USEPA, 2000b; Dietz and Schnoor, 2001; Pilon-Smits, 2005).

Uptake has primary control over translocation, metabolism and phytotoxic action because the total amount of xenobiotic available for these processes is determined by the amount of compound absorbed by the plant. Metabolism influences both xenobiotic uptake and their phytotoxic action by either rendering the compound less or more active (Dietz and Schnoor, 2001; Kvesitadze et al., 2006).

3.1.1. What happens to organic xenobiotics once taken up by the plant?

Organic xenobiotics taken up by roots are translocated into different organs of the plants as a result of the physiological processes involved in the transport of nutrients. The main forces involved in this transport are related to the transpiration stream, i.e. transport of water and dissolved substances from roots to shoots, passing through vessels and tracheids located in the xylem (Kvesitadze et al., 2006).

The importance of the transpiration stream for the uptake and translocation of organics by plants is expressed in the following equation (Briggs et al., 1983; Dietz and Schnoor, 2001):

$$U = (TSCF)\,(T)\,(C) \qquad (3.1)$$

where U is the rate of organic compound assimilation (mg day^{-1}); T, the rate of plant transpiration, (L day^{-1}); C, the organic compound concentration in the water phase (mg L^{-1}); $TSCF$, the transpiration stream concentration factor, is a dimensionless ratio between the concentration of the organic compound in the liquid of the transpiration stream (xylem sap) and the bulk concentration in the root zone solution (Dietz and Schnoor, 2001; Doucette et al., 2005; Kvesitadze et al., 2006).

The *TSCF* has been extensively used in modeling uptake and translocation of organic compounds in plants. With the possible exception of some hormone-like chemicals such as the phenoxy acid herbicides, there is no evidence of active uptake ($TSCF > 1$) of xenobiotic organic chemicals (Doucette et al., 2005). A chemical is said to be excluded ($TSCF < 1$) when uptake is not directly proportional to water uptake ($TSCF = 1$), although the mechanism of uptake is still thought to be a passive process. However, factors such as membrane permeability and xylem sap solubility of the contaminant may limit the extent or kinetics of passive uptake (Doucette et al., 2005). Sorption and rapid metabolism of contaminants within the plant may also reduce xylem concentrations and keep measured *TSCF* values from reaching one (Doucette et al., 2005).

For organic chemicals, several empirical relationships between *TSCF* and the logarithm octanol/water partition coefficient (log K_{ow}) have been reported in which the *TSCF* and the log K_{ow} are related by characteristic bell-shaped gaussian curves (Briggs et al., 1983; Hsu et al., 1990; Sicbaldi et al., 1997; Burken and Schnoor, 1998; de Carvalho et al., 2007; Paraiba, 2007). These, again, suggest an optimal lipophilicity (corresponding to the maxima of the Gaussian curves) for uptake and translocation and infer that compounds which are either highly polar (log $K_{ow} < 0.5$) or are highly lipophilic (log $K_{ow} > 3.5$) will not be significantly taken up by plants. However, laboratory and field experiments with some xenobiotics such as 1,4-dioxane, MTBE, sulfolane and diisopropanolamine also suggest that these predictive schemes may not be applicable for some non-ionizable, highly water soluble organics (Aitchison et al., 2000; Rubin and Ramaswami, 2001; Groom et al., 2002; Doucette et al., 2005; Chard et al., 2006).

3.1.2. Plant detoxification processes

Organic xenobiotics which penetrate the plant cells are exposed to plant's metabolic transformations that may lead to their partial or complete degradation or through which they may be transformed in less toxic compounds and bound in plant tissues (Korte et al., 2000; Kvesitadze et al., 2006).

Metabolism of foreign compounds in plant systems is generally considered to be a "detoxification" process that is similar to the metabolism of xenobiotic compounds in humans, hence the name "green liver" (Sandermann, 1994). Once an organic xenobiotic is taken up and translocated, it undergoes one or several phases of metabolic transformation, as is illustrated by the diagram in Figure 3.1.

Three possible phases of metabolic transformation of organic compounds in higher plants can be identified (Sandermann, 1994):

- ***Phase I – Functionalization:*** involves a conversion/activation (oxidation, reduction and hydrolysis) of lipophilic xenobiotic compounds (Komives and Gullner, 2005; Eapen et al., 2007); in this phase the molecules of the hydrophobic compound acquire a hydrophilic functional group (e.g. hydroxyl, amine, carboxyl, sulphydryl) through enzymatic transformations. The polarity and water solubility of the compound increase as a result of these processes, which also causes an increased affinity to enzymes catalyzing further transformation (conjugation or deep oxidation (Korte et al., 2000; Kvesitadze et al., 2006)) by the addition or exposure of the appropriate functional groups. In the case of a low concentration, oxidative degradation of some xenobiotics to common metabolites of the cell and CO_2 may take place. Following this pathway, a plant cell not only detoxifies the compound but also assimilates the resulting carbon atoms for cell needs. In case of a high concentration, full detoxification is not achieved and the contaminant is exposed to conjugation (Korte et al., 2000).

  During this phase several different groups of enzymes are known to play an important role (Sandermann, 1992; Sandermann, 1994; Macek et al., 2000; Eapen et al., 2007). In plants, oxidative metabolism of the xenobiotics is mediated mainly by cytochrome P450 monooxygenase which is of crucial importance in the oxidative processes to bioactivate the xenobiotics into chemically reactive electrophilic compounds which subsequently form conjugates during Phase II. Peroxidases are another important group of enzymes, which help in the conversion

of some of the xenobiotics and peroxygenases may also be involved in the oxidation of some compounds. Nitroreductase is needed for the degradation of nitroaromatics and laccase for breaking up aromatic ring structures.

Phase I reactions are the first step needed to make a xenobiotic less toxic; those reactions modify the molecule to be ready for Phase II and Phase III reactions which further detoxify the chemical. However, if it already has a functional group suitable for Phase II metabolism, the compound can directly be used for Phase II without entering Phase I.

- ***Phase II – Conjugation***: involves conjugation of xenobiotic metabolites of Phase I (or the xenobiotics themselves when they already contain appropriate functional groups) to endogenous molecules (proteins, peptides, amino acids, organic acids, mono-, oligo- and polysaccharides, pectins, lignin, etc.) (Coleman et al., 1997; Korte et al., 2000; Dietz and Schnoor, 2001; Eapen et al., 2007); as result of conjugation, compounds of higher molecular weight are formed with greatly reduced biological activity and usually reduced mobility. The end products of Phase II are usually less toxic than the original molecules or Phase I derivatives.

  Conjugation is catalyzed by transferases. Enzymes such as glutathione-S-transferases, glucosyl transferase and *N*-malonyl transferases are associated with Phase II (Eapen et al., 2007).

  Conjugation of Phase I metabolites takes place in the cytosol, but it is harmful to accumulate these compounds in cytosol (Eapen et al., 2007).

- ***Phase III – Compartmentalization***: involves modified xenobiotics getting compartmentalized in vacuoles or getting bound to cell wall components such as lignin or hemicellulose (Coleman et al., 1997; Dietz and Schnoor, 2001; Eapen et al., 2007). In this phase, a potential final step in the non-oxidative utilization of xenobiotics, the conjugates are removed from vulnerable sites in cytosol and transported to sites where they may not interfere with cellular metabolism: soluble conjugates (with peptides, sugars, amino acids, etc.) are accumulated in vacuoles, whereas insoluble conjugates (coupled with pectin, lignin, xylan and other polysaccharide) are taken out of the cell and accumulated in plant cell walls (Sandermann, 1987; Sandermann, 1992; Sandermann, 1994; Eapen et al., 2007).

  Phase III reactions are unique to plants because they do not excrete xenobiotics as animals do. Plants therefore, need to somehow remove the xenobiotic within their

own system. ATP driven vacuolar transporters are the main enzymes involved in this phase and further processing of conjugates may take place in the vacuolar matrix (Eapen et al., 2007).

It is assumed that Phase III products are no longer toxic; however, this area of xenobiotic fate in plants is poorly understood, especially with reference to the identity of sequestered products and any subsequent fate in herbivores who might consume those plants.

Metabolism of pesticides has already been extensively studied (Casida and Lykken, 1969; Chaudhry et al., 2002; Coleman et al., 2002; Eapen et al., 2007). More recently, the metabolism of non-agricultural xenobiotics such as trichloroethylene (TCE), TNT, glyceroltrinitrate (GTN), PAHs, PCBs and other organic compounds has also been studied (Görge et al., 1994; Salt et al., 1998; Alkorta and Garbisu, 2001; Hannink et al., 2002; Harvey et al., 2002; Eapen et al., 2007). It has been shown that most of these compounds are metabolized, but only a few chemicals appear to be fully mineralized. Studies applied to pharmaceutical substances, however, are very scarce until now (Huber et al., 2009) in spite of the great interest that such data represents for phytoremediation applications.

3.2. Xenobiotic phytotoxicity

All plants have defense mechanisms to protect them from the negative effects of small quantities of foreign compounds. The relative rates of organic xenobiotics uptake, translocation, and metabolism usually determines whether or not these compounds will induce a plant response, which can be inferred from the plant's physiological, biochemical and molecular responses.

Physiological responses such as growth reduction, chlorosis and necrosis of tissues can usually be observed easily. Quantitative parameters can also be evaluated, which can provide an assessment of physiological toxic responses to xenobiotic stress, namely the plant's relative growth rate (RGR) or the photosynthetic pigments concentration in plant tissues. Alteration of these two parameters, have been observed for plants exposed to xenobiotics, sometimes compromising plant viability (Mishra et al., 2006).

The RGR can be calculated according to the equation:

$$RGR = \frac{(\ln W_1 - \ln W_0)}{t_1 - t_0} \qquad (3.1)$$

where W_0 and W_1 are, respectively, the initial and final weights of plants and $(t_1 - t_0)$ is the period during which the plant is exposed to the xenobiotics.

The photosynthetic apparatus is one of the most important targets of stress in plants. Indeed, most of the metabolic responses induced by stress conditions have consequences on the aptitude of the plant to maintain an efficient light energy conversion (Rmiki et al., 1999). Alteration in the chlorophylls (total, *a* and *b*) and carotenoids contents have been reported in plants subjected to stress conditions (Ferrat et al., 2003). In general stressed plants tend to increase their carotenoid content to provide protection against the formation of free oxygen radicals. A decrease in total chlorophyll and in the ratio chlorophyll/carotenoids are often observed (Ferrat et al., 2003). These variations in photosynthetic pigments under exposure to trace metals and organic xenobiotics such as herbicides have been observed for various species (Ferrat et al., 2003).

Biochemical alterations are also induced by the presence of organic xenobiotics which lead to a production of reactive oxygen species (ROS). These chemical species are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of the triplet O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two or three electrons to O_2 to form, respectively, superoxide radicals ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) or O_2^{3-} (that dismutates into water and hydroxyl radicals, $^{\bullet}OH$) (Wojtaszek, 1997; Mittler, 2002; Apel and Hirt, 2004; Smirnoff, 2005). These are usually produced by plants as by-products of various metabolic pathways (such as photosynthesis and respiration) localized in different cellular compartments (predominantly in chloroplasts, mitochondria and peroxisomes) (Apel and Hirt, 2004). Under physiological steady state conditions these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments (Apel and Hirt, 2004). However, their overproduction can be triggered by external stress factors such as xenobiotics exposure. When exposed to xenobiotics, plants activate pathways to metabolize these foreign compounds which produce large amounts of ROS and may perturb the normal equilibrium between production and scavenging of ROS. As a result of these disturbances, intracellular levels of ROS may rapidly rise thus posing a threat to the cell viability (Mittler, 2002; Masella et al., 2005). The rapid and transient production of high quantities of ROS results in what is called "oxidative burst" (Wojtaszek, 1997; Apel and Hirt, 2004). ROS, unlike the atmospheric oxygen, are capable of unrestricted oxidation of various cellular components and, if not controlled,

have the ability to damage biomolecules (e.g. membrane lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage) and that can ultimately lead to the oxidative destruction of the cell (Mittler, 2002; Masella et al., 2005). For these reasons, the ROS levels within the cells must be strictly controlled and kept within a narrow range. Plants cells have developed mechanisms to monitor and scavenge excessive amounts of ROS. Plants tolerance to pollutants is related to their capacity to cope with ROS over production.

Despite these problems, a steady-state of ROS is required within the cells because they also act as a signal for the activation of stress response and defense pathways. Thus, ROS can be viewed as cellular indicators of stress, and as secondary messengers involved in the stress-response signal transduction pathway. The measurement of antioxidant enzymes activities is nowadays being frequently used to assess environmental stress induced in plants by various pollutants (Mittler, 2002; Apel and Hirt, 2004).

Two different mechanisms are involved in ROS control: one that will enable the fine modulation of low levels of ROS for signaling purposes, and one that will enable the detoxification of excess ROS, especially during stress. Mechanisms of ROS detoxification exist in all plants and can be categorized as non-enzymatic (e.g. by flavanones, anthocyanins, carotenoids and ascorbic acid (AsA)) or as enzymatic. Major enzymatic ROS scavengers in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) (Figure 3.2) (Mittler, 2002; Apel and Hirt, 2004; Geoffroy et al., 2004; Ashraf, 2009). The enzymes involved are present in different cell compartment and their expression is genetically controlled and regulated both by developmental and environmental stimuli, according to the necessity to remove the ROS produced in the cells (De Gara et al., 2003).

SODs act as the first line of defense against ROS, dismutating superoxide (O_2^-) to H_2O_2 (Figure 3.2a). APX, GPX, and CAT subsequently detoxify H_2O_2.

CAT, present in the peroxisomes of nearly all aerobic cells, can protect the cell from H_2O_2 by catalysing its decomposition into O_2 and H_2O (Mittler, 2002; Apel and Hirt, 2004) (Figure 3.2b). In contrast to CAT, APX requires an ascorbate and glutathione (GSH) regeneration system, the ascorbate-glutathione cycle (Figure 3.2d). Detoxifiying H_2O_2 to H_2O by APX occurs by oxidation of ascorbate to monodehydroascorbate (MDA) (Equation 1 in Figure 3.2d), which can be regenerated by MDA reductase (MDAR) using NAD(P)H as reducing agent (Equation 2 in Figure 3.2d). MDA can

spontaneously dismutate into ascorbate and dehydroascorbate (DHA). Ascorbate regeneration is mediated by dehydroascorbate reductase (DHAR) driven by the oxidation of GSH to oxidized glutathione (GSSG) (Equation 3 in Figure 3.2d). Finally, glutathione reductase (GR) can regenerate GSH from GSSG using NAD(P)H as a reducing agent (Equation 4 in Figure 3.2d). Like APX, GPX also detoxifies H_2O_2 to H_2O, but uses GSH directly as a reducing agent (Equation 1 in Figure 3.2c). The GPX cycle is closed by regeneration of GSH from GSSG by GR using NAD(P)H (Equation 2 in Figure 3.2c) (Wojtaszek, 1997; Mittler, 2002)

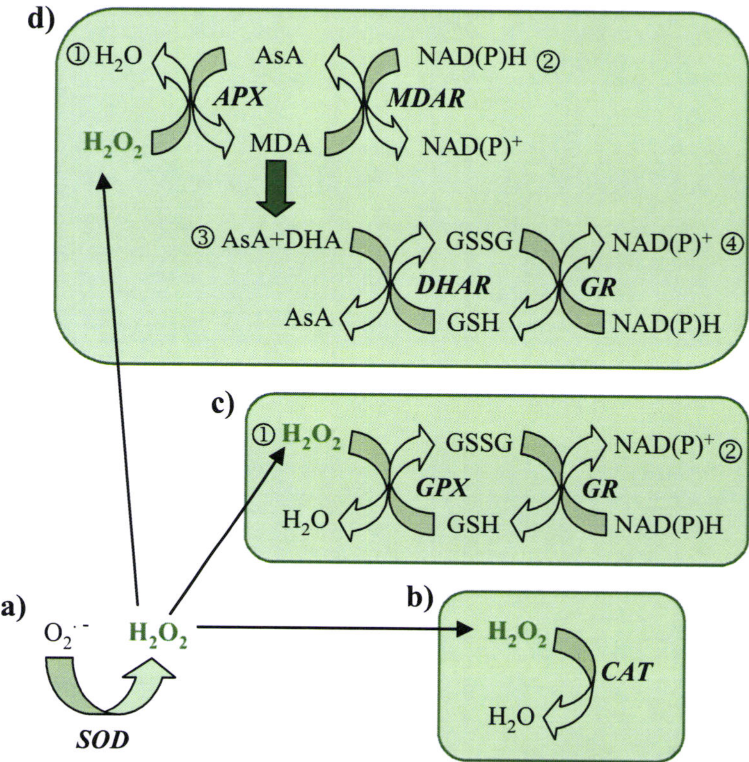

Figure 3.2. The main cellular pathways for reactive oxygen scavenging in plants.
(a) Superoxide dismutase (SOD). (b) Catalase (CAT). (c) The glutathione peroxidase cycle. (d) The ascorbate-glutathione cycle. Abbreviations: AsA, ascorbate; APX, ascorbate peroxidase; MDA, monodehydroascorbate; MDAR, monodehydroascorbate reductase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GSH, glutathione; GSSG, oxidized glutathione; GPX, glutathione peroxidase; GR, glutathione reductase. (Adapted from Mittler (2002)).

Unlike most organisms, plants have multiple genes encoding SOD and APX. Different isoforms are specifically targeted to chloroplasts, mitochondria, peroxisomes, as well as to the cytosol and apoplast (Table 3.1). Whereas GPX is located in cytosol, CAT is located mainly in peroxisomes (Table 3.1).

The extent of oxidative stress in a cell is determined by the concentrations of superoxide, hydrogen peroxide, and hydroxyl radicals. Therefore, the balance of SOD,

APX, and CAT activities will be crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms (Mittler, 2002; Apel and Hirt, 2004; Smirnoff, 2005).

In Table 3.1 a summary is presented of the several enzymes and reactions involved in the enzymatic ROS control and detoxification processes.

Table 3.1. Detoxifying enzymes and respective ROS scavenging reactions (Wojtaszek, 1997; Mittler, 2002; Blokhina et al., 2003; Ashraf, 2009)

Enzyme	EC number	Localization	Reaction catalyzed
Superoxide dismutase	1.15.1.1	Chol, Cyt, Mit, Per, Apo	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow 2\ H_2O_2 + O_2$
Catalase	1.11.1.6	Per	$2\ H_2O_2 \rightarrow O_2 + 2H_2O$
Ascorbate peroxidase	1.11.1.11	Chol, Cyt, Mit, Per, Apo	$2Asa + H_2O_2 \rightarrow 2MDA + 2H_2O$ ($2MDA \rightarrow AsA + DHA$)
Guaiacol peroxidase	1.11.1.7	Cyt	Donor + $H_2O_2 \leftrightarrow$ oxidized donor + $2H_2O$
Glutathione peroxidase	1.11.1.12	Cyt	$2GSH + H_2O_2 \leftrightarrow GSSG + 2H_2O$
Glutathione S-transferase	2.5.1.18	Cyt, Mit	$RX+GSH \leftrightarrow HX + R\text{-}S\text{-}GSH$*
MDA reductase	1.6.5.4	Chl, Mit, Per, Cyt	$NADPH + MDA \leftrightarrow NAD(P)^+ + AsA$
DHA reductase	1.8.5.1	Chl, Mit, Per	$2GSH + DHA \leftrightarrow GSSG + AsA$
Glutathione reductase	1.6.4.2	Chl, Cyt, Mit	$2NADPH + GSSG \leftrightarrow 2NADP^+ + 2GSH$

Abbreviations: Apo, apoplast; Chl, chloroplast; Cyt, cytosol; Mit, mitochondria; Per, peroxisome; * R may be an aliphatic, aromatic or herocyclic group; X may be a sulfate, nitrite or halite group

In the processes of detoxification and response against oxidative stress, phenolic compounds, which are ubiquitous plant secondary metabolites, may have an important role both in enzymatic as well as non-enzymatic mechanisms. Many phenolic compounds are used as substrates for antioxidant enzymes (and the guaiacol peroxidase in particular) (Smirnoff, 2005). In addition, phenolic compounds have antioxidant properties on their own because their chemical structure enables them to quench radicals, which makes them also important players in the control of the oxidative burst (Smirnoff, 2005).

The alteration of the levels of phenolic compounds is, therefore, a quantitative parameter which is complimentary to the evaluation of antioxidant enzymes activities to assess the oxidative stress induced by the plants exposure to xenobiotics.

The toxic effects caused in plants (including *Typha* spp.) by specific types of xenobiotic organic compounds (especially pesticides, but including other classes of compounds as well) have been extensively studied (Langan and Hoagland, 1996; Wilson et al., 2000;

Amaya-Chavez et al., 2006; Olette et al., 2008). However, once again, oxidative stress induced by pharmaceuticals is still poorly studied and characterized, thus contributing to the general scarcity of data available for adequate design of phytoremediation solutions for pharmaceuticals contamination (Pomati et al., 2004; Boxall et al., 2006; Kong et al., 2007).

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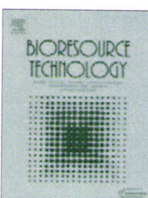

Toxicity and removal efficiency of pharmaceutical metabolite clofibric acid by *Typha* spp. – Potential use for phytoremediation?

Ana V. Dordio [a,*], Cátia Duarte [a], Margarida Barreiros [a], A.J. Palace Carvalho [a,b], A.P. Pinto [a,c], Cristina Teixeira da Costa [a,b]

[a] *Departamento de Química, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal*
[b] *Centro de Química de Évora, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal*
[c] *ICAM – Instituto de Ciências Agrárias e Mediterrâneas, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal*

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ABSTRACT

A study was conducted to assess *Typha* spp.'s ability to withstand and remove, from water, a metabolite of blood lipid regulator drugs, clofibric acid (CA). At a concentration of 20 μg L^{-1}, *Typha* had removed >50% of CA within the first 48 h, reaching a maximum of 80% by the end of the assay. Experimental conditions assured that photodegradation, adsorption to vessel walls and microbial degradation did not contribute to the removal. Exposure to higher CA concentrations did not affect *Typha*'s photosynthetic pigments but the overall increase in enzyme activity (ascorbate and guaiacol peroxidases, catalase, superoxide dismutase) indicates that both roots and leaves were affected by the xenobiotic. Eventually, *Typha* seemed able to cope with the CA's induced oxidative damage suggesting its ability for phytoremediation of CA contaminated waters.

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1. Introduction

Pharmaceutical active compounds (PhACs) are a class of emergent pollutants which are being continuously introduced in the environment mainly due to improper disposal of unused or expired drugs or through excretion and inefficient removal in sewage treatment plants (STPs) (Fent et al., 2006).

Low concentrations of PhACs in the environment, which are typically at trace levels, and lack of suitable sensitive methods of analysis in the past, have been the main reasons for only recently the interest in these compounds having started to emerge. However, despite low environmental concentrations, PhACs can still induce adverse effects due to cumulative effects and continuous exposure that occurs especially in aquatic ecosystems (Fent et al., 2006; Hernando et al., 2007).

Clofibric acid (CA) is one of the most frequently found and reported pharmaceutical residues in ground, surface and drinking waters all over the world (Tauxe-Wuersch et al., 2005; Fent et al., 2006; Hernando et al., 2007). This compound is an active metabolite from a series of widely used blood lipid lowering agents, the fibrates. These drugs are typically used in relatively high therapeutic dosages (1–2 g d^{-1}) and for extended periods of time, leading to excretion of large amounts of the metabolite. CA is non-biodegradable, highly mobile and very persistent in the environment, with a half-life of 21 years and a water residence time of 1–2 years (Zuccato et al., 2000). Several studies identify CA as a refractory contaminant of municipal STP influents and effluents (Tauxe-Wuersch et al., 2005; Fent et al., 2006) because conventional wastewater treatment processes are not designed to deal in general with these types of organic substances.

Optimization of STP processes to decrease PhACs amounts in the effluents has been tried by increasing sludge residence times, use of membrane bioreactors and advanced oxidation processes (Larsen et al., 2004; Urase et al., 2005; Carballa et al., 2007; Sirés et al., 2007). Many of these treatment options tend to reduce PhACs effluent loads but they are not widely used due to the higher costs involved.

Subsurface flow constructed wetland systems (SSF-CWs) are low cost wastewater treatment systems usually used to provide a form of secondary or tertiary treatment for wastewater, and have been already used with success to remove some organic recalcitrant compounds such as pesticides from contaminated waters (Haberl et al., 2003; Dordio et al., 2008). However, only very few studies have been carried out recently focusing on the removal of PhACs such as CA by SSF-CWs, and they all confirmed the CA recalcitrant behavior (Matamoros et al., 2005, 2008).

* Corresponding author. Tel./fax: +351 266 745343.
E-mail address: avbd@uevora.pt (A.V. Dordio).

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Depuration in SSF-CWs is achieved by the concerted action between plant rhizomes, microorganisms and the solid media components. This technology can exploit the ability of macrophytes to adsorb, uptake and concentrate elements and organic xenobiotics which contaminate the environment, as well as to release root exudates that enhance compound biotransformation and microbial degradation (Stottmeister et al., 2003). In fact, macrophytes are fairly robust plants and several studies have shown not only their capacity to withstand fairly high concentrations of organic xenobiotics but also their ability to remove them from contaminated waters (Williams, 2002; Haberl et al., 2003; Amaya-Chávez et al., 2006; Kong et al., 2007). Due to these characteristics macrophytes are frequently chosen for the application of phytoremediation technologies to treat waters contaminated with organic pollutants (Williams, 2002; Haberl et al., 2003).

In order to find suitable macrophytes for removal of PhACs such as CA from a contaminated environment we need a wide range of knowledge concerning the physiological and biochemical features of potentially useful species. The xenobiotic's toxicity can be inferred from the plant's physiological response. Alteration of photosynthetic pigments concentration in plant tissues and of plant growth rates have been observed for plants exposed to xenobiotics, sometimes compromising plant viability (Mishra et al., 2006). Xenobiotics also induce the formation of an excess of reactive oxygen intermediates (ROIs) which have the capacity to damage biomolecules (lipids, proteins, DNA) and can ultimately cause cell death (Mittler, 2002). Plant tolerance to pollutants is related to their capacity to cope with the ROIs production by the activation of the enzymatic systems capable of scavenging and eliminating them. Activation of enzymes like catalase, superoxide dismutase, and ascorbate and guaiacol peroxidases have been reported for plants subjected to different sources of abiotic stress (drought, excessive light, xenobiotic) (Mittler, 2002). Phenolic compounds and other antioxidant compounds can also be used to cope with the excessive ROIs production (Schützendübel et al., 2001; Strycharz and Shetty, 2002).

In this study, the ability of *Typha* spp. to remove CA from contaminated water was evaluated. Biochemical and physiological parameters were also determined in order to shed some light into the tolerance mechanisms developed by *Typha* spp. in the presence of this xenobiotic. This characterization of *Typha*'s behavior towards its exposure to CA can be useful for the purpose of assessing the potential of this macrophyte species for the depuration of this xenobiotic from contaminated wastewaters in SSF-CWs.

2. Methods

2.1. Plant collection and acclimation

Typha spp. rhizomes with shoots were collected in water streams in Alentejo, Portugal, during March 2007. The rhizomes were thoroughly washed to remove any soil/sediment particles attached to the plant surfaces. Roots were submersed for a short period in dilute hypochlorite solution in order to diminish the native microbial population. The plants were then placed in 20 L vessels with aerated modified Hoagland nutrient solution adapted from Fediuc and Erdei (2002). The nutrient solution was prepared with the following chemical composition: 2.5 mmol L^{-1} K^+, 2 mmol L^{-1} Mg^{2+}, 2 mmol L^{-1} Ca^{2+}, 2 mmol L^{-1} SO_4^{2-}, 6 mmol L^{-1} NO_3^-, 0.5 mmol L^{-1} $H_2PO_4^-$, 10 µmol L^{-1} Fe^{3+}, 10 µmol L^{-1} H_3BO_3, 1 µmol L^{-1} Mn^{2+}, 0.5 µmol L^{-1} Cu^{2+}, 0.1 µmol L^{-1} MoO_4^{2-}. The pH was adjusted to 6.0 and the nutrient solution was replaced twice every week. Plants were grown in a growth chamber (Fitoclima, Portugal) at 22 °C, with 70% relative humidity and a light-dark cycle of 12:12 h. The photon flux density was 270 µmol m^{-2} s^{-1}. After 6 weeks, when new roots and leaves had developed, plants of uniform size (approximately 30 cm height) were selected to be used in the assay setup.

2.2. Assay setup

2.2.1. CA removal assay

Selected plants, whose roots were submersed for a short period in dilute hypochlorite solution in order to diminish the native microbial population, were transferred to 3 L plastic vessels (3 plants per vessel) which contained aerated modified Hoagland nutrient solution spiked with 20 µg L^{-1} of CA. A control assay, without plants, was used to evaluate CA photodegradation and another one was setup in the dark to evaluate possible compound adsorption on the plastic vessel walls. All assays were done in triplicate. Water samples were collected after 6, 12, 18 and 24 h of exposure during the first day, and then every 24 h during a period of 7 days and, finally, after 14 and 21 days.

2.2.2. Toxicity assays

A setup similar to the one just described was used in toxicity assays, but in this case nutrient solutions were spiked with CA at 0.5, 1.0 and 2.0 mg L^{-1} concentrations, prepared from a stock aqueous solution of 50 mg L^{-1}. Control plants were grown in nutrient solution without CA. Water samples were collected after 7, 14 and 21 days of exposure. For each CA concentration three replicate assays were setup and the containers were arranged as a completely randomized factorial design. At the end of the experiments the plants were removed, leaves and roots were separated, immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analyses.

2.3. Quantification of CA in nutrient solutions

Samples were collected, filtered through a 0.45 µm PTFE filter (Macherey-Nagel, Germany) and analyzed using high performance liquid chromatography (Agilant 1100 series, Hitachi, Japan) with UV detection at 230 nm. The reverse phase analytical column used was a Zorbax Eclipse XDB-C8 with 5 µm particle size. Separation was performed in isocratic mode, and the mobile phase used was composed by 75:25 methanol:water. Water contained 2.5% (v/v) of methanol and was acidified with 0.1% (v/v) phosphoric acid. The flow rate was 1.0 mL min^{-1}, and injection volume was 20 µL. Retention time for CA was 2.9 min (the CA identity in this peak was confirmed by HPLC–MS analysis using electron spray ionization in negative mode). Three replicate injections were made for each treatment vessel.

A calibration curve was constructed for CA. A standard solution of 100.0 mg L^{-1} was used to prepare the standards of 0.25, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}. Three replicates were made for each standard solution, and each solution was injected five times. The average areas of the CA peaks were plotted against the standards concentration resulting in a linear correlation with $R^2 = 0.999$.

Whenever the measured CA concentration was below the limit of quantification of 0.1 mg L^{-1} (LOQ, calculated as the blank signal plus 10 standard deviations of the blank, as in Miller and Miller (2000)), samples were pre-concentrated on Sep-Pak Vac 3cc (500 mg) SPE cartridges (Waters, USA) according to a procedure for which >95% recoveries were obtained. Before sample application on the SPE cartridges, water samples were filtered through 0.45 µm PTFE filter (Macherey-Nagel, Germany) and the pH was adjusted to 2 by the addition of concentrated phosphoric acid. Cartridges were pre-conditioned with 7.5 mL of methanol and an identical volume of distilled water. A variable sample volume (depending on the initial assay concentration) was applied to the cartridge. Cartridges were air dried for about 15 min under vacuum

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to remove excess water. The analyte (CA) retained was eluted with 5.0 mL of methanol. Following elution, the solution was evaporated on a rotary evaporator at 30 °C to dryness and redissolved with 1.0 mL of distilled water.

2.4. Plant growth parameters

Fresh-plant weights and leaf and root sizes were measured before and after a 21-day period of CA exposure. Visual inspection of injury symptoms was also recorded. Relative growth rates (RGR) were calculated according to the equation

$$RGR = (\ln W_1 - \ln W_0)/(t_1 - t_0) \quad (1)$$

where W_0 and W_1 are, respectively, the initial and final weights of plants and $(t_1 - t_0)$ is the duration of the experiment.

2.5. Chlorophyll and carotenoids contents

Concentrations of chlorophyll and carotenoids were determined in an 80% (v/v) aqueous acetone extract using a modified method of Lichtenthaler and Wellburn (1983). Chlorophyll content results are expressed in mg g^{-1} of fresh weight (FW) and calculated using extinction coefficients and equations given by Porra (2002). Carotenoids content results are expressed in mg g^{-1} FW and calculated using extinction coefficients and equations given by Lichtenthaler and Wellburn (1983). Three replicate measurements were done for each treatment vessel.

2.6. Extraction and determination of total phenolic content of plant extracts

Phenolic compounds were extracted by the sea sand disruption method (SSDM) according to Teixeira et al. (2006). Amounts of total phenolics in the extracts were determined spectrophotometrically according to the Folin-Ciocalteu method (Singleton et al., 1999). A standard curve was prepared with gallic acid ($R^2 = 0.999$), and the total phenolic content was expressed as gallic acid equivalents (GAE, mg g^{-1} FW). The estimation of phenolic compounds in the extracts was carried out in triplicate.

2.7. Activities of antioxidant enzymes

2.7.1. Enzyme extraction procedure

Plant material, both leaves and roots (1.0 g each from each treatment vessel), was ground to a fine powder in a cold glass mortar with 4.0 g of acid washed sand and homogenized in 100 mM *Tris*-HCl buffer solution (pH 7.5) containing 1 mM EDTA, 3 mM 1,4-dithiothreitol and 2% (w/v) polyvinylpolypyrrolidone. Homogenates were centrifuged at 12,000g for 30 min at 4 °C and the resulting supernatants were used for the determinations of enzymes activities. All steps in the preparation of enzymes extracts were carried out at 0–4 °C.

2.7.2. Catalase (CAT) (EC 1.11.1.6)

CAT activity was measured according to the method of Aebi (1984). Activity was determined by monitoring the decrease in absorbance due to H_2O_2 reduction at 240 nm for 2 min. CAT activity was defined as the amount of enzyme required to catalyze the dismutation of 1 μmol of H_2O_2 per minute and the results were expressed as U mg^{-1} FW.

2.7.3. Superoxide dismutase (SOD) (EC 1.15.1.1)

SOD activity was assayed by the Ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969). SOD activity was measured as the inhibition of the rate of reduction of Cytochrome *c* by the superoxide radical, observed at 550 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the Cytochrome *c* reduction and the results were expressed as U mg^{-1} FW.

2.7.4. Guaiacol peroxidase (GPX) (EC 1.11.1.7)

GPX activity was determined according to the method adopted by Bergmeyer (1983). Activity was determined by monitoring the increase in absorbance due to formation of the reaction product tetraguaiacol at 420 nm for 2 min. One unit of GPX activity is defined as the amount of enzyme required for catalyzing the oxidation of 1 μmol of guaiacol in 1 min and the results were expressed as U mg^{-1} FW.

2.7.5. Ascorbate peroxidase (APX) (EC 1.11.1.11)

For the determination of APX activity, this enzyme was extracted using a procedure similar to that described in Section 2.7.1 but with ascorbate (10 mM) being added to the extraction buffer solution. APX activity was determined according to the method of Nakano and Asada (1981) by the estimation of the ascorbate oxidation rate (extinction coefficient 2.8 mM^{-1} cm^{-1}). Change in absorbance was monitored at 290 nm and enzyme activity was expressed in terms of μmol of ascorbate oxidized min^{-1} g^{-1} FW.

2.8. Statistical analysis

Data were analyzed through one-way analysis of variance (ANOVA) for comparison of the effects of CA exposure on physiological and biochemical parameters (RGR, photosynthetic pigments, phenolic contents and enzymatic activities) with those of the control assays. Comparisons were considered significantly different for $P < 0.05$.

3. Results and discussion

Typha spp. (cattail) is an emergent macrophyte which has been frequently used in SSF-CWs to depurate water contaminated with organic compounds and has shown a good tolerance when exposed to some xenobiotic substances (Williams, 2002; Haberl et al., 2003; Amaya-Chávez et al., 2006). In order to assess its ability to remove CA from contaminated water, an assay was conducted by spiking the cattail growth media with CA in a concentration of 20 μg L^{-1} which is within the range of concentrations typically used in biological removal studies for this kind of pollutants (Zwiener and Frimmel, 2003; Matamoros et al., 2005, 2008; Urase et al., 2005).

CA removal in hydroponic systems may be attributed to abiotic and biotic processes. Abiotic processes include volatilization, photodegradation and hydrolysis. When leaving a CA aqueous solution in a plastic vessel for a long period of time, its concentration remained approximately constant thus indicating that adsorption to the walls of the plastic vessels was negligible as well as other phenomena such as photodegradation, hydrolysis and volatilization. Therefore, these abiotic processes are not responsible for any CA removal from the growth media. Given the reported non-biodegradability of this compound (Zwiener and Frimmel, 2003), and the care taken to diminish the microbial populations in the plants rhizosphere when the assays were setup, microbial degradation should also have a very limited contribution. Therefore, any CA removal observed in the plants assays should result, mainly, from adsorption on the rhizomes and/or from plant uptake.

Cattail was able to partially remove the CA present in the growth media, whose concentration diminished by 50% within the first 48 h and achieving a maximum of 80% removal after a 21-day period of exposure (see Electronic Annex 1 in the online version of this article).

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The CA removal kinetics is characterized by a fast initial stage occurring within the first period of 96 h, which can be described as a first-order process that fits the equation

$$\ln[\mathrm{CA}]_t = \ln[\mathrm{CA}]_0 - 0.0071\ \mathrm{h}^{-1}t \quad (R^2 = 0.995)$$

where $[\mathrm{CA}]_t$ and $[\mathrm{CA}]_0$ are the CA concentrations (μg L^{-1}) at any time (t) and at the beginning, respectively.

This is then followed by a slower stage, where removal yield gradually tends to stabilize around 80% by the end of the assay. This behavior is frequently observed in other studies on organic xenobiotics removal by *Typha* and other plant species (Amaya-Chávez et al., 2006; Olette et al., 2008).

In order to assess CA toxicity towards *Typha* at increasing CA concentrations, three new assays using the same conditions tested before but with growth media spiked with CA at 0.5, 1.0 and 2.0 mg L^{-1} were established. Since symptoms of toxicity (chlorosis) are visually evident when plants are exposed to CA concentrations of 4 mg L^{-1} (data not shown), these concentrations were chosen as a set of test cases bordering visually evident CA toxicity.

For this range of concentrations no visual signs of toxicity were observed in *Typha* spp. and, in fact, cattail was not only able to cope with the large amounts of CA in their growth media but continue to partially remove it. Maximum percentage of CA removal in these assays ranged from 46% for the CA initial concentration of 2.0 mg L^{-1}, to 64% for the assay with CA initial concentration of 0.5 mg L^{-1}. When comparing these removals with that of 20 μg L^{-1} assay, they clearly fit a linear relationship ($R^2 > 0.99$) between the initial CA concentrations and percent removal at every corresponding exposure times, with the exception of the 2.0 mg L^{-1} assays (see Electronic Annex 2 in the online version of this article).

Relative growth rate of the plants exposed to the tested concentrations of CA was higher than the control plants (Table 1), mainly because the root system of the plants exposed to CA was much more developed than the control plants (data not shown). Carotenoid and chlorophyll contents of the plants exposed to the high CA concentrations were also not significantly affected in biological terms (Fig. 1). The plant growth spur which occurs mainly on the root region also helps explain why there were no observed differences in the photosynthetic pigments. Other authors have already reported that chlorophyll pigments of *Typha* plants are not affected by other organic xenobiotics like methyl parathion (Amaya-Chávez et al., 2006).

Plants respond in different ways to abiotic stress in their root environment. However, in the course of that response, an excess of reactive oxygen intermediates (ROIs) is frequently produced. ROIs are toxic by-products of aerobic metabolism whose enhanced production during stress can be viewed as a cell signal for the activation of stress-response and defense pathways. Although a steady-state level of ROIs can be used by cells to monitor their stress, the cellular amounts need to be kept under tight control because over-accumulation of ROIs can ultimately cause cell death (Wojtaszek, 1997; Mittler, 2002).

The fact that *Typha* seemed not to be negatively affected by the presence of the high amounts of CA in the growth media indicated that the expected production of excessive ROIs was effectively

Table 1
Relative growth rate (RGR) and total phenolic contents of *Typha* spp. (averages ± SD, $n = 9$)

CA concentration (mg L^{-1})	RGR (d^{-1})	Phenolic contents (mg L^{-1})
Control	0.022^a ± 0.004	54.4^a ± 2.0
0.5	0.034^b ± 0.005	41.8^b ± 1.4
1.0	0.035^b ± 0.005	40.2^c ± 0.7
2.0	0.032^b ± 0.003	41.5^b ± 1.0

ANOVA significant at $P < 0.05$ when compared with control. Different letters indicate significantly different values.

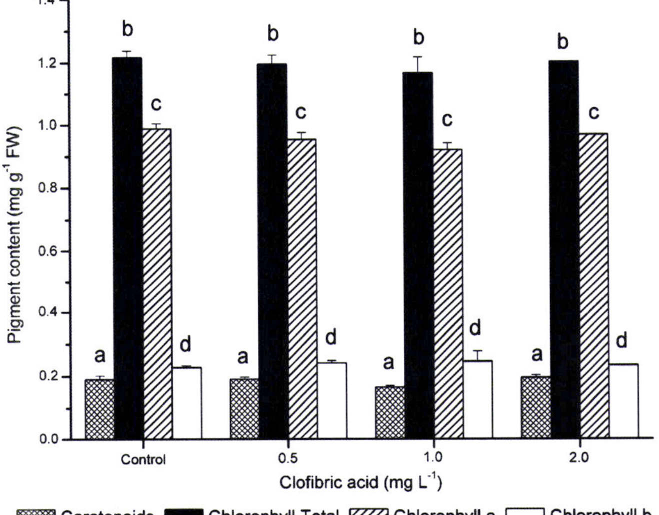

Fig. 1. Effects of clofibric acid exposure in photosynthetic pigments of *Typha* spp. Vertical error bars indicate ±SD ($n = 9$). ANOVA significant at $P < 0.05$ when compared to control. Different letters indicate significantly different values.

counteracted by antioxidant compounds and enzymes which are known to be involved in the sequestration of ROIs. SOD, CAT, GPX and APX are reportedly key enzymes in the first line of defense against the oxidative burst induced by the presence of organic xenobiotics (Wojtaszek, 1997; Mittler, 2002). Activities of these enzymes were measured in plant tissues from different assay concentrations and compared to those of the control plants.

Enzymes activities measured showed that not only the roots seem to be affected by the increase in ROIs but also the leaf tissues experience an enzymatic activity increase indicating that the so-called oxidative burst also affected them (Fig. 2).

SOD is considered to be in the first line of defense against ROIs damage. This enzyme acts against the superoxide radicals, which are formed in different cell compartments and can act as precursors of other ROIs (Wojtaszek, 1997; Mittler, 2002). SOD dismutates two superoxide radicals to H_2O_2 and O_2, maintaining the lower levels of superoxide radicals within the cell. The increase in SOD activity observed in both roots and leaves – but higher in the leaves – (Fig. 2D) can be attributed to an increase in superoxide radicals within the cells.

Uncontrolled levels of H_2O_2 produced either by the action of SOD on superoxide radicals or due to photorespiration can lead to generation of $OH^{\cdot}$ via Haber-Weiss reactions and these can lead to other chain reactions which can cause serious damage within the cells. The enzymes CAT, APX and GPX, functioning in different cell compartments, can be activated in order to maintain the appropriate H_2O_2 levels (Wojtaszek, 1997; Mittler, 2002). Overall the CAT, GPX and APX enzyme activities were higher in the plants exposed to CA than in the control, but different concentration-response relationships were observed.

CAT is an enzyme present in the peroxisomes and mitochondria where it decomposes H_2O_2 into water and oxygen (Wojtaszek, 1997; Mittler, 2002). Increase in CAT activity is believed to be explained by a plant adaptive mechanism which tries to maintain the H_2O_2 steady-state level within the cells. Enzyme activity increase observed in the leaves and roots (Fig. 2A) seems to indicate that higher amounts of CA on growth media induce higher concentrations of H_2O_2 in the tissues, leading to an enzyme activity increase in order to diminish the intra-cellular level of H_2O_2.

APX is an enzyme of the Halliwell–Asada pathway which catalyzes the reduction of hydrogen peroxide into water using ascorbic acid as a donor of electrons (Wojtaszek, 1997; Mittler, 2002). The

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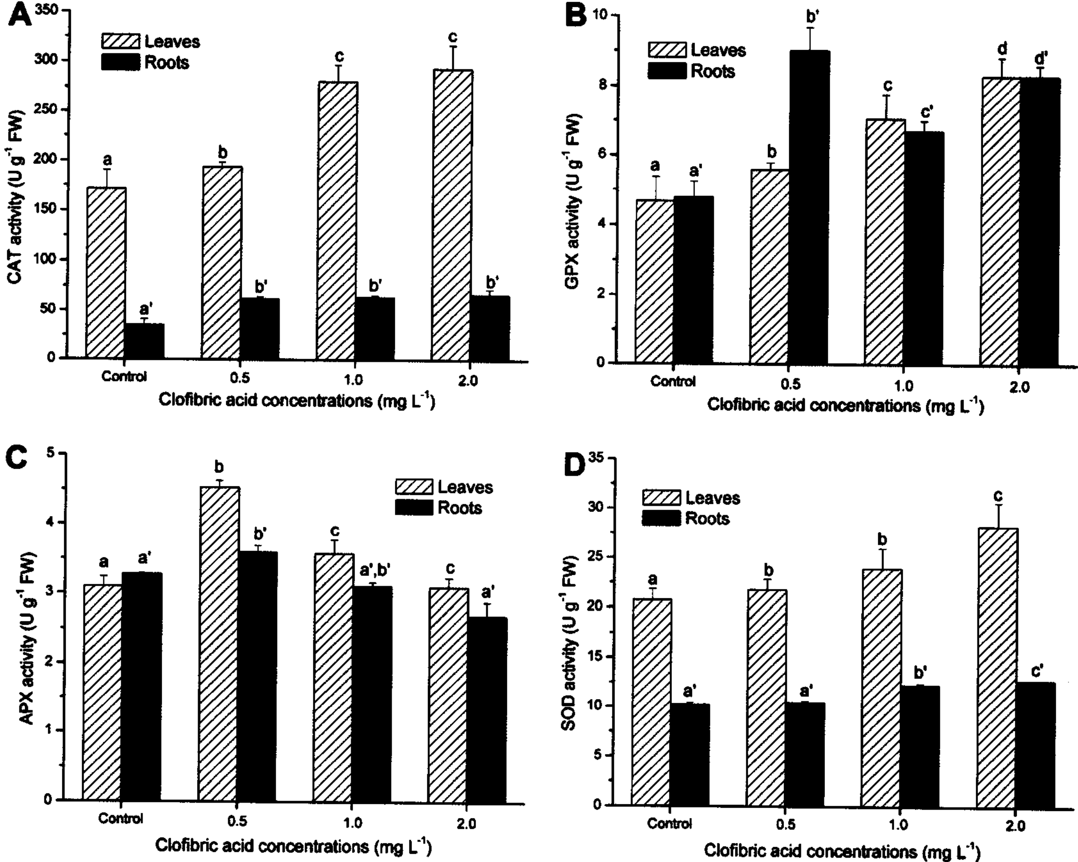

Fig. 2. Effects of clofibric acid treatment on the enzymatic activities of the antioxidant enzymes catalase (A), guaiacol peroxidase (B), ascorbate peroxidase (C) and superoxide dismutase (D) of *Typha* spp. Side by side are represented the activities measured in the leaves (lighter color) and in the roots (darker color). Vertical error bars indicate ±SD ($n = 9$). ANOVA significant at $P < 0.05$ when compared to the control assays, but no comparisons are made between the activities in the roots and those in the leaves. Different letters indicate significantly different values.

finding that the Halliwell–Asada pathway is present in almost all the cellular compartments tested, together with the high affinity of APX for H_2O_2, suggests that this enzyme plays a crucial role in controlling the level of ROIs in these compartments. Maximum increase in the APX activity was observed for 0.5 mg L^{-1} (Fig. 2C) and the relative decrease observed for higher CA concentrations might be due to early signs of toxicity. In the case of the leaves, the APX activity was nevertheless always higher in the plants exposed to CA. In the root tissues, APX activity measured at higher concentrations (1.0 and 2.0 mg L^{-1}) was not statistically different from that of the control. Roots can be considered the primary site of contact with the xenobiotic, and several reports indicate that toxicity is more severe in their tissues (Wang and Zhou, 2005). It is possible that the APX enzyme is inhibited in the presence of larger amounts of CA and it is in the roots tissues that this effect is more evident.

GPX is located in the cytosol, cell wall, vacuole and extra-cellular spaces and it can be considered a stress marker enzyme having a broad specificity for phenolic substrates and a higher affinity for H_2O_2 than CAT (Wojtaszek, 1997; Mittler, 2002). GPX activity in leaves presented a significant increase with increasing CA concentration in growth media (Fig. 2B). In the roots, the GPX activity was also significantly higher for the plants exposed to CA than for the control (Fig. 2B). However, the largest increase in GPX activity in the roots was observed for 0.5 mg L^{-1} of CA while plants exposed to 1.0 and 2.0 mg L^{-1} of CA showed a significant decline in the enzyme activity. The decline in activity (also observed in the roots APX activity) might result from the fact that the enzymatic system is starting to fail at higher CA concentrations.

The increase in the GPX activity in the leaves of *Typha* spp. was accompanied by a concomitant reduction on the free phenolic compounds measured (Table 1). It has been shown that GPX can use H_2O_2 and phenolic substrates to produce cell wall components like lignin. The increase of lignin has been observed when plants have been exposed to heavy metals (Schützendübel et al., 2001) and organic xenobiotics (Strycharz and Shetty, 2002). It is likely that the observed decrease in total phenolic compounds might be due to the fact that some of the lower molecular weight phenolics (i.e. cinnamic acids and alcohols) have been used as electron donors for the GPX resulting in the formation of phenoxyl radicals that polymerize to form lignin.

Organic xenobiotic compounds, for which no specific transporters exist in cell membranes, tend to move into and within the plant tissues driven simply by diffusion, dependent on their chemical properties, especially on their hydrophobicity (Stottmeister et al., 2003; Pilon-Smits, 2005). Compounds with $\log K_{ow}$ between 1 and 3.5 are highly bioavailable to roots of vascular plants such as cattails because they are lipophilic enough to move through the lipid bilayer of membranes, and still water soluble enough to travel into the cell fluids (Pilon-Smits, 2005). Considering how the antioxidant enzymatic activity in the *Typha* tissues (leaves and roots) was affected by the exposure to CA and also CA's only moderate hydrophobicity ($\log K_{ow}$ 2.57 – 2.84 (Hernando et al., 2007)), we are led to believe that absorption of this compound into the plant occurs and the removal of CA from water is not simply due to an adsorption process on the roots surface alone. However, the data presented here do not allow for any conclusions to be made on the extent of either phenomena and additional studies which are ongo-

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ing will be required to better characterize the mechanisms involved in CA's removal by *Typha*.

4. Conclusion

Typha spp. was able to remove 80% of CA after 21 days of exposure to a solution spiked with 20 $\mu g\ L^{-1}$, with over 50% being removed just within the first 24–48 h. The CA removal was achieved without any obvious visual symptoms of toxicity even when the plants were subjected to concentrations several orders of magnitude higher. Relative plant growth rates indicate that the higher amounts of CA stimulate the growth of the roots while the photosynthetic pigments are not significantly affected by the xenobiotic. However, there was an increase in the activity of the antioxidant enzymes studied, CAT, APX, GPX and SOD, indicating that CA induces an oxidative burst in the *Typha* tissues (leaves and roots). The antioxidant enzymatic system seems to be able to cope with CA concentrations up to 0.5 $mg\ L^{-1}$ but, for higher concentrations, the loss of enzyme activity may constitute an early sign of toxicity.

These results illustrate the potential of *Typha* to remove CA from contaminated water. In addition, CA behavior may serve as a model for other PhACs or other organic xenobiotics with similar chemical properties, and thus suggest the potential use of SSF-CWs planted with *Typha* for removing a wider range of related compounds from wastewaters.

Nevertheless, further studies are needed in order to better understand the mechanisms involved in CA removal at the plant level. This knowledge may be useful for optimizing the role of this plant in a SSF-CW.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2008.08.034.

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Potential of *Typha* spp. for the phytotreatment of water contaminated with Ibuprofen

Ana Dordio[a]*, Raquel Ferro[a], Dora Teixeira[a,b], A.J. Palace Carvalho[a,c], A. P. Pinto[a,b], Cristina Barrocas Dias[a,c]

[a] *Departamento de Química, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal.*

[b] *ICAAM – Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal.*

[c] *Centro de Química de Évora, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal.*

Keywords: Constructed wetlands, Oxidative stress, Pharmaceuticals, Phytoremediation, *Typha* spp.

Abstract

Several studies on phytotoxic effects caused by organic xenobiotics and their removal from water by macrophytes have already been performed to evaluate the usefulness of these plants for phytoremediation technologies. In this context, a study was conducted to assess *Typha* spp.'s ability to withstand and remove, from water, the non-steroidal anti-inflammatory drug ibuprofen. For an initial ibuprofen concentration of 20 µg/L, *Typha* removed nearly 60% of it within the first 24h, attaining over 99% removal by the end of the assay. Exposure to higher ibuprofen concentrations did affect *Typha*'s growth but, by the end of the assays, plant's growth as well as photosynthetic pigments approached normal values. An alteration in antioxidant enzymes' activities (superoxide dismutase, catalase, guaiacol peroxidase) indicated that both roots and leaves were affected by the xenobiotic. Eventually, *Typha* seemed able to cope with ibuprofen's induced oxidative damage suggesting its ability for phytotreatment of waters contaminated with ibuprofen.

* Corresponding author. E-mail: avbd@uevora.pt

1. Introduction

At present, water quality is one of the most relevant topics in environmental chemistry. Contamination with human and veterinary drugs and their metabolites has been emerging as a major issue due to the ever increasing amounts and variety of substances of this type which are excreted or improperly disposed of in the sewage systems. Wastewater treatment plants (WWTP) do not guarantee their effective removal from wastewaters due to the general inefficiency of the conventional treatment processes in dealing with this type of compounds, resulting most of the times, in the discharge of effluents from the WWTPs which are still contaminated with some pharmaceutical residues (Fent et al., 2006; Petrovic and Barceló, 2007; Aga, 2008).

One of the pharmaceuticals most frequently found in water resources (Fent et al., 2006; Petrovic and Barceló, 2007; Aga, 2008) is ibuprofen (IB) (2-[4-(2-methylpropyl)phenyl]propanoic acid), a non-steroidal anti-inflammatory drug used in the treatment of rheumatic disorders, pain and fever. This non-prescription drug is among the most consumed pharmaceuticals all over the world and environmental contamination with this substance is a result of the very high amounts entering the WWTPs which, despite the also high removal efficiencies (up to 90%) (Fent et al., 2006; Aga, 2008), still results in the discharge of contaminated effluent.

The foreseeable environmental consequences resulting from the presence of pharmaceuticals in aquatic systems points out to the urgent need of finding ways to retain and remove these pollutants before they reach the waterbodies. Optimization of WWTP processes to decrease pharmaceuticals amounts in the effluents has been tried by increasing sludge residence times, use of membrane bioreactors and advanced oxidation processes, among others (Larsen et al., 2004; Petrovic and Barceló, 2007; Carballa et al., 2007; Aga, 2008). Many of these treatment options tend to reduce pharmaceuticals load in effluents but they are not widely used due to the high costs involved.

Biological treatments based on phytoremediation have been used with success to remove some organic xenobiotics from wastewaters (Dietz and Schnoor, 2001; Haberl et al., 2003; Pilon-Smits, 2005). Such technology attempts to exploit the ability of plants to adsorb, uptake and concentrate elements and organic xenobiotics, as well as to release root exudates that enhance compound biotransformation and microbial degradation (Dietz and Schnoor, 2001; Stottmeister et al., 2003; Pilon-Smits, 2005)

Constructed wetlands (CW) are low-cost wastewater treatment systems designed to mimic the water depurative functions of natural wetlands by the use of processes involving wetlands vegetation, microorganisms and solid matrix components (Vymazal et al., 1998). These systems are increasingly being used to provide a form of secondary or tertiary treatment for wastewater, and have already been used with success to remove some organic recalcitrant compounds from contaminated waters (Williams, 2002; Haberl et al., 2003; Pilon-Smits, 2005). However, only very few studies have been carried out recently focusing on the removal of pharmaceuticals by CWs (Matamoros et al., 2005; 2008).

The role of vegetation in CW is a key issue under study. It has already been established that the binding of xenobiotics to roots occurs by adsorption followed by absorption. In fact, many current studies indicate that not only the exoenzymes excreted by plant roots and the microorganisms enzymes are important for the degradation of organic xenobiotics but also that this may take place within plant tissues through its own intrinsic enzymatic machinery (Dietz and Schnoor, 2001; Pilon-Smits, 2005).

In plants exposed to xenobiotics, however, alterations have been observed of photosynthetic pigments concentrations in plant tissues and of plant growth rates, sometimes compromising plant viability. Exposure to xenobiotics is a source of abiotic stress which can induce the formation of an excess of reactive oxygen intermediates (ROIs) with a potential to damage biomolecules (lipids, proteins, DNA) and ultimately cause cell death (Mittler, 2002).

The existence of plants tolerant to high concentrations of organic xenobiotics suggests their ability to cope with the ROIs production by the activation of the enzymatic systems capable of scavenging and eliminating them. Activation of enzymes like catalase, superoxide dismutase, and peroxidases have been reported for plants subjected to different sources of abiotic stress (drought, excessive light, xenobiotic) (Mittler, 2002).

The main goal of this study is the evaluation of the ability of *Typha* spp. (cattail) to tolerate and remove IB from contaminated water with the purpose of testing the usefulness of this macrophyte species for the depuration of IB contaminated wastewaters in CW systems. As an important preliminary step, a methodology for the quantification of IB and sample pre-treatment was developed and optimized. In addition, biochemical and physiological parameters were also determined to shed some light on the tolerance mechanisms developed by *Typha* spp. in the presence of IB.

2. Experimental

2.1. Reagents and Materials

Ibuprofen (IB) (99.8% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals and solvents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Panreac Quimica SA (Barcelona, Spain). Ultra-pure water was obtained with a Milli-Q water purification system (Simplicity® UV, Millipore Corp., France).

Solid phase extraction (SPE) cartridges used were LiChrolut® RP-18 (500 mg, 3 mL) from Merck (Darmstadt, Germany), and Sep-Pak® Vac (500 mg, 3 mL), Oasis® HLB (200mg, 6 mL) and Oasis® MCX (150 mg, 6 mL) all from Waters Corporation (Milford, MA, USA). Filters with 0.45 μm nylon membrane were purchased from VWR International (West Chester, PA, USA).

2.2. Quantification of IB in nutrient solutions

2.2.1. Sample preconcentration by solid phase extraction

Several SPE cartridges were tested with plant nutrient solutions spiked with IB for the choice of the optimal conditions for IB recovery in SPE preconcentration: LiChrolut® RP-18, Sep-Pak® Vac and Oasis® MCX (all conditioned with 7.5 mL of methanol and 7.5 mL of water), and Oasis® HLB (conditioned with 3.0 mL of methanol and 3.0 mL of water). After sample pH adjustment to values of 2, 5 and 7 using either H_3PO_4 or NaOH solutions, the spiked plant nutrient solutions were percolated through the SPE cartridges. The cartridges were then air dried for about 15 min under vacuum to remove excess water. The analyte retained in the cartridges was eluted with 5.0 mL of methanol. Following elution, the solutions were evaporated on a rotary evaporator at 30ºC to dryness and redissolved with 1.0 mL of Milli-Q water. Five replicates were done for every tested cartridge and experimental condition.

Additionally, possible negative effects due to using large sample volumes were evaluated using, according to the results obtained, the best SPE cartridges (LiChrolut® RP-18) and conditions (pH adjusted to 7). Thus, a series of trials were performed using varying volumes of solution (between 2 mL and 200 mL) adjusted to pH 7 and all

containing the same amount of the analyte, which was, at the end, redissolved to a final concentration of 1.0 mg L^{-1}. Three replicates were done of each sample volume assay. Considering the results obtained, all samples were henceforth prepared by adjusting their pH to 7 and preconcentrated in LiChrolut® cartridges.

2.2.2. Quantification and analytical method validation

IB in nutrient solutions was quantified by the high performance liquid chromatography (HPLC) technique. Analyses were performed on an Elite LaChrom HPLC system with UV detection (Hitachi, Japan). The reversed phase analytical column used was a Zorbax Eclipse XDB-C18 with 5 μm particle size. Chromatographic separation was performed in isocratic mode, and the mobile phase used was composed by 75:25 (%, v/v) acetonitrile:water, at a flow rate of 1.0 mL min^{-1}. Water was acidified with 0.1% (v/v) phosphoric acid. The UV detector wavelength was set at 222 nm. Five replicate injections were made for each sample previously filtered through a 0.45 μm filter.

Calibration curves were constructed using a set of IB standard solutions with concentrations of 0.25, 0.5, 1.0, 2.0 and 5.0 mg L^{-1}. Instrumental detection and quantification limits (IDL and IQL) for the chromatographic measurement were obtained by determining the concentrations corresponding to signal-to-noise ratios of 3 and 10 respectively, according to Miller and Miller (2000). The repeatability and reproducibility of the HPLC-UV system were evaluated, respectively, by consecutive injections of the same standard solution using the same mobile phase, for the former, or by performing injections of different standard solutions in different days, in the latter case, and were expressed as the dispersion (relative standard deviation) of the measured peak areas.

The limit of quantification (LOQ) of the entire analytical method (including SPE preconcentration) was calculated resorting to the following equation (Vieno et al., 2006):

$$\text{LOQ} = (\text{IQL} \times 100)/(\text{Rec}(\%) \times C) \quad (1)$$

where IQL is the instrumental quantification limit (mg L^{-1}), Rec (%) is the average SPE recovery of IB from the plant nutrient solution and C is the concentration factor (a maximum value of 200 was used).

The reproducibility of the entire analytical method was determined by performing, in different days, quantification of IB recovered from five spiked plant nutrient solutions with different IB concentrations (5-500 μg L^{-1}) and sample volumes (2-200 mL).

Reproducibility was expressed as the dispersion (relative standard deviation) of IB recoveries.

2.3. Assay setup

2.3.1. Plants collection and acclimation

Typha spp. rhizomes with shoots were collected in water streams in Alentejo, Portugal, during April 2007. The rhizomes were thoroughly washed to remove any soil/sediment particles attached to the plant surfaces. The plants were then placed in 20 L vessels with aerated modified Hoagland nutrient solution whose composition was adapted from Fediuc and Erdei (2002). The nutrient solution was prepared with the following chemical composition: 2.5 mmol L^{-1} K^+, 2 mmol L^{-1} Mg^{2+}, 2 mmol L^{-1} Ca^{2+}, 2 mmol L^{-1} SO_4^{2-}, 6 mmol L^{-1} NO_3^-, 0.5 mmol L^{-1} $H_2PO_4^-$, 10 μmol L^{-1} Fe^{3+}, 10 μmol L^{-1} H_3BO_3, 1 μmol L^{-1} Mn^{2+}, 0.5 μmol L^{-1} Cu^{2+}, 0.1 μmol L^{-1} MoO_4^{2-}. The pH was adjusted to 6.0 and the nutrient solution was replaced twice every week. Plants were grown in a growth chamber (Fitoclima, Portugal) at 22° C, with 70% of relative humidity and a light-dark cycle of 12:12 h. The photon flux density was 270 mol m^{-2} s^{-1}. After 6 weeks, when new roots and leaves had developed, plants of uniform size (approximately 30 cm height) were selected to be used in the assay setup.

2.3.2. IB removal assay

Selected plants, whose roots were rinsed with a dilute hypochlorite solution in order to diminish the native microbial population, were transferred to 3 L plastic vessels (3 plants per vessel) which contained aerated modified Hoagland nutrient solution spiked with 20 μg L^{-1} of IB. A control assay, without plants, was set up to evaluate IB photodegradation and the possible effect of IB adsorption on the plastic vessel walls. All assays were done in triplicate. Samples of nutrient solution were collected after 6h, 12h, 18h and 24h of exposure during the first day, and then every 24h during a period of 7 days and, finally, after 14 and 21 days. IB removal by the plants was evaluated along these periods by quantification of remaining IB in the collected samples, following the optimized analytical methodology that had been previously developed.

2.3.3. Toxicity assays

A setup similar to the one just described was used in toxicity assays, but in this case nutrient solutions were spiked with IB at 0.5, 1.0 and 2.0 mg L^{-1} concentrations, prepared from a stock aqueous solution of 50 mg L^{-1}. Control plants were grown in nutrient solution without IB. For each of the three IB concentrations as well as for the control, three assays were setup corresponding to each one of the three exposure times studied (7, 14 and 21 days), thus making a total of twelve assays. Each assay corresponding to a single exposure time was performed using three replicate vessels. The vessels were arranged as a completely randomized factorial design. At the end of each exposure time the nutrient solution samples were analyzed and plants were removed, leaves and roots were separated, immediately frozen in liquid nitrogen and stored at -80°C for posterior analyses.

2.4. Plant growth parameters

Fresh-plant weights were measured at different IB exposure times (7, 14 and 21 days) for each of the different initial IB concentrations tested. Visual inspection of injury symptoms was also recorded. Relative growth rates (RGR) were calculated according to the equation:

$$RGR = (\ln W_1 - \ln W_0)/(t_1 - t_0) \qquad (2)$$

where W_0 and W_1 are, respectively, the initial and final weights of plants and $(t_1 - t_0)$ is the duration of the experiment.

2.5. Chlorophyll and carotenoids contents

Concentrations of chlorophyll and carotenoids were determined in an 80% (v/v) aqueous acetone extract using a modified method of Lichtenthaler and Wellburn (1983). Chlorophyll content results are expressed in mg g^{-1} of fresh weight (FW) and calculated using extinction coefficients and the equations given by Porra (2002). Carotenoids content results are expressed in mg g^{-1} FW and calculated using extinction coefficients and the equations given by Lichtenthaler and Wellburn (1983). Three replicate measurements were done for each treatment vessel.

2.6. Activities of antioxidant enzymes

2.6.1. Enzyme extraction procedure

Plant material, both leaves and roots, (1.0 g each from each treatment vessel) was ground to a fine powder in a cold glass mortar with 4.0 g of acid washed sand and homogenized in 100 mM Tris-HCl buffer solution (pH = 7.5) containing 1 mM EDTA, 3 mM 1,4–dithiothreitol and 2% (w/v) polyvinylpolypyrrolidone. The homogenates were centrifuged at 12 000 *g* for 30 minutes at 4° C and the resulting supernatants were used for the determinations of the enzymes activities. All steps in the preparation of the enzymes extracts were carried out at 0 – 4° C.

2.6.2. Catalase (CAT) (EC 1.11.1.6)

CAT activity was measured according to the method of Aebi (1984). The activity was determined by monitoring the decrease in absorbance due to H_2O_2 reduction at 240 nm for 2 minutes. CAT activity was defined as the amount of enzyme required to catalyze the dismutation of 1 μmol of H_2O_2 per minute and the results were expressed as U mg^{-1} FW.

2.6.3. Superoxide dismutase (SOD) (EC 1.15.1.1)

SOD activity was assayed by the Ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich, 1969). SOD activity was measured as the inhibition of the rate of reduction of Cytochrome C by the superoxide radical, observed at 550 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the Cytochrome C reduction and the results were expressed as U mg^{-1} FW.

2.6.4. Guaiacol peroxidase (GPX) (EC 1.11.1.7)

GPX activity was determined according to the method adopted by Bergmeyer (1983). The activity was determined by monitoring the increase in absorbance due to the formation of the reaction product tetraguaiacol at 420 nm for 2 minutes. One unit of GPX activity is defined as the amount of enzyme required for catalyzing the oxidation of 1 μmol of guaiacol in 1 minute and the results were expressed as U mg^{-1} FW.

2.7. Statistical analysis

Data were analyzed through one-way analysis of variance (ANOVA) for comparison of recoveries obtained by different SPE cartridges and conditions as well as for comparisons of effects due to IB exposure on physiological and biochemical parameters

(RGR, photosynthetic pigments and enzymatic activities) with those of the control assays. Comparisons were considered significantly different for $P < 0.05$.

3. Results and discussion

3.1. Development and validation of the analytical methodology

The evaluation of IB removal by *Typha* requires the setup of a chromatographic method for quantification of remaining IB in the plant's nutrient solutions. Nowadays, the mass spectrometry (MS) detector is usually preferred to other liquid chromatography (LC) detectors due to the lower instrumental quantification limits and possibility for analyte identification. However, a significant drawback associated with the use of the MS detector is that the electrospray interface (ESI) is highly susceptible to other components in the matrix, which may result in signal suppression (more often) or signal enhancement leading to erroneous results (Gros et al., 2006; Petrovic and Barceló, 2007). HPLC-UV when coupled with an appropriate method for analyte concentration such as SPE can be a suitable and less expensive alternative for determination of trace organics such as IB (Gros et al., 2006; Petrovic and Barceló, 2007), and was used throughout this work.

Different SPE sorbent materials for performing analyte preconcentration were thus tested, including a non polar sorbent (LiChrolut® RP-18 and Sep-Pak® Vac), a polymeric sorbent (Oasis® HLB) and a mixed polymeric and cation-exchange sorbent (Oasis® MCX). As shown in Figure 1, the behavior of the silica based SPE cartridges was different from the Oasis™ polymer cartridges with the first ones yielding better analyte recoveries at higher pH values and performing equally poor at the pH of 2. The highest IB recoveries were attained with the silica based cartridges with sample pH of 7. Lowering the sample pH lead to lower recoveries for the silica based cartridges while the best recoveries attained for the Oasis™ cartridges was achieved at sample pH of 5, which were nevertheless still lower than those obtained with the silica based cartridges. This was a somehow unexpected result because previously published results indicate that Oasis® HLB cartridges achieve better yields for the majority of the pharmaceutical residues extracted from water samples, including IB (Gros et al., 2006; Petrovic and Barceló, 2007). Lowering the sample pH for the extraction of acidic compounds is not always required and can even have a negative effect in the analytes recoveries as was also previously observed by Gros et al. (2006).

Taking into consideration the obtained results, LiChrolut® cartridges were chosen to be used throughout this study as it was the less expensive option of the two silica based SPE cartridges, given that both yielded essentially the same IB recoveries. For further validation of the SPE procedure, additional tests were performed with the select cartridge type and using different sample volumes. These tests showed (Table 1) that increasing sample volumes up to a factor of 100 (thereby increasing the amount of salts percolated through the SPE cartridge) has a negligible influence in the IB recoveries.
The HLPC methodology developed was found to have high repeatability and reproducibility (RSD < 1% in both cases) as well as low values of IQL and IDL (60.0 and 18.0 μg L^{-1} respectively). However, this IQL was not sufficiently low to perform the desired IB quantification. When coupled with the SPE pre-concentration, the limit of quantification of the entire analytical method, LOQ (calculated with equation 1) was found to be 0.3 μg L^{-1}, which is low enough for IB quantification in nutrient solutions. The entire analytical methodology was also found to be highly reproducible (RSD < 1%).

3.2 Removal efficiency and toxicity of ibuprofen

Typha spp. is an emergent macrophyte which has been frequently used in CWs to depurate water contaminated with organic compounds and has shown a good tolerance when exposed to some xenobiotic substances including some pharmaceuticals (Wilson et al., 2000; Williams, 2002; Haberl et al., 2003; Amaya-Chávez et al., 2006; Dordio et al., 2008). In order to assess its ability to remove IB from contaminated water, an assay was conducted by spiking the cattail nutrient solution with 20 μg L^{-1} of IB which is a value within the range of IB concentrations detected in the environment (Fent et al., 2006; Santos et al., 2007; Petrovic and Barceló, 2007; Aga, 2008) and typically used in biological removal studies for this kind of pollutants (Matamoros et al., 2005; Matamoros et al., 2008).
As shown in Figure 2A, cattail was able to remove nearly all IB present in the nutrient solution. In fact, just within the first 24 h as much as 58% of IB was readily removed by *Typha* and over 95% after 96 h. By the end of the assay (after 21 days of exposure) IB had been almost completely removed from the nutrient solution (> 99% removal). When compared to the removal capacity displayed by *Typha* for other acidic pharmaceutical compounds (Dordio et al., 2008), IB's removal by this plant species is exceptionally

high, suggesting the potential of *Typha* for removal of this type of compounds from water.

IB removal kinetics, as is also shown in Figure 2A, is characterized by a fast initial stage occurring within the first period of 96 hours, which can be described as a first-order process that fits the equation:

$$\ln[\mathrm{IB}]_t = \ln[\mathrm{IB}]_0 - 0.032\,\mathrm{h}^{-1}\,t \qquad (R^2 = 0.994)$$

where $[\mathrm{IB}]_t$ and $[\mathrm{IB}]_0$ are the IB concentrations (μg L^{-1}) at any given time (t) and at the beginning, respectively.

After 96 h IB removal proceeds at a much slower rate, the curve in Figure 2A being nearly flat beyond this period. A slight increase in removal efficiency (from 95% to 99%) is obtained only if exposure time is extended over a much longer period (up to 504 h). This kinetic behavior is frequently observed in other studies on organic xenobiotics removal by *Typha* and other plant species (Sun et al., 2004; Amaya-Chávez et al., 2006; Olette et al., 2008). The kinetic profile of IB removal is very similar to that observed for the removal of another pharmaceutical, clofibric acid, using the same plant (Dordio et al., 2008) but IB removal progressed at a faster rate.

Xenobiotics removal in hydroponic systems may be attributed to abiotic and biotic processes. However, according to the observations in control assays without plants (where variation of IB concentration in the nutrient solution was negligible for long periods of time), the influence of abiotic processes such as volatilization, adsorption to vessel walls, photodegradation and hydrolysis was minimal under the assays conditions. IB removal should, therefore, result mainly from adsorption on the plant rhizomes and from biotic processes such as plant uptake. Microbial degradation should also play an important role under ordinary conditions (and may even be enhanced by rhizostimulation) but, in the current study, care was taken to diminish the microorganisms populations by sterilization of the roots and materials used. Therefore, microbial degradation should have a very limited contribution in the removal efficiencies reported in these studies, and the system's behavior can be mainly attributed to the plants action.

Synthetic organic compounds like IB are xenobiotic to plants, which do not have specific transporters in their cell membranes for these compounds. Therefore, organic pollutants tend to move into and within the plant tissues driven simply by diffusion, dependent on their chemical properties, especially on their hydrophobicity (Dietz and

Schnoor, 2001; Stottmeister et al., 2003; Pilon-Smits, 2005). Compounds with log K_{ow} between 1 and 3.5 are highly bioavailable to roots of vascular plants such as cattails because they are lipophilic enough to move through the lipid bilayer of membranes, and still water soluble enough to travel into the cell fluids (Dietz and Schnoor, 2001; Pilon-Smits, 2005). IB is a compound only moderately hydrophobic with a log K_{ow} of 2.48 (Scheytt et al., 2005) and, probably, it is partially adsorbed onto roots, taken up and translocated within the plant, where it is accumulated or transformed by the plants metabolic system.

The exposure to xenobiotics is a cause of abiotic stress which induces an excessive production of reactive oxygen intermediates (ROIs). ROIs are toxic by-products of aerobic metabolism whose enhanced production during stress can be viewed as a cell signal for the activation of stress-response and defense pathways, namely the enzymatic antioxidant system. Measurements of the alterations in key antioxidant enzymes activities in different types of plant tissues may therefore be a useful indication, not only of the plant's reaction to the oxidative stress, but also an evidence for the xenobiotics translocation within the plant.

In order to assess cattail toxicity at increasing IB concentrations, 3 new assays were thus set up using the same conditions tested before but with nutrient solution spiked with IB at concentrations of 0.5, 1.0 and 2.0 mg L^{-1}. These concentrations were chosen as a set of test cases bordering visually evident IB toxicity, as visual symptoms of toxicity (chlorosis) begin to appear when plants are exposed to IB concentrations slightly above these levels (data not shown). However, for the tested range of concentrations no visual signs of toxicity were observed in *Typha* spp. and, in fact, cattail was not only able to cope with the large amounts of IB in their nutrient solutions but continued to remove it. Maximum percentage of IB removal in these high concentration assays ranged from 95% for the IB initial concentration of 2.0 mg L^{-1}, to 98% for the assay with IB initial concentration of 0.5 mg L^{-1} by the end of the assays. When comparing these removals with those of 20 μg L^{-1} assay, they clearly fit a linear relationship between the initial IB concentrations and the IB removed at every corresponding exposure time, with the exception of the values for the 7-day period of exposure for the most concentrated (2.0 mg L^{-1}) IB solution (Figure 2B). As will be shown later, the lower IB removal observed is in agreement with the toxicity effects inferred from the plant's lower relative growth rates and the measured enzymatic activity for this period of exposure.

Relative growth rates (RGR) of the plants exposed to the tested concentrations of IB were significantly below those of the control plants after 7 days of exposure and, with exception of the 0.5 mg L^{-1} assay, were still smaller even after 14 and 21 days of exposure (Figure 3). However, for increasing exposure periods, the RGR of the plants exposed to IB showed a trend of approach towards the average RGR levels of the control plants that were not exposed to IB. This indicates that the presence of IB affects the normal plant's growth but plants seem to be able to cope with the toxic effects of this substance because not only do they remove it extensively from the solution (Figure 2B) but also they show an evolution towards a recovery to normal growth rates beyond a period of 21 days of exposure (Figure 3).

Chlorophyll (total, *a* and *b*) and carotenoid contents were also determined at the end of the experiments (Table 2). No statistically significant differences were found in the carotenoid and chlorophyll *b* contents of the plants exposed to IB from those of the control assay. However, for chlorophyll *a* and total chlorophyll contents, statistically significant differences were observed between the plants exposed to IB concentrations of 2.0 mg L^{-1} and those of the control assay, whereas the assays at 0.5 mg L^{-1} and 1.0 mg L^{-1} of IB remained not statistically different from the control. The lower contents of these pigments in the 2.0 mg L^{-1} assay may be a sign of toxicity which still subsisted after 21 days of exposure when almost all the IB had been removed from solution.

The enzymatic antioxidant system is one of the protective mechanisms to eliminate or reduce ROIs excess, whose activity levels may serve as an indication of the stress levels to which the plants are subjected. The fact that *Typha* seemed to cope with high amounts of IB in the nutrient solution may be revealing that the expected production of excessive ROIs was effectively counteracted by the antioxidant enzymatic system. This involves the sequential and simultaneous actions of a number of enzymes including SOD, CAT and peroxidases (POX).

In order to better characterize the antioxidant response put forward by *Typha* in reaction to the stress caused by the plant's exposure to IB, the enzyme activities of SOD, CAT and the GPX were measured in root and leaf tissues of those plants and compared to those of the control assays' plants.

SOD is considered to be in the first line of defense against ROIs damage. This enzyme occurs in various cell compartments and acts against the superoxide radicals (O_2^-, which are formed in different cell compartments and can be precursors to other ROIs) by catalyzing their dismutation into H_2O_2 and O_2 (Alscher et al., 2002). Actually,

superoxide dismutase is a group of enzymes (several isoforms exist in plant species), so a general activity of all these together is measured in plant extracts. An increase in SOD activity was observed in the plants exposed to IB solutions in comparison with that of the control plants. This increase, which was observed both in leaves and roots (Figures 4A and 4B), can be attributed to an increase in superoxide radicals within the cells. This enzyme's activity is higher for the highest IB concentration and for the longest exposure time. The continuous increase in SOD activity may be an evidence of the stress caused by the uptake and accumulation of the xenobiotic or its degradation products within the plant tissues leading to the consequent production of superoxide radicals which stimulates the enzymatic response.

The cooperation between SOD and H_2O_2-eliminating enzymes plays an important role in the resistance of plants to environmental stress (Apel and Hirt, 2004). Uncontrolled levels of H_2O_2 produced by the action of SOD can lead to the generation of $OH^{\bullet}$ via Haber-Weiss reactions and these can lead to other chain reactions which can cause serious damage within the cells. CAT and peroxidases functioning in different cell compartments can be activated in order to maintain appropriate H_2O_2 levels (Mittler, 2002; Apel and Hirt, 2004). CAT is found mainly in peroxisomes where it decomposes H_2O_2 into H_2O and O_2 (Alscher et al., 2002). Peroxidases can participate in lignin biosynthesis and convert H_2O_2 to water. In this process, a wide range of electron donor substrates can be used, for example guaiacol or ascorbic acid, and correspondingly there are several different peroxidases such as GPX or ascorbate peroxidase (Mittler, 2002). Among these, GPX can be considered one of the key enzymes since both of its extra- and intracellular forms participate in the breakdown of H_2O_2 (Foyer et al., 1994).

The activities of CAT and GPX are affected in a way which is different from that observed for SOD (Figures 4 C, D, E and F). An increase in CAT and GPX activity is observed in leaves and roots of the plants subjected to 0.5 mg L^{-1} of IB. Top activities of CAT and GPX are attained at this IB concentration, and increasing the concentration beyond 0.5 mg L^{-1} almost always leads to a diminishing of both enzymes activities when compared to the 0.5 mg L^{-1} assay. Nevertheless, CAT and GPX activities in plants exposed to 1.0 mg L^{-1} of IB are in general still higher than those of the control plants. On the other hand, for plants exposed to 2.0 mg L^{-1} of IB, the activities of these enzymes are in some cases lower than the control activities levels, which reveals some inhibition induced by IB's presence. CAT and GPX inhibition is also more evident at the 7-day exposure, when IB concentrations in the nutrient solution are higher.

GPX seems to be more affected by increasing amounts of IB in the nutrient solution, as the differences in the enzyme activity between the 0.5 and 1.0 mg L^{-1} assays are more evident than those observed for the CAT enzyme (see Figure 4 C, D, E and F).

The strong induction of CAT and GPX enzyme activity at the lower IB concentration followed by the decrease of the activity at higher IB concentrations suggests that the enzymatic system is failing and IB toxicity is affecting the plant. This observation comes in agreement with the observed diminishment in RGR and photosynthetic pigments content in plants grown in the most IB concentrated nutrient solutions.

The trends observed in the enzyme activity alterations are not very different in the roots and in the leaves of the plants exposed to IB, suggesting that both tissues are affected by the xenobiotic. It is also interesting to note that despite the fact that there are only small amounts of IB in the nutrient solution during the third week of assays, the enzyme activity of the plants is still altered when compared to the control and overall presents the same trend observed at 7 and 14 days. This fact is illustrative of the long term effects caused by IB which might be an indication that either IB or its degradation products were in fact uptaken by the plant roots. In fact, considering how the antioxidant enzymatic activity in the *Typha* tissues (leaves and roots) was affected by the exposure to IB and noticing the moderate value of IB's log K_{ow}, we are led to believe that absorption of this compound into the plant occurs and the removal of IB from water is not simply due to an adsorption process on the roots surface alone. In fact, previous studies on the removal, also by *Typha*, of another acidic pharmaceutical, clofibric acid, which has a similar log K_{ow} (2.57 – 2.84 (Hernando et al., 2007)), had presented a similar behavior with leaf tissues (and not just root tissues) also exhibiting significant alterations of antioxidant enzymes activities (Dordio et al., 2008). This may be an indication that this type of compounds, with moderate values of log K_{ow}, may be readily absorbed by *Typha* roots and translocated to upper parts.

4. Conclusion

The macrophyte *Typha* spp. was able to remove nearly all IB (> 99%) after 21 days of exposure to a solution spiked with 20 μg L^{-1}, with over 58% being removed just within the first 24 h and over 95% after 96 h. Even when the plants were subjected to concentrations several orders of magnitude higher, still high IB removal efficiencies were observed. In the tested IB concentrations range, a linear relationship was observed

between initial and removed amounts of IB, except in the case where IB toxicity effects become more adverse to *Typha* (2 mg L^{-1}).

IB removal was achieved without obvious visual symptoms of toxicity, even for the highest IB concentrations to which the plants were subjected. However, relative growth rates indicate that IB inhibits *Typha*'s growth, especially for the highest concentrations in the initial exposure periods.

The enzymatic response to the abiotic stress induced by IB was assessed. SOD enzyme seems to cope with the expected oxidative burst due to the increasing amounts of IB in the nutrient solution showing a concomitant increase in its activity. CAT and GPX enzyme activities, however, do not show the same trend. The strong increase in the activity observed in the plant tissues from the 0.5 mg L^{-1} assay is less evident at the higher IB concentrations, and even an inhibition of enzymes activities was observed in some cases for the 2.0 mg L^{-1} assay, which may be an early sign of toxicity. This also comes in agreement with the diminishment in photosynthetic pigments content observed at the end of the assays for the plants grown in the solutions with 2.0 mg L^{-1} of IB.

The results presented here show the potential of *Typha* to remove IB from contaminated waters, and come in agreement with the results obtained with clofibric acid, which is another acidic pharmaceutical compound (Dordio et al., 2008).

The physical-chemical similarities between the compounds already studied suggests the potential use of *Typha* in phytoremediation technologies such as CWs for removal of a wide range of related xenobiotics from contaminated waters.

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Table 1

Influence of sample volume on the IB recoveries (± SD, n = 3) from spiked nutrient solutions obtained using LiChrolut® RP-18 cartridges. ANOVA significant at $P < 0.05$ when compared with control. Different letters indicate significantly different values.

Sample volume	2 mL	10 mL	20 mL	50 mL	200 mL
% IB Recovery	99.3 ^{a} ± 1.0	98.6 ^{a} ± 1.2	97.8 ^{a} ± 2.5	99.4 ^{a} ± 1.8	98.6 ^{a} ± 0.9

Table 2

Average values of photosynthetic pigments contents of *Typha* spp. after 21 of exposure to IB (n = 9). ANOVA significant at $P < 0.05$ when compared to control. Different letters indicate significantly different values.

IB concentration (mg L^{-1})	Chlorophyll total (mg g^{-1} FW)	Chlorophyll *a* (mg g^{-1} FW)	Chlorophyll *b* (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
0	1.6813 ^{a} ± 0.0185	1.444 ^{a} ± 0.032	0.237 ^{a} ± 0.031	0.397 ^{a} ± 0.013
0.5	1.6966 ^{a} ± 0.0068	1.429 ^{a} ± 0.019	0.268 ^{a} ± 0.017	0.406 ^{a} ± 0.010
1.0	1.6855 ^{a} ± 0.0087	1.419 ^{a} ± 0.021	0.267 ^{a} ± 0.019	0.416 ^{a} ± 0.010
2.0	1.2037 ^{b} ± 0.0004	1.314 ^{b} ± 0.009	0.233 ^{a} ± 0.011	0.396 ^{a} ± 0.005

Figures

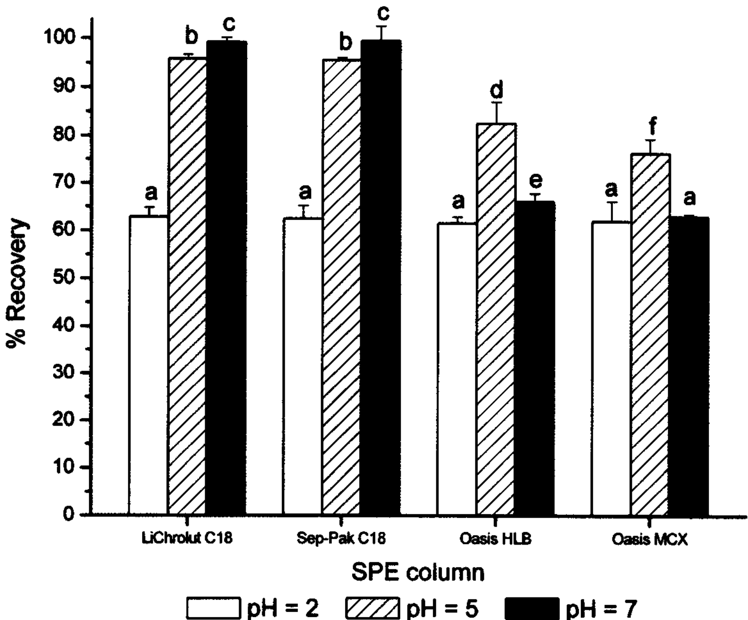

Fig. 1. Influence of different SPE materials and pH adjustment (2, 5 and 7), on the ibuprofen recovery from plant nutrient solution. Vertical error bars indicate ±SD (*n* = 5). ANOVA significant at P < 0.05 when compared with control. Different letters indicate significantly different values.

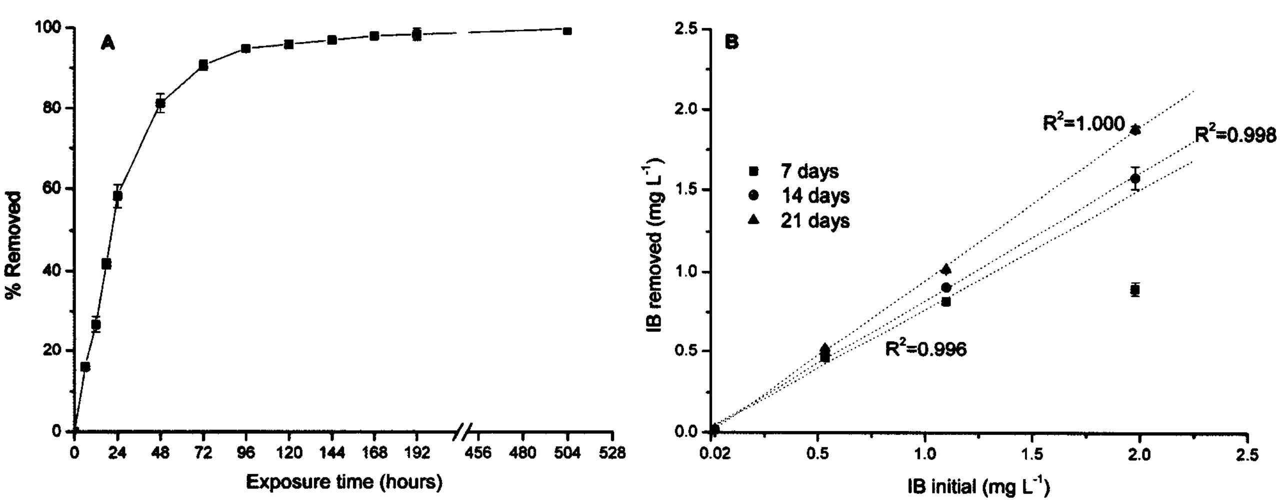

Fig. 2. Ibuprofen removal by *Typha* spp.: (A) percent removal as function of the exposure time, for an initial concentration of 20 μg L^{-1}; (B) IB removed after 7, 14 and 21 days versus the initial IB concentration. Error bars represent ± SD (n = 9).

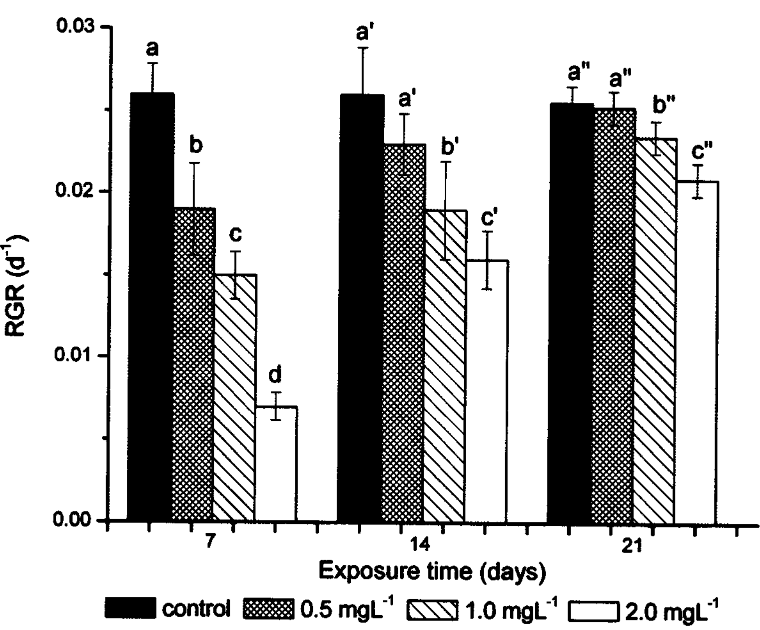

Fig. 3. Relative growth rates of plants exposed to IB at concentrations of 0.5, 1.0 and 2.0 mg L^{-1}. Vertical error bars indicate ±SD (*n* = 9). ANOVA significant at P < 0.05 when compared with control. Different letters indicate significantly different values.

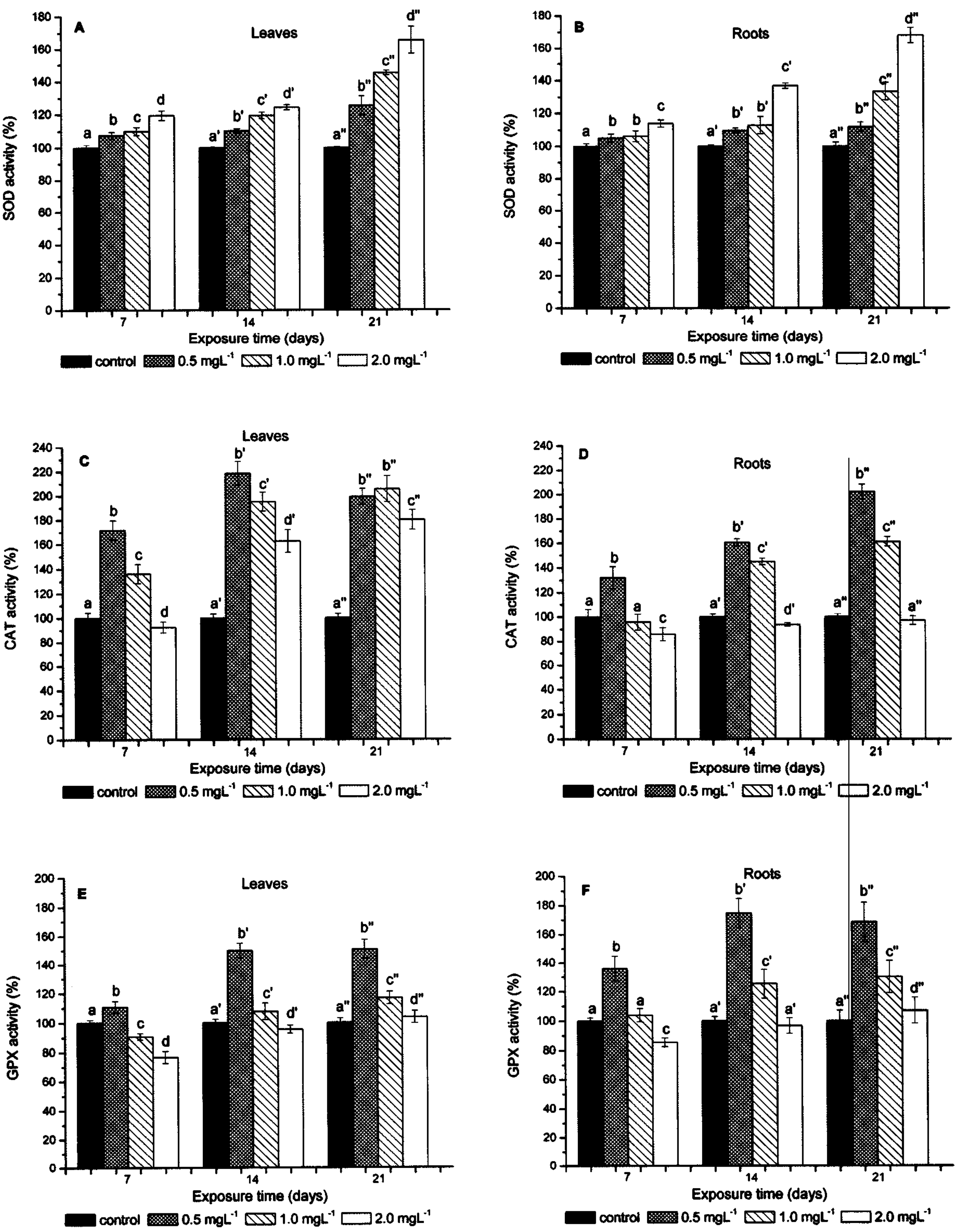

Fig. 4. Effects of ibuprofen treatment on the enzymatic activities of the antioxidant enzymes superoxide dismutase (A, B), catalase (C, D) and guaiacol peroxidase (E, F) of *Typha* spp. Side by side are represented the activities measured in the leaves (left) and in the roots (right). The activity was expressed relative to the activity in control plants (100%). Vertical error bars indicate ± SD (n = 9). For each exposure time, ANOVA significant at $P < 0.05$ when compared with control; different letters indicate significantly different values.

Chapter 4

4. Studies for the evaluation of pharmaceuticals removal efficiency in SSF-CW microcosms based on LECA and planted with *Typha* spp.

Article 1

Title: Removal of pharmaceuticals in microcosm constructed wetlands using *Typha* spp. and LECA

Authors: Ana Dordio, A. J. Palace Carvalho, Dora Martins Teixeira, Cristina Barrocas Dias, Ana Paula Pinto

Submitted to: Bioresource Technology (BITE-S-09-02355)

Article 2

Title: Atenolol removal in microcosm constructed wetlands

Authors: Ana Dordio, José Pinto, Cristina Barrocas Dias, Ana Paula Pinto, A. J. Palace Carvalho, Dora Martins Teixeira

Accepted for publication by: International Journal of Environmental Analytical Chemistry

Motivations

The removal of pollutants in a constructed wetlands system is achieved through the concerted action of all its components. The overall efficiency of the whole system is higher than of all its components' considered separately. The careful selection of its components is an important aspect of a constructed wetlands system optimization.

The present chapter discusses the efficiency of a microcosm constructed wetlands system (CWS) to remove selected pharmaceuticals, namely clofibric acid, carbamazepine, ibuprofen and atenolol from contaminated wastewaters. The CWS was assembled with the components evaluated in previous studies, i.e. using LECA as solid matrix, and planted with *Typha* spp. plants. The performance of planted and unplanted beds is compared in order to assess the relative importance of the solid matrix and the vegetation components in the overall system's performance. In order to evaluate the effects of seasonal variability in the performance of these microcosm systems, assays were conducted for clofibric acid, carbamazepine and ibuprofen in two different periods, one corresponding to a warm dry season and the other to a cold humid one.

For the quantifications of the studied pharmaceuticals in spiked wastewater samples, an analytical methodology was developed and optimized. The method used for quantification of studied pharmaceuticals in aqueous samples included the following steps: isolation and pre-concentration with solid phase extraction (whenever necessary), separation by liquid chromatography (HPLC) and detection/quantification by UV/Vis spectrometry.

4.1. Pharmaceuticals removal in CWS

In spite of the scarce number of studies conducted so far on pharmaceuticals removal by CWS, it is, nevertheless, possible to make some observations regarding the behavior of some compounds already studied, considering in particular the distinction between biodegradable and non-biodegradable pharmaceuticals, and establishing comparisons with the behavior of the same compounds in conventional WWTPs.

Easily biodegradable pharmaceuticals such as ibuprofen, naproxen, acetylsalicylic acid, caffeine and acetaminophen are eliminated in very similar (high) degree in CWS and in WWTPs (Table 4.1). Removal of these compound in WWTPs is mostly attributed to the action of the microorganisms. In CWS they are most effectively removed by the biotic components which may include the action of the rhizostimulated microbial populations, but also their uptake by plants, depending on the compounds properties (Matamoros et al., 2005; Park et al., 2009). Some moderately biodegradable compounds such as atenolol, show an increased removal in CWS in comparison with WWTPs which may

be attributed to an enhancement of biodegradation conditions or the contribution of additional routes of elimination provided by the vegetation or sorption to the solid matrix (Conkle et al., 2008; Park et al., 2009).

On the other hand, pharmaceuticals that are not as easily biodegraded and tend to exhibit a recalcitrant behavior in WWTPs, such as carbamazepine, clofibric acid or diclofenac may also, in some cases, have reasonable removals in CWS (Table 4.1). For these compounds, sorption processes appear as the suggested main removal mechanism although other processes, such as plant uptake, may also contribute with a significant role if pharmaceutical physico-chemical properties and environmental conditions are adequate (Matamoros et al., 2005; Imfeld et al., 2009; Park et al., 2009).

In general, it is observed that pharmaceuticals are removed in CWS at least with similar efficiencies as in WWTPs or higher (Table 4.1). Biodegradable compounds are more extensively removed than non-biodegradable ones, but since essentially the same processes are responsible for removal of biodegradable compounds in WWTPs and in CWS, the potential advantages of CWS processes are more noticeable in the removal of some non-biodegradable pharmaceuticals (Imfeld et al., 2009).

In some studies the effect of seasonal climate conditions has been evaluated and it was observed to also affect the overall efficiency of CWS (Matamoros et al., 2008b). These effects are, in general, more pronounced for biodegradable pharmaceuticals than non-biodegradable ones.

Higher temperatures usually lead to improved removals especially in the cases where biodegradation is the main mechanism, which can be attributed to the effect of increasing microbial activity. Higher relative humidity, on the other hand, has been associated with lower uptake by plants which is related to a decrease in transpiration rates. Seasons with longer sun periods and more intense solar radiation may also have a relevant contribution to the removal of less photostable compounds through photodegradation, especially in FWS-CWS (Matamoros et al., 2008b). In subsurface flow systems, however, as the water level is below the matrix level, this effect is less pronounced or even negligible. Seasonal efficiency variations are more attenuated in CWS than in WWTPs as in the former case the whole system (the solid matrix, the vegetation, accumulation of plant debris on the bed's surface) provide a moderation of the environmental conditions.

The prevalence of aerobic conditions in the wetlands promote biochemical pathways, such as aerobic respiration, that are more efficient in removing most compounds than

anaerobic pathways. In some cases, the existence of aerobic conditions is considered as vital for ensuring a high degree of removal of some compounds such as ibuprofen in SSF-CWS (Matamoros et al., 2005; Matamoros et al., 2008a). Therefore, the level of dissolved oxygen is an important parameter for the CWS performance, and this is also influenced by climatic conditions, especially temperature, as well as the type of CWS (FWS or SSF) and operating conditions such as the flooding rate.

Studies conducted so far on the removal of pharmaceuticals in CWS have shown the potential of these systems to remove a wide variety of compounds (Table 4.1). However, there is still ample work of optimization to be carried out on these systems, which must be based on a better understanding of how the several processes involved perform their functions and interoperate. A more profound characterization of the roles played by each CWS component in the overall pharmaceuticals removal efficiency, related with the properties of each substance involved, is also necessary to guide an optimal selection of each component. The available studies on this subject, although providing valuable information, are still scarce and further work is still necessary.

Table 4.1. Pharmaceuticals removal in different types of CWS

Organic compound	Physico-chemical properties			Type of CWS [c]	Type of substrate /plant [d]	% Removed [e]	Removal processes suggested by authors	References
	S_W [*,a] (25°C) (mg L^{-1})	$\log K_{ow}$ [*,b] (25° C)	pKa [*]					
Ibuprofen	21	3.97	4.9	HSSF	Gravel/ *Phragmites australis*	48 (deep) 81 (shallow)	Microbial degradation, sorption	(Matamoros et al., 2005)
				HSSF	Gravel/ *Phragmites australis*	71	ibid.	(Matamoros and Bayona, 2006)
				VSSF	Gravel/ *Phragmites australis*	99	ibid.	(Matamoros et al., 2007a)
				HSSF	Gravel/ *Phragmites australis*	52	ibid.	(Matamoros et al., 2008a)
				HSSF	n.d.	65	ibid.	(Matamoros et al., 2009)
				VSSF	n.d.	89	ibid.	
				FWS	*Typha* spp. + *Phragmites australis*	95 (Winter) 96 (Summer)	ibid.	(Matamoros et al., 2008b)
				Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	> 99	n. d.	(Conkle et al., 2008)
				WWTP (a.s.)	-	60-100	-	(see Table 1.4, page 22)
Carbamazepine	17.7	2.45	14	HSSF	Gravel/ *Phragmites australis*	26 (deep) 16 (shallow)	Sorption	(Matamoros et al., 2005)
				HSSF	Gravel/ *Phragmites australis*	16	ibid.	(Matamoros and Bayona, 2006)
				VSSF	Gravel/ *Phragmites australis*	26	ibid.	(Matamoros et al., 2007a)
				HSSF	Gravel/ *Phragmites australis*		ibid.	(Matamoros et al., 2008a)
				HSSF	n.d.	38	ibid.	(Matamoros et al., 2009)
				FWS	*Typha* spp. + *Phragmites australis*		ibid.	(Matamoros et al., 2008b)
				FWS	*Acorus* + *Typha* spp.	65	Plant uptake	(Park et al., 2009)
				WWTP (a.s.)	-	0-45	-	(see Table 1.4, page 22)

* PHYSPROP, 2009; [a] S_w=Water solubility; [b] K_{ow}=Octanol-water partition coefficient; [c] CWS: Constructed wetlands system; HSSF: Horizontal subsurface flow; VSSF: Vertical subsurface flow; FWS: Free Water Surface; [d] n.d.: not detailed; [e] n.r.: not removed

Table 4.1. Pharmaceuticals removal in different types of CWS (cont.)

Organic compound	Physico-chemical properties			Type of CWS [c]	Type of substrate /plant [d]	% Removed [e]	Removal processes suggested by authors	References
	S_W [*,a] (25°C) (mg L^{-1})	$\log K_{ow}$ [*,b] (25° C)	pKa [*]					
Clofibric acid	583	2.57	3.18	HSSF	Gravel/ *Phragmites australis*	n.r.	-	(Matamoros et al., 2005)
				FWS	*Typha* spp. + *Phragmites australis*	32 (Winter) 36 (Summer)	n.d	(Matamoros et al., 2008b)
				WWTP (a.s.)	-	0-40	-	(see Table 1.4, page 22)
Atenolol	13300	0.16	9.6	Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	> 99	n.d.	(Conkle et al., 2008)
				FWS	*Acorus* + *Typha* spp.	97	n.d	(Park et al., 2009)
				WWTP (a.s.)	-	0-76	-	(see Table 1.4, page 22)
Naproxen	15.9	3.18	4.15	HSSF	Gravel/ *Phragmites australis*	85	Microbial degradation, sorption	(Matamoros and Bayona, 2006)
				VSSF	Gravel/ *Phragmites australis*	89	ibid.	(Matamoros et al., 2007a)
				HSSF	n.d.	45	ibid.	(Matamoros et al., 2009)
				VSSF	n.d.	92	ibid.	
				FWS	*Typha* spp. + *Phragmites australis*	52 (Winter) 92 (Summer)	ibid.	(Matamoros et al., 2008b)
				WWTP (a.s.)	-	40-55; 82	-	(Matamoros et al., 2009) [f]
Ketoprofen	51	3.12	4.45	HSSF	Gravel/ *Phragmites australis*	38	Sorption	(Matamoros and Bayona, 2006)
				HSSF	n.d.	90	ibid.	(Matamoros et al., 2009)
				VSSF	n.d.	n.r.	ibid.	
				FWS	*Typha* spp. + *Phragmites australis*	97 (Winter) 99 (Summer)	Photodegradation	(Matamoros et al., 2008b)
				WWTP (a.s.)	-	54	-	(Matamoros et al., 2009) [f]

* PHYSPROP, 2009; [a] S_w=Water solubility; [b] K_{ow}=Octanol-water partition coefficient; [c] CWS: Constructed wetlands system; HSSF: Horizontal subsurface flow; VSSF: Vertical subsurface flow; FWS: Free Water Surface; [d] n.d.: not detailed; [e] n.r.: not removed [f] and references therein

Table 4.1. Pharmaceuticals removal in different types of CWS (cont.)

Organic compound	Physico-chemical properties			Type of CWS [c]	Type of substrate /plant [d]	% Removed [e]	Removal processes suggested by authors	References
	S_W [*,a] (25°C) (mg L^{-1})	$\log K_{ow}$ [*,b] (25° C)	pKa [*]					
Diclofenac	2.4	4.51	4.15	HSSF	Gravel/ Phragmites australis	15	Sorption	(Matamoros and Bayona, 2006)
				VSSF	Gravel/ Phragmites australis	73	ibid.	(Matamoros et al., 2007a)
				HSSF	n.d.	21	ibid.	(Matamoros et al., 2009)
				FWS	Typha spp. + Phragmites australis	73 (Winter) 96 (Summer)	ibid.	(Matamoros et al., 2008b)
				WWTP (a.s.)	-	17	-	(Matamoros et al., 2009) [f]
Caffeine	21600	-0.07	10.4	HSSF	Gravel/ *Phragmites australis*	97	Microbial degradation, sorption	(Matamoros and Bayona, 2006)
				VSSF	Gravel/ *Phragmites australis*	99	ibid.	(Matamoros et al., 2007a)
				HSSF	n.d.	97	ibid.	(Matamoros et al., 2009)
				VSSF	n.d.	99	ibid.	
				Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	> 99	n.d.	(Conkle et al., 2008)
				WWTP (a.s.)		99	-	(Matamoros et al., 2009) [f]
Salicylic acid	2240	2.26	2.97	HSSF	Gravel/ *Phragmites australis*	96	Microbial degradation, sorption	(Matamoros and Bayona, 2006)
				VSSF	Gravel/ *Phragmites australis*	98	ibid.	(Matamoros et al., 2007a)
				HSSF	n.d.	95	ibid.	(Matamoros et al., 2009)
				VSSF	n.d.	87	ibid.	
				WWTP (a.s.)	-	99	-	(Matamoros et al., 2009) [f]

* PHYSPROP, 2009; [a] S_w=Water solubility; [b] K_{ow}=Octanol-water partition coefficient; [c] CWS: Constructed wetlands system; HSSF: Horizontal subsurface flow; VSSF: Vertical subsurface flow; FWS: Free Water Surface; [d] n.d.: not detailed; [e] n.r.: not removed [f] and references therein

Table 4.1. Pharmaceuticals removal in different types of CWS (cont.)

Organic compound	Physico-chemical properties			Type of CWS [c]	Type of substrate /plant [d]	% Removed [e]	Removal processes suggested by authors	References
	S_W [*,a] (25°C) (mg L^{-1})	$\log K_{ow}$ [*,b] (25° C)	pKa [*]					
Sulfamethoxazole	610	0.89		Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	91	n.d.	(Conkle et al., 2008)
				FWS	*Acorus* + *Typha* spp.	30		(Park et al., 2009)
Metoprolol	16900	1.88	9.6	Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	92	n.d.	(Conkle et al., 2008)
Sotalol	5510	0.24		Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	30	n.d.	(Conkle et al., 2008)
Acetaminophen	14000	0.46	9.38	Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	100	n.d.	(Conkle et al., 2008)
Gemfibrozil	10.9	4.77		Lagoon + VSSF	Gravel/ *Hydrocottle* spp. + *Phragmites australis*	64	n.d.	(Conkle et al., 2008)

* PHYSPROP, 2009; [a] S_w=Water solubility; [b] K_{ow}=Octanol-water partition coefficient; [c] CWS: Constructed wetlands system; HSSF: Horizontal subsurface flow; VSSF: Vertical subsurface flow; FWS: Free Water Surface; [d] n.d.: not detailed; [e] n.r.: not removed

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Title: Removal of pharmaceuticals in microcosm constructed wetlands using Typha spp. and LECA

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Keywords: Carbamazepine; Clofibric acid; Constructed Wetlands; Ibuprofen; LECA

Corresponding Author: Eng. Ana Dordio,

Corresponding Author's Institution: Universidade de Evora

First Author: Ana Dordio

Order of Authors: Ana Dordio; Alfredo P Carvalho; Dora M Teixeira; Cristina B Dias; Ana P Pinto

Abstract: Microcosm constructed wetlands systems established with a matrix of light expanded clay aggregates (LECA) and planted with Typha spp. were used to evaluate their ability to remove pharmaceuticals ibuprofen, carbamazepine and clofibric acid from wastewaters. Seasonal variability of these systems' performances was also evaluated. Overall, removal efficiencies of 96%, 97% and 75% for ibuprofen, carbamazepine and clofibric acid, respectively, were achieved under summer conditions after a retention time of 7 days. In the winter, a maximum loss of 26% in removal efficiency was observed for clofibric acid. Removal kinetics was characterized by a fast initial step (>50% removal within 6 hours) mainly due to adsorption on LECA but, on a larger timescale, plants also contributed significantly to the system's performance. Despite the fact that further tests using larger-scale systems are required, this study points to the possible application of these low-cost wastewater treatment systems for dealing with pharmaceuticals contaminated wastewater.

Suggested Reviewers: Josep M Bayona

Removal of pharmaceuticals in microcosm constructed wetlands using *Typha* spp. and LECA

Ana Dordio[a,b] *, A. J. Palace Carvalho[a,c], Dora Martins Teixeira[a,d], Cristina Barrocas Dias[a,c], Ana Paula Pinto[a,d]

[a]*Chemistry Department. University of Évora, Rua Romão Ramalho, 59, 7000-676 Évora (Portugal).*

[b]*IMAR - Institute of Marine Research Consortium, Environmental Biogeochemistry Group. University of Évora, Rua Romão Ramalho, 59, 7000-676 Évora (Portugal).*

[c]*CQE - Évora Chemistry Centre. University of Évora, Rua Romão Ramalho, 59, 7000-676 Évora (Portugal).*

[d]*ICAM - Institute of Mediterranean Agricultural Sciences. University of Évora, Herdade Experimental da Mitra, 7000-676 Évora (Portugal).*

* Corresponding author: *Tel:* +351 - 266 745343; *E-mail address*: avbd@uevora.pt

Abstract

Microcosm constructed wetlands systems established with a matrix of light expanded clay aggregates (LECA) and planted with *Typha* spp. were used to evaluate their ability to remove pharmaceuticals ibuprofen, carbamazepine and clofibric acid from wastewaters. Seasonal variability of these systems' performances was also evaluated. Overall, removal efficiencies of 96%, 97% and 75% for ibuprofen, carbamazepine and clofibric acid, respectively, were achieved under summer conditions after a retention time of 7 days. In the winter, a maximum loss of 26% in removal efficiency was observed for clofibric acid. Removal kinetics was characterized by a fast initial step (>50% removal within 6 hours) mainly due to adsorption on LECA but, on a larger timescale, plants also contributed significantly to the system's performance. Despite the fact that further tests using larger-scale systems are required, this study points to the possible application of these low-cost wastewater treatment systems for dealing with pharmaceuticals contaminated wastewater.

Keywords: Carbamazepine; Clofibric acid; Constructed Wetlands; Ibuprofen; LECA

1. Introduction

In recent years, the occurrence and fate of pharmaceutical compounds in the aquatic environment has been recognized as one of the emerging issues in environmental chemistry. Pharmaceuticals and their metabolites have been detected in wastewater, surface water, groundwater and even in drinking water worldwide (Heberer, 2002; Fent et al., 2006; Aga, 2008).

Municipal wastewater treatment plants (WWTPs) nowadays receive wastewaters that contain a lot of different trace polluting compounds, for which conventional treatment technologies have not been specifically designed. Several studies have presented evidence that some pharmaceuticals are not efficiently removed in the municipal WWTPs and are, thus, discharged as contaminants into the receiving water bodies (Heberer, 2002; Fent et al., 2006; Aga, 2008).

The widespread use of some drugs and their generally inefficient removal from wastewaters in WWTPs are the main reasons for the frequent detection of compounds such as clofibric acid (CA), carbamazepine (CB) and ibuprofen (IB) in many water monitoring studies (Heberer, 2002; Fent et al., 2006; Santos et al., 2007; Aga, 2008).

With the aim of improving the efficiency of WWTPs in removing pharmaceuticals such as these drugs, optimization of wastewater treatment processes has been attempted, e.g. by increasing hydraulic and solids residence times (Clara et al., 2005; Aga, 2008). Some advanced technologies have also been evaluated such as advanced oxidative processes, activated carbon adsorption, membrane filtration and membrane bioreactors (Fent et al., 2006; Radjenovic et al., 2007; Kim et al., 2007; Esplugas et al., 2007; Snyder et al., 2007; Benner et al., 2008; Aga, 2008). However, despite the sometimes high removal efficiencies attained, these processes are not widely used mainly for reasons of cost effectiveness (Fent et al., 2006). Consequently, there is a growing need for alternative

wastewater treatment processes for removing pharmaceuticals from waters that have higher efficiencies at reasonable costs of operation/maintenance.

Subsurface flow constructed wetland (SSFCW) systems are low cost wastewater treatment systems consisting of inundated vegetated beds, designed to emulate the well-known water depurative capacity of natural wetlands (Vymazal et al., 1998). These systems are increasingly being used to provide a form of secondary or tertiary treatment for wastewaters, and have already been used with success to remove some organic recalcitrant compounds such as pesticides, PAHs or explosives from contaminated waters (Williams, 2002; Haberl et al., 2003; Grove and Stein, 2005; Imfeld et al., 2009). However, until now research on the behavior of pharmaceuticals in SSFCWs has been scarce (Matamoros and Bayona, 2006; Matamoros et al., 2007; Matamoros et al., 2008) probably due to the only recent awareness and concern with these water contaminants.

Depuration of wastewaters in SSFCWs is achieved through the concerted action of plants, microorganisms and the solid matrix components. SSFCWs' efficiency can be significantly improved by careful selection of components and optimization of the operation conditions. The performance of these systems is highly dependent on the solid matrix materials and the plant species chosen.

Macrophytes play a central role in the removal mechanisms occurring in a SSFCW as they provide support for the growth of microorganisms and promote the removal of a variety of pollutants by their adsorption, uptake and/or degradation (Imfeld et al., 2009). Previous studies have shown that *Typha* spp., one of the most commonly used macrophytes in constructed wetlands, presents a high capacity to tolerate and remove some pharmaceuticals from contaminated waters (Park et al., 2009; Dordio et al., 2009b).

The support matrix is also a very important component in SSFCWs. Not only does it support the growth of macrophytes and microorganisms but it can also promote a series of chemical and physical processes which assist in the wastewater depuration. Among such processes, sorption by the solid matrix plays an important role in contaminant retention. Thus, choice of a material with high sorption capacity is of some importance when designing a SSFCW. Previous studies have shown that light expanded clay aggregates (LECA) are able to remove by sorption pharmaceutical compounds such as CA, CB and IB from water and wastewater (Dordio et al., 2007; Dordio et al., 2009a).

The aim of the present work was to evaluate the efficiency of microcosm constructed wetlands systems to remove three pharmaceuticals compounds, namely IB, CB and CA from contaminated wastewaters, using LECA as the solid matrix, and planted with *Typha* spp. plants. In order to evaluate the effects of seasonal variability in the performance of these systems, assays were performed in two different periods, one corresponding to a warm dry season and the other to a cold humid one.

2. Materials and Methods

2.1. Reagents and materials

Clofibric acid (CA) (97% purity), ibuprofen (IB) (99.8% purity) and carbamazepine (CB) (> 99% purity) were purchased from Sigma Aldrich (Steinheim, Germany).

All other high purity chemicals and solvents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Panreac Quimica SA (Barcelona, Spain), and were used without further purification. Ultra-pure water was obtained with a Milli-Q water purification system (Simplicity® UV, Millipore Corp., France).

For solid phase extraction (SPE), the following sorbent cartridges were used: LiChrolut® RP-18 (500 mg, 3 mL) from Merck (Darmstadt, Germany), and Oasis® HLB (200mg, 6 mL) from Waters Corporation (Milford, MA, USA). Filters with 0.45 μm nylon membrane were purchased from VWR International (West Chester, PA, USA).

LECA (granulometric grade 2/4), that was used as the solid matrix for the SSFCW microcosms, was supplied by MaxitGroup, Portugal. The commercially available LECA contains considerable amounts of fine materials which were significantly reduced by washing LECA with Millipore water until no further suspended materials were visible. The washed media were then air dried and used throughout this study. Physical and chemical characterization of this material was conducted in a previous study (Dordio et al., 2009a).

2.2. Pharmaceuticals removal by microcosm wetlands systems

2.2.1. Plant collection and acclimation

Typha spp. plants were collected in water streams in Alentejo, Portugal, during April 2007. The rhizomes were thoroughly washed to remove any soil/sediment particles attached to the plant surfaces and then were placed in vessels for acclimation. An aerated modified Hoagland nutrient solution, adapted from Fediuc and Erdei (2002), and having the following starting chemical composition, with pH adjusted to 6.0 was used: 2.5 mmol L^{-1} K^{+}, 2 mmol L^{-1} Mg^{2+}, 2 mmol L^{-1} Ca^{2+}, 2 mmol L^{-1} SO_4^{2-}, 6 mmol L^{-1} NO_3^{-}, 0.5 mmol L^{-1} $H_2PO_4^{-}$, 10 μmol L^{-1} Fe^{3+}, 10 μmol L^{-1} H_3BO_3, 1 μmol L^{-1} Mn^{2+}, 0.5 μmol L^{-1} Cu^{2+}, 0.1 μmol L^{-1} MoO_4^{2-}. The nutrient solution was renewed twice every week.

Plants were grown in a growth chamber (Fitoclima, Portugal) at 22° C, with 70% of relative humidity and a light-dark cycle of 12:12 h. The photon flux density was 270

139 μmol m^{-2} s^{-1}. After 6 weeks, when new roots and leaves had developed, plants of
140 uniform size were selected and planted in LECA beds, in a greenhouse, where they were
141 set for a period of acclimation of approximately one year.

142

143 *2.2.2. Setup of microcosm wetlands assays*

144 Six microcosms were set up using PVC containers (0.6 m long × 0.5 m wide × 0.4 m
145 deep) filled with washed LECA (2/4) with a depth of 0.3 m. Water level was maintained
146 just below the LECA surface, corresponding to a flooding rate of approximately 100%.
147 Three beds were planted with pre-grown cattails (density of 20 plants/m^2) and another
148 three were left unplanted. In addition to these six microcosms, three additional vessels
149 were used, filled only with the tested wastewater solutions and without any plants or
150 solid media, for the purpose of assessing the biodegradation of pharmaceuticals in the
151 wastewater alone.

152

153 *2.2.3. Microcosm wetlands systems operation and sample collection*

154 Both the six microscosms as well as the three additional empty vessels described in
155 section 2.2.2 were filled with a wastewater spiked with CB, CA and IB pharmaceuticals
156 at a concentration of 1 μg mL^{-1} each. The solutions were prepared with wastewater
157 collected after a secondary treatment stage in a WWTP serving a small rural community
158 population of *ca.* 400 inhabitants. The treatment processes used in this WWTP include
159 screening, primary sedimentation and conventional activated sludge treatment. The
160 collected wastewater was spiked with the pharmaceuticals by dissolution of a standard
161 solution of 100 μg mL^{-1} containing the three compounds.
162 The systems were operated in a batch mode, i. e. with the initial load of the spiked
163 wastewater and without any running flow during the assays.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

During the assays, samples of the spiked wastewater were collected, during the first day, after the periods of 6, 12 and 24 hours of contact and, in the following days, with a daily periodicity for a total of 7 days of retention time. Sample collection was made at half depth of the beds and at random points on the beds surface. After collection, the samples were kept refrigerated until the time of analysis, which were always carried out within 2 hours from the time of collection.

The pH of the solutions was monitored during the assays. Evaporation (and transpiration in planted beds) was also daily controlled and the water volumes lost were restored with distilled water.

The assays were repeated in summer and in winter conditions in order to observe the influence of seasonal conditions (especially temperature, relative air humidity and vegetative stage of plants) on the system behavior. Effects due to rainfall, however, were excluded from this study as the systems were placed in covered facilities.

2.3. Analytical methodology

2.3.1. Wastewater characterization

The collected WWTP effluent was characterized by the determination of the following wastewater quality parameters, according to the APHA-AWWA-WPCF methods (Clescerl et al., 1998): total suspended solids (TSS), pH and total and soluble chemical oxygen demand (COD_t and COD_s) of samples filtered through 0.45 μm filters.

2.3.2. Development and implementation of a SPE method

Two different SPE cartridges were tested with water and wastewater spiked with CB, IB and CA for the choice of the optimal conditions for pharmaceuticals recoveries in the SPE preconcentration step: LiChrolut® RP-18 (conditioned with 7.5 mL of methanol

189 and 7.5 mL of water), and Oasis® HLB (conditioned with 3.0 mL of methanol and 3.0
190 mL of water). After sample pH adjustment to values of 2 and 7 using either H_3PO_4 or
191 NaOH solutions, the samples were percolated through the SPE cartridges. Afterwards
192 some cartridges were rinsed with 5.0 mL of Milli-Q water with pH adjusted at 2 in order
193 to test the influence of a washing step. The cartridges were then air dried for about 15
194 min under vacuum to remove excess water. The analytes (CB, IB and CA) retained in
195 the columns were eluted with 5.0 mL of methanol. Following elution, the solutions were
196 evaporated on a rotary evaporator at 30ºC to dryness and redissolved with 1.0 mL of
197 Milli-Q water. Five replicates were done for every tested cartridge and experimental
198 condition.

199 According to the results obtained with the previous tests, all following samples were
200 prepared by adjusting the pH to 7 and preconcentrating them in LiChrolut® RP-18
201 cartridges without a washing step.

202 In order to test for possible negative effects on the LiChrolut® RP-18 cartridges
203 performance due to the use of large wastewater sample volumes, a series of trials were
204 also performed using varying volumes of solution (between 5 mL and 100 mL) all
205 containing the same amount of the analytes. The effect of pharmaceuticals concentration
206 was also investigated using wastewater samples spiked with CA, IB and CB at
207 concentration levels of 0.5, 1.5 and 2.5 $\mu g\ mL^{-1}$, for the same volume (5.0 mL) of
208 sample percolated through the SPE column. Three replicates were done of each sample
209 volume assay.

210

211 *2.3.3. Quantification and analytical method validation*

212 Pharmaceuticals quantifications were performed on an Elite LaChrom HPLC system
213 with UV detection (Hitachi, Japan). The reversed phase analytical column used was a

Zorbax Eclipse XDB-C18 with 5 μm particle size. Chromatographic separation was performed in gradient mode using the following gradient program: linear from 50% to 75% of acetonitrile in water between 0 and 3 minutes and isocratic at 75% of acetonitrile after 3 minutes. The flow rate was 1.0 mL min^{-1} and the total run time was 8 minutes. The water was acidified with 0.1% (v/v) phosphoric acid. The UV detector wavelength was at 210, 222 and 227 nm for CB, IB and CA, respectively. Five replicate injections were made for each sample, which was previously filtered through a 0.45 μm filter.

Calibration curves were constructed for analytes quantification. Three replicates were done for each standard and three HPLC analysis were performed for each standard.

Instrumental detection and quantification limits (IDL and IQL) were obtained by determining the concentrations corresponding, respectively, to signal-to-noise ratios of 3 and 10 for the chromatographic measurements (Miller and Miller, 2000) and the limit of quantification (LOQ) of the entire analytical method (including SPE preconcentration) was calculated resorting to the following equation (Vieno et al., 2006):

$$\mathrm{LOQ} = (\mathrm{IQL} \times 100)/(\mathrm{Rec}(\%) \times C) \quad (1)$$

where IQL is the instrumental quantification limit, Rec (%) is the average SPE recovery of pharmaceuticals from the wastewater and C is the preconcentration factor obtained with SPE.

The repeatability of the HPLC-DAD system was tested by performing six consecutive replicate injections of same standard solution using the same mobile phase, and it was evaluated as the dispersion (relative standard deviation) of the measured peak areas. The reproducibility of the HPLC-DAD system was determined by performing injections of six different standard solutions in different days always using fresh solvent as the

239 mobile phase each day, and it was evaluated as the dispersion (relative standard
240 deviation) of the measured peak areas.
241
242 *2.4. Statistical analysis*
243 Data were analyzed by the analysis of variance method (ANOVA, single factor) at
244 different significance levels. Comparisons were considered significantly different for P
245 < 0.05.
246
247 **3. Results and Discussion**
248 *3.1. Development and validation of the analytical methodology*
249 The HLPC methodology developed for the quantification of CA, CB and IB in water
250 and wastewater samples presents a high repeatability and reproducibility (RSD < 1% in
251 all cases) as well as low values of IQL (< 0.092 $\mu g\ mL^{-1}$ for all compounds). However,
252 these IQL were still above those required for some of the samples and it was be
253 necessary to carry out a pre-concentration step of those samples prior to their
254 chromatographic analysis.
255 SPE technique was chosen for sample preconcentration and, in order to optimize the
256 methodology, two different cartridges were tested: a hydrophilic-lipophilic balanced
257 polymeric sorbent (Oasis HLB®) and a reversed phase alkyl bonded-silica cartridge
258 (Merck LiChrolut® RP-18). The performance of both SPE cartridges was compared,
259 first using water and then wastewater spiked with the three pharmaceuticals under study
260 (CB, IB and CA).
261 CA and IB are acidic pharmaceutical compounds (pKa of 3.18 and 4.91 respectively)
262 and at low pH values, they are predominantly in the neutral protonated form, which
263 should increase their affinities to the hydrophobic SPE sorbents. In this work, the

efficiency of recovery of the different SPE columns was compared at the pH values of 2 and 7. The results obtained are presented in table 1.

For all pharmaceuticals, the highest recoveries were attained with both types of cartridges at sample pH of 7 and both columns have similar recoveries at this pH value. The marginally better recovery values obtained with Lichrolut® RP-18 are not statistically different from those obtained with Oasis® HLB. However, because Lichrolut® RP-18 cartridges are less expensive and show similar performance, they were chosen to be used throughout this study.

A washing step with water, after the percolation of the sample through the cartridge is sometimes beneficial for further cleaning up the sample of matrix interfering compounds. The effect of this procedure was evaluated for the Lichrolut® RP-18 columns with wastewater samples spiked with pharmaceuticals at a pH of 7. Despite the water pH, the washing step leads to a decrease in the pharmaceuticals recoveries, except for CB whose recoveries are mostly unaffected (data not shown). Assay samples were therefore preconcentrated without the washing step.

In order to test the possible detrimental effect on the pharmaceuticals recoveries when large volumes of wastewater, on one hand, and larger concentrations of the pharmaceuticals, on the other, were percolated through the LiChrolut® RP-18, a series of SPE experiments were established. Firstly, when varying the sample volumes percolated but maintaining the same total pharmaceuticals amount, it was observed that increasing volumes up to a factor of 100 had negligible influence in the recoveries of pharmaceuticals (data not shown). Additionally, higher concentrations of pharmaceuticals in the samples did not seem to decrease the recoveries of the SPE method either (data not shown).

288 Coupled to a SPE pre-concentration step, LOQ for the entire analytical method as low
289 as 0.00093 μg mL^{-1} could be achieved for all compounds, which was enough for the
290 requirements of this study. The entire analytical methodology was also found to be
291 highly reproducible (RSD < 1%, for all pharmaceuticals).
292

293 *3.2. Pharmaceuticals removal by microcosm wetlands systems*

294 *3.2.1. Relevant conditions for the realization of the assays*

295 The wastewater used in the assays was collected after a secondary treatment stage in a
296 WWTP of a small rural community. Wastewater was collected in December for the
297 assays in winter conditions (average temperature 12° C), and in June for the assays in
298 summer conditions (average temperature 26° C). Some of the most common parameters
299 used to characterize wastewater quality were evaluated and are presented in Table 2. In
300 general, organic loads and suspended solids for both wastewaters were, at the time of
301 collection, somewhat high but still within the Portuguese legal limits for discharge into
302 water bodies.

303 Plants during the summer period showed a good development, presenting an average
304 height of 1.5 – 2 meters. Conversely, during the winter, plants presented very little
305 aerial parts as most dry leaves had been previously harvested.

306 The evaporation/evapotranspiration rates were estimated daily through the water
307 balance at the beds, i.e. the volume of water loss corresponds to the added amount of
308 water necessary to maintain the water column height at the beds. These determinations
309 are presented in Table 2.

310 In unplanted beds, the rate of water evaporation is mainly dependent on the temperature
311 and relative air humidity and, thus, is highest in the summer season. In planted beds, in
312 addition to evaporation there is a major contribution of the plants' transpiration rate to

313 the water loss. In this case, the type of the plants and their vegetative stage is
314 determinant to evapotranspiration rates. The higher plant size and their more intense
315 activity during the summer also contribute to an increased evapotranspiration rate of
316 microcosm wetlands in this season.
317

318 *3.2.2. Kinetics of pharmaceuticals removal*

319 The effects of contact time and of the seasonal environmental conditions (with the
320 exception of rainfall) in the removal of CA, IB and CB from spiked wastewater in
321 planted and unplanted LECA beds are shown in Figure 1.

322 In every assay the kinetics are characterized by an initial fast step (first 6 hours) through
323 which, in almost every case, more than half of the initial pharmaceuticals amounts are
324 removed. During this initial step, removal of each compound is similar in planted and
325 unplanted beds. Therefore, removal should be due, essentially, to adsorption over the
326 LECA's surface (although, in the planted beds, some adsorption onto the plant's roots is
327 likely to occur as well). No significant differences are visible at this initial period
328 between summer and winter assays.

329 After 6 hours, a second stage of additional compounds removal occurs at a slower rate,
330 which is even slower in the case of the unplanted beds than it is in planted beds (Fig. 1).
331 In the period between 96 and 120 hours, pharmaceuticals removal tends to stabilize,
332 with exception of CA in the planted summer assays, whose removal is still increasing by
333 the end of the experiment.

334 In the period of 6 to 96 hours, in all cases, the processes of removal follow a kinetic
335 behavior that fits well first-order rate equations. Details of the fitted equations for each
336 assay are presented in Table 3. From the analysis of the kinetic data in Table 3, in
337 particular the rate constant, it is clear that overall faster removal rates are achieved in

planted beds when compared to the unplanted ones. This corresponds to the expected behavior, considering that the same sorption processes occurring in the unplanted beds also occur in planted ones, but, in the latter, additional biological processes also contribute for the quicker removal of pharmaceuticals. Summer removal rates also are higher than in winter, which is also according to the expected behavior as the higher summer temperatures in general have an effect of increasing the kinetics of most processes involved in the removal of the pharmaceuticals (i.e., faster physical sorption, more active vegetative stage, higher plant transpiration rates and higher microbial activity).

3.2.3. Pharmaceuticals removal efficiency by the microcosm constructed wetlands

In comparison with conventional treatment, removal of all pharmaceuticals in planted beds was generally high (Table 4). Between winter and summer conditions, removal efficiencies were as high as 88.2-96.7%, 81.9-96.2% and 48.3-74.5% for CB, IB and CA respectively. A significant removal of IB could be observed even in the wastewater only assays (Table 4), especially in the summer period, which shows the high biodegradability of this compound comparatively to the other two. Therefore, biodegradation should contribute significantly to the removal of IB obtained in microcosm constructed wetlands. The importance of biodegradation processes in the removal of IB makes the efficiency of constructed wetlands comparable to that obtained in WWTPs as the removal in both systems is attained essentially through similar processes (Table 4).

On the other hand, the removal of CB and CA in the wastewater only systems was low (usually < 12%), which reaffirms their non-biodegradability, as is widely reported in the literature (Fent et al., 2006; Santos et al., 2007; Khetan and Collins, 2007; Aga, 2008).

363 For these compounds, the removal efficiencies obtained in the microcosm wetlands are
364 considerably higher than those typically observed in WWTPs (Table 4).
365 An important role in the removal processes in the wetlands microcosms is played by
366 LECA due to pharmaceutical sorption to this solid matrix. Such effect has been
367 evaluated through the assays performed in the unplanted beds. CB was observed to be
368 extensively sorbed by the LECA matrix (Table 4). IB was highly removed in unplanted
369 systems, although in this case removal has to be attributed to the combination of
370 sorption and biodegradation. CA, on the other hand, was only moderately removed by
371 sorption. These results are, to some extent, consistent with laboratory results reported
372 earlier (Dordio et al., 2009a), although some small loss of efficiency observed here may
373 be attributed to the longer age of operation of these microcosm systems and the
374 establishment of biofilm over the LECA medium.
375 The planted beds showed a clear improvement of the removal efficiencies relatively to
376 the unplanted beds for all compounds, especially in the case of CA during the summer.
377 The benefits provided by the presence of plants is especially evident in the case of the
378 compound less removed by sorption, CA. In previous studies conducted in hydroponic
379 conditions, *Typha* showed some capacity to remove CA from nutrient solutions without
380 any symptoms of toxicity up to concentrations of 2 $\mu g\ mL^{-1}$ (Dordio et al., 2009b).
381 Due to the moderate lipophilicity of the three pharmaceuticals, as indicated by their
382 octanol-water partition coefficient ($\log K_{ow}$ ranging from 2.45, for CB, to 2.57, for CA),
383 it is likely that plants have an active role in the removal of these compounds through
384 direct uptake. In fact, it is widely considered that organic compounds with $0.5 < \log K_{ow}$
385 < 3 have adequate properties to move through cell membranes and enter the plant's
386 transpiration stream, thereby being easily taken up by the plants (Pilon-Smits, 2005). In
387 addition, an increase of the amounts of oxygen released by the plant's roots in the

388 rhizosphere favors the occurrence of aerobic biodegradation processes which are more
389 efficient than anaerobic ones (Zwiener et al., 2002; Matamoros et al., 2008) and may
390 also be responsible for the improvement of the removal efficiencies of the most
391 biodegradable pharmaceutical IB.

392 Seasonal variability of the efficiency of the microcosm wetlands systems was also
393 characterized by comparison of pharmaceuticals removal obtained in the planted beds
394 (and unplanted ones as well) during the summer and winter assays. Two major
395 conditions may contribute to such variability, namely temperature and a more active
396 vegetative stage of plants. Transpiration rate is a key variable that determines the rate of
397 organics uptake by the plants; the higher transpiration rates in this season can, therefore,
398 contribute with a significant enhancement of their uptake of pharmaceuticals (Pilon-
399 Smits, 2005).

400 A significantly higher elimination of IB was observed for all systems (wastewater only,
401 unplanted beds and *Typha* planted beds) during the warm season in comparison to the
402 cold season. In several studies such a similar trend was observed for the elimination of
403 IB in WWTPs (Vieno et al., 2005; Castiglioni et al., 2006; Aga, 2008). The increased
404 removal of IB in the warm season was probably due primarily to the more efficient
405 biodegradation of the compound caused by the enhanced microbial activity at higher
406 wastewater temperatures.

407 Two different effects of seasonal conditions are observed in planted beds in the case of
408 the other two hardly biodegradable pharmaceuticals, CA and CB, namely strikingly
409 different efficiencies in the summer and winter seasons (75% and 48%, respectively) in
410 the case of the former compound, and more subtle ones (97% and 88%, respectively) in
411 the case of the latter. Given the recalcitrant behavior of those compounds, the vegetative
412 stage of the plants seems to be the most important factor that contributes to the

413 differences observed. In the winter, the removal rates from the planted beds are not very
414 different from those of the unplanted beds. On the other hand, during the summer, the
415 higher transpiration rates lead to a rapid uptake of the compounds which is most evident
416 in the higher removal efficiencies obtained in the planted beds for CA and CB in this
417 season and which cannot be attributed, for these compounds, to enhanced
418 biodegradation.
419
420 **4. Conclusion**
421 In the present study, a LECA-based microcosm CW planted with *Typha* spp. was tested
422 for its ability to remove the pharmaceuticals CA, CB and IB from wastewaters. The
423 study provides an assessment of the potential of these systems to deal with
424 pharmaceutical contamination in wastewater treatment.
425 The removal efficiencies obtained in planted beds for the studies pharmaceuticals were
426 significantly high with equilibrium attained within 96 – 120 hours of contact time.
427 The material LECA, used as solid matrix in the tested microcosms, was responsible for
428 most of pharmaceuticals removal from the wastewater, but the presence of the *Typha*
429 plants used in this study did influence with an additional contribution of 6-32% in the
430 summer (although of only 2-8% in the winter) to the overall removal by the planted
431 systems. In addition, removal of pharmaceuticals was made significantly more rapid in
432 the presence of plants. Influence of vegetation may be attributed to direct uptake of
433 some of the compounds as well as to the enhancement of biodegradation processes'
434 efficiency.
435 The comparison between assays conducted during a summer period with those
436 performed in the winter show that there is some loss of efficiency in the latter assays,
437 which is highest for the removal of CA but is also significant for IB, because biological

processes are important in both cases (probably extensive plant uptake for CA and, most certainly, biodegradation for IB). The compound least affected by seasonal variability was CB, and this may be due to the fact that sorption is the major process of removal for this pharmaceutical which is less affected by temperature variations than the activities of the biotic components (plants and microorganisms). As expected, the rates of removal are also increased during the summer.

In order to fully evaluate these systems under more realistic conditions, further tests are still necessary, namely for determining the impact on performance caused by a longer period of operation with several cycles of loading in a microcosm wetland, changing from a discontinuous feed (as is used in this work) over to a continuous feed mode. A possible decrease in performance when going to a full scale wetland should also be evaluated. Nevertheless, the *Typha* + LECA microcosm wetlands studied in this work sufficiently showed the potentially high efficiency of these systems in removing the three pharmaceuticals and possibly other similar organic compounds from wastewaters, suggesting that larger scale SSFCW systems may be efficient and cost-effective alternatives to other high-cost technologies, such as ozonation or membrane bioreactors, to be used as a tertiary treatment stage.

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558 **Figure Captions**

559 **Fig.1.** Kinetics of carbamazepine (a), clofibric acid (b) and ibuprofen (c) removal by the
560 *Typha* planted beds as well as the unplanted LECA beds in summer and winter
561 conditions. Also depicted is the kinetics of pharmaceuticals biodegradation in the
562 wastewater only. Vertical error bars correspond to an interval of ± SD (n = 3).

Table 1.

Analyte recoveries (average ± SD, *n* = 5) in SPE for different cartridges and pH conditions. ANOVA comparisons significant at P < 0.05. Different letters indicate significantly different values among recoveries of each compound

Cartridges	Sample pH	% recoveries of pharmaceuticals		
		CB	CA	IB
Lichrolut® RP-18	2	97.2 ^{a} ± 1.3	88.3 $^{a'}$ ± 4.7	74.5 $^{a''}$ ± 2.3
	7	95.8 ^{a} ± 0.74	99.0 $^{b'}$ ± 0.89	98.9 $^{b''}$ ± 2.0
Oasis® HLB	2	89.3 ^{b} ± 1.8	88.2 $^{a'}$ ± 2.0	74.5 $^{a''}$ ± 6.0
	7	94.2 ^{a} ± 4.8	98.1 $^{b'}$ ± 2.0	98.1 $^{b''}$ ± 1.7

Table 2.

Relevant conditions for the realization of the assays

		Summer conditions	Winter conditions
Medium composition	pH	8.29 ± 0.05	7.31 ± 0.10
	SST (mg L^{-1})	57 ± 3	68 ± 3
	COD_t (mg L^{-1})	133 ± 2	146 ± 3
	COD_s (mg L^{-1})	82 ± 2	90 ± 4
Mean air temperature during assays		26° C	12° C
Mean plant height at the beginning of the assays		1.5-2 meters	After harvesting
Water loss (mL d^{-1})	Unplanted beds	111 ± 13	45 ± 5
	Planted beds	552 ± 63	50 ± 6

Table 3.

Rate equations fitted to the kinetic behavior of the assays in the period of 6-96 hours

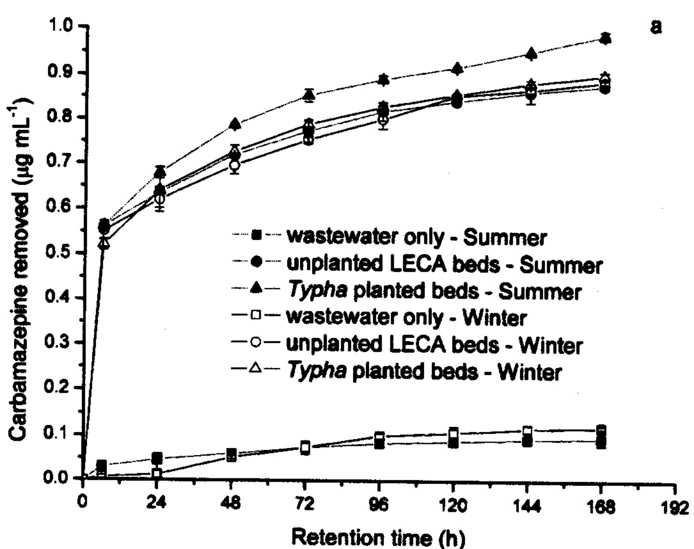
Pharmaceuticals	System type	Season	equation	R^2
CB	Planted beds	Summer	$\ln[CB](t) = -0.73 - 0.014\ h^{-1}\ t$	0.994
		Winter	$\ln[CB](t) = -0.70 - 0.010\ h^{-1}\ t$	0.992
	Unplanted beds	Summer	$\ln[CB](t) = -0.73 - 0.0091\ h^{-1}\ t$	0.998
		Winter	$\ln[CB](t) = -0.71 - 0.0084\ h^{-1}\ t$	0.999
CA	Planted beds	Summer	$\ln[CA](t) = -0.31 - 0.006\ h^{-1}\ t$	0.996
		Winter	$\ln[CA](t) = -0.40 - 0.0019\ h^{-1}\ t$	0.985
	Unplanted beds	Summer	$\ln[CA](t) = -0.17 - 0.0031\ h^{-1}\ t$	0.993
		Winter	$\ln[CA](t) = -0.27 - 0.0017\ h^{-1}\ t$	0.991
IB	Planted beds	Summer	$\ln[IB](t) = -0.67 - 0.019\ h^{-1}\ t$	0.997
		Winter	$\ln[IB](t) = -0.82 - 0.0058\ h^{-1}\ t$	0.999
	Unplanted beds	Summer	$\ln[IB](t) = -0.67 - 0.011\ h^{-1}\ t$	0.993
		Winter	$\ln[IB](t) = -0.80 - 0.0045\ h^{-1}\ t$	0.985

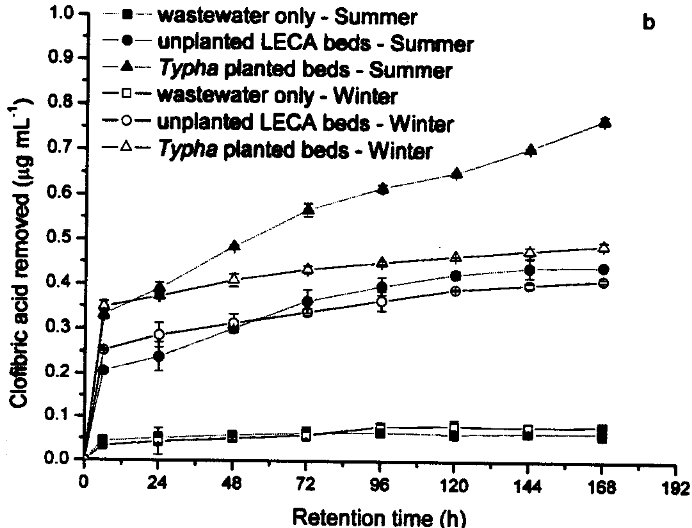
Table 4.

Removal efficiencies (average ± SD, n = 3) of pharmaceuticals in wetland microcosm assays as well as in the unplanted LECA beds and in the wastewater only, after 168 h of retention time

System type	Season	Pharmaceuticals removed (%)		
		CB	CA	IB
Typha planted beds	Summer	96.7 ± 2.6	74.5 ± 2.5	96.2 ± 2.9
	Winter	88.2 ± 2.4	48.3 ± 1.6	81.9 ± 3.5
Unplanted planted beds	Summer	85.8 ± 2.2	43.0 ± 1.4	90.6 ± 2.6
	Winter	86.7 ± 2.3	40.7 ± 0.6	73.5 ± 3.1
Wastewater only	Summer	9.4 ± 1.4	6.3 ± 1.3	75.2 ± 2.6
	Winter	11.8 ± 0.9	7.9 ±0.4	38.2 ± 1.7
Conventional WWTPs	-	8[a]; < 20[b,c]; 0-25[d]	<0[a]; 15-34[e]; 20-40[c]	60-70[f], 80[c]; 80-100[b]; 88-93[d]

[a](Heberer, 2002); [b](Clara et al., 2005); [c](Miège et al., 2009); [d](Santos et al., 2007); [e](Stumpf et al., 1999); [f](Carballa et al., 2004)





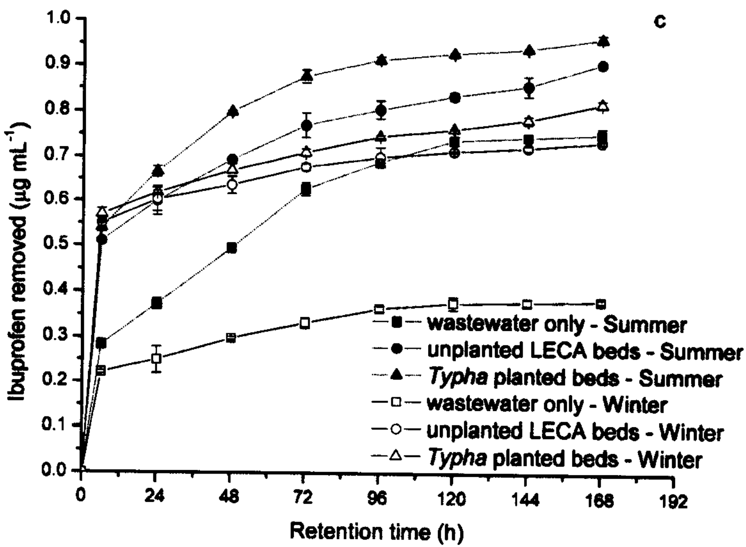


Figure 1

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Atenolol removal in microcosm constructed wetlands

Ana Dordio[ab], José Pinto[a], Cristina Teixeira da Costa[ac], Ana Paula Pinto[ad], Alfredo Carvalho[ac] and Dora Martins Teixeira[ad]*

[a]*Chemistry Department, University of Évora, Rua Romão Ramalho, 59, 7000-676 Évora,*
5 *Portugal;* [b]*IMAR – Institute of Marine Research Consortium, Environmental Biogeochemistry Group, University of Évora, Rua Romão Ramalho, 59, 7000-676 Évora, Portugal;* [c]*CQE – Évora Chemistry Centre, University of Évora, Rua Romão Ramalho, 59, 7000-676 Évora, Portugal;* [d]*ICAM – Institute of Mediterranean Agricultural Sciences, University of Évora, Herdade Experimental da Mitra, 7000-676 Évora, Portugal*

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Microcosm constructed wetland systems established with a matrix of light expanded clay aggregates (LECA) and *Typha* spp. or *Phragmites australis* were used to evaluate their ability to remove atenolol from wastewater. Combined with an efficient SPE concentration step, the use of HPLC-DAD yielded an analytical
15 method for atenolol quantification with very low LOQ (9 $ng\,mL^{-1}$) and high reproducibility (RSD < 4%). Overall removal efficiencies of 92.5% and 94.5% were achieved after a retention time of only 4 days with the microcosm systems planted with *Phragmites australis* and *Typha* spp. respectively. The removal kinetics was characterised by an initial fast step (removal of about 75% after just
20 24 h) which is mainly attributable to adsorption on the LECA matrix. Atenolol removal in LECA beds continues to increase in a steady pace up to the end of the assay (8 days) being nevertheless about 5–10% lower than those observed in the planted beds after the first 4 days. For the retention time of 4 days most of the atenolol is removed by the LECA matrix but an additional 12–14% of the overall
25 removal efficiency can be attributed to the *Typha* and *Phragmites* plants, which is in agreement with other published reports. Despite the fact that further tests using larger-scale flowing systems are required to evaluate fully the atenolol behaviour in constructed wetlands, this study points to the possible application of these low-cost wastewater systems to treat atenolol contaminated wastewater.

30 **Keywords:** pharmaceuticals; atenolol; subsurface flow constructed wetlands (SSFCWs); LECA; *Phragmites australis*; *Typha* spp.

1. Introduction

Contamination of aquatic systems with pharmaceutical residues has emerged recently as one of the key issues in environmental chemistry. Analytical techniques made available in
35 the past few years have significantly lowered the detection and quantification limits for organic substances in environmental matrices. Thanks to these advances numerous monitoring studies have been conducted lately that revealed a wide range of pharmaceutical active compounds (PhACs) present in low amounts but in great diversity in water bodies [1–4].

*Corresponding author. Email: dmt@uevora.pt

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In modern society, an ever increasing number of pharmaceuticals are used for the treatment and prevention of various diseases. Ingested drugs are only partially absorbed by the body. In addition to their increasingly high global consumption rates, this results in a wide variety of pharmaceuticals along with their metabolites being continuously introduced in domestic wastewaters. Studies have shown that PhACs are only partially removed by conventional wastewater treatment processes used in wastewater treatment plants (WWTPs) resulting in the discharge of contaminated effluent into the receiving water bodies [1,5,6].

β-blockers like atenolol are used in the treatment of high blood pressure as well as in recovery from heart attacks. In several studies, traces of these substances were detected in wastewaters, as well as in surface and groundwaters, indicating their incomplete removal in WWTPs [3,5–8]. Despite the low concentrations detected, those studies also show some damaging effects of these compounds on aquatic ecosystems [6,8].

With the aim of improving the efficiency of WWTPs in removing PhACs such as atenolol and others, optimisation of wastewater treatment processes has been attempted, e.g. by increasing sludge residence times. Some advanced technologies have also been evaluated such as advanced oxidative processes, activated carbon adsorption, membrane filtration and membrane bioreactors [1,9–13]. However, despite the sometimes high removal efficiencies attained, these processes are generally not cost-effective on a large scale [1]. Consequently, there is a growing need for new wastewater treatment systems for removing PhACs from waters that have higher efficiencies at reasonable costs of operation/maintenance.

Subsurface flow constructed wetland (SSFCW) systems are low cost wastewater treatment systems consisting of inundated vegetated beds, designed to emulate the well-known water depurative capacity of natural wetlands [14]. These systems are increasingly being used to provide a form of secondary or tertiary treatment for wastewaters. They have already been used with success to remove some organic recalcitrant compounds from contaminated waters such as pesticides, PAHs, organic solvents or explosives [15–17]. However, only a few studies have until now been conducted on the removal of pharmaceutical residues [18–23] probably due to the only recent awareness and concern with these water contaminants.

Wastewater depuration in SSFCWs is achieved by the concerted action between plant rhizomes, microorganisms and the support matrix components. SSFCWs' efficiency can be significantly improved by optimisation of the operation conditions. The performance of these systems is highly dependent on the solid matrix materials and the plant species chosen. When designing a SSFCW it is important to select a matrix with a high sorption capacity, which will depend on the physico-chemical properties of the material chosen. Previous studies have shown that light expanded clay aggregates (LECA) are able to remove, by sorption, other pharmaceuticals from water [23,24]. With respect to the vegetation, aquatic plants play a central role in the depuration mechanisms occurring in a SSFCW. They provide support for the growth of microorganisms and promote the removal of a variety of pollutants by adsorption on the roots, uptake or degradation [14,25]. The most commonly used emergent vegetation in SSFCWs includes macrophyte species such as the cattail (*Typha* spp.) and the common reed (*Phragmites australis*) [14,26,27].

The aim of the present work was to evaluate the efficiency of microcosm constructed wetland systems to remove atenolol from contaminated wastewater, using LECA as the solid matrix, and planted with *Phragmites australis* or *Typha* spp. plants.

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For pre-concentration of the samples, a SPE (solid phase extraction) method was developed and optimised using water and wastewater spiked with atenolol which was
90 quantified using high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) instrumentation.

2. Experimental

2.1 *Atenolol removal by microcosm SSFCW systems*

2.1.1 *SSFCW microcosm assays setup*

95 Nine SSFCW microcosms were built using PVC containers (0.6 m long × 0.5 m wide × 0.4 m deep) filled with washed LECA (2/4) with a depth of 0.3 m. Water level was maintained just below the LECA surface, corresponding to a flooding rate of approximately 100%. Three beds were planted with pre-grown reeds (density of 20 plants/m^2), three beds were planted with pre-grown cattails (density of 20 plants/m^2) and another
100 three were left unplanted. In addition to the nine SSFCW microcosms, three additional vessels were used, filled only with the tested wastewater solutions and without any plants or solid media, for the purpose of assessing the biodegradation of atenolol in the wastewater alone (see Figure 1).

2.1.2 *Physical and chemical characterisation of the support matrix*

105 In this study, LECA with a granulometric grade of 2/4 (commercial name Filtralite® NR 2-4), that was used as the solid matrix for the SSFCW microcosms, was supplied by MaxitGroup Portugal.

The commercially available media contain considerable amounts of fine materials which were significantly reduced by washing the LECA material with Millipore water
110 (Simplicity® UV, Millipore Corp.) until no further suspended materials were visible. The washed media were then air dried and used throughout this study.

The particle-size distribution on a weight basis was analysed in triplicate by the conventional dry-sieving technique [28]. Grain-size distribution plots were used to estimate d_{10} (effective grain size) and d_{60}, and the uniformity coefficient ($U = d_{60}/d_{10}$). The apparent
115 porosity (void space) of the media was determined from the amount of water needed to saturate a known volume of the solid [29,30]. Bulk density was determined based on the

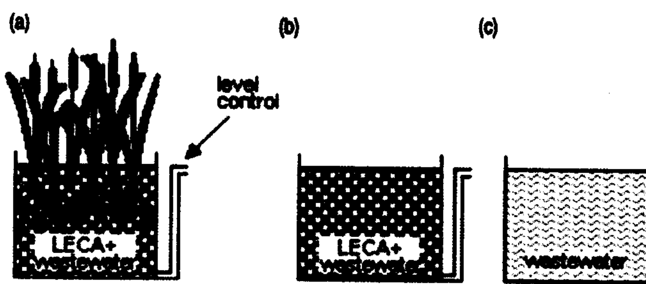

Figure 1. Schematic diagram of the experimental setup used in the atenolol removal assays; in the diagram are depicted (a) the planted beds (with either *Phragmites* or *Typha*), (b) unplanted LECA beds and (c) the wastewater only systems. Three replicates of each system were constructed.

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ratio between the dry weight and the bulk volume of the media [29]. Hydraulic conductivity was measured as described by Cooper [27]. All these measurements were replicated five times.

120 The mineralogical composition of the media was studied by X-ray diffraction (XRD) using a Bruker AXS-D8 Advance diffractometer with Cu $K\alpha$ radiation and a speed of 0.05 °/s, from 3 to 75°: 2θ, after grinding the samples so as to pass a 106 µm sieve.

2.1.3 *Plant collection and acclimation*

Phragmites australis and *Typha* spp. rhizomes with shoots were collected in water streams
125 in Alentejo, Portugal, during April 2007. The rhizomes were thoroughly washed to remove any soil/sediment particles attached to the plant surfaces and then were placed in vessels for acclimation. An aerated modified Hoagland nutrient solution was used, adapted from Fediuc and Erdei [31], having the following starting chemical composition, with pH adjusted to 6.0 : 2.5 mmol L^{-1} K^+, 2 mmol L^{-1} Mg^{2+}, 2 mmol L^{-1} Ca^{2+}, 2 mmol L^{-1} SO_4^{2-},
130 6 mmol L^{-1} NO_3^-, 0.5 mmol L^{-1} $H_2PO_4^-$, 10 µmol L^{-1} Fe^{3+}, 10 µmol L^{-1} H_3BO_3, 1 µmol L^{-1} Mn^{2+}, 0.5 µmol L^{-1} Cu^{2+}, 0.1 µmol L^{-1} MoO_4^{2-}. In the case of *Typha* this solution was used without any dilution, but in the case of *Phragmites* better shoot development was obtained with a dilution to 10% of the starting Hoagland solution. The nutrient solution was replaced twice every week.

135 Plants were grown in a growth chamber (Fitoclima, Portugal) at 22°C, with 70% of relative humidity and a light-dark cycle of 12 : 12 h. The photon flux density was 270 µmol $m^{-2}s^{-1}$. After 6 weeks, when new roots and leaves had developed, plants of uniform size were selected and planted in LECA beds, in a greenhouse, where they were set for a period of acclimation of approximately one year.

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2.1.4 *SSFCW microcosms operation and sample collection*

Both the SSFCW microcosms as well as the three additional empty vessels described in (2.1.1) were filled with wastewater spiked with atenolol at 0.78 µg mL^{-1}. This solution was prepared from a wastewater collected at a secondary treatment stage in a WWTP serving a small rural community population of *ca.* 400 inhabitants. The treatment processes used
145 in this WWTP include screening, primary sedimentation and conventional activated sludge treatment. The wastewater collected at the WWTP was spiked with atenolol by dissolution of a stock aqueous solution of 100 µg mL^{-1} of atenolol.

The systems were operated in a batch mode, i.e. with the initial load of the solution and without any solution flow during the assay.

150 During the assays, samples of atenolol solution were collected after the periods of 6, 12 and 24 hours for the first day and then with a daily periodicity for a total of 8 days of retention time. Collection of the liquid was made at half depth of the beds at random points on the beds surface. After collection, the samples were kept refrigerated until the time of analysis, which was always carried out within 2 hours from the time of collection.

155 Evapotranspiration in the beds was daily controlled and the water volumes lost through evapotranspiration were restored with distilled water.

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Table 1. Relevant physical-chemical properties of atenolol [5].

Common name	Atenolol
IUPAC name	2-[4-[2-hydroxy-3-(1-methylethylamino) propoxy]phenyl]ethanamide OR 2-(4-(2-hydroxy-3-(isopropylamino) propoxy)phenyl)acetamide
CAS number	29122-68-7
Pharmacological activity	Active metabolite (β-Blocker) 90% excreted unchanged
Structure	
Molecular weight ($g\,mol^{-1}$)	266.34
Melting point (°C)	152.0
Ionisation constant, pKa	9.6
Water solubility (25°C) ($mg\,L^{-1}$)	13,300
Log Kow	0.16

2.2 *Reagents and materials*

Atenolol (≥98% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). Some of the most relevant physical-chemical properties of this pharmaceutical are listed in Table 1. HPLC gradient grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). All other high purity chemicals and solvents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Panreac Quimica SA (Barcelona, Spain), and were used without further purification. Ultra-pure water was obtained with a Milli-Q water purification system (Simplicity® UV, Millipore Corp., France).

The cartridges used for solid phase extraction were: LiChrolut® C_{18} (500 mg, 3 mL) from Merck (Darmstadt, Germany), and Sep-Pak® Vac (500 mg, 3 mL) and Oasis® HLB (200 mg, 6 mL) from Waters Corporation (Milford, MA, USA). Filters with 0.45 μm nylon membrane were purchased from VWR International (West Chester, PA, USA).

2.3 *Analytical methods*

2.3.1 *Wastewater characterisation*

The collected WWTP effluent was characterised by the determination of the following wastewater quality parameters, according to the APHA-AWWA-WPCF methods [32]: total suspended solids (TSS), pH and total and soluble chemical oxygen demand (COD_t and COD_s) of samples filtered through 0.45 μm filters.

2.3.2 *Solid phase extraction*

Several SPE cartridges were tested with spiked water and wastewater for the extraction of atenolol: LiChrolut® C_{18}, Sep-Pak® Vac and Oasis® HLB (conditioned with 10.0 mL of

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methanol and 10.0 mL of water). All columns were tested at two different sample pH conditions, namely pH = 12 (adjusted with NaOH) and without any pH adjustment (pH ~ 7–8). Three replicates were done for every test.

After sample filtration through 0.45 μm filters, the samples were percolated through the cartridges. Afterwards some cartridges were rinsed with 5.0 mL of Milli-Q water to test the influence of a washing step. The cartridges were then air dried for about 15 min under vacuum to remove excess water. The analyte (atenolol) retained in the columns was eluted with 5.0 mL of methanol. Following elution, the solutions were evaporated on a rotary evaporator at 30°C to dryness and redissolved with 1.0 mL of Milli-Q water.

The optimised SPE conditions used for the analysis of the remaining atenolol in the SSFCW microcosm assays were as follows: the samples were filtered through 0.45 μm filters and their pH adjusted to 12 before being percolated throughout LiChrolut® C_{18}. The cartridges were then rinsed with 5.0 mL of Milli-Q water and dried under vacuum conditions for 15 min after which the atenolol was eluted with 5.0 mL of methanol. Solutions were evaporated on a rotary evaporator at 30°C to dryness and redissolved with 1.0 mL of Milli-Q water. Three replicate analyses were done for each plant assay.

2.3.3 *Quantification of atenolol and analytical method validation*

Analysis was performed using HPLC equipment Agilent 1100 with a DAD detector (Agilent Technologies, Germany). The reversed phase analytical column used was a Zorbax Elipse XDB-C_8 (4.6 mm × 150 mm) with 5 μm particle size. The DAD detector was scanned from 200 to 500 nm, and the chromatographic profile was recorded at 230 nm.

The separation was performed in isocratic mode, and the mobile phase used was composed of 10 : 90 (v/v) acetonitrile : water, at a flow rate of 1.0 mL min^{-1}. The water was acidified with 0.1% (v/v) phosphoric acid. All analyses were performed at room temperature and the injection volume was 20 μL. Three replicate injections were made for each sample previously filtered through a 0.45 μm filter.

Identification of the atenolol peak in the HPLC-DAD chromatogram was achieved by comparing the retention time and UV spectra of each sample with that of the corresponding atenolol reference and, whenever necessary, co-elution studies were performed. Calibration curves were constructed using a standard solution of 100.0 μg mL^{-1} of atenolol to prepare the standards of 0.20, 0.25, 0.50, 0.75, 1.0, 2.0, 3.0 and 5.0 μg mL^{-1}. Three replicates were made for each standard solution, and each solution was injected five times. Instrumental quantification and detection limits (IQL and IDL) for the chromatographic measurement were determined as the analyte concentrations giving a signal equal to the blank signal plus ten standard deviations, and plus three standard deviations, respectively [33]. The repeatability of the HPLC-DAD system was tested by performing six consecutive replicate injections of the same standard solution using the same mobile phase, and it was evaluated as the dispersion (relative standard deviation) of the measured peak areas. The reproducibility of the HPLC-DAD system was determined by performing injections of six different standard solutions in different days always using fresh solvent as the mobile phase each day, and it was evaluated as the dispersion (relative standard deviation) of the measured peak areas.

In order to test for possible negative effects on the LiChrolut® C_{18} column's performance due to the use of large sample volumes, a series of trials were also performed using varying volumes of solution (5, 50 and 100 mL) all containing the same amount of the analyte. The effect of atenolol concentration was also investigated using wastewater

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225 samples spiked with atenolol at concentration levels of 0.5, 1.5 and 2.5 μg mL^{-1}, for the same volume (5.0 mL) of sample percolated through the SPE column. Three replicate analyses were performed for each sample volume percolated and atenolol concentration level. Atenolol absolute recoveries were calculated as the ratio of the peak areas obtained by HPLC-DAD in the solid phase extracted sample and in the non-extracted standard.
230 The average absolute recovery percentage obtained for the different volume assays was used to calculate the analytical methods LOQ.

Limits of quantification of the entire analytical methods (LOQ) were calculated following the equation [34]:

$$LOQ = (IQL \times 100)/(Rec(\%) \times C)$$

where IQL is the instrumental quantification limit (μg mL^{-1}), Rec (%) is the average
235 absolute recovery of the atenolol in wastewater samples and C is the concentration factor (100).

The reproducibility of the entire analytical method was determined by performing, in different days, quantification of atenolol recovered from five spiked wastewater solutions with different atenolol concentrations (0.5–2.5 μg mL^{-1}) and sample volumes (5–100 mL),
240 always using fresh solvent as the mobile phase each day. Reproducibility was evaluated as the dispersion (relative standard deviation) of atenolol recoveries.

3. Results and discussion

3.1 *Analytical method for the quantification of atenolol*

3.1.1 *Chromatographic analysis*

245 The quantification of pharmaceuticals such as atenolol is mainly performed using chromatographic techniques like liquid chromatography coupled with a mass spectrometer using electrospray ionisation (LC-ESI-MS), LC-ESI-MS tandem and gas chromatography coupled with a mass spectrometer (GC-MS) because of the lower quantification limits that these techniques can achieve (ng L^{-1} range) [35]. However, the ESI-MS detector has a
250 significant drawback associated with the ESI ionisation which is highly susceptible to other components in the matrix that may induce signal suppression (more often) or signal enhancement leading to erroneous results [5]. The determination of pharmaceuticals in water samples using GC-MS always requires sample derivatisation. In the β-blockers case, a two-step derivatisation by silylation of the hydroxy groups and trifluoroacetylation
255 of the secondary amino moieties is needed. Since the derivatisation of the hydroxy groups can be incomplete, the use of GC-MS can become inappropriate for the quantification of those pharmaceuticals in water samples [35]. HPLC-DAD, when coupled with an appropriate method of analyte concentration, can be a suitable and less expensive alternative for the determination of trace organics such as atenolol. In this work, an
260 HPLC-DAD method was developed to evaluate the efficiency of SSFCW microcosms to remove atenolol from contaminated wastewater. To optimise the chromatographic separation, a series of preliminary experiments were performed, testing different mobile phases consisting of methanol, acetonitrile or mixtures of methanol and acetonitrile as organic solvent and water with different additives, such as formic acid and phosphoric
265 acid. The best quantification conditions were achieved using isocratic separation with the mobile phase composed by 10 : 90 (v/v) acetonitrile : water acidified with 0.1% (v/v) phosphoric acid. The linearity range ($R^2 > 0.99$) determined for the atenolol standards,

Table 2. Method validation parameters.

	LC-UV system				Entire analytical method	
Linearity range (μg mL^{-1}) (R^2)	IQL (μg mL^{-1})	IDL (μg mL^{-1})	Repeatability % RSD	Reproducibility % RSD	LOQ (μg mL^{-1})	Reproducibility % RSD
0.2–60 (0.991)	0.81	0.24	1.52	2.51	0.009	3.69

Notes: IDL and IQL: instrumental detection and quantification limits; LOQ: limit of quantification of the entire method.

the IDL and IQL of the chromatographic separations calculated according to Miller and Miller [33], the repeatability, the reproducibility and the LOQ of the analytical method
270 developed for the HPLC-DAD equipment, calculated according to Vieno *et al.* [34] are presented in Table 2.

3.1.2 *SPE method optimisation*

In order to optimise a solid phase extraction method for the pre-concentration of atenolol in wastewater samples, several SPE columns were tested, including one polymeric sorbent
275 (Oasis HLB®) and two apolar cartridges (Merck LiChrolut® C_{18} and Waters Sep Pak® C_{18}). The performance of the different SPE columns was compared first using water spiked with atenolol at a concentration level of 0.5 μg mL^{-1}. Atenolol is a basic pharmaceutical compound (pKa = 9.6) [5] and at basic pH values, it should be predominantly at the non protonated form, increasing its affinity to the SPE sorbent. In 2006, M. Gros *et al.* [5]
280 tested several SPE columns to pre-concentrate a number of pharmaceutical compounds, including atenolol, and they concluded that at lower pH values (pH = 2) the recoveries of atenolol from an Oasis HLB® column were significantly lower than those obtained if no pH adjustment was done (neutral pH). However, in that work, the recoveries at more basic pH values of the sample were not evaluated. In our work, the efficiency of recovery of the
285 different SPE columns was compared at pH 12 and without pH adjustment of the samples (pH about 7). There are several references [5,36] suggesting that a washing step with water, after the percolation of the sample through the cartridge, can clean up the matrix of some interfering compounds and improve the recoveries of the pharmaceuticals in SPE pre-concentration methods. This parameter was evaluated as well. The results obtained with
290 the four different SPE methods tested are shown in Figure 2.

There was no improvement in the SPE recoveries when the sample pH was adjusted to 12. However, when an additional washing step was added to the procedure a significant improvement was observed in the atenolol recoveries, only on the samples where the pH had been previously adjusted to 12. For the optimal conditions, the three tested columns
295 did not show significant differences in terms of atenolol recovery. Therefore, Merck Lichrolut® C_{18} columns were chosen to be used throughout this study because they were less expensive.

SPE method validation was performed using the optimised conditions (sample at pH 12 and using a washing step with 5 mL of water) and wastewater samples spiked with
300 atenolol. In order to test the possible detrimental effect on the atenolol recoveries when large volumes of wastewater were percolated through the LiChrolut® C_{18}, a series of SPE experiments were established, varying the sample volumes but maintaining the same

XML Template (2009) [5.5.2009-6:35pm] [1-14]
{TANDF_FPP}GEAC/GEAC_A_396422.3d (GEAC) [FPP Stage]

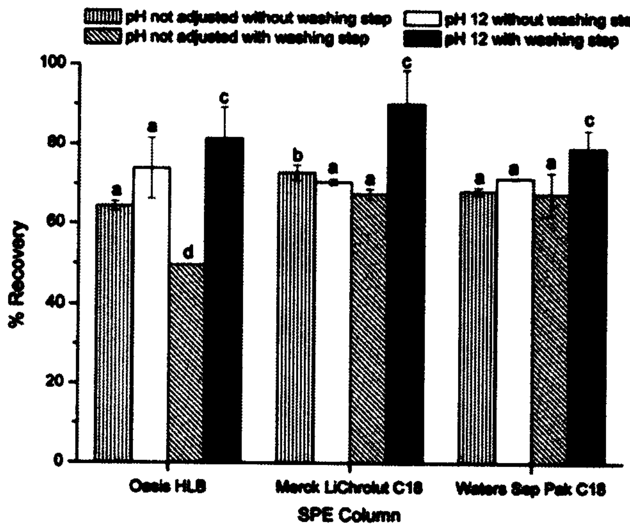

Figure 2. Influence of different SPE materials, pH adjustment (7 and 12), and wash step on the atenolol recovery from water samples. Vertical error bars indicate ± SD ($n = 3$). ANOVA significant at $P < 0.05$ when compared with control. Different letters indicate significantly different values.

Table 3. Influence of sample volume and concentration on the atenolol recoveries from spiked wastewater (average ± SD, $n = 3$) obtained using LiChrolut® C_{18} cartridges. ANOVA significant at $P < 0.05$ when compared with control. Different letters indicate significantly different values.

	% Recovery		
Atenolol concentration ($\mu g\,mL^{-1}$)	0.5	1.5	2.5
	$83.85^{a} \pm 2.33$	$84.77^{a} \pm 1.93$	$85.59^{a} \pm 3.91$
Volume (mL)	5	50	100
	$85.59^{a} \pm 4.01$	$85.58^{a} \pm 3.75$	$83.26^{a} \pm 1.36$

atenolol amount. Data presented in Table 3 prove that increasing volumes by a factor of 100 has negligible influence in the recoveries of atenolol. Additionally, higher concentrations of atenolol in the samples (up to $2.5\,\mu g\,mL^{-1}$) did not seem to decrease the recoveries of the SPE method.

Overall the low quantification limit of the entire analytical method (calculated according to Vieno [34] and found to be $9\,ng\,mL^{-1}$, see Table 2), along with the high reproducibility of the analytical method (RSD < 4%, see Table 2) proved that the developed analytical method was suitable to be used in the determination of atenolol in wastewater.

3.2 *Atenolol removal by SSFCW microcosms*

3.2.1 *Physical and chemical characterisation of solid matrix and wastewater*

LECA (2/4) used in the assays was quite uniform in terms of particle size ($U = 1.38$) with most of its particles (95%) having diameters within 2.83–5.00 mm. LECA presented

XML Template (2009) [5.5.2009–6:35pm] [1–14]
{TANDF_FPP}GEAC/GEAC_A_396422.3d (GEAC) [FPP Stage]

a pronounced alkalinity (pH in water of 9.01 ± 0.02 and PZC of 9.67 ± 0.03). These characteristics may be attributed to the presence of alkaline components such as metal oxides and carbonates, as was verified in a media mineralogical characterisation by X-ray diffraction (data not shown). The apparent porosity (or void space) of LECA is
320 quite large (48% ± 1), which may contribute to the good hydraulic conductivity measured ($7.7 \times 10^{-3}\,m^3\,m^{-2}\,s^{-1}$).

The wastewater used in the assays was collected after a secondary treatment stage in a WWTP of a small rural community. Some of the most common parameters used to characterise wastewater quality were evaluated and are presented in Table 4. Organic load
325 and suspended solids for this wastewater were, at the time of collection, somewhat high but still within the legal limits for discharge into water streams.

Table 4. Physical and chemical properties (±SD, $n = 5$) of the treated wastewater used in the assays.

Parameters	Treated wastewater
pH	8.06 ± 0.05
TSS ($mg\,L^{-1}$)	47 ± 3
COD_t ($mg\,L^{-1}$)	127 ± 2
COD_s ($mg\,L^{-1}$)	76 ± 2

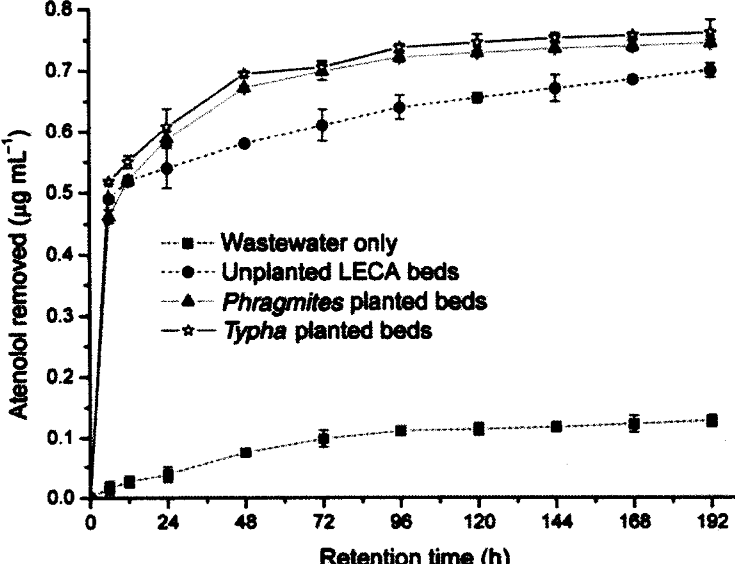

Figure 3. Kinetics of atenolol removal by the *Phragmites* and *Typha* planted beds as well as the unplanted LECA beds. Also depicted is the kinetics of atenolol biodegradation in the wastewater only. Vertical error bars indicate ± SD ($n = 3$).

3.2.2 *Kinetics of atenolol removal*

The effect of contact time and type of vegetation (*Phragmites* or *Typha*) in the removal of atenolol from spiked wastewater is shown in Figure 3 and compared with unplanted
330 LECA beds. In the same figure, the kinetics of the biodegradation of atenolol in the wastewater (i.e. not in contact with planted or LECA beds) is also presented.

In every assay the kinetics are characterised by an initial fast step (first 6 hours) that is mostly due to adsorption over the LECA's surface (although, in the planted beds, some adsorption onto the plant's roots is likely to occur also) through which more than half the
335 initial atenolol is removed within this short period. Subsequently, a slower process is responsible for additional compound removal. At this stage, removal in the unplanted LECA beds is slower than in planted ones. After 96 hours, almost no further atenolol is removed in planted beds while it continues to be removed in unplanted LECA beds until the end of the assays. However the amounts of atenolol removed in the planted beds at
340 96 hours are higher than those removed in the unplanted LECA beds at 192 hours (see Figure 3).

The kinetic behaviour observed for the sorption process occurring in the unplanted LECA beds has similarities with that observed for other compounds sorbed on the same material, namely other PhACs such as clofibric acid, ibuprofen and carbamazepine [23,24].
345 After the first step of 6 hours, the process has been observed, in the period of 6 to 96 hours, to follow a first-order kinetics which fits the equation

$$\ln[\text{atenolol}](t) = -1.23 - 0.0077\,\text{h}^{-1}t \quad R^2 = 0.994 \qquad (1)$$

In first-order kinetics the half-lives of the consumed species are independent of their initial concentrations for a given removal rate constant. For this process, therefore, a similar profile should be followed by the time evolution profile of relative concentrations removed
350 ($[\text{PhAC}]_t/[\text{PhAC}]_0$) for all the initial concentrations of the compound.

Considering the profile of the atenolol removal kinetics, very little advantage can be obtained in terms of percent removal by extending beyond 96 hours the retention time of the atenolol spiked wastewater in the planted systems.

3.2.3 *Atenolol removal efficiency by the SSFCW microcosms*

355 Within the period up to 96 hours, as much as 93% and 95% of atenolol were removed by the *Phragmites* systems and the *Typha* systems respectively. A decrease in atenolol concentration is also observed (Table 5) in the wastewater only, not submitted to any treatment, which may be indicative of the atenolol biodegradability by the microorganisms

Table 5. Removal efficiencies ± 1 SD ($n = 3$) of atenolol in SSFCW microcosm assays as well as in the unplanted LECA beds and in the wastewater only, after 96 h of retention time.

System	Atenolol removed (%)
Wastewater only	14.3 ± 1.2
Unplanted LECA beds	82.0 ± 1.5
Phragmites planted beds	92.5 ± 0.6
Typha planted beds	94.5 ± 1.1

present in the wastewater. As can be observed, the biodegradability of atenolol is rather
360 low, as has already been reported in other studies [1,4,6].

For the same period of 96 hours, the unplanted LECA beds were able to achieve high removal efficiencies (82%, Table 5) that are indicative of the strong sorption capacity of this material towards atenolol. The good sorbent characteristics of LECA have already been reported for other pharmaceuticals such as the acidic compounds clofibric acid and
365 ibuprofen as well as the neutral carbamazepine for which the sorption by LECA is also high [24]. Electrostatic interactions for the case of the acidic pharmaceuticals and van der Waals interactions for the case of the neutral compounds have been hypothesised as being responsible for the affinities of these compounds towards LECA's surface. However, considering that both LECA and atenolol have alkaline nature and both are positively
370 charged at working pH conditions (pH ~ 8) the influence of electrostatic interactions doesn't explain the strong sorption of atenolol onto LECA. Probably, for this compound, ion exchange phenomena may be responsible to some extent for the efficiency of atenolol removal by LECA. This mechanism is known to be responsible for the removal into clay materials of other charged organic compounds such as some pesticides [37–39].

375 Enhanced efficiency is achieved by the planted LECA beds is enhanced in comparison with that observed for the unplanted beds (Table 5). Even though most atenolol is retained at the solid matrix, the presence of the plants, either *Typha* or *Phragmites*, contributes with additional 12–14% efficiency in comparison with that due to the LECA material alone. This contribution by the plants is consistent with the contributions reported by other
380 authors [14]. In addition to other benefits for the SSFCW system operation which derive from the presence of plants [14,25,27], these results demonstrate the equally important and active role played by the vegetation in the removal of atenolol, which enables the performance of planted systems to surpass that of a simpler LECA filter setup.

The comparison between the performance attained with *Phragmites* and *Typha* planted
385 beds seems to suggest that the latter species is somewhat more efficient than the former one. However, it should be noted although both *Typha* and *Phragmites* plants were planted in the beds at the same time, it was observed (by visual inspection) that during the acclimation period aerial parts of *Typha* were slightly more grown than were those of *Phragmites*. Also, probably due to a more developed aerial part, the transpiration rate
390 of *Typha* was higher than for *Phragmites* (data not shown) which may have contributed to the slightly higher efficiency of the *Typha*.

The overall performance of these SSFCW systems, either planted with *Typha* or *Phragmites*, is largely superior to that reported for atenolol removal by the conventional processes used in WWTP [1,3,4]. These types of systems are being increasingly used as an
395 alternative form of tertiary treatment stage in WWTPs and the present results suggest that it may be an efficient and cost-effective solution to deal with contamination of wastewaters by pharmaceuticals.

4. Conclusion

In the present study, two types of LECA-based SSFCW microcosm planted with two
400 different macrophyte species, *Typha* and *Phragmites*, were tested for their atenolol removal capabilities from wastewaters. The study provides an assessment of the potential of these systems to deal with pharmaceutical contamination.

A chromatographic method of analysis was established to measure atenolol in wastewater. Combined with an efficient SPE concentration step, the use of the widely available and inexpensive DAD detector yielded an analytical method with very low LOQ (9 ng mL^{-1}) and high reproducibility (RSD < 4%).

The material LECA, used as solid matrix in the tested microcosms, was responsible for most of atenolol removal from the wastewater, but both plant species used did contribute with an additional 12–14% to the overall removal by the planted systems. In addition, plants significantly increased the rate of atenolol removal, which enables retention time of the wastewater in the planted beds to be reduced to 96 hours in comparison to the much longer times needed to attain the same efficiency in a LECA filter bed. Although *Typha* presented a slightly higher efficiency than *Phragmites*, this may be related to the more advanced stage of development of the former species at the beginning of the assays.

Further tests are still required for assessing the impact on performance caused by more realistic conditions such as the removal during a longer time period with several cycles of loading in a microcosm wetland. Differences in changing from a discontinuous feed (batch mode), such as is used in this work, over to a continuous mode of feed and also a possible decrease in performance when going to a full scale wetland should also be evaluated. Nevertheless, the studied microcosms showed the high efficiency of these systems in removing atenolol and possibly other similar organic compounds from wastewaters and suggest that larger scale SSFCW systems may be efficient and cost-effective alternatives to other high-cost technologies, such as ozonisation or membrane bioreactors, to be used as a tertiary treatment stage.

Another aspect requiring further research is the potential risk of toxicity for the aquatic ecosystems by the residual concentrations still present in the effluents of the planted or the unplanted beds, after the treatment. In fact, even if large percentage removals are attained that does not ensure non toxic levels of the pharmaceuticals in the treated effluent.

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Chapter 5

5. Conclusion and future perspectives

The work conducted in this thesis presents an approach to the potential use of optimized constructed wetland systems (CWS) for the removal of selected pharmaceutical compounds from contaminated wastewaters.

In CWS, removal of pollutants is achieved through the concerted action of its components and, therefore, a significant improvement of the system's performance can be obtained by a careful selection of the system's components in order to maximize the role played by each one in the overall removal of the contaminants. Thus, as preliminary steps of this study, a major attention was devoted to the selection of the solid matrix materials and to the evaluation of the selected plant species' ability to withstand and remove the contaminants.

In respect to the matrix component, LECA was shown to be the most suitable material among all those tested to be used as support matrix in a CWS for the removal of the studied pharmaceuticals, namely clofibric acid, carbamazepine and ibuprofen. Other materials tested included sand, exfoliated vermiculite, sepiolite and expanded perlite. In addition, two granulometries of LECA (2/4 and 3/8) were compared. From these tests it could be concluded that LECA, in particular of the 2/4 granulometry, presents important advantages, namely a good sorption capacity for all the studied pharmaceuticals, a pH buffering capacity and a suited hydraulic permeability (7.7×10^{-3} m^3 m^{-2} s^{-1}) and porosity (46%). For several different conditions (aqueous solutions of the single compounds; aqueous solutions of clofibric acid, carbamazepine and ibuprofen mixtures; and treated wastewater spiked with the three compounds) LECA 2/4 removed the pharmaceuticals at initial concentrations of 1 mg L^{-1} in large extent (50-95%). The following decreasing order was observed for the removed amounts of each pharmaceutical: carbamazepine > ibuprofen > clofibric acid. The kinetics of the sorption process was characterized by an initial fast step, with most pharmaceuticals being removed within the first 24 h. Equilibrium was attained, in general, after approximately 72–96 h, and the kinetic behavior was similar in all tested conditions.

Despite the fact that a good option for a support matrix material was suggested by the obtained results, the choice of appropriate support matrix materials is not, however, a closed issue. In fact, one of the clay materials tested, vermiculite, exhibited a higher sorption capacity than LECA for the more recalcitrant compound, clofibric acid. However, some vermiculite's properties other than its sorbent qualities (e.g. its low density and mechanical properties) make it a less suitable material for an application as CWS's support matrix. Nevertheless, it can be worthwhile to proceed in the future with further tests using other materials which can still lead to improvements of the sorption qualities of the matrix for the studied pharmaceuticals and, possibly, for a wider range of compounds. Among other potential materials, tests can be extended to industrial by-products and agricultural wastes that can result in efficient and cost-effective solutions. As an example, cork is a local natural material which has an important role in the local economy and it would be interesting to find applications for its residues, thereby increasing its value.

In respect to the vegetation component, *Typha* spp. showed a good ability to remove the pharmaceuticals clofibric acid, carbamazepine and ibuprofen from aqueous solutions and to cope with their toxicity. In assays performed in hydroponic conditions, under controlled temperature and humidity, *Typha* spp. showed a remarkable capability to uptake the studied pharmaceuticals, being able to remove 80%, 95% and > 99% of clofibric acid, carbamazepine and ibuprofen respectively, after 21 days of exposure to a solution spiked with 20 $\mu g\ L^{-1}$ of each compound. Moreover, just within the initial period of 24–48 h, over 50% of the initial amounts of the compounds were removed in all cases, and even when the plants were subjected to concentrations several orders of magnitude higher (up to 2000 $\mu g\ L^{-1}$), still high removal efficiencies were observed. In the tested concentrations range, a linear relationship was observed between initial and removed amounts of the compounds, except in the assay performed with the highest concentration of ibuprofen.

One strong possibility for the fate of pharmaceuticals as they are removed from water is their uptake by the plants roots, translocation to aerial plant parts and consequent metabolization in leaf tissues. In fact, such route could be established for carbamazepine as this pharmaceutical was detected in leaf tissues of *Typha* spp. plants grown in hydroponic carbamazepine solutions, for all carbamazepine concentrations tested, and at increasing amounts for increasing initial carbamazepine concentrations. These results confirmed not only the uptake of the pharmaceutical but also its concomitant

translocation to the plants' leaves. Furthermore, the metabolite 10,11-dihydro-10,11-epoxycarbamazepine was tentatively identified in *Typha* leaf extracts which may be indicative of carbamazepine metabolization. The identification of other carbamazepine metabolites and conjugates remains to be carried out as a future continuation of this study in order to better understand the metabolism of this compound by *Typha* spp. The knowledge and understanding of the fate of pharmaceuticals in the plants is quite relevant for deciding how the vegetation should be maintained, for example to decide the need for periodic harvesting of the aerial plant parts to prevent reintroduction of contaminated material as dead plant parts decay.

In respect to the pharmaceuticals phytotoxicity, in general *Typha* spp. was able to cope with the toxic effects caused by the exposure to the studied compounds in nutrient solutions and exhibited an appropriate reaction to the pharmaceuticals' induced oxidative burst (as determined by the alteration of antioxidant enzymes activities both in roots and leaves) except in the case of the highest concentrations of some of the pharmaceuticals, where the inhibition of some antioxidant enzymes might be regarded as an early sign of toxicity. However, it should be noted that such cases corresponded to pharmaceuticals concentrations that were largely above normal environmental concentration levels. During the experiments, plants' growth rates were also affected by the exposure to the pharmaceuticals, but by the end of the assays they had, in general, approached normal values.

Overall, given the qualities exhibited by *Typha* spp., the species should be considered as a good candidate for an application in phytoremediation of waters contaminated with the studied pharmaceuticals. However, and in spite of the positive results obtained in these assays, the consideration of other plant species (e.g. *Phragmites australis*) for further future studies should not be discarded. The possibility of constructing systems with mixtures of several plant species may also be regarded as a possible additional optimization of these phytotreatment systems, for example if plants have different abilities to uptake different types and amounts of xenobiotics.

A microcosm constructed wetlands system established with a matrix of LECA and planted with *Typha* spp. was highly efficient in removing the pharmaceuticals atenolol, clofibric acid, carbamazepine and ibuprofen from spiked wastewater, thus showing the potential of these low-cost wastewater treatment systems for dealing with pharmaceuticals contamination. It was observed that the solid matrix composed by LECA 2/4 was responsible for most of the pharmaceuticals removal efficiency from

wastewater, but the presence of the *Typha* spp. plants made an additional contribution which was, in some cases, as high as ~ 30%, especially when plants were at their most active vegetative stage. *Typha* spp. also contributed to a significant increase in the rate of the pharmaceuticals removal. Influence of vegetation may be attributed to direct uptake of some of the compounds (as was proven in this work for carbamazepine) as well as to the enhancement of biodegradation processes' efficiency in comparison with other types of biological treatment. Removal kinetics in the microcosm was characterized by a fast initial step (>50% removal within 6 hours) with equilibrium attained within 96 – 120 hours of contact time.

Seasonal variability of the system's performance was observed with some loss of efficiency and slower removal rates during the winter season, in particular for the compounds mainly removed through some form of biological process (plant uptake or biodegradation). On the other hand, removal by sorption was influenced very lightly by seasonal alterations of environmental conditions. In fact, this process remained one of the most relevant, even during the winter, and removal of pharmaceuticals in which sorption had the major contribution (e.g. carbamazepine) were observed to be minimally affected by seasonal variations. These results show primarily that the support matrix plays an important role in smoothing the seasonal variability of the performance by providing abiotic removal processes that are less sensitive than biotic components to variations of environmental conditions. In addition, an overall optimization of these systems in this regard may include the selection and introduction of several plant species that have peaks of vegetative activity in non-overlapping periods of the year, minimizing in this way the seasonal variations in the performance of the biotic components of a CWS.

Future work on the evaluation of these systems' efficiencies must necessarily include tests under more realistic conditions. In particular, it is of primary importance to assess the impact on performance caused by a longer period of operation with several cycles of influent loading in the microcosm wetland, moving from a discontinuous mode of feed (as was used in this work) over to a continuous mode of feed and evaluating a possible decrease in the overall performance when going to a full-scale wetland. Additional optimizations should be addressed when scaling up these systems, including the evaluation of hydraulic retention times when the system is operating in intermittent or continuous feed conditions as well as the assessment of optimal flooding rates.

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Appendices

Appendix A

A. Pharmaceutical substances

Most of the information in the Appendices A.1 to A.4 was obtained from databases that were made accessible on the world wide web. Whenever bibliography is not cited directly in the text, data was obtained from one of these sources:

- ChemFinder.com, a scientific databases (http://chemfinder.cambridgesoft.com/reference/chemfinder.asp)
- DrugBank database (http://www.drugbank.ca/)
- INFARMED - Autoridade Nacional do Medicamento e Produtos de Saúde, I. P., Portuguese database on drug (http://www.infarmed.pt/portal/page/portal/INFARMED)
- PubChem Text Search (http://pubchem.ncbi.nlm.nih.gov/)
- RxList, the internet drug index (http://www.rxlist.com)
- The Physical Properties Database (PHYSPROP) of Syracuse Research Corporation (http://www.srcinc.com/what-we-do/databaseforms.aspx?id=386)
- TOXNET - Databases on toxicology, hazardous chemicals, environmental health, and toxic releases (http://toxnet.nlm.nih.gov/)

A.1. Clofibric acid

General data

- IUPAC Name: 2-(4-chlorophenoxy)-2-methyl propanoic acid
- CAS Number: 882-09-7
- Molecular formula: $C_{10}H_{11}ClO_3$
- Molecular weight: 214.65
- Vapor pressure @25°C (bar): 1.51×10^{-7} [a]
- Henry's law constant @25°C (atm m^3 mol^{-1}): 2.19×10^{-8} [a]
- Ionization constant, pKa: 2.5 [a,b], 3.18 [c]
- λ_{max} (absorption in water): 227 nm
- Water solubility @25°C (mg L^{-1}): 583 [a, d]
- log K_{ow}: 2.57 [a, d], 2.88 [e]
- Soil sorption coefficient, log K_{oc}: (0.9 – 1.36) [e], 1.88 [c]

[a] (SRC, 2009) [b] (Drillia et al., 2005) [c] (Packer et al., 2003)
[d] (Ferrari et al., 2003) [e] (Scheytt et al., 2005a)

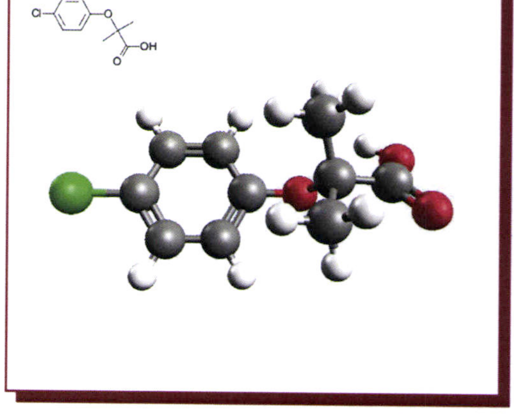


Figure A-1. Molecular structure of clofibric acid.

Clofibric acid is an active metabolite of clofibrate, etofibrate and etophyllinclofibrate which are drugs used as blood lipid regulators. These substances are used to decrease the plasmatic concentration of cholesterol and triglycerides. The mechanism of action in humans has not been established definitively. Clofibric acid is also classified as a plant growth regulator (antiauxin) pesticide (Wood, 2009).

Dosage

Patients typically ingest a dose of 500 mg of clofibrate 1-4 times per day (Leikin and Paloucek, 2007).

Metabolism and Elimination

Clofibric acid is the main active metabolite which results, after an oral administration of the parent drug, of a rapid de-esterification of clofibrate in the gastro-intestinal tract.

Between 95% and 99% of an oral dose of clofibrate is excreted in the urine as free clofibric acid and conjugated clofibric-*O*-*β*-hydroxyglucoronide (Winkler et al., 2001; Khetan and Collins, 2007).

The half-life of elimination of clofibric acid in normal volunteers averages between 18 and 22 hours (full range between 14 and 35 hours) but can vary by up to 7 hours in the same subject at different times.

Biodegradability

Clofibric acid has been reported in many studies to resist microbial degradation with several types of microorganisms (Winkler et al., 2001; Tixier et al., 2003; Khetan and Collins, 2007; Hernando et al., 2007a; Aga, 2008; Evangelista et al., 2008).

Analytical methods for clofibric acid quantification

Water and wastewater matrices: Solid phase extraction (SPE) followed by HPLC-UV/Vis, LC-MS/MS or GC-MS are commonly used (Petrovic and Barceló, 2007; Aga, 2008). Currently, LC-MS/MS is the method of choice because it has been shown to have lower limits of detection and better selectivity (Gros et al., 2006a; Gros et al., 2006b; Hernando et al., 2007a).

Occurrence in the environment

Clofibric acid was the first prescription drug metabolite reported in environmental studies (Garrison et al., 1976). It is still one of the most frequently found and reported drugs in wastewater treatment plants (WWTPs) effluents (Heberer, 2002a; Fent et al., 2006; Khetan and Collins, 2007; Miège et al., 2009; Kasprzyk-Hordern et al., 2009). In the aquatic environment, it has been detected in surface waters (rivers, lakes), ground waters and drinking waters all over the world in concentrations ranging from ng-µg L^{-1} levels (Ternes, 1998; Tixier et al., 2003; Ashton et al., 2004; Fent et al., 2006; Petrovic and Barceló, 2007; Aga, 2008).

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna (Invertebrates)	EC_{50} = 106 mg L^{-1}	(Webb, 2004)
Brachyderio rerio (Fish embryos)	EC_{50} (48h) = 86 mg L^{-1}	(Webb, 2004)
Scenedesmus subspicatus (Algae)	EC_{50} (72h) = 89 mg L^{-1}	(Webb, 2004)
Daphnia magna (Invertebrates)	EC_{50} (48h) > 200 mg L^{-1}	(Ferrari et al., 2003)
Ceriodaphnia dubia (Invertebrates)	EC_{50} (48h) > 200 mg L^{-1}	(Ferrari et al., 2003)
P. subcapitata (Algae)	NOEC (96h) = 75 mg L^{-1}	(Ferrari et al., 2003)
B. Calyciflorus (Invertebrates)	NOEC (48h) = 0.246 mg L^{-1}	(Ferrari et al., 2003)
Ceriodaphnia dubia (Invertebrates)	NOEC (7d) = 0.640 mg L^{-1}	(Ferrari et al., 2003)
Brachyderio rerio (Fish embryos)	NOEC (10d) = 70 mg L^{-1}	(Ferrari et al., 2003)

EC_{50}: median Effect Concentration or 50% Effective Concentration
NOEC: No Observed Effect Concentration

A.2. Ibuprofen

General data

- IUPAC Name: 2-(4-isobutylphenyl) propanoic acid
- CAS Number: 15687-27-1
- Molecular formula: $C_{13}H_{18}O_2$
- Molecular weight: 206.28
- Vapor pressure @25°C (bar): 2.5×10^{-7} [a]
- Henry's law constant @25°C (atm m^3 mol^{-1}): 1.57×10^{-7} [a]
- Ionization constant, pKa: 4.42 [b], 4.91 [a]
- λ_{max} (absorption in water): 222 nm
- Water solubility @25°C (mgL^{-1}): 21 [a], 49 [b]
- log K_{ow}: 2.48 [c], 3.5 [d], 3.97 [a], 4.13 [b]
- Soil sorption coefficient, log K_{oc}: (2.14 – 2.21) [c]

[a] (SRC, 2009) [b] (Avdeef et al., 2000) [c] (Scheytt et al., 2005a) [d] (Jones et al., 2002)

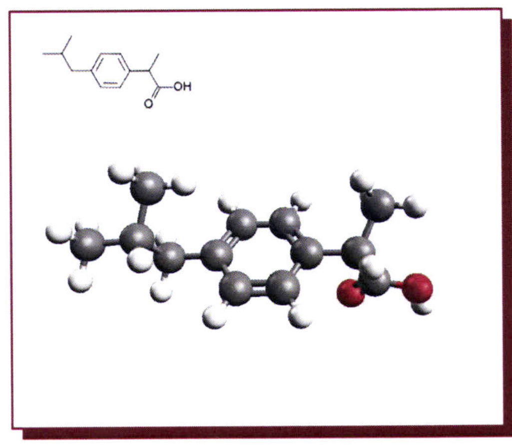


Figure A-2. Molecular structure of ibuprofen.

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID). It has analgesic and antipyretic properties but its mode of action, like that of other NSAIDs, is not completely understood, though it is believed that it may be related to prostaglandin synthetase inhibition. Ibuprofen is indicated for inflammatory diseases and rheumatoid disorders including juvenile rheumatoid arthritis, mild-to-moderate pain, fever and dysmenorrhea (Leikin and Paloucek, 2007). It is one of the most important pharmaceuticals in terms of consumption amounts.

Dosage

The suggested dosage is 1200-3200 mg daily, and must not exceed a 3200 mg total daily dose. Usual oral doses for an adult are between 200-400 mg/dose every 4-6 hours (analgesia/pain/fever/dysmenorrhea) and 400-800 mg/dose 3-4 times/day (inflammatory diseases, e.g. rheumatoid arthritis, osteoarthritis) (Leikin and Paloucek, 2007).

Metabolism and Elimination

Ibuprofen is rapidly absorbed when administered orally. Studies have shown that within 24 hours following ingestion of the drug, 45% to 79% of the dose was recovered in the urine as the metabolites: 25% as 2-hydroxyibuprofen (IB-2OH), and 37% as carboxyibuprofen (IB-CA) (Table A-1); the percentages of free and conjugated ibuprofen were 1-8% and 14%, respectively (Ternes, 1998; Winkler et al., 2001; Leikin and Paloucek, 2007). In humans, the parent drug as well as the metabolites are found to be conjugated with glucuronic acid, and glucuronidation has, in all cases, taken place at the carboxyl group in the propanoic acid side chain (Khetan and Collins, 2007).

Table A-1. Main metabolites of ibuprofen (Buser et al., 1999; Winkler et al., 2001; Petrovic and Barceló, 2007; Aga, 2008)

Structure	Compound	Abbreviation	Molecular formula	Molecular weight
	1-Hydroxyibuprofen	IB-OH	$C_{13}H_{18}O_3$	222
	2-Hydroxyibuprofen	IB-2OH	$C_{13}H_{18}O_3$	222
	3-Hydroxyibuprofen	IB-3OH	$C_{13}H_{18}O_3$	222
	Carboxyibuprofen	IB-CA	$C_{13}H_{16}O_4$	236

Biodegradability

Ibuprofen's good biodegradability during wastewater treatment has been reported by several authors (Buser et al., 1999; Zwiener et al., 2002; Kümmerer, 2008).

Analytical methods for ibuprofen quantification

Water and wastewater matrices: SPE followed by HPLC-UV, LC-MS/MS or GC-MS were used in several studies (Petrovic and Barceló, 2007; Aga, 2008). Recently LC-MS/MS has become the common methodology for separation and detection of anti-inflamatories such as ibuprofen (Gros et al., 2006a; Gros et al., 2006b; Aga, 2008).

Occurrence in the environment

Ibuprofen has been detected in surface waters as well as in effluents of wastewater treatment plants (WWTPs) worldwide at concentrations up to μg L^{-1} levels (Ternes, 1998; Petrovic and Barceló, 2007; Aga, 2008; Kasprzyk-Hordern et al., 2009). In raw wastewater, ibuprofen is found together with its main metabolites, IB-OH and IB-CA (Zwiener et al., 2002; Aga, 2008). In some studies a significant removal of ibuprofen was observed and especially of IB-CA during wastewater treatment, whereas the concentration of IB-OH in the WWTPs effluents was almost similar to those in the influents. Thus, IB-OH was found in surface water at much higher concentrations than ibuprofen or IB-CA (Jones et al., 2002; Zwiener et al., 2002).

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna (Invertebrates)	EC_{50} (48h) = 9.06 mg L^{-1}	(Webb, 2004)
Lepomis macrochirus (Fish)	EC_{50} (96h) = 173 mg L^{-1}	(Webb, 2004)
Skeletonema costatum (Algae)	EC_{50} (96h) = 7.1 mg L^{-1}	(Webb, 2004)
Planorbis carinatus (Mollusc)	EC_{50} (48 and 72h) = 17.08 mg L^{-1}	(Pounds et al., 2008)

EC_{50}: median Effect Concentration or 50% Effective Concentration

Adverse effects on humans

The most frequent type of adverse reactions occurring with ibuprofen is gastrointestinal (e.g. abdominal cramps, dyspepsia, vomiting, GI ulceration, constipation or diarrhea). Effects on the central nervous system (e.g. dizziness, headache, psychosis, cognitive dysfunction or coma) and dermatologic effects (rash, urticaria, pruritus) were observed during controlled clinical trials at an incidence greater than 1% (Leikin and Paloucek, 2007). Because of the known effects of Ibuprofen on the fetal cardiovascular system (closure of ductus arteriosus), use during late pregnancy should be avoided (Leikin and Paloucek, 2007).

Medicines on sale in Portugal

Anadvil®, Arfen®, Baroc®, Brufen®, Calbrun®, Dorifen®, Trifene®, Ozonol®, etc. (INFARMED, 2008).

A.3. Carbamazepine

General data

- IUPAC Name: benzo[b][1]benzazepine-11-carboxamide
- CAS Number: 298-46-4
- Molecular formula: $C_{15}H_{12}N_2O$
- Molecular weight: 236.27
- Vapor pressure @25°C (bar): 2.45×10^{-10} [a]
- Henry's law constant @25°C (atm m^3 mol^{-1}): 1.08×10^{-10} [a]
- Ionization constant, pKa: 13.9 [b], 14 [c]
- λ_{max} (absorption in water): 210 nm
- Water solubility @25°C (mg L^{-1}): 17.7 [c], 112 [a]
- log K_{ow}: 2.25 [c], 2.45 [a]
- Soil sorption coefficient, log K_{oc}: (2.00 - 3.42) [d]

[a] (SRC, 2009) [b] (Jones et al., 2002) [c] (Scheytt et al., 2005b) [d] (Scheytt et al., 2005a)

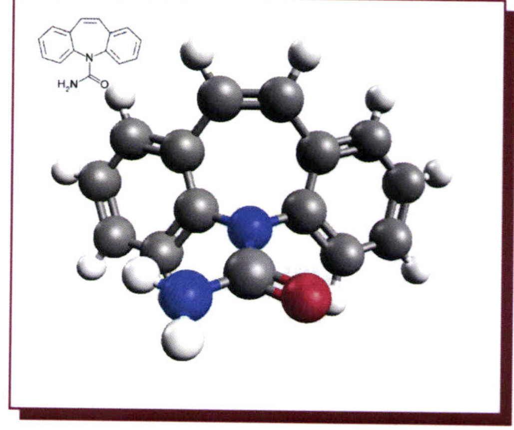


Figure A-3. Molecular structure of carbamazepine.

Carbamazepine is an anticonvulsant and mood stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder. It is also used to treat other affective disorders such as resistant schizophrenia, ethanol withdrawal, restless leg syndrome, psychotic behavior associated with dementia and post-traumatic stress disorders (Leikin and Paloucek, 2007).

Dosage

Usual adult dose is 400-1200 mg/day in 2-4 divided doses. Maximum dose for children of ages between 12-15 years is 1000 mg/day, and for individuals over 15 years is 1200 mg/day; however, some patients have required up to 1600-2400 mg/day (Leikin and Paloucek, 2007).

Metabolism and Elimination

Carbamazepine undergoes extensive hepatic metabolism by the cytochrome P-450 system. Thirty-three metabolites of carbamazepine have been identified from human and rat urine (Miao and Metcalfe, 2003; Aga, 2008; Leclercq et al., 2009), the main

ones of which are presented in Table A-2. The main metabolic pathway of carbamazepine is oxidation to 10,11-epoxycarbamazepine (CB-EP), hydration to 10,11-dihydroxycarbamazepine (CB-DiOH) and 10-hydroxycarbamazepine (CB-10OH), then conjugation of these compounds with glucuronide. The second minor distinct pathway for the biotransformation of carbamazepine, catalyzed by cytochrome P-450, involves the oxidation to 2-hydroxycarbamazepine (CB-2OH) and 3-hydroxycarbamazepine (CB-3OH), and subsequent conjugation with glucuronide (Miao and Metcalfe, 2003; Miao et al., 2005; Leclercq et al., 2009).

Table A-2. Main metabolites of carbamazepine (Miao and Metcalfe, 2003; Miao et al., 2005; Leclercq et al., 2009)

Structure	Compound	Abbreviation	Molecular formula	Molecular weight
	10,11-dihydro-10,11-epoxycarbamazepine	CB-EP	$C_{15}H_{12}N_2O_2$	252.09
	10,11-dihydro-10,11-dihydroxycarbamazepine	CB-DiOH	$C_{15}H_{14}N_2O_3$	270.10
	2 - hydroxycarbamazepine	CB-2OH	$C_{15}H_{12}N_2O_2$	252.09
	3 - hydroxycarbamazepine	CB-3OH	$C_{15}H_{12}N_2O_2$	252.09
	10,11-dihydro-10-hydroxycarbamazepine	CB-10OH	$C_{15}H_{14}N_2O_2$	254.10

Biodegradability

Carbamazepine is highly resistant either to biodegradation in the aquatic environment and in biological processes of wastewater treatment (Stamatelatou et al., 2003; Petrovic and Barceló, 2007; Aga, 2008; Zhang et al., 2008; Kümmerer, 2008; Leclercq et al., 2009).

Analytical methods for carbamazepine quantification

Water and wastewater matrices: SPE followed by HPLC-UV/Vis, LC-MS/MS or GC-MS were used in several studies (Petrovic and Barceló, 2007; Aga, 2008). Currently, LC-MS/MS is the method of choice because it has been shown to have better limits of detection and better selectivity (Gros et al., 2006a; Gros et al., 2006b; Aga, 2008).

Occurrence in the environment

Carbamazepine has frequently been detected in wastewater, surface water and ground water samples all over the world (Ternes, 1998; Heberer, 2002a; Petrovic and Barceló, 2007; Khetan and Collins, 2007; Aga, 2008; Zhang et al., 2008). Analyses of influents and effluents from different municipal wastewater treatment plants (WWTPs) have shown that carbamazepine is not significantly removed during wastewater treatment. As a result, carbamazepine has been frequently detected in environmental samples (Heberer, 2002a; Petrovic and Barceló, 2007; Aga, 2008; Zhang et al., 2008). In addition, five metabolites of carbamazepine have already been detected in WWTPs influent and effluent samples (Miao and Metcalfe, 2003; Miao et al., 2005; Petrovic and Barceló, 2007; Aga, 2008; Zhang et al., 2008; Leclercq et al., 2009). However only carbamazepine and CB-DiOH have been detected in surface water (Miao and Metcalfe, 2003).

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna (Invertebrates)	EC_{50} (48h) > 13.8 mg L^{-1}	(Ferrari et al., 2003)
Ceriodaphnia dubia (Invertebrates)	EC_{50} (48h) > 77.7 mg L^{-1}	(Ferrari et al., 2003)
P. subcapitata (Algae)	NOEC (96h) = 100 mg L^{-1}	(Ferrari et al., 2003)
B. Calyciflorus (Invertebrates)	NOEC (48h) = 0.377 mg L^{-1}	(Ferrari et al., 2003)
Ceriodaphnia dubia (Invertebrates)	NOEC (7d) = 0.025 mg L^{-1}	(Ferrari et al., 2003)
Brachyderio rerio (Fish embryos)	NOEC (10d) = 25mg L^{-1}	(Ferrari et al., 2003)

EC_{50}: median Effect Concentration or 50% Effective Concentration
NOEC: No Observed Effect Concentration

Adverse effects on humans

The most frequent type of adverse reaction occurring with carbamazepine is gastrointestinal (nausea, vomiting, gastric distress, anorexia, abdominal pain, diarrhea, constipation, pancreatitis) and on the central nervous system (sedation, dizziness, fatigue, ataxia, confusion, headache, aseptic meningitis) (Leikin and Paloucek, 2007).

Medicines on sale in Portugal

Tegrol® and some generic medicines such as Carbamazepina Alter, Carbamazepina Generis, Carbamazepina Mylan, Carbamazepina Normon (INFARMED, 2008).

A.4. Atenolol

General data

- IUPAC Name: 2-[4-[2-hydroxy-3-(1-methylethylamino) propoxy]phenyl]ethanamide
- CAS Number: 29122-68-7
- Molecular formula: $C_{14}H_{22}N_2O_3$
- Molecular weight: 266.34
- Vapor pressure @25ºC (bar): 3.89×10^{-13} [a]
- Henry's law constant @25ºC (atm m^3 mol^{-1}): 1.37×10^{-18} [a]
- Ionization constant, pK_a: 9.6 [a]
- λ_{max} (absorption in water): 230 nm
- Water solubility @25ºC (mg L^{-1}): 13300 [a]
- log K_{ow}: 0.16 [a] – 0.46 [b]

[a](SRC, 2009) [b](Hernando et al., 2007b)

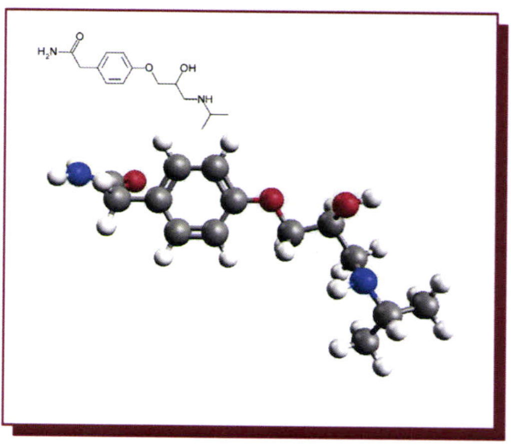


Figure A-4. Molecular structure of atenolol.

Atenolol is a specific antagonist of beta-1 receptors, a drug belonging to the group of beta-blockers, a class of drugs used primarily in cardiovascular diseases. Atenolol can be used to treat cardiovascular diseases and conditions such as hypertension, coronary heart disease, arrhythmias, angina (chest pain) and to treat and reduce the risk of heart complications following myocardial infarction (heart attack).

Dosage

Atenolol dosage can vary from as little as 25 mg to 200 mg a day. In cases of doses over 100 mg, the dosage is usually divided and taken twice daily (Leikin and Paloucek, 2007).

In patients with normal renal function, the usual daily dose is 25-50 mg for the management of hypertension, depending on the indication and severity of the disease whereas, for the management of angina, daily 100 mg may be given (Leikin and Paloucek, 2007).

Maximum daily dose of atenolol for high blood pressure control is 100 mg, while the maximum daily dose for treating angina symptoms is 200 mg.

Metabolism and Elimination

Absorption of an oral dose is rapid and consistent but incomplete. After ingestion, approximately 50% of an oral dose is absorbed from the gastrointestinal tract, the remainder being excreted via urine mainly as an unchanged compound (90%), with a small percentage of atenolol-glucuronide (0.8%-4.4%) and hydroxyatenolol (1.1-4.4%) (Escher et al., 2006; Radjenovic et al., 2008).

Biodegradability

Atenolol has been found to be biodegradable in wastewater treatment plants (WWTPs) (Maurer et al., 2007). However the degradation rates are too low for the entire biodegradation of the compound and therefore, elimination in WWTPs has been found to be incomplete (Paxeus, 2004; Hernando et al., 2007b; Palmer et al., 2008; Wick et al., 2009).

Analytical methods for atenolol quantification

Water and wastewater matrices: SPE followed by HPLC-UV/Vis, LC-MS/MS or GC-MS were used in several studies (Petrovic and Barceló, 2007; Aga, 2008). Some authors recommended use of LC rather than GC for the analysis of these polar molecules in the environment, because the derivatization of the hydroxyl groups required for GC-MS analysis was incomplete (Gros et al., 2006a; Aga, 2008).

Occurrence in the environment

Atenolol was frequently detected in WWTP influents at ng L^{-1} concentration levels, whereas, in some cases, comparable concentrations in the treated effluent were noticed as well as in natural water bodies (Maurer et al., 2007; Hernando et al., 2007b; Palmer et al., 2008; Snyder, 2008; Wick et al., 2009).

Ecotoxicity

Organisms	Toxicity	References
Daphnia magna (Invertebrates)	EC_{50} = 313 mg L^{-1}	(Cleuvers, 2005)
Desmodesmus subsicatus (Algae)	EC_{50} = 620 mg L^{-1}	(Cleuvers, 2005)
Daphnia magna (Invertebrates)	EC_{50} = 200 mg L^{-1}	(Hernando et al., 2004)

EC_{50}: median Effect Concentration or 50% Effective Concentration

Adverse effects on humans

Common side effects of atenolol include general fatigue, dizziness, impotence, loss of libido, nausea. Rare side effects of atenolol may include abdominal cramps, constipation, diarrhea, insomnia, depression, dizziness upon standing up, drowsiness, lightheadedness, tiredness, vertigo, wheezing (Leikin and Paloucek, 2007).

Medicines on sale in Portugal

Generic medicines: Atenolol 1 Apharma, Atenolol Alter, Atenolol Angenérico, Atenolol Azevedos, Atenolol Bril, Atenolol Corzil (INFARMED, 2008).

A.5. Consumption of drugs in Portugal

Table A-3. Consumption of the studied pharmaceuticals (INFARMED, 2004; 2005; 2006; 2007; 2008)

Active substances *	2003	2004	2005	2006	2007
	Number of packages				
Ibuprofen	1260328	1255318	1438448	1416708	1633842
Carbamazepine	410837	418143	412375	398455	391173
Atenolol	380193	382521	371534	83340	106988

* data is not available for clofibric acid as it is a metabolite and not an active substance

Table A-4. Distribution of National Health Service medicines sales (packages) sorted by pharmacotherapeutic groups (INFARMED, 2004; 2005; 2006; 2007; 2008)

Pharmacotherapeutic groups	2003	2004	2005	2006	2007
	Number of packages				
Anti-Infectives products	10188198	9600421	9810826	9232659	9497408
Central Nervous system	29354466	31732022	32423760	32333681	31243105
Cardiovascular system	26746108	28072004	29644933	30850049	31964322
Blood	3319826	4009423	4404933	4784683	5260979
Respiratory System	4677456	4547502	4805030	4438143	4637884
Digestive system	6008896	6281984	6317664	6528670	6608505
Genitourinary system	1924604	2672294	2797267	2944602	2960154
Endocrine system	12348181	10843762	11079805	11263476	11572179
Locomotor System	11749914	13708812	14113415	13908827	14162621
Antiallergic Medication	2217169	2401901	2571682	2480729	2681278
Nutrition	2171065	2230217	1526964	1347597	1207521
Electrostatic and Fluid Balance Regulation Agents	321705	125082	120671	104515	96137
Dermatological Agents	2199028	2036420	1855862	1797353	1725407
Oropharyngeal drugs	685359	752397	762476	783463	857237
Drugs for Ophthalmologic use	3094487	3265850	3292905	3266419	3274084
Antineoplastic and immunomodulators agents	203380	238204	236780	215526	209224
Anti-Poisoning agents	107185	44949	38360	33719	30458
Vaccines and immunoglobulins	1959694	1768222	1797138	1225649	1178821
Remaining		77028	104019	69986	56335

Appendix B

B. Support materials

Most of the information in the Appendices B.1 to B.5 was obtained from databases that are accessible on the world wide web. Whenever bibliography is not cited directly in the text, data was obtained from one of these sources:

- Aguiar & Mello, Lda (http://www.aguiaremello.pt/)
- Incon Corporation's Perlite.info website. (http://www.perlite.info/hbk/0031401.htm)
- Maxit Portugal (http://www.maxit.pt/)
- Mindat.org's database and reference website for mineral and mineralogical data (http://www.mindat.org/index.php)
- Mineral information institute (http://www.mii.org/index.html)
- Mineralogy Database (http://www.webmineral.com/)
- Sepiolsa (http://www.sepiolsa.com/)
- The Perlite Institute Inc (http://www.perlite.org/perlite_info.htm)
- The Vermiculite Institute, sponsored by The Schundler Company (http://www.vermiculite.net/)

B.1. Light Expanded Clay Aggregates (LECA)

Description

LECA is a processed natural material that is produced by subjecting clay aggregates to a high temperature treatment. This material is manufactured by running pelletized clay aggregates through rotary kilns at 1200°C, causing injected CO_2 to expand within the clay aggregates and thus creating highly porous, lightweight ceramic pebbles.

Figure B-1. Typical aspect of LECA grains.

Typical chemical composition

Constituent	Percentage present by weight (%)			
SiO_2	70	58	51.8	62
Al_2O_3	20	27	15.6	18
K_2O	-	2.3	1.3	4
Na_2O	-	0.3	1.2	2
CaO	} 1.3	0.2	9.3	3
MgO		0.4	6.1	3
Fe_2O_3	-	1.0	13.0	7
TiO_2	-	1.3	1.7	-
FeO	8.7	-	-	-
Data source	(Haque et al., 2008)	(Arioz et al., 2008)		(maxit Portugal, 2009)

Typical physico-chemical properties

Parameters	Value
Porosity (%)	56
Bulk density (g cm^{-3})	0.47
K_s (m day^{-1})	1310 ± 60
U (d_{60}/d_{10})	3.9
pH (H_2O)	7.69
Data source	(Brix et al., 2001)

K_s, hydraulic condutivity; U= d_{60}/d_{10}, uniformity coefficient

Nominal sizes of particles

The standard grades normally available are:

Commercial designation	Nominal Size (mm)
Leca® Areia (0.5/3)	0.5-3
Leca® Godo (2/4)	1-5
Leca® Enchimento bombagem (3/8 F)	4-12.5
Leca® Enchimento manual (3/8 C)	10-20
Leca® Isolamento (8/16)	10-20
Leca® (10/20)	10-20
Data source	(maxit Portugal, 2009)

Applications

LECA is mainly used for construction purposes (such as in building blocks and insulating material). However over the last years it is also being used for different types of water and wastewater treatment processes such as filter processes and constructed wetlands systems (Zhu et al., 1997; Brix et al., 2001; Scholz and Xu, 2002; Heistad et al., 2006; Calheiros et al., 2008).

Distribution in Portugal

maxit-Argilas Expandidas, SA (maxitGroup) (Portugal)

B.2. Exfoliated Vermiculite

Description

Vermiculite forms a group of hydrated laminar minerals which are aluminum-iron-magnesium silicates resembling mica in appearance. Crude vermiculite is found in various parts of the world, and its commercial form consists of thin golden-brown flakes classified into grades. Vermiculite has the property of exfoliating or expanding when subjected to sudden intense heat, due to the interlaminar vaporization of the internal structure hydration water and consequent generation of steam. Exfoliation occurs at right angles to the cleavage planes, causing the flakes to expand into concertina-shaped granules.

Figure B-2. Typical aspect of vermiculite grains.

Typical chemical composition

Constituent	Percentage present by weight (%)				
SiO_2	39	40.87	38-46	39.37	38-40
Al_2O_3	12	19.46	10-16	12.08	8-9.5
MgO	20	18.41	16-35	23.37	24.5-27
K_2O	4	8.31	1-6	2.46	4-6
Na_2O	-	0.04	-	0.80	-
CaO	3	0.22	1-5	1.46	2
MnO_2	-	0.04	-	0.30	-
Fe_2O_3	8	-	6-13	5.45	5-6
P_2O_5	-	-	-	0.15	0.06
TiO_2	-	1.02	1-3	-	0.75
FeO	-	6.50	-	1.17	-
BaO	-	0.01	-	0.03	-
ZnO	-	0.01	-	-	-
Others	-	-	0.2-1.2	< 1.83	4.133
Loss on ignition	-	5.01	-	-	14.24
Data source	(Panuccio et al., 2009)	(Malandrino et al., 2006)	(Mysore et al., 2005)	(Mathialagan and Viraraghavan, 2003)	(Aguiar & Mello, 2009)

Typical physico-chemical properties

Parameters	Value			
Surface area ($m^2\ g^{-1}$)	-	-	134.4	-
Bulk density ($g\ cm^{-3}$)	2.6	-	0.07	2.4
Cation exchange capacity (meq/100 g)	100	40.08	-	-
pH (in water)	7	8.63 (PZC)	7.3	7.0
Porosity (%)	-	-	75	-
Data source	(Panuccio et al., 2009)	(Malandrino et al., 2006)	(Mysore et al., 2005)	(Mathialagan and Viraraghavan, 2003)

Nominal sizes of particles

The standard grades normally available are:

Commercial designation	Nominal Size (mm)
Vermiculite V0 Micron	0.1 - 1.0
Vermiculite V1 Superfine	0.5 - 2.0
Vermiculite V2 Fine	1.0 - 3.0
Vermiculite V3 Medium	2.0 - 5.0
Vermiculite V4 Coarse	5.0 - 15.0

Note: Nominal particle size indicates the 80% band i.e. up to 10% above or below the specified range.

Applications

Notwithstanding the several different applications, vermiculite has been used mostly in agriculture, in particular horticulture – where exfoliated vermiculite improves soil aeration and moisture retention and is also used as a carrier for fertilizers and pesticides. In construction, it is used in general building plasters and fillers to enhance acoustic, fire rating, and insulation performance. Recently, several studies have been carried out on the use of vermiculite, usually after chemical modification (i.e. organovermiculite), as an adsorbent of organic compounds from water (da Silva Jr. et al., 2003; Abate and Masini, 2005; Mysore et al., 2005).

Distribution in Portugal

Aguiar & Mello, Lda (Portugal)

B.3. Expanded Perlite

Description

Perlite is a glassy volcanic rock which is essentially a metastable amorphous aluminum silicate. It has a relatively high water content, typically formed by the hydration of obsidian. This material occurs naturally and has the unusual property of greatly expanding when sufficiently heated. When it reaches temperatures of 850–900°C, perlite softens (since it is a glass) and the water trapped in the structure of the material vaporizes and escapes. This causes the expansion of the material to 7–16 times its original volume and results in glasslike material with a cellular structure. The expanded material is a brilliant white, due to the reflectivity of the trapped bubbles.

Figure B-3. Typical aspect of perlite grains.

Typical chemical composition

Constituent	Percentage present by weight (%)			
SiO_2	72.44	71-75	72.75	75.22
Al_2O_3	14.5	12-16	13.56	13.08
K_2O	5.087	4-5	4.93	4.95
Na_2O	4.652	2.9-4.0	2.92	3.00
CaO	0.831	0.5-2.0	1.10	1.43
MnO	0.093	0.0-0.1	-	0.06
MgO	<0.083	0.03-0.5	0.29	0.10
Fe_2O_3	< 0.729	0.5-1.45	0.83	1.83
P_2O_5	<0.115	-	-	0.02
TiO_2	<0.059	0.03-0.2	-	0.13
SO_3	-	0.0-0.1	-	-
FeO	-	0.0-0.1	-	-
Ba	-	0.0-0.1	-	-
PbO	-	0.0-0.5	-	-
Loss on ignition	36.8	-	3.63	-
Data source	(Mathialagan and Viraraghavan, 2002)	(Acemioglu, 2005)	(Alkan et al., 2005)	(Chakir et al., 2002)

Typical physico-chemical properties

Parameters	Value		
Surface area (m^2g^{-1})	-	1.88	2.30
Density (g cm^{-3})	2.4	-	2.24
Cation exchange capacity (meq/100 g)	-	34.25	33
pH (in water)	7-8	6.6-8.0	-
Data source	(Mathialagan and Viraraghavan, 2002)	(Acemioglu, 2005)	(Alkan et al., 2005)

Nominal sizes of particles

There are ten grades of expanded perlite currently available:

Commercial designation	Nominal Size (mm)
P05 Ultrafine	0.01 - 1.0
P3 Cryogenic	0.75 - 1.2
P6 Extra Fine	0.10 - 1.70
P10 Super Fine	0.15 - 2.0
P15 Medium	0.15 - 3.0
P25 Special	1.0 - 3.0
P35 Standard	1.0 - 5.0
PCLG Chimney Lining Grade	1.0 - 5.0
P321 Through Grade	0.15 - 6.0
P40 Extra Coarse	3.0 - 6.0
P45 Super Coarse	1.0 - 6.0

Note: Nominal particle size indicates the 80% band i.e. up to 10% above or below the specified range.

Applications

The uses of expanded perlite are many and varied. Expanded perlite is an excellent insulator, both thermal and acoustic, resists fire and is classified as an ultralightweight material. Due to these properties, this material finds its major use in the construction industry. In addition, when mixed with soil, perlite increases the amount of air and water retained in the soil which obviously improves the growing conditions for plants and, therefore, another important use for perlite is found in horticultural applications (Mathialagan and Viraraghavan, 2002). As perlite contains a high silica content, usually

greater than 70%, it is chemically inert in many environments and hence it is also an excellent filter aid and filler in various industrial processes.

Recently, the properties of perlite for adsorption of water contaminants have been investigated in a number of papers (Chakir et al., 2002; Mathialagan and Viraraghavan, 2002; Alkan et al., 2005).

Distribution in Portugal

Hubel (Portugal)

B.4. Sepiolite

Description

Sepiolite, which forms an important group of clay minerals, is a natural hydrated magnesium silicate. Structurally, it is formed by blocks and channels extending in a fibrous texture containing a significant number of silanol (Si–OH) groups at the surface of the mineral. These groups can play an important role in the adsorption capacity and the removal mechanism of the adsorbates.

Figure B-4. Typical aspect of sepiolite grains

Typical chemical composition

Constituent	Percentage present by weight (%)		
SiO_2	53.47	51.17	53.0
MgO	23.55	25.50	28.6
CaO	0.71	7.52	0.22
Al_2O_3	0.19	1.04	0.5
K_2O	-	0.80	0.07
Na_2O	-	0.54	0.01
Fe_2O_3	0.16	0.40	0.95
NiO	0.43	-	-
TiO_2	-	0.05	-
Loss on ignition	21.49	12.98	15.6
Data source	(Alkan et al., 2004; Tekin et al., 2006; Dogan et al., 2007)	(Ozcan et al., 2004)	(Lazarevic et al., 2007)

Typical physico-chemical properties

Parameters	Value
Surface area (m^2g^{-1})	342
Density ($g\ cm^{-3}$)	2.5
Cation exchange capacity (meq/100 g)	25
pH	7.8-8.3
Porosity (%)	50.8
Data source	(Alkan et al., 2004; Dogan et al., 2007)

Nominal sizes of particles

The standard grades normally available are:

Commercial designation	Grade
Powder	< 100
Super Fine	60/100
Very Fine	30/60
Fine	15/30
Coarse	4/30
Data source	(Sepiolsa, 2009)

Applications

Sepiolite has a very popular application in pet litters due to its light weight, high liquid absorption capacity and dehydrating effect which minimizes bad odors and inhibits bacteria proliferation. Sepiolite also finds applications in waste treatment, where it can be used to absorb toxic and hazardous wastes in stabilization or inertization treatments. As an industrial absorbent, it is used to absorb liquid spills and leaks, keeping work and transit areas dry and safe. Several studies have also been conducted using sepiolite as a catalyst support, in wastewater and solid wastes treatment, and in reducing the toxic effect of some pollutants (Rytwo et al., 2002; Rajakovic et al., 2007; Dogan et al., 2007; Ugurlu, 2008; Donat, 2009).

Distribution in Portugal

ActivPet(Portugal), Sepiolsa (Espanha)

B.5. Sand

Description

Sand is a naturally occurring granular material composed of finely divided rock and mineral particles. The composition of sand is highly variable, depending on the local rock sources and conditions, but typically contains high amounts of silica usually in the form of quartz, and, especially in the case of sea sand, also some carbonates such as calcite whose sources may be parts of mollusk shells.

Figure B-5. Typical aspect of sand grains

Applications

A common application of sand is in water treatment plants as a medium used in filtration processes and as a support matrix in constructed wetlands (USEPA and USDA-NRCS, 1995; Cooper et al., 1996; Vymazal et al., 1998; Brix et al., 2001; Kadlec and Wallace, 2009). A very common application is also in construction as a concrete filler material.

Sources

Sand may be obtained commercially from a variety of distributors, either in a raw state or following a thermal and/or chemical treatment. This material is, however, also widely available to be collected at river banks and in coastal areas. The sand used in some assays of this work was collected in Faro Beach, Portugal.

Appendix C

C. Vegetation

Most of the information in Appendix C.1 was obtained from databases that were made accessible on the world wide web. Whenever bibliography is not cited directly in the text, data was obtained from one of these sources:

- BayScience Foundation, Inc (http://zipcodezoo.com)
- Base de Dados "Flora Digital de Portugal", Jardim Botânico da UTAD (http://www.jb.utad.pt/pt/herbario/cons_reg.asp)
- Plants For A Future - Database Search (http://www.ibiblio.org/pfaf/D_search.html)
- University of Texas at Austin – Native Plant Database (http://www.wildflower.org/plants/result.php?id_plant=TYDO)
- USDA Forest Service (http://www.fs.fed.us/database/feis/plants/)
- USDA-Natural Resources Conservation Service's Plants Database (http://plants.usda.gov/)

C.1. *Typha* spp.

Name: *Typha*

Common names

Typha spp. plants are known in British English as cattail, bulrush or bullrush (cattails should not be confused with the bulrush of the genus Scirpus) or, in some older British texts, as reedmace. In American English they are known as cattail, punks or corndog grass. Common names for *Typha* in other languages are: tábua (Portuguese), espadaña, junco de la pasión, macío (Spanish), rohrkolben (German), massette (French), mazza sorda, lisca, tifa and stiancia d´acqua (Italian).

Figure C-1. *Typha* spp.

Taxonomy

Kingdom: *Plantae* – Plants
- Subkingdom: *Tracheobionta* – Vascular plants
  - Superdivision: *Spermatophyta* – Seed plants
    - Division: *Magnoliophyta* – Flowering plants
      - Class: *Liliopsida* – Monocotyledons
        - Subclass: *Commelinidae*
          - Order: *Typhales*
            - Family: *Typhaceae* – Cat-tail family
              - Genus: *Typha* L. - Cattail

There are several species of the genus *Typha*, but the most widespread ones are *Typha latifolia*, *Typha angustifolia* and *Typha domingensis*.

Description

Typha is a genus of perennial herbaceous plants in the Typhaceae family, characterized by long (typically 1 to 7 m tall), spongy, strap-like leaves (Figure C-1).

The most distinctive feature of this plant, however, is the dark brown busby-like flowering head known as "spadix" that develops at the top of a central vertical stem (see detail in Figure C-2). This flowering head is composed by two parts, a male region and a female region, that in some species are touching while in other species are separated by a few centimeters of bare stem. In these two cylindrical flowering spikes, the individual flowers are tiny, closely packed and surrounded by slender hairs; female flowers, which produce seeds, are situated towards the bottom of the spadix and the male flowers are located towards the top. *Typha* is wind-pollinated: seeds are minute (about 0.2 mm long), and attached to a thin hair or stalk, which effects wind dispersal.

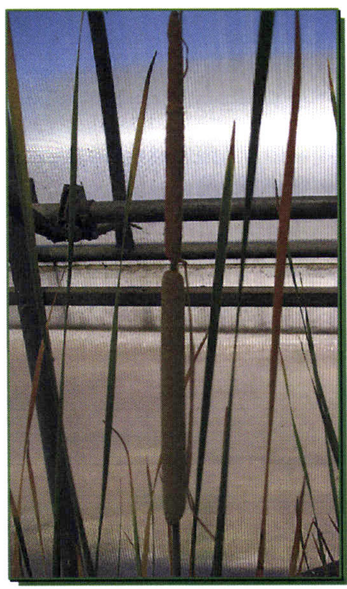


Figure C-2. Detail of *Typha* flowering head

Typha's rhizomes spread horizontally beneath the surface of muddy ground to start new upright growth, and the spread of cattails is an important part of the process of open water bodies being converted to vegetated marshland and eventually dry land. *Typha* are often among the first wetland plants to colonize areas of newly exposed wet mud.

In Table C-1 some data is presented concerning typical development characteristics of *Typha* plants.

Table C-1. Development characteristics of Typha spp. (Cooper et al., 1996)

Emergent species	Growth rate (cover 1 st year)	Typical spacing (m)	Typical root penetration in gravel (m)	Annual yield (mt/ha) dry weight	Habitat value
Typha	Rapid (dense)	0.6	0.3-0.4	30	Good nesting cover and food source for wetland birds

Propagation

Typha reproduces by seed and by rhizomes. Vegetative reproduction occurs through an extensive rhizomes system and is responsible for the maintenance and expansion of existing stands.

Life cycle

Perennial

Habitat

Typha plants show a preference for slow moving streams and sites that are rich in nutrients. They grow on mud or in shallow water at the margins of lakes, ditches, ponds and canals, and less commonly beside streams and rivers. They can develop on a wide range of substrate types. Wet pure sand, peat, clay and loamy soils have been documented under cattail stands.

In Table C-2 some data is presented concerning typical environmental conditions appropriate for adaptation and development of *Typha* spp. plants.

Table C-2. Environmental conditions for Typha spp. development (Cooper et al., 1996)

Emergent species	**Temperature °C**		**Optimum pH**	**Max. salinity tolerance (mg L^{-1})**
	Desirable	Survival*		
Typha	10-30	12-24	4-10	30,000

* Temperature range for seed germination; roots and rhizomes can survive in frozen soils

World Distribution

Cattails have a cosmopolitan distribution and a wide ecological amplitude. They are common in wetland areas in temperate and cold regions of both the Northern and the Southern Hemispheres, in North America, Europe, Asia, Africa, and Australia. The most widespread species is *Typha latifolia*, extending across the entire temperate northern hemisphere. *T. angustifolia* is nearly as widespread, but does not extend so far

north. *T. domingensis* is a more southerly American species, extending from the U.S. to South America, while *T. laxmannii*, *T. minima* and *T. shuttleworthii* are largely restricted to Asia and parts of southern Europe.

Distribution in Portugal

The two most important species of the *Typha* genus, *Typha latifolia* and *Typha angustifolia*, have a slightly different distribution in Portugal. *Typha latifolia* is more abundant in Northern and Central Portugal (Figure C-3a) while *Typha angustifolia* is more frequently found in Central and Southern Portugal (Figure C-3b).

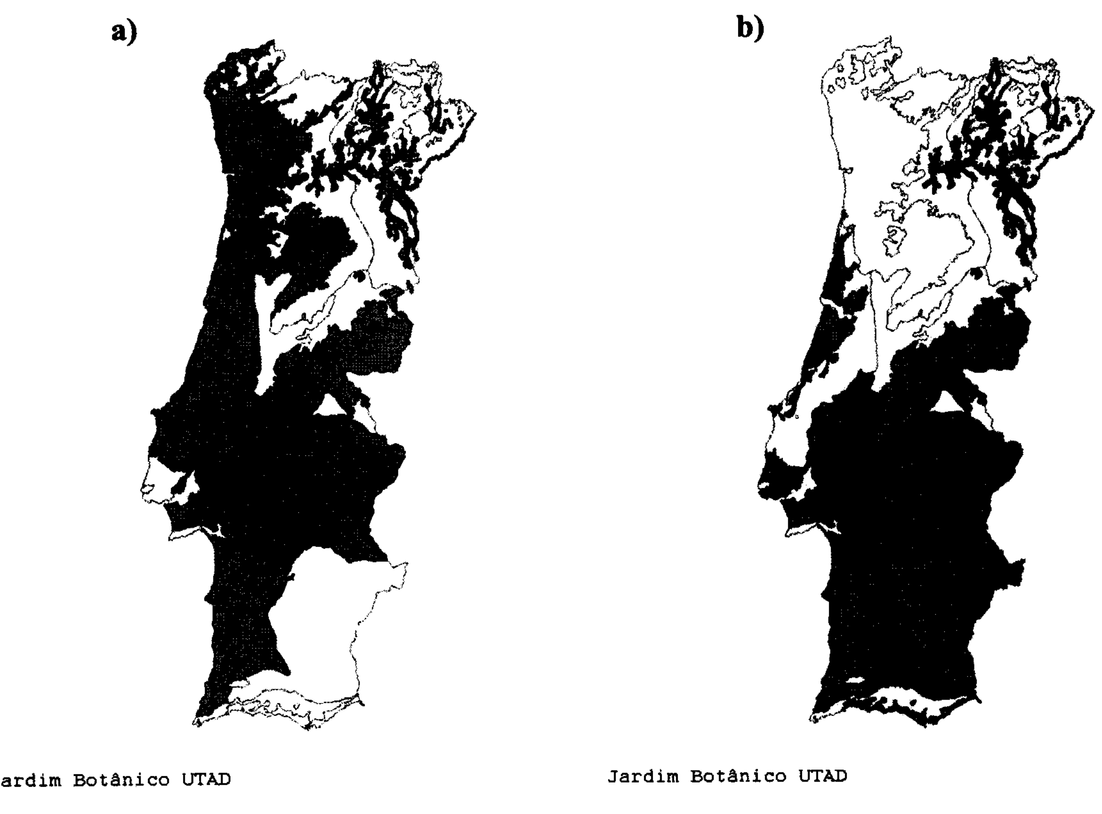


Figure C-3. Distribution of *Typha latifolia* (a) and *Typha angustifolia* (b) in Portugal (source: Flora Digital de Portugal)

Uses

Typha species have been (and still are) utilized in numerous ways worldwide. Leaves are used for dwellings (walls, roof thatch, floor coverings); for mats, baskets, and other handicraft objects; for caning chairs; and for caulking barrels, boats, and houses. "Fluff" from fruiting spikes is used for tinder and insulation; for dressing burns; and for stuffing pillows, quilts, mattresses, life preservers, toys, and diapers. Young shoot bases, young rhizomes, starch from mature rhizomes, staminate flowers before anthesis, and pollen are all minor sources of food. *Typha* is valuable as habitat and food for many kinds of wildlife. It is also useful as a potential source of fiber for paper and other products; and a potential source of energy, e.g., for alcohol production. The seeds comprise about 18-20% of an edible oil (69% linoleic acid). Several species are cultivated as ornamentals. The north american species are often sold commercially and planted for wildlife habitat and in wetland restoration.

It is also frequently used in constructed wetlands systems (CWS) for removal of various kinds of pollutants. In fact, *Typha* spp. is one of the most frequently used species in CWS for treating different types of wastewaters, especially in North America.

Applications in CWS

In particular, the use of *Typha* for the removal of organic xenobiotics from wastewaters in CWS is an important application since these type of compounds are frequently refractory to conventional wastewater treatment processes. In the main text (Table 1.5) an overview is presented of studies conducted regarding this type of application.

Appendix D

D. Complementary results

D.1. Uptake of carbamazepine from contaminated waters by *Typha* spp. Potential use for phytoremediation

D.1.1. Abstract

One option for the removal of pharmaceuticals from effluents of wastewater treatment plants is the implementation of phytoremediation technologies such as constructed wetlands systems. In this context, a study was conducted to assess the ability of the macrophyte *Typha* spp. to withstand and remove the anti-epileptic drug carbamazepine from water.

As an important preliminary step, analytical methodologies for quantification of carbamazepine in nutrient solutions and in plant tissues samples were developed and optimized for each type of sample. For nutrient solutions the methodology comprised, whenever necessary, an optimized step of pre-concentration with solid phase extraction (SPE), chromatographic separation by high performance liquid chromatography (HPLC), and detection/quantification by UV/Vis spectrometry. In plant tissues, a methodology was developed for the extraction of carbamazepine from leaf tissues by an optimized sea sand disruption method (SSDM), chromatographic separation by liquid chromatography and selective detection/quantification by quadrupole ion trap mass spectrometry with an electrospray interface (LC-ESI-MS/MS).

The evaluation of the carbamazepine removal efficiency by *Typha* spp. was conducted in hydroponic assays, where the plants were grown in a nutrient solution containing carbamazepine at initial concentrations ranging from 0.5 to 2.0 mg L^{-1} for a maximum period of 21 days. By the end of the assays, maximum carbamazepine removal ranged from 56% for the carbamazepine initial concentration of 2.0 mg L^{-1}, to 82% for the assay with carbamazepine initial concentration of 0.5 mg L^{-1}. Carbamazepine and some metabolites were detected in leaf tissues of *Typha* plants thus indicating carbamazepine

translocation to aerial parts and its metabolization. Exposure to higher carbamazepine concentrations (up to 2.0 mg L^{-1}) did affect *Typha*'s growth but, by the end of the assays, plant's growth as well as photosynthetic pigments approached normal values. An alteration in antioxidant enzymes' activities (superoxide dismutase, catalase, guaiacol peroxidase) indicated that plants were affected by the xenobiotic.
Eventually, *Typha* seemed able to cope with carbamazepine induced oxidative damage, suggesting its ability for phytotreatment of waters contaminated with this contaminant.

D.1.2. Experimental Section

D.1.2.1. Reagents and Materials

Carbamazepine (CB) (> 99% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). All other high purity chemicals and solvents were purchased from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Panreac Quimica SA (Barcelona, Spain), and were used without further purification. Ultra-pure water was obtained with a Milli-Q water purification system (Simplicity® UV, Millipore Corp., France).
For solid phase extraction (SPE) two different sorbent cartridges were tested, namely LiChrolut® RP-18 (500 mg, 3 mL) from Merck (Darmstadt, Germany), and Oasis® HLB (200mg, 6 mL) from Waters Corporation (Milford, MA, USA). Filters with 0.45 μm nylon membrane were purchased from VWR International (West Chester, PA, USA).

D.1.2.2. Carbamazepine removal assays

D.1.2.2.1. Plants collection and acclimation

Typha spp. plants were collected in water streams in Alentejo, Portugal, during April 2008. The rhizomes were thoroughly washed to remove any soil/sediment particles attached to the plant surfaces. The plants were then placed in 20 L vessels with aerated modified Hoagland nutrient solution whose composition was adapted from Fediuc and Erdei (2002). The nutrient solution was prepared with the following chemical composition: 2.5 mmol L^{-1} K^{+}, 2 mmol L^{-1} Mg^{2+}, 2 mmol L^{-1} Ca^{2+}, 2 mmol L^{-1} SO_4^{2-}, 6 mmol L^{-1} NO_3^{-}, 0.5 mmol L^{-1} $H_2PO_4^{-}$, 10 μmol L^{-1} Fe^{3+}, 10 μmol L^{-1} H_3BO_3, 1 μmol L^{-1} Mn^{2+}, 0.5 μmol L^{-1} Cu^{2+}, 0.1 μmol L^{-1} MoO_4^{2-}. The pH was adjusted to 6.0 and the nutrient solution was replaced twice every week. Plants were grown in a growth chamber (Fitoclima, Portugal) at 22° C, with 70% of relative humidity and a light-dark

cycle of 12:12 h. The photon flux density was 270 mol m^{-2} s^{-1}. After 6 weeks, when new roots and leaves had developed, plants of uniform size (approximately 30 cm height) were selected to be used in the assay setup.

D.1.2.2.2. Assays setup

Selected plants were transferred to 3 L plastic vessels (3 plants per vessel) which contained aerated modified Hoagland nutrient solution spiked with CB at 0.5, 1.0 and 2.0 mg L^{-1} concentrations, prepared from a stock aqueous solution of 50 mg L^{-1}. Control plants were grown in nutrient solution without CB. For each of the three CB concentrations as well as for the control, three assays were setup corresponding to each one of the three exposure times studied (7, 14 and 21 days), thus making a total of twelve assays. Each assay corresponding to a single exposure time and concentration was performed using three replicate vessels. The vessels were arranged as a completely randomized factorial design. Another control assay, without plants, was set up to evaluate CB photodegradation and the possible effect of CB adsorption on the plastic vessel walls.

For each exposure time and for each CB concentration level the assays were dismantled and the remaining nutrient solution was analyzed for CB quantification, while plant tissues (roots and leaves) were immediately frozen in liquid nitrogen and stored at -80ºC for posterior analyses.

D.1.2.3. Quantification of carbamazepine in nutrient solutions

D.1.2.3.1. Sample pre-concentration by solid phase extraction

The two SPE cartridges (LiChrolut® RP-18 and Oasis® HLB) were tested with nutrient solutions spiked with CB to find the optimal conditions for CB recovery. Sample pH was adjusted to values of either 2, 5 or 7 using H_3PO_4 or NaOH solutions prior to the percolation through the SPE cartridges. The analyte retained in the cartridges was eluted with 5.0 mL of methanol and redissolved with 1.0 mL of Milli-Q water following the same procedure as described in Dordio et al. (2009a). Five replicates were done for every tested cartridge and experimental condition. Additionally, the SPE pre-concentration method under the selected optimized conditions was subsequently validated against varying sample volumes and amounts of analyte.

D.1.2.3.2. HPLC-UV/Vis analysis and method validation

High performance liquid chromatography (HPLC) with UV/Vis spectrometry detection, using an Elite LaChrom HPLC system equipment (Hitachi, Japan) was used to quantity CB in the nutrient solution. The separation was performed in isocratic mode, with a mobile phase composed by 75:25 (%, v/v) acetonitrile:water (acidified with phosphoric acid 0.1%, v/v), at a flow rate of 1.0 mL min^{-1}, and using a reversed phase analytical column Zorbax Eclipse XDB-C18 with 5 μm particle size. The UV detector wavelength was set at 210 nm. Five replicate injections were made for each sample previously filtered through a 0.45 μm filter.

Calibration curves were constructed using a set of CB standard solutions with concentrations ranging from 0.25 to 5.0 mg L^{-1}. Instrumental detection and quantification limits (IDL and IQL) for the chromatographic measurement were obtained by determining the concentrations corresponding to signal-to-noise ratios of 3 and 10 respectively, according to Miller and Miller (2000).

The limit of quantification (LOQ) of the entire analytical method (including SPE pre-concentration) was calculated resorting to the following equation (Vieno et al., 2006):

$$\text{LOQ} = (\text{IQL} \times 100)/(\text{Rec}(\%) \times C) \quad (1)$$

where IQL is the instrumental quantification limit (mg L^{-1}), Rec (%) is the average SPE recovery of CB from the plant nutrient solution and C is the concentration factor (a maximum value of 50 was used).

The repeatability and reproducibility of the HPLC-UV system as well as of the entire analytical method were also evaluated.

D.1.2.4. Quantification of carbamazepine in plant tissues

D.1.2.4.1. Extraction of carbamazepine and metabolites

In this study both MSPD (Barker, 2000; Barker, 2007) and SSDM (Teixeira and Costa, 2005) were tested as methods to extract CB from plant tissues. Two different support materials were tested, namely C_{18} silica and sea sand. Both materials were cleaned before use: C_{18} silica was washed three times with methanol and the sea sand was washed several times with deionized water, and three times with methanol.

The recovery of the extraction method was assessed by measuring the recovery of 400 μL of the CB stock solution (equivalent to 0.02 mg of CB) after it was added to a

mortar containing 0.5 g of leaf tissues never exposed to the pharmaceutical, 2 g of C_{18} silica, sea sand or mixture sand:C_{18} silica (1:1, w/w), and 2 mL of *n*-hexane. The materials were mixed in the glass mortar using a glass pestle under liquid nitrogen to obtain a homogenous material suitable for column packing. The blend was then packed into a 5 mL syringe following the procedure described in Teixeira and Costa (2005). CB was eluted with three different eluents: 10 mL of methanol, acetonitrile or chloroform. All extracts were dried under vacuum, redissolved in 5 mL of a water:methanol mixture (10:1, v/v), and filtered through a 0.45 µm PTFE filter (Macherey- Nagel, Germany). This procedure was repeated three times and three replicate analyses were performed on each extract.

D.1.2.4.1.1. Optimization of extraction conditions

The determination of the optimal elution volume was made using sea sand as solid support and methanol as elution media. Three different elution volumes of methanol were tested: 5.0, 10.0 and 15.0 mL. All extracts were dried under vacuum, redissolved in 5 mL of a mixture water:methanol (10:1, v/v), and filtered through a 0.45 µm PTFE filter (Macherey-Nagel, Germany).

The determination of the optimal redissolution solvent was made using sea sand as solid support and 10 mL of methanol as elution media. All extracts were dried under vacuum, redissolved in 5 mL of three different methanol:water mixtures (in proportions of 1:10; 1:20 and 1:30, v/v).

Assays were repeated three times and three replica analyses were performed on each extract.

D.1.2.4.2. LC-ESI-MS/MS analysis

LC-ESI-MS/MS analyses were carried out in a LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionization source and using an ion trap mass analyzer. The conditions of analysis were: capillary temperature of 275° C; source voltage of 5.0 kV, source current of 100.0 µA, and capillary voltage of 15.0V in positive ion mode.

The mass spectrometer equipment was coupled to an HPLC system with autosampler (Surveyor ThermoFinnigan). The analytical column was a reversed phase Thermo Hypersil gold (C_{18}, particle size 5 µm, 150 mm × 2.1 mm). The quantification of CB was performed with an isocratic program using methanol as eluent A and water

acidified with 0.1% (v/v) formic acid as eluent B. The mobile phase was composed by 75% eluent A:25 % eluent B (v/v) at a flow rate of 0.3 mL min^{-1}. For the identification of the CB metabolites, separation was performed with mobile phase at a flow rate of 0.2 mL min^{-1} and using the following elution program: linear gradient from 0 to 80% of A (0-20 min), isocratic at 80% of A (20-35 min), linear gradient from 80 to 100% of A (35-55 min) and isocratic at 100% of A (55-60 min).

D.1.2.5. Phytotoxicity studies

D.1.2.5.1. Plant growth parameters

Fresh-plant weights were measured at different CB exposure times (7, 14 and 21 days) for each of the different initial CB concentrations tested. Visual inspection of injury symptoms was also recorded. Relative growth rates (RGR) were calculated according to the equation:

$$RGR = (\ln W_1 - \ln W_0)/(t_1 - t_0) \qquad (2)$$

where W_0 and W_1 are, respectively, the initial and final weights of plants and $(t_1 - t_0)$ is the duration of the experiment.

D.1.2.5.2. Chlorophyll and carotenoids contents

Concentrations of chlorophyll and carotenoids were determined in samples of plant leaf tissues according to the procedures described in Dordio et al. (2009b).

D.1.2.5.3. Activities of antioxidant enzymes

Catalase (CAT), superoxide dismutase (SOD) and guaiacol peroxidase (GPX) were extracted from samples of leaf tissues of the plants and these enzymes' activities were measured according to the procedures described in Dordio et al. (2009b).

D.1.2.6. Statistical analysis

Data were analyzed by the analysis of variance method (ANOVA, single factor) at different significance levels. Comparisons were considered significantly different for $P < 0.05$.

D.1.3. Results and discussion

D.1.3.1. Optimization of analytical methodologies

D.1.3.1.1. CB quantification in nutrient solutions

The HPLC-UV/Vis methodology developed for the quantification of CB in nutrient solution samples presented a high repeatability and reproducibility (RSD < 1%) as well as low values of IQL (< 0.09 mg L^{-1}). However, this IQL was still above the lowest levels required for some of the samples and, therefore, it was necessary to carry out a pre-concentration step of those samples prior to their chromatographic analysis.

A SPE technique was chosen for sample pre-concentration and, in order to optimize the methodology, the performance of two different cartridges was evaluated: a hydrophilic-lipophilic balanced polymeric sorbent (Oasis HLB®) and a reversed phase alkyl bonded-silica cartridge (Merck LiChrolut® RP-18). Additionally, for each cartridge, two different sample pH conditions were tested.

The highest recoveries were attained with both types of cartridges at sample pH of 7 and both columns had similar recoveries at this pH value (~ 95%) (data not shown). The marginally better recovery values obtained with Lichrolut® RP-18 are not statistically different from those obtained with Oasis® HLB. However, because Lichrolut® RP-18 cartridges are less expensive and show similar performance, they were chosen to be used throughout this study.

Coupled to a SPE pre-concentration step, the LOQ for the entire analytical method was 0.0019 mg L^{-1} which was low enough for the requirements of this study. The entire analytical methodology was also found to be highly reproducible (RSD < 1%) (data not shown).

D.1.3.1.2. CB quantification in plant tissues

Optimization of CB extraction procedure

Different procedures were tested for the extraction of CB from spiked leaf tissues of *Typha* spp. in order to evaluate recoveries and select the most adequate procedure. Two support materials were evaluated, C_{18} silica and sea sand, which were used either alone or in a 1:1 (w/w) mixture of C_{18} silica:sand. Several elution solvents (methanol, acetonitrile and chloroform) were also tested for quantitative extraction of CB. Different

methanol:water mixtures (in proportions of 1:10, 1:5, and 1:3, v/v) were tried for redissolution of the extracted CB.

From the analysis of the recovery efficiencies obtained (Figure D-1) methanol was found to be the most efficient eluent for all extraction procedures tested. However, for the same eluent, the recovery efficiencies obtained for the different support materials were not significantly different (Figure D-1).

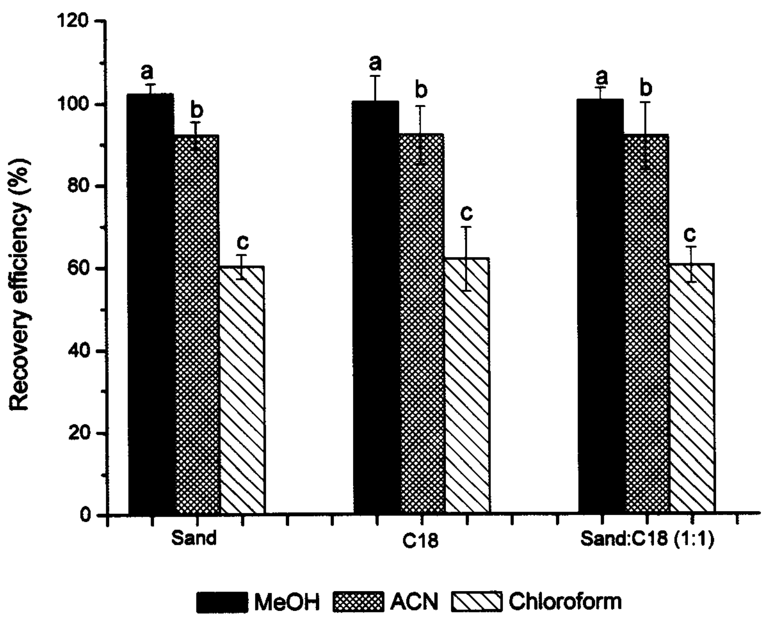


Figure D-1. Comparison of different procedures for extraction of CB from leaf tissues. Vertical error bars indicate ± SD (n = 9). For each exposure time, ANOVA significant at $P < 0.05$ when compared with control; different letters indicate significantly different values.

Considering the results of the tests, and taking into account the cost of the materials, sea sand was selected as the support material together with methanol as elution solvent to extract the assays samples. The recoveries obtained with the SSDM were also overall more reproducible, being affected by smaller SD than the other tested procedures. These observations are consistent with results obtained in previous works where the SSDM method has also shown more reproducible efficiencies for other compounds and in other matrices (Teixeira and Costa, 2005; Manhita et al., 2006).

For optimizing the conditions for SSDM extraction, different methanol elution volumes were tested (5, 10 and 15 mL) and different methanol:water mixtures in several proportions (1:10, 1:5 and 1:3, v/v) were also tested for redissolution of extracted CB.

The optimized conditions consisted of 10 mL of solvent and a mixture of methanol:water in proportion of 1:10 for redissolution of CB (data not shown).
For the optimized extraction procedure, the average CB recovery was determined to be 102 ± 3%.

Validation of the LC-ESI-MS/MS methodology

The study of matrix effects, analyte extraction recoveries, calibration linearity and method precision were considered as the criteria for the validation of the analytical methodology developed for CB quantification in leaf tissues.
Quantitative analysis by ESI-MS has a significant drawback which is the occurrence of matrix effects. This is caused by the high susceptibility of the ESI source to other components present in the matrix, which may result in the suppression or enhancement of the signal, thus leading to erroneous results. Several strategies may be adopted in an attempt to reduce these effects, e.g. selective extraction, effective sample cleanup after the extraction, or improvement of the chromatographic separation. However, these approaches have the drawbacks of sometimes leading to analyte losses as well as needing longer analysis times (Gros et al., 2006b; Kang et al., 2007). Other effective strategies reported in the literature (Kloepfer et al., 2005; Gros et al., 2006b; Kang et al., 2007) consist of the use of suitable calibration approaches, such as matrix-matched calibration standards (often referred to as external matrix-matched calibration), use of an internal standard or the method of standard addition, as well as the dilution of sample extracts. The use of internal standards is a very reliable technique, however appropriate internal standards (structurally similar unlabeled compounds or isotopically labeled standards) are not always commercially available or they are very expensive. On the other hand, standard addition is also a reliable method but it is very time-consuming, and the sample extract dilution could lead to a considerable decrease in sensitivity, which is an important drawback that should be taken into account.
In order to assess possible matrix effects and to what extent CB quantification was sensitive to it, the signal enhancement as function of increasing concentrations observed for a set of standards prepared in a plain methanol:water (10:90) mixture (as a control for the absence of matrix effects) was compared with that of the same standards in leaf extracts which were obtained with the optimized extraction method procedure (SSDM using methanol as elution solvent) and from plant tissues not exposed to CB (in order to reproduce the effects of the matrix). The comparison between the slopes of the two lines

can thus provide such assessment, i.e. when both lines are parallel and totally overlapped, compounds are not subjected to ion suppression or enhancement due to matrix effects. As it can be observed in Figure D-2, the spiked leaf extract produces a higher slope, indicating that CB is susceptible to signal enhancement (~ 41 %).

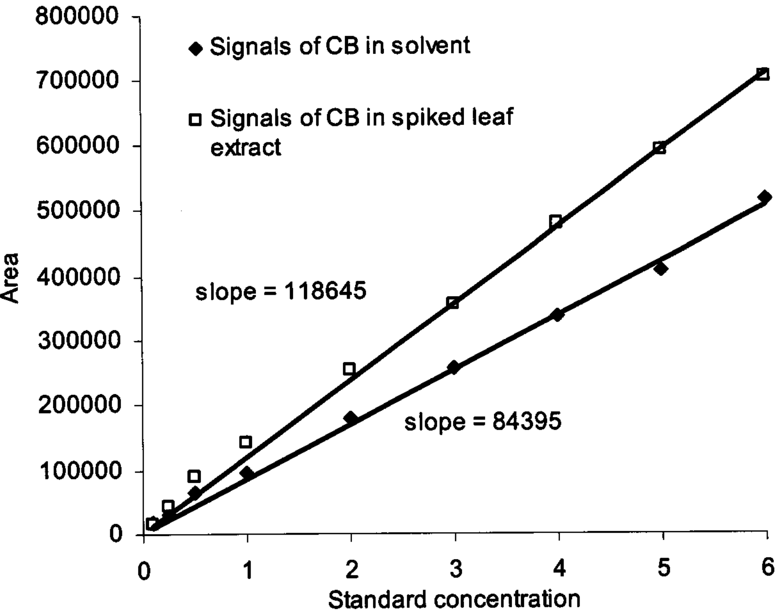


Figure D-2. Evaluation of matrix effects in CB quantification.

In order to mitigate the matrix effects observed, a calibration curve was obtained with standards dissolved in a leaf extract matrix (external matrix-matched calibration) which was, then, used in all CB quantifications in leaf extracts. This curve was linear ($R^2 > 0.996$) over the established concentrations range (0.1 – 5 mg L^{-1}). The precision of the methodology, as determined by the relative standard deviation of the determinations of several CB spiked leaf extracts, was evaluated as 4 %.

D.1.3.2. Removal efficiency and phytotoxicity of carbamazepine

Typha spp. (cattail) is an emergent macrophyte which has been frequently used in phytoremediation technologies such as CWS to depurate water contaminated with organic compounds and has shown a good tolerance when exposed to some xenobiotic substances (Williams, 2002; Haberl et al., 2003; Amaya-Chavez et al., 2006) .

Xenobiotics removal in hydroponic systems may be attributed to abiotic and biotic processes. However, according to the observations in control assays without plants where variation of the CB concentration in the nutrient solution was negligible for long periods of time (data not shown), the influence of abiotic processes such as

volatilization, adsorption to vessel walls, photodegradation and hydrolysis was minimal under the assays conditions.

Given the reported high resistance to biodegradability of this compound (Zhang et al., 2008; Kümmerer, 2008), and the care taken to diminish the microbial populations in the plants rhizosphere when the assays were setup, microbial degradation should be considered to have also a very limited contribution. Therefore, any CB removal observed in the plants assays should result, mainly, from adsorption on the rhizomes and/or from plant uptake.

Synthetic organic compounds like CB are xenobiotic to plants, which do not have specific transporters in their cell membranes for these compounds, and therefore tend to move into and within the plant tissues driven simply by diffusion (Pilon-Smits, 2005). Their mobility is dependent on their chemical properties, especially on their hydrophobicity (Dietz and Schnoor, 2001; Pilon-Smits, 2005). In general, it is considered that compounds with log K_{ow} between 0.5 and 3.5 are lipophilic enough to move through the lipid bilayer of membranes, and still water soluble enough to travel into the cell fluids (Pilon-Smits, 2005). CB is a compound only moderately hydrophobic with a log K_{ow} of 2.45 (SRC, 2009) and, probably, it is partially adsorbed onto roots, but it can also be taken up and translocated within the plant, where it can be accumulated or transformed by the plants metabolic system.

In this work assays were performed in order to assess *Typha*'s ability to uptake CB from aqueous solutions, translocate the compound to its aerial parts and to cope with the compound's toxicity.

The assays were conducted with CB solutions at concentrations of 0.5, 1.0 and 2.0 mg L^{-1}. This set of concentrations was chosen as they correspond to conditions bordering visually evident CB toxicity, since visual symptoms of toxicity (chlorosis) begin to appear when plants are exposed to CB concentrations slightly above these levels (data not shown). However, for the tested range of concentrations no visual signs of toxicity were observed in *Typha* spp. and, in fact, cattail was not only able to cope with the large amounts of CB in their nutrient solutions but, as can be seen below, it continued to remove the pharmaceutical from nutrient solution for the whole assay duration.

By the end of the assays, maximum efficiency of CB removal ranged from 56% for the CB initial concentration of 2.0 mg L^{-1}, to 82% for the assay with CB initial concentration of 0.5 mg L^{-1} (Figure D-3). CB was steadily removed from aqueous solutions by *Typha* for the duration of the whole assay and not just in an initial period,

as data collected after 7-days periods can show (Figure D-3). This observation reveals a capacity of the plant to cope with a steady input of the xenobiotic. Furthermore, at every corresponding exposure time, the initial CB concentrations and the CB removed clearly fit a linear relationship (in all cases, the R^2 of the fit is always higher than 0.9985, data not shown).

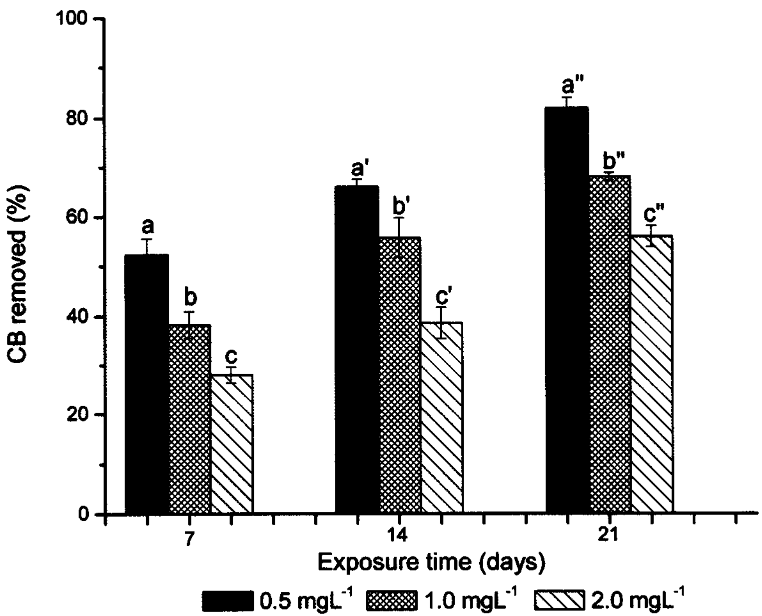


Figure D-3. Carbamazepine removal from nutrient solutions by *Typha* spp. at different exposure periods.
Vertical error bars indicate ± SD (n = 9). For each exposure time, ANOVA significant at $P < 0.05$ compared with control; different letters indicate significantly different values.

In order to study the fate of CB after being removed from the aqueous solution, the pharmaceutical was quantified in the leaf tissue samples of the plants exposed to carbamazepine spiked nutrient solutions. In fact, CB could be detected in *Typha* leaf tissues for all the concentrations tested and at increasing amounts for increasing initial CB concentrations (Figure D-4). The amounts of the non-conjugate CB were found to increase in the period from 7 days to 14 days of exposure, which shows that this pharmaceutical is taken up by the plants' roots and translocated to the leaves. The metabolization of CB in the plants cells seems to be slow which leads to accumulation of the xenobiotic during this period. Between 14 and 21 days, a substantial decrease in the amounts of CB found in the leaves was observed. This may be due to a decrease in uptake rate (Figure D-3) and simultaneous occurrence of metabolization/conjugation of the pharmaceutical accumulated in the leaves, thus causing the net reduction observed.

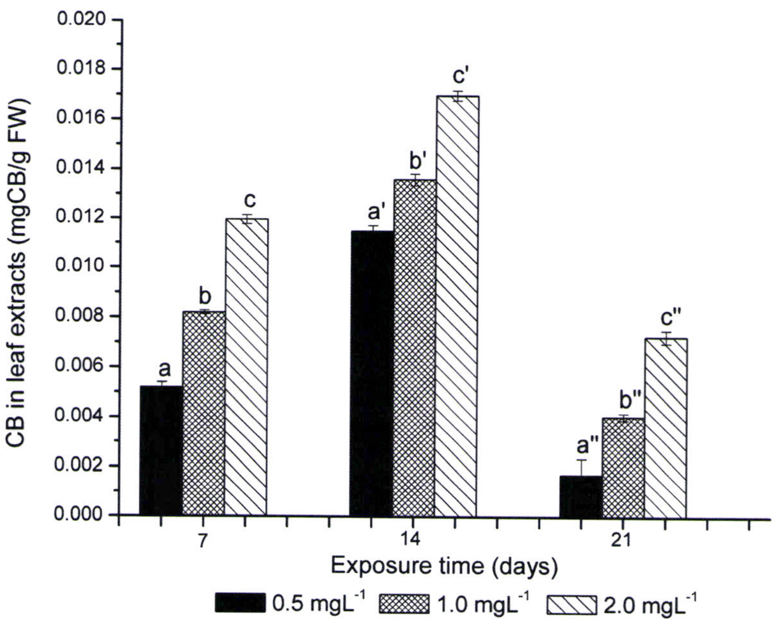


Figure D-4. Amounts of carbamazepine detected in leaf tissues of *Typha* spp. plants exposed to spiked nutrient solutions, for different exposure periods.
Vertical error bars indicate ± SD (n = 9). For each exposure time, ANOVA significant at P < 0.05 compared with control; different letters indicate significantly different values.

In order to find evidence for the occurrence of CB metabolization in *Typha*'s tissues, an LC-ESI-MS and MS/MS analysis of some samples was conducted to enable a preliminary identification of some compounds. The chromatogram obtained showed several peaks which can be attributed to the natural compounds present in the plant (Figure D-5). The peak with retention time (r_t) 25.69 min was identified as carbamazepine since the full MS and MS^2 spectra are coincident with the ESI-MS spectra obtained for a carbamazepine standard solution. The peak obtained at r_t 22.57 min corresponds probably to one of the CB metabolites, 10,1-dihydro-10,11-epoxycarbamazepine (CB-EP). The full MS spectra shows a ion signal at *m/z* 253, with abundance of 100%, corresponding to the $[M + H]^+$ parent ion, and at *m/z* 275 and 291 the peaks corresponding to the sodium $[M + Na]^+$ and potassium $[M+ K]^+$ aducts, respectively (data not shown). The MS^2 spectrum of the parent ion at *m/z* 253 is presented in (Figure D-6). This fragmentation yielded ions at *m/z* 253 $[M +H]^+$, *m/z* 236 $[M +H-NH_3]^+$, *m/z* 210 $[M +H-HNCO]^+$, and *m/z* 180 which can be attributed to the loss of H_2CO or 2CO (Figure D-6). The MS^2 spectrum and the fragment pattern

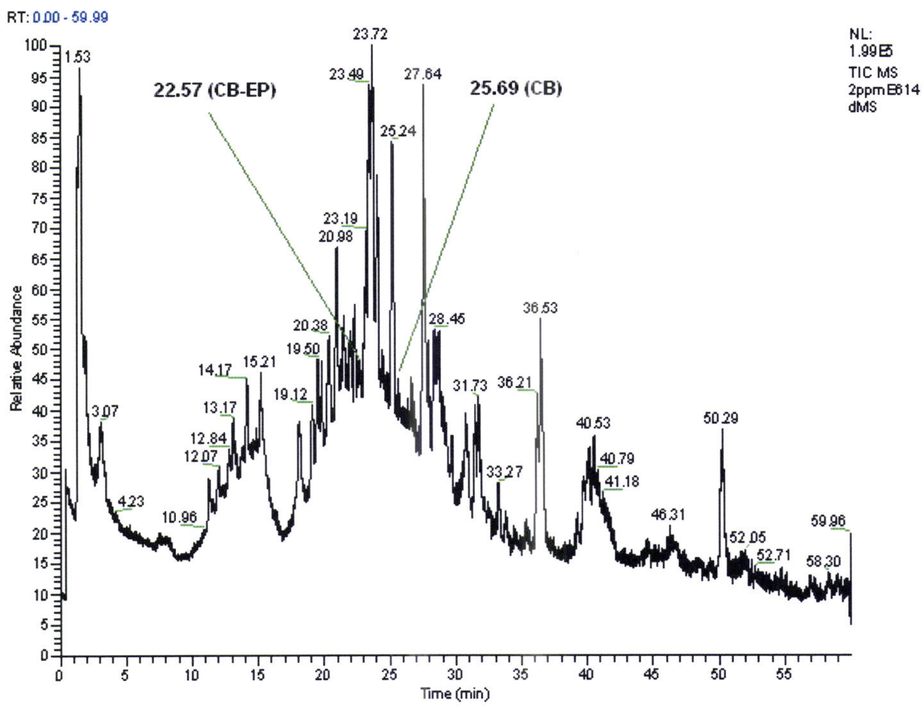


Figure D-5. Total ion current chromatogram obtained for the leaf samples of *Typha* spp., exposed to carbamazepine spiked nutrient solutions.

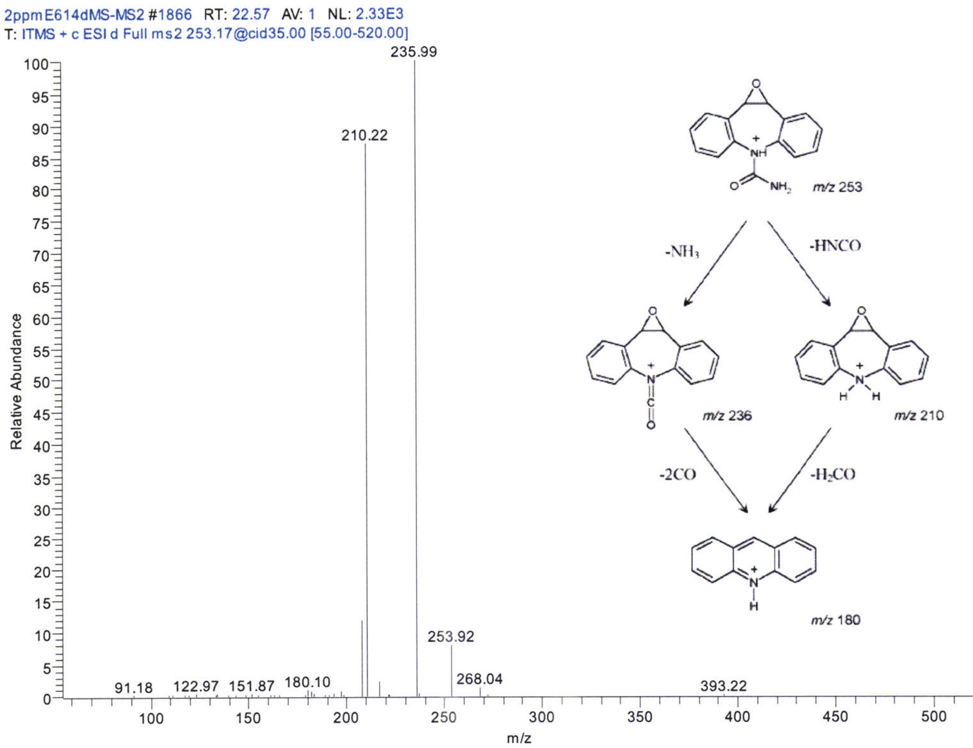


Figure D-6. LC-ESI-MS^2 spectrum obtained for parent ion with *m/z* 253.
The scheme in overlay presents the fragmentation pattern proposed for parent ion with *m/z* 253, i.e. 10,1-dihydro-10,11-epoxycarbamazepine (CB-EP) (adapted from Miao and Metcalfe (2003)).

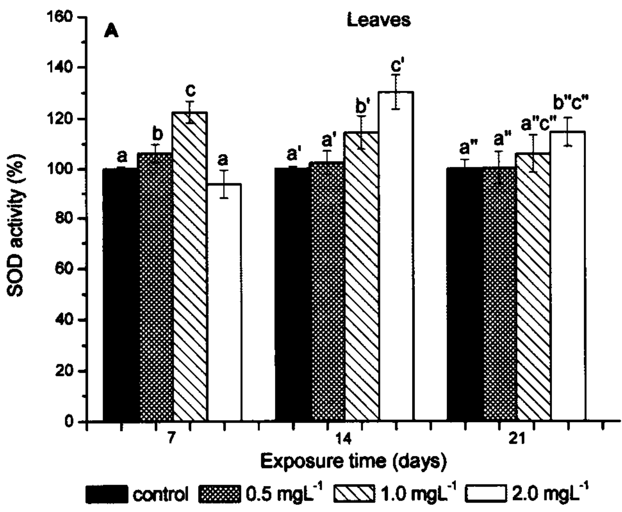
obtained for the peak with *m/z* 253 is similar to those proposed by Miao and Metcalfe (2003) for CB-EP. Those authors identified CB and five of its metabolites in contaminated aqueous samples collected from effluents of wastewater treatment plants (WWTP) and surface water samples.

In humans, CB undergoes extensive hepatic metabolism by the cytochrome P450 system (Kerr et al., 1994; Valentine et al., 1996). Thirty-three CB metabolites have been identified from human and rat urine (Lertratanangkoon and Horning, 1982). The main metabolic pathway of CB is oxidation to 10,11-dihydro-10,11-epoxycarbamazepine, followed by hydration to 10,11-dihydro-10,11-dihydroxycarbamazepine, and subsequent conjugation with glucuronide. There are no reports in the literature about the metabolic pathways of CB in plants. However, the eventual presence of CB-EP in leaf tissues of *Typha* spp. seem to indicate that CB oxidation to 10,11-dihydro-10,11-epoxycarbamazepine is one of the possible metabolization processes, that lead to a substantial decrease in the amounts of the pharmaceutical observed in those tissues between 14 and 21 days.

The exposure of plants to xenobiotic compounds such as CB induces a so-called oxidative burst, with over-production of reactive oxygen species (ROS) which have the potential to damage cells. The enzymatic antioxidant system is one of the protective mechanisms to eliminate or reduce ROS excess, whose activity levels may serve as an indication of the levels of stress to which the plants are subjected. The fact that *Typha* seemed to cope with high amounts of CB in the nutrient solution may be revealing that the expected production of excessive ROS was effectively counteracted by the antioxidant enzymatic system. This involves the sequential and simultaneous actions of a number of enzymes including SOD, CAT and peroxidases (POX). The biochemical pathways through which these enzymes act and the cell compartments where they are present predominantly are described in much detail in some reviews on xenobiotic phytotoxicity (Mittler, 2002; Apel and Hirt, 2004).

In order to better characterize the antioxidant response put forward by *Typha* spp. in reaction to the stress caused by the plant's exposure to CB, the enzyme activities of SOD, CAT and the GPX were measured in leaf tissues of those plants and compared to those of the control assays' plants (Figure D-7).

SOD is considered to be in the first line of defense against ROS damage, acting against the superoxide radicals which are formed in different cell compartments and are



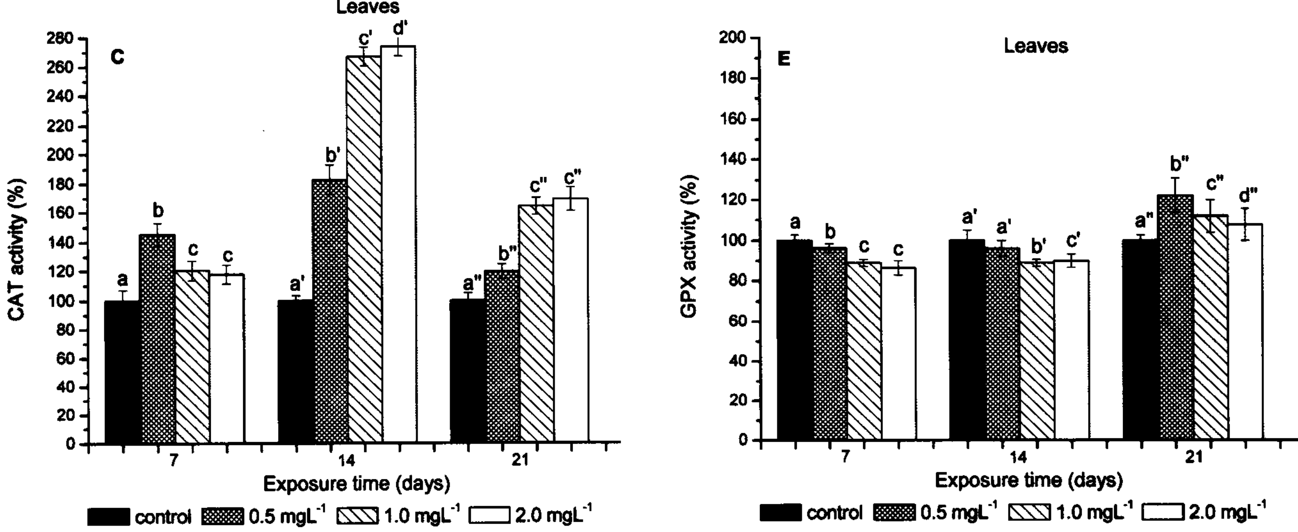


Figure D-7. Effects of carbamazepine exposure on the activities of antioxidant enzymes in leaf tissues: superoxide dismutase (A), catalase (B) and guaiacol peroxidase (C). Activity expressed relative to the activity in control plants (100%). Vertical error bars indicate ± SD (n = 9). For each exposure time, ANOVA significant at $P < 0.05$ compared with control; different letters indicate significantly different values.

precursors of other ROS. In general, an increase in SOD activity was observed in the plants exposed to CB solutions in comparison with that of the control plants, which overall is larger as the initial CB concentrations increase (Figure D-7A). This increase of enzymatic activity may be an evidence of the stress caused by the uptake and accumulation of the xenobiotic within the plant tissues leading to the consequent production of superoxide radicals which stimulates the enzymatic response. An exception was observed for the enzyme's activity level after the first 7-day period for the highest (2.0 mg L^{-1}) CB concentration, where the SOD activity was actually not

statistically different from that in the control plants. This may be due to an inhibition of the enzyme at the highest xenobiotic uptake rates (Figure D-7A). After 14 days, however, SOD activity in the 2.0 mg L^{-1} assay was already higher than in the control and in all other assays as well. In this period, for the other assays, SOD activity decreased in comparison with the previous period and, in the case of the 0.5 mg L^{-1} assays, it cannot even be considered statistically different from the control's. This period, therefore, corresponds to a turning point in the oxidative burst. After 21 days, SOD activities had fallen back to normal levels, which reveal that the plant was capable to cope with CB toxicity, except for the 2.0 mg L^{-1} assay where the activity of the enzyme remains somewhat higher than in the control but nevertheless approaching normal levels.

The cooperation between SOD and H_2O_2-eliminating enzymes plays an important role in the resistance of plants to abiotic stress. CAT and peroxidases functioning in different cell compartments can be activated in order to maintain appropriate H_2O_2 levels. CAT activity was observed to increase as consequence of CB exposure, attaining top activity after a 14-day period and with a subsequent decline towards the end of the 21-day period of exposure (Figure D-7B). Higher increase in CAT activity was observed for higher initial CB concentrations in general, but for the initial 7-day period the most concentrated CB solutions induced a lower CAT activity increase than for the 0.5 mg L^{-1} case. This may be explained by some inhibition of CAT or by a concomitant reduced peroxide production by SOD catalyzed reactions due to the inhibition of this enzyme as observed earlier. CAT activity increase, therefore, seems to be correlated with higher amounts of non-conjugated CB in plant tissues (Figure D-7B) and as a delayed effect of SOD activity alterations which induce higher concentrations of H_2O_2, thus leading to an enzyme activity increase in order to diminish the intra-cellular level of peroxide.

The activity of GPX is very negatively affected by the exposure to CB, displaying the effects of inhibition of this enzyme up to a period of 14 days (the activities of GPX reduced in comparison with the control) and with an extent of the enzyme inhibition which seems to be dependent on the initial concentration of CB (Figure D-7C). Only by the end of the assays does GPX respond with an increase of its activity to CB-induced stress, and even then with a lower activity increase observed in the assays with the highest CB concentrations.

The toxic effects of CB towards *Typha* were also evaluated by measurements of relative growth rates (RGR) of the plants exposed to CB. After 7 days, RGR of exposed plants were significantly below those of the control plants and, with exception of those subjected to the 0.5 mg L^{-1} CB solution, exposed plants were still underdeveloped even after 14 days. However, after 21 days of exposure, the RGR of the plants exposed to CB showed a trend of approach towards the average RGR levels of the control plants that were not exposed to CB. This indicates that the presence of CB affects the normal plant's growth but plants seem to be able to cope with the toxic effects of this substance because not only do they remove it extensively from the solution (Figure D-8) but also they show an evolution towards a recovery to normal growth rates beyond a period of 21 days of exposure (Figure D-8).

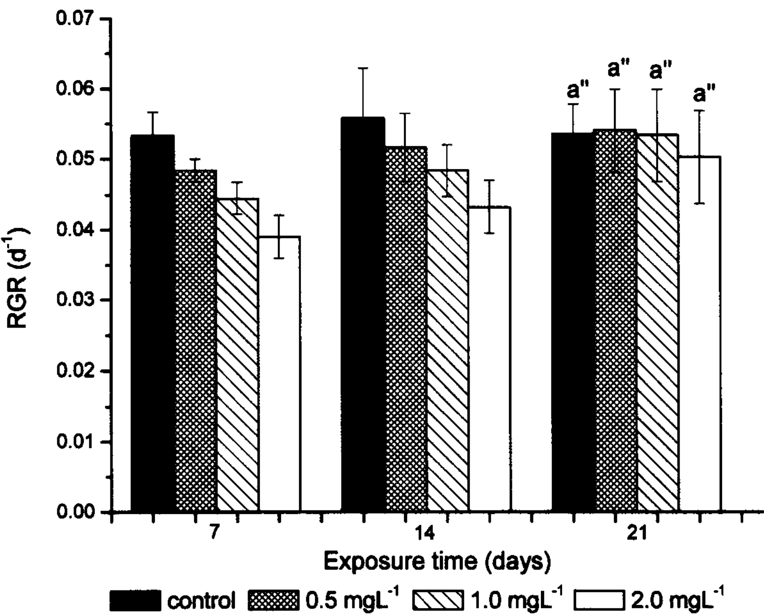


Figure D-8. Relative growth rates of plants exposed to CB at concentrations of 0.5, 1.0 and 2.0 mg L^{-1}.
Vertical error bars indicate ±SD ($n = 9$). ANOVA significant at $P < 0.05$ when compared with control. Different letters indicate significantly different values.

Chlorophyll (total, *a* and *b*) and carotenoid contents were also determined at the end of the experiments and the results are presented in Table D-1. No statistically significant differences were found in the chlorophyll *b* contents of the plants exposed to CB from those of the control assay. However, for chlorophyll *a* and total chlorophyll and carotenoid contents, statistically significant differences were observed between the plants exposed to CB concentrations and those of the control assay. The lower contents

of these chlorophyll pigments as well as the higher contents of carotenoids in the plants exposed to different CB concentrations assays compared with control plants may be a sign of toxicity which still subsisted after 21 days of exposure when almost all the CB had been removed from solution.

Table D-1. Average values of photosynthetic pigments contents of *Typha* spp. after 21 of exposure to CB (n = 9)
ANOVA significant at P < 0.05 when compared to control. Different letters indicate significantly different values.

CB concentration ($mg\ L^{-1}$)	Chlorophyll total ($mg\ g^{-1}$ FW)	Chlorophyll *a* ($mg\ g^{-1}$ FW)	Chlorophyll *b* ($mg\ g^{-1}$ FW)	Carotenoids ($mg\ g^{-1}$ FW)
0	$1.40^{a} \pm 0.008$	$1.19^{a} \pm 0.02$	$0.21^{a} \pm 0.01$	$0.34^{a} \pm 0.001$
0.5	$1.39^{b} \pm 0.007$	$1.17^{b} \pm 0.01$	$0.22^{a} \pm 0.005$	$0.35^{b} \pm 0.004$
1.0	$1.38^{c} \pm 0.007$	$1.16^{b} \pm 0.01$	$0.22^{a} \pm 0.003$	$0.37^{c} \pm 0.002$
2.0	$1.37^{c} \pm 0.01$	$1.16^{b} \pm 0.003$	$0.21^{a} \pm 0.008$	$0.39^{d} \pm 0.003$

The results presented here show the ability of *Typha* spp. to remove, translocate and metabolize CB from contaminated waters. Eventually, *Typha* seemed able to cope with CB's induced oxidative damage suggesting its ability for phytotreatment of waters contaminated with CB.