ANALYSIS OF CHEMOTHERAPY DRUGS AND RELATED COMPOUNDS IN AQUATIC ENVIRONMENT: REMOVAL, TRANSFORMATION AND RISK EVALUATION IN ECO-FRIENDLY AND ADVANCED TECHNOLOGIES

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Doctoral Thesis

Analysis of chemotherapy drugs and related compounds in aquatic environment: removal, transformation and risk evaluation in eco-friendly and advanced technologies.

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Certifiquen

Que el treball titulat "*Analysis of chemotherapy drugs and related compounds in aquatic environment: removal, transformation and risk evaluation in eco-friendly and advanced technologies."* que presenta Laura Ferrando Climent per a l'obtenció del títol de Doctor per compendi d`articles (prèviament publicats, acceptats o enviats per a la seva publicació), ha estat realitzat sota la nostra direcció i que compleix els requeriments per poder optar a la Menció Internacional.

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Dedicado a la luz de mi vida,
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Summary

During the last decades pharmaceutical compounds have become a concerning group of emerging pollutants according to a large number of studies. The fact that a high amount of these drugs reach the environment via urban sewage system put in evidence that conventional wastewater treatment plants (WWTPs) are inefficient in their removal. Pharmaceutical compounds are designed to have target effects in human body but it is still not very well-known how these substances can affect other organisms in the natural environment as well as their indirect impact on human health. Chemotherapy drugs are a specific group of pharmaceutical compounds used to treat cancer diseases. They are often called ***anticancer drugs*** and have been shown to have potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor, and/or teratogenic effects in several organisms, since they have been mainly designed to disrupt or prevent cellular proliferation, usually by interfering in DNA synthesis. Nowadays there is a lack of information about the occurrence and fate of these substances in the environment although their consumption has increased in the last years and it is foreseen to further increase in the future due to cancer incidence.

In order to evaluate the increasing environmental and human risk of anticancer drugs in the environment, more information needs to be gathered about their presence, their toxicity, bioaccumulation and persistence. In addition, their removal performance in conventional wastewater treatment facilities and in alternative treatments needs further investigation efforts.

The aim of this thesis is to fill knowledge gaps and provide tools for a better assessment about the presence and fate of anticancer drugs in the urban water cycle. The work presented hereby includes three main objectives: i) to develop analytical methodologies for target and non-target analysis of anticancer drugs in aquatic environment, ii) to assess the occurrence of anticancer drugs in urban systems and iii) to appraise different technological alternatives to remove anticancer drugs from polluted effluents.

Based on these objectives, the thesis work is developed through three blocks: The first block is devoted to the analytical method development (including target and suspect screening analysis) by ultra-high chromatography coupled to triple quadrupole-ion trap mass spectrometer (UPLC-QqLiT). A feasible and reliable analytical methodology was

developed for the quantification of 10 selected anticancer drugs and for the screening of human metabolites in water.

Along the second block, the analytical methodology was successfully applied (with some modifications) for the assessment of the presence of anticancer drugs in selected urban systems in Spain and Norway following two approaches: target analysis in the case of Spanish urban system and further tentative identification in both case studies. Additionally, an evaluation of the potential risk of these drugs for the environment was also carried out based on the occurrence data obtained in these studies as well as on ecotoxicological data generated in other studies.

The third block concerns to the evaluation of two different alternative wastewater treatments for anticancer drugs removal: a) a non-conventional biological treatment using white rot fungi (WRF) and b) advanced oxidation processes (AOP) using the combination of ozone, UV radiation and/or hydrogen peroxide. The occurrence of a target anticancer drug (Tamoxifen) and their sub-products generated along the water treatments were assessed by high resolution mass spectrometry (HRMS). This data was correlated with the toxicity data obtained through the standard Microtox® assay. Tamoxifen was selected as model anticancer drug based on information collected from the first two blocks and the literature available regarding its occurrence, persistence, toxicity and potential bioaccumulation in the environment.

The results derived from this thesis provide a novel dataset about the occurrence of anticancer drugs in urban and natural environment. This is valuable information, principally as roadmap for further studies about the impact of anticancer drugs in the aquatic environment

Resumen

Durante las última década y en base al gran número de estudios realizados hasta la fecha, los compuestos farmacéuticos se han convertido en un grupo de contaminantes emergentes preocupantes. El hecho de que una gran cantidad de estos fármacos puedan alcanzar el medio ambiente a través de la red urbana de aguas residuales ha puesto de manifiesto que las plantas de tratamiento de aguas residuales actuales (EDARs) no pueden eliminar eficientemente este tipo específico de sustancias. Los compuestos farmacéuticos están diseñados para ejercer ciertos efectos en los seres humanos, sin embargo no se conoce bien la forma en la que estas sustancias pueden afectar a otros organismos en el medio ambiente ni las consecuencias indirectas en la salud humana. Los fármacos quimioterápicos son un grupo específico de compuestos farmacéuticos utilizados para el tratamiento del cáncer. A menudo conocidos como ***medicamentos antitumorales***, han demostrado tener además un potente efecto citotóxico, genotóxico, mutagénico, carcinogénico, disruptor endocrino y/o efectos teratogénicos en varios organismos. Ello se debe a que se trata de sustancias diseñadas principalmente para interrumpir o impedir la proliferación celular, interfiriendo en muchas ocasiones en la propia síntesis del ADN. En la actualidad existe escasa información sobre la presencia y el destino de estas sustancias en el medio ambiente, a pesar de que su consumo no ha hecho más que aumentar en los últimos años y de que se prevé un incremento adicional en el futuro debido al aumento de la incidencia de cáncer en la población.

Con el fin de evaluar el incremento del riesgo ambiental y para los seres humanos por la presencia de los medicamentos contra el cáncer en el medio ambiente y, finalmente, proponer una futura mejora de las EDAR convencionales para eliminar estos compuestos, se hace imprescindible obtener más información acerca de su presencia en las aguas residuales y naturales, su toxicidad, bioacumulación y persistencia en el medio ambiente, su eliminación en EDAR convencionales y en tratamientos alternativos, así como los sub-productos que podrían ser generados a lo largo del ciclo de vida de las aguas residuales urbanas y en masas de agua naturales.

El objetivo de esta tesis doctoral es proporcionar información relevante sobre la presencia y el destino de los medicamentos contra el cáncer en el ciclo urbano del agua con el fin de evaluar su importancia ambiental. El trabajo aquí presentado incluye tres objetivos principales: i) desarrollar metodologías analíticas para análisis “target” y “no-

target" de fármacos antitumorales en el medio acuático ii) evaluar la presencia de estos medicamentos contra el cáncer en los sistemas de aguas residuales urbanos y iii) evaluar diferentes alternativas tecnológicas para eliminar fármacos contra el cáncer de efluentes contaminados.

En base a estos objetivos definidos, el trabajo de tesis se desarrolla a través de tres bloques: el primer bloque está dedicado al desarrollo de métodos analíticos (incluyendo análisis "target" y cribado de "sospechosos") mediante cromatografía de ultra-alta resolución acoplada a espectrometría de masas con trampa de iones y triple cuadrupolo (UPLC-QqLiT). Con esta tecnología se desarrolló un método que permitía la cuantificación de 10 fármacos antitumorales y la identificación de sus metabolitos humanos en agua.

A lo largo del segundo bloque, la metodología analítica previamente establecida se aplicó con éxito (con ligeras modificaciones) en la evaluación de la presencia de medicamentos contra el cáncer en 2 diferentes sistemas urbanos de aguas residuales en España y Noruega mediante dos enfoques: cuantificación de los compuestos escogidos en al caso del sistema urbano español e identificación tentativa de una serie de compuestos "sospechosos" para ambos sistemas. Además, se realizó una evaluación del riesgo toxicológico medioambiental en base a los datos obtenidos en el análisis cuantitativo y a los datos ecotoxicológicos generados en otros estudios.

El tercer bloque está dedicado a la evaluación de dos tratamientos alternativos a los convencionales para la eliminación de estos compuestos en aguas residuales: a) un tratamiento biológico no convencional basado en el hongo *Trametes versicolor* y b) procesos de oxidación avanzada (AOP) usando combinaciones de ozono, radiación UV y/o peróxido de hidrógeno. En este bloque se evaluó la presencia de un medicamento contra el cáncer tamoxifen y de los sub-productos generados a lo largo del tratamiento AOP y con el hongo, mediante espectrometría de masas de alta resolución (HRMS). Los datos obtenidos se correlacionaron con la toxicidad del efluente, obtenida mediante el ensayo estándar Microtox®. El tamoxifeno fue seleccionado como fármaco modelo debido a su relevancia ambiental: Los resultados obtenidos en los dos primeros bloques de la tesis y la bibliografía disponible hasta el momento, señalan al tamoxifeno como una substancia preocupante por su presencia, ubicuidad, baja eliminación, peligrosidad y potencial de bioacumulación en el medio ambiente.

Los resultados derivados de esta tesis proporcionan una nueva y valiosa base de datos acerca de la presencia de los medicamentos contra el cáncer en el ambiente urbano y natural. Se trata de una información de gran utilidad, principalmente como hoja de ruta a la hora de realizar nuevos estudios sobre el impacto de los medicamentos contra el cáncer en el medio acuático.

Resum

Durant la darrera dècada, els compostos farmacèutics s'han convertit en un grup de contaminants emergents preocupants segons un gran nombre resultats i estudis bibliogràfics. El fet que una gran quantitat d'aquests fàrmacs puguin arribar al medi ambient a través del sistema de clavegueram urbà ha posat de manifest que les plantes de tractament d'aigües residuals actuals (EDAR) no poden eliminar eficientment aquest tipus específic de substàncies. Els compostos farmacèutics estan dissenyats per exercir els seus efectes en els éssers humans, però no es coneix bé la forma en què aquestes substàncies poden afectar altres organismes en el medi ambient ni les conseqüències indirectes d'aquests efectes ambientals en la vida humana.

Els fàrmacs quimioteràpics són un grup específic de compostos farmacèutics utilitzats per al tractament del càncer. Sovint coneguts com a ***medicaments antitumorals***, han demostrat tenir un potent efecte citotòxic, genotòxic, mutagènic, carcinogen, disruptor endocrí i/o efectes teratogènics en diversos organismes. Això es deu al fet de que es tracta de substàncies dissenyades principalment per interrompre o impedir la proliferació cel·lular, interferint en moltes ocasions en la pròpia síntesi de l'ADN. En l'actualitat hi manca informació sobre la presència i la destinació d'aquestes substàncies en el medi ambient, tot i que el seu consum no ha fet més que augmentar en els darrers anys i es preveu un increment addicional en el futur a causa de l'augment de la incidència de càncer en la població.

Per tal d'avaluar el increment del risc ambiental i per als éssers humans debut a la presència dels medicaments contra el càncer en el medi ambient i, finalment, proposar una futura millora de les EDAR convencionals per eliminar aquests compostos, es fa imprescindible obtenir més informació sobre de la seva presència a les aigües residuals i naturals, la seva toxicitat, bioacumulació i persistència en el medi ambient, la seva eliminació en EDAR convencionals, així com els sub-productes que podrien ser generats al llarg del cicle de vida de les aigües residuals urbanes i en masses d'aigua naturals.

L'objectiu d'aquesta tesi doctoral és proporcionar informació rellevant sobre la presència i el destí dels medicaments contra el càncer en el cicle urbà de l'aigua per tal d'avaluar la seva importància ambiental. El treball presentat aquí inclou tres objectius principals: i) desenvolupar metodologies analítiques per a anàlisi dirigit i no dirigit de

fàrmacs antitumorals en el medi aquàtic ii) avaluar la presència de medicaments contra el càncer en els sistemes urbans i iii) avaluar diferents alternatives tecnològiques per eliminar-hi els fàrmacs contra el càncer d'efluents contaminats.

Aquests objectius es desenvolupen a través de tres blocs: el primer bloc està dedicat al desenvolupament de metodologia analítica (incloent-hi anàlisi dirigit i investigació de sospitosos) mitjançant cromatografia d'ultra-alta resolució acoblada a espectrometria de masses amb trampa d'ions i triple quadrupol (UPLC-QqLiT). Amb aquesta tecnologia es va desenvolupar un mètode que permetia la quantificació de 10 fàrmacs antitumorals i la identificació dels seus metabòlits humans en aigua.

Al llarg del segon bloc, la metodologia analítica prèviament establerta es va aplicar amb èxit (amb lleugeres modificacions) en l'avaluació de la presència de medicaments contra el càncer en sistemes urbans seleccionats a Espanya i Noruega seguint dos enfocaments: anàlisi de quantificació al sistema urbà espanyol i identificació temptativa ambdós casos. Addicionalment es va realitzar una avaluació del risc toxicològic mediambiental en base a les dades obtingudes en l'anàlisi quantitativa i els estudis ecotoxicològics previs reportats per diversos autors.

El tercer bloc està dedicat a l'avaluació de dos tractaments alternatius als convencionals per a l'eliminació d'aquests compostos: a) un tractament biològic no convencional basat en l'espècie de fong anomenada *Trametes versicolor* i b) processos d'oxidació avançada (AOP) usant combinacions de ozó, radiació UV i/o peròxid d'hidrogen. En aquest bloc, els medicaments anticàncer seleccionats com a objectiu i els sub-productes generats al llarg del tractament van ser avaluats per espectrometria de masses d'alta resolució (HRMS). Les dades obtingudes es van correlacionar amb la toxicitat de l'efluent obtinguda mitjançant l'assaig estàndard Microtox®. El tamoxifè va ser seleccionat com a fàrmac model fonamentat en els resultats dels dos primers blocs i de la literatura disponible fins la data, lo que el posiciona com a substancia preocupant per la seva presència, ubiqüitat, baixa eliminació, perillositat i potencial de bioacumulació en el medi ambient.

Els resultats derivats d'aquesta tesi proporcionen una nova i valuosa base de dades sobre la presència dels medicaments contra el càncer en l'ambient urbà i natural. Es tracta d'una informació de gran utilitat, principalment com a full de ruta per fer nous estudis sobre medicaments contra el càncer en el medi aquàtic.

Abbreviations and acronyms

AOPs	*Advanced Oxidation Processes*
ASE	*Accelerated solid extraction*
CECs	*Contaminants of Emerging Concern*
CHAFEA	*Consumer, Health and Food Executive Agency*
CAFOs	*Concentrated animal feeding operations*
CAS	*Conventional activated sludge*
DDD	*Defined daily dosage*
EACR	*The European Association for Cancer Research*
EC50	*50% Effect Concentration*
EDAR	*Estación Depuradora de Aguas Residuales*
EU	*European Union*
HRMS	*High Resolution Mass Spectrometer*
IDA	*Information Dependent Acquisition*
LogP	*Partition coefficient dependant of pH*
LC	*Liquid chromatography*
LOEC	*Lowest observed-effect concentration*
MS	*Mass spectrometer*
MBR	*Membrane bioreactor*
NORDP	*Norwegian databases for medical prescription*
PEC	*Predicted environmental concentration*
PhACs	*Pharmaceutical Active Compounds*
QqLiT	*Triple Quadrupole-Ion Trap Mass Spectrometer.*
QTOF	*Quadrupole-Time-of-flight Mass Spectrometer*
SEFH	*Sociedad Española de Farmacia Hospitalaria*
SPE	*Solid phase extraction*
TPs	*Transformation Products*
UPLC	*Ultra-High Performance Liquid Chromatography*
WHO	*World health organization*
WRF	*White Rot Fungi*
WWTPs	*Waste Water Treatment Plants.*

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Honestamente, la inmensa mayoría de la gente que hace una tesis doctoral tiende a pensar que ha perdido un tiempo valioso de su vida y que habría sido más provechoso dedicarse a cualquier otra actividad. La realidad es que, al margen de la propia riqueza intelectual que una Tesis pueda proveer (de la cual no eres consciente en el transcurso de la misma) y el grado de Doctor que obtienes (aunque venga acompañado de un billete de avión con cualquier destino fuera del territorio Español), lo que si es cierto es que te proporciona grandes enseñanzas; las frustraciones, fracasos, errores, aciertos, satisfacciones que sientes durante la Tesis son algo que forma parte de la vida misma. Y sobre todo las personas que viven contigo esos momentos. Es algo muy valioso como para pensar que se ha perdido el tiempo. No obstante también creo, que hacer una Tesis tiene que significar algo para uno mismo, si no se cree en lo que se hace entonces no se debe dudar, tu "lugar" puede estar en otro sitio. Siempre que la vida lo permita, la razón y el corazón deben ir a la par porque si no el riesgo de insatisfacción es muy alto. Nunca toméis decisiones importantes promovidas sin pasión. En mi caso la Tesis fue algo que me permitió liberar la mente, una forma no encorsetada de hacer y de pensar. Será, probablemente, el único momento de vuestra vida en el que podréis decir, hacer y pensar casi lo que queráis (no hay protocolos, es sólo ciencia) y lo mejor es que seguramente alguien os escuchará. Así es como debe ser una Tesis, y así es como ha sido la mía, un río de ideas que fluyen hacia algún lugar.

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List of publications

This thesis is presented as a compendium of scientific articles published, accepted or submitted to recognized scientific journals (quality criteria: Impact factor):

1. Ferrando-Climent, L., Rodriguez-Mozaz, S., Barceló D. (2013). "Development of a UPLC-MS/MS method for the determination of 10 anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information dependant acquisition tool (IDA) in sewage samples." Analytical and Bionalytical Chemistry 405(18): 5937-5952. Impact Factor 2015:3.125

2. Ferrando-Climent, L, Reid, M.J., Rodriguez-Mozaz, S., Barceló, D., Thomas, K.V.. "Identification of markers of cancer in urban sewage through the use of a suspect screening approach". Journal of Pharmacology and biomedical analysis 129 (2016) 571–580.Impact factor 2015: 3.169

3. Ferrando-Climent, L., Rodriguez-Mozaz, S. , Barceló, D.. (2014). "Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment." Environmental Pollution 193(0): 216-223. Impact factor 2015: 4.839

4. Ferrando-Climent, L., Cruz-Morató, C. Marco-Urrea, E., Vicens, T., Sarrà, M. Rodriguez-Mozaz, S., Barceló, D.. (2015). "Non conventional biological treatment based on *Trametes versicolor* for the elimination of recalcitrant anticancer drugs in hospital wastewater." Chemosphere 136: 9-19. Impact factor 2015: 3.698

5. Ferrando-Climent, L, Gonzalez, R., Anfruns A., Americh, I. Corominas, L., Barceló, D. Rodriguez-Mozaz, S. "Elimination study of the chemotherapy drug Tamoxifen by different advanced oxidation processes: transformation products and toxicity". Submitted to Chemosphere (July 2016). Impact factor 2015: 3.698

Life happens
Livet skjer
La vida ocurre

CHAPTER 1

1. Introduction

1.1. Pharmaceuticals in the environment

The presence of pharmaceutical active compounds (PhACs) in the environment such as antibiotics, analgesics, and psychiatric drugs, -among others- are considered a serious threat to water quality [1-5]. As contaminants of emerging concern (CECs) PhACs have boosted the interest of the scientific community all over the world in the last couple of decades. Although, there are almost no regulations in force that deal with the discharge of these kind of micropollutants to the environment, some efforts are ongoing in an attempt to set new policies regarding the occurrence of these substances in the environment. This growing awareness is contributing to create a framework for controlling the release of these compounds. A critical assessment on the environmental fate and effects of the PhACs will contribute to the future enforcement of regulations as well as providing a set of best management practices related to water quality. The European Union, in the Directive 2008/105/EC under the decision (EU) 2015/495 of 20 March 2015 has recently established a watch list of substances for monitoring Union-wide; it establishes that those substances found to pose a significant risk should be considered for inclusion in a priority substances list [6]. In the watch list it is possible to find some macrolide antibiotics such as erythromycin or the analgesic diclofenac.

Pharmaceuticals, their metabolites and transformation products are discharged in the environment during their life cycle (figure 1 and 2). After pharmaceuticals are administered or consumed, they are excreted as a mixture of the parent compounds and their metabolites (both biologically active and/or inactive). The inappropriate management and disposal of pharmaceuticals from the household scenario is another relevant pollution source [7]: When people do not completely follow a medical prescription or clean out the medicine leftovers, sometimes they deliver and flush them into the toilet. The drugs present in the wastewater are then transported through the sewage system and reach the wastewater treatment plant. Depending on the characteristics of the drug, the regular treatment might not achieve its total removal being then discharged in the receiving surface waters. The compounds can enter also terrestrial systems through sewage effluent and/or sludge, when used for irrigation or as a fertiliser to agricultural crops [8].

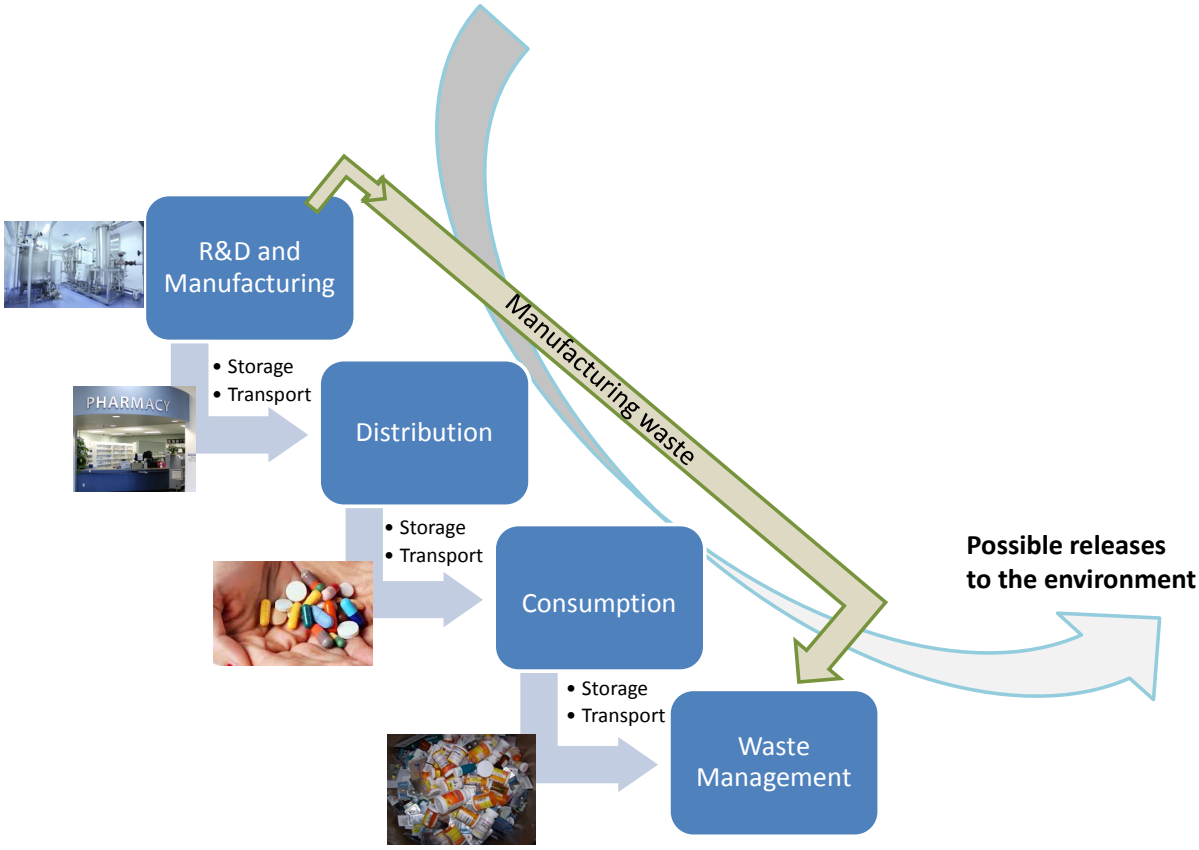

Figure 1. Overview of the life-cycle of medicinal products (adapted from "Study on the environmental risks of medicinal products" CHAFEA 2013 [9])

Veterinary medicinal products are released also as parent compounds and metabolites to the environment either directly -from use in aquaculture and treatment of pasture animals- or indirectly -during the land application of manure and slurry from livestock facilities- [10]. PhACs can also be released from industrial facilities from, i.e., leakages and/or manufacturing waste.

The relative relevance of the sources of PhACs and contamination pathways along the water cycle depends on whether veterinary or human medicinal products are considered and whether both point sources (pharmaceutical factories or waste treatment plants) and diffuse sources are taken into account [9]. Figure 2 summarizes in a diagram the known contamination pathways related to the use phase for both human and veterinary medicinal products. The nature and amount of medicinal residues released after consumption mainly depend on the volumes and nature of the administered substances, the modes of administration and the metabolism rates [9]. According to the "Study on the environmental risks of medicinal products" reported by the European Consumer, Health and Food Executive Agency (CHAFEA), the consumption

stage both, human and veterinary, is the most important contribution to the emissions for medicinal products into the environment. It may sometimes be difficult to attribute human or veterinary origins to the residues detected in the environment because some medicinal products can be used in both, humans and animals.

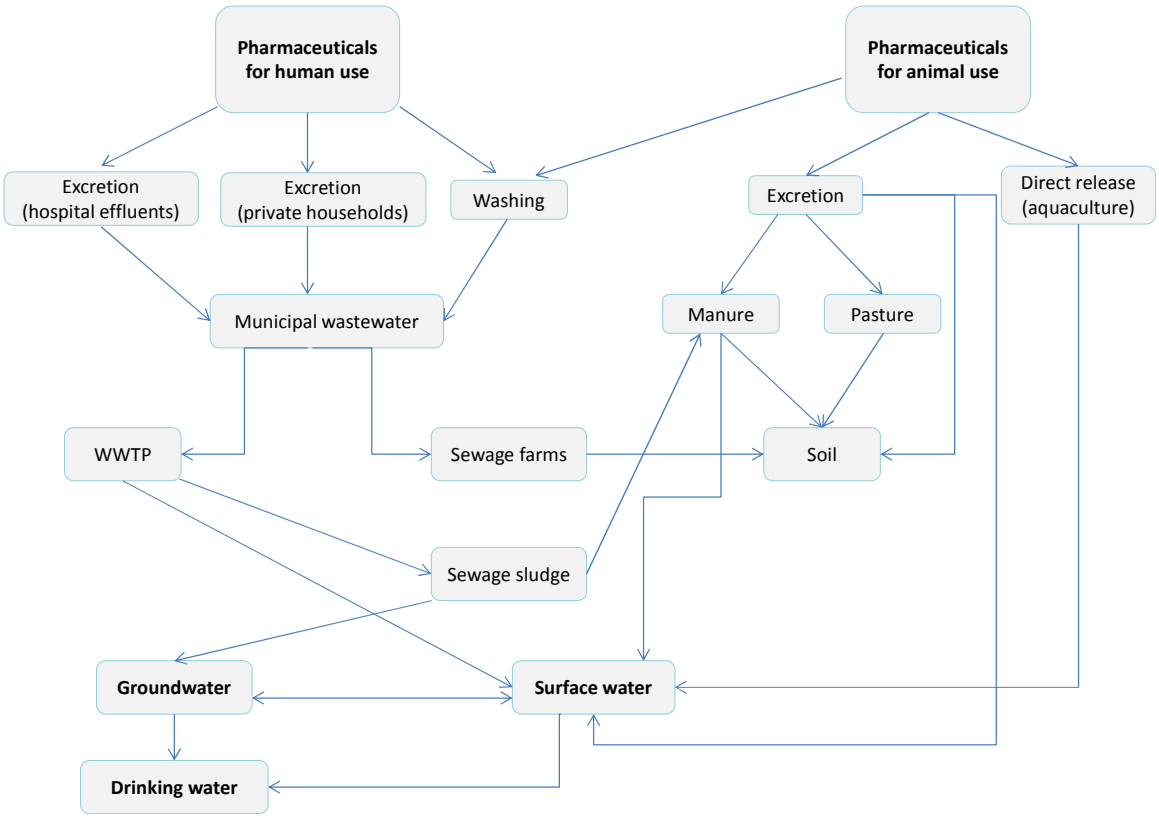

Figure 2. Emission pathways linked to the use-phase of pharmaceutical compounds (adapted from "Study on the environmental risks of medicinal products" CHAFEA 2013 [9])

In addition, the discharge of pharmaceutical compounds to the urban wastewater is increasing due to the population aging, the increase in population density and, in some geographic areas, due to water scarcity associated with climate change [11-13]. Furthermore, the huge global demand of meat at affordable prices contributes to increase the production pressure in the farms. In order to satisfy the market demand, the farms have increased the consumption of pharmaceutical compounds (mainly antibiotics and hormones) to increase production yield. An inappropriate management of the wastewater produced in farms is considered a major issue nowadays, since these veterinary drugs may find as final sink surface or underground water bodies.

1.2. Anticancer drugs

Cancer is one of the most concerning diseases in western countries and quite a lot of resources are devoted to investigate its treatment and eventually its cure. Despite the chemotherapeutic treatments have been improved in the last decades and are now more effective and patient specific, cancer is still one of the most harmful diseases worldwide. World Health Organization (WHO) has published a world health report where cancer is ranked as the second cause of death (21%) after cardiovascular illness (48%) and followed by respiratory diseases (12%) in the sector of non-communicable diseases; namely diseases caused by non-infectious and non-transmissible medical conditions (www.who. int). American Cancer Society has foreseen a total of 1.638.910 of new cases and 577.190 deaths in United States of America in 2012 whereas annual cancer mortality is expected to decrease only a 1% [14].

The huge increment of cancer disease in the population has led to an enlargement on drugs consumption and it can be foreseen an even higher discharge of these kind of substances into the environment in the coming years. Despite the chemotherapy treatments are administrated mainly at hospital facilities, it is quite usual that patients leave the hospital right of just a few hours after getting the treatment. Additionally, there are types of cancer such prostate cancer whose treatments are delivered in ambulatory facilities or even at home. This means that anticancer drugs, their metabolites and related compounds (biomarkers, etc) are excreted by humans via hospital and/or domestic wastewaters being the immediate recipient the urban sewage system. It is necessary to remark that the a**nticancer drugs** have been shown to have potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor, and/or teratogenic effects in several organisms, since they have been mainly designed to disrupt or prevent cellular proliferation, usually by interfering in DNA synthesis. From this point of view, it is also important to highlight the relevance of the metabolites, since they have often equal or even greater activity than the parent compound. This is the case of the main metabolite of the anticancer drug tamoxifen, the hydroxy-tamoxifen, whose estrogenic potential is even higher than the observed for the drug itself [15].

Veterinary facilities should be also be considered as a relevant source of anticancer drugs, since most of these drugs are also used in veterinary applications, mainly in the treatment of cancer in dogs and cats [16].

1.3. Description of Chemotherapy drugs

Chemotherapy uses powerful chemicals to kill fast-growing cells in the body and can be used alone or combined to treat a wide variety of cancer diseases. The anticancer drugs include a large number of compounds which belongs to different chemical families: They can be divided into several groups based on factors such as the mode of action, chemical structure, their relationship to another drug and/or if they come from natural sources. For instance, some anticancer drugs are grouped together because they were derived from the same herbal although they have different ways of action.

Based on different health organisations (WHO, SEFH, Mayo Clinic, American Cancer Society and EACR), the anticancer drugs can be structured in ten groups: i) Alkylating agents, ii) Antimetabolites, iii) Anti-tumour antibiotics, iv) Topoisomerase inhibitors, v) Mitotic inhibitors, vi) Corticosteroids, vii) Miscellaneous chemotherapy drugs, viii) Hormone, ix) Anti-tumour antiretroviral and x) Immunotherapy drugs (Table 1). There are relevant scientific advances in chemotherapy, based sometimes on more targeted treatments or even on the prevention of the cancer disease such as the cancer vaccines or use of the genetic profiling to take early preventive measures. However there are many conventional drugs described here which are - to date - still necessary based on their well-proven effectiveness in a large number of cancers.

Table 1. Chemotherapy drugs classified by mode of action (table adapted from SEFH).

Mechanism of action	***Chemical group***	***Compound***
Alkylanting agents	*Nitrogen mustards*	Cyclophosphamide Chlorambucil Ifosfamide Melphalan Trofosfamide Mechlorethamine
	Nitrosoureas	Carmustine Estramustine Lomustine Streptozocin
	Alkyl sulfonates	Busulfan
	Triazines	Dacarbazine Procarbazine Temozolomide
	Ethylenimines	Altretamine Thiotepa
	Platinum drugs	Cisplatin Carboplatin Oxalaplatin
Antimetabolites	*Pyrimidine antagonist*	Cytarabine

		Tegafur Floxuridine Azatadine 5-flurouracil Ftorafur (Tegafur/Uracil) Gemcitabine
	Purina antagonist	Thioguanine Azathioprine Mercatopurine Cladribine
	Adenosine antagonist	Fludarabine Pentostatine
	Folic acid antagonist	Methotrexate Trimetrexate Raltitrexed
Anti-tumor antibiotics	*Anthracyclines*	Daunorubicin Doxorubicin Epirubicin Idarubicin Mitoxantrone Pirarubicin Amsacrine
	Others	Actinomycin-D Bleomycin Mitomycin-C Ciprofloxacin
Topoisomerase inhibitors	*Derived from camptothecin (natural source)*	Topotecan Irinotecan
	Derived from podophyllotoxin (natural source)	Etoposide Teniposide
Mitotic Inhibitors	*Taxanes (natural source)*	Paclitaxel Docetaxel
	Vinca alkaloid (natural source)	Vinblastine Vincristine Vinorelbine
Corticosteroids/anti-emetic	*Steroids*	Prednisone Methylprednisolone Dexamethasone
Miscellaneous chemotherapy drugs	*Enzymes*	L-asparaginase
Hormone	*Anti-estrogens*	Fulvestrant Tamoxifen Toremifene
	Aromatase inhibitors	Anastrozole Exemestane Letrozole
	Progestins	Megestrol acetate
	Anti-androgens	Bicalutamide Flutamide Nilutamde
	Gonadotropin-releasing hormone	Leuprolide Goserelin

Anti-tumor antiretroviral	*HIV-protease inhibitors*	Ritonavir Saquinavir Indinavir Nelfinavir Atazanavir
Immunotherapy	*Monoclonal antibody therapy*	Rituximab Alemtuzumab
	Non-specific immunotherapies and adjuvants	BCG Interleukin-2 (IL-2) Interferon-alfa
	Immunomodulating drugs	Thalidomide Lenalidomide
	Cancer vaccines	Provenge® vaccine for advanced prostate cancer

1.4. Occurrence of anticancer drugs in the environment

Reports on occurrence of cytostatic compounds in the environment are very recent but still scarce [17-53] and the concentration found in wastewater and natural samples are very low compared with other common pharmaceuticals. Table 2 intends to summarize the data available in the literature regarding the presence of these drugs in different aquatic compartments and matrices. To date, most of the studies reported in literature have been performed in wastewater, particularly in hospital effluents as a potential source of these micropollutants. Certainly, there is almost no information of these compounds in surface water, groundwater and drinking water neither in activated sludge or natural sediments. Up to 14 studies have reported incidence of anticancer drugs in hospital effluents, 10 studies in wastewater influents, 12 studies in wastewater effluents, 7 studies in surface waters, 2 studies in drinking water and 1 study in activated sludge. Seven of these studies have been performed in Germany, four in Spain, four in Austria, three in China, three in France, three in United Kingdom, two in Switzerland, and three in Italy. Most of the studies have assessed one or two compounds. Only Yin. et al 2009 had studied up to six compounds in Chinese hospital effluents before this thesis has begun [27] . During the thesis timeframe, Negreira et al. 2014 also evaluated the presence of 13 anticancer drugs and 4 metabolites in municipal and hospital wastewaters in Spain [30].

Cyclophosphamide and ifosfamide are the most studied anticancer drugs being as well the most consumed according to the National Health System of Spain [54]. Levels of cyclophosphamide ranged from 6 till 143 ng/L and from 19 until 4,500 ng/L in urban

and hospital wastewaters, respectively, whereas levels between 6 and 20 ng/L were found in wastewater effluents (Table 2) [25, 26]. This wide range in occurrence levels can be attributed to the limited number of studies available and it is in accordance with the variability observed in wastewater concentrations for the drugs with relative low consumption. Only in very few cases, some anticancer drugs were detected at relatively high levels. This is the case of epirubicin, which was found up to 24800 ng/L in an effluent wastewater and 5-fluorouracil detected up to 124 000 ng/L in a hospital effluent (Table 2)

Most of the anticancer drugs have never been analyzed in surface water.This is the case of anastrozole, azathioprine, capecitebine, docetaxel, doxorubicin, doxorubicinol, epirubicin, etoposide, irinitecan, letrozole, methotrexate, among others (Table 2). Just the occurrence of one anticancer drug, bleomycin, has been studied in drinking water where the concentration found varied from 5 to 17 ng L^{-1}. Also, only cyclophosphamide and ifosfamide have been analyzed once in activated sludge (Table 2).

Furthermore there is a huge gap when it comes to information about the presence of human metabolites of anticancer drugs in the aquatic environment. So far, only Negreira et al. have reported the presence of paclitaxel and tamoxifen human metabolites in wastewaters (Table 2).

According to up-to-date literature data, it can be concluded that there is a lack of information regarding the sources, fate and occurrence of anticancer drugs. It becomes a challenging and difficult task identifying whether the main sources of contamination are hospital effluents or urban influents. In fact, there are no studies that comprehensive gather the occurrence of a representative number of anticancer drugs in the whole urban water cycle. The scarcity of information about environmental levels of these compounds is partially explained by the lack of analytical methods suited to environmental applications [28, 50, 51]: the anticancer drugs belong to different chemical families and developing a multi-residue method for all of them is an analytical challenge. Furthermore, the high cost of chemotherapy pharmaceutical reference standards - often produced through expensive synthesis - and their particular health and safety hazards, makes the conventional target analysis of these compounds difficult in most of the environmental laboratories. This is mainly due to the special and expensive safety conditions required for their handling (analyst training for cytotoxic handling and spills, personal protective equipment, biological safety cabinet or similar category hood, specific containers for residues, etc).

Table 2. Literature review about the occurrence of anticancer drug in aquatic environment

Compound	Reference	Location	Hospital Effluent ng L^{-1}	WWTP Influent ng L^{-1}	WWTP Effluent ng L^{-1}	Surface water ng L^{-1}	Groundwater ng L^{-1}	Drinking water ng L^{-1}	Sludge ng g^{-1}
Anastrozole	[29]	China	0.3 - 3.7	0.12 – 0.32	0.3	-	-	-	-
	This Thesis	**Norway**	**-**	**nd(sp)**	**-**	**-**	**-**	**-**	**-**
Azathioprine	[24]	China	15	-	-	-	-	-	-
	This Thesis	**Spain**	**blq-188**	**nd-20**	**nd**	**nd**	**-**	**-**	**-**
Capecitebine	[32]	Spain	-	8.2 -27.0	-	-	-	-	-
	[30]	Spain	-	nd – 72.6	Nd - 36	-	-	-	-
Cyclophosphamide	[25]	Germany	146	-	-	-	-	-	-
	[31]	Germany	19- 4500	6 -143	6 - 17	-	-	-	-
	[33]	Germany	-	-	-	-	-	-	20
	[53]	Italy	-	-	2.1 - 9	-	-	-	-
	[34]	Germany	-	-	10 - 20	< 10	-	-	-
	[35]	Italy	-	-	-	2.2 -10	-	-	-
	[36]	Romania	-	-	-	nd – 64.8	-	-	-
	[26]	France	30 - 900	-	300	-	-	-	-
	[19]	Switzerland	-	2 – 11	-	0.05 – 0.17	-	-	-
	[20]	Spain	5300	13100	-	-	-	-	-
	[27]	China	6 - 2000	-	-	-	-	-	-
	[30]	Spain	-	n.d – 43.8	n.d – 25.0	-	-	-	-
	This Thesis	**Spain**	**blq-200.7**	**nd-26**	**7-25**	**blq-20**	**-**	**-**	**-**
Carboxyphosphamide	**This Thesis**	**Spain**	**TI**	**TI**	**-**	**-**	**-**	**-**	**-**

Compound	Reference	Location	Hospital Effluent ng L^{-1}	WWTP Influent ng L^{-1}	WWTP Effluent ng L^{-1}	Surface water ng L^{-1}	Groundwater ng L^{-1}	Drinking water ng L^{-1}	Sludge ng g^{-1}
Platinum compounds*	[37]	Austria	3000- 250, 000	-	-	-	-	-	-
	[38]	France	350	-	-	-	-	-	-
	[39]	Austria	1700	-	-	-	-	-	-
Bleomycin	[40]	Germany	-	-	-	-	-	5- 13	-
	[41]	U.K.	-	15.8	11 -19	< 5 – 17	-	< 5 - 17	-
	[26]	France	< 30	-	-	-	-	-	-
Cytarabine	[42]	Spain	-	9.2	14	13	-	-	-
Daunorubicin	[43]	Austria	< 60	-	-	-	-	-	-
Docetaxel	**This Thesis**	**Spain**	**nd-98**	**nd-219**	**nd**	**nd**	**-**	**-**	**-**
Doxorubicin	[43]	Austria	260 -1350	-	-	-	-	-	-
	[42]	Spain	-	4.5	-	-	-	-	-
Doxorubicinol	[27]	China	< 10	-	-	-	-	-	-
Epirubicin	[20]	Spain	-	-	24800	-	-	-	-
Etoposide	[27]	China	5 – 380	-	-	-	-	-	-
	[26]	France	110 - 300	-	-	-	-	-	-
	[42]	Spain	-	15	3.4	-	-	-	-
	This Thesis	**Spain**	**nd-714**	**nd-175**	**nd**	**nd**	**-**	**-**	**-**
5-Fluorouracil	[44]	Austria	8600 – 124, 000	-	-	-	-	-	-
	[51]	Switzerland	<5.0 – 27	-	-	-	-	-	-
	[43]	Austria	20, 000-122, 000	-	-	-	-	-	-
2, 2- difluoro-deoxyuridine[(m)]	[51]	Switzerland	< 9.0 - 840	-	-	-	-	-	-
Gemcitarabine	[51]	Switzerland	<0.9 – 38	-	-	-	-	-	-
	[42]	Spain	-	9.3	7.0	2.4	-	-	-

Compound	Reference	Location	Hospital Effluent ng L^{-1}	WWTP Influent ng L^{-1}	WWTP Effluent ng L^{-1}	Surface water ng L^{-1}	Groundwater ng L^{-1}	Drinking water ng L^{-1}	Sludge ng g^{-1}
Ifosfamide	[25]	Germany	24	-	-	-	-	-	-
	[17]	Germany	6 - 1914	6 – 29	6 - 43	-	-	-	-
	[33]	Germany	-	-	-	-	-	-	20
	[34]	Germany	-	-	10 - 2900	< 10	-	-	-
	[19]	Germany	-	1.4 – 6	-	0.05 – 0.14	-	-	-
	[27]	China	4 - 10647	-	-	-	-	-	-
	[42]	Spain	-	3.5	1.2	-	-	-	-
	[30]	Spain	-	nd -27.9	nd – 15.9	-	-	-	-
	This Thesis	**Spain**	**nd-228**	**nd-130**	**nd**	**nd**	**-**	**-**	**-**
Irinitecan	[30]	Spain	-	nd – 21.3	nd -16.8	-	-	-	-
Letrozole	[29]	China	0.20 – 2.38	0.28 – 0.8	0.27 – 0.60	-	-	-	-
	This Thesis	**Norway**	**-**	**nd(sp)**	**-**	**-**	**-**	**-**	**-**
Methotrexate	[46]	U.K.	1000	-	-	-	-	-	-
	[53]	Italy	-	-	12.6	-	-	-	-
	[27]	China	2 - 4689	-	-	-	-	-	-
	This Thesis	**Spain**	**nd-19**	**nd-26**	**nd-6**	**nd**	**-**	**-**	**-**
Paclita-el	**This Thesis**	**Spain**	**blq-100**	**nd-18**	**nd-blq**	**nd**	**-**	**-**	**-**
Hydroxy-paclitaxel	[30]	Spain	-	nd -18.5	nd - 3.7	-	-	-	-
Procarbazine	[27]	China	< 5	-	-	-	-	-	-
Tamoxifen	[47]	U.K.	-	-	-	27 - 212	-	-	-
	[48]	France	-	-	< 102	< 25	-	-	-
	[49]	U.K.	-	-	< 42	-	-	-	-
	[29]	China	0.2- 8.2	0.28	-	-	-	-	-
	[30]	Spain	-	110 – 147	nd –180.6	-	-	-	-

Compound	Reference	Location	Hospital Effluent ng L^{-1}	WWTP Influent ng L^{-1}	WWTP Effluent ng L^{-1}	Surface water ng L^{-1}	Groundwater ng L^{-1}	Drinking water ng L^{-1}	Sludge ng g^{-1}
Tamoxifen	This Thesis	Spain	26-170	nd-58	11-42	25-38	-	-	-
Hydroxy-tamoxifen[m]	[30]	Spain	-	-	nd - 5.8	-	-	-	-
	This Thesis	Spain	TI	TI	-	-	-	-	-
4-hydroxy-N-desmethyltamoxifen[m]	[30]	Spain	-	-	91.6	-	-	-	-
4,4-dihydroxy desmethyltamoxifen[m]	This Thesis	Spain	TI	TI	-	-	-	-	-
Vincristine	[27]	China	< 20	-	-	-	-	-	-
	This Thesis	Spain	nd-49	nd-23	nd	nd	-	-	-
Vinorelbine	[42]	Spain	-	-	9.1	-	-	-	-
Special section									
Atazanavir[i]	This Thesis	Norway	-	TI	-	-	-	-	-
Ciprofloxacin[i]	This Thesis	Spain	679-14826	1172-1558	36-147	7-103	-	-	-
	This Thesis	Norway	-	nd	-	-	-	-	-
Citalopram[i]	This Thesis	Norway	-	TI	-	-	-	-	-
Inositol[i,ii]	This Thesis	Norway	-	TI	-	-	-	-	-
Medroxyprogesterone	This Thesis	Norway	-	TI	-	-	-	-	-
Megestrol[i]	This Thesis	Norway	-	TI	-	-	-	-	-
Iomeprol&Iopamidol[i]	This Thesis	Norway	-	TI	-	-	-	-	-
Viridiflorine	This Thesis	Norway	-	TI	-	-	-	-	-

* This group includes several compounds such as Cisplatin, Carboplatin, Oxiliplatin and/or Lobaplatin very frequent estimated as a total platinum concentration.

(-)= not available data; nd = not detected; TI= tentatively identified; blq=below limit of quantification.; [m]Human metabolite; [sp]Suspect screening; [ta]Target analysis; [i]Refers to compounds which have other therapeutic applications however they have been included in this study since they are highly relevant in the chemotherapy treatments. The author has avoided including other bibliographic references due to the large number of studies for some of these compounds ;[ii]Cancer biomarker.

1.5. Environmental risk of anticancer drugs

As previously mentioned, the anticancer drugs are, in general terms, regarded as very hazardous compounds, since they are designed to kill or to provoke severe damage on cells. These processes may cause as side-effects acute disorders and alterations on normal functions of the organisms exposed to them (endocrine system, immunologic system, etc). On the other hand, ecotoxicological studies carried out with cytotoxic substances such as 5- fluorouracil, have revealed that the lowest observed-effect concentration (LOEC) in algae and bacterial assays was about 10 μg L^{-1}, close to the concentration found in several sewage effluents [55]. In another example, the LOEC obtained for tamoxifen in freshwater fish was 5.6 μg L^{-1} [56]. This concentration is only one fold higher than those found in wastewaters nowadays (about 0.2 μg L^{-1}) [30, 47]. Recent studies have revealed also that mixtures of anticancer drugs in real samples possess an important toxic effect, even higher than the expected by addition of the toxicity of the individual drugs [57]. Therefore, potential synergy should not be neglected when it comes to toxicity of "cocktails" of drugs in water.

Furthermore, several authors have pointed out that some pharmaceutical substances, frequently discharged in hospital effluents, might be assessed in regard to their bioaccumulative potential in the aquatic environment. This is the case of one anticancer drug, Tamoxifen, which was included in a list of priority substances by Jean et al 2012 due to strong evidences based on its bioconcentration potential (accumulation of a chemical in an organism when the source of chemical is solely water), consumption data, biodegradability and excretion factor [58]. These authors concluded with the necessity of measuring accumulated dose levels of some PhACs – including Tamoxifen - in species of different trophic levels.

1.6. Conventional and advanced treatment for the removal of anticancer drugs from wastewater

In view of the aforementioned (section 1.4 and 1.5), it becomes then essential to pay special attention to the discharge of anticancer drugs in the urban sewage system- focusing in their removal at urban WWTPs - as well as to their occurrence in the aquatic environment. The majority of full-scale wastewater treatment plants nowadays were designed with the aim of removing the content of organic matter (which may cause oxygen depletion in receiving surface water) and to reduce nutrients such as nitrogen and phosphorus (which can lead to the over-fertilisation of receiving surface waters and

eventually of the sea). Once used, pharmaceuticals and their metabolites enter the wastewater system and the wastewater treatment plants, where their fate is governed by their physical, chemical and biological properties. The pharmaceuticals, in some cases, can be biologically degraded if the process conditions in the plant are favourable. Furthermore, some drugs with hydrophobic/lipophilic nature can be adsorbed to the sludge so that the drugs are distributed between the output liquid stream and the solid sludge effluent [59-61]. To date, the scarce literature available reveals that anticancer drugs have in most cases very low or even no biodegradability in conventional activated sludge technology (CAS) employed in wastewater treatment facilities [17, 37, 45, 62]. Since they are not efficiently removed from the wastewater, they can reach the aquatic environment, which acts as a final sink of urban sewage effluents. Knowing the limitations of conventional wastewater treatments to eliminate anticancer drugs and other recalcitrant micropollutants, different technologies and strategies for their removal are currently being studied. One of these new technologies is based on the use of membranes, often combined with biological treatments. An example of these coupled technologies is the membrane bioreactor (MBR), which combines permeation selectivity through a physical barrier with biological removal (biosorption and/or biodegradation) to improve the performance of the overall water treatment. This technology has been successfully used as an alternative treatment for some of these anticancer drugs. That is the case of 5-fluorouracil or doxorubicin, both almost completely removed from hospital effluents by an MBR process [37]. However, other authors have found low removal percentages for anticancer drugs using MBR: c.a. 60% for cytostatic drugs based on platinum organometallic compounds [37] or less than 20% of elimination for cyclophosphamide [52] . Despite MBR system seems a promising technology for many substances, it is not clear its suitability for the removal of anticancer drugs and it is still an expensive treatment due to the special maintenance required for the membranes, their frequent replacement and electricity consumption.

Non-conventional biological treatments such as those based on the use of white rot fungi (WRF) for the degradation of PhACs have also been evaluated with positive results. WRF such as *Trametes versicolor* (Picture 1) have shown a potent biodegradation capacity, which makes them candidates for effective bioremediation strategies. Their success is related to their unspecific oxidative enzymatic system, which includes lignin-modifying enzymes, especially laccase and peroxidases, but also to their intracellular enzymatic complexes (e.g., cytochrome P450) [63]. Main chemical reactions involved in the biotransformation of pharmaceutical compounds are hydroxylation, formylation,

deamination and dehalogenation [64]. The removal efficacy of WRF against some PhACs such as β-blockers, anti-inflammatory drugs, antibiotics and psychiatric drugs has been proved: degradation was reached after some minutes to few days for Ibuprofen, Clofibric acid and Carbamazepine[65-68] or after periods even longer than a week in the case of some antimicrobial agents, estrogens and iodinated contrast agents [69, 70]. At the time of starting this thesis, there were no studies regarding the use of this biological treatment (based on *Trametes versicolor*) for the removal of anticancer drugs and WRF seemed to be a promising technology to treat these recalcitrant compounds.

Picture 1. *Trametes versicolor* (picture obtained from ww.mykoweb.com)

On the other hand, tertiary treatments (placed after the regular biological processes employed in WWTPs) can contribute substantially to decontamination of the waste effluent when the biological treatment alone is unable to remove efficiently micropollutants load [71]. In fact, the objective of tertiary treatments, so-often called "effluent polishing", is usually to improve the quality of the effluent right before being discharged into the aquatic environment, particularly when it is a highly sensitive or fragile ecosystem (estuaries, low-flow rivers, coral reefs, etc). There are many types and combinations of tertiary treatment processes which can be used in a WWTP. Among them, disinfection is one of the most widely used and always placed at the end of the process, with the goal of substantially decrease the number of microorganisms in the wastewater before being emitted into the environment [72-74]. Nowadays, some of these disinfection techniques are currently based in advanced oxidation processes (AOP). These processes refer to a set of chemical treatment procedures designed to

remove organic (and occasionally inorganic) substances from water and waste water by oxidation through reactions with hydroxyl radicals (•OH). AOPs have demonstrated their effectiveness not only for disinfection purposes, but also for the removal of a large number of compounds, being feasible their implementation as tertiary treatment in wastewater treatment plants [75].

Hydroxyl radicals are very reactive and short half-life oxidants (10 μs at a 10^{-4} M concentration) which acts in a non-selective way [76, 77]. The activation of •OH radicals is a very complex process, which can take place according to a variety of different reaction mechanisms. However, these radicals need to be generated on site in order to drive oxidation/reduction reactions with the organic molecules present in wastewater. Hydroxyl radicals might be generated by using different treatments such ultraviolet radiation/hydrogen peroxide, ozone/hydrogen peroxide, ultraviolet radiation/ozone, Fenton's process (ferrous iron and hydrogen peroxide), titanium dioxide/ultraviolet radiation, etc. In the case of ozone based processes, the disintegration of ozone in water produces •OH radicals, with higher oxidation potential than ozone itself. In the reaction mechanism shown below (mechanism 1), ozone is combined with hydrogen peroxide, both of them being very reactive and short half-life oxidants [73, 76, 77].

i) $H_2O_2 \rightarrow H_2O + O_2$ (spontaneous decomposition)

ii) $H_2O_2 \rightarrow HO_2^- + H^+$

$2\ O_3 + H_2O_2 \rightarrow 2\ OH^{\bullet} + 3\ O_2$ (O_2 reduces to H_2O in presence of H^+)

Mechanism 1. Hydrogen peroxide splits in water according to these simultaneous reactions.

Direct or/and indirect oxidation reactions can take place between radicals and organic matter depending on various factors, such as temperature, pH and other chemical substances present in the water. Typically direct reactions are cyclo-addition (Criegee mechanism), electrophilic reactions, and nucleophilic reactions (figure 3) [76, 77].

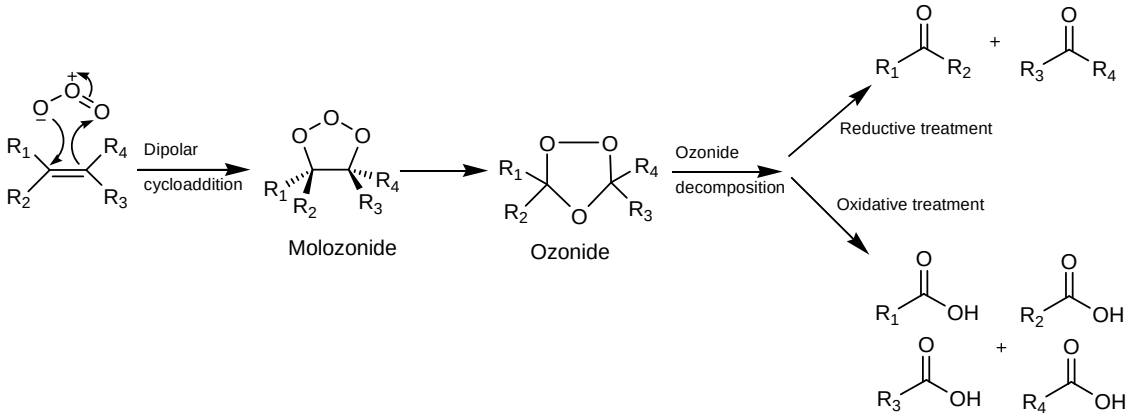

Figure 3. Ozone oxidation of olefins: 1,3-cycloaddition; retro 1,3-cycloaddition and ozonide decomposition.

Indirect reactions (by radical generation) in an ozone based oxidation process can be very complex. The radical generation follows different steps involving initiation, radical chain-reaction and termination (such as mechanisms 2) [76, 77]

i) $O_3 + OH^- \rightarrow O_2^{\bullet -} + HO_2^{\bullet}$

$HO_2^{\bullet} \rightarrow O_2^{\bullet -} + H^+$ (pKa = 4,8)

ii) $O_3 + O_2^{\bullet -} \rightarrow O_3^{\bullet -} + O_2$

$O_3^{\bullet -} + H^+ \rightarrow HO_3^{\bullet}$ (pH < ≈ 8)

iii) $OH^{\bullet} + O_3 \rightarrow HO_4^{\bullet}$

$HO_4^{\bullet} \rightarrow O_2 + HO_2^{\bullet}$

Mechanism 2. Indirect reactions in ozone based processes.

So far, very few studies have evaluated the potential removal of anticancer drugs by AOPs [78-80]. Garcia-Ac et al. (2010) showed that methotrexate can be rapidly and effectively removed from drinking water by ozonation, whereas the removal of cyclophosphamide needs much higher ozone dose and longer contact time; thought identification of the potential ozonation by-products was not followed up in this study [81]. On the other hand Somensi et al. (2012) demonstrated that a combination of sonolysis and ozonolysis is a more efficient process than ozonolysis alone for the degradation of doxorubicin, while methotrexate can easily be degraded by ozonolysis alone or sonolysis/ozonolysis methodologies [82]. Based on the encouraging results previously reported by several authors, it becomes mandatory to further study AOPs as

potential alternatives for the removal of these substances from sewage, paying special attention to the sub-products generated during the oxidation processes.

Although the processes aforementioned -AOP and the non-conventional biological treatment- have different purposes than the removal of micropollutants, both of them may nowadays be implemented in the existing WWPTs, according to different possible configurations (figure 4). The most frequent configuration, which is already in use in several WWTPs, involves placing the AOPs after the biological process as a tertiary treatment (see figure 4 dotted green square). A second potential configuration would involve substituting the conventional biological treatment, based in activated sludge, by a non-conventional biological process (MBR, fungi, algae, and/or consortium of microorganism). In a potential third configuration, the activated sludge process of the regular scheme of the WWTP would be upgraded using sequential changes of microbial community composition installing sequential bioreactors. In this approach, another bioreactor (based on non-conventional biological process) would be installed right after the secondary clarifier (figure 4 dotted red square) [83]. Other possibility might involve placing the AOPs units right after the primary clarifier and before the biological treatment (figure 4 dotted blue square). By doing so, the biodegradability of micropollutants could be enhanced, being then the substances more readily available to enter the microbial metabolism [84] .

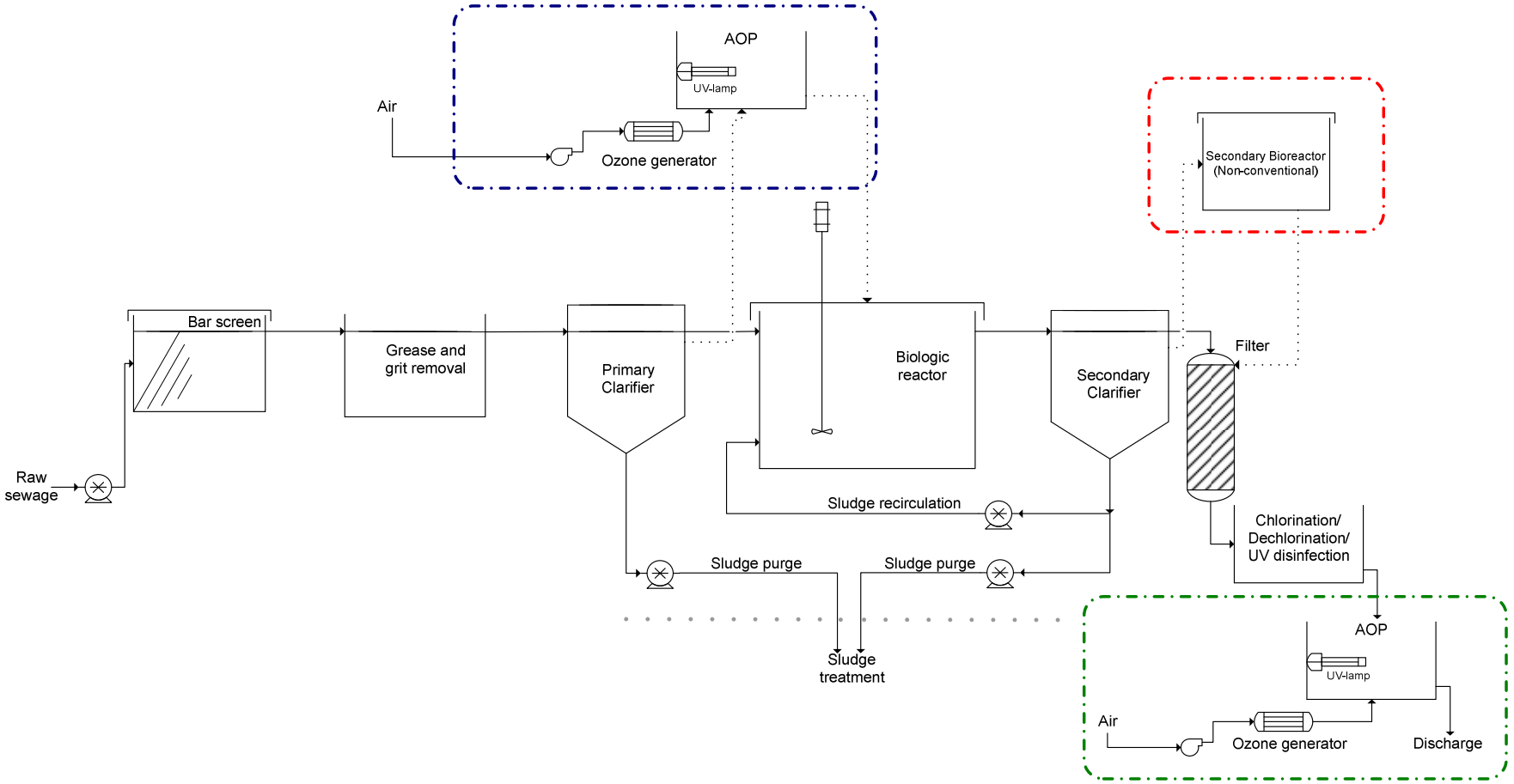

Figure 4. Layout of a conventional WWTP with the potential upgrades : i) AOP placed in conventional configuration green line, ii) AOP placed before biological treatment in blue line and iii) secondary bioreactor with non-conventional biological treatment in red line (figure adapted from Tratamientos de Aguas by Jose Ferrer Polo y Aurora Seco Torrecillas 2004, Edt. Universitat Politecnica de Valencia).

1.7. Hypothesis and thesis motivation

The lack of information about the occurrence of anticancer drugs in the aquatic environment, their inherent toxicity and their presumable persistence after conventional WWTPs motivated the performance of a dedicated research in basic aspects such as: incidence, ecotoxicity and elimination of anticancer drugs from wastewater by conventional systems as well as by new technological alternatives in order to ensure a safe discharge of polluted effluents in the environment.

The occurrence of anticancer drugs along the urban system ought to be assessed, although it could be foreseen that their concentration would be noticeable because of their consumption increase in the last decades. In order to achieve this goal, different analytical strategies based in liquid chromatography (LC) coupled to mass spectrometry (MS) needed to be applied. The most concerning substances occurring in the aquatic environment could thus be identified based on their concentrations, removal under the usual WWTP schemes as well as their potential ecotoxicological effect, Eventually, the right management of the effluents polluted with these substances will be able to be established.

In this thesis, several disciplines -analytical chemistry, ecotoxicology, engineering and microbiology- join forces to provide a holistic approach covering important aspects such as the monitoring of the occurrence of these substances in relevant environmental scenarios or the assessment of novel water treatment technologies as potential candidates for future upgrading of WWTPs.

The following figure 5 summarizes the structure of this thesis covering thoroughly the topics described in the introduction.

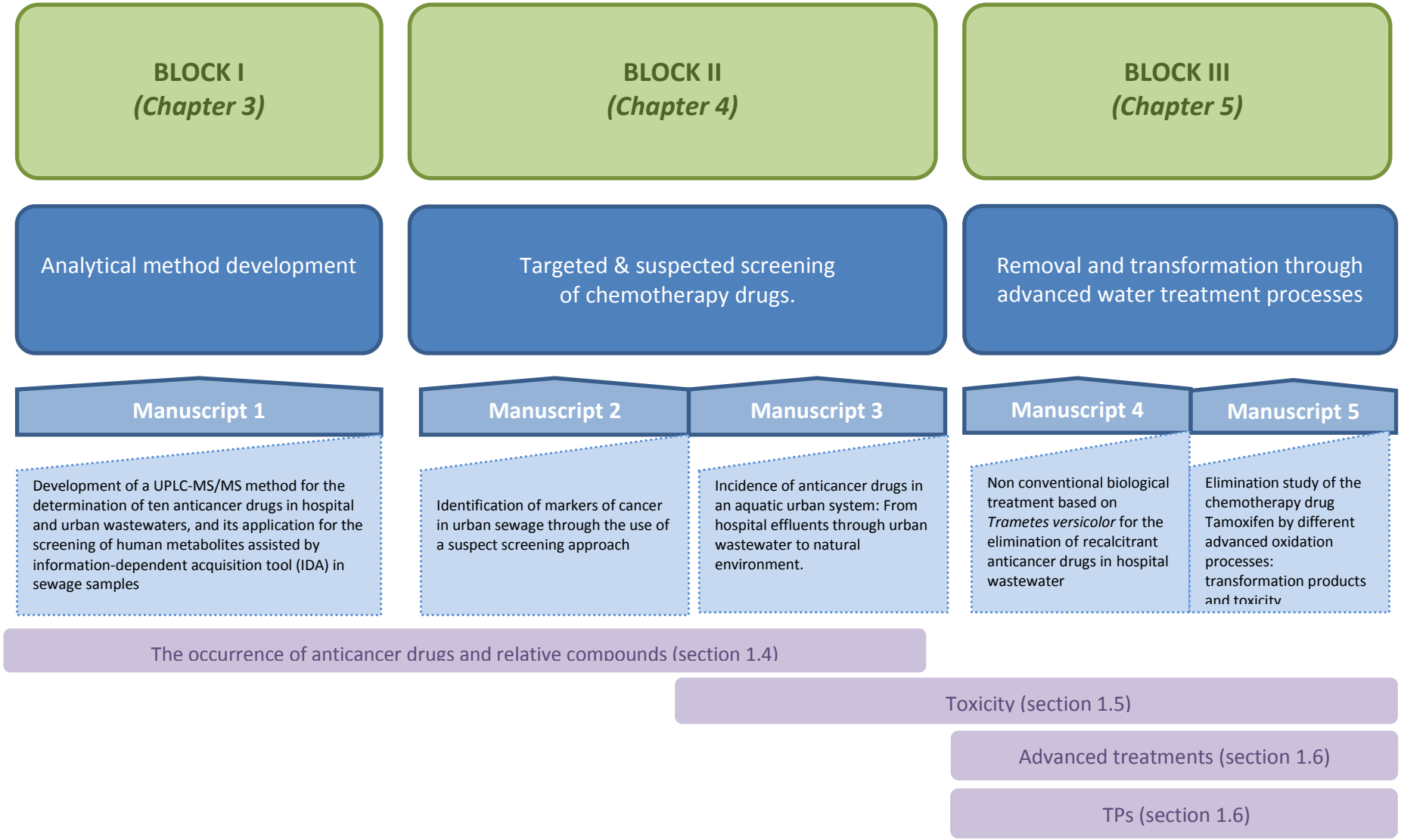

Figure 5. Thesis work flow diagram

CHAPTER 2

2. Objectives

The main goal of this thesis was to evaluate the occurrence of some selected anticancer drugs in the aquatic environment, paying attention to potential alternatives for their removal from wastewater as well as to the presence of transformation products of the drugs (TPs) generated along the processes studied. In order to achieve this, the following specific objectives were defined:

1. To develop analytical methodologies and strategies for the quantitative determination as well as for the tentative identification of anticancer drugs in water.
2. To assess the incidence of anticancer drugs in different urban systems: Spain and Norway.
3. To assess the environmental risk that anticancer drugs pose to the environment
4. To evaluate different alternatives for the elimination of anticancer drugs: non-conventional biological treatment based on fungi and advanced oxidation processes (AOP).
5. To study the elimination, generation or TPs and the toxicity of tamoxifen, as a model anticancer drug, in the alternative wastewater processes studied.

CHAPTER 3

3. Block I: Analytical method development

3.1. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples.

L Ferrando-Climent, S Rodríguez-Mozaz, D. Barceló. 2013

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Published online: 6 March 2013

Abstract

In the present work, the development, optimization, and validation (including a whole stability study) of a fast, reliable, and comprehensive method for the analysis of ten anticancer drugs in hospital and urban wastewater is described. Extraction of these pharmaceutical compounds was performed using automated off-line solid-phase extraction followed by their determination by ultra-performance liquid chromatography coupled to a triple quadrupole–linear ion trap mass spectrometer. Target compounds include nine cytotoxic agents: cyclophosphamide, ifosfamide, docetaxel, paclitaxel, etoposide, vincristine, tamoxifen, methotrexate, and azathioprine; and the cytotoxic quinolone, ciprofloxacin. Method detection limits (MDL) ranged from 0.8 to 24 ng/L. Levels found of cytostatic agents in the hospital and wastewater influents did not differ significantly, and therefore, hospitals cannot be considered as the primary source of this type of contaminants. All the target compounds were detected in at least one of the influent samples analyzed: Ciprofloxacin, cyclophosphamide, tamoxifen, and azathioprine were found in most of them and achieving maximum levels of 14.725, 0.201, 0.133, and 0.188 μg/L, respectively. The rest of target cancer drugs were less frequently detected and at values ranging between MDL and 0.406 μg/L. Furthermore, a feasible, useful, and advantageous approach based on information acquisition tool (information-dependent acquisition) was used for the screening of human metabolites in hospital effluents, where the hydroxy tamoxifen, endoxifen, and carboxyphosphamide were detected.

Keywords: Cytotoxic. Anticancer drugs. Metabolites. Hospital effluent. UPLC-QqLit. IDA

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CHAPTER 4

4. Block II: Targeted and suspected screening of chemotherapy drugs.

4.1. Identification of markers of cancer in urban sewage through the use of a suspect screening approach

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Abstract

The administration of anticancer drugs during chemotherapy treatments has increased considerably in recent years, and based on the growing incidence of cancer worldwide there is a foreseen increase in their use over the coming years. Many anticancer drugs are not removed by conventional wastewater treatment plants (WWTPs) and can therefore reach the aquatic environment and potentially threaten aquatic life. The objective of this work was to apply a *suspect screening* methodology to detect chemotherapy and radiotherapy drugs and their related compounds such metabolites and/or biomarkers in wastewater. The use of logical pre-determined criteria to refine the suspect list down to a relatively small number of relevant compounds greatly improved the efficiency of the analysis. Mass accuracy, isotopic patterns and predicted retention time were used to tentatively identify the suspects. Successful identification of cancer-related 1suspects included two antineoplastic hormones, two X-ray contrast agents and a pyrrolizidine alkaloid related to an herbal medicine. This is the first time that a suspect screening paradigm has been successfully applied to the identification of pharmaceuticals and biomarkers related to chemotherapy in wastewater.

Keywords

Suspect screening; retrospective analysis; liquid chromatography coupled to high resolution mass spectrometry; chemotherapy drugs; metabolites; biomarkers.

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Identification of markers of cancer in urban sewage through the use of a suspect screening approach

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ABSTRACT

The administration of anticancer drugs during chemotherapy treatments has increased considerably in recent years, and based on the growing incidence of cancer worldwide there is a foreseen increase in their use over the coming years. Many anticancer drugs are not removed by conventional wastewater treatment plants (WWTPs) and can therefore reach the aquatic environment and potentially threaten aquatic life. The objective of this work was to apply a *suspect screening* methodology to detect chemotherapy and radiotherapy drugs and their related compounds such metabolites and/or biomarkers in wastewater. The use of logical pre-determined criteria to refine the suspect list down to a relatively small number of relevant compounds greatly improved the efficiency of the analysis. Mass accuracy, isotopic patterns and predicted retention time were used to tentatively identify the suspects. Successful identification of cancer-related suspects included two antineoplastic hormones, two X-ray contrast agents and a pyrrolizidine alkaloid related to an herbal medicine. This is the first time that a suspect screening paradigm has been successfully applied to the identification of pharmaceuticals and biomarkers related to chemotherapy in wastewater.

1. Introduction

Growth in the incidence of cancer in the human population has led to an increase in the use of chemotherapy drugs with a further rise in the use of these drugs foreseen in the coming years [1]. As a result, greater attention needs to be paid to the occurrence of anticancer drugs in the environment and any potential ecological consequences. Although chemotherapy drugs are typically administered in hospitals, 75% of the treatments are outpatient therapies [2] and therefore cytostatic compounds can reach the aquatic environment via hospital or domestic wastewater with wastewater treatment plants (WWTPs) being the ultimate source [3,4] It is also important to take into account that cytostatic compounds, as with many other pharmaceuticals, are excreted as both parent compound and as metabolites that may have the same mode of action as the parent cytostatic compound or potentially even greater activity. This is the case for 4-hydroxytamoxifen, a metabolite of tamoxifen, which is a more potent estrogen receptor antagonist than the parent compound [5]. Likewise, specific biomarkers produced by the body whilst suffering from cancer and during oncologic treatments, such α-fenotroin (prostate cancer), inositol (hepatocellular carcinoma), normethanephrine (hepatocellular carcinoma) [6], among other compounds, are present at higher or lower levels in urine. For instance α-fenotroin is present at concentrations of up to 50 ng/mL in the serum/urine of patients with liver cancer [6]. Since these substances are concomitant with chemotherapy drugs they have the potential to provide useful information about the prevalence of different types of cancer as well as about the chemotherapy drugs consumed in the population served by a WWTP.

The high cost of chemotherapy pharmaceutical reference standards, often based on a challenging and expensive synthesis, and especially their particular hazard to health or safety, makes conventional target analysis of these compounds difficult in most of the environmental laboratories due to the special and expensive safety conditions required for their handling (analyst training for cytotoxic handling and spills, personal protective equipment, bio-

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0731-7085/

logical safety cabinet or similar category hood, specific containers for residues, etc). Nevertheless different analytical methodologies without the use of standards have recently been developed for the screening of micropollutants in water samples as a qualitative mode of assessment. In general, these novel approaches are based on high-resolution mass spectrometry (HRMS) using time-of-flight (TOF) or Orbitrap mass spectrometers often coupled to liquid chromatography (LC) [7,8]. LC-HRMS allows the wide-scope screening of organic pollutants without the pre-selection of analytes and without using authentic reference standards [9]. Known compounds suspected of being present in environmental samples can be retrospectively screened following data acquisition [10].

In this work, a "*suspect screening*" approach was applied to wastewater samples collected from Oslo, Norway for the purposes of detecting and identifying (bio-)markers associated with prostate cancer and breast cancer.

Oslo is an ideal sampling location because Norway has a strictly controlled system for the prescription and dispensing of medications that is well-managed by the Norwegian Institute of Public Health (NIPH) through the Norwegian prescription database (NORDP) (www.nordp.no). This database provides accurate information on the use of therapeutic agents in any given region/city.

Prostate cancer and breast cancer were selected as the targets because a review of the World Health Organization databases reveals that these two cancers are the most frequently diagnosed in Norway (www.who.com).,

2. Material and Methods

2.1. Chemical reagents

LC-MS grade water and acetonitrile were supplied by Merck (Darmstadt, Germany). Reagents, such as formic acid 98% (HCOOH), were supplied by Sigma-Aldrich (HPLC-grade) and leucine encephalin and ethylenediaminetetraacetic acid disodium salt (EDTA) was supplied by Sigma-Aldrich (Germany).

2.2. Sampling campaign

The urban area selected for this study, Oslo, has a population of approximately 647,000 residents. Both VEAS and Bekkelaget WWTPs, which are located on the Oslofjord, receive the urban wastewater from the city and various surrounding municipalities. VEAS receives wastewater from different sources such as hospitals, including the region's largest hospital where most of the chemotherapy treatments are administered, and industry in addition to domestic sewage from the west of city. Bekkelaget WWTP receives wastewater from the east urban collectors including small health centres such a psychiatric clinic (Lovisenberg Diakonale Sykehus Psykiatrisk). The volume of wastewater processed by both WWTPs is estimated to be between 100 and 110 million m^3 $year^{-1}$ for VEAS and about 42 millon m^3 $year^{-1}$ for Bekkelaget. In total, 24 samples (four-hourly time-proportional composite samples of 1 L, providing a total of six samples per day over a period of four weekdays) were collected from the wastewater influent of both WWTPs. All the samples were collected in amber glass bottles that were pre-rinsed with Milli-Q water and were analysed immediately after the sampling.

2.3. Sample pre-treatment

The analytical methodology previously developed by Ferrando-Climent et al. was adapted for sample pre-treatment [3]. Sample pre-concentration was performed using solid disk sorbents in an automated system (SP-DESK® 4790, was provided by Horizont Technologies (New Hampshire, U.S.). This system allows the use of high flow rates during sample loading allowing increased sensitivity since the volume loaded is higher than that of a conventional SPE cartridge.

EDTA was added to the sample to obtain a final concentration of 0.1% (g solute g^{-1} solution), as it is well known that it improves the extraction of certain organic compounds that may be bound to residual metals [3,11–13]. Each sample (800 mL) was loaded by the automatic system (60 mL·min^{-1}) onto a hydrophilic-lipophilic balance (HLB) medium size disk (Atlantic ® SPE disk 47 mm and 500 mg of sorbent) with a glass fibre disk pre-filter just placed onto the extraction disk (Atlantic pre-filter ® fast flow, 50 mm). Both disks were first conditioned using 6 mL of methanol (2 × 3 mL) and followed by 3 mL 0.1% formic acid solution (2 × 3 mL). Elution was performed using 15 mL (3 × 5 mL) of pure methanol. The extract was evaporated to dryness under gentle nitrogen stream and reconstituted with 1 mL of methanol-water (10:90, v/v). Samples were subsequently filtered through 0.45 μm PVFD filter (Sartorius) before injection. Note however that in order to recover any non-polar molecules that may have adsorbed to the filter, 1 mL of methanol was passed through the filter after the sample filtration. Both sample extracts, aqueous and organic, were collected for analysis.

There is a common limitation in the methods based on non-target analysis: the estimation of the limit of detection, recovery or ionization parameters are not possible to assess since the analytical methodology is based on the screening of suspected molecules and (frequently) no reference standards are available.

2.4. UPLC-QTOF Method

Analysis was performed using ultra-performance liquid chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, coupled to a quadrupole-time of flight mass spectrometer (Xevo-G2 S QTOF, Waters Corp., Mildford, MA, USA). Chromatographic separation was performed using an Acquity BEH C18 column (150 mm x 2,1 mm i.d. 1,7 μm particle size; Waters Corp. Mildford, MA, USA) where the separation was performed in 15 minutes using a binary mobile phase of formic acid 0.1% (solvent A) and acetonitrile (solvent B) at 0.4 mL/min with a standardised gradient for blind screenings [14]. The gradient elution starts with 87% A and then increasing B to 95% in 15 min: Solvent A, held for 0.5 min; 0.5–10 linear rate to 50% B, 10–10.75 linear rate to 95% B, held for 0.5 min; reconditioning with a linear rate to 87% A, 12.50–15 min. 10 μL of extracted samples (both organic and aqueous extracts) were injected in the system.

The MS analysis was performed using positive electrospray ionization (ESI +) over the range of 50 − 800 Da with a scan time of 0.2 s and an inter-scan delay of 0.05 s. Two simultaneous acquisition modes with different collision energies were used for the MS experiments: (i) a low energy mode (LE) where collision energy of 4 eV was selected for detection of molecular ions [M-H]+, and (ii) a high energy (HE) mode with a collision energy ramp from 15 eV to 40 eV for detection of fragment ions.

2.5. Suspect list

The suspect list was based on two cancer types, prostate and breast since they are the most frequently diagnosed in Norway (www.who.com). The chemotherapy drugs associated with the treatment of these cancers, together with a large group of pharmaceuticals/therapies such mitotic inhibitors, anti-metabolites, hormones and immunotherapeutic agents were identified with the NORDP databases. Following the selection of specific therapies (> 1420 compounds), only the pharmaceuticals used during 2013 in Oslo for both males and females that were prescribed at sufficiently

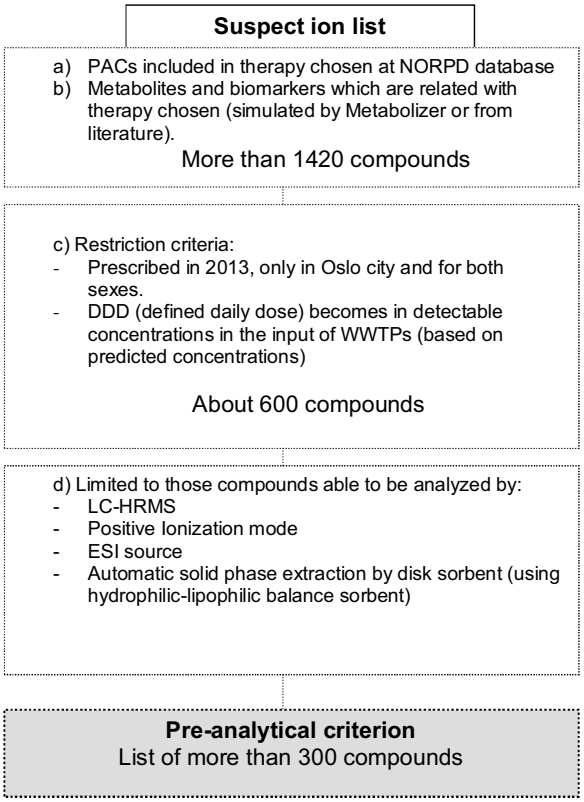


Fig. 1. Diagram for the generation of a list of compounds suspected to be present in the input of WWTPs.

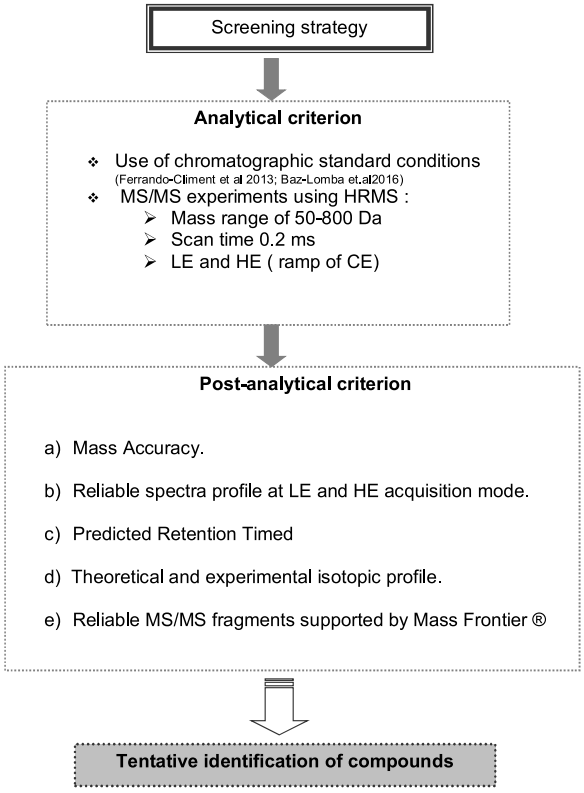


Fig. 2. Strategy diagram of MS/MS screening for known-unknown compounds.

high doses were selected for post-target screening. The latter criteria takes into account the defined daily dose (DDD) and the dilution factor in the sewage in order to assure that the appropriate compound is likely to be detectable in the influent of the WWPT (based on Predicted Concentration). Human metabolites derived from pharmaceuticals, as well as biomarkers related with prostate and breast cancer, were also selected (based on Metabolizer JChem software and literature sources) and included in the suspect list. In total, 600 compounds, including pharmaceuticals, xenobiotic metabolites and biomarkers, were selected as suitable targets for the suspect list [15–27] (www.drugbank.ca; www.rxlist.com).

Predicted concentrations (PEC) were calculated based on: a) the defined-daily-dose (DDD) (mg/day) of each pharmaceutical (from WHO databases), b) the turnover by dosage (TD; number of total doses per year) prescribed by NIPH (from NORDP databases) and c) the dilution factor at the entrance of WWTP ($3.024 \cdot 10^8$ L per day, data provided by wastewater treatment company) following the equation 1.

$$PEC = \frac{DDD(mg/dosage/day) \times TD(dosage)}{365(days)} \times \frac{1}{3.024 \cdot 10^8 (L/day)} = (mg/L/day) = 10^6 (ng/L/day) \quad (1)$$

The suspect list was further refined to account for limitations in the analytical methodology. Only those compounds that fall within specific analytical conditions were included in the list. These limitations were: i) the analyte should be compatible with LC, ii) the analyte should be ionizable with electrospray (ESI) in positive mode [3], iii) the analyte should be a small-molecule in the mass-range 100–800 Da, rather than a structure like a protein (such immunotherapy or new generation of drugs) which necessitate other analytical requirements. These final limitations reduced the suspect list to about 300 compounds (Fig. 1). Finally some herbal medicines which are not registered at NORDP data bases were added to the suspect list. Particularly two natural products associated to cancer disease as non-regulated palliative treatment were included: Viridiflorine and Cannabis. These substances are commonly used as alternative medicines for population from subtropical regions such as Bandar-e Anzali in Iran or Hanoi in Vietnam. Immigrant population in Oslo city pose a 30.4% of the total population, being the biggest groups of immigrant people from: Pakistan, Sweden, Somalia, Poland, Sri Lanka, Vietnam, Turkey, Morocco, Iraq and Iran.

Because of the large number of compounds, the authors has avoided to include all the names of these suspected molecules, however it can be extracted from webpage www.norpd.no (see an example on supplementary material Fig. S2)

2.6. Post-analytical criterion

The compounds included in the suspect list were screened for in the wastewater influent samples using the following approach (Fig. 2). The monoisotopic mass was calculated (as the [M-H]+ ion) for each compound in the "suspected ion list", and the appropriate chromatogram extracted from the LE trace from all samples. All chromatographic peaks were then scrutinised according to the criteria below.

2.6.1. Mass Accuracy

A tolerance of 5 ppm was used to test all detected ions. The calculation of the accuracy (ppm) was done using Equation 2, where M_{theo} is the theoretical mass and M_{exp} is the experimental mass:

$$\Delta m(ppm) = . \frac{M_{exp} - M_{theo}}{M_{theo}} \cdot 10^6 \quad (1)$$

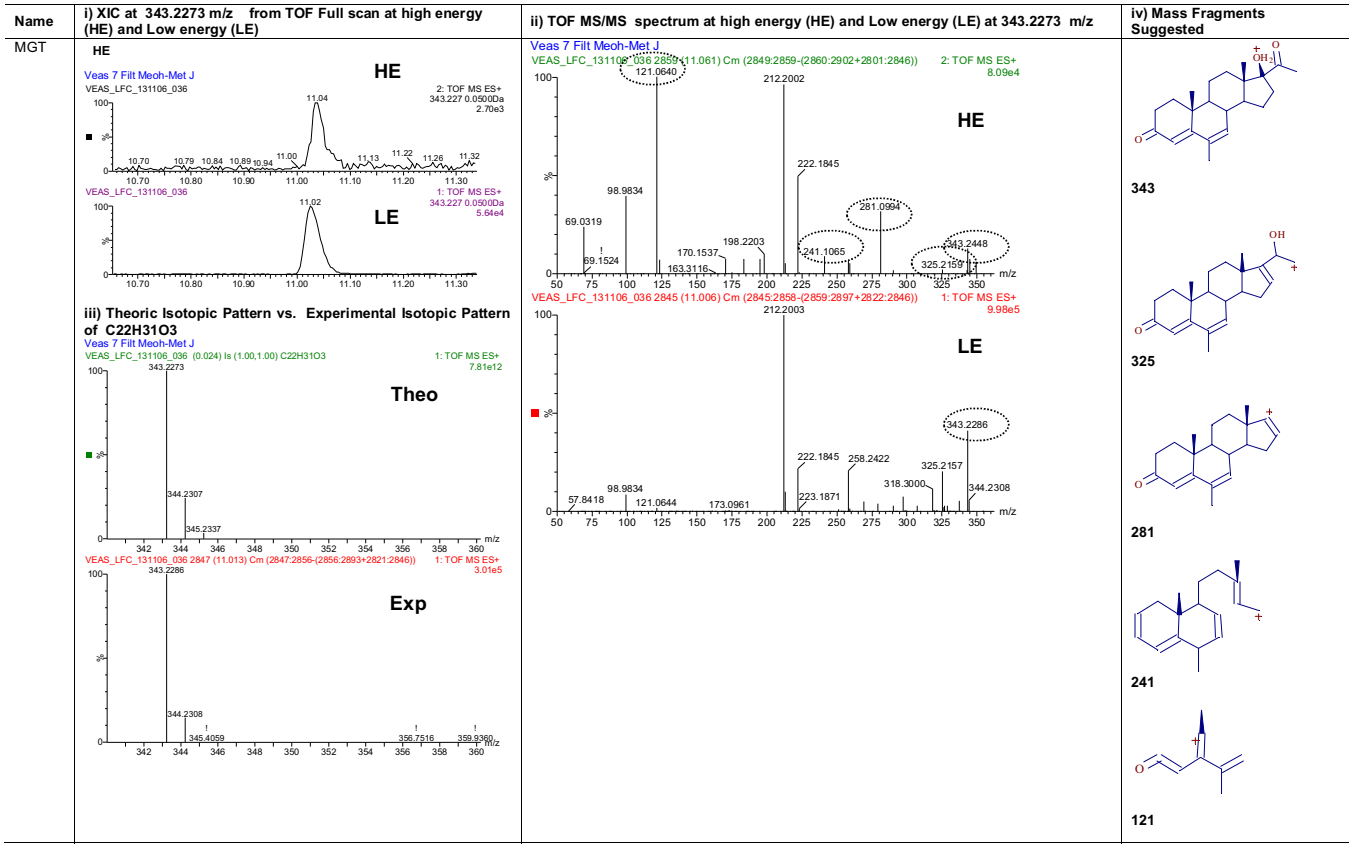


Fig. 3. Mass spectrometry criterion for Megestrol identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier.

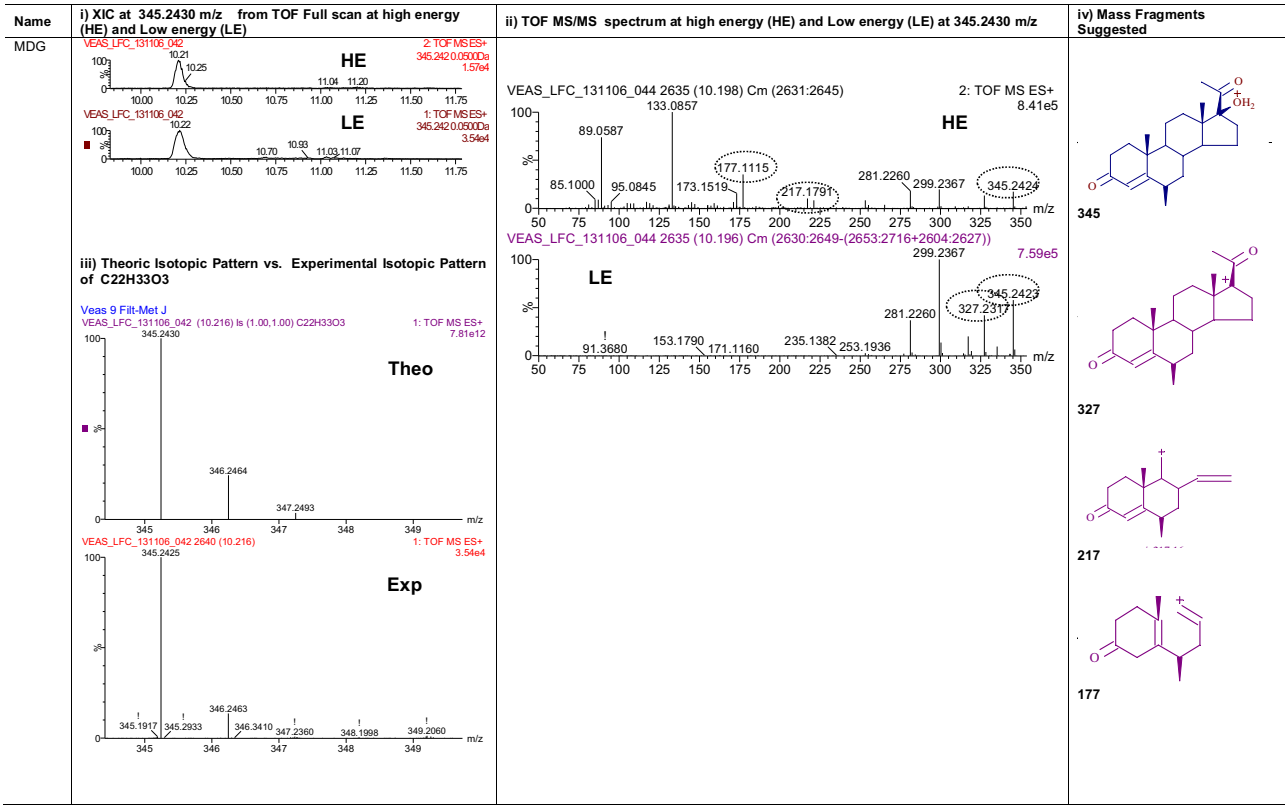


Fig. 4. Mass spectrometric criterion for Medroxyprogesterone identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier.

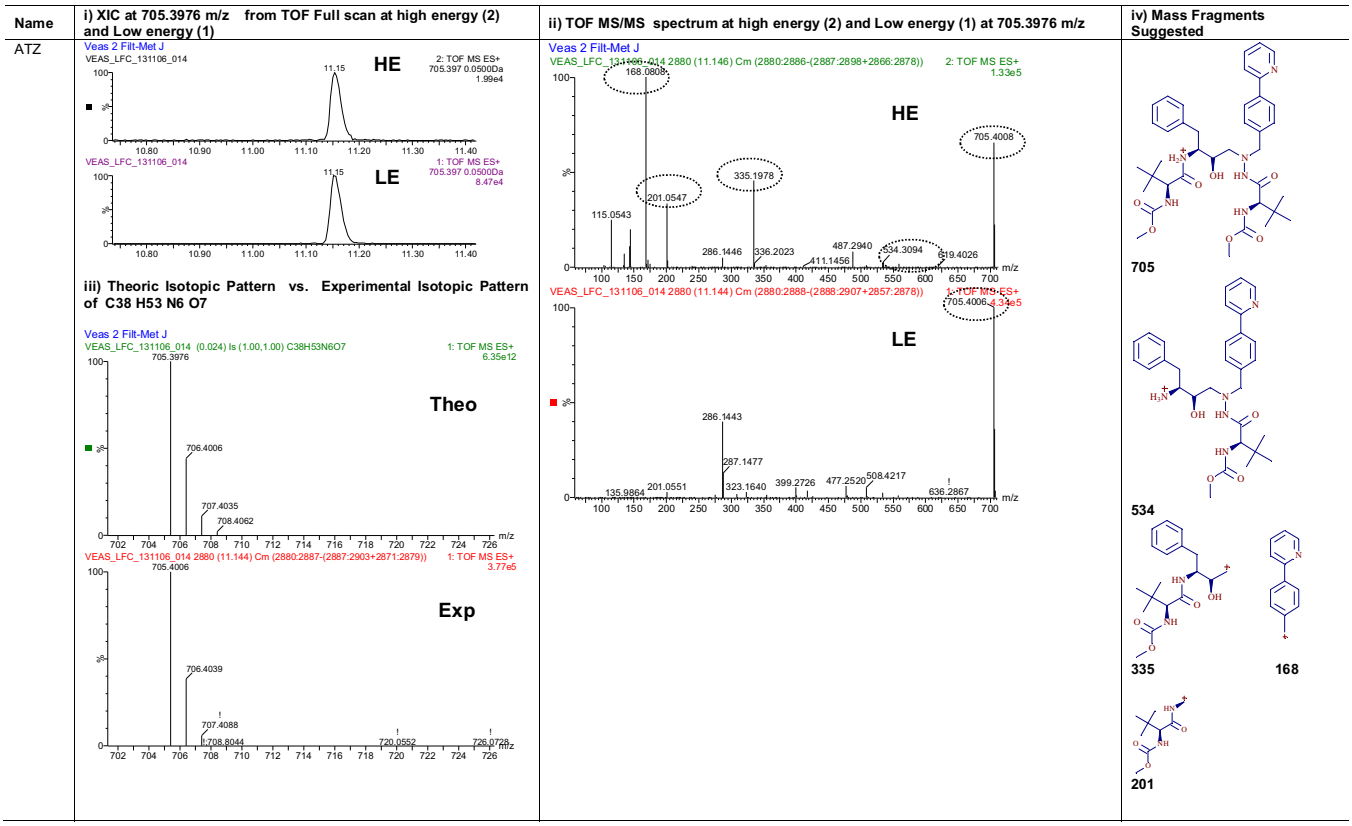


Fig. 5. Mass spectrometric criterion for Atazanavir identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier.

2.6.2. Reliable spectra profile in both LE and HE acquisition modes.

The spectra corresponding to the chromatographic peaks in both the LE and HE traces were investigated to ensure that data was of sufficient quality to aid identification. It is expected, for example, that the signal intensity of the proposed molecular ion [M-H]+ should be lower in the HE trace because of fragmentation. Higher signal intensities in the HE trace relative to the LE trace are indicative of fragmentation, and it may therefore be concluded that the ion is actually a fragment of a larger ion and not the proposed molecular ion [M-H]+.

Retention time and chromatographic peak shape for each ion in the HE spectra were compared with that of the molecular ion [M-H]+ in the LE trace. All HE ions with retention time and peak shape that precisely matched that of the molecular ion are considered as potential fragments of the molecular ion which can later be used for structural elucidation/confirmation (below).

2.6.3. Predicted Retention Time

The chromatographic retention time (RT) of the suspected ion precursor was then checked against the calculated LogD for this compound estimated by Marvin Sketch software taking to account the pH of the chromatographic medium (pH = 3). This approach was proven by Kern et al., 2009 and Ferrando-Climent et al., 2013 for the screening of organic contaminants (further information can be found in both works) [3,28]. The model built by Ferrando-Climent et al., 2013 has been used here. It is expected that polar substances elute earlier than non-polar substances. The retention time of polar substances therefore had to be close to the first part of the chromatogram (form 0 until 5–6 minutes when the aqueous phase is high), whereas for non-polar molecules the RT has to be in the latter half of the chromatogram (from 6–7 minutes when the organic phase is being increased). Only chromatographic peaks lying within the expected retention time windows were considered for further investigation.

2.6.4. Theoretical and experimental isotopic profile.

The isotopic pattern of the molecular ion and fragments were then compared against the theoretical isotopic profile for a given molecular formula (obtained using the simulation tool of Waters' MassLynx software). The matching criteria include the number of isotopes, the mass accuracy of the isotopes and relative abundance of the isotopes. Only those suspects with isotopic patterns that matched the model were considered for further investigation.

2.6.5. Reliable MS/MS fragments supported by Mass Frontier ®

The fragmentation profile obtained from the high-energy spectra aids in the structural elucidation/confirmation of the suspect ion. HE fragments identified in b) (above) were then checked against those predicted by Mass Frontier (HighChem software) which enables theoretical generation of mass fragments based on a proposed chemical structure. Only suspects showing fragmentation patterns that were supported by the software model and/or from previous literature were considered for further investigation.

3. Results

3.1. Data processing and evaluation

Eight substances that are directly linked with the chemotherapeutic treatment of prostate and breast cancer were tentatively identified (Table 1).

Two antineoplasic hormones, megestrol (MGT) and medroxyprogesterone (MDG), were identified in 3 and 7 samples

Table 1
Molecules tentatively identified using the post-analytical criterion: molecular formula proposed, chemical structure, accurate mass (theoretic and experimental), accuracy (in ppm), mass fragments, LogD, Rt (min), Predicted concentration (PEC) (ng/L). (n.a = not available).

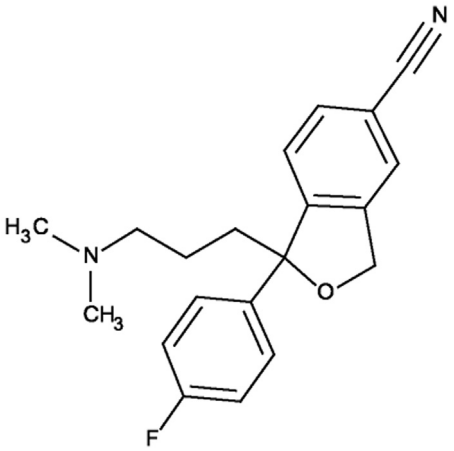
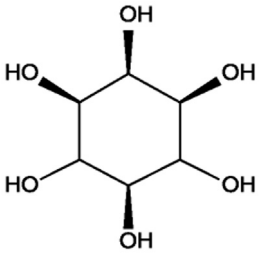
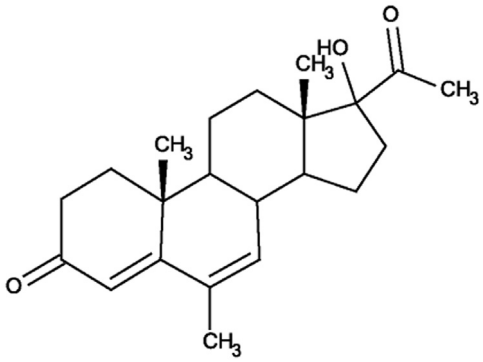
Name	Type of compound	Molecular Formula proposed	CAS	Chemical structure	Experimental ion precursor [MH^+]	Theoric ion precursor [MH^+]	Accuracy (ppm)	Molecular Fragments (Mass Frontier 7.0)	LogD	Rt (min)	Sample extract	PEC (ng/L)
Citalopram (CTM)	Antidepressant drug	C20 H21N2OF	59729-33-8		325.1712	325.1716	−1,2	325-262325-109	3.76	6.88	Organic	138.2
Inositol (INO)	Liver tumor Biomarker & Cutting agent of illicit drugs	C6 H12 O6	87-89-8		181.0715	181.0712	1,6	181-163 181-89	−2.11	1.19	Aqueous	n.a.
Megestrol (MGT)	Antineoplasic & Hormone	C22H30O3	3562-63-8		343.2286	343.2273	3,8	343-325, 343-281 343-241 343-121	3.22	11.02	Organic	2.58

Table 1 (*Continued*)

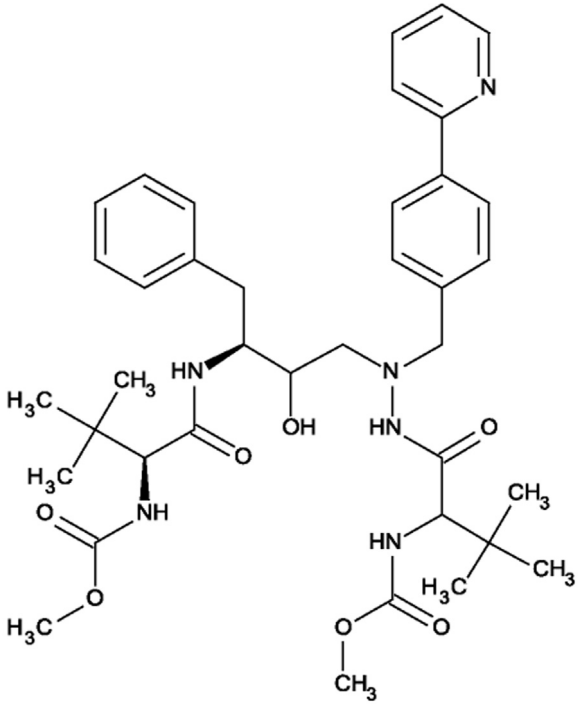
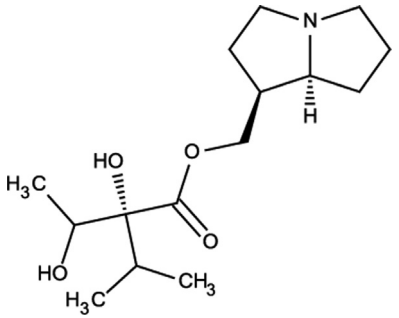
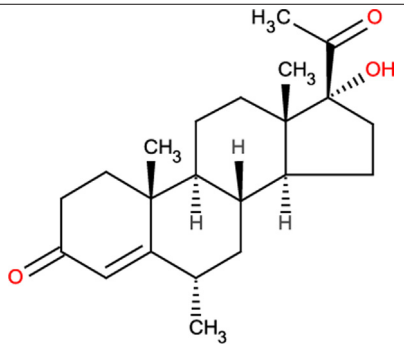
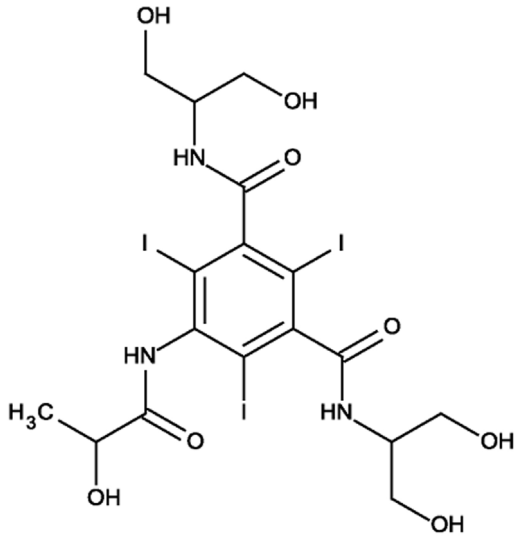
Name	Type of compound	Molecular Formula proposed	CAS	Chemical structure	Experimental ion precursor [MH^+]	Theoric ion precursor [MH^+]	Accuracy (ppm)	Molecular Fragments (Mass Frontier 7.0)	LogD	Rt (min)	Sample extract	PEC (ng/L)
Atazanavir (ATZ)	Antiviral & antitumor agent	C38 H52 N6 O7	198904-31-3		705.4006	705.3976	4,2	705-534 705-335 705-201 705-168	4.54	11.15	Aqueous& Organic	510.1
Viridiflorine (VRD)	Pyrrolizidine Alkaloid – Phytophar-mac.	C15 H27 N O4	n.a.		286.2004	286.2018	−4.9	286-85	−2.59	5.46	Aqueous	n.a.

Table 1 (*Continued*)

Name	Type of compound	Molecular Formula proposed	CAS	Chemical structure	Experimental ion precursor [MH^+]	Theoric ion precursor [MH^+]	Accuracy (ppm)	Molecular Fragments (Mass Frontier 7.0)	LogD	Rt (min)	Sample extract	PEC (ng/L)
Medroxypro-gesterone (MDG)	Hormone therapy	C22H32O3	520-85-4		345.2423	345.2430	−2.0	327-217 327-177	3.69	10.21	Aqueous& Organic	6.85
Iomeprol & Iopamidol (IOM& IOP) (isomers)	X Ray contrast (neuroradiology including myelography such lumbar, thoracic, cervical, etc)	C17H22I3N3O8	78649-41-9 & 62883-00-5		777.8625	777.8619	0.7	777-686 777-558 777-405	−0.74	1.05	Aqueous	n.a.

respectively (Table 1, Fig. 3 and 4 respectively). One antiviral and antitumor agent, atazanavir (ATZ) [29,30], was identified in 7 samples (Table 1 and Fig. 5). One liver tumour biomarker, inositol (INO), was identified in 4 samples (Table 1 and Supplementary S3) although it should be noted that this compound is not specific to liver cancer as it is also used as cutting agent for illicit drugs [6]. However the authors has taken in consideration this finding since it was found only at input samples from VEAS WWTP which receive the effluent from the biggest hospital of the city, Rikshospitalet. Two X-ray contrast agents, iomeprol (IOM) and iopamidol (IOP), were identified in 10 samples. The identification of these two compounds is complicated by the fact that they are positional isomers and therefore have the same monoisotopic mass for the ion precursor. The process is further complicated because the chromatographic peaks are unresolved. Tentatively identification of both compounds is possible however because their mass fragmentation pathways differ and they produce unique characteristic fragments (IOM: 777-686, 777-405 and IOP: 777-559). Note that fragments suggested by both MassFrontier and those fragments found experimentally by other authors [31,32] were taken into account for the identification (Table 1 and supplementary S4).

In addition, one antidepressant drug, Citalopram (CTM), was identified in 4 samples analysed (Table 1 and supplementary S5). This drug is often used as antidepressant in ambulatory cancer patients [33]. The pyrrolizidine alkaloid viridiflorine (VRD) was also identified in 3 samples (Table 1 and supplementary S6). To the author's knowledge, this is the first time that this compound is reported in wastewater. Interestingly this phytopharmac is used as a traditional medicine in tropical and subtropical countries for the treatment of a large number of conditions and is described as having antioxidant, antimicrobial, anticancer, antiplas-modial, anti-inflammatory and larvicidal properties (among others) [34,35]. Products containing this active compound are commercially available in Norway (http://www.rolv.no).

Human metabolites, paying special attention to those related with parent compound tentatively identified, were subsequently screened for in wastewater samples, but were not detected in any of the samples (supplementary material S1 shows information about these metabolites).

The pharmaceuticals that are most often dispensed in hospitals (alone), such as X-ray contrast agents and antineoplastic hormones, were exclusively detected at in the one WTTP (VEAS) that process wastewater from the region's largest hospital. Other more widely dispensed pharmaceuticals were detected in samples from both WWTPs.

It is important to note that only those pharmaceutical compounds with a predicted concentration (PEC) in the ng/L–μg/L range were identified. It is expected that a large number of additional compounds would be present in the wastewater, but at lower concentrations. The identification of these compounds is challenging in municipal wastewater due to the dilution of micropollutants in the sewage system.

4. Conclusions

This is the first time that a suspect screening paradigm has been applied to the identification of pharmaceuticals and biomarkers related to chemotherapy in wastewater.

A list of suspect ions (> 1420 compounds) was generated from a review of the chemotherapeutic agents, mitotic inhibitors, antimetabolites, hormones and immunotherapeutic agents identified within the NORDP databases. Dispensing rates and limitations in the analytical technique reduced this suspect list to approximately 300 compounds. Mass accuracy, isotopic patterns and predicted retention times based on LogD were then used to confirm the tentative identification of 8 suspects in samples of wastewater from Oslo. This analytical strategy has demonstrated to be a successful, fast and an economically advantageous tool for the screening of micropollutants in wastewater. The use of logical pre-determined criteria to refine the suspect list down to a relatively small number of compounds (as described here) provides an efficient workflow where resources can be focused on the identification/quantification of compounds that are most likely to be found.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.08.001.

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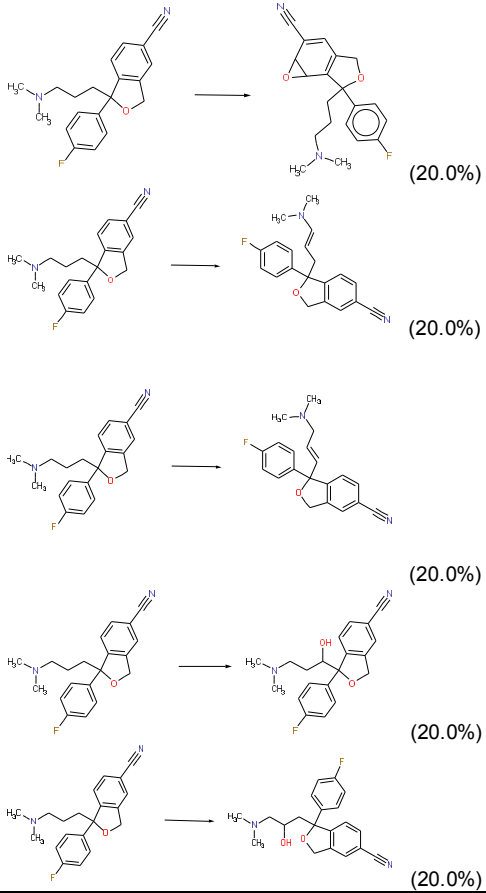
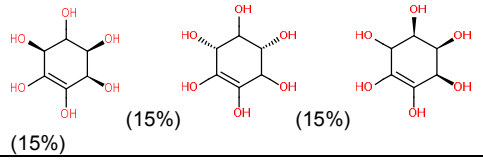
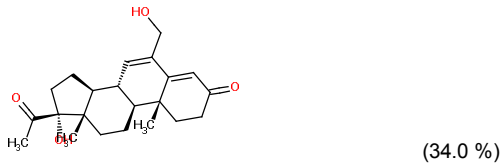
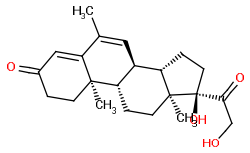
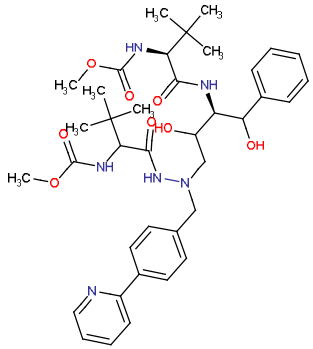
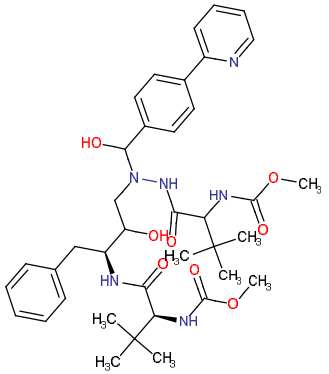
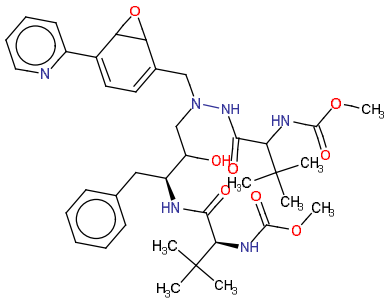
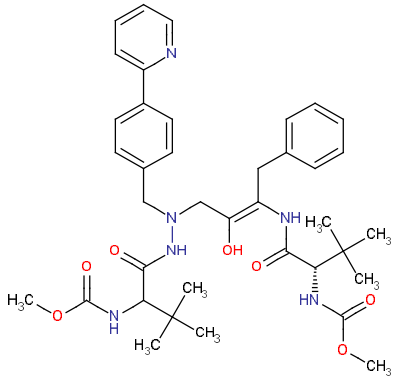
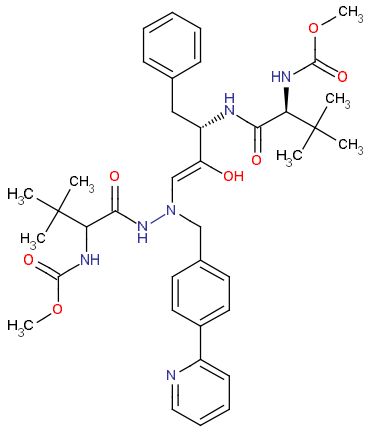
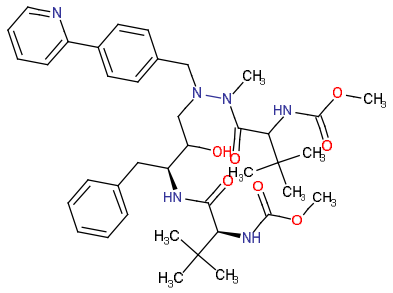
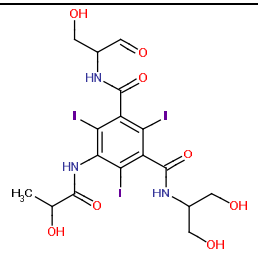
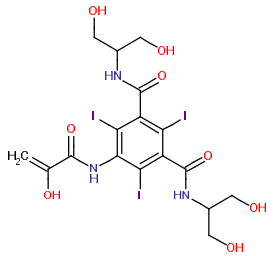
www.who.com.
www.nordp.no.
www.drugbank.ca.
www.rxlist.com.

S1. Data shown the human metabolites from those compounds tentatively identified which were post-screened in the samples analyzed.

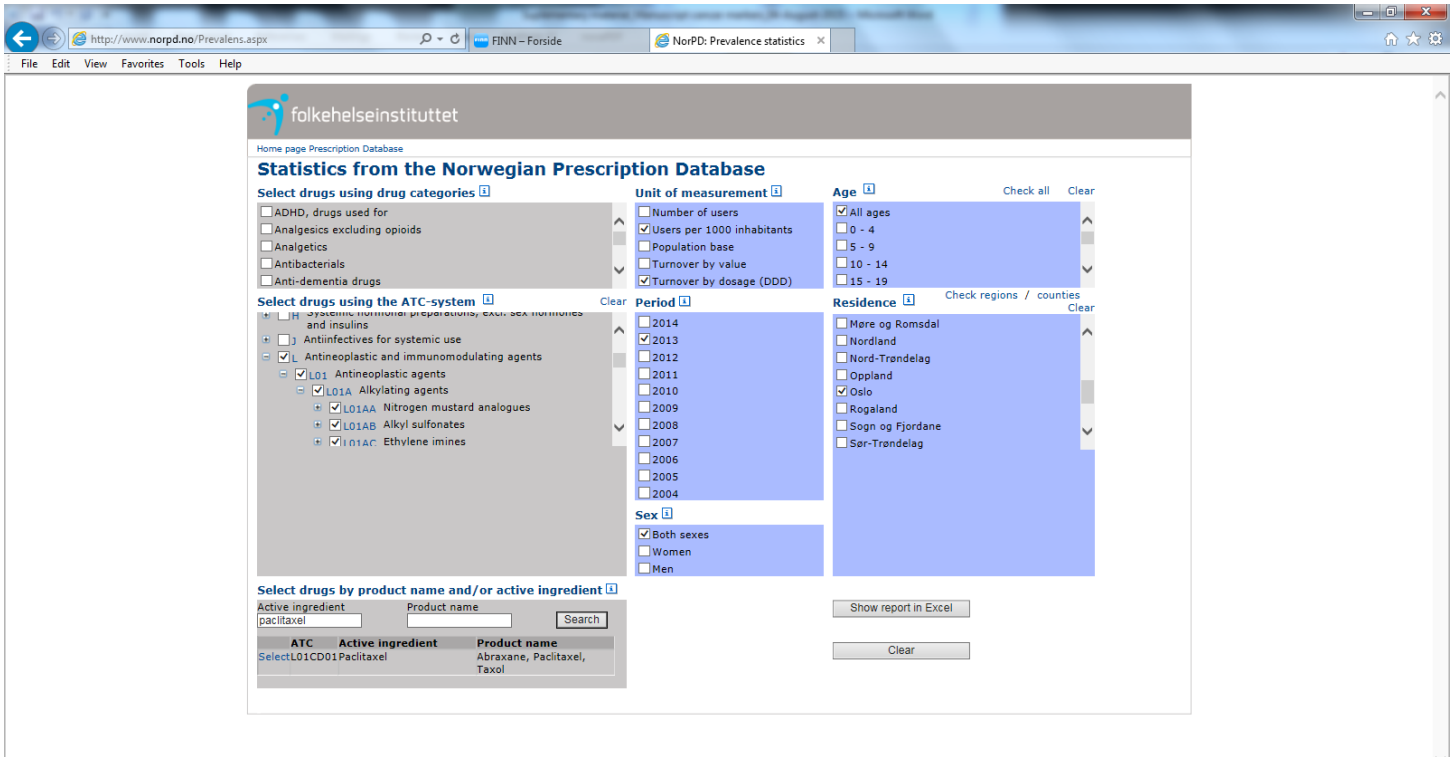
Name	Type of compound	Human Metabolism from Literature	Simulated metabolites by Metabolizer JChem (% of formation probability)	Metabolites detected (YES/NO)	Literature
Citalopram (CTM)	Antidepressant drug	Desmethylcitalopram Didesmethylcitalopram		NO	Herrlin et al. 2003
Inositol (INO)	Liver tumor Biomarker & Cutting agent of illicit drugs	unknown		NO	n.a.
Megestrol (MGT)	Antineoplasic & Hormone	Megestrol metabolites which were identified in urine constituted 5% to 8% of the dose administered: 17α-acetoxy-11β-hydroxy-6-methyl-pregna-4,6-diene-3,20-dione 17,18-dihydroxy-6-methylpregna-4,6-diene-3,20-dione		NO	www.drugbank.ca Cooke and Vallance 1968

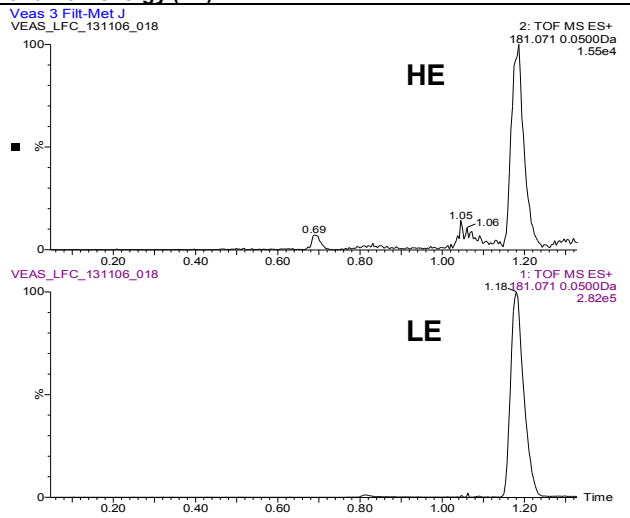
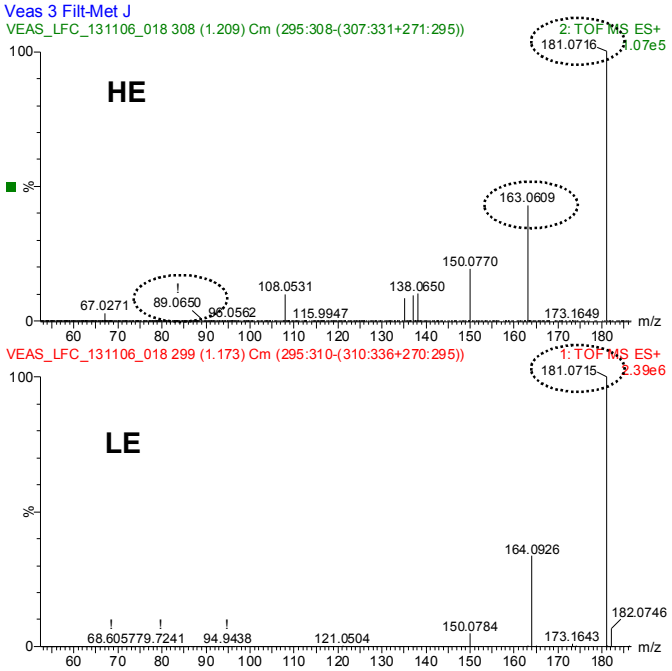
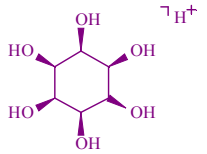
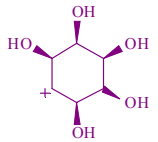
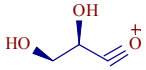
		LOW METABOLISM	 (34.0 %)		
Atazanavir (ATZ)	Antiviral & antitumor agent	N-dealkylation product (M1) two metabolites resulting from carbamate hydrolysis (M2 and M3) ydroxylated product (M4) keto-metabolite(M5)	 (43.0%)  (43.0%)  (4.3 %)	NO	Heine et al 2009

			 (4.3 %)  (4.3%)  (4.3%)		

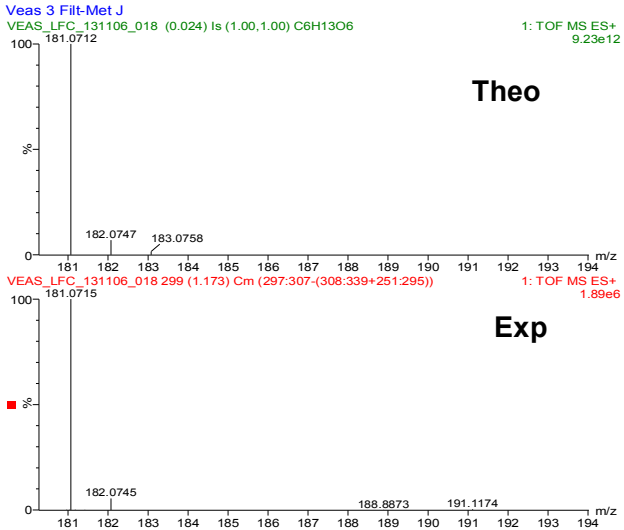
Iomeprol & Iopamidol (positional isomers) (IOM & IOP)	X Ray contrast	Between 72 and 85% of injected Iopamidol is excreted within 72h of injection No significant metabolism, deiodination, or biotransformation occurs. Iomeprol is not metabolized and did not bind to plasma proteins. LOW METABOLISM	 (90.0%)  (9%)	NO	Pitré et al. 1983 Rossati 1994 www.rxlist.com

S2.Screenshot from norweguian prescription database (NORPD) webpage.

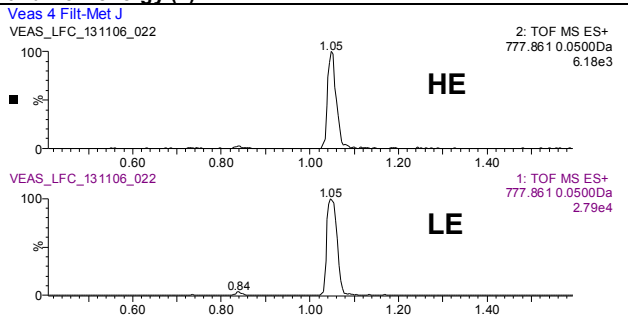
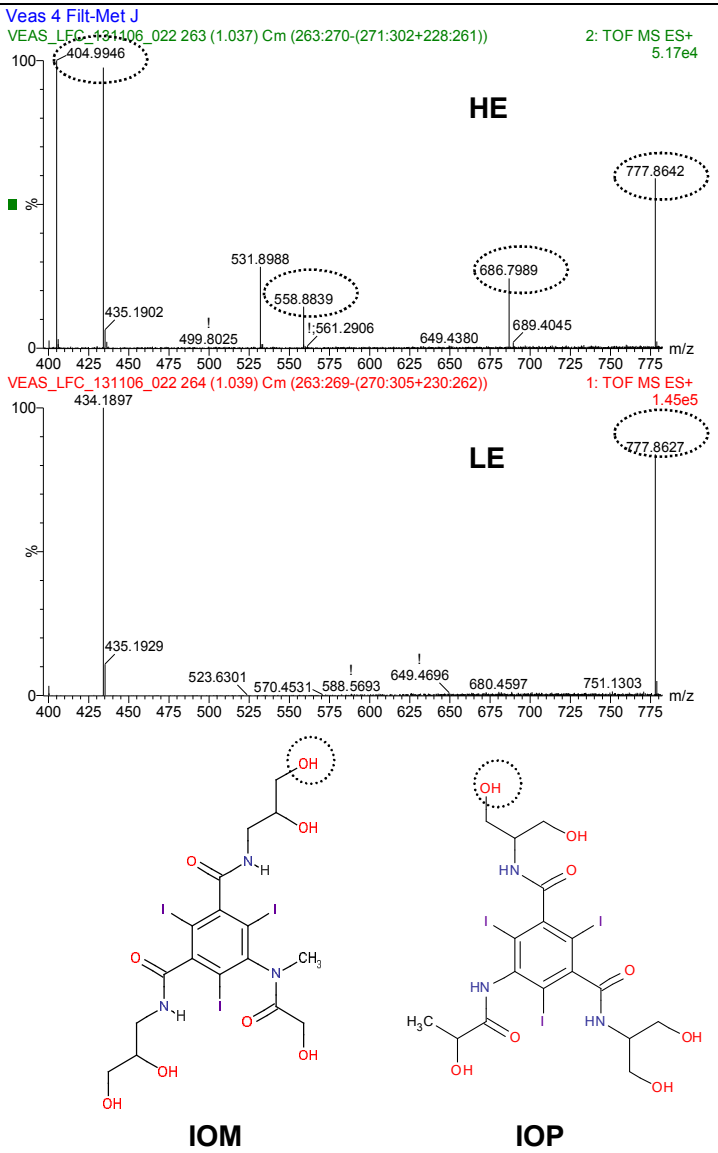
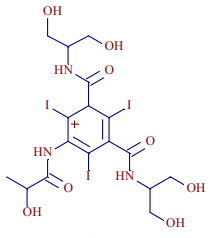
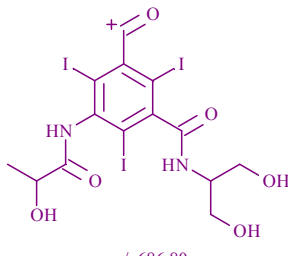
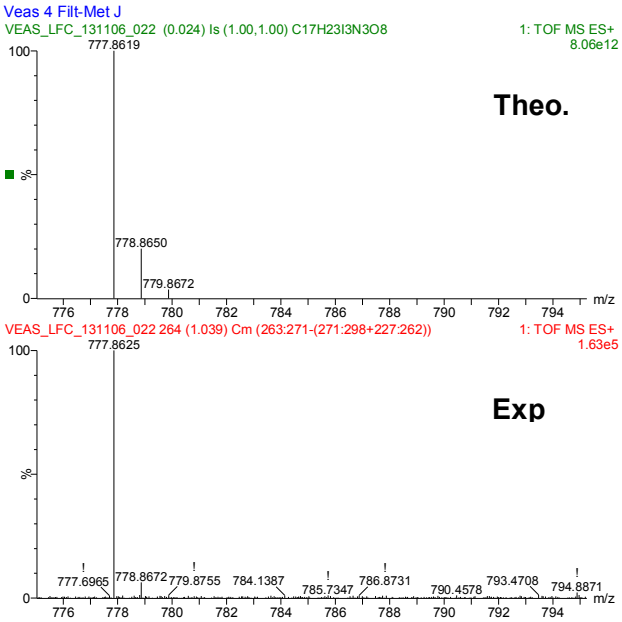


Name	i) XIC at 181.0712 m/z from TOF Full scan at high energy (HE) and Low energy (LE)	ii) TOF MS/MS spectrum at high energy (HE) and Low energy (LE) at 181.0712 m/z	iv) Mass Fragments Suggested
INO			 181  163  89

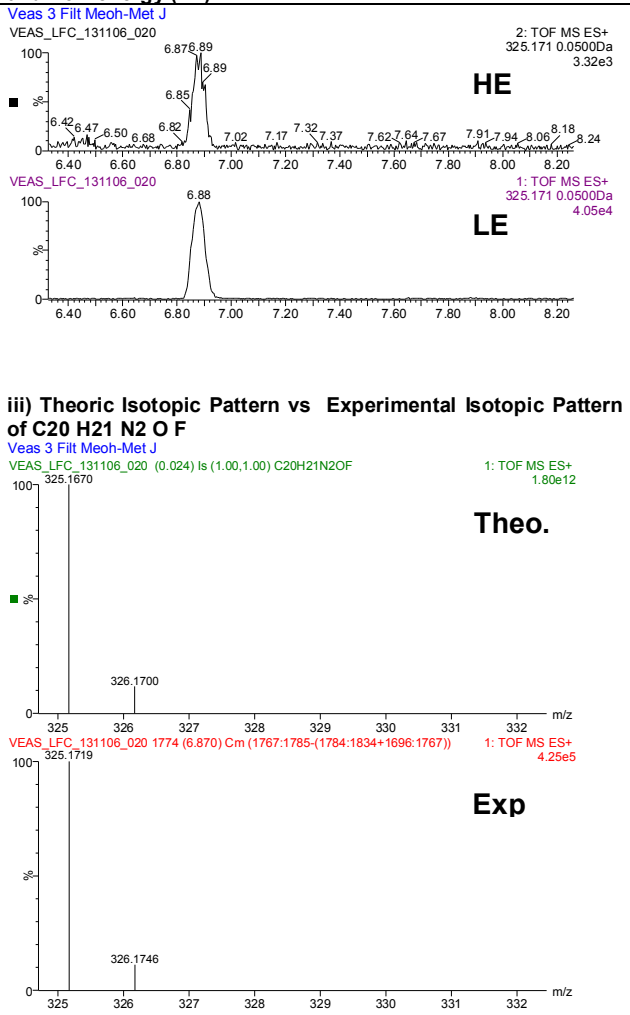
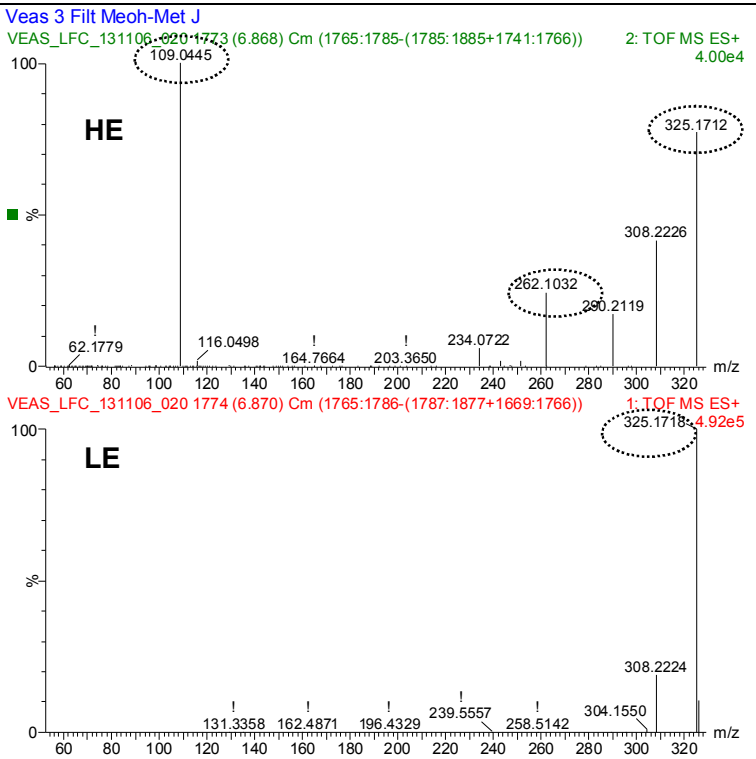
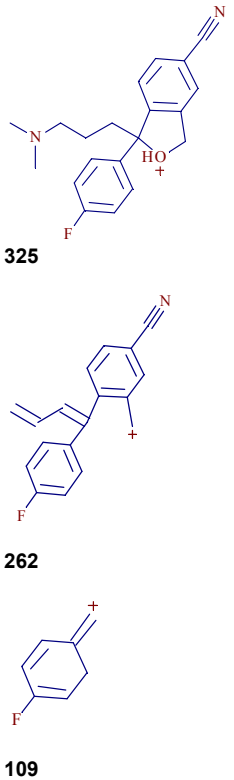
iii)Theoric Isotopic Pattern vs Experimental Isotopic Pattern of C6 H13 O6



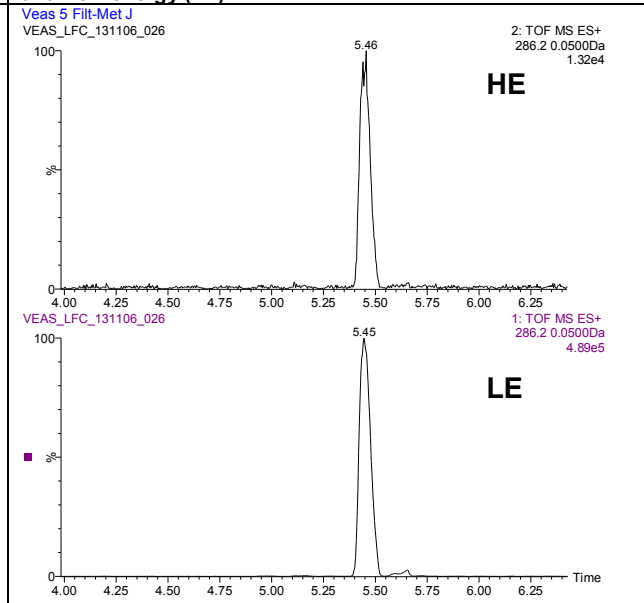
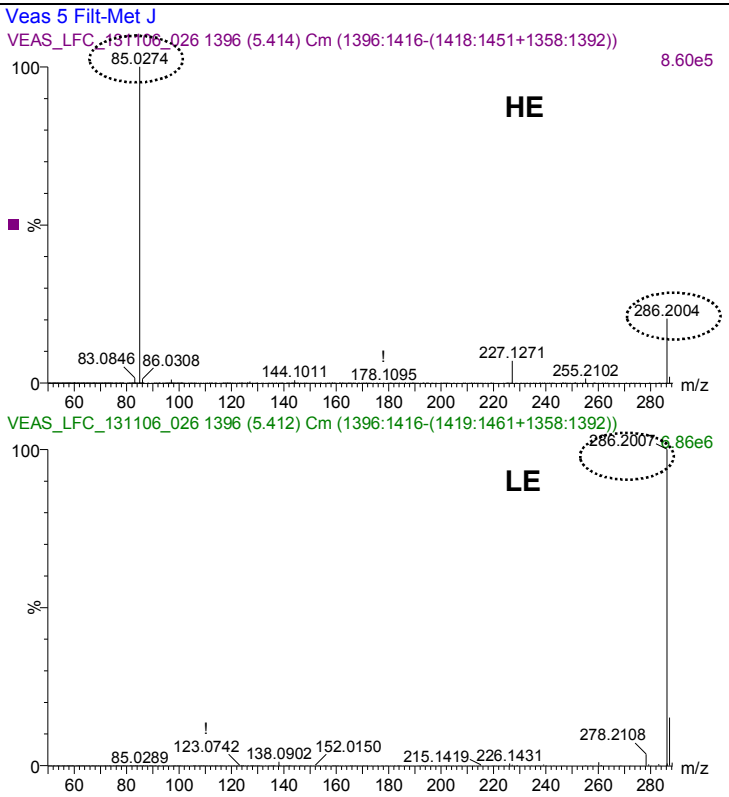
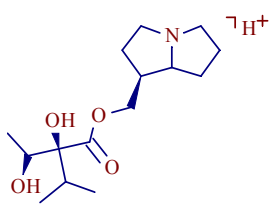
S3. Mass spectrometric criterion for Inositol identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier

Name	i) XIC at 777.8619 m/z from TOF Full scan at high energy (2) and Low energy (1)	ii) TOF MS/MS spectrum at high energy (2) and Low energy (1) at 777.8619 m/z	iv) Mass Fragments Suggested
IOM & IOP			 **777**  **686** **Fragments from literature:** **559 (Wittrig et al. 2010 ABSciex reports)** **405 (Ternes et al 2005)** **Transitions IOM:** **777- 686** **777- 405** **Transitions IOP:** **777- 559**
	iii) **Theoric Isotopic Pattern vs. Experimental Isotopic Pattern of C17H23I3N3O8** 		

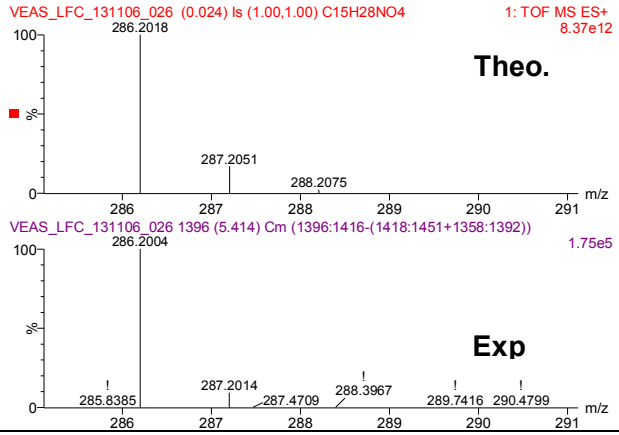
S4. Mass spectrometric criterion for Iomeprol and Iopamidol identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier

Name	i) XIC at 325.1716 m/z from TOF Full scan at high energy (HE) and Low energy (LE) iii) Theoric Isotopic Pattern vs Experimental Isotopic Pattern of C20 H21 N2 O F	ii) TOF MS/MS at high energy (HE) and Low energy (LE) at 325.1716 m/z	iv) Mass Fragments Suggested
CTM			

S5. Mass spectrometric criterion for Citalopram identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier

Name	i) XIC at 286.2018 m/z from TOF Full scan at high energy (HE) and Low energy (LE)	ii) TOF MS/MS at high energy (HE) and Low energy (LE) at 286.2018 m/z	iv) Mass Fragments Suggested
VRD			 **286**  **85**

iii) Theoric Isotopic Pattern vs Experimental Isotopic Pattern of C15H28NO4



S6. Mass spectrometric criterion for Viridiflorine identification: i) Peak mass detection by accurate mass, ii) spectra profile at LE and HE acquisition mode, iii) theoretical and experimental isotopic profile and iv) MS/MS fragments supported by Mass Frontier

4.2. Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment

L Ferrando-Climent, S Rodríguez-Mozaz, D. Barceló.

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Abstract

The presence of 10 anticancer drugs was studied along the entire urban water cycle -from hospital effluents through urban wastewater treatment plant till surface waters- and their potential environmental risk was assessed. Azathioprine, etoposide, docetaxel, paclitaxel, methotrexate, cyclophosphamide, tamoxifen and ciprofloxacin were detected in hospital effluent and in the urban influent of the sewage treatment plant although most of them were totally eliminated after WWTP. Only cyclophosphamide, tamoxifen and ciprofloxacin were found in both WWTP effluent and in the receiving river at a concentration range between nd-20 ng L 1, 25e38 ng L 1 and 7e103 ng L 1 respectively. Tamoxifen and ciprofloxacin, commonly used for veterinary practices, were also detected in the river upstream the sewage discharge. In addition, they both were considered to pose a potential risk to the environment based on the levels found in the WWTP effluent together with their ecotoxicological impact in selected organisms.

Keywords: Cytotoxic. Anticancer drugs. Hospital effluent. Wastewater. Surface water. UPLC-QqLiT

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Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment

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ABSTRACT

The presence of 10 anticancer drugs was studied along the entire urban water cycle -from hospital effluents through urban wastewater treatment plant till surface waters- and their potential environmental risk was assessed. Azathioprine, etoposide, docetaxel, paclitaxel, methotrexate, cyclophosphamide, tamoxifen and ciprofloxacin were detected in hospital effluent and in the urban influent of the sewage treatment plant although most of them were totally eliminated after WWTP. Only cyclophosphamide, tamoxifen and ciprofloxacin were found in both WWTP effluent and in the receiving river at a concentration range between nd-20 ng L^{-1}, 25–38 ng L^{-1} and 7–103 ng L^{-1} respectively. Tamoxifen and ciprofloxacin, commonly used for veterinary practices, were also detected in the river upstream the sewage discharge. In addition, they both were considered to pose a potential risk to the environment based on the levels found in the WWTP effluent together with their ecotoxicological impact in selected organisms.

1. Introduction

Cancer is one of the most concerning diseases in western countries and quite a lot of resources are devoted to investigate its treatment and eventually its cure. Despite the chemotherapeutic treatments have been improved in the last decades and are now more effective and patient specific, cancer is still one of the most harmful diseases worldwide. World Health Organization (WHO) has recently published a world health report where cancer is ranked as the second cause of death (21%) after cardiovascular illness (48%) and followed by respiratory diseases (12%) in the sector of non-communicable diseases; namely diseases caused by non-infectious and non-transmissible medical conditions (www.who.int). American Cancer Society has foreseen a total of 1.638.910 of new cases and 577.190 deaths in United States of America in 2012 whereas annual cancer mortality is expected to decrease only a 1% (Avendaño-López, 2012). Since the cancer incidence is increasing in the so-called “modern societies”, the consumption of anticancer drugs has consequently augmented in the last years.

Anticancer drugs have been shown to have potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor and/or teratogenic effects in several organisms, since they have been designed to disrupt or prevent cellular proliferation, usually by interfering in DNA synthesis. Some ecotoxicological studies with anticancer drugs, on the other hand, such as in the case of for 5-Fluorouracil, have shown that the lowest observed-effect concentration (LOEC) in alga and bacterial assays (10 μg L^{-1}) was close to the concentration found in sewage effluents (Zounkova et al., 2007). In another example, LOEC obtained for Tamoxifen in freshwater fish was 5.6 μg L^{-1} being this concentration slightly higher than those found in wastewaters till the moment (Williams et al., 2007). Recent studies have revealed that mixtures of anticancer drugs in real samples possess an important toxicological effect comparing with the individual drug (Mater et al., 2014).

Although anticancer drugs seem to be equally released via hospital or domestic wastewater in previous studies (Ferrando-Climent et al., 2013), other authors have found that hospitals are, in general, the main source of some pharmaceuticals (Verlicchi et al., 2010, 2012). In any case, anticancer drugs have shown to be recalcitrant in wastewater: they are not removed by conventional wastewater treatments, and also have proven to be a challenge for the non-conventional technologies of water decontamination (Zhang et al., 2013). Therefore, there is a high probability that

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anticancer drugs reach the environment and their occurrence in wastewater, surface water and potential presence in drinking water is cause of concern (Kümmerer et al., 1997; Johnson et al., 2008; Liu et al., 2010; Booker et al., 2014).

Chemotherapy drugs are thus considered a group of emerging pollutants, which could be impacting the aquatic life in WWTP effluents receiving waters. Most of the studies till date report relatively high levels of these compounds in urban wastewaters (up to 146 ng L^{-1} for Cyclophosphamide and up to 42 ng L^{-1} for Tamoxifen) and some others have also found them (i.e. Tamoxifen) in natural waters up to 200 ng L^{-1}(Roberts and Thomas, 2006; Coetsier et al., 2009; Kosjek and Heath, 2011; Martín et al., 2011; Yin et al., 2010; Ferrando-Climent et al., 2013).

In this work, the occurrence of anticancer drugs through the entire urban sewage system was performed to clearly assess their fate in sewage system as well as their input and potential risk onto the aquatic environment Ten selected anticancer drugs were measured during a sampling campaign in the effluent of the main hospital of Girona (north-east of Spain), in the influent and effluent of Girona wastewater treatment plant (WWTP) (which receives hospital loads) and also in the river Ter where the WWTP discharges the treated water. Target anticancer drugs, selected due to their importance, consumption, inherent cytotoxic activity, and potential risk to the environment were Azathioprine (AZA), Cyclophosphamide (CY), Ciprofloxacin (CIP), Docetaxel (DOC), Etoposide (ETO), Ifosfamide (IF), Methotrexate (MTX), Paclitaxel (PAC), Tamoxifen (TAM) and Vincristine (VIN). Finally, the risk that these compounds can pose to the environment was assessed based on the results derived from their occurrence in the wastewater effluents as well as their ecotoxicological effects described in the literature.

2. Material and methods

Ciprofloxacin HCl, Cyclophosphamide, Ifosfamide, Methotrexate, Azathioprine, Etoposide, Docetaxel, Paclitaxel, Vincristine Sulfate and Tamoxifen Citrate were purchased by European Directorate for the Quality of Medicines and Healthcare (EDQM) Reference Standards (Strasbourg, France). Isotopically labeled compounds, used as internal standards, [$^{2}H_4$]-Cyclophosphamide, [$^{13}C_6$]-Tamoxifen Citrate, [$^{2}H_3$]-Etoposide, [$^{2}H_3$]-Methotrexate, [$^{2}H_3$]-Vincristine Sulfate, [$^{13}C_4$]-Azathioprine were purchased from Toronto Chemical Research Inc. (Canada) and [$^{2}H_8$]-Ciprofloxacin from EDQM Reference Standards (Strasbourg, France). HPLC-grade Water and HPLC-grade acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany). Reagents like Formic acid 98% (HCOOH) were provided by Scharlab (HPLC-grade) and the NH_3 30% by Panreac. Ethylenediaminetetraacetic Acid Disodium Salt 0.1 M solution (SV) was provided by Panreac.

2.1. Samples and standards preparation

Individual stock standard solutions of each target compound were prepared on a weight basis in methanol at 1 mg mL^{-1} and kept frozen at −20 °C. A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. Stock solutions of internal standards were also prepared in methanol and were stored at −20 °C. A mixture of these internal standards was also prepared by diluting the individual stock solution in methanol.

Calibration standard solutions were prepared based on methodology previously developed (Ferrando-Climent et al., 2013) using a matrix match approach by appropriate dilution in extracted samples of the stock solution of target compounds.

2.2. Sampling campaign

Girona (north-east of Spain), the urban area selected for this study, has approximately 96,000 habitants and the main hospital of the region is located also in this municipality: Dr. Josep Trueta Hospital, which counts with around 400 beds, receives indeed most of the oncologic patients of this area. Municipal wastewater treatment plant (WWTP) of Girona, receives the urban wastewater from the main city and also from diverse municipals nearby (Salt, Sarrià de Ter, Sant Julià de Ramis, Aiguaviva, Vilablareix and Fornells de la Selva). Not only domestic sewage water but also wastewater from different sources: health centres (including the Dr. Josep Trueta hospital), industrial zone, etc. are discharging in the WWTP. Wastewater volume processed by this WWTP is estimated between 40,000 and 50,000 m^3/day (data provided by TRARGISA, trading company which manages the plant).

The sampling campaign was performed along consecutive months: November, December and January. Water was collected from hospital effluent through wastewater influent and effluent, till surface water, from Ter River at 500 m upstream and downstream of the wastewater treatment plant as presumably non-impacted and impacted sampling points respectively (Fig. 1).

The hospital effluent and the influent of the WWTP were collected within the same day while the wastewater effluent samples were collected the day after, together with the surface water samples, taking into account the hydraulic retention time (27 h) of WWTP.

Samples from the river, were taken 500 m upstream and downstream of the discharge of WWTP into the river. Both samples were taken same day that WWTP effluent was sampled. Samples were taken for specific days (work days), at same hours (morning) according with the urban sewage timings.

All the samples were collected in amber glass bottles which were pre-rinsed with Milli-Q water. They were vacuum filtered through 1 μm glass fiber filters followed by 0.45 μm nylon membrane filters (from Whatman, Teknokroma, Barcelona, Spain). The samples were kept frozen at −20 °C in amber PET containers, a period always inferior than 1 month, until their analysis based on the stability studies described at Ferrando-Climent et al. (2013), that establishes that 1 month is the maximum time that the target cancer drugs can be stored in those conditions before some degradation or lost of contaminants is observed.

General physico-chemical parameters of each sample as temperature, pH, oxygen amount and conductivity were measured *in situ* during sampling. A portable pH-meter (Crison; Model GLP 21) and an oximeter (YSI; model ProODO handlhed) were used for this purpose.

2.3. Toxicity assay

Toxicity of each sample was perform using the bacterial bioluminescence assay from Microtox™ (Carlsbad, CA, USA) based on the ISO 11348-3 standard protocol (UNE-EN ISO 11348-3:2007, 2009). Quantitative information about the toxicity of the samples was obtained by calculating the toxicity in terms of EC_{50} and toxicity units ($TU = 100/EC_{50}$). In case of testing the toxicity real solutions it is not possible to present results based on units such the concentration (ng/L) since there are many known and unknown contaminants in the sample. Therefore, Microtox results obtained are derived from the real sample as the most concentrated solution (45%) and its consequent dilution following the microtox protocol. A wide range of dilutions of each sample were measured using saline solution where the initial volume was 2.5 mL (45, 22.5, 11.25 and 5.63% of sample dilution), inhibition curves were performed, and the corresponding 50% effective concentrations (EC_{50}) were calculated. The analysis was carried out tempered at 15 °C. To enhance test performance, the salt contain was adjusted in order to reach 2% of salinity in sample. Bacterial reagents were reconstituted just prior to analysis and the pre-incubation times used before luminescence measurements were those given in microtox protocols. The concentration of toxicant in the test that caused a 50% reduction in light (inhibition = 50%) after exposure for 15 and 30 min.

2.4. Sample pre-treatment

The analytical methodology previously developed by Ferrando-Climent et al. (2013) was applied for sample pretreatment. A suitable volume of the chelating agent EDTA was added to all of them to a final concentration of 0.1% (g solute g^{-1} solution), as it is well known that it improves the extraction of some antibiotics such ciprofloxacin(Cha et al., 2006; Gros et al., 2012; Hernandez et al., 2007). Preconcentration of samples was performed by solid phase extraction (SPE) by the automatic extraction system GX-271 ASPEC™ (Gilson, Villiers le Bel, France). 50 mL of each sample was loaded at 1 mL min^{-1} in the Oasis HLB (200 mg, 6 mL) cartridge previously conditioned using 5 mL of methanol followed by 5 mL 0.1% formic acid solution at 2 mL min^{-1}. Elution was performed with 10 mL at a flow rate of 2 mL min^{-1}, using pure methanol. The extract was evaporated under gentle nitrogen stream using a Reacti-Therm 18824 System (Thermo Scientific) and reconstituted with 500 μL of methanol-water (10:90, v/v). Finally, internal standard mix to compensate possible matrix effect was added in the sample extract for internal standard calibration reaching a concentration of 10 μg L^{-1}.

2.5. UPLC-QqLit method

Chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3 column (50 mm × 2.1 mm i.d. 1.7 μm particle size; Waters Corp. Mildford, MA, USA) under positive electrospray ionization (PI). The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All transitions were recorded by using Multiple Reactive Monitoring Mode (MRM) and the data were acquired and processed using Analyst 2.1 software. Analytical parameters as limits of detection and quantification are shown in previous work (Ferrando-Climent et al., 2013).

2.6. Risk assessment

In order to assess the risk that the presence of these cancer drugs can pose into the environment, "Risk Characterization Ratio" (RCR) was calculated for each compound. RCR are calculated according to the EU guidelines (93/67/EEC, 2003) as the ratio

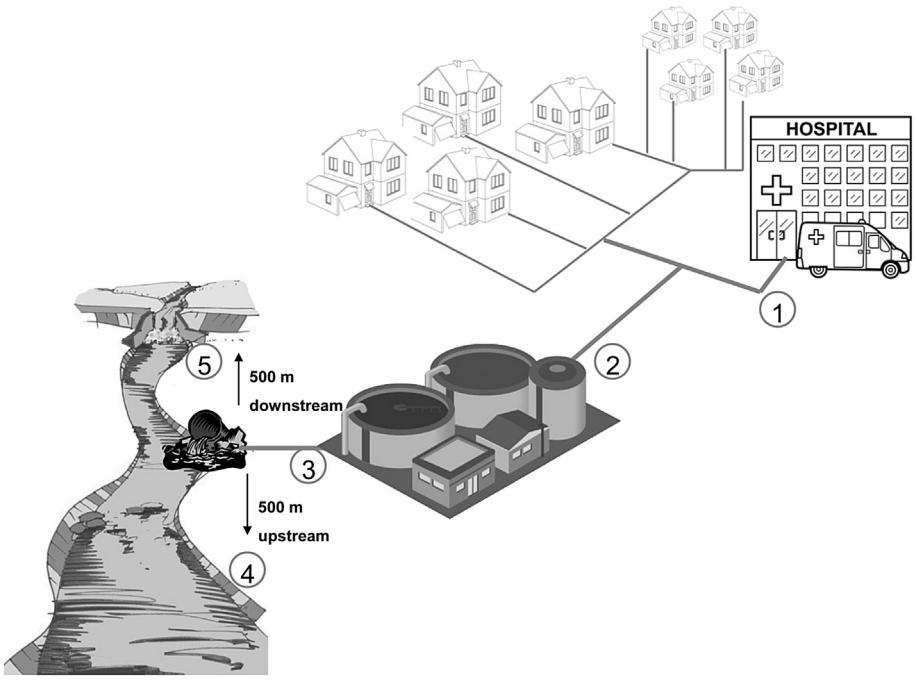

Fig. 1. Sampling points from the urban system evaluated: from hospital effluent (1), through to wastewater influent (2) and wastewater effluent (3) till river water before (4) and after (5) the WWTP discharge.

between predicted environmental concentration (PEC) and predicted no effect concentration (PNEC). However, in order to obtain a most realistic scenario, occurrence data obtained in this study, namely "measured environmental concentrations" (MEC) were used instead of PEC values for the estimation of RCR. PNEC on the other hand is based on environmental effect data, such as toxicity to fish, Daphnia or algae and is determined by applying a "safety factor". For acute studies, the safety factor of 1000 is applied to the EC_{50} value, ie. a Daphnia EC_{50} following 48 h exposure of 50 mg L^{-1} would lead to a PNEC of 0.05 mg L^{-1}, sub-acute studies (medium-term) require a safety factor of 100, and for chronic studies (longer-term) a safety factor of 10 (93/67/EEC, 2003).

Under the current European Directive, RCR parameter has several meanings depending of the value obtained, for values inferior to 1 "it is not a cause of concern for the moment", for values from 1 to 10 "it is cause of concern depending the volume increase", for values from 10 to 100 "further data is required" and finally if the RCR value is above 100 "it is recommended to reduce the risk immediately".

3. Results and discussion

3.1. General parameters

Temperature, pH, conductivity and dissolved oxygen were measured *in situ* along the sampling campaign. As it is shown in Table 1, wastewater treatment plant has a significant influence onto the river temperature, since it showed an increase of 4° compared to water temperature upstream after the sewage effluent discharge. Temperature of wastewater effluent is usually higher than the river temperature, although in this particular case the high temperature of effluent is also due to the specific use of WWTP effluent: a portion of effluent water, (450 m^3 h^{-1}; 0.12 m^3 s^{-1}) is continuously recirculated to refrigerate a waste incinerator nearby, just before being discharged into the river. The highest conductivity was always found for the hospital effluents (except in case of December campaign) due to the huge level of electrolytic substances (urine, body fluids in general), with higher contribution in hospitals effluents than in urban effluents. Conductivity was indeed decreasing along the water treatment being surface water, the samples with the lowest conductivity. Dissolved oxygen ranged from 1.09 to 13.21 mg L^{-1} being the influent samples the ones with the lowest values. Organic components from wastewater in the sewage system are easily oxidized and consequently aerobically degraded by microbial community, thus leading to the consumption of a big amount of water solved oxygen.

3.2. Toxicity measurements

Acute toxicity (to V. fischeri) of water samples was measured as EC_{50} and TU (toxicity units) obtained after 15 min and 30 min of

Table 1
Physico-chemical parameters and ecotoxicity values obtained for the samples along three sampling periods in hospital effluent (WWH), wastewater treatment plant (WWI and WWE) and surface water (SW) (n.t = no toxic; $n = 3$ replicates).

Parameters	Month 1 (November)					Month 2 (December)					Month 3 (January)				
	WWH	WWI	WWE	SW Before WWTP	SW After WWTP	WWH	WWI	WWE	SW Before WWTP	SW After WWTP	WWH	WWI	WWE	SW Before WWTP	SW After WWTP
pH	7.97	7.76	7.29	8.20	**7.50**	8.22	8.09	7.55	8.19	**7.97**	8.43	8.29	7.55	8.13	8.00
Temperature (°C)	22.7	18.6	18.6	14.2	18.3	14.4	16.5	16.7	8.8	12.8	18.8	15.1	16.1	9.7	14.3
O_2 (mg/L)	5.52	1.09	5.41	10.28	8.90	8.49	3.95	6.11	13.21	11.38	4.01	1.41	6.03	11.92	10.15
Conductivity (μS/cm)	1376	1156	891	426	755	891	1256	951	514	706	1822	1208	904	525	750
Flow River Ter (m^3/s)	–	–	–	115.95		–	–	–	52.29		–	–	–	10.07	
Acute Toxicity EC_{50} (%) (15 min)	34	25	45	n.t.	n.t.	16	20	52	n.t.	n.t.	12	25	47	n.t.	n.t.
UT (Toxicity Units)	3.0	4.0	2.2	n.t.	n.t.	6.3	4.9	1.9	n.t.	n.t.	8.3	3.9	2.1	n.t.	n.t.

Bold highlights the value which is lower than pH = 8.

exposure. Results of 15 min are shown in Table 1 since both results (15 and 30 min) were almost identical. In general, samples from hospital effluents showed more toxicity than influent wastewater samples and toxicity values decreased from hospital effluents to surface water. Test performed, based on toxicity to *V. fisheri*, is a general toxicity assay rather than a specific toxicity assay for cancer drugs. However it allows to study and compare the ecotoxicological effects of each particular effluent with the corresponding load of chemical contaminants, in this case anticancer drugs. Hospital effluents were the most contaminated water samples both in terms of ecotox response as well as presence of chemical contaminants (see Tables 1 and 3). Despite the low specificity of Microtox analysis, this test allows having important information about the general toxicity in contaminated mixture samples and it reveals the potential ecotoxicological impact due to a low or high number/concentration of pollutants. However further studies are necessary for evaluating the specific toxicity of each contaminant or type of contaminant in real samples. This can be achieved by approaches such as the so-called effect directed analysis (EDA) (Brack, 2011) and by applying specific tox assays for this type of contaminants.

3.3. Analytical parameters

Analytical parameters such method detection limit (MDL), method quantification limit (MQL) and recoveries were evaluated for the different matrices studied in the present work, except for the case of hospital effluent and wastewater influent which were already calculated in previous studies (Ferrando-Climent et al., 2013) .

MDL and MQL were determined as the minimum detectable amount of each analyte with a signal-to-noise of 3 and 10, respectively. MDL and MQLs were calculated from spiked real samples for the aqueous matrices studied. Values of MDL and MQL for all the matrices are shown in Table 2.

Recoveries were also calculated for wastewater effluent and surface waters. In order to calculate them, samples were spiked in triplicate at two spiking levels (0.5 and 0.1 ng mL^{-1}) using a standard mixture solution containing all target compounds. These levels were selected based on the usual concentration of target compound found in the water matrices under study according to scientific literature (Kosjek and Heath, 2011; Besse et al., 2012; Ferrando-Climent et al., 2013; Negreira et al., 2013) (Table 2). The recoveries showed at Table 2 are those obtained at spiking level of 0.1 ng mL^{-1} since most of the compounds were found at concentrations close to this level.

3.4. Incidence of anticancer drugs

The occurrence of anticancer drugs in surface water, influent and effluent wastewater, and hospital effluent, are shown in Fig. 2 and Table 3. VIN and IF were either not detected or were found below limit of quantification in almost all the samples analyzed whereas the rest of target anticancer drugs were detected in the three sampling periods in most of the wastewaters and in some of the river waters analyzed.

CIP was detected at huge levels in hospital effluents (from 3089 till 14,826 ng L^{-1}), sewage samples (from 1172 till 1558 ng L^{-1}) and even in surface waters both upstream (from 8 till 56 ng L^{-1}) and downstream (from 7 till 103 ng L^{-1}) of the WWTP discharge. These high values can not only be attributed to their use in chemotherapy treatment since CIP is also used as antibiotic to treat a number of human infections as well as for veterinary treatments (Sissi and Palumbo, 2003). CIP is also used in farms of poultry, pigs, rabbits etc; and indeed there is a farm a few meters upstream of the river near to the WWTP, which could be responsible of the levels of CIP found in surface water before the discharge of the WWTP. Nevertheless, the high amount of CIP in the WWTP effluent can be pointed out as the major contribution to the overall CIP contamination in the river.

TAM was found in all the samples studied and at higher levels (up to 170, 51 and 42 ng L^{-1} in hospital effluents, urban influents and effluents respectively) than reported in most of existing studies in sewage samples till date, such in that of Liu et al. (2010), who found up to 8.2 ng L^{-1} and 0.28 ng L^{-1} of TAM in hospital and sewage influent respectively (Liu et al., 2010), or Ashton et al., who found up to 42 ng L^{-1} at sewage effluents (Ashton et al., 2004). TAM can be classified as a recalcitrant compound because it was not removed from wastewater in the WWTP and its concentration remains almost unaltered. TAM was also found in the river before the WWTP discharge (from 12 till 36 ng L^{-1}) at very similar levels than those found downstream. This drug is used not only for human treatments but also for veterinary treatments such reproductive control or hormonal treatments (for cats, dogs, rabbits, etc) (Virbac-España, 2011; Corrada et al., 2003). Thereby, these findings in upstream water might be due to the farms located along to the river before the sewage discharge. The presence of TAM in rivers needs to be taken very much into consideration in terms of environmental risk assessment since it is suspected to provoke endocrine disruption effects as well as to have a high bioaccumulation potential (Jean et al., 2012). TAM has been recently included with 13 other substances as priority compounds that cumulate several risk factors for aquatic ecosystems (Besse et al., 2012).

CY, one of the most studied cytostatic agents so far, was found at low levels in this study (from blq till 43 ng L^{-1} in hospital effluents, from 8 till 26 ng L^{-1} in influents, from 7 till 25 ng L^{-1} in effluents and from 0 till 20 ng L^{-1} in downstream river). CY is a contaminant of known toxicity (Table 4), persistence and ubiquity in the environment: it was indeed detected in almost all the samples analyzed in this study. Moreover, and in the same way that TAM, it remains unaltered during WWTP. These finding confirm previous studies

Table 2
Basic analytical parameters for the 10 anticancer drugs studied: Method detection limits (MDL), Method quantification limits (MQL) and Recoveries (%) for the target compounds in the different matrices studied: WWH (hospital effluent), WWI (wastewater influent), WWE (wastewater effluent seasons) and SW (surface water) ($n = 3$ replicates).

Target compounds	MDL (ng/L)				MQL (ng/L)				% Recoveries (% RSD) spiked at 0.1 μg/L	
	WWH	WWI	WWE	SW	WWH	WWI	WWE	SW	WWE	SW
AZA	3.8	3.5	1.8	1.2	12.7	11.7	6.1	3.9	87.5 (8%)	93.4 (11%)
CIP	2.4	1.6	1.8	0.6	8.2	5.2	5.8	2.0	76.5 (11%)	74.5 (10%)
CY	1.1	2.2	0.6	0.3	3.6	7.3	2.1	0.9	70.9 (9%)	74.9 (2%)
DOC	7.5	6.3	3.8	3.8	20.9	20.8	12.7	12.7	108.7 (15%)	94.0 (1%)
ETO	24.0	28.8	22.7	21.7	80.0	96.2	75.7	72.5	127.0 (3%)	129.7 (7%)
IF	1.7	1.0	0.4	0.3	5.8	3.2	1.3	1.1	76.5 (9%)	82.7 (6%)
MTX	1.8	1.5	1.2	0.2	5.9	4.8	4.1	0.8	81.4 (13%)	84.5 (8%)
PAC	5.5	2.7	2.6	0.9	18.4	8.9	8.7	2.9	126.5 (12%)	121.3 (1%)
TAM	0.8	2.1	0.6	0.3	2.7	7.0	1.8	1.1	56.5 (15%)	58.4 (8%)
VIN	7.4	14.3	7.1	6.4	24.5	47.6	23.5	21.3	84.8 (6%)	87.2 (3%)

Table 3
Occurrence of target compounds (ng L^{-1}) for the three months studied along the sampling points selected: WWH (hospital effluent), WWI (wastewater influent), WWE (wastewater effluent seasons), SW (surface water) before and after WWTP. (blq = below limit of quantification; n.d = not detected; * values used for risk assessment; $n = 3$ replicates).

Target compounds (ng L^{-1})	Month 1 (November)					Month 2 (December)					Month 3 (January)				
	WWH	WWI	WWE	SW Before WWTP	SW After WWTP	WWH	WWI	WWE	SW Before WWTP	SW After WWTP	WWH	WWI	WWE	SW Before WWTP	SW After WWTP
AZA	90 ± 3	20 ± 1	n.d.	n.d.	n.d.	blq	n.d.	n.d.	n.d.	n.d.	20 ± 2	19 ± 2	n.d.	n.d.	n.d.
CIP	7973 ± 210	1172 ± 74	78 ± 11	56 ± 12	102 ± 10	3089 ± 307	1472 ± 97	36 ± 3	8 ± 1	7 ± 1	14,826 ± 2260	1558 ± 164	147 ± 13*	10 ± 1	103 ± 9
CY	36 ± 4	26 ± 2	25 ± 3*	n.d.	20 ± 4	blq	8 ± 0.2	7 ± 0.3	n.d.	blq	43 ± 4	14 ± 4	15 ± 3	n.d.	9 ± 0.2
DOC	79 ± 25	175 ± 39	n.d.	n.d.	n.d.	n.d.	65 ± 18	n.d.	n.d.	n.d.	61 ± 4	219 ± 57	n.d.	n.d.	n.d.
ETO	714 ± 37	blq	n.d.	n.d.	n.d.	blq	blq	n.d.	n.d.	n.d.	367 ± 68	n.d.	n.d.	n.d.	n.d.
IF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MTX	6 ± 1	11 ± 2	6 ± 0.1	n.d.	5 ± 0.2	n.d.	23 ± 2	n.d.	n.d.	n.d.	19 ± 5	blq	n.d.	n.d.	n.d.
PAC	59 ± 7	n.d.	n.d.	n.d.	n.d.	blq	18 ± 3	blq	n.d.	n.d.	100 ± 14	n.d.	n.d.	n.d.	n.d.
TAM	95 ± 2	51 ± 1	42 ± 3*	36 ± 3	38 ± 2	36 ± 1	15 ± 3	11 ± 0.2	12 ± 0.3	25 ± 0.3	170 ± 0.2	58 ± 5	33 ± 0.4	30 ± 0.1	34 ± 0.2
VIN	n.d.	n.d.	n.d.	n.d.	n.d.	blq	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Fig. 2. Selected anticancer drugs in the whole aquatic urban system: WWH (hospital), WWI (influent), WWE (effluent), SW downstream/upstream (surface waters) during the three months studied (Month 1, 2 and 3).

(Buerge et al., 2006; Castiglioni et al., 2005; Steger-Hartmann et al., 1996; Verlicchi et al., 2012) where this drug could not be eliminated from wastewater by conventional sewage treatments being thus susceptible to reach the surface waters, as it is the case in this study, where CY was not found upstream but downstream of the WWTP.

In this study AZA was only found in raw wastewater influents in both Hospital wastewater as well in influent wastewater at levels ranging between 20–90 and 19–20 ng L^{-1} respectively. AZA has

Table 4
Ecotoxicological data for target anticancer drugs detected in wastewater effluents: Predicted No Effect Concentration (PNEC), Measured Environmental Concentration (MEC) and Risk Characterization Ratio (RCR), calculated under European Frame Directive as the ratio between the MEC and PNEC values for each species reported in the table.

Anticancer drug	Type of toxicity test	Species	Critical effect	Ecotoxicity value	Concentration ($mg\ L^{-1}$)	Reference	Safety factor for PNEC calculation	PNEC ($mg\ L^{-1}$)	MEC ($mg\ L^{-1}$)	RCR
Cyclophosphamide	ISO 6341:199	*Daphnia magna (Crustacean)*	Immobilization	EC_{50} 48 h	>1000	Zounkova et al., 2007	1000	1	$2.5\cdot10^{-5}$	$2.5\cdot10^{-5}$
	ISO 8692:1989	*Pseudokirchneriella subcapitata (Freshwater green alga)*	Growth inhibition	EC_{50} 72 h	930	Zounkova et al., 2007	1000	0.9	$2.5\cdot10^{-5}$	$2.8\cdot10^{-5}$
Ciprofloxacin	OECD guideline	*Daphnia magna*	Fecundity	EC_{50} 21d	12.8	Martins et al., 2012	10	1.28	$1.5\cdot10^{-4}$	0.0001
	OECD guideline	*Gambusia holbrooki*	Mortality	EC_{50} 96 h	>60	Martins et al., 2012	1000	0.06	$1.5\cdot10^{-4}$	$2.5\cdot10^{-3}$
	ASTM, static toxicity	*Lemna gibba (Duckweed)*	Growth inhibition	EC_{50} 7 days	0.697	Brain et al. 2004 (a)	100	$6.97\cdot10^{-3}$	$1.5\cdot10^{-4}$	0.02
	OECD guideline	*Lemna minor*	Growth rate	EC_{50} 7 days	3.75	Martins et al., 2012	100	0.0375	$1.5\cdot10^{-4}$	0.05
		Microcystis aeruginosa (Freshwater cyanobacteria)	Growth and reproduction	EC_{50} 5 days	0.017	Robinson et al., 2005	100	0.00002	$1.5\cdot10^{-4}$	7.5
		Microcystis aeruginosa (Freshwater cyanobacteria)	Growth and reproduction	EC_{50} 5 days	0.005	Halling-Sorensen et al., 2000	100	$5\cdot10^{-5}$	$1.5\cdot10^{-4}$	3
	OECD.201 (1984)	*Selenastrum capricornutum (Freshwater green algae)*		EC_{50}	2.97	Halling-Sorensen et al., 2000	1000	0.00297	$1.5\cdot10^{-4}$	0.05
	ISO 15522 (1998)	*Sludge bacteria*		EC_{50}	0.61	Halling-Sorensen et al., 2000	1000	0.00061	$1.5\cdot10^{-4}$	0.25
	Microtox test	*Vibrio fischeri*	Acutely toxic	EC_{50}	11.5	Martins et al., 2012	1000	0.0115	$1.5\cdot10^{-4}$	0.01
Methotrexate	OECD Guideline	*Bluegill sunfish cells (BF-2 cells)*	cell density	EC_{50}	3	Henschel et al., 1997	1000	0.003	$6\cdot10^{-6}$	$2\cdot10^{-3}$
	OECD Guideline	*Brachydanio rerio (zebra fish)*	pulse rate	EC_{50} 48 h	142	Henschel et al., 1997	1000	0.142	$6\cdot10^{-6}$	$4\cdot10^{-5}$
	OECD Guideline	*Brachydanio rerio (zebra fish)*	mortality	EC_{50} 48 h	85	Henschel et al., 1997	1000	0.085	$6\cdot10^{-6}$	$7\cdot10^{-5}$
	OECD Guideline	*Daphnia magna (Crustacean)*	immobilization	EC_{50} 48 h	>1000	Henschel et al., 1997	1000	1	$6\cdot10^{-6}$	$6\cdot10^{-6}$
	OECD Guideline	*Scenedesmus subspicatus (Green algae)*	growth inhibition	EC_{50} 72 h	260	Henschel et al., 1997	1000	0.26	$6\cdot10^{-6}$	$2\cdot10^{-5}$
	OECD draft (1996)	*Tetrahymena pyriformis(Ciliate)*	growth inhibition	EC_{50} 48 h	45	Henschel et al., 1997	1000	0.045	$6\cdot10^{-6}$	$1\cdot10^{-4}$
	DIN Guideline 38412 L34	*Vibrio fischeri (Bacteria)*	luminescence inhibition	EC_{50}	1220	Henschel et al., 1997	1000	1.22	$6\cdot10^{-6}$	$5\cdot10^{-6}$
Tamoxifen	NR assay	*PLHC-1 fish cell line (Poeciliopsis lucida hepatocytes)*	cell viability	EC_{50} 24 h	1.72	Caminada et al., 2008	1000	0.00172	$4.2\cdot10^{-5}$	0.024
	MTT assay	*PLHC-1 fish cell line (Poeciliopsis lucida hepatocytes)*	cell viability	EC_{50} 24 h	5.12	Caminada et al., 2008	1000	0.00512	$4.2\cdot10^{-5}$	$8.2\cdot10^{-3}$
	MTT assay	*RTG-2 cell line (Rainbow trout gonad)*	cell viability	EC_{50} 24 h	5.38	Caminada et al., 2008	1000	0.00538	$4.2\cdot10^{-5}$	$8.2\cdot10^{-3}$
	MTT assay	*RTG-2 cell line (Rainbow trout gonad)*	cell viability	EC_{50} 24 h	7.09	Caminada et al., 2008	1000	0.00709	$4.2\cdot10^{-5}$	$5.92\cdot10^{-3}$
		Pimephales promelas	F1 growth	112 days	0.00001	Williams et al., 2007	10	$1\cdot10^{-6}$	$4.2\cdot10^{-5}$	42
		Pimephales promelas	F1 larvae growth significant decrease	28 days	0.00008	Williams et al., 2007	10	$8\cdot10^{-6}$	$4.2\cdot10^{-5}$	5.3
		Pimephales promelas	Increase in vitellogenin in F1 males	112 days	0.00001	Williams et al., 2007	10	$1\cdot10^{-6}$	$4.2\cdot10^{-5}$	42
		Acartia tonsa	Larval development	EC_{50} 5days	49	Williams et al., 2007	100	0.49	$4.2\cdot10^{-5}$	$8.6\cdot10^{-5}$
	OECD Guideline 201 (2002)	*Microalgae S. capricornutum*	growth inhibition	72 h	0.001	Mater et al., 2014	1000	$1\cdot10^{-6}$	$4.2\cdot10^{-5}$	42

not been taken into account in analytical methodologies developed so far, therefore there was no information about the occurrence of this compound in wastewater. This is indeed the first time to the authors' knowledge that AZA has been found in such effluents. AZA seems to be easily removed from wastewater through biodegradation or sorption processes and thus it does not seem to arrive to receiving river waters and should not pose a risk for the aquatic life.

DOC was the only cancer drug found at higher levels in influent sewage than in hospital effluent. This particular behavior can be explained by its slow metabolism in human organism: DOC is metabolized through the oxidative metabolism of the tert-butyl ester mediated by cytochrome P450, and it is excreted in seven days through both urine and faeces (6% and 75% respectively). Around 80% of this drug is excreted during the first 48 h in the form of inactive metabolites as well as low amounts of unchanged product (www.ema.europa.eu) consequently the patient is already at home and the excretion usually takes place there. Slow human metabolism is also described for ETO: approximately 63% and 31% in faeces after c.a. 80 h (http://www.aemps.gob.es). However, ETO was found at high concentrations in hospital effluent (up to 714 ng L^{-1}) whereas it was not detected (or it was found below limit of quantification) in urban sewage. This can be explained because of ETO is used for kinds of cancer that usually requires hospitalization of the patients (cancer bounds, Wilm's Tumor, Neuroblastome and retinoblastoma in children, etc) and excretion takes place thus mainly at the hospital. PAC is mostly used for prostate cancers and others that, in general, do not require staying at hospital (www.who.com), however it was mainly detected at hospital effluent. This fact might be explained because of the fast elimination of this drug from the human organism, in less than 27 h (www.ema.europa.eu), a much faster metabolization than for ETO and DOC. Therefore PAC could possibly reach wastewater before patient leaves the hospital.

In general, MTX was found at similar concentrations in hospital effluents (from "non-detected" till 19 ng L^{-1}) than in sewage input (from non-detected till 23 ng L^{-1}) where it was completely removed from wastewater in all the samples analyzed. Despite MTX was originally designed as a chemotherapy drug, it is also used for the treatment of some autoimmune diseases, including rheumatoid arthritis, psoriasis, psoriatic arthritis, etc., which are normally consumed at home (Sweetman and Martindale, 2011). This should be the reason why there is no significant differences in the levels found for this compound in hospital and domestic wastewaters.

During the three months study, target compounds exhibited similar occurrence profile and, except by the case of DOC as explained above, the levels found in wastewater were higher in hospital effluents than in urban WWTP influents. However, there are important differences in the levels found for these contaminants in the three periods analyzed: the lowest amount of pollutants were found before Christmas season (December 2011) whereas the highest amount was found in January, after this holiday season (Fig. 2 and Table 3). This fact could be due to that some treatments for non-hospitalized patients might be stopped at Christmas time and restarted after this period.

AZA, MTX, DOC and PAC were removed from wastewater whereas CIP, CY and TAM have shown to be recalcitrant since WWTP were inefficient in eliminating them. Therefore, WWTP discharges are contributing to river contamination with CY, CIP and TAM although the two latest were already present upstream. This fact can be attributed to sources of contamination such farms located before the sewage discharge.

Sorption processes of target compounds into particulate matter, sludge, sediment, etc. should be considered in further studies in order to study the distribution of these pollutant in both phases (aqueous and solid).

3.5. Environmental risk assessment

In order to evaluate the risks that the presence of anticancer drugs poses into the environment, ecotoxicological data obtained from different bibliographic sources were collected (www.wikipharma.org,; Brezovšek et al., 2014; Caminada et al., 2008; Canty et al., 2009; Halling-Sorensen et al., 2000; Hartmann et al., 1999; Henschel et al., 1997; Martins et al., 2012; Mater et al., 2014; Parrella et al., in press; Robinson et al., 2005; Williams et al., 2007; Zounkova et al., 2007). PNEC values were calculated for different trophic levels representative of aquatic ecosystem (daphnids, algae, fish, etc) applying a "safety factor" onto the EC_{50} parameter (from literature) mentioned in Section 2.6 (93/67/EEC, 2003) (Table 4). MEC values selected were those related to the output of wastewater treatment plant as they are the levels expected in river in a worst case water scarcity scenario, when almost 100% of river flow can be originated from WWTP effluents. Risk characterization ratio (RCR) values were calculated as explained in Section 2.6. As the ratio between MEC and PNEC and results are presented in Table 4. According to the wastewater effluent results, cyclophosphamide and methotrexate showed RCR values lower than 1, within the range of the substances that are not of immediate concern. However RCR values for ciprofloxacin and tamoxifen were often higher, depending the organism/species considered: Particularly ciprofloxacin showed RCR values between 1 and 10 for *Freshwater cyanobacteria*. In the case of tamoxifen, it showed RCR values below 1 for species belonging to different trophic level such *Acartia tonsa* or the *Rainbow trout gonad*, nonetheless for the *Pimephales promelas*, which showed values from 1 to 100 or *S. capricornutum microalgae* which showed values from 10 to 100 (Table 4). Special attention must thus be paid to this compound in further studies, based on the high RCR values encountered in this study.

4. Conclusions

The present study has contributed to put in the spotlight the real occurrence and impact of anti-cancer drugs in the environment. Among the cancer drugs studied, Ciprofloxacin and Tamoxifen have shown, based on the risk assessment study performed, to pose a potential impact into the aquatic environment. This fact highlights the importance of improving the conventional wastewater treatments as well as applying decentralized solutions to treat hospital effluent "on-site" (before being discharged into the urban sewage collection system) as other authors have recommended (Verlicchi et al., 2010). Even the management of the human excretions (urine and faecal) from oncologic patients as a separate waste with potential environmental impact, might be a solution to reduce the impact of these compounds in the environment.

Furthermore, considering aspects such presence, toxicity, persistence and bioaccumulation of some of anticancer drugs as well as the fact that these drugs are excreted by humans not only in their original form, future studies should focus in the screening of the metabolites and transformation products (which could also be present in wastewaters) since most of these compounds can have equal or even more activity than the parent compound (Custodio et al., 1994; Kiffmeyer et al., 1998) .

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CHAPTER 5

5. Block III: Removal and transformation through advanced processes

5.1. Non conventional biological treatment based on *Trametes versicolor* fungi for the elimination of anticancer drugs in wastewater. Ferrando-Climent et al. (Chemosphere 2015)

Laura Ferrando-Climent, Carles Cruz-Morató, Ernest Marco-Urrea, Teresa Vicent, Montserrat Sarrà, Sara Rodríguez-Mozaz, Damià Barceló

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Abstract

This work presents a study about the elimination of anticancer drugs, a group of pollutants considered recalcitrant during conventional activated sludge wastewater treatment, using a biological treatment based on the fungus *Trametes versicolor*. A 10-L fluidized bed bioreactor inoculated with this fungus was set up in order to evaluate the removal of 10 selected anticancer drugs in real hospital wastewater. Almost all the tested anticancer drugs were completely removed from the wastewater at the end of the batch experiment (8 days) with the exception of Ifosfamide and Tamoxifen. These two recalcitrant compounds, together with Cyclophosphamide, were selected for further studies to test their degradability by *T. versicolor* under optimal growth conditions. Cyclophosphamide and Ifosfamide were inalterable during batch experiments both at high and low concentration, whereas Tamoxifen exhibited a decrease in its concentration along the treatment. Two positional isomers of a hydroxylated form of Tamoxifen were identified during this experiment using a high resolution mass spectrometry based on ultra-high performance chromatography coupled to an Orbitrap detector (LTQ-Velos Orbitrap). Finally the identified transformation products of Tamoxifen were monitored in the bioreactor run with real hospital wastewater.

Keywords: Cytotoxic. Anticancer drugs. Hospital effluent. Removal. *Trametes versicolor*. HRMS

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Non conventional biological treatment based on *Trametes versicolor* for the elimination of recalcitrant anticancer drugs in hospital wastewater

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HIGHLIGHTS

- Removals from 48 till 100% of anticancer drugs in hospital effluent treated by fungi.
- Cyclophosphamide and Ifosfamide remained unalterable whereas Tamoxifen was totally removed.
- Two hydroxilated positional isomers of Tamoxifen detected for first time in treated effluents.

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ABSTRACT

This work presents a study about the elimination of anticancer drugs, a group of pollutants considered recalcitrant during conventional activated sludge wastewater treatment, using a biological treatment based on the fungus *Trametes versicolor*. A 10-L fluidized bed bioreactor inoculated with this fungus was set up in order to evaluate the removal of 10 selected anticancer drugs in real hospital wastewater. Almost all the tested anticancer drugs were completely removed from the wastewater at the end of the batch experiment (8 days) with the exception of Ifosfamide and Tamoxifen. These two recalcitrant compounds, together with Cyclophosphamide, were selected for further studies to test their degradability by *T. versicolor* under optimal growth conditions. Cyclophosphamide and Ifosfamide were inalterable during batch experiments both at high and low concentration, whereas Tamoxifen exhibited a decrease in its concentration along the treatment. Two positional isomers of a hydroxylated form of Tamoxifen were identified during this experiment using a high resolution mass spectrometry based on ultra-high performance chromatography coupled to an Orbitrap detector (LTQ-Velos Orbitrap). Finally the identified transformation products of Tamoxifen were monitored in the bioreactor run with real hospital wastewater.

1. Introduction

Cancer is ranked (year 2012) in the second place (21%) of non-communicable diseases (this means non-infectious and non-transmissible medical conditions) which are causing deaths, after cardiovascular illness (48%) and followed by respiratory diseases (12%) (www.who.int); for that reason the high consumption of the drugs for chemotherapy treatments has became a cause of concern. These specific drugs have been shown to have potent cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor and/or teratogenic effects in several organisms, since they have been designed to disrupt or prevent cellular proliferation, usually by interfering in DNA synthesis or disrupting the endocrine system. The occurrences of these drugs in the aquatic environment could be especially critical since they are intrinsically hazardous. Several ecotoxicological studies have shown that in some cases such as for the cancer drug 5-Fluorouracil, the lowest observed-effect concentration values (in algal and bacterial assays) were close to the concentration found in sewage effluents (Zounkova et al., 2007). More recently, studies have revealed that mixtures of anticancer drugs in real samples possess an important toxicological effect comparing with the individual drug (Mater et al., 2014).

In general these so-called anticancer drugs can be released to the aquatic environment via hospital or domestic wastewater

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(Kovalova, 2009; Kosjek and Heath, 2011; Ferrando-Climent et al., 2013, 2014; Negreira et al., 2013) since there is a large number of them not removed from the wastewaters neither by biological conventional treatments (Kümmerer et al., 1997; Ferrando-Climent et al., 2014) nor by advanced technologies studied so far, such membranes bioreactors (Lenz et al., 2007; Kovalova et al., 2012), electrolysis, and advanced oxidation processes (ozonization, UV, H_2O_2) (Chen et al., 2008; Zhang et al., 2013). Therefore there is a need of development and application of new technological alternatives for wastewater treatment, and for the removal of the anticancer drugs from sewage.

In this work the performance of an alternative biological treatment based on white-rot fungi (WRF) was explored to eliminate selected anticancer drugs. *Trametes versicolor* has been already shown to have a special capacity to remove a wide amount of pharmaceutical compounds (Cruz-Morató et al., 2013) including β-blockers, antibiotics, anti-inflammatory and psychiatric drugs and achieving even the mineralization of some compounds such Diclofenac and Ketoprofen (Marco-Urrea et al., 2010a,b,c,d; Prieto et al., 2011; Jelic et al., 2012; Rodriguez-Rodriguez et al., 2012). WRF has an unspecific oxidative enzymatic system which includes lignin-modifiers enzymes, in particular laccase and peroxidases (extra-cellular enzymes), and also intracellular enzymatic complexes (cytochrome P450) (Asgher et al., 2008). Hydroxylation, formylation, deamination and dehalogenation mechanisms in the anthropogenic pollutants take place during the fungi metabolism (Harms et al., 2011; Cruz-Morató et al., 2012) and enable the degradation of the parent compound. However, detoxification does not necessarily occur since transformation products (TPs) of parent compounds can be in occasions more recalcitrant or even more toxic than the parent compound.

The objective of this work was to study the potential ability of WRF *T. versicolor* to eliminate selected anticancer drugs from real hospital effluents. 10 anticancer drugs, selected because of their use, ubiquity, non-biodegradability and also their potential bioaccumulation in the environment (Besse et al., 2012), were monitored along the experiment performed in a fluidized bed bioreactor. Further individual degradation experiments were performed for Cyclophosphamide, Ifosfamide and Tamoxifen in order to assess their possible degradation by this fungus under optimal growth conditions and to identify transformation products generated in the experiments by high resolution mass spectrometry (HRMS). These three compounds were selected as target pollutants for individual studies due to their ubiquity in wastewater, low biodegradability as well as high toxicity.

2. Materials and methods

2.1. Fungus preparation

T. versicolor (ATCC#42530) was provided by the American Type Culture Collection. It was kept by subculturing on 2% malt extract agar slants (pH 4.5) at room temperature. Subcultures were routinely made every 30 d. *T. versicolor* was grown in form of pellets as previously described (Blánquez et al., 2004) and subsequently the pellets were washed with sterile deionized water before its use.

2.2. Standard preparation and reagents

Ciprofloxacin HCl, Cyclophosphamide, Ifosfamide, Methotrexate, Azathioprine, Etoposide, Docetaxel, Paclitaxel, Vincristine Sulphate and Tamoxifen Citrate were purchased by European Directorate for the Quality of Medicines and Healthcare (EDQM) Reference Standards (Strasbourg, France). Isotopically labeled compounds, used as internal standards, [^{2}H4]-Cyclophosphamide, [^{13}C6]-Tamoxifen Citrate, [^{2}H3]-Etoposide, [^{2}H3]-Methotrexate, [^{2}H3]-Vincristine Sulphate, [^{13}C4]-Azathioprine were purchased from Toronto Chemical Research Inc. (Canada) and [^{2}H8]-Ciprofloxacin from EDQM Reference Standards (Strasbourg, France). HPLC-grade Water and HPLC-grade acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany). Reagents like Formic acid 98% (HCOOH) were provided by Sharlab (HPLC-grade). Ethylenediaminetetraacetic acid disodium Salt 0.1 M solution (SV) and NH_3 30% was provided by Panreac (Barcelona, Spain).

The cartridges used for solid phase extraction were Oasis HLB (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA). Glass fiber filters (1 μm) and nylon membrane filters (0.45 μm) were purchased from Whatman (U.K.). Glucose, ammonium tartrate dibasic and malt extract were purchased from Sigma–Aldrich (Barcelona, Spain).

Individual stock standard solutions of each target compound were prepared on a weight basis in methanol at 1 mg mL^{-1} and kept frozen at −20 °C. A mixture of all pharmaceutical standards was prepared by appropriate dilution of individual stock solutions. Stock solutions of internal standards were also prepared in methanol and were stored at −20 °C. A mixture of these internal standards was also prepared by diluting the individual stock solution in methanol.

2.3. Hospital wastewater samples

The main hospital of Girona, Dr. Josep Trueta, was selected for this study. This municipality, which is located in the north of Spain, has approximately 96.000 habitants and the hospital, which counts with around 400 beds, receives the major of the oncologic patients of this area. Two non-consecutive samplings (Sample 1 and 2) were performed at hospital wastewater effluent prior to the connection with the wastewater treatment plant (WWTP).

In order to isolate the effect of *T. versicolor* onto the pollutants so discarding the activity from the rest of microorganism present in the wastewater, two treatments were tested in the wastewaters: Sample 1 was sterilized previous to all the experimental set-up while Sample 2 was not sterilized.

2.4. Biodegradation experiments

2.4.1. Degradation of anticancer drugs in bioreactors fed with real hospital effluents

A glass fluidized bed bioreactor with a working volume of 10 L (Blanquez et al., 2008) was used to carry out both sterile (Sample 1) and non-sterile (Sample 2) hospital wastewater treatment in batch mode. Approximately, 2.0 g dry weight (d.w.) pellets L^{-1} were inoculated in both sterile and non-sterile treatments. Fungal biomass was maintained fluidized by air pulses generated by an electrovalve. The electrovalve was controlled by a cyclic timer (1 s open, 5 s close) and the air flow was 12 L h^{-1}. The bioreactor was equipped with a pH controller in order to keep pH at 4.5 and the temperature was maintained at 25 °C. Glucose and ammonium tartrate were fed continuously from their stock solution (300 g L^{-1} and 675 mg L^{-1}, respectively) at a flow rate to ensure an uptake rate of 0.31 g glucose g^{-1} d.w. pellets d^{-1} and 2 mg ammonium tartrate g^{-1} d.w. pellets d^{-1}. For sterile conditions the bioreactor and the wastewater (Sample 1) were autoclaved at 121 °C for 30 min. Samples of 250 mL were taken periodically. All the samples were filtered with 0.45 μm filters. 200 mL were stored at −20 °C to be further analyzed by UPLC coupled to a triple quadrupole-ion trap mass spectrometer (QqLIT). 50 mL from each sample were used to measure glucose concentration, COD, N—NH_4^+ and laccase.

2.4.2. Degradation of Cyclophosphamide, Ifosfamide and Tamoxifen in synthetic water

Individual degradation experiments for Cyclophosphamide, Ifosfamide and Tamoxifen were performed in 500 mL Erlenmeyer flask containing appropriate amounts of mycelial pellets (0.6 g d. w.) in a total volume of 100 mL of Kirk medium (pH 4.5) (Kirk et al., 1978). Stock solution of Cyclophosphamide, Ifosfamide and Tamoxifen were prepared in ethanol and they were spiked into the flasks reaching the desired concentration (approximately 10, 10 and 0.3 mg L^{-1} respectively). The concentration selected for Tamoxifen was limited for its solubility into water solutions (maximum 0.3 mg L^{-1}).

All these experiments were performed under sterile conditions using autoclave at 121 °C for 30 min before adding the WRF. The flasks were incubated under darkness in an orbital shaker (135 rpm) at 25 °C. The whole content of the flasks was collected for its analysis at times 30 min, 6 h, 1 d, 2 d, 3 d, 6 d and 9 d, and filtered through 0.45 μm glass fiber filter GF/A from (Whatman, Spain).

In parallel, two types of control experiments were performed. One experimental blank, was prepared with target compound in the same conditions that in the experimental cultures (feed, pH, etc.) but without inoculation of *T. versicolor*. This control sample was used to assess the potential photodegradation of the micropollutants as well as the matrix effect onto the contaminants from the experimental conditions. Another control consisted in heat-killed cultures by autoclave (121 °C for 30 min) under identical conditions to those of the experimental cultures. This control was used to evaluate potential fungal sorption processes that could be taking place in time-courses degradation experiments. The amount of adsorbed pollutant was determined from the difference in the Cyclophosphamide, Ifosfamide and Tamoxifen concentration between the non-inoculated and heat-killed control.

Samples were analyzed by UPLC-QqLiT to evaluate the quantitative degradation of each compound using the analytical method previously developed (Ferrando-Climent et al., 2013). Samples were also analyzed by high resolution mass spectrometry (HRMS) technology in order to identify the potential transformation products of parent cancer drugs.

2.5. Analysis of anticancer drugs

Anticancer drugs were quantified in wastewater samples using an analytical methodology previously described (Ferrando-Climent et al., 2013) for 10 target compounds: Azathioprine (AZA), Cyclophosphamide (CY), Ciprofloxacin (CIP), Docetaxel (DOC), Etoposide (ETO), Ifosfamide (IF), Methotrexate (MTX), Paclitaxel (PAC), Tamoxifen (TAM) and Vincristine (VIN).

Briefly, samples were filtered through 0.45 μm nylon membrane filters (Whatman, U.K.). A suitable volume of the chelating agent EDTA was added to all of them to a final concentration of 0.1% (g solute g^{-1} solution), as it is well known that it improves the extraction of some antibiotics such Ciprofloxacin (Cha et al., 2006; Hernandez et al., 2007; Gros et al., 2012). Pre-concentration of samples was performed by solid phase extraction (SPE) by the automatically extract system GX-271 ASPECTM (Gilson, Villiers le Bel, France). 50 mL of each sample was loaded at 1 mL min^{-1} in the Oasis HLB (200 mg, 6 mL) cartridge previously conditioned using 5 mL of methanol followed by 5 mL 0.1% formic acid solution at 2 mL min^{-1}. Elution was performed with 10 mL at a flow rate of 2 mL min^{-1} using pure methanol. The extract was evaporated under gentle nitrogen stream using a Reacti-Therm 18824 System (Thermo Scientific) and reconstituted with 500 μL of methanol–water (10:90, v/v). Finally, 5 μL of standard of internal standard mix at 10 ng μL^{-1} was added in the extract for internal standard calibration and to compensate possible matrix effect.

Chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3 column (50 mm × 2,1 mm i.d. 1,7 μm particle size; Waters Corp. Mildford, MA, USA) under positive electrospray ionization (PI). The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All transitions were recorded by using Multiple Reactive Monitoring Mode (MRM) and the data were acquired and processed using Analyst 2.1 software.

2.6. Identification of TPs of anticancer drugs

Analysis were performed using an UPLC (Accela 1250 chromatograph with autosampler Thermopal PAL AS) coupled to a LTQ-Velos Orbitrap from Thermo Scientific. The mass (MS) analysis was performed with an electrospray ionization (ESI) interface in positive ionization mode. Samples were injected in the system either after appropriate dilution (in the case of bench-scale experiments) or after off-line SPE pretreatment (Ferrando-Climent et al., 2013) (in the case of real wastewater samples obtained from bioreactor along the experiment).

Samples obtained from the bench-scale experiments were injected after dilution in the system and MS full-scan was acquired. Chromatograms obtained were compared in order to identify new chromatographic peaks generated during the biodegradation of the target compounds selected. Only those peaks produced along the experiments, that were not present in control samples, were considered as a TP candidate for further evaluation. Accurate masses of those candidates were selected included in a mass list to use it in a second set of analysis where samples extracts were acquired using data-dependant acquisition where only if the masses included in the candidate mass list were triggering; MS/MS experiments. MS/MS spectra obtained were carefully studied in order to propose a chemical structure. The assignment of fragmentation profile detected in MS/MS spectra to each TP candidate was supported by Mass Frontier (software from Thermo Science) which has enable the theoretical generation of mass fragments based on a proposed chemical structure.

2.7. Toxicity assays

A Microtox bioassay was used to perform toxicity test. This method is based on the percent decrease in the amount of light emitted by the bioluminescent bacterium *V. fischeri* upon contact with a filtered sample at pH 7. The half maximal effective concentration (EC50), was measured after 15 min. The toxicity of the liquid medium was expressed in percentages of EC50. Toxicity of samples from Erlenmeyer flasks treatments were assessed during the experiments.

2.8. Other analysis

Laccase activity was assessed during the experiments using an adapted procedure from a method for the determination of manganese peroxidase (MnP) previously described (Kaal et al., 1993). The reaction mixture used consisted in 200 μL of 250 mM sodium malonate at pH 4.5, 50 μL of 20 mM 2,6-dimethoxiphenol (DMP) and 600 μL of sample. DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV–vis spectrophotometer at 30 °C. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1} (Wariishi et al., 1992).

Biomass pellets dry weight was determined after vacuum-filtering the cultures through pre-weighed glass-fiber filters (Whatman GF/A, Barcelona, Spain). The filters containing the biomass pellets were dried at 105 °C to constant weight.

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instrument and Co (Yellow Springs, OH, USA).

3. Results

3.1. Fluidized bed bioreactor treatment of hospital effluent

The method detection limits (MDL) and the initial concentration of the anticancer drugs analyzed in the hospital wastewaters are shown in Table 1. The presence of anticancer drugs in the samples collected from the hospital in two occasions was dissimilar since chemotherapy drugs dosed per patient can vary from day to day. Only three anticancer drugs were initially detected in the Sample 1 (sterilized wastewater), the cytotoxic quinolone Ciprofloxacin, Tamoxifen and Etoposide (below limit of quantification). Concentration found for Ciprofloxacin (7000 ng L^{-1}) was similar of those detected by other authors in similar effluents (Gros et al., 2012; Verlicchi et al., 2012; Ferrando-Climent et al., 2013). Tamoxifen was detected at very high concentration (970 ng L^{-1}), much higher than ever found in other wastewater effluents (Langford and Thomas, 2009; Liu et al., 2010; Kosjek and Heath, 2011; Ferrando-Climent et al., 2013). Removals achieved for Ciprofloxacin and Tamoxifen at the end of the batch degradation experiments (8 d) were quite high, 84% and 91% respectively (Table 1 and Fig. 1).

In the case of the non-sterile hospital sample (Sample 2), Azathioprine, Ifosfamide and Etoposide were also detected besides Ciprofloxacin and Tamoxifen. Concentrations found in Sample 2 were in the range of those detected previously in hospital wastewater (Ferrando-Climent et al., 2013): 55 ng L^{-1} (Azathioprine), 2179 ng L^{-1} (Ciprofloxacin), 77 ng L^{-1} (Ifosfamide), 198 ng L^{-1} (Etoposide) and 45 ng L^{-1} (Tamoxifen) (Table 1).

In this case, the removals from wastewater at the end of the experiment were also high for Azathioprine (100%), Etoposide (100%), Ciprofloxacin (97%), Ifosfamide (61%) and Tamoxifen (48%) (Table 1).

Ciprofloxacin was eliminated more efficiently at non-sterile conditions, which can be attributed to a synergic degrading contribution of fungi and common fecal bacteria. In the case of tamoxifen, higher elimination was obtained under sterile conditions. Although tamoxifen sorption processes are playing a crucial role in the overall compound removal, other factors such the initial concentration (970 and 45 ng L^{-1} at sterile and non-sterile experiments respectively) in both samples and/or even an increase of fungi capacity for the elimination of this drug in absence of other microorganisms might explain these differences in the removals.

In both sterile and non-sterile treatments, the highest laccase activity (about 100 U L^{-1}) was reached at the end of the experiment. Although glucose was accumulated during the first hours, it was almost completely consumed after 24 h. The glucose and nitrogen consumption and the laccase production observed indicate that *T. versicolor* was active through the experiment (data not shown). Biomass concentration was constant during the treatment (2.3 g d.w. L^{-1} at the end) because the process was carried out at growth-limited conditions.

Table 1
Method detection limits (MDL), initial concentration in hospital effluents and removal of anticancer drugs after the batch bioreactor treatment in sterile (sample 1) and non sterile (sample 2) conditions (blq = below limit of quantification, ND = non detected).

Type of agent	Compound	MDL (ng L^{-1})	Sample 1 (sterilized wastewater)		Sample 2 (non-sterilized wastewater)	
			Concentration (ng L^{-1})	Removal (%)	Concentration (ng L^{-1})	Removal (%)
Cytotoxic quinolone	Ciprofloxacin	0.6	7000 ± 686	84	2179 ± 214	97
Antiestrogenic	Tamoxifen	1.0	970 ± 74	91	45 ± 3	48
Alquilant agent	Ifosfamide	1.7	ND	–	77 ± 6	61
	Cyclophosphamide	1.3	ND	–	ND	–
	Vincristine	9.2	ND	–	ND	–
Plant alkaloid (antimicrotubule agent)	Docetaxel	0.7	ND	–	ND	–
	Paclitaxel	5.5	ND	–	ND	–
Plant alkaloid (topoisomerase II inhibitor)	Etoposide	48	blq	100	198 ± 12	100
Anti-metabolites	Methotrexate	2.1	ND	–	ND	–
	Azathioprine	3.8	ND	–	55 ± 2	100

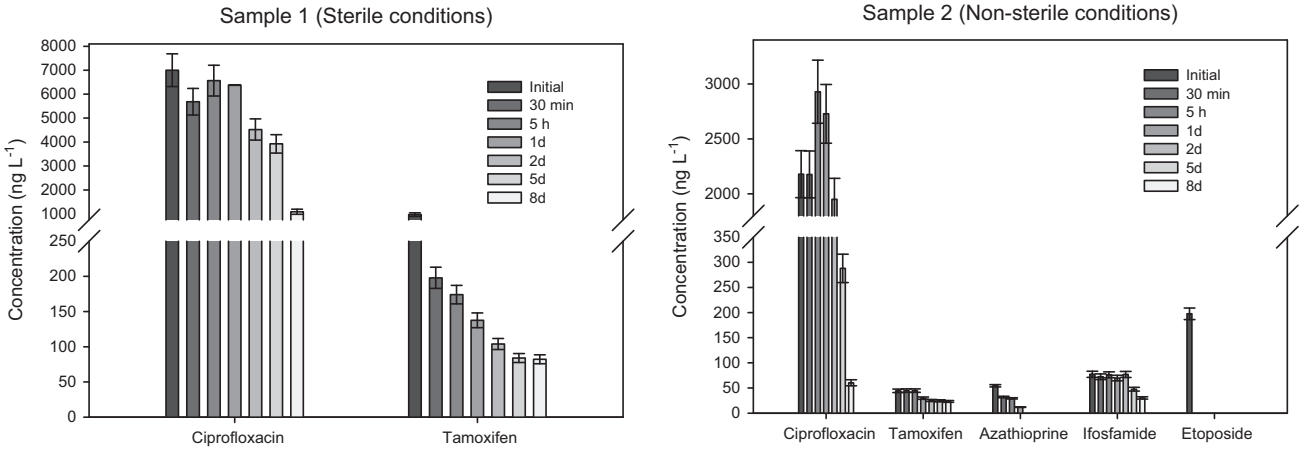

Fig. 1. Degradation of anti-cancer drugs present in both hospital wastewater sterile (samples 1) and non-sterile (sample 2) by *T. versicolor* batch fluidized bed reactor.

3.2. Degradation of Cyclophosphamide, Ifosfamide and Tamoxifen in experiments with synthetic water: Target and non-target analysis

In the light of results obtained from both hospital samples which were treated by the fluidized bed bioreactor, where Ifosfamide and Tamoxifen were partially eliminated from hospital wastewater, three anticancer drugs were selected for further comprehensive study of their degradation by *T. versicolor*: Ifosfamide, Tamoxifen and Cyclophosphamide. Despite Cyclophosphamide was not present in any of hospital wastewater samples analyzed

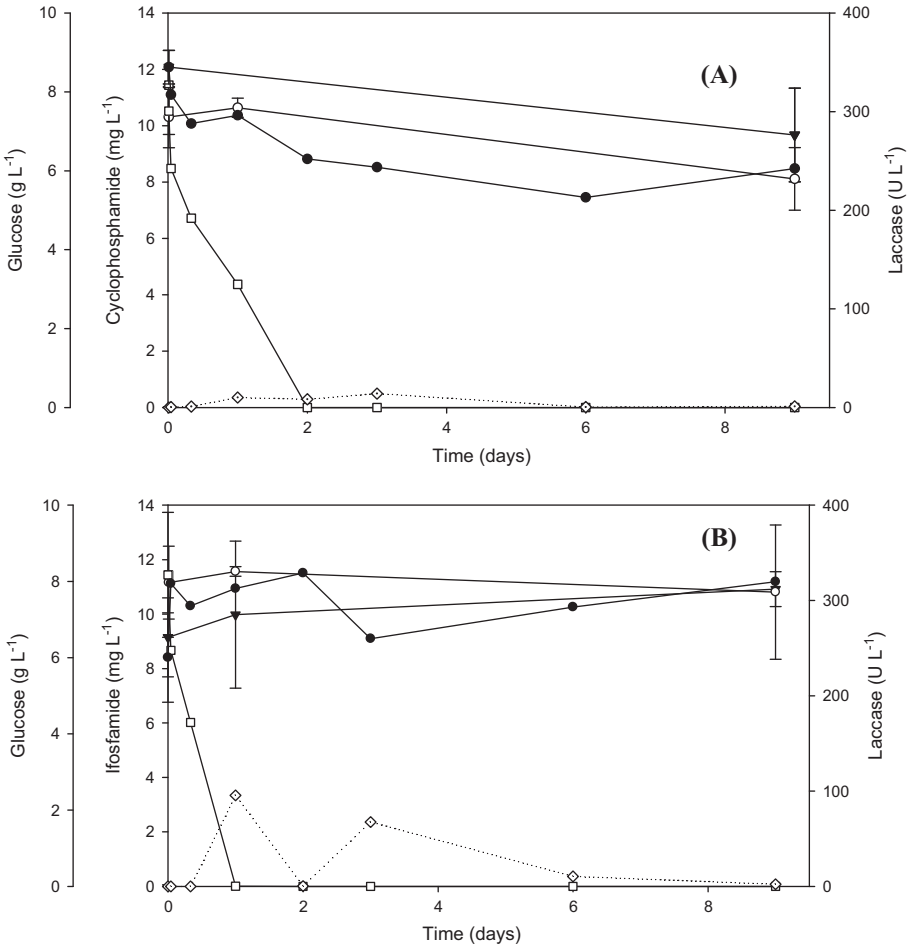

Fig. 2. (A) Time-course of Cyclophosphamide (CY) degradation spiked at 10 mg L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Uninoculated control (▼), heat-killed experiment (○), inoculated experiment (●). Levels of Glucose (□) and laccase activity (◇). (B) Time-course of Ifosfamide (IF) degradation spiked at 10 mg L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Symbology: uninoculated controls (▼), heat-killed (○), Glucose (□), laccase activity (◇), and experimental cultures (●).

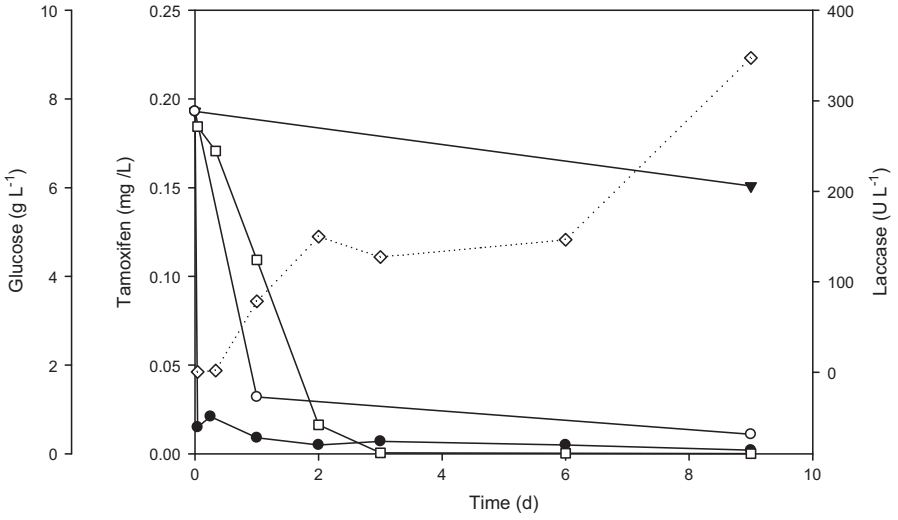

Fig. 3. Time-course of Tamoxifen (TAM) degradation spiked at 0.3 mg L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Symbology: uninoculated controls (▼), heat-killed (○), Glucose (□), laccase activity (◇), and experimental cultures (●).

in this work, it was selected for a more detailed study since it is still one of the most important anticancer drugs: it has been detected in wastewaters (both hospitals effluents and influents of WWTPs) (Buerge et al., 2006; Gómez-Canela et al., 2012; Ferrando-Climent et al., 2013, 2014) and their elimination during conventional activated sludge treatments has been described as negligible (Zhang et al., 2013; Ferrando-Climent et al., 2014). This is the first time that the removal of this drug is studied with *T. versicolor*.

Individual experiments with two control samples (non-inoculated and heat-killed) in addition to the inoculated samples spiked with Cyclophosphamide, Ifosfamide or Tamoxifen were performed in triplicate in order to study the degradation of these compounds as well as the formation of their potential TPs. Cyclophosphamide and Ifosfamide showed neither degradation nor sorption by *T. versicolor* at 10 mg L^{-1} (Fig. 2A and B respectively). Cyclophosphamide showed a similar very slight decrease in both controls and culture, which might be due to some kind of affinity of this compound for the glass material of the recipient (Fig. 2). Photodegradation of these compounds is discarded as all the experiments were performed in darkness. These results are in contrast with the results previously described, where Ifosfamide was slowly removed from the hospital effluent in the experiment performed with the reactor at non-sterile conditions after 5 d of treatment. Concentration of Ifosfamide in real wastewater was in the range of ng L^{-1} whereas it was spiked at 10 mg L^{-1} in the synthetic water for the individual experiments. The high concentration of the pollutant in the experiments with synthetic water could have inhibited WRF degradation potential. In order to discard this possibility, a second set of experiments with Ifosfamide spiked in synthetic samples was performed but using lower concentration (100 μg L^{-1}). No degradation was also observed in this experiment (supplementary material S1) and therefore high removal in real wastewater experiments should be attributed to other processes in the non-sterile reactor such synergic biodegradation pathways by WRF with other microorganisms (bacteria), which are present in the hospital effluent (Haia et al., 2012; Nguyen et al., 2013). The elimination of Ifosfamide observed in bioreactor experiments only began after 5 d, although it was not completely removed. Ifosfamide and Cyclophosphamide contained halogenated atoms (Cl, F) in their molecular structure and they may contribute to hinder aerobic biodegradation, since halogenated functional groups decrease the electron density of the reaction site (Tadkaew et al., 2011).

In the case of Tamoxifen, it showed an elimination of 92% and 99% after 1 h and 9 d respectively in the experiments performed with synthetic wastewater spiked at 0.3 mg L^{-1} and treated with *T. versicolor* (Figs. 3 and S2). Most of the removal observed can be attributed to sorption processes since the heat-killed experiment showed 83% and 94% of elimination from the water after 24 h and 9 days respectively (Fig. 3). In fact, Tamoxifen, has a high hydrophobicity ($\log K_{ow} = 6.30$), and is quite prone to be sorbed onto the fungi surface because of its physical–chemical characteristics. Nevertheless, after the sorption process, biodegradation is likely taking place since two potential TPs, with related chemical structure of Tamoxifen, were found in the liquid phase during the experiment. In particular, two suspected hydroxylated forms of Tamoxifen (TP 388 A and B) were detected after 30 min of fungal treatment and their concentration were increasing along the experiment while Tamoxifen was at very low concentration in the liquid phase (Figs. 4 and S2). These hydroxylated compounds were not detected in the heat-killed controls. A possible degradation pathway would involve the intracellular transformation of Tamoxifen by the cytochrome P-450 system, which typically yields hydroxylated metabolites as the products, and further excretion to the liquid medium (Cerniglia, 1997). In contrast to Cyclophosphamide and Ifosfamide, the rapid degradation of

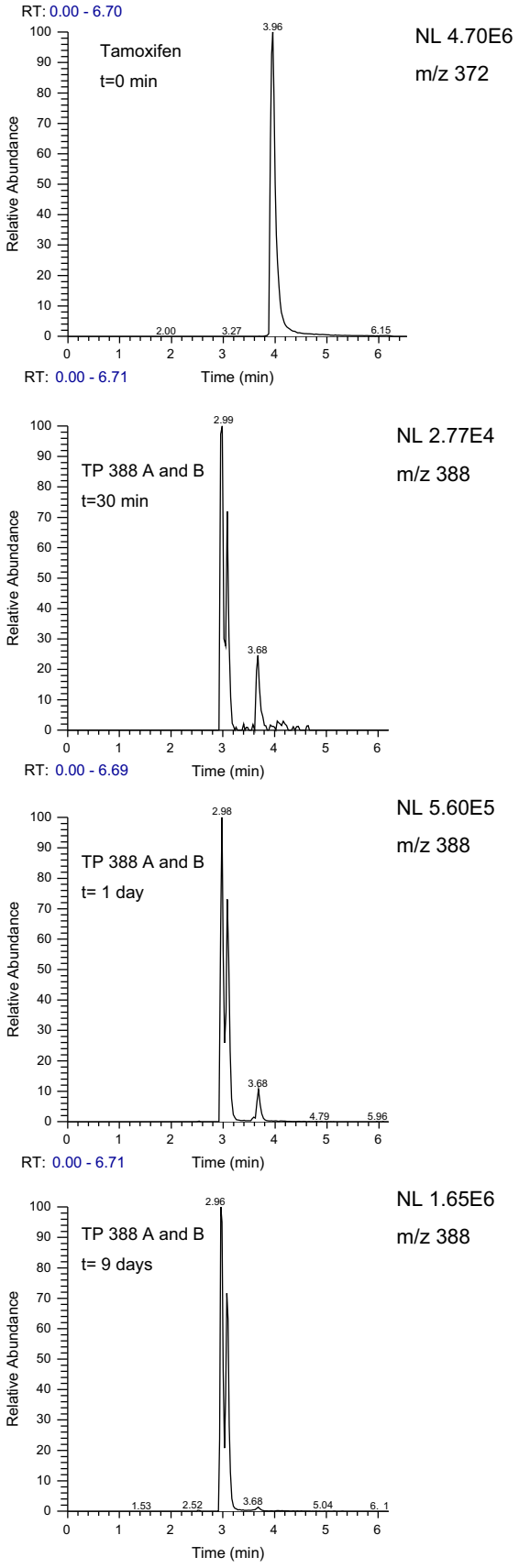

Fig. 4. Extract ion chromatograms for the tamoxifen at initial conditions and the two suspected transformation products (TP 388 A and B) which have the theoretical *m*/*z* of 388.22710 during the culture experiment ($t = 0$; $t = 30$ min, $t = 1$ d and $t = 9$ d).

Tamoxiflen would be in accordance to the absence of halogenated functional groups in the structure and to the presence of electron-donating group (amino group), which decreases the electron deficiency and increases aerobic biodegradation rates (Tadkaew et al., 2011).

In order to identify the molecular ions of these TPs, to propose their empirical formula and to elucidate their chemical structure, data-dependent experiments combining full-scan MS data (at 60.000 units of resolutions) with the product ion spectra were acquired using a quadrupole-orbitrap MS instrument. Samples diluted (1:1) from the synthetic water experiments were used for this purpose.

Tamoxifen has a *m*/*z* of 372.2322 as the protonated form with a formula of $C_{26}H_{30}ON$. The hydroxylated form of Tamoxifen which is proposed here has the molecular formula of $C_{26}H_{30}O_2N$ (the hydroxylation has an increment of 16 Da) for the protonated form with a *m*/*z* of 388.2271 as theoretic mass. As it is shown in Fig. 5a, two hydroxylated forms are suspected to be present in during the experiment, peak called "A" (RT = 2.96 min) corresponds with the *m*/*z* 388.2268 (TP 388 A) and the peak called "B" (RT = 3.08 min) corresponds with the *m*/*z* 388.2272 (TP 388 B). The molecular formula for both peaks where proposed based on the calculation of the elemental composition for peak A (*m*/*z* 388.2269) and peak B (*m*/*z* 388.2272). Accurate molecular masses of peak A and B were

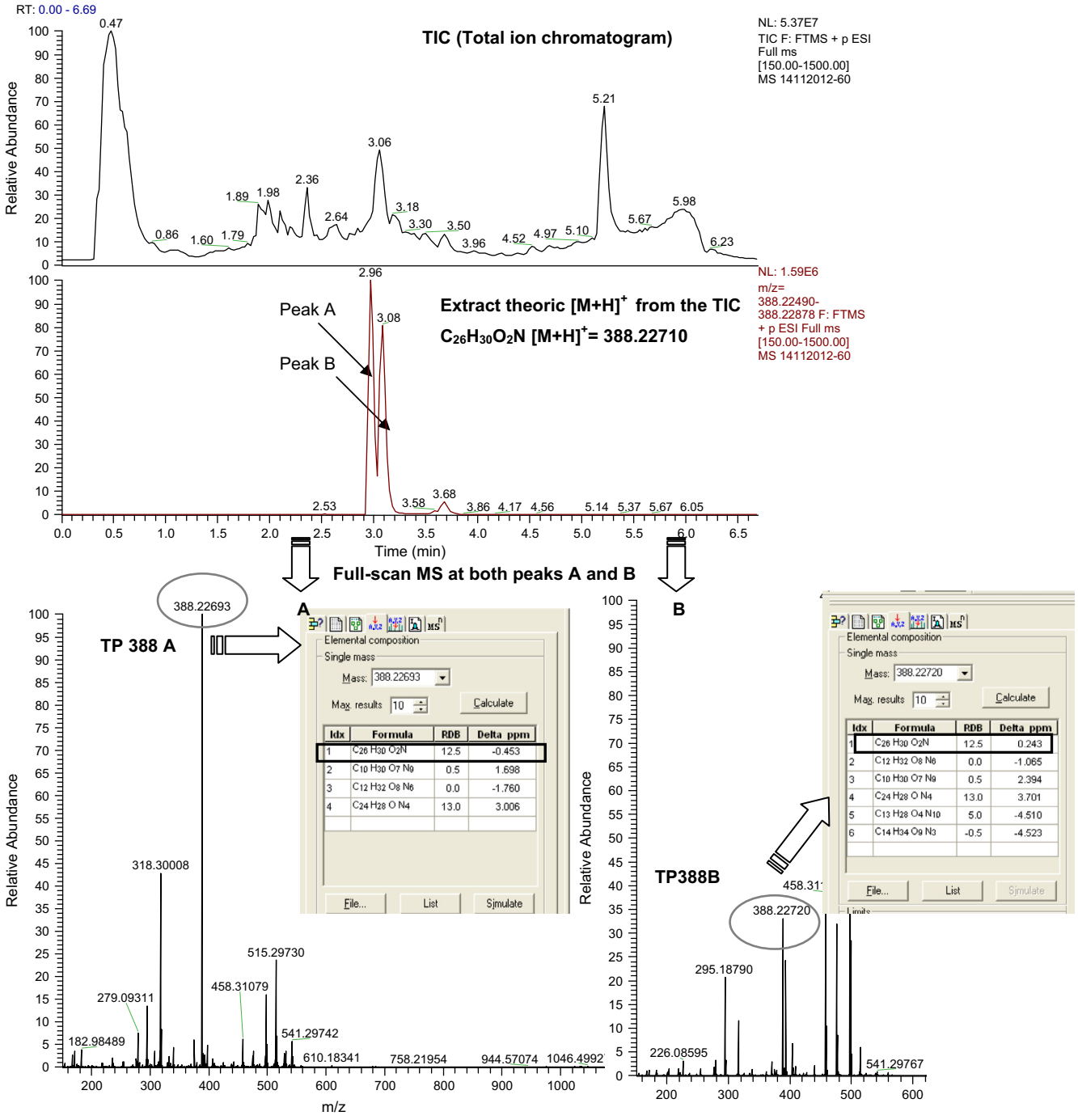

Fig. 5a. Identification of TP 388 A (Peak A) and 388 B (Peak B). Extraction of the exact masses $[M + H]^+$ from the TIC, evaluation of full-scan MS at both peaks (A and B) and calculation of the elemental composition at 388.2269 *m*/*z* (A)/388.2272 *m*/*z* (B) and 388.2271 *m*/*z* (theoretical) where the difference between the measured and theoretical exact mass has to be lower than 5 ppm.

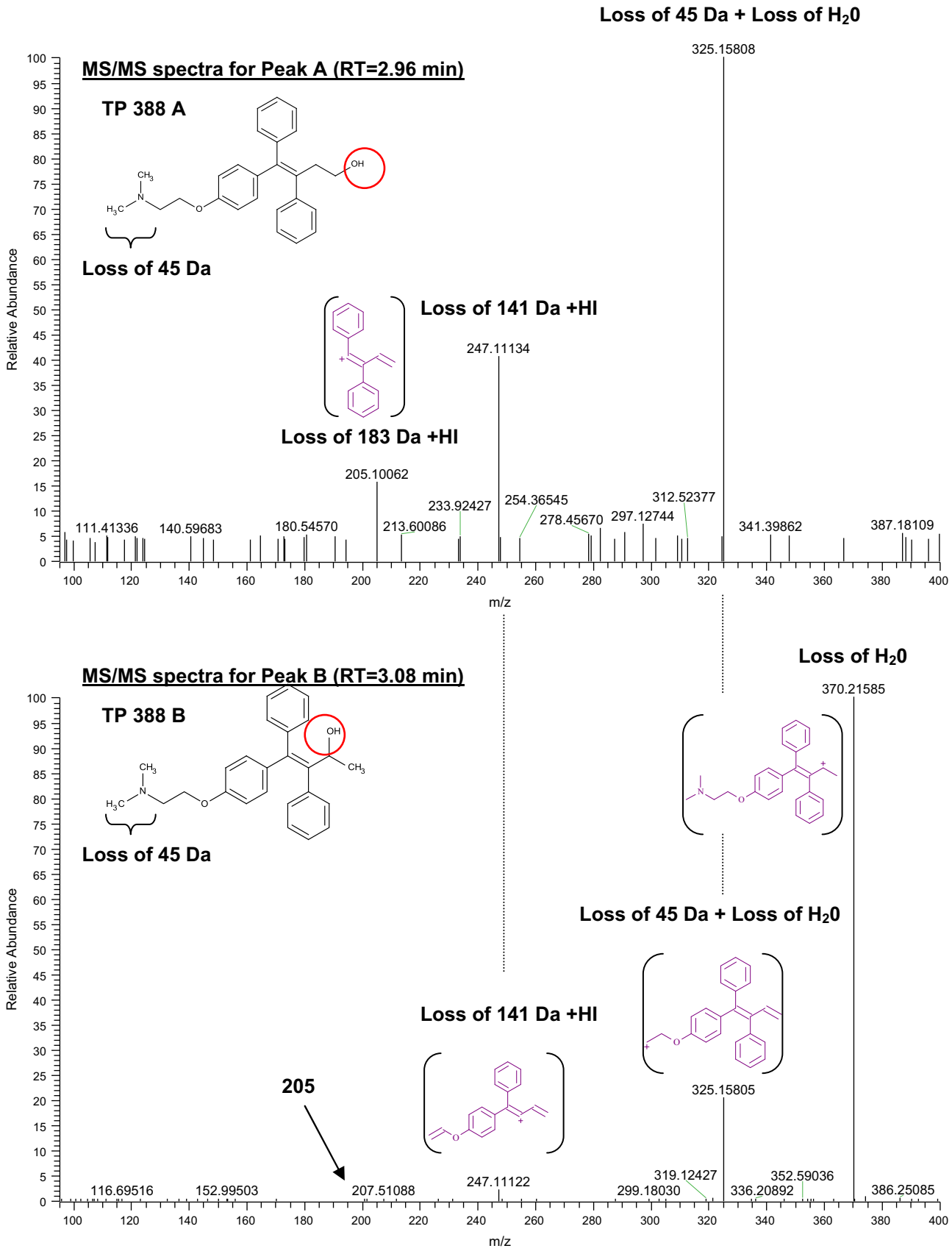

Fig. 5b. Evaluation of the MS/MS spectra for the transformation products proposed: TP 388 A (Peak A) and 388 B (Peak B). Both TPs have the same mass $[M + H]^+$ but they shown differences in the abundance and presence of some the fragments due to the different position on the group hydroxyl which is affecting the mechanism of fragmentation.

compared with the theoretical mass (*m*/*z* 388.2272) of hidroxytamoxifen and a difference lower than 5 ppm (−0.45 and 0.24 ppm respectively) were found.

The next step for the identification of these potential TPs was the evaluation of the MS/MS spectra where the fragmentation profile for both peaks was quite similar.

The mass fragments obtained for the TP 388 A were 325, 247 and 205 (Fig. 5b). The fragment 325 correspond to the loss of the amine side chain (45 Da) through the fragment 343 which immediately loss H_2O (18 Da), showing a total loss of 63 Da. The fragment 247 correspond to a loss of benzene unit (78 Da) from the fragment 325 with a total loss of 141 Da and finally the fragment 205 correspond with a loss of 42 Da (ketone HC—HC=O) (view supplementary material, S3). In case of TP 388 B, the mass fragments obtained were, as in the case of TP 388 A, 325, 247 and 205 (Fig. 5b), but also fragment 370 was detected. This last fragment might be formed due to the different position of the hydroxyl group (—OH) in the molecule proposed for the TP 388 B. Based on the different fragmentation spectra of the TPs, two positional isomers (position of hydroxyl group; —OH) were proposed (Fig. 5b). In the case of TP388 B the suggested position of the —OH may be that place where it is conjugated with the olefin and the two aromatic rings of the molecule (Fig. 5b). This means that a loss of H_2O (18 Da) may be possible because of the charge generated onto the molecule in this position is stabilized by resonance with olefin and the aromatic rings whose can delocalized this charge. Therefore the fragment of 370 is generated by a loss of 18 Da but only in case of TP 388 B. On the contrary, the suggested position of hydroxyl in the TP 388 A is not conjugated with the olefin, and it cannot be possible to generate this additional stability which allows the observation of the fragment 370 (see supplementary material S3). Thereby the profile of both MS/MS spectra confirms the presence of two positional isomers with the protonated mass of 388.2271 *m*/*z*. The mass fragments obtained in the MS/MS spectra were supported by the same findings obtained using the software Mass Frontier (Thermo Science) (Fig. S2) which allows simulating the potential mass fragments in the working conditions (ionization source, mode, etc.) for the molecule proposed.

Based on a semi-quantification approach (using the chromatographic areas) (Rubirola et al., 2014), relative removal percentage values of tamoxifen removal (0.72% at the end) and relative formation percentage of TPs formation (nothing at initial and TP 388 A: 14.62% and TP 388 B: 10.70% at the end) were calculated. It might be concluded that, at the end of the synthetic water experiments, 25–30% of the total removal of the target compound is due to the TPs formation (supplementary material S3).

In the time-course of the synthetic water experiments, the activity of laccase was measured reaching different values for the three tested compounds. Laccase activity was around 100 U L^{-1} for Cyclophosphamide and Ifosfamide while it reached values up to 350 U L^{-1} for the Tamoxifen. Both Cyclophosphamide and Ifosfamide are perhaps affecting the enzymatic system of the WRF but not killing the microorganism since the glucose is almost consumed at the end of the experiment for three target compounds. Laccase activity can play an important role in the biodegradation of different pharmaceuticals such as analgesics (diclofenac and naproxen) (Marco-Urrea et al., 2009, 2010) or endocrine disruptors (Jonsson, 1990; Catapane et al., 2013), but here we cannot establish a direct correlation between the degradability of the tested anticancer drugs with this enzyme.

Finally, the Tamoxifen TPs found at synthetic water experiments were screened in the real hospital samples treated with WRF at the bioreactor. Cytotoxic drugs are found in hospital effluents at low concentrations (Table 1) and therefore, previous to the MS analysis, SPE preconcentration of these samples was performed following the methodology described at Ferrando-Climent et al., 2013. The SPE extracts were analyzed employing the same non-target approach mentioned in this section. However it was not possible to detect any of these TPs in the real samples maybe due to the low concentration of Tamoxifen. Taking into account its initial concentration in hospital wastewater (970 and 45 ng L^{-1}) and the relative concentrations of its TPs at synthetic water experiments (10–14%), the levels of TPs that might be present in these effluents were probably close to limit of detection. Unfortunately, it is not possible to assess the efficiency of the SPE process for the preconcentration of TPs recoveries because of the absence of reference standards to perform recovery studies.

3.3. Toxicity evaluation

The bioassay with bacteria *V. fischeri* (Microtox test) was performed in order to evaluate the feasibility of the treatment as well as the potential toxicity of the TPs generated along the process with WRF. Microtox results can only refer to the total toxicity of the liquid medium contained in the flasks during the batch experiment, without distinguishing individual toxicity of each compound present in the sample. Despite of this, it provides useful information about the toxicity of the process comparing the initial samples, the controls and the liquid medium at the end of the experiment.

Abiotic controls of Cyclophosphamide and Ifosfamide as well as the samples taken at initial time and at the end of the synthetic water experiments with *T. versicolor*, showed a EC_{50} (15 min) below than 5%. These compounds are intrinsically cytotoxics consequently the value of the EC_{50} exhibited a high toxicity not only at the beginning but also at the end of the experiment where these compounds remained inalterable.

In the case of Tamoxifen, abiotic controls and the samples taken during the synthetic water experiment with *T. versicolor* showed a EC_{50} (15 min) around 43%, which means that this compound and also their transformation products have no toxicity for the bacteria *V. fischeri* at the concentrations where these experiments were performed. However, it could be interesting in further work, to study other type of effects such endocrine disruptor effects which is more related with the pharmaceutical activity of this anticancer drug (Williams et al., 2007).

4. Conclusions

Most of the studied anticancer drugs were removed form hospital wastewater using *T. versicolor* in comparison with the conventional biological treatments, which are inefficient in eliminating these compounds. Fungal treatment was not efficient in eliminating Cyclophosphamide and Ifosfamide, which might require more specific biodegrading systems (bacteria, another fungi, etc.) able to degrade this type of chemical structures. Conversely, Tamoxifen showed a total removal from the wastewater (by combined sorption-biodegradation processes). Two compounds (tamoxifen hydroxylated positional isomers) were identified as derived from biodegradation of Tamoxifen through the WRF activity.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.03.051.

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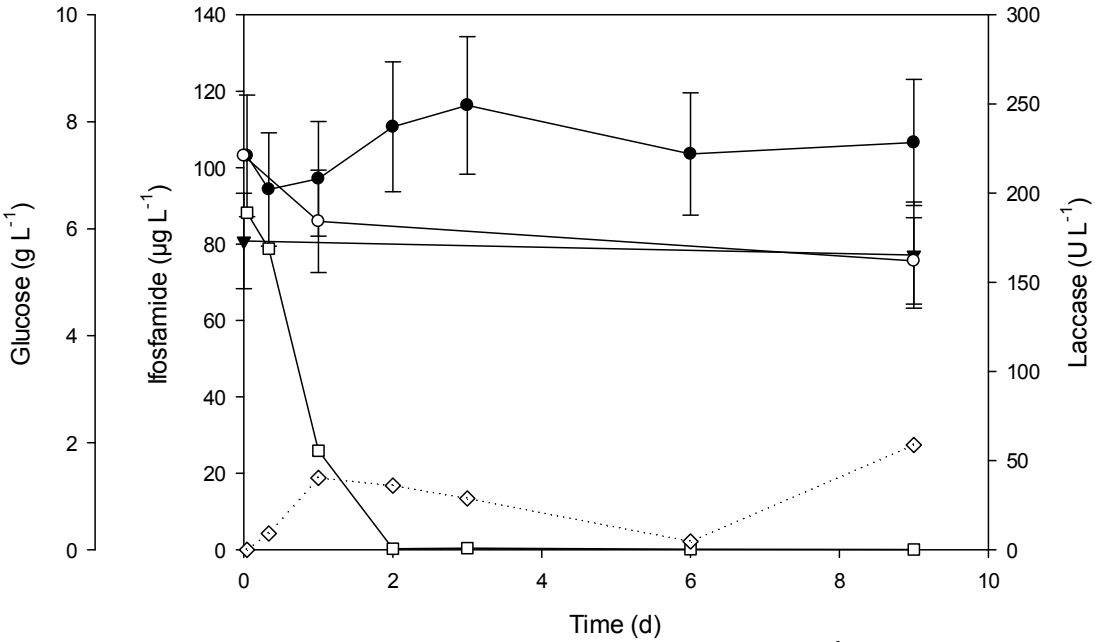
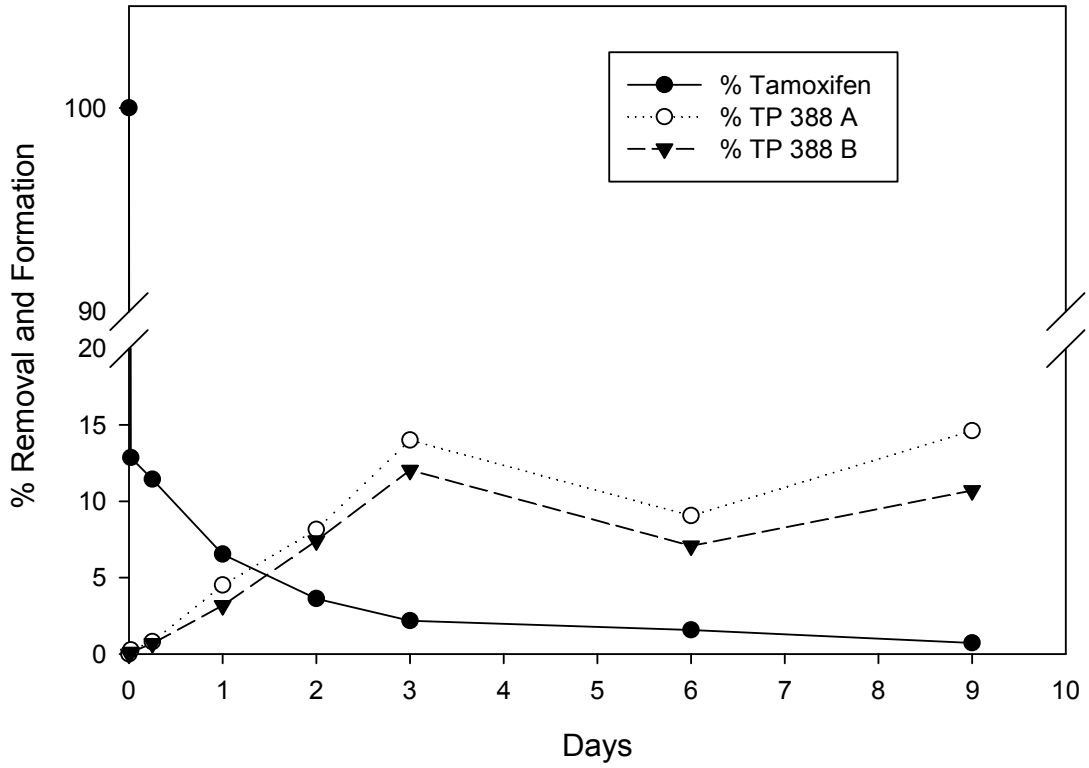
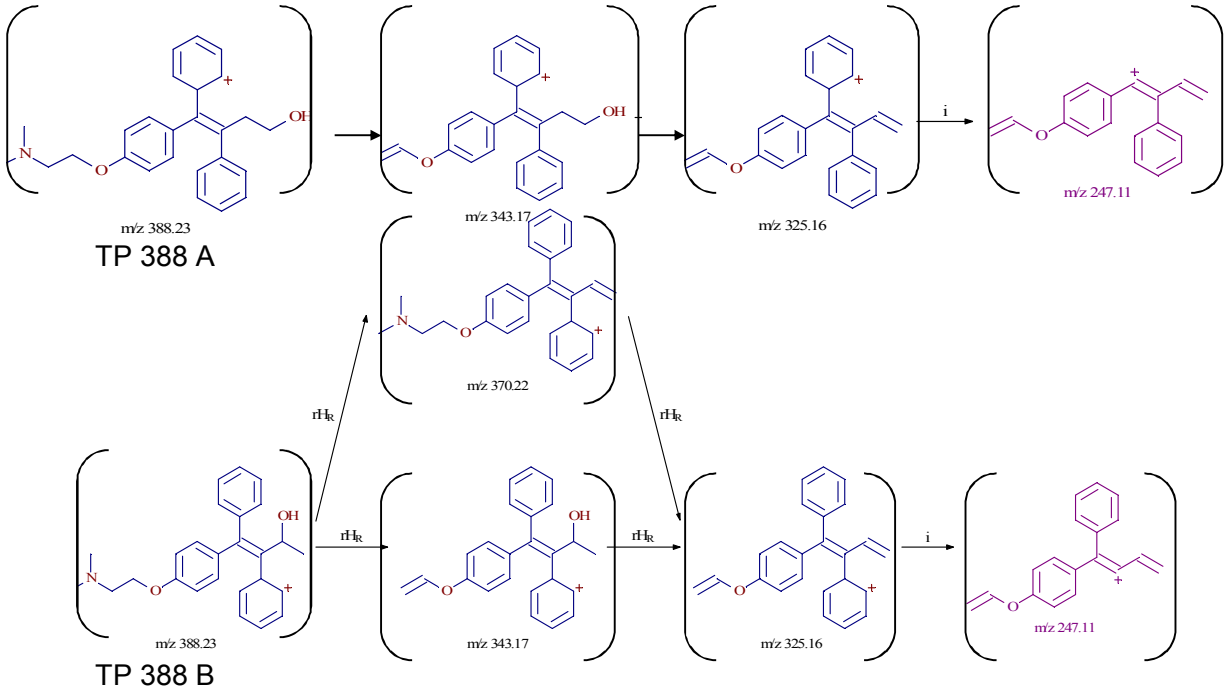


S 1. Time-course of Ifosfamide (IF) degradation spiked at 100 µ L^{-1} by *Trametes versicolor* pellets in Erlenmeyer flask. Symbology: uninoculated controls (▼) , heat-killed (○), Glucose (□), laccase activity (◇), and experimental cultures (◆).



S 2. Elimination of tamoxifen in batch experiment and formation of TPs during the experiment performed using *T. versicolor* at initial tamoxifen concentration of 0.3 mg/l. For each compound, relative areas to the initial MTP area were calculated and represented along the time using percentage values.



S 3. Fragmentation pathway suggested by Mass Frontier software for the proposed TP 388 A and B.

5.2. Elimination study of the chemotherapy drug Tamoxifen by different advanced oxidation processes: transformation products and toxicity assessment. Ferrando-Climent et al.

Laura Ferrando-Climent[a,b], Rafael González-Olmos[c], Alba Anfruns[d], Ignasi Aymerich[a], Lluis Corominas[a] Damià Barceló[a, e] , Sara Rodríguez-Mozaz[a*]

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Abstract

Tamoxifen is a chemotherapy drug considered as recalcitrant contaminant (by its low biodegradability in conventional activated sludge wastewater treatment), bioaccumulative, ubiquitous, and potentially hazardous for the environment. This work studies the removal of Tamoxifen from water by advanced oxidation processes, paying special attention to the formation of transformation products (TPs) and to the evolution of toxicity (using the Microtox® bioassay) during the oxidation processes. Five types of treatments were evaluated combining different technologies based on ozone, hydrogen peroxide and UV radiation: i) O3, ii) O3/UV, iii) O3/H2O2 (peroxone), iv) UV and v) UV/H2O2. Complete removal of tamoxifen was achieved after 30 minutes for all the treatments carried out with O3 while a residual concentration (about 10% of initial one) was observed in the treatments based on UV and UV/H2O2 after 4 hours of reaction. An increase of toxicity was observed during the oxidation processes, indicating that TPs were formed which were more toxic than tamoxifen. 8 TPs were tentatively identified and one (non-ionizable molecule) was suspected to be present by using ultra high performance liquid chromatography coupled to high resolution mass spectrometry. In the case of ozone-based treatments the increase in toxicity was attributed to the presence of some of the TPs identified, whereas in the case of UV-based treatments there was no clear correlation between toxicity and the identified TPs.

Keywords: anticancer drugs; Tamoxifen; persistent pollutant; HRMS; advanced oxidation processes.

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Editor of Chemosphere

Girone, July 18th, 2015

Dear Editor,

Herewith I enclose the manuscript entitled " Elimination study of the chemotherapy drug tamoxifen by different advanced oxidation processes: transformation products and toxicity assessment", by Laura Ferrando-Climent, Rafael Gonzalez-Olmos, Alba Anfruns, Ignasi Aymerich, Lluis Corominas, Damià Barceló and Sara Rodriguez-Mozaz to be considered for publication in Chemosphere Journal. With the submission of this manuscript I would like to point out that it has not been published, accepted for publication or under editorial review for publication elsewhere.

The present study describes the removal of Tamoxifen from water by advanced oxidation processes, paying special attention to the formation of transformation products (TPs) and to the evolution of toxicity (using the Microtox® bioassay) during the oxidation processes. Five types of treatments were evaluated combining different technologies based on ozone, hydrogen peroxide and UV radiation: i) O3, ii) O3/UV, iii) O3/H2O2 (peroxone), iv) UV and v) UV/H2O2. Complete removal of tamoxifen was achieved. 8 TPs were tentatively identified and one (non-ionizable molecule) was suspected to be present by using ultra high performance liquid chromatography coupled to high resolution mass spectrometry. An increase of toxicity was observed during the oxidation processes, indicating that TPs were formed which were more toxic than Tamoxifen.

To the author knowledge's, this is the first time that tamoxifen degradation has been studied under advanced oxidation processes integrating the removal, identification and semiquantitation of TPs as well as the assessment of toxicity along the process. Moreover, this is the first time that these TPs have been detected and identified, except one previously identified tamoxifen TP.

I hope that this manuscript is of right scope and merits for publication in Chemosphere Journal as a regular research paper.

Thank you for your consideration

Sincerely yours,

PhD Sara Rodriguez-Mozaz

Research scientist

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Highlights

- Complete removal of tamoxifen in all the AOP studied.
- 8 TPs were tentatively identified for the first time.
- Drastic increase of toxicity along all the experiments.
- At least one non-ionizable molecule was suspected to be present.
- Toxicity is due to the presence of TPs

Elimination study of the chemotherapy drug tamoxifen by different advanced oxidation processes: transformation products and toxicity assessment.

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Keywords: anticancer drugs; tamoxifen; persistent pollutant; HRMS; advanced oxidation processes; PCA.

Abstract

Tamoxifen is a chemotherapy drug considered as recalcitrant contaminant (by its low biodegradability in conventional activated sludge wastewater treatment), bioaccumulative, ubiquitous, and potentially hazardous for the environment. This work studies the removal of Tamoxifen from water by advanced oxidation processes, paying special attention to the formation of transformation products (TPs) and to the evolution of toxicity (using the Microtox® bioassay) during the oxidation processes. Five types of treatments were evaluated combining different technologies based on ozone, hydrogen peroxide and UV radiation: i) O_3, ii) O_3/UV, iii) O_3/H_2O_2 (peroxone), iv) UV and v) UV/H_2O_2. Complete removal of tamoxifen was achieved after 30 minutes for all the treatments carried out with O_3 while a residual concentration (about 10% of initial one) was observed in the treatments based on UV and UV/H_2O_2 after 4 hours of reaction. An increase of toxicity was observed during the oxidation processes, indicating that TPs were formed which were more toxic than tamoxifen. 8 TPs were tentatively identified and one (non-ionizable molecule) was suspected to be present by using ultra high performance liquid chromatography coupled to high resolution mass spectrometry. In the case of ozone-based treatments the increase in toxicity was attributed to the presence of some of the TPs identified, whereas in the case of UV-based treatments there was no clear correlation between toxicity and the identified TPs.

1. Introduction

Tamoxifen is a non-steroidal anti-estrogen frequently used to treat breast cancer as well as a prophylactic agent in women with a significant risk of developing the disease (DellaGreca et al., 2007) . Several studies have shown the occurrence of this compound in hospital effluents, sewage (input and output) and surface waters at different range of concentrations (51 - 200 ng/L) in almost all the samples analyzed (Roberts and Thomas, 2006);(Besse et al., 2012);(Ashton et al., 2004);(Ferrando-Climent et al., 2015);(Ferrando-Climent et al., 2013);(Ferrando-Climent et al., 2014). Tamoxifen is being released in the aquatic environment since it is well-known for being non-biodegradable during conventional wastewater treatments (Ferrando-Climent et al., 2015);(Ferrando-Climent et al., 2014). The presence of this drug in the aquatic environment is concerning due to its known toxicity, endocrine disruption effects and bioaccumulation potential (Jean et al., 2012). Moreover, recent studies have revealed that mixtures of tamoxifen with other anticancer drugs in real samples possess higher toxicity than the individual drug (Mater et al., 2014).

Tamoxifen is released in the sewer system via hospital and domestic wastewater and, since it is not totally removed from the wastewaters during conventional biological treatments, is further discharged into natural receiving waters (Kovalova, 2009);(Kosjek and Heath, 2011);(Ferrando-Climent et al., 2013);(Negreira et al., 2013);(Ferrando-Climent et al., 2014). A tertiary treatment of WWTP effluents might be a solution for an efficient removal of tamoxifen, avoiding its entrance into the aquatic environment. However, very few studies have evaluated the elimination of tamoxifen by non-biological

90 treatments (Chen et al., 2008);(Zhang et al., 2013);(DellaGreca et al., 2007);(Negreira et
91 al., 2015). DellaGreca et al. (2007) reported only a 50% of conversion of tamoxifen to
92 other sub-products under sunlight exposure whereas several subproducts were identified
93 after one month experiment (DellaGreca et al., 2007). Negreira et al. (2015) reported the
94 persistence of tamoxifen along a chlorination treatment (Negreira et al., 2015) whereas
95 their human metabolites were almost removed. They also identified several disinfection
96 by-products of tamoxifen.
97 Advanced oxidation processes (AOPs) have demonstrated their effectiveness for the
98 removal of large number of contaminants, being feasible their implementation at
99 wastewater treatment plants, frequently as a tertiary treatment (Klavarioti et al., 2009).
100 AOPs are based in the power of hydroxyl radicals ($^{\cdot}OH$), which are very reactive, short
101 half-life oxidants. Hydroxyl radicals can be generated by using combined treatments,
102 such ultraviolet radiation/hydrogen peroxide (UV/H_2O_2), ozone/hydrogen peroxide
103 (O_3/H_2O_2), ultraviolet radiation/ozone (UV/O_3), Fenton's process (ferrous iron and
104 hydrogen peroxide), titanium dioxide/ultraviolet radiation (TiO_2/UV), etc. Till the date,
105 only two studies have evaluated the removal of tamoxifen using AOPs. Chen et al. 2008
106 have studied the removal of tamoxifen using either ozone or ultraviolet
107 radiation/hydrogen peroxide (Chen et al., 2008). In their work, tamoxifen was completely
108 eliminated from synthetic water. Very recently W. Li et al. 2016 have studied the removal
109 of tamoxifen under different doses of ozone being the anticancer drug completely
110 eliminated from the wastewater using 0.6 mg of ozone per mg of dissolved organic matter
111 (Li et al., 2016). However, neither transformation products (TPs) generated along the
112 time-course experiments nor the toxicity of the treated water were studied in both works

(Chen et al., 2008; Li et al., 2016). In this regard, further comprehensive studies on the removal of this anticancer drug from sewage using alternative water treatments are needed.

In this work the performance of advanced oxidation treatments based on combination of ozone, ultraviolet radiation (UV) and hydrogen peroxide was studied for the elimination of tamoxifen; where the potential detoxification of the effluent derived from these treatments was explored paying special attention in the generation of transformation products. The objective of this work was to study the efficiency of five treatments: i) Ozone (O_3), ii) Ozone/UV (O_3/UV), iii) Ozone/Hydrogen peroxide (O_3/H_2O_2), iv) UV and v) UV/Hydrogen peroxide (UV/H_2O_2), for the elimination of tamoxifen from water. Tamoxifen was monitored by LC-MS/MS along the five experiments performed. Identification of tamoxifen TPs was also studied in the same samples by high resolution mass spectrometry (HRMS). Toxicity of the effluent was assessed by Microtox® analysis in all the time-course experiments. Principal components analysis (PCA) was applied to the experimental data to identify correlations between toxicity and the TPs generated during the oxidation processes. To the best of our knowledge, this is the first time that identification and toxicity assessment of the TPs of tamoxifen along AOPs processes has been studied under all these conditions.

2. Materials and methods

2.1. Standard preparation and reagents.

Tamoxifen Citrate was purchased from the European Directorate for the Quality of Medicines and Healthcare (EDQM) Reference Standards (Strasbourg, France).

Isotopically labelled compound, used as internal standard, [$^{13}C_6$]-Tamoxifen Citrate was purchased from Toronto Chemical Research Inc. (Canada). HPLC-grade Water and HPLC-grade acetonitrile and water (LiChrosolv) were supplied by Merck (Darmstadt, Germany). Chromatographic reagents i.e. formic acid 98% (HCOOH) were provided by Scharlab (HPLC-grade). Hydrogen peroxide solution ACS reagent (30 w.t. % in H_2O), Titanium (IV) oxysulfate solution (Fluka) and Sodium thiosulfate (Fluka) were purchased from Sigma- Aldrich. Individual stock standard solution of tamoxifen as well as the internal standard were prepared on a weight basis in methanol at 1 mg/mL and kept frozen at -20ºC.

2.2. Advanced oxidation experiments

The degradation of tamoxifen was assessed in five advanced oxidation processes: : i) O_3, ii) O_3/UV, iii) O_3/H_2O_2, iv) UV and v) UV/H_2O_2. All the experiments were performed in 500 mL MQ water spiked with tamoxifen at 100 $\mu g \cdot L^{-1}$, higher level than those found in environment ($ng \cdot L^{-1}$ levels) with the aim of assessing the potential generation of TPs with enough sensitivity and also taking into account the low solubility of tamoxifen in water (300 $\mu g \cdot L^{-1}$) (Roberts and Thomas, 2006; Ferrando-Climent et al., 2013, 2014). All the experiments were performed in a cylindrical glass reactor covered by aluminum film at room temperature. The sampling was set-up at times: 5 min, 15 min, 30 min, 1 h, 2 h, 3 h and 4 h and sodium thiosulfate was added to each sample at a concentration of 24 $mg \cdot L^{-1}$ to consume remaining hydrogen peroxide and quench $^{\cdot}OH$ radicals, guaranteeing the end of the oxidation reactions.

For UV treatments, a low-pressure mercury lamp emitting at 254 nm was used. In the ozonation treatments, the ozone stream was generated by an ozone generator (Anseros COM-AD-02, Actualia) coupled with an oxygen concentrator (Actualia HCM 2003). The ozone stream was 125 $L.h^{-1}$ with an ozone concentration of 48 $g.cm^{-3}$ (within the recommended range of ozone disinfection processes) ()). In the assays with H_2O_2 initial concentration was set at 20 $mg \cdot L^{-1}$ and kept constant along the experiments (further H_2O_2 addition) in order to keep the concentration of the $^{\cdot}OH$ radical constant in the reactor. Concentration of hydrogen peroxide was monitored during the experiments at 45 min with the aim of keeping the amount of hydroxyl radicals. For this purpose, samples were measured at 405 nm by UV spectrometry using titanium oxysulfate solution for determination of peroxides (Vogel, 2000).

2.3. Quantification of tamoxifen

Tamoxifen was quantified directly in synthetic samples from bench scale-experiments using an analytical methodology previously described (Ferrando-Climent et al., 2013). Chromatographic separation was carried out with a Ultra-Performance liquid chromatography system (Waters Corp. Mildford, MA, USA) equipped with a binary solvent system (Mildford, MA, USA) and a sample manager, using an Acquity HSS T3 column (50mm x 2,1mm i.d. 1,7 μm particle size; Waters Corp. Mildford, MA, USA) under positive electrospray ionization (PI). The UPLC instrument was coupled to 5500 QqLit, triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) with a Turbo V ion spray source. All transitions were recorded by using Multiple Reactive Monitoring Mode (MRM) and the data were acquired and processed using Analyst 2.1 software.

2.4. Non target analysis of TPs of tamoxifen

Analysis was performed using an UPLC (Accela 1250 chromatograph with autosampler Thermopal PAL AS) coupled to a LTQ-Velos Orbitrap from Thermo Scientific. The MS analysis was done with an electrospray ionization (ESI) interface in positive ionization mode. Samples obtained from the bench-scale experiments were directly injected in the system.

For the MS detection, four acquisition methods in full scan mode (60 0000 resolution power) were performed by using narrow range of masses: i) 50 -200 Da, ii) 200-300 Da, iii) 300-400 Da and iv) 400-800 Da, in order to increase the sensitivity without compromising the selectivity of the method.. Parallel to full-scan MS acquisition, data-dependant acquisition was used where threshold of intensity (1000 counts) was used for triggering the ion masses to a MS/MS experiment (30 000 resolution power). Chromatograms obtained were compared in order to identify new chromatographic peaks generated during the degradation of the tamoxifen. Only those peaks produced along the experiments that were not present at the beginning of the experiments, were considered as TP candidates for further evaluation. MS/MS spectra obtained were carefully studied in order to propose a chemical structure. The assignment of fragmentation profile detected in MS/MS spectra to each TP candidate was supported by Mass Frontier (software from Thermo Science) which has enable the theoretical generation of mass fragments based on a proposed chemical structure.

2.5. Toxicity assays.

A Microtox® bioassay was used to assess the toxicity of the effluent through the tests. This method is based on the percent of decay in the intensity of light emitted by the bioluminescent bacterium *V. fischeri* upon contact with a filtered sample at pH 7. The effective concentration, EC50, was measured after 15 min. The toxicity of the liquid medium was expressed in percentages of EC50. The toxicity of a reference standard containing tamoxifen at 100 µg L^{-1} (prepared in Milli-Q water) was evaluated as the initial toxicity of the effluent. The toxicity of the mixture of tamoxifen and the quenching agent sodium thiosulfate in Milli-Q water was also tested. Non significant difference was found compared to the toxicity of tamoxifen with the added quenching agent sodium thiosulfate.

2.6. Statistical Analysis

Principal component analysis (PCA) was used to better identify correlations between toxicity of the water samples collected along the AOPs experiments and the presence of the water contaminants (tamoxifen and TPs). PCA was conducted independently for 2 sets of data: i) from the AOPs experiments based on O3 and ii) from the AOPs experiments based on UV. The inputs to the PCA were the relative areas (area of the peaks detected in chromatogram divided by the area of the chromatographic peak of tamoxifen before the treatment) of tamoxifen and all identified TPs, and toxicity, measured at each sampling time.. Note that toxicity values for the PCA were expressed in Toxicity Units (TUs) in order to facilitate the correlation analysis between water contaminants and toxicity, defined as:

$$TUs = 100/EC50$$

For all calculations, the STATISTICA version 7 software (StatSoft Inc) was used.

2.7. UV-VIS spectra

Samples derived from all the experiments were analyzed by UV-vis spectrometry using 8453 UV-vis spectrometer from Agilent technologies. An automatic wavelength scanning (150-800 nm) was performed for all the samples.

3. Results and discussion

3.1. Degradation of tamoxifen.

The elimination of tamoxifen in the treatments followed a particular order : O3> O3/H2O2 > O3/UV > UV/H2O2 > UV.. Removal percentage of tamoxifen higher than 99% was achieved after 30 minutes in all experiments under ozonation conditions whereas almost total removal was only observed after 240 minutes in experiments where UV radiation is used as main oxidation source (figure 1). This is in line with the observations of other authors in previous studies, where it was observed that tamoxifen reacts faster under ozone exposure (Chen et al., 2008). The reactivity of ozone thus seems to be higher by itself than in combination with UV radiation or H_2O_2.. Despite of the high elimination efficiency of tamoxifen in all AOP treatments, none of them treatments achieved a complete mineralization of the target compound and the toxicity of treated water is superior than those found at initial values (see figure 1). Furthermore a mixture containing different organic by-products or TPs is formed (see figure 2). Based on the structures elucidated for the TPs in each of the treatment, different degradation pathways

were suggested, which are associated to diverse oxidative mechanisms: a degradation pathway for tamoxifen under ozonation conditions (O_3, O_3/UV and O_3/H_2O_2) was proposed and compared to the degradation pathways suggested under UV radiation conditions (UV and UV/H_2O_2). In total, 3 well-defined pathways as well as a set of transformation products associated are proposed and described in the next sub-sections.

Degradation pathway associated to O3, O3/UV and O3/H_2O_2 treatments

In the 3 experiments carried out with ozone– O_3 /UV irradiation and O_3/H_2O_2 – two degradation routes were identified. The first route involves the attack to the olefin bond and its scission to get the biphenyl ketone compound through a molozonide intermediate and leading to the formation of TP286 (figure 2). This product exhibits a protonated accurate mass of 286.14377, with a mass error of 3.21 ppm, from the theoretical mass for the composition $C_{17}H_{19}NO_3^+$ (Table 1). The MS/MS spectrum of TP286 is characterized by two characteristic fragments m/z: 268.1333, (loss of H_2O consistent with the structure $C_{17}H_{18}O_2N^+$, with a mass error of -1.7 ppm) and 225.0909 (loss of 43 Da consistent with the composition $C_{15}H_{13}O_2^+$ and a mass error of -0.2 ppm).

After the generation of TP286, the oxidation of the olefin bond occurs with oxidation of the aromatic ring in ortho position respect to the ethoxylated aliphatic chain. TP286 undergoes further oxidative reactions that lead to the formation of the dicarbonyl compound labelled as TP214. This TP214 was identified as m/z 214.14377, with a mass error of 3.77 ppm from the theoretical mass for the composition $C_{11}H_{19}NO_3^+$ (Table 1). The MS/MS spectrum of TP214 is characterized by fragments: m/z 196.3333 (loss of H_2O consistent with the structure $C_{11}H_{18}O_2N^+$, with a mass error of 2.1 ppm). The

transformation process from TP286 to TP214 occurs through oxidation of the aromatic ring, provoking the loose of its aromaticity and the formation of the cyclohexanone structure. TP214 is subsequently oxidized to the polyhydroxilated carboxylic acid depicted as TP224 and two carbon atoms are lost. This TP224 was identified as m/z 224.11286, with an error of 1.75 ppm. The MS/MS spectrum of TP224 is characterized by the fragments: 206.1026 (loss of H_2O consistent with the structure $C_8H_{16}O_5N^+$, with -0.8 ppm error), 146.0814 (loss of 60 Da consistent with $C_6H_{12}O_3N^+$, with -1.9 ppm error) and 128.0709 (loss of H_2O consistent with $C_6H_{10}O_2N^+$, with -1.8 ppm error).

From the further oxidization of TP224, the release of the dihydroxilated tertiary amine indicated as TP106 takes place. This compound was identified as m/z 106.08625, with an error of -1.93 ppm. The MS/MS spectrum is characterized by two small fragments: m/z 88.0760 ($C_4H_{10}ON^+$) and 58.0653 ($C_3H_8N^+$) identified with errors of -2.7 and -1.9 ppm respectively.

The second degradation route of tamoxifen in these set of treatments begins with the monohydroxylation of the aliphatic ethoxylated chain in the C adjacent to the oxygen position to produce the structure labelled as TP388. This TP was identified as m/z 388.22711 with a mass error of only 0.16 ppm. There is no MS/MS pattern for TP388 since its presence along the process was under the threshold previously established in the full-scan for triggering ions to the MS/MS. Probably this TP is generated and transformed to the subsequent TPs very quick. TP388 undergoes further oxidative reactions to produce the structures indicated as TP106 (common to the route 1) and TP284 (suspected of being present). In the scission process of TP388, an internal cyclization is suggested for taking place, enhancing the aromaticity of the system and leading to the formation of

289 TP284 and the release of the aliphatic chain as the dyhydroxilated tertiary amine
290 (previously labelled as TP106). Despite TP284 was not detected (due to its lack of
291 ionization in the electrospray source), its presence can be hypothesized due to the large
292 increase on the absorbance of the solution (supplementary material S1) and following the
293 route proposed. The formation of this molecule might be supported by the increase of the
294 toxicity of the solution, since TP284 shows a tentative structure similar to
295 alkylphenylphenanthrene, (EC50 phenanthrene= 33 mg/kg) (Amorim et al., 2011)

296 **Degradation pathway associated to UV and UV/H_2O_2 treatments**

297 In the experiments carried out under both, UV irradiation and UV combined with H_2O_2
298 addition, the transformation of tamoxifen begins with an internal radical cyclization that
299 leads to the formation of TP370. This process involves the loss of just 2 H atoms and the
300 formation of the protonated molecule found at m/z 370.21649. The MS/MS spectrum of
301 TP370 is characterized by fragment m/z 325.1596 which is consistent with the structure
302 $C_{24}H_{21}O^+$ (with a mass error of 1.1 ppm). This TP was previously described for
303 tamoxifen under sunlight conditions by Della Greca et al 2007 (DellaGreca et al., 2007).

304 TP370 undergoes transformation in two parallel oxidative sub-paths. The first one
305 involves the monohydroxilation of C located in the O adjacent position of the aliphatic
306 chain to produce the structure labelled as TP386. This TP was identified as m/z
307 386.21145 with a mass error of -0.87 ppm. The MS/MS spectra is characterized by
308 fragment 221.09683 (loss of 165 Da consistent with the structure $C_{16}H_{13}O^+$ with an error
309 of 0.8 ppm).

The second degradation sub-path of TP370 involves its radical dihydroxilation of both ortho positions of the aromatic ring to produce the structure indicated as TP402. This TP was characterized by m/z 402.20637 with an error of 3.45 ppm. The MS/MS spectrum is characterized by one fragment: m/z 384.19724 (loss of H_2O consistent with the structure $C_{26}H_{26}O_2N^+$, with a mass error of 2.3 ppm).

Due to the increase of aromaticity (by internal cyclation) in the molecules resulting from route 3, it is observed an augment of absorbance of solution in UV an d UV/ H_2O_2 processes (supplementary material S1)

The formation of all MS/MS fragments in all the routes described was further supported by Mass Frontier tool. The experimental isotopic profile found for each TP in all the samples was successfully compared with the theoretic isotopic profile.

3.2. Toxicity assessment: Generation of tamoxifen TPs in time-course experiments.

Monitoring of the toxicity for each of the treatment conditions at all sampling times was performed by measuring EC_{50} values (expressed in percentage units). The toxicity of samples along the tamoxifen batch experiments were always either equal or higher than the tamoxifen solution before any treatment takes place. Figure 1 shows the observed decay in tamoxifen concentration as well as the measured EC_{50} along the experiment with the 5 treatment scenarios assessed. Tamoxifen concentration was severely reduced in the first minutes of every treatment. Therefore, the increase of toxicity observed should be attributed to the formation of other substances with higher toxicity than the parent compound or to synergy effect of the mix of all the compounds in the sample (including

332 both, tamoxifen and TPs). Special attention should be payed to the dramatic enhancement
333 of the toxicity in the experiments performed with O_3 and O_3/ UV (figure 1 and 2a and
334 2b). Although toxicity values are derived from the mixture of contaminants present in
335 each sample, the measurement of the toxicity of each single TP generated is not feasible.
336 However, EC_{50} values reported at each sampling time were used to extract some potential
337 explanation and conclusion about the toxicity of the TPs. Toxicity evolution (EC_{50}) along
338 the treatment, as well as the relative area in percentage values (relative area to the
339 tamoxifen initial area) of TPs generated are shown in figure 2 for each of the treatments
340 considered.

341 *Experiments based on O3 treatment*

342 **O_3 process:** In the experiment carried out with O_3 (figure 2a), the toxicity of the effluent
343 follows an increasing trend, from being almost not toxic to reach a value of 2% EC_{50} at
344 the end of the experiment (after 240 minutes). Since tamoxifen concentration is
345 completely removed in approximately 20-30 minutes, increase of toxicity can only be
346 attributed to the toxicity of the transformation products formed: TP286, TP214, TP224
347 and TP106 were identified in this experiment. TP286 generates in the first minutes of the
348 treatment, reaching its maximum concentration in approximately 10 minutes (58 %) and
349 decreasing along the experiment. In contrast TP214, TP224 and TP106 are produced and
350 accumulated in the solution, being TP224 the major TP at final time (34 %). This fact is
351 in agreement with the sequential transformation mechanism proposed as Route 1.

352 **O_3/ H_2O_2 process:** In the degradation assays carried out with O_3 / H_2O_2 (figure 2b), the
353 toxicity followed a general increasing trend from the beginning of the experiment,

achieving 15% EC_{50} after 120 minutes of the treatment. In this experiment as well as in the O_3 experiment, it is evident the fast generation of TP286 (originated from the oxidative rupture of the olefin bond between the aromatic rings), which in this case reaches the maximum concentration in 5 minutes (65%). As in the case of O3 treatment, the concentration of this TP gradually decreases to a final relative concentration of 10%. At the end of the experiment, a mixture of TP286, TP214, TP224, TP106 and TP388 (same TPs detected in the O3 process) was observed whereas tamoxifen was not detected. Tamoxifen indeed almost disappears in the first minutes of the treatment.

O_3/ UV process: A similar toxicity profile can be observed in the experiments performed with O_3 under UV irradiation (figure 2c). The toxicity dramatically increases in the first minutes of the experiments reaching a final value of 1% EC50, whereas tamoxifen is not present. Same TPs than those described in the experiments with O3 and O3/UV are found along this experiment but unlike them, the most prevalent TP was TP106, which is generated in the first stages of the treatment and reaches a constant concentration (about 10%) after approximately 30 minutes. After 4h ozonation under UV irradiation, total removal of tamoxifen was observed. The final solution contained a mixture of TP214, TP224 and TP106.

PCA results obtained for the different O3 based experiments can be found in the supporting information (Table S1 and Figure S6). The two first principal components (PCs) were used to study correlations between toxicity and water contaminants by accounting for more than 72% of the total variance (52% for PC1 and 20% for PC2) (see

376 Table S1). PCA loadings (Figure S6a) confirm a direct correlation between toxicity (TU)
377 and the relative areas of TP224, TP214 and TP105 (> 0.8 contribution over PC1),
378 whereas an inverse correlation between toxicity and tamoxifen, TP388 and TP286 is also
379 observed. PC1 and PC2 scores of the analyzed samples (Figure S6b) show that samples
380 collected before 240 min coincide with the appearance of TP286 and TP388. At the end
381 of the experiments (samples taken at 240 minutes), O3 and O3/UV based experiments
382 exhibit the highest toxicity and also the highest relative areas for TP224, TP214 and
383 TP106, confirming the findings from Figure 2a and 2b. In addition, relative areas of
384 TP224, TP214 and TP106 in the samples collected at 240 minutes of treatment were
385 much lower in the case of O_3/H_2O_2 experiments than those measured in the experiments
386 with O_3 and O_3/UV. This is in line with the lower toxicity observed for the water samples
387 in O_3/H_2O_2 experiments at the end of experiment

388 *Experiments based on UV treatment*

389 **UV process:** In the experiments performed under UV irradiation, the overall toxicity of
390 the effluent follows a general increasing trend until approximately 120 minutes, reaching
391 an EC_{50} value about 15% (figure 2d). Carrying out the experiments beyond 120 minutes
392 provokes a further decay in toxicity, reaching a value about 33% after 240 minutes of
393 continuous of exposure to UV radiation. In the process, formation and removal of TPs
394 occurs according to the scheme presented as Route 3 (Figure 3). Kinetics reveals a very
395 fast production of TP370 in the first minutes of irradiation, which leads to the formation
396 of its highest concentration after 5 minutes (about 10%). Subsequently the concentration
397 of this TP smoothly decays to reach total disappearing after 240 minutes. According to
398 the transformation Route 3, after formation of TP370, this compound is further oxidized

399 to either TP386 (by hydroxylation of the aliphatic chain) or TP402 (by
400 monohydroxylation of both O-ortho positions). The kinetics observed are in excellent
401 agreement with the proposed mechanistic scheme, since the maximum concentration of
402 both TPs is achieved in 15 minutes irradiation (10 % for TP386 and 11 % for TP402), 10
403 minutes later than the maximum production of their precursor, TP370. After this
404 maximum, the concentration of both TPs decreases, being TP386 almost completely
405 removed after 240 minutes irradiation. After 4 hours, a non-transformed tamoxifen
406 concentration about 10 ppb remained in solution.

407 **UV/ H_2O_2 process:** In the assays performed with H_2O_2 under UV irradiation, the toxicity
408 slightly increases during the first 5 minutes of the process getting close to its initial value
409 after 15 minutes (figure 2e). Afterwards, constantly increases to reach an EC_{50} about 12
410 % in 120 minutes. From this point, the EC_{50} of the effluent increases to reach a final value
411 about 35%, indicating that the toxicity of the effluent is more toxic than the raw
412 tamoxifen solution (47 %). Despite in the first 5 minutes, TP370 (0.5%), TP386 (3%) and
413 TP402 (about 8%) were detected, it is remarkable a biggest production of TP402
414 compared to the other two. This observation is coherent with the expected higher
415 concentrations in $^{\cdot}OH$ radicals in this experiments compared to the assay performed with
416 exposure to only UV radiation. Since H_2O_2 is added to the reactor, the dihydroxylation
417 reaction that converts TP370 into TP402 is expected to happen in higher extent. As in the
418 case of the assays carried out only with UV radiation, a residual non-transformed
419 concentration or tamoxifen (about 7 $\mu g\ L^{-1}$) was detected after 4 hours.

420 PCA results obtained for the different UV based experiments can be found in the
421 supporting information (Table S1 and Figure S7). The two first PC accounted for 81% of

422 the total variance (54% for PC1 and 27% for PC2) (see Table S1). PCA loadings (Figure
423 S7a) show there is no correlation between toxicity and any of the identified TPs (TP402,
424 TP386 or TP370). PC1 and PC2 scores of the analyzed samples (Figure S7b) show that
425 samples taken after 120 and 240 min sit together and are the ones that show a relationship
426 with toxicity. Thus, it can be hypothesized that toxicity increase in these experiments
427 would be attributed to the formation of other TPs not identified in the experiments or to
428 an synergic effect of the compounds present in the mixture along the experiments.

429
430 The high toxicity of the effluent at the end of the different experimental conditions
431 evaluated put into evidence that despite the quite efficient removal of tamoxifen (almost
432 100% removal in all cases at the end of the experiments) a total mineralization is not
433 achieved. Overall toxicity is thus the result of the presence of a cocktail of TPs that are
434 being generated and also degraded along each treatment. The sum of TPs (in percentage
435 values) detected in the last sample (240 minutes) was between 0.6 - 6.5 % for the UV-
436 based processes and between 27-81% for the ozone-based processes. Based on this, it
437 might be hypothesized that UV treatments have achieved total mineralization of
438 tamoxifen. However, the high toxicity and the absorbance found from the treated water
439 most likely indicate the presence of other non-detected compound/s. The internal
440 cyclation during UV radiation experiments, the proposed presence of TP284 in the
441 ozonation process (section 3.1) and the increment of absorbance along almost all the
442 experiments (supplementary material S1-S5) suggests an increase of the aromaticity of
443 the tamoxifen molecule therefore a potential increase in the toxicity. The use of statistics
444 tools, such as the principal components analysis was applied in order to help to figure out

445 whether the source of this toxicity is due to the cocktail of TPs, to a particular TP
446 tentatively identified or to the existence of other substance/s not detected here.

447

448 **4. Conclusions**

449 The results obtained in this study suggest that advanced oxidation processes based on
450 combination of ozone, UV irradiation and hydrogen peroxide are efficient in the removal
451 of tamoxifen in aqueous solutions. The use of these treatments offers the possibility of
452 not only increasing the efficiency of removal of tamoxifen but also to reduce the reaction
453 time in comparison with conventional biodegradation system. Processes under
454 ozonization can be considered the most efficient process for the removal of tamoxifen.
455 However, several transformation products of tamoxifen were found along time-course
456 experiment being the treated water always much more toxic than tamoxifen. Tamoxifen
457 TPs are result of oxidative transformation reactions involving non-selective hydroxyl
458 radicals in the different treatments where 3 routes were identified: two occurring under
459 ozonization processes and one only related to UV irradiation. Four TPs were identified in
460 ozone-based treatments and one was suspected of being present, whereas three other
461 different TPs were identified in UV-based treatments. In the case of ozone-based
462 treatments the toxicity was attributed to the presence of some of the TPs identified,
463 however in the UV-based treatments the presence of another/s TP is suspected according
464 to the increase of the toxicity despite of the low levels of the identified TPs.
465 To the author knowledge's, this is the first time that tamoxifen degradation has been
466 studied under advanced oxidation processes integrating the removal, identification and

semiquantitation of TPs as well as the assessment of toxicity along the process. Moreover, this is the first time that these TPs have been detected and identified, except one previously identified tamoxifen TP.

This study concludes that despite some micropollutants can be effectively removed with advanced oxidation processes, some other compounds with higher toxicity can be generated, as it is the case of transformation products of tamoxifen.

Acknowledgments

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553

Table

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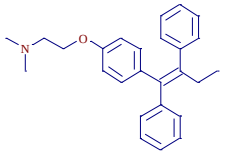
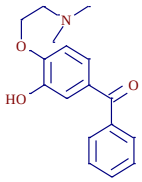
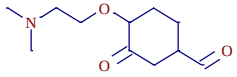
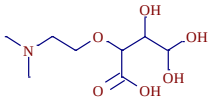
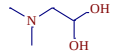
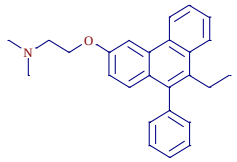
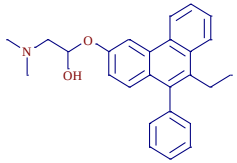
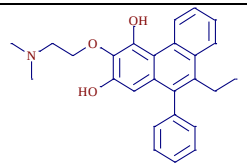
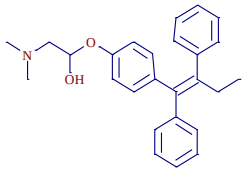
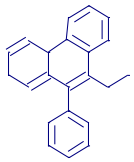
Table 1. Transformation products of Tamoxifen identified by HRMS during the advanced oxidation processes (AOP) (na=not available;n.i.=non ionizable molecule)

Comp.	Structure	RT (min)	AOP Process	Molecular Formula	$[M+H]^+$ Theor.	$[M+H]^+$ Exp.	Error (ppm)	Fragment Ions	Molecular Formula for Fragments (error in ppm)	Fragments supported by Mass Frontier	Isotopic profile(**)	First Time detected
TAM		5.63	O_3 (O_3/H_2O_2) (O_3/UV) (UV) (UV/H_2O_2)	$C_{26}H_{29}NO$	372.23219	372.23239	0.53	327.1752 249.1279 207.1175	$[C_{24}H_{23}O]^+$ (2.6) $[C_{18}H_{17}O]^+$ (2.2) $[C_{16}H_{15}]^+$ (3.3)	Yes	Yes	-------
TP286		5.49	O_3 (O_3/H_2O_2) (O_3/UV)	$C_{17}H_{19}NO_3$	286.14377	286.14285	3.21	268.1333 225.0909	$[C_{17}H_{18}O_2N]^+$ (-1.7) $[C_{15}H_{13}O_2]^+$(-0.2)	Yes	Yes	Yes
TP214		5.53	O_3 (O_3/H_2O_2) (O_3/UV)	$C_{11}H_{19}NO_3$	214.14377	214.14351	3.77	196.3333	$[C_{11}H_{18}O_2N]^+$ (2.1)	Yes	Yes	Yes
TP224		0.55	O_3 (O_3/H_2O_2) (O_3/UV)	$C_8H_{17}NO_6$	224.11286	224.11256	1.75	206.1026 146.0814 128.0709	$[C_8H_{16}O_5N]^+$ (-0.8) $[C_6H_{12}O_3N]^+$ (-1.9) $[C_6H_{10}O_2N]^+$(-1.8)	Yes	Yes	Yes
TP106		0.52	O_3 (O_3/H_2O_2) (O_3/UV)	$C_4H_{11}NO_2$	106.08625	106.08605	-1.93	88.0760 58.0653	$[C_4H_{10}ON]^+$(-2.7) $[C_3H_8N]^+$(-1.9)	Yes	Yes	Yes
TP370		5.64	(UV) (UV/H_2O_2)	$C_{26}H_{27}NO$	370.21654	370.21649	-1.69	325.1596	$[C_{24}H_{21}O]^+$ (1.1)	Yes	Yes	No
TP386		5.55	(UV) (UV/H_2O_2)	$C_{26}H_{27}NO_2$	386.21145	386.21167	-0.87	221.09683	$[C_{16}H_{13}O]^+$ (0.8)	Yes	Yes	Yes

TP402		5.48	(UV) (UV/H_2O_2)	$C_{26}H_{27}NO_3$	402.20637	402.20831	3.45	384.19724	$[C_{26}H_{26}O_2N]^+$ (2.3)	Yes	Yes	Yes
TP388		5.60	O_3 (O_3/H_2O_2) (O_3/UV)	$C_{26}H_{29}NO_2$	388.227105	388.22717	0.16	n.a.(***)	n.a(***)	n.a. (***)	Yes	Yes
TP284*		n.i./n.a.	O_3 (O_3/H_2O_2) (O_3/UV)	$C_{22}H_{19}$	284.15595	n.i.	n.i.	n.i.	n.i.	n.a.	n.a.	Yes

*Based on the presence of TP106 and TP388 as well as the increment of UV absorbance values along the experiment, the TP284 is candidate to be present in O3, O3/H2O2 and O3/UV treatments. However this compound has a non ionizable structure being impossible to get MS/MS spectra for its identification. This compound might be the responsible of the high toxicity of these processes as the structure proposed is close to the polycyclic aromatic hydrocarbon alkylphenylphenanthrene.

**Theoretical isotopic profile was compared with those found at experimental conditions. All of the suggested molecules fitted with the theoretical isotopic profile.

*** Not available due to the very low limit of detection.

Figure
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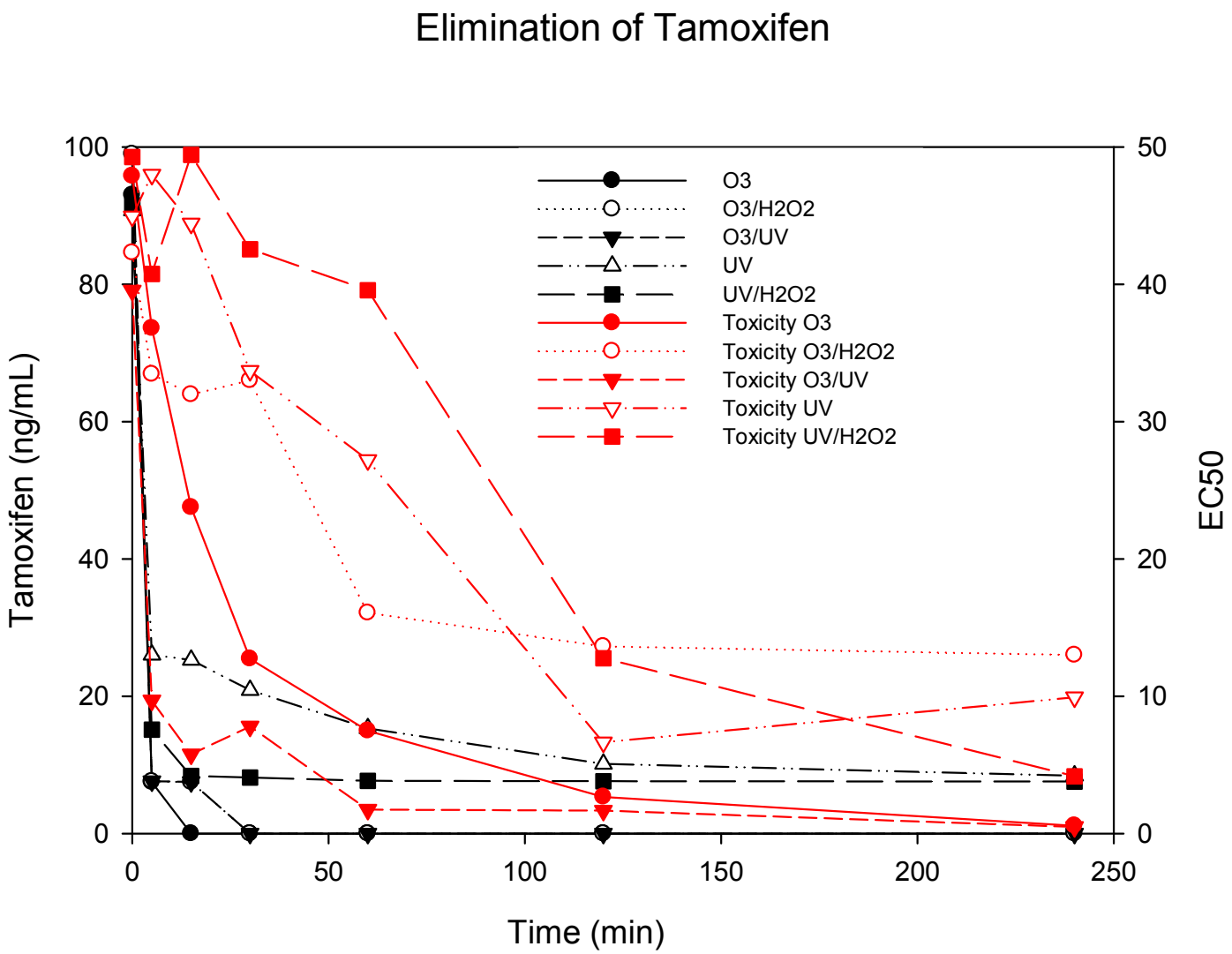
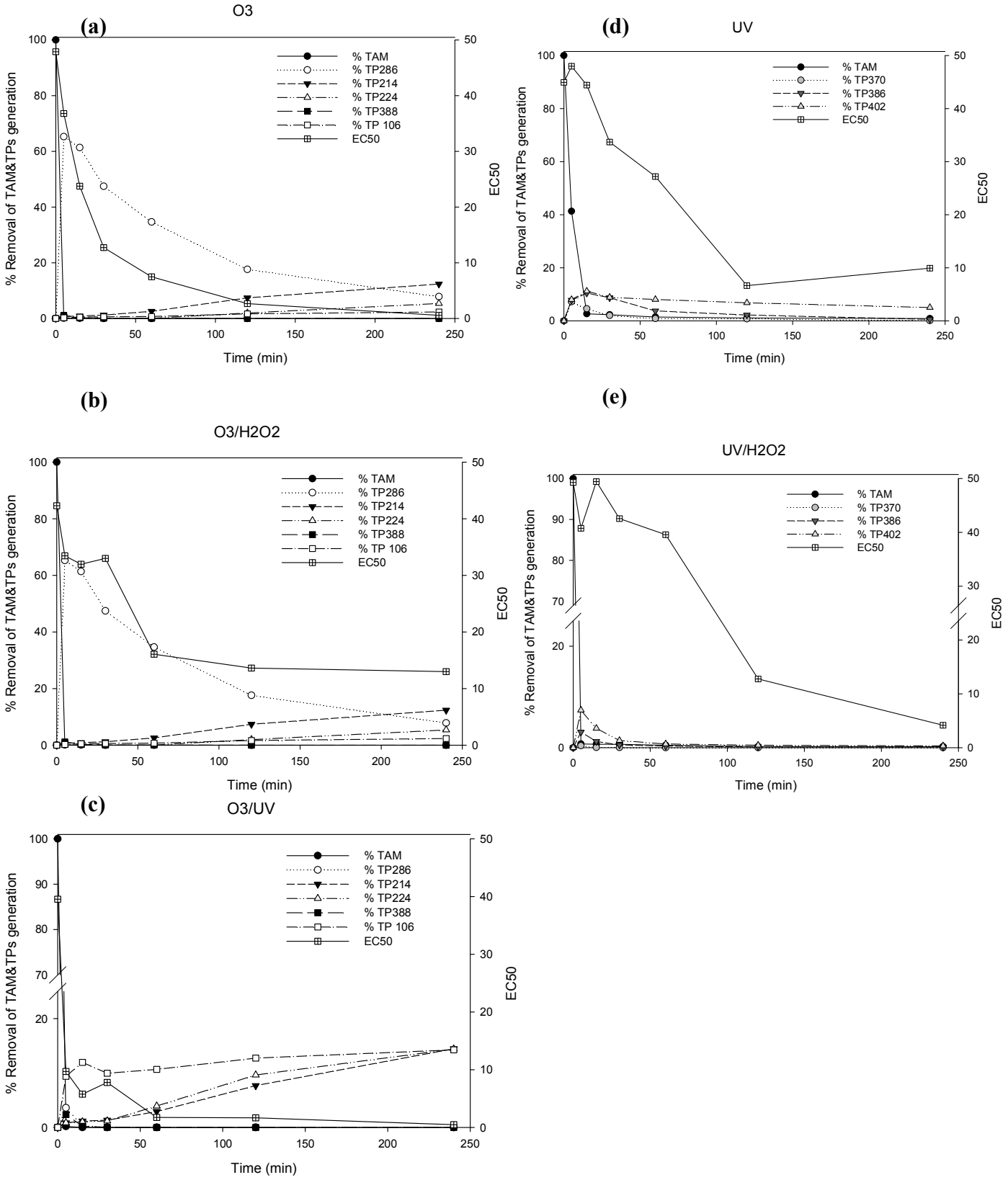


Figure 1. Removal of Tamoxifen by advanced oxidation treatments (O_3,O_3/H_2O_2,O_3/UV,UV and UV/H_2O_2) and the toxicity generated during the processes (EC_{50}). $C_{Tx,0}$: 100 ng·mL^{-1}.

Figure 2. Time-course formation of transformation products (% relative area to tamoxifen), toxicity (EC_{50}) and removal of tamoxifen (% area) in the treatments studied: O_3 (a), O_3/H_2O_2 (b), O_3/UV (c), UV(d) and UV/H_2O_2 (e).



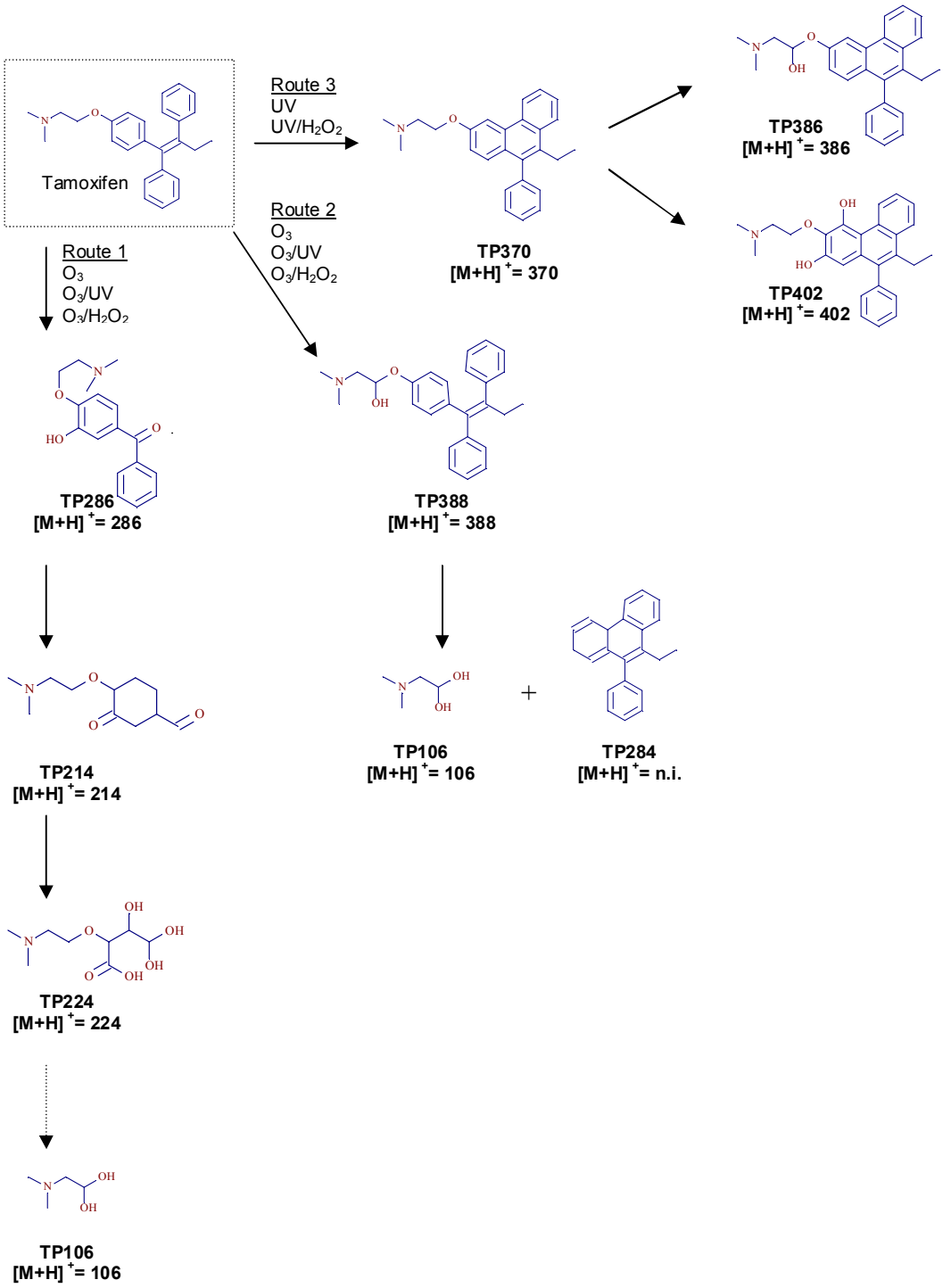


Figure 3. Proposed degradation pathway of Tamoxifen for all the advanced oxidation treatments.

Supplementary Material

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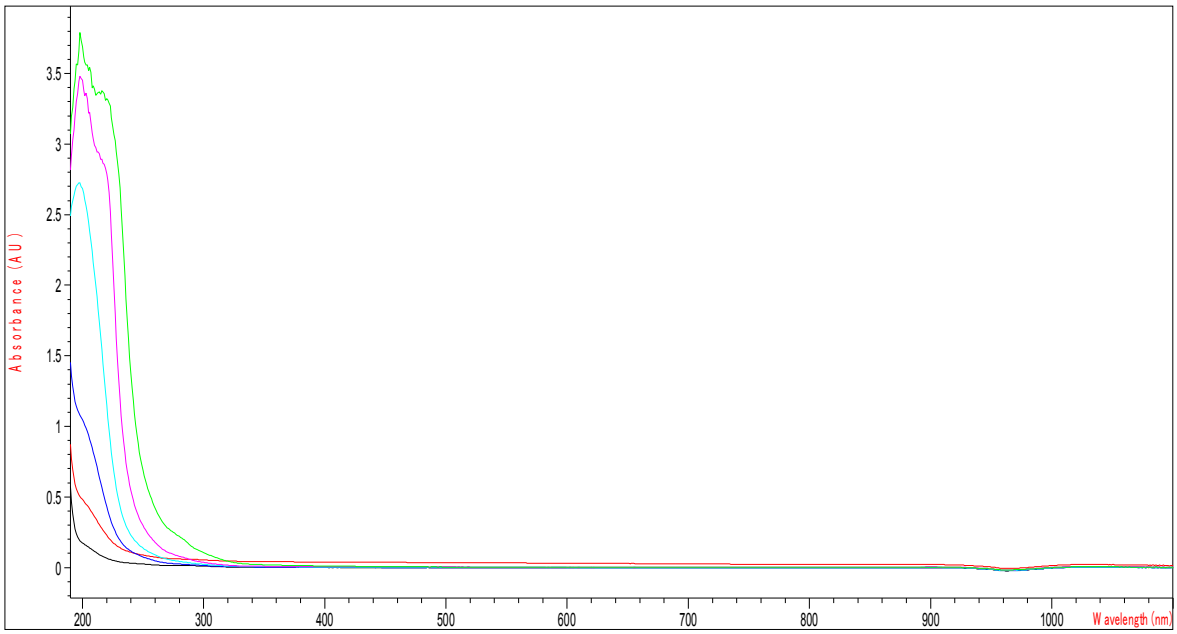

Figure S1. Spectrum of absorbance of treated samples from ozone experiments: black line (initial), red line (15 minutes), blue (30 minutes), sky blue (1 hour), pink (2 hours) and green (3 hours).

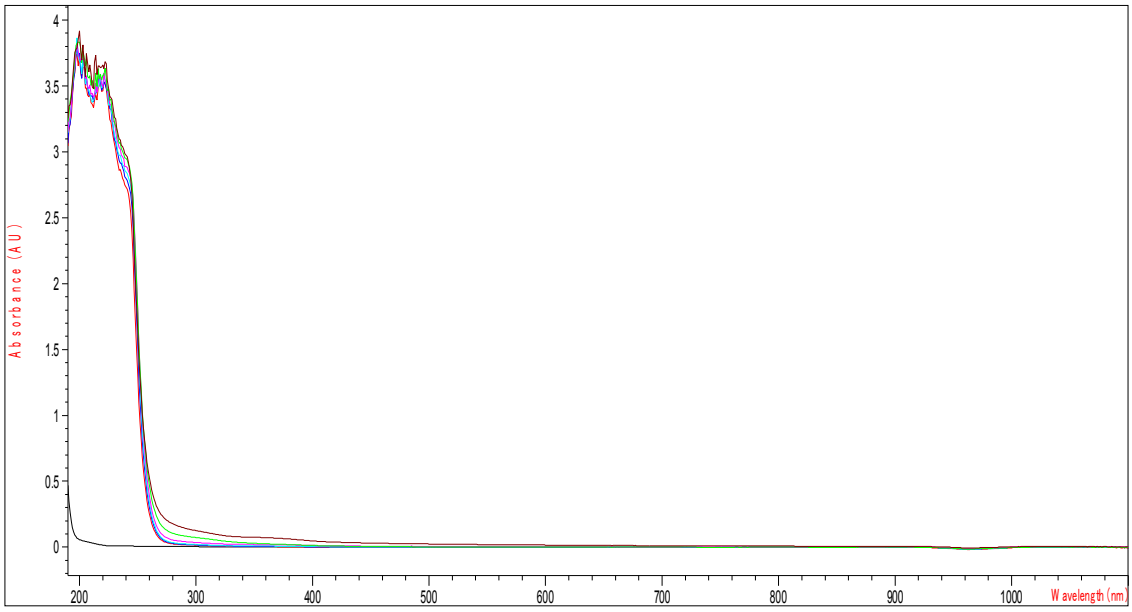

Figure S2. Spectrum of absorbance of treated samples from ozone/H_2O_2 experiments: black line (initial), red line (15 minutes), blue line (30 minutes), sky blue line(1 hour), pink line (2 hours) and green line (4hours)

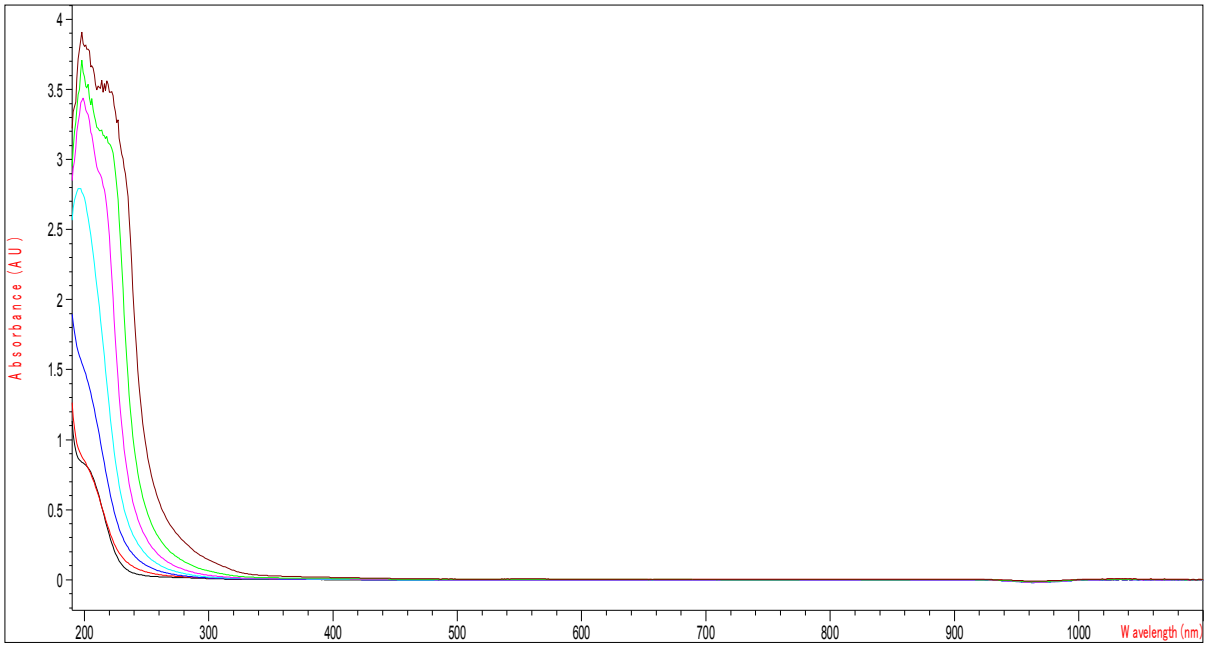

Figure S3. Spectrum of absorbance of treated samples from ozone/UV experiments: black line (initial), red line (15 minutes), blue (30 minutes), sky blue (1 hour), pink (2 hours), green (3 hours) and brown (4hours).

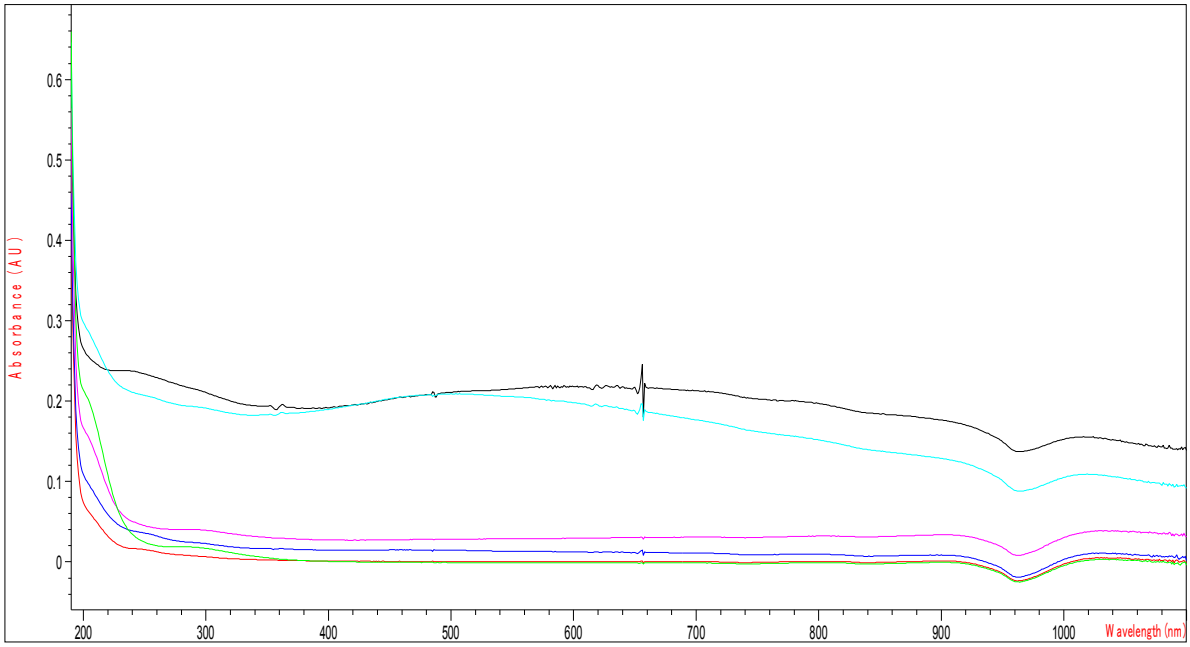

Figure S4. Spectrum of absorbance of treated samples from UV experiments: black line (initial), sky blue line (15 minutes),pink line (30 minutes), blue line (1 hour),green line (2 hours), and red line (4hours).

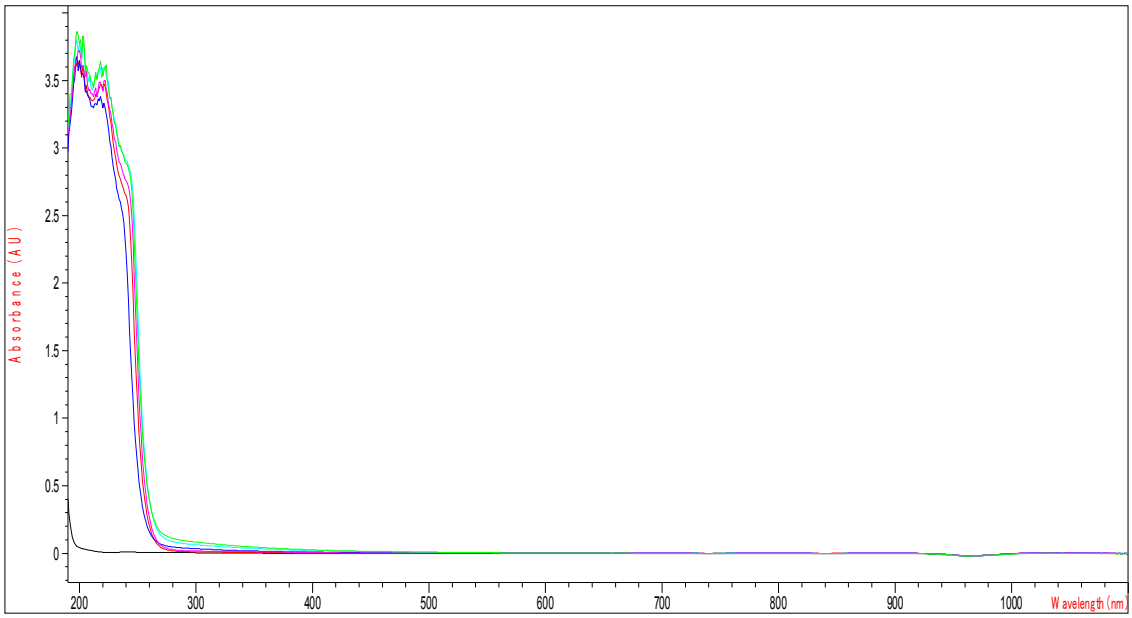

Figure S5. Spectrum of absorbance of treated samples fromUV/H_2O_2 experiments: black line (initial), red line (15 minutes), blue line (30 minutes), sky blue line (1 hour), pink line (2 hours), and green (4 hours) .

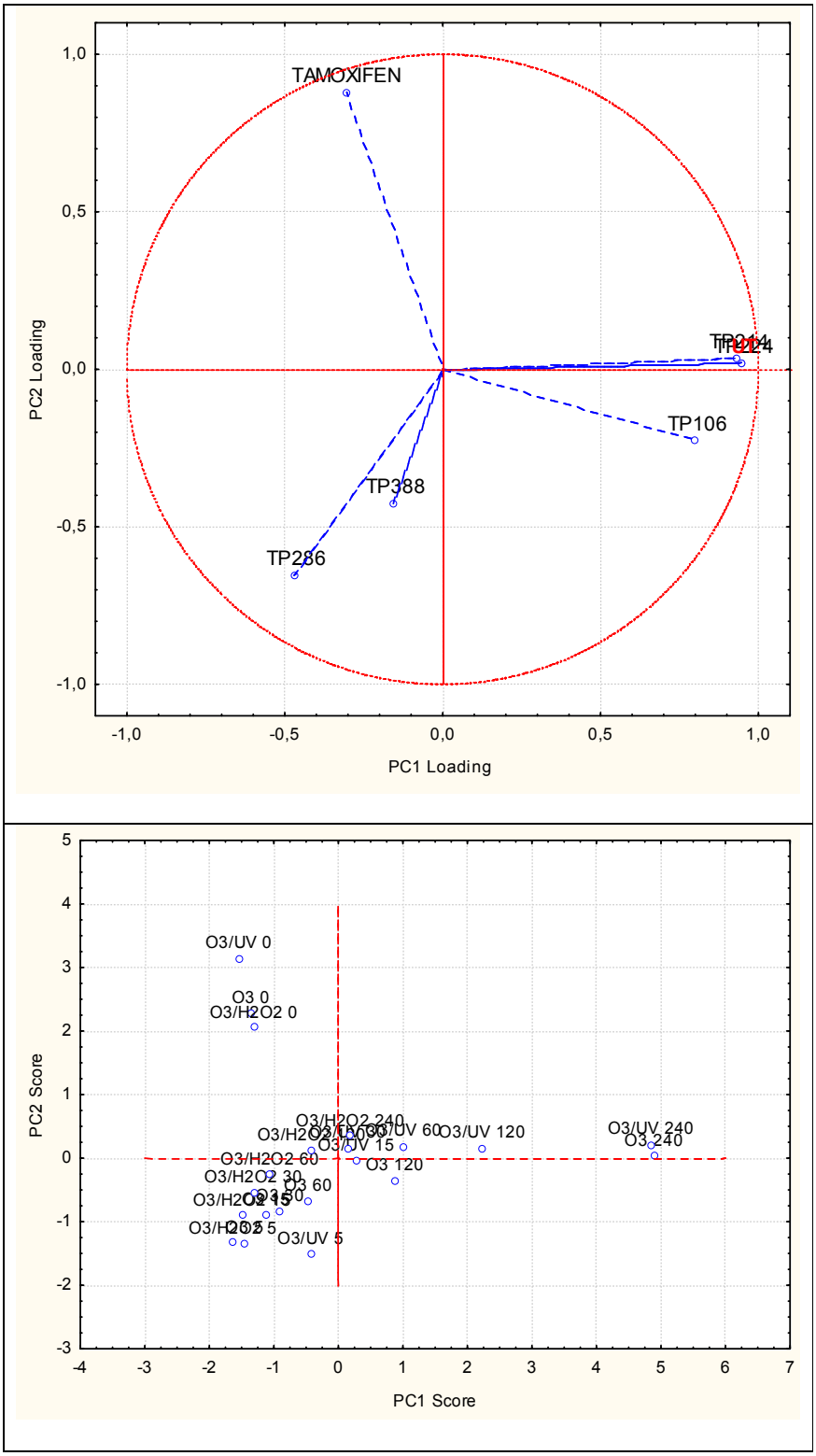

Figure S6. PCA results obtained from the O3 experiments a) PC1 versus PC2 loadings of the measured variables b) PC1 versus PC2 scores of the observed cases. UT is marked in red.

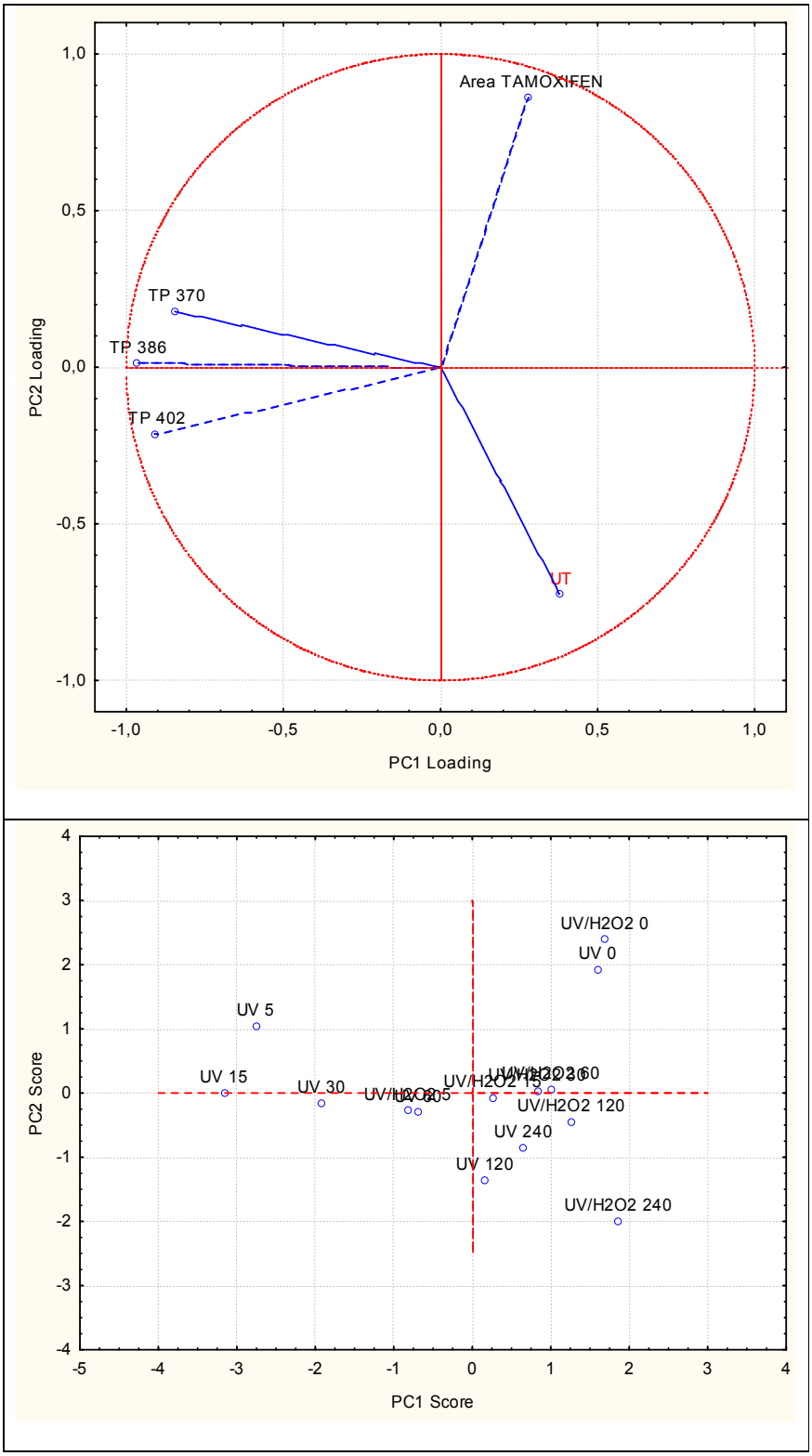

Figure S7. PCA results obtained from the O3 experiments a) PC1 versus PC2 loadings of the measured variables b) PC1 versus PC2 scores of the observed cases. UT is marked in red.

Table S1. Table of the PCA results for the O3 and UV based experiments with the eigenvalues, total variance, and the corresponding cumulative of the different PCs.

Eigenvalues of correlation matrix, and related statistics to O3 based experiments				
	Eigenvalue	% Total	Cumulative	Cumulative
1	3,637138	51,95912	3,637138	51,9591
2	1,434839	20,49769	5,071977	72,4568
3	1,031930	14,74185	6,103907	87,1987
4	0,507207	7,24581	6,611113	94,4445
5	0,274306	3,91866	6,885419	98,3631
6	0,079473	1,13533	6,964892	99,4985
7	0,035108	0,50154	7,000000	100,0000

Eigenvalues of correlation matrix, and related statistics to UV based experiments				
	Eigenvalue	% Total	Cumulative	Cumulative
1	2,706426	54,12851	2,706426	54,1285
2	1,342113	26,84225	4,048538	80,9708
3	0,638369	12,76737	4,686907	93,7381
4	0,235992	4,71985	4,922899	98,4580
5	0,077101	1,54201	5,000000	100,0000

CHAPTER 6

6. Results and discussion

This PhD thesis encloses a compendium of publications which is organized following a rational order under the scope and objectives established at the beginning of this research work. Each research manuscript (chapters 3-5) contains a section with results and discussions. Hence, the aim of the present chapter is to provide a comprehensive summary of the main results of the thesis and provide a holistic discussion.

6.1. Analysis of anticancer drugs, metabolites and transformation products

During the thesis, a bunch of data was generated about the occurrence of anticancer drugs, their metabolites, TPs as well as related compounds (such as cancer biomarkers) in different scenarios: from hospitals, through wastewaters until natural waters (river water). All this information, which can be extracted from the manuscripts 1, 2 and 3 (block I and II) is summarized and discussed in this section. On the other hand, analytical strategies applied with this main purpose are also described and discussed here in two different sub-sections: (i) target analysis, where a multi-residue method was developed and applied for the quantification of target pollutants in several water matrices and (ii) non-target analysis, where different approaches were employed for the tentative identification of suspected molecules (manuscript 1 and 2) and for the identification of unknown substances (manuscript 4 and 5).

6.1.1. Target analysis

A comprehensive optimization of an off-line SPE-UPLC-MS/MS method was performed for the determination of ten selected anticancer drugs in water matrices (manuscript 1). The method was combined with an IDA (Information- Dependent Acquisition) detection tool, which was applied for the screening of target drugs metabolites in real samples; and which will also be discussed in section 6.1.2. The method developed here has several advantages compared to other methods developed for anticancer drugs so far: (1) wide and representative group of target compounds; (2) complete method validation, including a stability study of target compounds, a target confirmation strategy by a novel ion-ratio approach and a thorough matrix effects study; (3) a very advantageous approach to, in addition, search known-unknown compounds in wastewater samples.

Results from target analysis – of samples collected along 3 months – revealed the presence of several anticancer drugs such as ciprofloxacin, cyclophosphamide, ifosfamide, etoposide, methotrexate, tamoxifen and vincristine in hospital effluents and influent wastewater in the range of concentrations that other studies have reported so far [24-26, 32, 85-87]. Docetaxel and paclitaxel were detected for the first time in hospital effluents and wastewater influents. Also azathioprine was detected for the first time in the inlet stream of a WWTP.

Only tamoxifen, ciprofloxacin, cyclophosphamide and occasionally methotrexate were found in wastewater effluents, indicating that these substances are not successfully removed in the conventional treatment scheme of WWTPs. Tamoxifen, ciprofloxacin and cyclophosphamide were indeed being discharged through WWTP effluents into the aquatic environment at a concentration in the range of ng per Litre.

Special attention was paid to tamoxifen and ciprofloxacin, since they were found in all the water samples analysed, even in the river water upstream of the WWTPs discharge: Both tamoxifen and ciprofloxacin are also used for veterinary applications and therefore its presence in upstream river waters could have been caused by non-point sources of pharmaceutical contamination such as concentrated animal feeding operations (CAFOs) located along the river. Ciprofloxacin was present at very high levels particularly in the hospitals effluents (up to 14826 ng L^{-1}) as well as in influent wastewaters (up to 1558 ng L^{-1}). These high values can not only be attributed to chemotherapy treatments, since ciprofloxacin is also used as antibiotic in the treatment of a number of human infections and in veterinary treatments, as mentioned before.

On the other hand, cyclophosphamide and ifosfamide are among the most studied anticancer agents so far and they were detected in almost all the hospitals effluents as well as in the WWTP inlet. Despite of the low levels found in this study, they are still relevant contaminants because of their known toxicity, environmental persistence and ubiquity:

Based on discrete samples (manuscript 1), no significant differences in the detected levels of anticancer drugs were identified between the effluents from hospitals and regular urban effluents and, therefore, hospitals might not be considered as the primary source of this type of contaminants. As pointed out previously, most of the patients

treated with anticancer drugs leave the hospital facilities after the administration of the drugs and therefore the cytostatic compounds are likewise released via hospital and domestic wastewater. However the sampling campaign study (manuscript 3) suggests that the anticancer drugs were predominant in hospital effluents (in 2 out of the 3 sampling campaign performed). It should however be remarked that these studies were performed in 2012 in Catalonia, when the annual consumption (in kg of anticancer drugs) was rather similar for pharmacies and hospitals [88]. The contribution from the hospital and domestic effluents to the urban sewage might vary between years due to the differences in administration protocols, as Franquet-Griell et al. 2015 have reported in a recent study.

This is the first time that several anticancer drugs have been studied in different aquatic environments: i) docetaxel and paclitaxel in hospital effluents, ii) azathioprine, docetaxel, etoposide, paclitaxel and vincristine in wastewater at WWTPs and iii) azthioprine, docetaxel, etoposide, methotrexate, paclitaxel and vincristine in surface waters.

6.1.2. Non-target analysis of anticancer drugs, their metabolites and transformation products

Concerning the non-target analysis of the compounds of interest, three analytical approaches were applied and evaluated along this thesis based on different mass spectrometry techniques: i) UPLC coupled to a quadrupole-linear ion trap detector (QqLiT), ii) UPLC coupled to a quadrupole-time-of-flight detector (QTOF) and iii) UPLC coupled to a high resolution mass spectrometry based on Orbitrap detector (LTQ Orbitrap Velos). The (i) and (ii) methodologies were employed for a "suspect screening" of anticancer drugs and/or related compounds in real samples; while the methodology (iii) was used for screening and identification of "unknown" compounds, potential transformation products generated from the selected anticancer drug tamoxifen to different removal processes. A more detailed description and discussion about the three approaches are provided below:

i) *UPLC coupled to a quadrupole-linear ion trap detector (QqLiT):* A fast, and feasible approach based on the information acquisition tool (IDA) was used in the screening of human metabolites and transformation products of anticancer drugs in hospital wastewater

samples (manuscript 1). The combined use of several tools for the identification of "known-unknown" compounds, such as the IDA acquisition mode, the mass fragmentation mechanism simulator (Mass Frontier) and the physico-chemical properties simulator (Marvin Sketch), has been proven to be a reliable alternative for the analyst when high-resolution mass spectrometry instrumentation is not available. The presence of three known metabolites of selected cancer drugs was screened through the chromatograms obtained from the analysis of hospital samples: 1) hydroxy-Tamoxifen, the main human metabolite known of tamoxifen; 2) 4,4-dihydroxy desmethyltamoxifen, substance that belongs to the same chemical and therapeutic family than tamoxifen and which is also a secondary human metabolite of tamoxifen [54] and 3) carboxyphosphamide, a known human metabolite of cyclophosphamide. The presence of these compounds was successfully explored by searching their theoretical molecular ions in the sample chromatograms acquired using the IDA method: ions m/z 389, m/z 390 and m/z 293 corresponding to hydroxy-Tamoxifen, 4,4-dihydroxy desmethyltamoxifen and carboxyphosphamide respectively.

ii) *UPLC coupled to a quadrupole-time-of-flight detector (QTOF)-:* A suspect screening method has been applied to the identification of pharmaceuticals and biomarkers related to chemotherapy in wastewater (manuscript 2). A list of suspect ions (>1420 compounds) was generated from a review of the chemotherapeutic drugs identified within the NORDP databases as well as their metabolites and related biomarkers. Dispensing rates and limitations in the analytical technique reduced this suspect list to approximately 300 compounds. Mass accuracy, isotopic patterns and predicted retention times based on LogP were then used to confirm the tentative identification of eight suspects in wastewater samples from Oslo. This analytical strategy demonstrated to be a successful, fast and an economically advantageous tool for the screening of micropollutants in wastewater when standards of the suspect compounds are not available. The use of logical pre-determined

criteria to refine the suspect list down to a relatively small number of compounds (as described here) provides an efficient workflow and therefore resources can focus on the identification/quantification of those compounds most likely to be found.

iii) *UPLC coupled to a high resolution mass spectrometry based on Orbitrap detector (LTQ Velos-Orbitrap):* Samples obtained from the WRF biodegradation experiments (manuscript 4) and also the bench-scale AOP experiments (manuscript 5) were analysed in the UPLC coupled to a LTQ-Velos Orbitrap (Thermo Scientific) for further identification of transformation products generated in time-course experiments. For the MS analysis, different acquisition steps in full scan mode were performed by using narrow range of masses in order to increase the sensitivity and the selectivity of the method. MS full-scan was acquired at 60 000 resolution power for all of these methods. Conditioned up to full-scan MS, a data-dependant acquisition (with a signal intensity threshold) was used to trigger MS/MS experiment (30 000 resolution power). For a first screening of potential TPs, those chromatographic peaks appearing in samples of treated water and that were not present in the samples before the treatment, were considered as candidate TPs and further evaluated. MS/MS spectra of these chromatographic peaks were carefully studied in order to propose a chemical structure. The assignment of fragmentation profile detected in MS/MS spectra to each TP candidate was supported by Mass Frontier (software from Thermo Science), which enabled the theoretical generation of mass fragments based on a proposed chemical structure. This strategy for the screening of unknown compounds allowed the identification of two TPs of tamoxifen in WRF experiments and nine new TPs after AOP experiments.

The above described analytical methodologies provide specific and novel analytical strategies for the identification of anticancer drugs and related compounds in environmental aquatic samples. These methodologies were applied to different laboratory and field experiments during this *thesis.* Through these approaches several human metabolites (i), (bio-) markers (ii) and TPs (iii) related to the consumption and

administration of cancer drugs were tentatively identified in wastewaters, giving an up-to-date view of cytostatic occurrence in the environment.

These results point out non-target methodologies out as key tools for the screening of a wide number of substances, without requiring the availability of reference standards. In any case, the confirmation and quantification of the substances require certified material reference (CMR) standard and/or NMR. The tentative identification and semi-quantification (through relative chromatographic areas) of "unknown" compounds through non-target approaches becomes crucial for the further selection of compounds of interest. Before the advent of non-target approaches in the last years, the selection of contaminants for monitoring through target analysis was limited by: (a) the commercial availability of reference standards, (b) the literature available regarding previous findings, metabolism and biodegradation studies, etc. and (c) the choice of the analyst based on the evaluation of predicted concentration values, suspected TPs, etc. In this regard, IDA tool (i) has also been proved to be an accessible and powerful alternative for the analyst when HRMS instrumentation is not available. In summary, non-target methodologies are excellent screening tools, which can either complement target analysis or support further development of such target methodologies.

6.1.3. Comparative assessment in the occurrence of anticancer drugs in the cases studied: Oslo (Norway) and Girona (Spain)

Regarding both sewage scenarios, Oslo (Norway) and Girona (Spain), studied in publication 2 and 3 respectively, there are clear differences in the presence of cytostatic compounds in raw wastewater. Although different analytical approaches were used (target analysis in Spain and suspect screening in Norway), it should be highlighted that those compounds found in Spain were unlikely not found in Norway. This is partially explained by year consumption reported for both countries. Particularly the annual consumption of tamoxifen in Spain during 2012 was 10 864 kg [54] while the total consumption in Norway was about 447 kg per year (www.nordp.no). These consumption data, along with population data of both countries allow to calculate, the predicted concentration (PEC) of tamoxifen in raw wastewaters. Based on these calculations, PEC from Norway was lower than in Spain: Taking into account the value of annual consumption in Norway, and the dilution factor applied 3.024 E+08 L day^{-1} (manuscript 2), PEC in Norway is about 1 ng L^{-1}, which is very close to the limit of detection of the analytical methodology (0.8 ng L^{-1}; manuscript 1). Special mention

deserves the occurrence of ciprofloxacin. This compound has both anti-tumour and antibiotic activity and is frequently used in Spain as a wide-spectrum antibiotic. However, the Norwegian policy regarding the dispensation of this type of antibiotics in hospitals or pharmacies is very stringent. Due to the concern related to the development of antibiotic resistances, substances such as quinolones (such as the ciprofloxacin) or macrolides are merely reserved for special situations, where classic antibiotics (such as the penicillin's group) are not effective. Norway has a well-controlled system for pharmaceutical dispensation wherein the dose and patient are electronically registered and managed in all the pharmacies of the country.

6.2. Risk assessment

The study reported in *manuscript 3* contributed to put in the spotlight not only the occurrence but also the impact of anti-cancer drugs in the environment. This study confirms that anticancer drugs should be considered as a significant group within the emerging pollutants, due to their occurrence in the aquatic environment as well as their potential ecotoxicological risk. Therefore, their presence in conventional WWTPs should be comprehensively studied as well as the development of alternative wastewater treatments to remove these drugs from effluents. Ciprofloxacin and tamoxifen showed, among the anticancer drugs studied, to pose a potential hazard to the aquatic environment. Tamoxifen, in particular, cumulates several risk factors for aquatic ecosystems, due to its proved toxicity, its suspected endocrine disruption effects and its high bioaccumulation potential.

6.3. Removal of anticancer drugs and generation of TPs through advanced processes

Due to the insufficient removal of cancer drugs after conventional WWTPs, two alternative treatment technologies were evaluated with the ultimate aim of effectively reduce the release of anticancer drugs into the environment. One process was based on biological treatment (manuscript 4) whereas the 2nd one was based on an oxidative treatment (manuscript 5). In the experiments and studies performed, water quality of the effluent was evaluated before and after the treatment in terms of contaminants removal and ecotoxicity. Tamoxifen was selected as a model compound for these studies due to its presence and environmental relevance (as concluded in BLOCK I and II). Tamoxifen was thus thoroughly investigated concerning the formation of TPs along the two wastewater treatment methodologies evaluated.

6.3.1. Fungal treatment for the elimination of anticancer drugs from wastewater.

Most of the target anticancer drugs were removed from hospital wastewater after non-conventional biological treatment based on *T. versicolor*. This kind of lignolytic fungi demonstrated a better performance than the conventional biological treatments, which are inefficient in eliminating these compounds. Special mention deserves its removal capacity for ciprofloxacin, etoposide and azathioprine, which were successfully removed from a hospital effluent using these fungi in a fluidized bed bioreactor configuration (at experimental conditions close to those potentially implemented in a full-scale scenario). . However, neither bioreactor (hospital wastewater) nor batch experiments (synthetic water) were efficient in the removal of cyclophosphamide and ifosfamide, which contain chemical structures that might require more specific biodegrading systems (bacteria, other fungal strains, etc.). Tamoxifen was almost removed (about 50-91% of elimination) when the bioreactor was conducted in real hospital wastewater. Conversely, and as previously mentioned, tamoxifen was almost totally removed from the synthetic water (by combined sorption-biodegradation processes) when the batch experiments were conducted. Two compounds (tamoxifen hydroxylated positional isomers) were tentatively identified in water samples as biotransformation product of tamoxifen due to the WRF activity. In fact, hidroxy tamoxifen had been previously detected in hospital effluents, based on suspect-screening technology in manuscript 1 (Block I) .

6.3.2. Elimination of tamoxifen by different advanced oxidation processes: removal kinetics, generation of transformation products and toxicity

The results obtained in this study (manuscript 5) suggest that the integration of advanced oxidation processes based on combinations of ozone, UV irradiation and hydrogen peroxide, can substantially improve the removal of tamoxifen from aqueous solutions. The use of these treatments increases the removal efficiency of tamoxifen as well as reduces the time of the treatment when compared to conventional biodegradation system. Processes under ozonisation

conditions can be considered, based on the results, the most efficient process for the removal of the drug tamoxifen.

However, as previously discussed in the manuscript submitted, several transformation products of tamoxifen were identified along the time-course experiments, being always the toxicity of the effluent higher than the observed in the initial solution, where only tamoxifen was present. The TPs are presumably a product of oxidative transformation reactions as well as radical internal cyclizations of tamoxifen. In most of the degradation processes, non-selective hydroxyl radicals are the main species involved in the transformation. 3 metabolic routes were identified: two occurring under ozonisation conditions and one taking place with UV irradiation.

These results confirm that oxidation processes are a powerful tool concerning the elimination of micropollutants. They can be applied directly into the raw wastewater or as a tertiary treatment to eliminate those recalcitrant compounds, such as tamoxifen, not completely eliminated through biological treatment. However, as it was observed, special attention should be paid to the potential formation of non-desirable by-products generated along the process.

Finally relevant issue refers to life cycle assessment (LCA) of wastewater treatment process that was not part of this thesis.

CHAPTER 7

7. General conclusions

The work carried out during this thesis has led to the following conclusions:

- ✓ A successful off-line-SPE-UPLC-QqLiT analytical method for both quantitative and qualitative purposes has been developed for 10 anticancer drugs belonging to different groups applied to: hospital effluents, wastewater influents and effluents as well as surface waters.

- ✓ An analytical strategy based on suspect screening and UPLC-Q-TOF was developed for the tentatively identification of "suspected" anticancer drugs and related compounds in the aquatic environment.

- ✓ Target analysis allowed to determine the presence of chemotherapy drugs in a selected sewage urban system after wastewaters as well as in the surface waters receiving WWTPs discharge.

- ✓ Environmental risk assessment of anticancer drugs in surface waters concluded that tamoxifen and ciprofloxacin pose an environmental hazard to the aquatic environment based on the risk characterization ratio (RCR) observed (>1).

- ✓ Significant differences were found when assessing the presence of cancer drugs in wastewater influents from Norway and Spain: Cancer drugs (azathioprine, cyclophosphamide, ifosfamide, ciprofloxacin, docetaxel, etoposide, methotrexate, paclitaxel, vincristine, tamoxifen and their human metabolites) were found in Spain unlikely not found in Norway.

- ✓ Presence of cancer biomarkers and human metabolites related to anticancer drugs in wastewater influents at Oslo sewage were unnoticeable. However diverse anticancer drugs type progestin's, such as megestrol, were found in several samples.

- ✓ Improved biological treatment methods, such as those based on *Trametes versicolor*, were more efficient than conventional WWTPs in the degradation of several persistent pharmaceutical compounds.

- ✓ After the study of several removal technologies (CAS, biodegradation based on *Trametes versicolor* and diverse AOPs), it can be concluded that cyclophosphamide and ifosfamide are recalcitrant to degradation.

- ✓ Tamoxifen was totally removed using a non-conventional treatment based on *Trametes versicolor*. Two TPs were found along the time-course experiments being the toxicity of the effluent no greater than the parent compound.

- ✓ AOPs based on UV radiation or its combination with oxidizing substances such as H_2O_2 and O_3 contribute to achieve total elimination of the anticancer drug tamoxifen, being the one based on ozone the most successful method to remove this drug. However the generation of TPs along these processes lead to increasing of the effluent toxicity, as already shown in other examples in the literature.

- ✓ When assessing oxidative processes for the removal of PhACs, it should be considered however that decay on the concentration of the target substance does not necessarily involve decay on the overall toxicity of the effluent. Some TPs or the overall cocktail can be even more toxic than the original solution. Ecotoxicity assessment of the treated effluent becomes then a powerful and mandatory tool to be explored previous to the full scale implementation of AOPs.

- ✓ Anticancer drugs are excreted by humans in their original form and as metabolites, which can have equal or even more activity than the parent compound. Therefore, future studies should focus on the screening not only of the parental contaminant but also of their metabolites and transformation products, which are also present in wastewaters.

- ✓ Based on the results of this thesis tamoxifen should be considered as a contaminant of emerging concern to be included in a future priority watch list of the Water Framework Directive.

Concluding remarks and future perspectives

The benefits of pharmaceuticals for the quality of human life, particularly of anticancer drugs, are obvious. However, the potential risks linked to the presence of these substances in the environment should be further investigated. Along this thesis it has been demonstrated that some of these substances show high persistency and serious difficulties to be mineralized or transformed in other substances less hazardous for the environment. Therefore, a non-appropriate handling of the wastes derived from cancer disease and its related drugs might pose a risk for the aquatic life once they are released in the natural aquatic environment. According to their observed concentrations (ng L^{-1}), and their ecotoxicity, risks are mostly related to possible cumulative effects in aquatic organisms due to long-term low-dose exposure than to acute health effects, as it is the case of tamoxifen and ciprofloxacin.

Most of the anticancer drugs are not removed from secondary treatment (biological degradation) in conventional WWTPs. Therefore, the concentration of these compounds in WWTPs effluents should be further decreased to avoid any risk for aquatic life in receiving natural water bodies and also to assure safe water reuse for a variety of purposes. An improvement in overall wastewater treatment is thus needed. Tertiary treatments, originally designed to disinfect WWTPs effluents before the discharge in natural environments, are not intended to remove micropollutants. As it has been shown in this thesis, tertiary treatments such UV radiation, ozone, hydrogen peroxide and/or their combinations, are not always the right answer to deal with these substances since the cocktail of drugs and transformation products generated after the treatment can sometimes be even more toxic than the raw untreated effluent. In contrast, the non-conventional biological treatment based on fungi is presented here as a promising alternative to the removal of recalcitrant compounds. Further studies are required to assess the combination of several microbiological communities (including *Trametes versicolor*) to achieve a better performance in the removal of micropollutants.

The results presented and discussed throughout the thesis support the development of alternative wastewater treatments as well as the application of decentralized systems to treat hospital effluent "on-site" (before being discharged into the urban sewage collection system). This is in agreement with the recommendations provided by other authors in previous works (Verlicchi et al., 2010). A more effective management of human excretions (urine and faeces) from oncologic patients, separating them from

other household-like streams and treating them as a hazardous waste with environmental impact, is proposed as a potential solution to reduce the input of these compounds in the sewage system and eventually to decrease their impact in the environment.

A rigorous monitoring of pharmaceuticals in the environment should not cover just target substances themselves; special attention should also be paid to the metabolites and other sub-products formed along water cycle. As it has been demonstrated, the toxicity of these TPs could even be higher than the parent compound. In order to assess properly the risks of pharmaceutical compounds in the environment, the study of the fate and occurrence of these sub-products is mandatory.

Finally, the occurrence of this group of contaminants in solid compartments such as the activated sludge, sediments, suspended solids or colloids has not been the object of this thesis. The analysis of anticancer drugs in solid samples requires the development and validation of other analytical methodologies, sometimes based on techniques such as the accelerated solid extraction (ASE). However it is obvious that the fate of anticancer drugs in the environment is not completed without having the levels of those compounds that might be retained through sorption processes. Special attention should be paid to those compounds with high partition coefficient such as the case of tamoxifen. This compound has shown a high sorption affinity onto biomass (manuscript 4). Moreover, Tamoxifen might be accumulated in activated sludge at WWTPs according to its slight water solubility (0.3 mg L^{-1}) and non-polar character. In addition to this, tamoxifen has to be considered as an ubiquitous pollutant, since it has been detected in large number of liquid samples gathered from many different environments (manuscripts 1 and 3).

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88. Franquet-Griell, H., et al., *Predicting concentrations of cytostatic drugs in sewage effluents and surface waters of Catalonia (NE Spain).* Environmental Research, 2015. **138**: p. 161-172.

Annexes

Curriculum Vitae

Curriculum Vitae

Personal Information	
Name/ Surname	**Laura Ferrando-Climent**
Address	Idas vei 77, Oslo, Postal Code 0981 (Norway) Mobile: + 47 930 400 46
e-mail	laurafc@ife.no
Nationality	Spanish
Date and Birth place	February on 22nd of 1980 in Valencia (Spain)
Civil State	Married
Academic Education	➢ **Degree in Chemistry** by University of Valencia, 2006 ➢ **Experimental Techniques in Chemistry Course** by University of Valencia, 2007 ➢ **Joint Master's in Chromatographic Techniques Degree** by University of Girona (Spain), University Jaume I (Castellón) and University Rovira i Virgili (Tarragona), at 2011. ➢ **PhD in Chemistry** by University of Girona (Spain), 2016. Supervised by Prof. Dr. Damià Barceló Culleres and Dr. Sara Rodriguez-Mozaz.
h-index	7 (excluding self-citations)
Score in researchgate	23.81
Professional Experience	
	❖ **Research Scientist**
Date	February the 2nd of 2015- Currently
Department	Tracer Technology
Functions /Tasks	Analytical chemistry. Evaluation of new tracer candidates. Environmental applications. Project manager.
Company/Institution	Institutt for Energiteknikk (IFE) Institutveien 18, 2007, Kjeller, Norway
	❖ **Yggdrasil Researcher**
Date	August the 1th of 2013 to February 28th of 2014
Department	Ecotoxicology - Research Analysis
Functions /Tasks	Evaluation of organic micropollutants as tracers of the water quality by UPLC-QTOF. Environmental markers permit to establish relations between them, to identify their aquatic distribution or even to predict their occurrence in aquatic systems.
Company/Institution	Norsk institutt for vannforskning (NIVA) Gaustadalléen 21, 0349 Oslo, Norway
	❖ **PhD Candidate**
Date	January the 11th of 2010 to January 2014
Department	Quality Area-Department of Chemical Contamination of Water Bodies

Functions /Tasks	Thesis: "*Analysis of chemotherapy drugs and related compounds in aquatic environment: removal, transformation and risk evaluation in eco-friendly and advanced technologies*"
Company/Institution	Catalan Institute for Water Research (ICRA), Girona (Spain) www.icra.cat
	❖ **Responsible for Quality Assurance**
Date	May the 1st of 2012 to October the 31st of 2012 (temporally substitution)
Department	Quality Control
Functions /Tasks	Responsible of chemical laboratory, sanitary regulations, documentation, SOPs, Quality of raw material and final product.
Company/Institution	Axati Flaires Cosmetics S.L. ; Manresa (Barcelona, Spain)
	❖ **Associated Professor**
Date	September the 1st of 2009 to December the 31st of 2009
Department	Chemical Engineering
Functions /Tasks	Supervision of practices in the Laboratory of Chemistry and Technology of cellulosic fibres.
Company/Institution	Superior School of Engineers ; University of Girona (UDG), Girona (Spain) www.udg.edu
	❖ **Responsible of In Process Control Group for Active Principle Ingredients (APIs) manufacturing. Management of Stability Studies of APIs**
Date	May the 7th of 2007 to January the 11th of 2010
Department	Quality Control Laboratory
Functions /Tasks	Responsible of "*In Process Control* "and "*Stability Testing*". Analytical techniques employed: HPLC-UV/Vis, GC, Automatic Titration, Automatic Karl Fischer, Laser Particle Size Determination (Malvern), IR, spectrophotometry and optical rotation.
Company/Institution	Medichem S.A. Celrà , Spain. www.medichem.es
	❖ **Technical Responsible for Analytical Development**
Date	May the 4th of 2006 to May the 4th of 2007 (AFAPHE scholarship for recent graduates)
Department	Pharmacokinetic Laboratory- Hospital Pharmacy
Functions /Tasks	Development of Analytical Methods for Antineoplastic pharmaceuticals and other chemotherapy drugs by HPLC-UV/Vis and Graphite Furnace Atomic Absorption Spectrophotometry–Zeeman. Stability Studies of binary and ternary pharmaceutical mixtures for hospital pharmacy.
Company/Institution	Foundation AFAHPE (Hospital Pharmacists Association of Dr. Peset) Universitary Hospital Dr. Peset, Valencia, Spain
Additional Formation	
2012	LTQ Orbitrap Velos Operations Training Course by Thermo Fisher Scientific Training Institute on 11th, 12th and 13th of April.
2008	Course of "*Empower- Level 1 Interface Quick start*" (12 h) by Waters Corporation (Barcelona).
2007	Course of "*Basic HPLC"* (20 h) by Waters Corporation (Barcelona).
Student supervision	
February 2016 - current	Supervision of Master student (UiO- Oslo, Norway) at IFE: Janaki Rajakumar

Publications

1. Book Chapter: "New Tendencies in Liquid chromatography for controlling PAHs Polluting" R. Herráez-Hernández, P. Campíns-Falcó, J. Verdú-Andrés, A. Sevillano-Cabeza and L. Ferrando-Climent. *Polycyclic aromatic hydrocarbons. 2009 Nova Science Publishers 2009*
2. Collado N., Buttiglieri G., Ferrando-Climent L., Rodriguez-Mozaz S., Barceló D., Comas J. Removal of ibuprofen and its transformation products: Experimental and simulation studies. *Science of the Total Environment 2012; 433: 296-301.*
3. Ferrando-Climent L., Collado N., Buttiglieri G., Gros M., Rodriguez-Roda I., Rodriguez-Mozaz S., Barceló D. Comprehensive study of ibuprofen and its metabolites in activated sludge batch experiments and aquatic environment. *Science of the Total Environment 2012; 438: 404-413.*
4. Ferrando-Climent L., Rodriguez-Mozaz S. and Barceló D. Development of a UPLC-MS/MS method for the determination of 10 anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information dependant acquisition tool (IDA) in sewage samples. *Analytical and Bioanalytical Chemistry 2013.*
5. N. Collado, G. Buttiglieri, E. Marti, L. Ferrando-Climent, S. Rodriguez-Mozaz, D. Barceló, J. Comas, I. Rodriguez-Roda. Effects on activated sludge bacterial community exposed to Sulfamethoxazole. *Chemosphere 2013*
6. Mira Petrovic, Biljana Skrbic, Jelena Zivancev, Laura Ferrando-Climent, Damià Barceló. Determination of 80 pharmaceutical drugs by high performance liquid chromatography coupled to mass spectrometry with hybrid triple quadrupole-linear ion trap in different type of water in Serbia. *Science Total Environment 2013.*
7. Carles Cruz-Morató, Laura Ferrando-Climent, Sara Rodriguez-Mozaz, Damià Barceló, Ernest Marco-Urrea, Teresa Vicent, Montserrat Sarrà. Degradation of pharmaceuticals in non-sterile urban wastewater by Trametes versicolor in a fluidized bed bioreactor. *Water Research 2013.*
8. Ferrando-Climent L., Rodriguez-Mozaz S. and Barceló D Incidence of anticancer drugs in an aquatic urban system: from hospital effluents through urban wastewater to natural environment. *Environmental Pollution2014*
9. Laura Ferrando-Climent, Carles Cruz-Morató, Ernest Marco-Urrea, Teresa Vicent, Montserrat Sarrà, Sara Rodriguez-Mozaz, Damià Barceló. Advanced biological treatment based on Trametes versicolor for persistent pharmaceuticals in wastewaters: the anticancer drugs. *Chemosphere 2015*
10. Marta Llorca; Sara Rodríguez-Mozaz; Daniel Lucas; Laura Ferrando-Climent; Marina Badia-Fabregat; Carles Cruz-Morató; Damià Barceló "Suspect screening of emerging pollutants and their major transformation products in wastewaters treated with fungi by liquid chromatography coupled to a high resolution mass spectrometry" . *Journal of Chromatography A 2016*
11. Elissavet Kassotaki · Gianluigi Buttiglieri · Laura Ferrando-Climent · Ignasi Rodriguez-Roda · Maite Pijuan. "Enhanced sulfamethoxazole degradation through ammonia oxidizing bacteria co-metabolism and fate of transformation products". *Water Research 2016.*
12. Heath, E., et al. "First inter-laboratory comparison exercise for the determination of anticancer drugs in aqueous samples." *Environmental Science and Pollution Research 2015*
13. Ferrando-Climent, L, Gonzalez, R., Anfruns A., Americh, I. Corominas, L., Barceló, D. Rodriguez-Mozaz, S. "Elimination study of the chemotherapy drug Tamoxifen by different advanced oxidation processes: transformation products and toxicity". *Submitted to Chemosphere 2016. (Received with minor revision at 11th August 2016)*
14. Ferrando-Climent, L, Reid, M.J., Rodriguez-Mozaz, S., Barceló, D., Thomas, K.V. "Identification of (bio-) markers of cancer in urban sewage through the use of a suspect screening approach". *Journal of Pharmacology and biomedical analysis 2016.*
15. Book chapter invitation: Ferrando-Climent,L. Escudero, C. Do Santos, LH.and Rodriguez-Mozaz, S. Occurrence and risk of cytostatics, antibiotics and contrast agents in hospital effluent. Book: Hospital Wastewaters - Characteristics, Management, Treatment and Environmental Risks, *ed. Springer by Paola Verlicchi. (In preparation September 2016)*
16. Krivokapic, A., Ferrando-Climent, L., Sayfritz,S., Huseby, O. and Dye, C. (2016) A Game-Change for Single Well Chemical Tracer Tests (SWCTT): Development and Qualification of a Novel Set of Tracers, *(In preparation)*

Conferences Contributions

1) Poster: Carril-Avilés M.M., Ferrando-Climent L., González-Navarro M., González-Valdivieso J., Romero-Crespo I., Jiménez-Torres N.V. Stability of Haloperidol-Butilescopolamine-Midazolam in perfusion systems for 24 hours (Register 1538); Congress: SEFH 2007 (Tenerife, Spain)

2) Poster: Carril-Avilés M., Ferrando-Climent L., González-Navarro M., Romero-Crespo, I., González-Valdivieso J., Jimenez-Torres N.V. Stability of the ondansetron-dexamethasone mixture in 0.9% NaCl solution. (Register 1257) Congress: SEFH 2007 (Tenerife, Spain)

3) Poster: L. Ferrando-Climent, N. Collado, G. Buttiglieri, I. Rodriguez-Roda. S. Rodriguez-Mozaz, D. Barceló. Elimination Processes of Ibuprofen in Activate Sludge Batch Experiments. (Register E18); Conference: ICCE 2011 (Zurich, Switzerland).

4) Poster: Laura Ferrando-Climent, Sara Rodriguez-Mozaz, Damià Barceló. Development of a UPLC-MS/MS method for the determination of 10 cancer drugs in hospital and urban wastewaters. Symposium of Chromatography 2012 (Barcelona, Spain)

5) Oral presentation: I. Quesada, C. Coetsier, C. Causserand, S. Rodriguez-Mozaz, L. Ferrando-Climent, D. Barcelò, O. Lorain, C. Albasi. Treatment of a membrane bioreactor permeate by nanofiltration or reverse osmosis for the removal of anti-cancer drugs. Conference GRUTTEE 2012 (Marseille, France)

6) Poster: Mater N., Faucet-Marquis V., Ferrando-Climent L., Rodriguez-Mozaz S., Barceló D., Pfohl-Leszkowicz A. In vitro bioassays for risk assessment induced by mixtures of anticancer drugs released in hospital waste water - Application to hospital effluents. Conference GRUTTEE 2012 (Marseille, France)

7) Poster: Carles Cruz-Morató, Laura Ferrando-Climent, Daniel Lucas, Meritxell Gros, Sara Rodriguez-Mozaz, Damià Barceló, , Ernest Marco-Urrea, Montserrat Sarrà, Teresa Vicent, Imad Touahar, Sidy Ba, Hubert Cabana. Fungal and enzymatic treatment of pharmaceuticals active compounds in real effluents. Conference ACQE 2012 (Sherbrooke, Canada).

8) Poster: Laura Ferrando-Climent, Sara Rodriguez-Mozaz, Damià Barceló. Incidence of ten anticancer drugs and its metabolites in the aquatic environment: targeted and non targeted analysis. Conference Pharmaceuticals in the environvent 2013 (Nimes, France)

9) Poster: L. Ferrando-Climent, C. Cruz-Morató, E. Marco-Urrea, M. Sarrà, T. Vicent, S. Rodriguez-Mozaz, D. Barceló Incidence of chemotherapy drugs in the aquatic environment and their elimination by non conventional wastewater treatment: Trametes versicolor. Conference ICCE 2013 (Barcelona, Spain)

10) Oral presentation: Paola Verlicchi , Meritxell Gros, Noelia Negreira, Lucia Helena Santos, Laura Ferrando-Climent, Miren Lopez de Alda,Sara Rodriguez-Mozaz, Damia Barcelo. Pharmaceuticals in hospital effluents: LC tandem MS Identification, Risks and Solutions. ASMS 2013 (California, USA)

11) Oral presentation: Rafael Gonzalez-Olmos, Laura Ferrando-Climent, Alba Anfruns, Sara Rodriguez-Mozaz, Damià Barceló. Removal of a recalcitrant anticancer drug from water with ozone based technologies. IOA 2015 (Barcelona, Spain)

Languages

Mother tongue	Spanish and Catalan
English	Proficiency
Norsk	Beginner

Project Managing

January 2016 -current	Project manager: *Application of tracer technology for the characterization of chemical contaminated areas*. Eurostars project (3 years)