OCCURRENCE, FATE AND EFFECTS OF CYTOSTATIC RESIDUES IN THE AQUEOUS ENVIRONMENT

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Doctoral Dissertation

POJAVNOST, KROŽENJE IN UČINKI OSTANKOV CITOSTATIKOV V VODNEM OKOLJU

Doktorska disertacija

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“Znanstveni napredek ni vedno premočrtna pot k resnici, temveč je poln vzponov in padcev, med katerimi pogosto naletimo na slepe ulice. … Odsotnost napak ni znamenje uspeha – ravno iz napak se namreč lahko največ naučimo.”

Andraž Matkovič, Delo, November 2015

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Abstract

Among numerous active pharmaceutical ingredients (APIs) registered for chemotherapy, two of the oldest, cyclophosphamide (CP) and ifosfamide (IF), are still widely prescribed. Their administration results in side effects, such as non-selective cytotoxicity, genotoxicity, mutagenicity and teratogenicity. Increasing cancer incidence is associated with growing consumption of such compounds, which are excreted *via* urine and faeces as parent compounds and/or metabolites, eventually reaching the environment *via* the sewerage system. Research into the occurrence and effects of various APIs in the environment has been ongoing, whereas focus on cytostatic residues is still novel. Concentrations of CP and IF in hospital wastewaters (WWs), wastewater treatment plant (WWTP) influents and effluents and surface waters (SWs) are in ng L^{-1} up to µg L^{-1} range, yet the occurrence of potentially active metabolites and transformation products (TPs) remains to be determined. To reduce the burden of CP and IF residues in WW and consequently SW and drinking water, the development and optimization of biological and abiotic water treatments technologies is essential. In addition, knowledge regarding the degradation pathways of these compounds is important since their TPs might show higher toxicity towards aquatic ecosystems than the parent compounds. These effects can be assessed with bioassays that include different trophic levels, e.g. bacteria, algae, plants or fish. The review of literature reveals limited information about the toxicity of CP and IF, whereas complex mixtures containing CP, IF, their metabolites and TPs remain to be investigated.

The first aim of this doctoral thesis was to investigate the occurrence of CP and IF, their human metabolites and TPs 4-ketocyclophosphamide (keto-CP), N-dechloroethylcyclophosphamide or 3-dechlorethylifosfamide (N-decl-CP) and carboxy-cyclophosphamide (carboxy-CP) in the Slovene aqueous environment. Within this task, the analytical method for determination of CP and IF in various aqueous matrices was optimized. In addition, an analytical method for determining keto-CP, N-decl-CP and carboxy-CP in WW was developed. Solid-phase extraction (SPE) of CP, IF and keto-CP was achieved using Oasis HLB^{TM} cartridges (N-vinylpyrrolidone and divinylbenzene copolymer), whereas for N-decl-CP and carboxy-CP Isolute ENV+^{TM} cartridges (hydroxylated polystyrene divinylbenzene copolymer) were used. Optimal derivatization of metabolites was achieved with N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide with 1 % tert-butyldimethylchlorosilane, whereas for CP and IF trifluoroacetic anhydride was used. Chemical analysis was performed by gas chromatography coupled to mass spectrometry (GC-MS). The methods were assessed in terms of SPE recovery, linearity, instrumental and method repeatability, accuracy and limits of detection (LODs) and quantification (LOQs). The SPE recoveries and LODs were 99 %, 93 %, 58 %, 87 % and 103 % and 2.3 ng L^{-1}, 4.8 ng L^{-1}, 23 ng L^{-1}, 13.1 ng L^{-1} and 2.0 ng L^{-1} for CP, IF, carboxy-CP, keto-CP and N-decl-CP, respectively. Furthermore, an interlaboratory comparison was performed for the parent compounds, which confirmed the reliability of the optimized analytical method for CP and IF analysis. Seven (for CP) and 6 (for IF) laboratories from Europe and Asia participated within this interlaboratory exercise. The smallest absolute differences between the spiked values and the measured values of CP and IF were observed in SW.

The optimized methods were used to analyse aqueous samples collected from different hospitals and their corresponding WWTPs. Cyclophosphamide was detected in two hospital WWs (14 ng L^{-1} – 22,000 ng L^{-1}), one WWTP influent (19 ng L^{-1} – 27 ng L^{-1}) and one effluent (17 ng L^{-1}), whereas IF was detected only in one hospital WW sample (48 – 6,800 ng L^{-1}). The concentrations of carboxy-CP, N-decl-CP and keto-CP were 213 ng L^{-1} – 13,202 ng L^{-1}, 60 ng L^{-1} – 2,099 ng L^{-1} and < LOD – 178 ng L^{-1} in one hospital WW, respectively, whereas in WWTP influent and effluent they were < LODs.

Various treatment techniques were then assessed in terms of CP and IF removal from WW. Besides advanced biological treatment using attached-growth biomass on Mutag^{TM} carriers, different abiotic treatments were examined, including UV irradiation, ozonation (O_3), hydrodynamic cavitation (HC) and combinations with hydrogen peroxide (H_2O_2) as advanced oxidation processes (AOPs). The removal efficiency of biological treatment in a flow through bioreactor was 42 % and 18 % for CP and IF, respectively. Hydrodynamic cavitation was the least successful treatment (< 14 % for CP and IF), whereas the highest removal efficiencies, 99 % for CP and 94 % for IF, were achieved by UV/O_3/H_2O_2 at 5 g L^{-1} treatment. This AOP, sequentially coupled with biological treatment, resulted in removal efficiencies > 99 % for both compounds.

The mechanism of TPs formation during selected treatments (UV and UV/H_2O_2) was investigated in Milli-Q water. The structural elucidation of TPs was achieved using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). The results showed the formation of four TPs of CP (N-decl-CP, keto-CP, CP-TP138a and CP-TP138b) and four TPs of IF (N-dechloroethyl-ifosfamide, keto-ifosfamide, imino-ifosfamide and IF-TP138) during UV treatment. The same TPs were observed during UV/H_2O_2 treatment along with an additional CP TP, imino-phosphamide. Three novel TPs, CP-TP138a, imino-ifosfamide and IF-TP138, were identified and the formation pathway of IF TPs during UV and UV/H_2O_2 treatment was proposed for the first time.

The final part of this thesis evaluates the toxicity of the investigated compounds by measuring their ecotoxicity towards two phytoplankton species and their genotoxicity using the SOS/*umu*C assay with *Salmonella typhimurium*. The parent compounds were non-toxic towards the algae *Pseudokirchneriella subcapitata* and cyanobacteria *Synecococcus leopoliensis* at concentrations up to 320 mg L^{-1}. Moreover, the toxicity of their metabolites/TPs and mixture (parent compounds + metabolites/TPs) were studied towards *S. leopoliensis.* Only carboxy-CP caused significant growth inhibition of cyanobacteria (EC_{50} = 17.1 mg L^{-1}). The toxicity of the complex mixture was predicted using a concentration addition (CA) model and compared to experimentally determined toxicity. The predicted (EC_{50} = 21.1 mg L^{-1}) was lower than the measured toxicity (EC_{50} = 11.5 mg L^{-1}). These results revealed a potentiation effect of the mixture. The SOS/*umu*C assay revealed genotoxic activity of CP, carboxy-CP and the mixture in the presence of S9 metabolic activation, whereas carboxy-CP was genotoxic also in the absence of S9. Finally, UV irradiation of samples containing CP and IF resulted in considerable removal of both compounds (CP = 69 %; IF = 63 %; t = 48 h) and remained non-toxic towards *S. leopoliensis*.

This thesis describes the occurrence, fate and toxicity of CP, IF, N-decl-CP, keto-CP and carboxy-CP in aqueous environment. The occurrence of these compounds and the removal efficiency of CP and IF during advanced biological treatment and HC were studied for the first time. Sequentially coupled AOP (UV/O_3/H_2O_2) with biological treatment resulted in high removal of CP and IF (> 99 %). This study also confirmed the presence of three novel TPs during UV and UV/H_2O_2 treatment. Finally, it was shown that metabolites/TPs can be more toxic than parent compounds and that the studied mixture exhibits potentiation effect.

Povzetek

Ciklofosfamid (CP) in ifosfamid (IF) sodita med najstarejše zdravilne učinkovine za zdravljenje raka, ki ju še vedno pogosto uporabljamo. Administracija tovrstnih zdravilnih učinkovin povzroča stranske učinke, kot so neselektivna citotoksičnost, genotoksičnost, mutagenost in teratogenost. Zaradi naraščajoče obolevnosti za rakom se veča tudi poraba zdravil za zdravljenje raka, ki se izločajo iz telesa z urinom in blatom v nespremenjeni obliki ali kot metaboliti ter tako vstopajo v okolje preko izpustov v kanalizacijsko omrežje. V literaturi najdemo številne raziskave o pojavnosti in učinkih ostankov zdravilnih učinkovin v okolju, bistveno manj pa je podatkov o ostankih zdravil z delovanjem na novotvorbe. Literaturni podatki kažejo, da se koncentracije CP in IF, določene v bolnišničnih odpadnih vodah, vtokih in iztokih iz čistilnih naprav ter celo v površinskih vodah, gibljejo v območju ng L^{-1} do µg L^{-1}, medtem ko pojavnost potencialno aktivnih metabolitov oziroma transformacijskih produktov (TPjev) še ni ovrednotena. Za zmanjšanje prisotnosti ostankov CP in IF v odpadnih in posledično tako v površinskih kot pitnih vodah sta nujna razvoj in optimizacija bioloških in abiotskih postopkov čiščenja, pri čemer je pomembno poznavanje mehanizma transformacij tovrstnih spojin. Transformacijski produkti so namreč lahko za vodne ekosisteme bolj strupeni kot izhodne spojine. Tovrstne učinke na vodne organizme ocenjujemo s strupenostnimi testi, ki vključujejo organizme na različnih trofičnih nivojih, npr. na bakterijah, algah, rastlinah ali ribah. Objavljenih je le nekaj raziskav, ki ocenjujejo strupenost starševskih spojin, medtem ko strupenost kompleksnih mešanic teh spojin z njihovimi metaboliti/TPji še ni raziskana.

V prvem delu doktorske disertacije sem preverjala, ali so CP in IF ter njuni metaboliti in TPji keto-ciklofosfamid (keto-CP), N-dekloroetil-ciklofosfamid oziroma 3-dekloroetil-ifosfamid (N-decl-CP) in karboksi-ciklofosfamid (carboxy-CP) prisotni v vzorcih slovenskih voda. V ta namen sem optimizirala analizno metodo za določanje CP in IF v različnih vodnih matricah. Poleg tega sem razvila tudi analizno metodo za določanje keto-CP, N-decl-CP in carboxy-CP v odpadnih vodah. Ciklofosfamid, IF in keto-CP sem ekstrahirala na trdnem nosilcu z Oasis HLB^{TM} kartušami (kopolimer N-vinilpirolidona in divinilbenzena), za N-decl-CP in carboxy-CP pa sem uporabila Isolute ENV+^{TM} kartuše (kopolimer hidroksiliranega polistirena in divinilbenzena). Predhodno derivatizirane vzorce sem analizirala s plinskim kromatografom z masno selektivnim detektorjem (GC-MSD). Za derivatizacijo CP in IF sem uporabila trifluoroacetanhidrid, za metabolite/TPje pa N-(terc-butildimethilsilil)-N-methiltrifluoroacetamidom z 1 % terc-butildimethilklorosilanom. Razvitim metodam sem določila izkoristke ekstrakcije, linearnost, ponovljivost instrumenta in celotne analizne metode, točnost ter meje detekcije (LOD) in kvantifikacije (LOQ). Izkoristki ekstrakcije ter LOD vrednosti so znašali 99 %, 93 %, 58 %, 87 % in 103 % ter 2,3 ng L^{-1}, 4,8 ng L^{-1}, 23 ng L^{-1}, 13,1 ng L^{-1} in 2,0 ng L^{-1} za CP, IF, carboxy-CP, keto-CP ter N-decl-CP. Za potrditev zanesljivosti analizne metode za določanje starševskih spojin sem sodelovala v medlaboratorijski primerjalni analizi, v katero so bili vključeni laboratoriji iz Evrope in Azije, ki so določali CP (7 laboratorijev) in IF (6 laboratorijev) v različnih vodnih matricah. Najmanjše razlike med koncentracijami CP in IF, ki so bile dodane v vsak vzorec, ter izmerjenimi vrednostmi so bile zaznane v matrici površinske vode.

Z optimiziranimi metodami sem analizirala vzorce odpadnih vod iz slovenskih bolnišnic in pripadajočih čistilnih naprav. Ciklofosfamid sem določila v dveh bolnišničnih odpadnih vodah (14 ng L^{-1} – 22.000 ng L^{-1}), v enem vtoku (19 ng L^{-1} – 27 ng L^{-1}) in enem iztoku iz čistilne naprave (17 ng L^{-1}), medtem ko sem IF določila le v eni bolnišnični odpadni vodi (48 – 6.800 ng L^{-1}). Koncentracije metabolitov/TPjev, ki sem jih določila v odpadni vodi ene bolnišnice, so bile 213 ng L^{-1} – 13.202 ng L^{-1} (carboxy-CP), 60 ng L^{-1} – 2.099 ng L^{-1} (N-decl-CP) in < LOD – 178 ng L^{-1} (keto-CP). V vzorcih vtoka in iztoka iz čistilne naprave pa so bile vrednosti < LOD.

Ocenila sem tudi učinkovitost odstranjevanja preučevanih starševskih spojin z različnimi postopki čiščenja odpadnih vod. Poleg naprednega biološkega čiščenja s pritrjeno biomaso na Mutag^{TM} nosilcih sem preučevala tudi različne abiotske postopke čiščenja (UV obsevanje, ozonacija, hidrodinamska kavitacija (HC) ter kombinacije navedenih postopkov z vodikovim peroksidom (H_2O_2), t. i. napredni oksidacijski postopki (Advanced Oxidation Processes, AOP)). Učinkovitost odstranitve v laboratorijskih pretočnih bioreaktorjih je bila 42 % za CP in 18 % za IF. Hidrodinamska kavitacija je bila najmanj učinkovita (< 14 % za obe spojini), najvišji delež odstranitve pa sem dosegla s postopkom UV/O_3/H_2O_2 pri 5 g L^{-1} (CP = 99 %; IF = 94 %). Z zaporedno vezanim naprednim biološkim in izbranim AOP postopkom čiščenja (UV/O_3/H_2O_2) sem uspešno odstranila več kot 99 % obeh spojin.

Mehanizem tvorbe TPjev iz CP ter IF sem preučevala med izbranima postopkoma čiščenja (UV ter UV/H_2O_2) v Milli-Q vodi s tekočinsko kromatografijo z visokoločljivo masno spektrometrijo (LC-HRMS). Določila sem štiri TPje, ki so se tvorili iz CP (N-decl-CP, keto-CP, CP-TP138a in CP-138b) in štiri TPje iz IF (N-dekloroetil-ifosfamid, keto-ifosfamid, imino-ifosfamid in IF-TP138) med UV obsevanjem. Poleg navedenih se je med potekom UV/H_2O_2 eksperimenta tvoril še en TP iz CP, in sicer imino-fosfamid. Med naštetimi TPji sem prva identificirala tri TPje (imino-ifosfamid, CP-TP138a in IF-TP138) ter predlagala potek nastanka TPjev iz IF tekom preučevanih postopkov čiščenja.

V zadnjem delu doktorske naloge sem preučevala strupenost preiskovanih spojin z ekotoksikološkimi testi na dveh organizmih (fitoplankton) in genotoksičnost z SOS/*umu*C testom na bakteriji *Salmonella typhimurium*. Rezultati so pokazali, da starševski spojini v koncentracijah do 320 mg L^{-1} ne zavirata rasti alge *Pseudokirchneriella subcapitata* in cianobakterije *Synecococcus leopoliensis*. Oceno strupenosti metabolitov/TPjev in njihove mešanice (starševski spojini z metaboliti/TPji) sem preučevala na *S. leopoliensis*. Statistično značilno inhibicijo rasti sem opazila le pri carboxy-CP z vrednostjo EC_{50} = 17,1 mg L^{-1}. Teoretično napovedano strupenost mešanice z uporabo modela »seštevka koncentracij« (CA model) sem primerjala z eksperimentalno določeno strupenostjo. Strupenost, napovedana po matematičnem CA modelu (EC_{50} = 21,1 mg L^{-1}) je bila nižja od eksperimentalno določene (EC_{50} = 11,5 mg L^{-1}). Ti rezultati nakazujejo potencirajoči učinek preiskovane mešanice. S testom SOS/*umu*C sem dokazala genotoksično aktivnost spojin CP in carboxy-CP ter mešanice v prisotnosti metabolne aktivacije z S9, carboxy-CP pa je bil genotoksičen tudi v odsotnosti S9. Uspešnost odstranitve CP in IF pri eksperimentu z 48-urnim UV obsevanjem vodnih vzorcev je bila 69 % za CP in 63 % za IF, pri čemer obsevana mešanica ni zavirala rasti alge *S. leopoliensis*.

V okviru doktorske disertacije sem preučevala pojavnost, usodo in učinke CP, IF ter njunih metabolitov/TPjev N-decl-CP, keto-CP in carboxy-CP v vodnem okolju. Pojavnost preiskovanih spojin in uspešnost odstranitve CP in IF z naprednim biološkim postopkom čiščenja ter HC sem preučevala prva v svetovnem merilu. S sekvenčnim eksperimentom, kjer je biološkemu čiščenju sledil AOP (UV/O_3/H_2O_2), sem dosegla > 99 % odstranitev CP in IF. Poleg tega sem potrdila tvorbo treh novih TPjev tekom UV in UV/H_2O_2 postopkov čiščenja. Dokazala sem tudi, da so metaboliti/TPji lahko bolj strupeni kot starševske spojine ter da preučevana mešanica izraža potencirajoči učinek.

Abbreviations

AC	=	activated carbon
ACN	=	acetonitrile
Ac_2O	=	acetic anhydride
AOP	=	advanced oxidation process
API	=	active pharmaceutical ingredient
APIon	=	atmospheric pressure ionization
APCI	=	atmospheric pressure chemical ionization
AS	=	activated sludge
ATC	=	Anatomical Therapeutic Chemical (ATC) Classification
BOD	=	biological oxygen demand
BCF	=	bioconcentration factor
CA	=	concentration addition
Carboxy-CP	=	carboxy-cyclophosphamide
CH_2N_2	=	diazomethane
CI	=	chemical ionization
CIn	=	combination index
COD	=	chemical oxygen demand
CO_2	=	carbon dioxide
CP	=	cyclophosphamide
CRM	=	certified reference material
DO	=	dissolved oxygen
DOC	=	dissolved organic carbon
EC_{50}	=	concentration that provokes 50 % of the maximum response
EE2	=	ethinyl estradiol
EI	=	electron-impact ionization
EMEA	=	European Medicines Agency
ERA	=	Environmental Risk Assessment
ESI	=	electrospray ionization
FDA	=	U. S. Food and Drug Administration
$Fe^{2+/3+}$	=	iron ions
FWMH	=	full width at half maximum
GC	=	gas chromatography
HC	=	hydrodynamic cavitation
HFBA	=	heptafluorobutyric anhydride
HFBI	=	N-heptafluorobutyrylimidazole
HRMS	=	high resolution mass spectrometry
H_2O	=	water
H_2O_2	=	hydrogen peroxide
IA	=	independent action
ICP-MS	=	inductively coupled plasma - mass spectrometry
IF	=	ifosfamide
I.F.	=	impact factor
IT	=	ion-trap

LC	=	liquid chromatography
LC_{50}	=	concentration that causes death of 50 % of the population
LIT	=	linear ion-trap
LLE	=	liquid-liquid extraction
LOD	=	limit of detection
LOEC	=	lowest observed effect concentration
K_{ow}	=	octanol/water partition coefficient
K_{oc}	=	organic carbon/water partition coefficient
LOQ	=	limit of quantification
LTQ Orbitrap	=	linear ion-trap Orbitrap mass spectrometer
K_d	=	sorption coefficient
Keto-CP	=	keto-cyclophosphamide
MeOH	=	methanol
MBTFA	=	N-methyl-bis(trifluoroacetamide)
MBBR	=	moving-bed bioreactor
MBR	=	membrane bioreactor
MP	=	mobile phase
MRM	=	multiple reaction monitoring
MS	=	mass spectrometry
MS/MS	=	tandem mass spectrometry
MS^n	=	multiple stage mass spectrometry
MTBSTFA	=	N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide
MSTFA	=	N-methyl-N-(trimethylsilyl)trifluoroacetamide)
m/z	=	mass-to-charge ratio
NF	=	nanofiltration
NH_4-N	=	ammonia ion
NOEC	=	no observed effect concentration
NO_2-N	=	nitrite ions
NO_3-N	=	nitrate ions
N-decl-CP	=	N-dechloroethyl-cyclophosphamide
OECD	=	Organization for Economic Cooperation and Development
•OH	=	hydroxyl radical
OH^-	=	hydroxide ions
$O_2^{\bullet-}$	=	superoxide radical
O_2	=	oxygen
O_3	=	ozone
PFBBr	=	2,3,4,5,6-pentafluorobenzyl bromide
PFBCl	=	2,3,4,5,6-pentafluorobenzoyl chloride
PFBHA	=	O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride
pK_a	=	dissociation constant
POCIS	=	Polar Organic Chemical Integrative Sampler
Q	=	quadrupole
QqQ	=	triple quadrupole
RO	=	reverse osmosis
RP	=	reversed phase
RSD	=	relative standard deviation
SD	=	standard deviation
SIM	=	selected ion monitoring
SPE	=	solid phase extraction
SPME	=	solid phase microextraction
SRM	=	selected reaction monitoring

SW	=	surface water
TBDMCS	=	tert-butyldimethylchlorosilane
TFAA	=	trifluoroacetic anhydride
TOF	=	time-of-flight
TP	=	transformation product
WW	=	wastewater
WWTP	=	wastewater treatment plant

1 Introduction

Active pharmaceutical ingredients (APIs) are compounds, intended for diagnosis, treatment or prevention of various diseases. They are used in human and veterinary treatment to prolong and improve the quality of life for people and animals (Zakon o zdravilih, 2014). Regardless, recent decades have seen a growing concern among environmental scientists and the general public about possible adverse effects of APIs on non-target organisms, once they appear in the environment (Ternes, 1998). And how do APIs enter the environment? Based on pharmacokinetics, APIs are, after administration, distributed within the body, then metabolized before finally being excreted in either an unchanged state or as metabolites. These species are, in the case of animal treatment, disposed of directly into the environment, whereas in human therapy, they mainly enter the sewerage system which is, in most cases, connected to a wastewater treatment plant (WWTP). Since conventional and existing WWTPs are not specifically designed to remove APIs' residues, sometimes only partial degradation of these compounds is achieved. It is obvious that incomplete degradation results in a release of such biologically active species to surface waters (SWs) like rivers and lakes and, finally, ground or drinking water (Kümmerer, 2008).

The consumption of pharmaceuticals is increasing globally, which is in accordance with population growth and the consequent higher demand for drugs used to treat ageing-related and chronic diseases (OECD, 2012). A group of the most widely prescribed and consumed pharmaceuticals are antihypertensives, cholesterol-lowering pharmaceuticals, antibiotics, antidiabetics and antidepressants (OECD, 2013), where the highest increase in consumption between 2000 to 2010 in Europe was reported for the latter two groups (Figure 1).

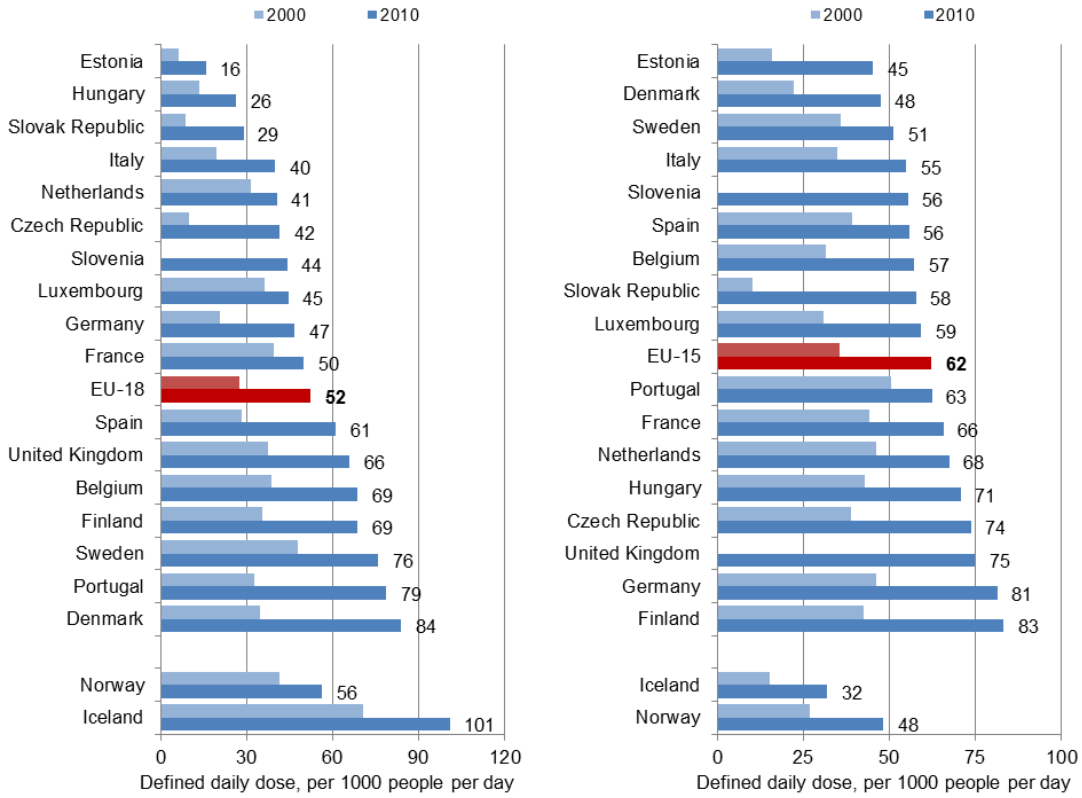


Figure 1: *The consumption of antidepressants (left) and antidiabetics (right) as defined daily dose (mg) in years 2000 and 2010 (OECD, 2012).*

These groups of pharmaceuticals represent the highest risk for aquatic organisms. For example, the emergence of antibiotic bacterial strains, resistant to antibiotics, was strongly correlated with the excessive or needless use of antibiotics in the past (Halling-Sørensen et al., 1998; OECD, 2013). Another study reports the adverse effects that the highly consumed analgesic, diclofenac, has on birds (Oaks, 2004). In India and Pakistan a decline in vulture populations (genus *Gyps*) that were feeding on carcasses of livestock previously treated with diclofenac was observed (Oaks, 2004). The general public is nowadays also aware of endocrine disrupting chemicals (EDCs), which can be any compound including pharmaceuticals that affects hormonal system of non-target organisms. One such compound is ethinyl estradiol (EE2), a widely used estrogen in oral contraceptives, which can cause the feminization of male fish (Bhandari et al., 2015).

Another group of concern are those used for treating cancer, i.e. cytostatic drugs. Kovalova et al. (2010) reported a comparison for Germany, where the annual consumptions of analgesics acetylsalicylic acid, paracetamol, and metamizol in 1999 in Land Brandenburg, Germany, were around 24,000, 7,000, and 5,000 kg year^{-1}, respectively, for antibiotics roxithromycin, ampicillin, and ciprofloxacin it was around 900, 500, and 400 kg year^{-1}, respectively, while for cytostatics 5-fluorouracil, cyclophosphamide and ifosfamide, the consumption was only 22, 8, and 8 kg year^{-1}, respectively. From these data, it is evident that cytostatics are consumed in much lower quantities. So why should we investigate their occurrence and potential adverse effects in the environment? The answer lies in their interaction with DNA in non-target organisms potentially having cytotoxic, mutagenic, genotoxic and teratogenic effects. These effects are characteristic for all cytostatics, which are mainly used in chemotherapy (*Centralna baza zdravil*, 2012). Two EU FP7 projects, CytoThreat (2011 – 2014) and Pharmas (2011 – 2013) revealed the high potency of these APIs and the risk posed to non-target organisms, including humans, despite their low abundance in the environment. Within

these projects, the occurrence, fate, transformations and the effects of these highly potent species in the environment were investigated. Their presence in the environment remains unregulated, although this may change in the future as more data regarding their occurrence and risk becomes available.

1.1 Cancer Treatment

Cancer represents a group of related diseases that can affect any part of the human body. It is defined as the uncontrolled rapid growth and spread of cells, which can invade into surrounding tissue or metastasize to distant sites in an organism. Numerous types of cancers can be prevented by avoiding exposure to common risk factors, e.g. cigarette smoke, however the genetics also plays an important role in the emergence of this disease (World Health Organization, 2014).

The recent project, GLOBOCAN (2012) addressed the incidence, mortality and prevalence of major types of cancer in 184 countries including Slovenia (Ferlay et al., 2015). Incidence is the number of new cases arising in a given period in a certain population, expressed as the number of cases per year, while mortality represents the number of deaths occurring in a given period in a specified population and is usually expressed as the number of deaths per year. The prevalence of a particular cancer is referred to as the number of persons in a given population, who have been diagnosed with a certain type of cancer and are still alive at the end of a given year. In 2012 there were approximately 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) globally (Ferlay et al., 2015). In Slovenia, 11,500 new cancer cases, 5,900 cancer-related deaths and approximately 28,900 prevalent cases were recorded in 2012 (International Agency for Research on Cancer, 2012). Based on these data, it is obvious that despite significant improvements in cancer treatment it remains one of the leading causes of death.

Cancer can be treated by surgery, radiotherapy and/or by the administration of various pharmaceuticals registered for cancer treatment. The type of treatment and the use of specific pharmaceuticals depend on the type of cancer, its position in the human body and patient condition e.g., age, gender, and accompanying diseases. There are numerous pharmaceuticals available for cancer treatment. Based on ATC classification, they belong to group L, namely Antineoplastic and Immunomodulating agents, which is divided into the following subgroups (World Health Organisation, 1975):

- L01 Antineoplastic agents:
    - L01A Alkylating agents
    - L01B Antimetabolites
    - L01C Plant alkaloids and other natural products
    - L01D Cytotoxic antibiotics and related substances
    - L01X Other antineoplastic agents
- L02 Endocrine therapy:
    - L02A Hormones and related agents
    - L02B Hormone antagonists and related agents
- L03 Immunostimulants
    - L03A Immunostimulants
- L04 Immunosuppressants
    - L04A Immunosuppressants

Some of the oldest and still widely prescribed antineoplastic agents (cytostatics) are cyclophosphamide (CP) and ifosfamide (IF), both included in group L01A (Kovalova, 2010; Jiefeng Zhang, Chang, Giannis, & Wang, 2013). These two drugs were registered by the Food and Drug Administration (FDA) in 1959 and 1988, respectively (*Drugs.com*, 2000). Since my thesis concerns these two APIs and their selected human metabolites/TPs in the aqueous environment, they are further described in the following chapters and subchapters.

1.1.1 Treatment, administration and consumption

Cyclophosphamide is used to treat different types of leukaemia, malignant lymphoma, some malignant solid tumors with or without metastases, Ewings' sarcoma, for various progressive autoimmune diseases (e.g. rheumatoid arthritis, erythematosus lupus, myasthenia gravis) and as immunosuppressive therapy after organ transplantations (*Centralna baza zdravil*, 2012). Ifosfamide is used to treat bronchial carcinoma, ovarian cancer, some testicular cancer tumors, soft tissue sarcomas, breast cancer, pancreatic carcinoma, renal cell cancer, carcinoma of the endometrium and malignant lymphomas (*Centralna baza zdravil*, 2012).

In Slovenia, CP is registered in two pharmaceutical forms, as a powder for infusion (200 mg, 500 mg and 1,000 mg) and as tablets for oral administration (50 mg), while IF (500 mg, 1 g and 2 g) is available only as a powder for infusion (*Centralna baza zdravil*, 2012).

The dosage for treatment is always determined individually for each patient but is typically administered as follows (*Centralna baza zdravil*, 2012):

- For continuous treatment of an adult: 3 – 6 mg kg^{-1} body weight per day
- For treatment in intervals: 10 – 15 mg kg^{-1} body weight every 2 – 5 days
- For treatment in intervals with high doses, e.g. 20 – 40 mg kg^{-1} body weight every 21 – 28 days.

For IF, typical intravenous administration is (*Centralna baza zdravil*, 2012):

- For continuous treatment of an adult: < 60 mg kg^{-1} body weight per day for 5 consecutive days (the infusion lasts 30 – 120 min)
- Single dosage: 125 – 200 mg kg^{-1} body weight as 24-h infusion.

The consumption of all pharmaceuticals for cancer treatment is increasing, however the trend strongly depends on the type of pharmaceutical (OECD, 2013). Table 1 gives the consumption data for CP and IF in various European countries or their regions. The average consumption is approximately 3 – 11 kg and 0.1 – 4 kg per million of population for CP and IF, respectively.

Table 1: *The consumption of CP and IF in various European countries or their parts*.

Country	Population	Year	CP cons. (kg)	IF cons. (kg)	Reference
Germany	82 M	2000	200 – 400		(Buerge, Buser, Poiger, & Müller, 2006)
France	66 M	2004	281	103	(Besse, Latour, & Garric, 2012)
		2008	305	95	
Austria	8 M	1997	39	18	(Buerge et al., 2006)
Switzerland	8 M	2002	55	12	(Buerge et al., 2006)
NW England	7 M	2010 – 2012	78	1	(Booker, Halsall, Llewellyn, Johnson, & Williams, 2014)
Slovenia	2 M	2009	6	3	(Cytothreat project, 2011)
		2010	6	3	
		2011	6	2	

1.1.2 Pharmacodynamics and pharmacokinetics

Cyclophosphamide and IF are pro-drugs, i.e. they represent in-active forms that are transformed into active metabolites in the human body after administration and metabolism. The primary mechanism of action for both APIs is the interaction with DNA material; in particular the active metabolites, 4-hydroxy-cyclophosphamide and 4-hydroxy-ifosfamide are capable of cross-linking the DNA in a tumor cell, which leads to its repressed division and final apoptosis (*Centralna baza zdravil*, 2012). Besides the active forms of CP and IF metabolites, also in-active species are formed *via* deactivation metabolic pathway, i.e. dealkylated forms of CP and IF.

Based on pharmacokinetics, all metabolites are, together with the parent compounds, excreted from the body *via* urine and faeces (negligible amounts). For CP approximately 62 % of the initial dosage is excreted *via* urine unaltered together with its metabolites when administration lasts over 48 h (Eitel, Scherrer, & Kümmerer, 2000). The total amount of IF and its metabolites excreted over a 24 h period *via* urine is approximately 50 % of the injected IF dose. The available data on excretion % for the investigated parent compounds and metabolites (relative to the dose applied) is presented in Table 2. These studies report that the stated excretion rates depend on numerous factors, i.e. age, gender, physical condition of the patient, the administered dosage, the duration of the intravenous administration and the accompanying therapy with other pharmaceuticals (Gilard, 1993; Joqueviel et al., 1998; Jing Zhang, Tian, & Zhou, 2006).

Table 2: *Excretion (%) of investigated parent compounds and metabolites.*

Compound	Excretion as % of the applied dose	Reference
CP	16 – 17; 10 – 40	(Joqueviel et al., 1998; Jing Zhang et al., 2006)
IF	18; 10 – 20	(Gilard, 1993; Jing Zhang et al., 2006)
carboxy-CP	11 – 23	(Joqueviel et al., 1998)
keto-CP	0.4 – 0.6	(Joqueviel et al., 1998; Jing Zhang et al., 2006)
N-decl-CP	2 – 3 (as CP metabolite) 11 (as IF metabolite)	(Gilard, 1993; Joqueviel et al., 1998)

Treatment with a certain API always comes with possible unwanted side effects and CP and IF are no exception. The main drawback of these APIs is their non-selectivity towards all fast-dividing cells in the human body, which can result in injuries to the gastrointestinal tract and renal system, hepatitis, alopecia, and the occurrence of new malignancies. Furthermore, due to a repressed immune system, patients receiving chemotherapy can suffer from various infections and myelosuppression (*Centralna baza zdravil*, 2012). Pre-clinical testing on animals revealed mutagenic, teratogenic and carcinogenic properties of CP and IF (*Centralna baza zdravil*, 2012). Therefore, the important question arises: Can these compounds, once in the environment, affect also non-target organisms, which are continuously exposed to them?

1.2 Cytostatic Residues in the Environment

The occurrence of CP and IF in aqueous environment, e.g. in wastewater (WW) and SW, has been studied in various European countries and other parts of the world, but as discussed in continuation, limited data on the occurrence of their metabolites or transformation products (TPs) exists.

1.2.1 Sources

To reduce the costs of cancer treatment, the current trend in chemotherapy is towards out-patient treatment, i.e. patients go home once they have received their therapy at the hospital. These patients may excrete cytostatics in the hospital since intravenous treatment can last several hours and at home due to the long half-life of administered drugs (Kosjek & Heath, 2011). In addition, there is still a significant number of hospitalized patients that receive chemotherapy, meaning that hospitals remain an important source of anticancer drug residues that end up in WW (Kümmerer, 2001).

Figure 2 shows the potential pathways of APIs and their metabolites into the environment. Once excreted, residues end up in either hospital or household WW. This WW then enters the sewerage system, which is connected to WWTP or is discharged directly into rivers (Figure 2). If not completely removed during WW treatment, the residues will enter SWs and potentially contaminate ground water and drinking water (Kümmerer, 2008).

The WW from the pharmaceutical industry is another potential source of API residues, where, as in the case of hospitals and households, the contaminated WW can be either treated in a WWTP or discharged directly into SW (Figure 2). Finally, animal farming, agriculture and leaching from landfills represent additional sources of environmental contamination; however, cytostatics are in this case of minor importance (Figure 2).

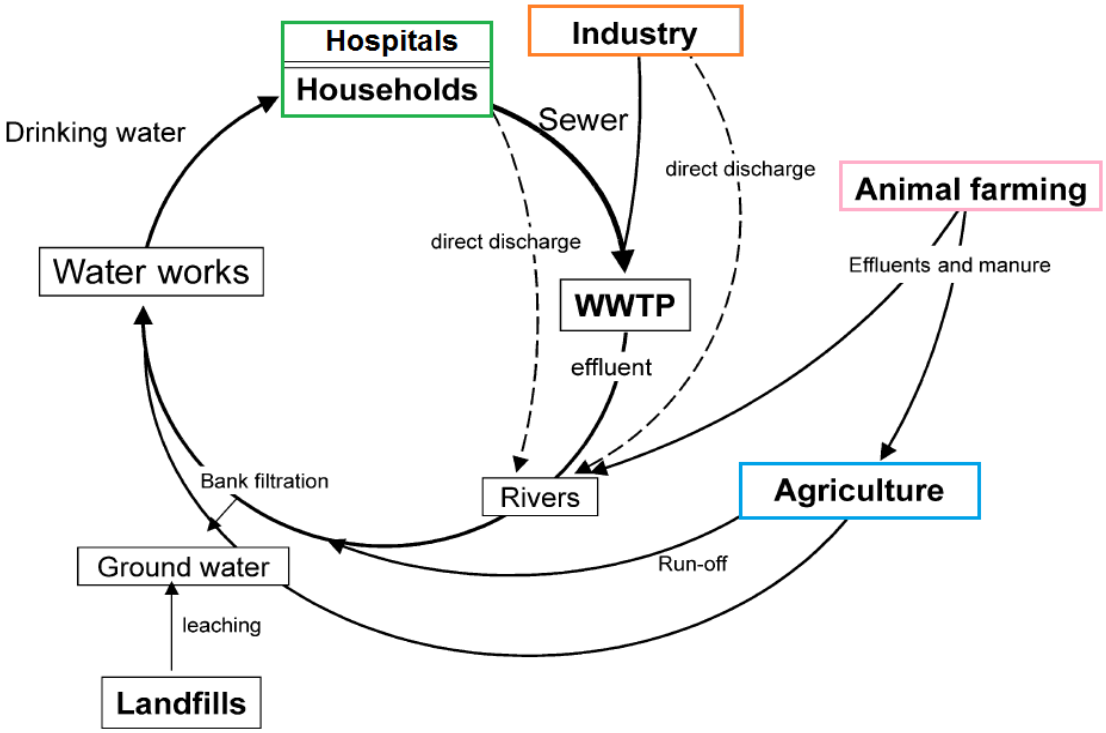


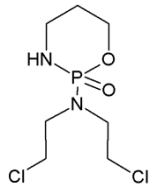
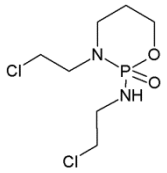
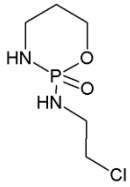
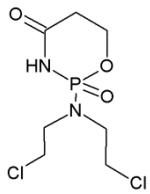
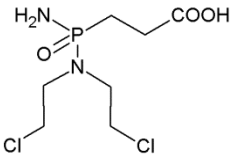
Figure 2: *The exposure pathways of APIs in the environment; adapted from Petrovič et al.(2003).*

1.2.2 Distribution

The structural characteristics and physico-chemical properties (i.e. solubility, dissociation constant (pK_a), bioconcentration factor (BCF), sorption constant (K_d), octanol-water (K_{ow}) and organic carbon-water (K_{oc}) partition coefficients, Henry's law constant etc.) of a certain compound dictates its distribution and consequently its fate in the environment. The solubility of CP and IF is higher than their typical environmental concentrations; hence, solubility does not limit their occurrence in the aquatic compartment (Table 3). Based on their pK_a values, both compounds act as weak acids and are partially dissociated in neutral water suggesting low sorption to organic matter, which is in accordance with their K_{oc} values suggesting that they only partially distribute into organic matter present in soil and sediment compartments, e.g. humus (Table 3). Moreover, the distribution of a compound between water and organic matter, in particular, lipids and fats, can also be determined by the log K_{ow} value. The log K_{ow} values for CP and IF are both below 1, indicating their high polarity and consequent distribution into the water phase (Table 3). In addition, the bioconcentration factor (BCF) predicts the potential accumulation of a compound in aquatic organisms (Kümmerer, 2008). Low BCF values for CP and IF indicate their low potential for bioaccumulation (Table 3). The data for sorption on solids (sludge, sediment, soil) can be described by the K_d, which has not been determined for the investigated compounds. However, it is known that CP and IF do not sorb onto sludge at WWTPs, suggesting limited elimination from WW by this mechanism (Kümmerer, Steger-Hartmann, & Meyer, 1997). Finally, the volatility of CP and IF is negligible since reported Henry's law constants are low (Table 3).

There are only slight structural differences between CP and IF and their metabolites (Table 3), hence it can be assumed that their physicochemical characteristics are similar. In, addition, since human metabolites are typically more polar than the parent compounds, these compounds might also distribute into the water phase and their potential for bioaccumulation will be even lower.

Table 3: *Physico-chemical characteristics of the investigated compounds*.

Compound (abbreviation and structure)	Solubility ($g\ L^{-1}$)	log K_{ow} and K_{oc}	K_d	Henry's law constant ($atm \times m^3\ mole^{-1}$)	pK_a	BCF	Reference
CP 	40	0.6352	n.a.	1.4×10^{-11}	6.00	3	(Kosjek & Heath, 2011; Mahoney, Raghunand, Baggett, & Gillies, 2003; "TOXNET: Toxicology Data Network," 2005)
IF 	38	0.8670	n.a.	1.36×10^{-11}	3.75	3	(Kosjek & Heath, 2011; Mahoney et al., 2003; "TOXNET: Toxicology Data Network," 2005)
N-decl-CP 	50	n.a.	n.a.	n.a.	n.a.	n.a.	Data provided by Niomech™
Keto-CP 	poor (≈ 0.5)	n.a.	n.a.	n.a.	n.a.	n.a.	Data provided by Niomech™
Carboxy-CP 	100	n.a.	n.a.	n.a.	n.a.	n.a.	data provided by Niomech™

Note 1: *n.a. = not available.*

1.2.3 Occurrence

A review of physico-chemical properties suggests that the studied compounds will occur mainly in the aqueous environment. Their occurrence in this compartment can be affected by a number of additional factors including their consumption, disposal, the pharmacokinetics of a patient receiving therapy with CP and/or IF, their fate during WW treatment and in the environment. Moreover, the sampling type (grab or time/flow proportional, position of sampling) and time along with the analytical methodology (sample preparation and type of analytical instrumentation used) can also affect the final result. In order to avoid many of these errors that originate from the applied methodology, the use of certified reference materials or interlaboratory comparisons can be applied.

Table 4 gives the occurrence of CP and IF in various WWs (hospital WW and WWTP influents and effluents) and SWs as determined concentration ranges or, where this data was not available, as mean value ± SD (standard deviation). The first studies, reporting the levels of CP and IF in SW and WW, were published almost 20 years ago (T. Steger-Hartmann, Kümmerer, & Hartmann, 1997; T. Steger-Hartmann, Kümmerer, & Schecker, 1996; Ternes, 1998). The presence of CP and IF in ground water or tap water is yet to be determined.

Except for Gómez-Canela et al. (2012), who reported levels of CP ≤ 13,100 ng L^{-1} in WWTP influent, reported concentrations in WWTPs influents and effluents ranged from below the LOD to ng L^{-1} range (Table 4). In addition, Buerge et al. (2006) reported comparable concentrations of CP and IF in both WWTP influents and effluents, suggesting only limited biodegradation of these compounds. The efficiency of this treatment is discussed in details in the Chapter 1.3.1. Table 4 shows higher concentrations of CP and IF detected in hospital WWs, WWTP influents and effluents (< LODs or LOQs to µg L^{-1}) than in SWs. Low concentrations of CP and IF in SWs (< LODs or LOQs to ng L^{-1}) can be attributed to the effect of dilution of WWTP effluent on entering SW, i.e. a river.

The occurrence of the metabolites keto-CP, N-decl-CP and carboxy-CP, with the exception of one study by Ferrando-Climent et al. (2013), who qualitatively confirmed the presence of a CP metabolite carboxy-CP in a hospital WW in Spain, is still to be assessed. Since these compounds are not well investigated in terms of their toxicity, it is necessary to study both, their environmental occurrence and toxicity.

Table 4: *The occurrence of CP and IF in WW and SW.*

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min – max; ng L^{-1})	Reference
CP	hospital WW	24 h time-proportional n = 7	19 – 4,500	(T. Steger-Hartmann et al., 1997)
		24 h time-proportional n = 1	146	(T. Steger-Hartmann et al., 1996)
		grab n = 12	< LOD (2) – 21	(Thomas, C., Schlabach, & Langford, 2007)
		grab n = 65 (21 hospitals)	6 – 2,000	(Yin, Shao, Zhang, & Li, 2010)
		24 h time-proportional n = 1	5,730	(Gómez-Canela et al., 2012)
		grab n = 1 (4 hospitals)	< LOQ (3.6) – 200.7	(L. Ferrando-Climent et al., 2013)
		24 h time-proportional n = 7	< LOQ (3.0) – 100.0	(Negreira, de Alda, & Barceló, 2014)

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min – max; ng L^{-1})	Reference
CP	WWTP influent	8 h time-proportional n = 2	< LOD (6) – 143 < LOD (6) – 8	(T. Steger-Hartmann et al., 1997)
		flow-proportional (24 h) n = 5 (3 WWTPs)	2 – 11	(Buerge et al., 2006)
		24 h time-proportional n = 2	< LOD (2)	(Thomas et al., 2007)
		24 h time-proportional n = 1	< LOQ (7.1)	(Martín, Camacho-Muñoz, Santos, Aparicio, & Alonso, 2011)
		24 h time-proportional n = 2 (3 WWTPs)	< LOD (0.35) – 13,100	(Gómez-Canela et al., 2012)
		grab n = 2 (3 WWTPs)	< LOQ (3.6) – 25.5	(L. Ferrando-Climent et al., 2013)
		24 h time-proportional n = 1 (12 WWTPs)	< LOQ (3.0) – 43.8	(Negreira et al., 2014)
	WWTP effluent	8 h time-proportional n = 2	6 – 17 8 – 15	(T. Steger-Hartmann et al., 1997)
		grab n = 1	< LOD (10) – 20	(Ternes, 1998)
		24 h time-proportional n = 9 (different WWTPs)	< LOQ (1.9) – 9	(Castiglioni, Bagnati, Calamari, Fanelli, & Zuccato, 2005)
		flow-proportional (24 h) n = 5 (3 WWTPs)	2 – 10	(Buerge et al., 2006)
		24 h time-proportional n = 1 (8 WWTPs)	median: 0.6	(Zuccato, Castiglioni, & Fanelli, 2005)
		24 h time-proportional n = 2	< LOD (2)	(Thomas et al., 2007)
		24 h time-proportional and grab n = 3 (2 WWTPs)	< LOQ (5)	(Busetti, Linge, & Heitz, 2009)
		24 h time-proportional n = 1	< LOQ (7.7)	(Martín et al., 2011)
		grab n = 3 (2 WWTPs)	0.19 – 3.7	(Llewellyn, Lloyd, Jürgens, & Johnson, 2011)
		24 h time-proportional n = 2 (3 WWTPs)	< LOD (0.35)	(Gómez-Canela et al., 2012)
		24 h time-proportional n = 1 (12 WWTPs)	< LOQ (SM) – 25.0	(Negreira et al., 2014)
	SW	grab n = 1	< LOD (10)	(Ternes, 1998)
		grab n = 5 (3 SWs)	0.05 – 0.17	(Buerge et al., 2006)
		2.5 h time-proportional n = 1 (2 SWs)	< LOD (not available)	(Zuccato et al., 2005)
		grab n = 2 (4 SWs)	< LOQ (30) – 65	(Moldovan, 2006)
		grab n = 1	< LOQ (5.5)	(Martín et al., 2011)
		grab n = 5 (5 rivers)	< LOD (3)	(Valcárcel, González Alonso, Rodríguez-Gil, Gil, & Catalá, 2011)

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min – max; ng L^{-1})	Reference
IF	hospital WW	grab	< LOD (6) – 1,914	(Kümmerer et al., 1997)
		24 h time-proportional n = 1	24	(T. Steger-Hartmann et al., 1996)
		grab n = 12	< LOD (2) – 338	(Thomas et al., 2007)
		grab n = 65 (21 hospitals)	4 – 10,647	(Yin, Shao, et al., 2010)
		grab n = 1 (4 hospitals)	< LOQ (5.8) – 227.9	(L. Ferrando-Climent et al., 2013)
		24 h time-proportional n = 7	< LOQ (2.0) – 19.4	(Negreira et al., 2014)
	WWTP influent	6 h time-proportional n = 2	7–29 < LOD (6) – 29	(Kümmerer et al., 1997)
		flow-proportional (24 h) n = 5 (3 WWTPs)	< LOD (0.3) – 15	(Buerge et al., 2006)
		24 h time-proportional n = 2	< LOD (2)	(Thomas et al., 2007)
		24 h time-proportional n = 1	3.5 ± 0.1 (mean ± SD)	(Martín et al., 2011)
		grab n = 2 (3 WWTPs)	< LOQ (5.8) – 130.1	(L. Ferrando-Climent et al., 2013)
		24 h time-proportional n = 1 (12 WWTPs)	< LOQ (2.0) – 27.9	(Negreira et al., 2014)
	WWTP effluent	6 h time-proportional n = 2	10 – 40 < LOD (6) – 43	(Kümmerer et al., 1997)
		grab n = 1	< LOD (10) – 2,900	(Ternes, 1998)
		flow-proportional (24 h) n = 5 (3 WWTPs)	1.7 – 6	(Buerge et al., 2006)
		24 h time-proportional n = 2	< LOD (2) – 71	(Thomas et al., 2007)
		24 h time-proportional and grab n = 3 (2 WWTPs)	< LOQ (25)	(Busetti et al., 2009)
		24 h time-proportional n = 1	1.2 ± 0.1 (mean ± SD)	(Martín et al., 2011)
		grab n = 3 (2 WWTPs)	< LOQ (0.24)	(Llewellyn et al., 2011)
		24 h time-proportional n = 1 (12 WWTPs)	< LOQ (2.0) – 15.9	(Negreira et al., 2014)
	SW	grab n = 1	< LOD (10)	(Ternes, 1998)
		grab n = 5 (3 SWs)	0.05 – 0.14	(Buerge et al., 2006)
		grab n = 1	< LOQ (4.4)	(Martín et al., 2011)
		grab n = 5 (5 rivers)	< LOD (1) – 41	(Valcárcel et al., 2011)

1.2.4 Transformations

The transformation of APIs in the environment depends to a great extent on their chemical structures. Active pharmaceutical ingredients can undergo chemical, physico-chemical and/or microbiological transformations in the environment (Mompelat, Le Bot, & Thomas, 2009). Common chemical transformations of APIs include the hydrolysis of esters and amides in water and the oxidation of alcohols to carboxylic acids in the presence of oxygen (O_2). Photodegradation under natural sunlight as a physico-chemical

transformation can modify the structure of APIs directly by solar absorption or indirectly through the formation of radicals (e.g. hydroxyl radicals, •OH) generated by solar irradiation of photosensitizers, i.e. fulvic acids and nitrate ions (NO_3-N), which are commonly present in natural waters. Alternatively, photoscavengers, such as dissolved organic carbon (DOC), carbonate and bicarbonate ions, can reduce the amount of •OH and consequently the photodegradation of the compounds (Buerge et al., 2006). Biodegradation is a microbial process, in which organic matter represents a source of carbon and energy for microorganisms. This process results in either complete mineralization to carbon dioxide (CO_2) and water (H_2O) or just in partial degradation resulting in the formation of TPs (Mompelat et al., 2009).

The rate at which chemical, microbiological and/or physico-chemical degradation occurs, depends on many factors, such as temperature, the amount of solar irradiation, the depth of the water body and pH of aqueous media. For example, Khetan (2007) found that seasonal variations in temperature and light intensity affect the fate of the investigated APIs in SW. As expected, an overall decrease in biodegradation and photolysis of the investigated APIs was observed during winter (Khetan, 2007).

The environmental fate of CP and IF has been rarely reported. Recently, Haddad et al. (2015) reviewed all the available data on TPs of cytostatics and no CP and IF TPs formed under environmental conditions are reported (Haddad et al., 2015). A further review of the literature revealed two studies concerning the environmental degradation of CP and/or IF in Switzerland and Taiwan, both in synthetic and natural SWs (Buerge et al., 2006; A. Y.-C. Lin, Wang, & Lee, 2013). Lin et al. (2013) investigated the degradation of CP, while Buerge et al. (2006) investigated the fate of both compounds. Both studies suggest only limited environmental biodegradation. In addition, it was shown that direct photodegradation plays only a minor (if any) role in the degradation of CP and/or IF in the environment. Moreover, an increase in photochemical degradation, which correlates to an increase in •OH formation in the presence of NO_3-N, a naturally present photosensitizer, is also reported. Finally, they conclude that the highest amount of degradation of CP and/or IF occurs in shallow, clear, NO_3-N rich natural waters (Buerge et al., 2006; A. Y.-C. Lin et al., 2013).

1.2.5 Legislation

The first regulation in Europe regarding the urban WW collection, treatment and discharge was established in May 1991 (Council Directive 91/271/EEC). Its objective is to protect human health and the environment from the adverse effects of WW discharges. The Directive requires the appropriate discharges of WW by setting maximum emission limit values of standard WW parameters, i.e. the presence of undissolved matter, the concentrations of ammonia, phosphorous and certain bacteria. Full implementation of the Directive is a pre-requisite for meeting the environmental objectives set out in the EU Water Framework Directive (2000), which is discussed in continuation. Implementation of this Directive has been challenging due to financial and planning aspects, therefore it is still far from complete and pollution problems persist. In Slovenia, an additional Decree on separation and treatment of urban and drainage WW was established in 2011, however the effluents are again regulated only in terms of standard WW parameters (Official Gazette of the Republic Slovenia, 2012).

It is obvious that these regulations do not cover specific pollutants such as cytostatic residues. The main reason for this is the lack of data on their occurrence and any adverse effects they might have on non-target organisms in the environment (Johnson et al., 2008). Regardless, in Europe, the European Medicinal Agency (EMEA) is responsible for the licensing of pharmaceuticals. Its mission is the protection and promotion of public and

animal health through the evaluation and supervision of pharmaceuticals for human and veterinary use (Johnson et al., 2008). In 2006, EMEA published guidance that an Environmental Risk Assessment (ERA) should accompany marketing authorization for new pharmaceuticals (EMEA, 2006).

Understanding the effects that pharmaceuticals have on aquatic organisms is important since such information will help us to determine the precautions necessary to prevent ecosystem damage (Nriagu, 2007). The Water Framework Directive (2000/60/EC) established a list of thirty-three priority substances (Decision No 2455/2001/EC) to be monitored and controlled in order to improve the quality of water in Europe. Later, the European Commission issued a Proposal for a Directive (COM (2011)876) to add an additional fifteen contaminants to the list of priority compounds, including estrogens EE2, 17β-estradiol and analgesic diclofenac. In 2015 Commission implemented Decision (EU) 2015/495, where these compounds along with three macrolide antibiotics (erythromycin, clarithromycin and azithromycin) are included in the watch list of seventeen substances for Union-wide monitoring (EU Decision 495/2015). If the presence of cytostatics is shown to be present in concentrations that may affect the environmental biota, their inclusion in the watch list will likely follow.

1.3 Treatment Technologies for the Removal of CP and IF

Various WW treatment technologies exist, which are designed to remove compounds, particles, dissolved gasses and pathogens from WW (Jjemba, 2008). Typically, a conventional WWTP includes mechanical and biological treatment processes. Mechanical treatment is the first step (primary treatment), where elimination of large solids by rakes and sieves is followed by a sedimentation process, where smaller particles are removed. Secondary treatment is a biological process, where activated sludge (AS), i.e. biomass, degrades biodegradable organic matter. Some WWTPs are upgraded with a tertiary treatment, where WW is further treated by abiotic or physical processes to either improve removal efficiency of compounds present and/or for its disinfection (Jjemba, 2008).

One of the reasons why WWTPs use biological treatment with suspended biomass (conventional treatment) is that it represents the least expensive option resulting in an effluent suitable for discharge into SW. Compounds that are resistant to biodegradation including some APIs can pass through the WWTPs either partially or completely unchanged and are as such released into the environment (Eggen, Vogelsang, & Eddy, 2015). Research towards upgrading existing conventional biological treatment has led to the development of new treatment technologies. Among them, advanced oxidation processes (AOPs), as novel treatment techniques for the removal of persistent APIs are promising, yet their full-scale application is limited due to their high cost (Andreozzi, Caprio, Insola, & Marotta, 1999).

1.3.1 Biological treatment

In biological treatment, WW is subjected to aerobic and sometimes also anaerobic microbial degradation. The treatment efficiency of AS is determined by continuously monitoring standard WW parameters including the following: total nitrogen (nitrate (NO_3-N), nitrite (NO_2-N) and ammonia (NH_4-N), total phosphorus, chemical oxygen demand (COD), biological oxygen demand (BOD) and suspended solids (Jjemba, 2008). The limit values for three different sizes of WWTPs are presented in Table 5. Under typical WWTP operating conditions, excess AS must be removed to maintain a constant AS to nutrient ratio. The excess AS can be either combusted or treated by e.g. anaerobic stabilisation in heated digesters with further drying to produce a useful waste product.

Table 5: *Limit values of WW parameters in Slovenia (Official Gazette of the Republic Slovenia, 2012).*

Parameter (mg L^{-1})	WWTP capability (as population equivalents; P.E.)		
	2,000 – 10,000	10,000 – 100,000	> 100,000
undissolved matter	60	35	35
NH_4-N	10	10	5
total nitrogen	25	25	20
total phosphorus	2	2	1
COD	125	110	100
BOD	25	20	20

Alternatives to conventional treatment with suspended biomass include membrane bioreactor (MBR) and moving-bed bioreactor (MBBR) system where biomass is attached to special carriers. The advantage of the MBBR is its simplicity, lower energy input and the smaller reactor volumes needed due to the higher surface area available for the growth of microorganisms (approximately 3,000 m^2 m^{-3}), the negligible hydraulic losses, the growth of both, aerobic and anaerobic organisms for nitrification and denitrification, respectively, and the higher resistance of microorganisms towards potent compounds (Multi Umwelttechnologie AG, 2009 ; Shammas & Wang, 2009). An MBR system also allows a higher biomass concentration since the filtration system prevents the biomass from being washed away. In addition, it prevents pathogenic bacteria and/or viruses passing through the treatment system, which is applicable in highly microbial-polluted WW, e.g. hospital WW. This treatment technology is therefore classified as a hybrid of biological and mechanical treatment. The main disadvantage is the cost of installation and maintenance (Melin et al., 2006).

The results of published studies, concerning the removal of CP and IF during biological treatment, are given in Table 6. In general, both compounds show limited removal under experimental conditions with either suspended biomass or fungi. Despite the different applied concentrations of CP and IF (ng L^{-1} to mg L^{-1}) the highest removal efficiencies reported for CP and IF during conventional treatment were 17 % and 3 %, respectively. In addition, these tests lasted from a few days to a few months, where no improvement in removal efficiency was observed within prolonged studies (Table 6). Three studies report the removal efficiency for CP using the MBR system with inconsistent results (Delgado et al., 2011; Kovalova, Siegrist, Singer, Wittmer, & McArdell, 2012; Köhler et al., 2012). Among them, only Delgado et al. (2011) reported significant removal (≤ 80 %). One reason for this discrepancy could be the use of different matrices, i.e. hospital WW *vs.* artificial WW. So far, no study concerning CP and/or IF removal using attached growth biomass has been reported.

Table 6: *The removal efficiency for CP and IF during various biological treatments*.

	Treatment type	Type of water	CP/IF conc.	Test duration	Removal	Reference
CP	modified Zahn-Wellens test (OECD 302 B)	OECD medium + AS from WWTP	160 mg L^{-1}	28 days	no elimination	(T. Steger-Hartmann et al., 1997)
	simulated WWTP	synthetic WW + AS from WWTP	10 µg L^{-1}	42 days	poor (≈ 17 %)	(T. Steger-Hartmann et al., 1997)
	OECD Confirmatory test (Degradation and Accumulation, 1992)	synthetic WW + AS from WWTP	375 mg L^{-1} 750 mg L^{-1}	10 days	no elimination (0 ± 5 %)	(Kiffmeyer, Götze, Jursch, & Lüders, 1998)
			150 mg L^{-1}	14 days		
	simulated WWTP	WWTP influent + AS from WWTP	90 ng L^{-1} 900 ng L^{-1}	24 h	no elimination	(Buerge et al., 2006)
	MBR	synthetic WW + AS from WWTP	5 µg L^{-1} addition of CP metabolites*	139 days 115 days	≤ 80 %	(Delgado et al., 2011)
	MBR	hospital WW	161 ng L^{-1}	1 year	< 20 %	(Kovalova et al., 2012)
	MBR	hospital WW	data not provided	5 days	≈ 12 %	(Köhler et al., 2012)
	biological treatment with fungi *Trametes versicolor*	hospital WW	10 mg L^{-1} 100 µg L^{-1}	8 days	no elimination	(L. Ferrando-Climent et al., 2015)
IF	modified Zahn-Wellens test (OECD 302 B)	drinking water + AS from WWTP	160 mg L^{-1}	42 days	no elimination	(Kümmerer et al., 1997)
		hospital WW + AS from WWTP	4.3 mg L^{-1}			
	simulated WWTP	WWTP effluent + AS from WWTP	11.4 µg L^{-1}	56 days	< 3 %	(Kümmerer et al., 1997)
	simulated WWTP	WWTP influent + AS from WWTP	120 ng L^{-1} 1,200 ng L^{-1}	24 h	no elimination	(Buerge et al., 2006)
	biological treatment with fungi *Trametes versicolor*	hospital WW	10 mg L^{-1} 100 µg L^{-1}	8 days	no elimination	(L. Ferrando-Climent et al., 2015)

Note 2: *The added CP metabolites were: acrolein (2,250 ng L^{-1}), phosphoramide mustard (8,880 ng L^{-1}), keto-CP (580 ng L^{-1}) and nitrogen mustard (517 ng L^{-1}).

1.3.2 Abiotic treatment

Various abiotic treatment technologies are available, i.e. UV irradiation, ozonation, chlorination, hydrodynamic cavitation (HC), treatment with plasma and AOPs, which can be used to disinfect and/or remove compounds from various aqueous matrices, such as drinking water and WW (Glaze, Kang, & Chapin, 1987; Huber, Korhonen, Ternes, & von Gunten, 2005; Legrini, Oliveros, & Braun, 1993). The most commonly applied treatments for the removal of organic contaminants are described in the following sections.

1.3.2.1 UV irradiation

UV irradiation can be used for disinfection and removal (complete or only partial degradation) of organic compounds present in water. The latter can be achieved by direct and/or indirect photolysis (Klavarioti, Mantzavinos, & Kassinos, 2009; Legrini et al., 1993). Direct photolysis occurs when the absorption spectrum of a compound overlaps with the spectrum of the UV irradiation. Therefore, the low pressure mercury lamps (LP) are frequently used for water treatment as they have an absorption maximum at either 185 nm or 254 nm, which represents the wavelengths at which the majority of organic compounds absorbs the photons. The degree of photons absorption and degradation depends on chemical structure, i.e. the compounds containing double C=C bonds, heteroatoms and aromatic rings are more prone to direct photolysis. Indirect photolysis is a more complex process, where photons are absorbed by the naturally present photosensitizers (nitrates and dissolved organic matter) in water (Fatta-Kassinos, Vasquez, & Kümmerer, 2011). This leads to the production of reactive species like •OH, singlet oxygen and other radicals. Besides photosensitizers, there are also species like humic acids, carbonate and bicarbonate ions, which are known to act in the opposite manner (radical scavengers); they "consume" photons with no further action (Fatta-Kassinos et al., 2011). The potential for indirect photolysis can be determined by the physico-chemical parameter called the atmospheric OH radical rate constant, which describes the reaction rate of a compound with •OH (Meylan & Howard, 1993). The reported values of atmospheric OH radical rate constants are 7.03×10^{-11} and 4.28×10^{-11} cm^3 mol^{-1} sec^{-1} for CP and IF, respectively, suggesting the low potential for indirect photolysis if compared to e.g. vinblastine, another cytostatic that has considerably higher atmospheric OH radical rate constant, i.e. 64.4×10^{-11} cm^3 mol^{-1} sec^{-1} (Kosjek & Heath, 2011).

Kim and Tanaka (2009) studied the degradation of CP by UV irradiation in pure water. The reported removal efficiency for CP among thirty APIs and personal care products was < 20 %. This agrees with the reported atmospheric OH rate radical constant value and chemical structure of CP.

1.3.2.2 Ozonation

Ozonation is a process, where ozone (O_3) is introduced into water. It can be used for disinfecting and/or removing compounds from drinking water or WW as a tertiary treatment. It can either be a direct or indirect degradation process. Direct ozonation is where O_3 reacts with the compounds present. In this case O_3 acts as a strong electrophile, attacking compounds that contain functional groups with a high electron density, i.e. double bonds, phenolic groups, amino or sulphur groups in their structures (U. von Gunten & von Sonntag, 2012). In indirect ozonation, O_3 in water decomposes to •OH *via* three fast and complex radical reaction steps (initiation, radical chain-reaction and terminalization). The produced •OH are very strong oxidants capable of non-selectively attacking all the compounds present (U. von Gunten & von Sonntag, 2012). Treatment efficiency can be affected by the matrix since •OH formation can be enhanced through

complex reactions of O_3 with the dissolved organic matter (U. von Gunten & Acero, 2001). The presence of •OH scavengers, like carbonates and bicarbonates reduces removal efficiency (Huber et al., 2005). Ozonation is also a pH dependant process. The formation of •OH increases with an increased pH value of medium since hydroxide ions (OH^-) act as initiators for the decay of ozone, which leads to a higher amount of •OH (U. von Gunten & von Sonntag, 2012). This phenomenon can be described as an AOP (Buffle, Schumacher, Meylan, Jekel, & von Gunten, 2006). In this thesis, only ozone based technologies with/without the addition of hydrogen peroxide (H_2O_2), which leads to the formation of •OH, are considered as AOPs.

Four studies report the removal efficiency of CP and/or IF by ozonation using comparable O_3 concentrations (Table 7). In general, removal efficiency above 60 % is achieved in less than 30 min regardless of the matrix type (deionized water or hospital WW), initial concentrations and pH values. The only exception is a study by Venta et al. (2005), who report a 20 % removal of CP at pH = 7, but their experiments lasted less than 15 min. Table 7 also shows how pH improves removal efficiency (Fernández et al., 2010; A. Y. H. Lin, J.Hong, P. K., 2015; Venta et al., 2005). These outcomes suggest that ozonation is a promising water treatment technique, especially for highly contaminated hospital WWs, however, installation and maintenance costs are high.

Table 7: *The removal efficiency for CP and IF during various ozonation treatment experiments*.

	O_3 concentration	Type of water	pH	CP/IF conc.	Removal	Reference
CP	45 mg L^{-1}	Milli-Q	7 9	261 mg L^{-1}	≈ 20 % (pH = 7, after 12 min); ≈ 60 % (pH = 9, after 12 min)	(Venta et al., 2005)
	32 mg L^{-1}	deionized water	5.6 9 11	5 mg L^{-1} 20 mg L^{-1}	61 % (pH = 5.6; after 30 min) – 100 % (pH = 11; after 5 min) not concentration-dependant	(A. Y. H. Lin, J.Hong, P. K., 2015)
		hospital WW	7.8	20 mg L^{-1}	100 % after 20 min	
	6 – 15 mg L^{-1}	ultrapure water	8.1	100 ng L^{-1}	87 % after 2 min 100 % after 30 min	(Garcia-Ac et al., 2010)
		drinking water	ambient		96 % after ≈ 5 min	
	30 mg L^{-1} 45 mg L^{-1}	buffered water	7 9 11	130.5 mg L^{-1} 261 mg L^{-1}	75 % (pH = 7) and 90 % (pH = 9 or 11) after 40 min not concentration-dependant	(Fernández et al., 2010)
IF	3 g O_3 h^{-1}	deionized water	5.6 9 11	5 mg L^{-1} 20 mg L^{-1}	79 % (pH = 5.6; after 30 min) – 100 % (pH = 11; after 5 min) not concentration-dependant	(A. Y. H. Lin, J.Hong, P. K., 2015)
		hospital WW	7.8	20 mg L^{-1}	100 % after 20 min	

1.3.2.3 Hydrodynamic cavitation

Cavitation is defined as the phenomenon where formation and subsequent collapse of bubbles in a liquid are accompanied by the release of high amounts of energy in a very small amount of time, i.e. microseconds (Ozonek, 2012; Saharan, Pinjari, Gogate, & Pandit, 2014). The increased fluid velocity and simultaneous decrease in static pressure results in bubble collapse, which is achieved when the fluid passes through a constriction, i.e. valves or gets a rotational impulse as in the case of hydraulic machines (Carlton, 2012; Ozonek, 2012). Although HC is associated with undesired consequences such as the damage caused to turbines and propellers, it is being applied in various fields as a promising treatment technique, i.e. cell disruption and lipid extraction from wet microalgae, the disintegration of AS, disinfection and degradation of compounds in water (Lee & Han, 2015; Mezule, Tsyfansky, Yakushevich, & Juhna, 2009; Petkovšek et al., 2015; Zupanc et al., 2014).

There are no published studies concerning the removal of cytostatic compounds by HC, however, since considerable removal efficiency was already reported for other APIs, i.e. analgesics and antiepileptics, this technique might be suitable for removing other classes of APIs as well (Zupanc et al., 2013; Zupanc et al., 2014).

1.3.2.4 Advanced oxidation processes

Glaze et al. (1987) defined advanced oxidation processes as “those which involve the generation of •OH in sufficient quantity to affect water purification”. They described only O_3/H_2O_2, UV/O_3 and UV/H_2O_2 as AOPs. As shown in Figure 3, also other AOPs such as UV/TiO_2, Fe^{2+}/H_2O_2 (Fenton), $UV/Fe^{2+}/H_2O_2$ (photo-assisted Fenton), $UV/O_3/H_2O_2$ and HC/H_2O_2 represent efficient AOP-based drinking water and WW treatment technologies nowadays (Linden & Mohseni, 2014; Saharan et al., 2014). During an AOP, the formation of •OH is followed by their reaction with the compounds present. These interactions lead to a series of complex oxidation reactions, which results in either their partial or complete degradation, i.e. mineralization (Saharan et al., 2014). Since high costs are involved with AOPs as WW treatment technologies, they are usually applied as pre- or post-treatments (before or after conventional WW treatment). In the former, AOPs degrade biologically non-degradable compounds into intermediates or TPs that might be more easily biodegraded, which lowers the price of the treatment. When AOP is applied as post-treatment (tertiary treatment), the easily biodegradable compounds do not compete for •OH with other compounds in the subsequent treatment step. Advance oxidation treatments are usually applied as a tertiary treatment for WW that contains high amounts of e.g. proteins or sugars, which are degraded during biological treatment, therefore the remaining organic matter can be degraded by an AOP (Oller, Malato, & Sánchez-Pérez, 2011).

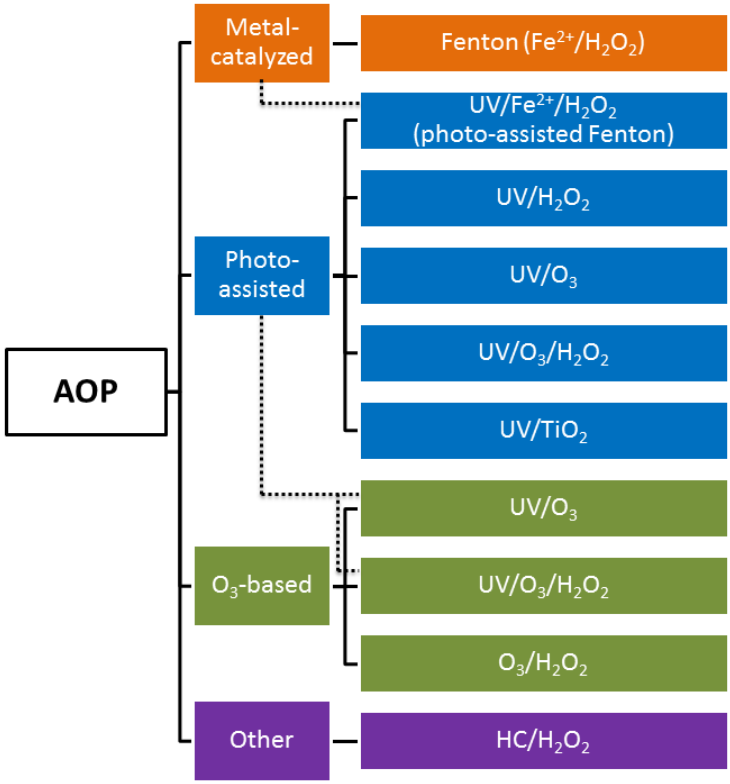


Figure 3: *Different types of AOP treatment*.

The formation of •OH is common to all AOPs, however, the mechanism of their "synthesis" differs. One option is Fenton process, Fe^{2+}/H_2O_2, where •OH are formed due to the oxidation of Fe^{2+} to Fe^{3+}. This is a metal-catalyzed oxidation, in which iron acts as a catalyst (Saharan et al., 2014).

There are numerous types of photo-assisted AOP treatments, such as UV/TiO_2, UV/H_2O_2, UV/O_3, UV/O_3/H_2O_2 and UV/Fe^{2+}/H_2O_2 (photo-assisted Fenton AOP), which is an advanced version of Fe^{2+}/H_2O_2 with a higher •OH formation rate (Andreozzi et al., 1999; Glaze et al., 1987; Legrini et al., 1993; Saharan et al., 2014). Except for UV/TiO_2, which is a photo-catalytic process, all others can be described as photo-activated chemical reactions, where interactions between photons with sufficient energy levels and H_2O_2 or O_3 result in the formation of free radicals, mostly •OH, which then further react with other compounds present (Saharan et al., 2014). To achieve homolytic cleavage of H_2O_2, an UV lamp irradiating with a wavelength of 254 nm is usually applied. Similarly, when UV is used in combination with O_3, it is also recommended to use UV light with a wavelength of 254 nm (Andreozzi et al., 1999). An alternative way to produce •OH is by photo-catalytic oxidation with UV/TiO_2, where •OH are formed on the surface of a semiconductor catalyst, e.g. titanium dioxide (TiO_2). The absorption of UV irradiation and consequent formation of electron-hole pairs on the catalyst's surface reduces the dissolved O_2 to the superoxide radical (O_2^-) ion and H_2O and OH^- to •OH (Saharan et al., 2014).

Besides UV/O_3, there are also other O_3-based AOPs: O_3/H_2O_2 and UV/O_3/H_2O_2. It is known, that decomposition of O_3 in an aqueous solution is accompanied by the formation of both, H_2O_2 and •OH (Legrini et al., 1993). The rate of •OH formation can be increased by adding H_2O_2 and by applying UV irradiation (Legrini et al., 1993). An example of in-house made laboratory-scale equipment, capable of all UV-based and O_3-based treatments is shown in Figure 4. One of the newest types of AOPs involves combining HC with

H_2O_2 (HC/H_2O_2). The formation of •OH in this case is a result of H_2O_2 cleavage under strong cavitational conditions (Saharan et al., 2014).

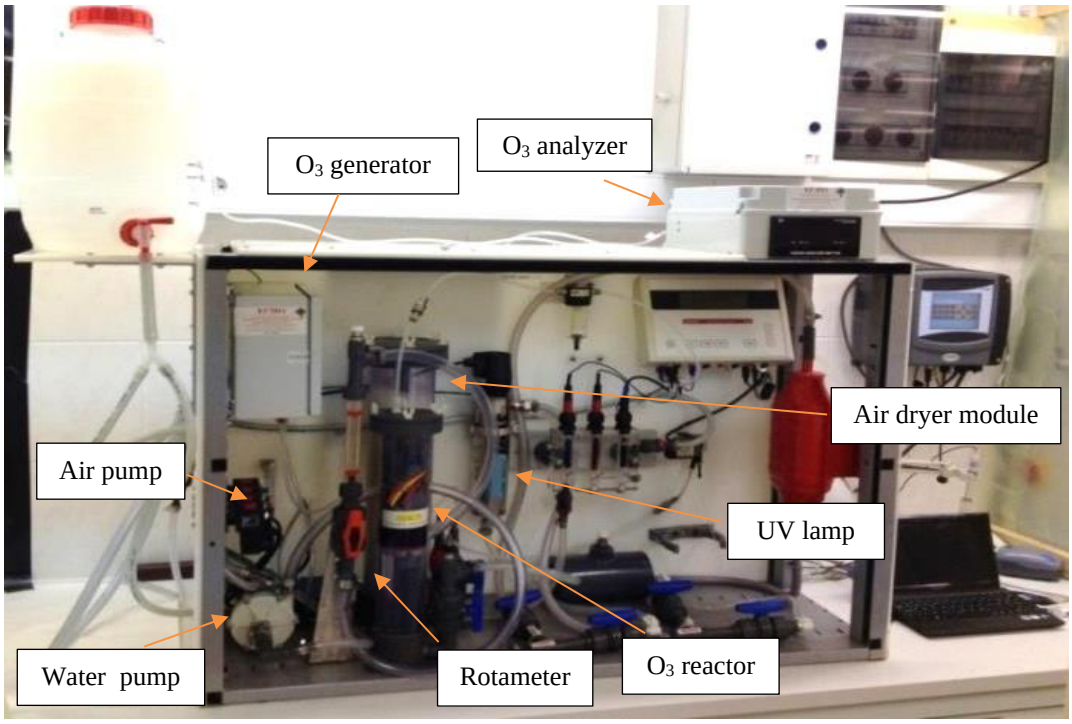


Figure 4: *Laboratory-scale AOP equipment for water treatment (courtesy of Prof. Kompare, Faculty of Civil and Geodetic Engineering, University of Ljubljana).*

To the author's knowledge, there are only two AOP-based studies, which report low removal efficiency of CP and/or IF (Lai, Lin, & Lin, 2015; Wols, Hofman-Caris, Harmsen, & Beerendonk, 2013). First is a study by Wols et al. (2013), who report 10 – 15 % (CP) and 10 – 30 % (IF) removal efficiency during UV/H_2O_2 treatment in tap water and WWTP effluent (Table 8). This contradicts Kim et al. (2009a), who used similar experimental conditions, i.e. WWTP effluent as a matrix, similar initial H_2O_2 concentration and UV dose, and reported ≤ 90 % CP removal (Table 8). However, the initial CP concentration reported by Kim et al. (2009a), 3 ng L^{-1}, is way below their reported LOD and LOQ values (170 ng L^{-1} and 560 ng L^{-1}, respectively), which questions the accuracy of their results. The two studies that report high CP removal efficiency (≈ 90 %) from WWTP effluent with similar initial CP concentrations used considerably higher UV and H_2O_2 doses (Table 8).

Table 8: *The removal efficiency of CP and IF under various AOPs*.

	AOP	CP/IF conc.	Fe^{2+}/TiO_2/ H_2O_2/O_3 conc.	Conditions	Matrix type	Removal	Reference
CP	UV/ H_2O_2	µg L^{-1} (not reported)	6 mg L^{-1}	8 W lamp	pure water	≈ 90 % (75 min; UV dose = 1,695 mJ cm^{-2})	(Kim, Yamashita, & Tanaka, 2009b)
			8.2 mg L^{-1}		WWTP effluent		
	UV/ H_2O_2	≈ 3 ng L^{-1}	7.8 mg L^{-1}	65 W lamp	WWTP effluent	≈ 90 % (5 min; UV dose = 923 mJ cm^{-2})	(Kim et al., 2009a)
	UV/ H_2O_2	1 µg L^{-1}	0.83 – 1.1 g L^{-1}	2-10 W lamp 250 W lamp	WWTP effluent	≤ 90 % (16 h with 4 kWh m^{-3} energy input)	(Köhler et al., 2012)
	UV/ H_2O_2	1 µg L^{-1}	5 – 20 mg L^{-1}	60 W lamp 2,000 W lamp	Milli-Q	80 – 100 % (500 mJ cm^{-2}; 5 – 20 mg L^{-1} H_2O_2	(Wols et al., 2013)
					tap water	10 – 15 % (1,000 mJ cm^{-2}; 10 mg L^{-1} H_2O_2)	
					WWTP effluent		
	UV/ H_2O_2	20 mg L^{-1}	333 mg L^{-1} 500 mg L^{-1} 666 mg L^{-1}	150 W	ultrapure water	100 % (8 min, 333 mg L^{-1})	(Lutterbeck et al., 2015)
	UV/ Fe^{2+}/ H_2O_2		434 mg L^{-1} Fe^{2+} 333 mg L^{-1} H_2O_2			100 % (< 2 min)	
	UV/ TiO_2		100 – 1,000 mg L^{-1}			100 % (32 min, 500 mg L^{-1})	
	UV/ TiO_2	100 µg L^{-1}	5 – 3000 mg L^{-1}	8 W; pH = 3 – 10	Milli-Q	100 % (2 h, pH = 5.8, 20 mg L^{-1} TiO_2)	(Hui-Hsiang Lin & Yu-Chen Lin, 2013)
		27.6 mg L^{-1}	20 mg L^{-1} 300 mg L^{-1}	8 W; pH not reported		100 % (4 h with 300 and 5 h with 20 mg L^{-1} TiO_2)	
	O_3/ H_2O_2	100 ng L^{-1}	10 mg L^{-1} O_3 2.5 mg L^{-1} H_2O_2	pH = 8	drinking water	100 % (2 min)	(Garcia-Ac et al., 2010)
	O_3/ H_2O_2	261 mg L^{-1}	30 – 45 mg L^{-1} O_3 34 mg L^{-1} H_2O_2	pH = 7	ultrapure water	70 – 95 % (15 min)	(Fernández et al., 2010)
			30 – 45 mg L^{-1} O_3 34 mg L^{-1} H_2O_2	pH = 9		60 – 80 % (15 min)	
	O_3/ H_2O_2	261 mg L^{-1}	45 mg L^{-1} O_3 4.7 – 12.5 mg L^{-1} H_2O_2	pH = 7	Milli-Q	100 % (15 min, all conditions)	(Venta et al., 2005)
				pH = 9			
				pH = 11			

	AOP	CP/IF conc.	Fe^{2+}/TiO_2/ H_2O_2/O_3 conc.	Conditions	Matrix type	Removal	Reference
IF	UV/ H_2O_2	1 µg L^{-1}	5 – 20 mg L^{-1}	60 W lamp 2,000 W lamp	Milli-Q	90 – 100 % (500 mJ cm^{-2}; 5 – 20 mg L^{-1} H_2O_2)	(Wols et al., 2013)
					tap water	10 – 30 % (1,000 mJ cm^{-2}; 10 mg L^{-1} H_2O_2)	
					WWTP effluent		
	UV/ TiO_2	100 – 20 mg L^{-1} (optimal: 100 µg L^{-1})	2 – 1,000 mg L^{-1} (optimal: 100 mg L^{-1})	8 W	ultrapure water	100 % after 10 min (100 mg L^{-1} TiO_2)	(Lai et al., 2015)
					hospital WW 1 and 2	1: 100 % after 60 min 2: 10 % after 120 min (DOC dependant)	
					pharma-ceutical industry WW 1 and 2	1 and 2: 40 % after 120 min	

Another study reporting low IF removal was performed by Lai et al. (2015), who investigated the removal efficiency of IF during UV/TiO_2 treatment in one hospital WW, whereas higher removal was achieved in another hospital WW, deionized water and two pharmaceutical industry WWs. The authors report a DOC-dependent removal efficiency, resulting from 10 % for hospital WW with highest DOC value (29 mg L^{-1}) to 100 % removal efficiency in deionized water with the lowest DOC value (data not provided) within 120 min of treatment (Lai et al., 2015). Interestingly, this study can be compared to that of Hui-Hsiang et al. (2013) since similar matrices (purified water) and initial concentrations of CP and IF were applied in the experiments with UV/TiO_2 treatment (Table 8). The only difference being the TiO_2 concentration (20 and 100 mg L^{-1}), which correlates to a decrease in the amount of time needed for 100 % removal of either CP (2 h) or IF (10 min; Table 8).

Within the UV-based AOPs (Table 8), the most efficient is the photo-Fenton type (UV/Fe^{2+}/H_2O_2). Cyclophosphamide was 100 % degraded within 2 min (Lutterbeck, Machado, & Kümmerer, 2015). This is comparable to O_3-based AOP, O_3/H_2O_2, conducted at an environmentally relevant initial CP concentration (Garcia-Ac et al., 2010). In the latter study, the amount of H_2O_2 used is smaller (2.5 mg L^{-1} compared to 333 mg L^{-1}), however O_3-based treatment technologies are more costly compared to UV-based AOPs (Saharan et al., 2014; U. von Gunten & von Sonntag, 2012). Fernandez et al. (2010), who investigated the removal of CP using O_3/H_2O_2 observed a decrease in removal efficiency at elevated pH values. This differs from using O_3 alone, where higher pH values result in more •OH being produced and consequently enhanced degradation (U. von Gunten & von Sonntag, 2012). They explain the opposite phenomenon observed within the experiments, where the added H_2O_2 was added with the behaviour of H_2O_2, which was acting as a scavenger of •OH produced at higher pH values. The same observation was reported by Venta et al. (2005), who reports complete CP removal within 15 min but with a lower amount of H_2O_2 compared to Fernandez et al. (2010).

1.3.3 Physical treatment

Adsorption on activated carbon (AC), microfiltration, ultrafiltration, nanofiltration (NF) and reverse osmosis (RO) are common physical treatment technologies, which can improve the quality of WW (Jjemba, 2008). All three technologies have been assessed in terms of removal efficiency for various groups of APIs, e.g. antibiotics, analgesics, antihyperthensives, antidepressants, contraceptives etc. (Calisto, Ferreira, Oliveira, Otero, & Esteves, 2015; Grover, Zhou, Frickers, & Readman, 2011; Miralles-Cuevas et al., 2014; Peeva, Burgal, Valtcheva, & Livingston, 2014; Ravikumar, Kalyani, Satyanarayana, & Sridhar, 2014; Urtiaga, Pérez, Ibáñez, & Ortiz, 2013).

The large surface area of AC enables the adsorption of high amount of compounds. Adsorption is a physical process, where hydrophobic interactions between the compound and the activated carbon are the main mechanism for retention, hence, non-polar and uncharged compounds are most readily adsorbed (Cheremisinoff, 2002). The main disadvantage is that the retained compounds are not degraded and further treatment is needed (Rakić, Rac, Krmar, Otman, & Auroux, 2015). De Ridder et al. (2009) reported a carbon dose dependent removal efficiency of CP and a correlation between matrix complexity and removal efficiency (Chen, Park, Herckes, & Westerhoff, 2008; de Ridder et al., 2009). The reported CP removal was between 28 % and 90 % (Table 9).

Table 9: *Removal efficiency of CP during various physical treatments (there are no data for IF).*

Treatment	CP conc.	Matrix type	Removal	Reference
AC	2 µg L^{-1}	deionized water	70 – 90 %	(Chen et al., 2008; de Ridder et al., 2009)
		SW	55 %	
		WWTP effluent	28 %	
NF	1 – 10 µg L^{-1}	ultrapure water	20 – 40 %	(L. Wang et al., 2009)
		WWTP effluent	60 %	
RO	1 – 10 µg L^{-1}	ultrapure water	> 90 %	(L. Wang et al., 2009)
		WWTP effluent	> 90 %	

While microfiltration and ultrafiltration are commonly used to remove e.g. bacteria, viruses and suspended solids from water passing the semipermeable membrane, NF and RO are used to purify drinking water or treat WW by removing the dissolved compounds. In case of NF, particles with a diameter > 1 nm are retained, whereas in RO, only particles < 0.1 nm pass through the membrane. Pre-treatment is necessary to remove any solid particles which could affect the rejection efficiency of NF and RO. Retention mechanisms are based on size exclusion, charge exclusion and physico-chemical interactions between the solute, solvent and the membrane (Radjenović, Petrović, Ventura, & Barceló, 2008; Ravikumar et al., 2014; Urs von Gunten, Janex-Habibi, Ternes, & Weber, 2006). Wang et al. (2009) studied the rejection efficiency of CP in ultrapure water and treated WW by NF and RO. For NF, the rejection efficiency was matrix dependent (Table 9). The higher rejection efficiency for treated WW was explained by membrane fouling with organic matter present in WW. The authors report over 90 % rejection efficiency of CP within the experiments with RO regardless of the matrix type (Table 9). Despite having a higher rejection efficiency, RO, like AC and NF, only prevent the compounds from being released in the effluent while the compounds

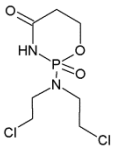
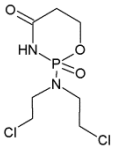
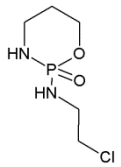
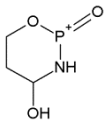
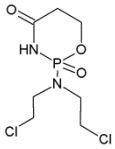
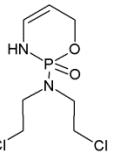
themselves still require treatment, which means that the overall costs are high (L. Wang et al., 2009).

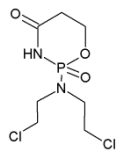
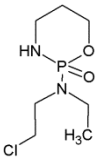
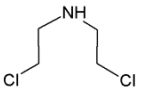
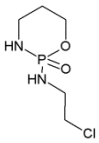
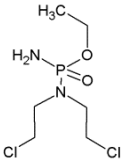
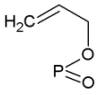
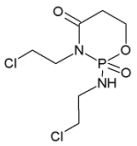
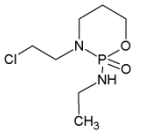
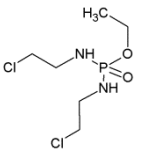
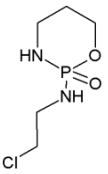
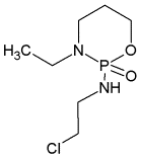
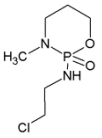
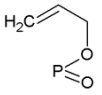
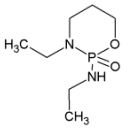
1.3.4 Transformations

Transformation products are formed when a compound is not completely mineralized. During water treatment compounds undergo similar transformation reactions as in the environment, i.e. chemical, physico-chemical and/or microbiological transformations. These processes are more intense during water treatment and strongly depend on treatment conditions (Mompelat et al., 2009; Saharan et al., 2014).

The transformations of CP and IF during biological treatment are not studied due to their poor biodegradability (Table 6), whereas TPs formed during AOPs have been addressed (Fernández et al., 2010; Lai et al., 2015; Lutterbeck et al., 2015; Venta et al., 2005) and are presented in Table 10. Four studies looked at CP degradation and identified 9 TPs (Fernández et al., 2010; Lai et al., 2015; Lutterbeck et al., 2015; Venta et al., 2005), while one study identified 9 IF TPs (Lai et al., 2015). It is evident that the formation of keto-CP (ketonization) occurs during O_3-based and UV-based AOPs. Keto-cyclophosphamide is also the most commonly reported TP. Moreover, some reported TPs (i.e. keto-CP, N-decl-CP, keto-IF and N-dechloroethyl-ifosfamide) are also known human metabolites of CP and IF (Li et al., 2010).

Table 10: *Reported TPs of CP and IF during various AOPs*.

	AOP	Identified TPs			Reference
CP	O_3/H_2O_2	keto-CP 			(Venta et al., 2005)
	O_3, pH = 9	keto-CP 			(Fernández et al., 2010)
	UV/H_2O_2 UV/TiO_2	N-decl-CP (only UV/H_2O_2)  TP 	keto-CP 	imino-cyclophosphamide 	(Lutterbeck et al., 2015)

AOP		Identified TPs			Reference
CP		keto-CP 	TP (loss of Cl) 	bis(2-chloroethyl)amine 	(Lai et al., 2015)
	UV/TiO_2	N-decl-CP 	TP (loss CH_2) 	2-chloroethylamine  TP 	
IF		keto-IF 	TP (loss 1 Cl) 	TP (loss CH_2) 	(Lai et al., 2015)
	UV/TiO_2	N-dechloroethyl-ifosfamide 	TP (loss 1 Cl) 	TP (loss CH_3-Cl) 	
		TP 	TP (loss 2 Cl) 	2-chloroethylamine 	

1.4 Effects of Cytostatic Residues

Active pharmaceutical ingredients are known xenobiotics that are primarily designed to have an effect on a target organism. These effects are well-studied prior to registration of every medicine available in the market (European Medicines Agency, 2001). On the contrary, there is still a dearth of information regarding the effects of numerous APIs on non-target organisms. This is especially concerning in the case of highly potent substances such as cytostatic residues.

The rise of ecotoxicology or environmental toxicology as a scientific field first appeared in the late 1960s. The first definition of ecotoxicology was published in 1971 and states: “Ecotoxicology is the study of nuisance effects on the relationships between organisms and their environment.” This definition has changed slightly overtime and now refers to the study of the effects of toxic substances in ecosystems (Férard & Blaise, 2013). The evaluation of the effects towards non-target organisms can be assessed by “conventional” ecotoxicological bioassays, where a measured endpoint is e.g., growth inhibition of a tested organism population (OECD TG 201, 2011). Another approach is to study the potential mechanism of action by performing *in vitro* bioassays, where, for example, genotoxicity and cytotoxicity of a compound is indirectly measured in the tested

organism (Žegura, Heath, Černoša, & Filipič, 2009).

1.4.1 Ecotoxicity

Since CP and IF accumulate in aquatic water bodies, this chapter addresses only their ecotoxicity in aqueous environment. Phytoplankton species are the main primary producers in the aquatic ecosystem and any disturbance of these communities' can affect communities at higher trophic levels (Geis et al., 2000; Pfleeger, 1990). To assess the toxicity of chemicals towards primary producers, tests based on OECD Guidelines, i.e. growth inhibition tests of algae and/or cyanobacteria are now commonly applied (OECD TG 201, 2011; Walker, Hopkin, Sibly, & Peakall, 2001). The parameter, which describes the compounds' toxicity, is the concentration that either provokes a response which is halfway between the baseline and maximum response on the dose-response curve (EC_{50}) or a concentration that causes 50 % death of a population (LC_{50}). In addition, also the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) are important parameters. The NOEC value is the concentration of a contaminant that will not trigger the measured effect on a tested organism, whereas LOEC represents the concentration that provokes a statistically significant effect on the exposed population of the test organism if compared to the controls (Walker et al., 2001). All of these parameters can be determined using computer software (e.g. GraphPad Prism). Figure 5 shows an example of a typical sigmoid dose-response curve with indicated NOEC, LOEC and EC_{50} or LC_{50}.

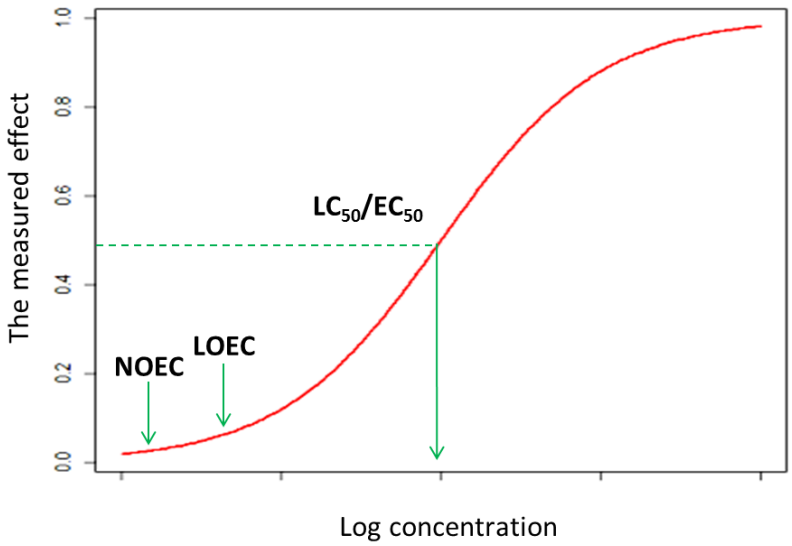


Figure 5: *Common parameters in toxicity evaluation of a compound.*

Such assays, where the described parameters are defined, are referred to as a short-term acute ecotoxicity assays that typically last from 24 h up to 96 h. Since aquatic organisms are exposed to compounds present in the environment over a life-time, chronic toxicity should be considered (Daughton & Ternes, 1998). Chronic toxicity tests examine the effects of a compound to a test organism over a prolonged period of time, where a significant part of the organisms' life cycle is monitored, e.g. several generations are exchanged within a single test. Growth inhibition tests with algae based on the OECD Guidelines (TG 201) can be therefore considered as chronic tests, since several generations exchange within the 72 h test. This was confirmed in the EU Technical Guidance Document (2003), which considers EC_{50} and NOEC values determined using

72-h (or longer) tests as short-term and long-term parameters, respectively (Isidori, Lavorgna, Nardelli, Pascarella, & Parrella, 2005; Radix et al., 2000).

The first studies addressing the ecotoxicity of APIs as environmental contaminants were published in the 1970s, and included analgesics (ibuprofen), antibiotics (ampicillin), hormones (estrogen) and lipid lowering agents (clofibric acid) on test organisms from various trophic levels, i.e. plants, algae and crustaceans (Halling-Sørensen et al., 1998). A further review of literature revealed that only two CP growth inhibition studies have been performed using the alga *Pseudokirchneriella subcapitata*, where NOEC values above 100 mg L^{-1} were reported (Grung, Källqvist, Sakshaug, Skurtveit, & Thomas, 2008; Zounkova et al., 2007). No studies have been reported neither for IF nor for CP and/or IF metabolites/TPs.

1.4.2 Genotoxicity

The debate regarding the morality of animal testing, especially vertebrates, among the general and the scientific public continues and is fuelling the search for humane alternatives (Goss & Sabourin, 1985). One alternative is the application of *in vitro* bioassays using cell lines, which are commercially available in kit forms and represent a simple and fast way to evaluate toxicity that maintains adequate quality and reliability of the results (Kokkali & van Delft, 2014). There is a number of commercially available test kits, where the selection of appropriate bioassay strongly depends on the purpose of the study as well as on the facilities of the research laboratory (Kokkali & van Delft, 2014). In some cases ecotoxicologists are replacing classical ecotoxicity tests with newer *in vitro* bacterial assays also since bacteria represent an integral part of the ecosystem (Parvez, Venkataraman, & Mukherji, 2006). Regardless, toxicity should be evaluated based on various representative test organisms since the mechanisms of action and therefore the biological responses can vary for different organisms like algae, cyanobacteria and bacteria (Brezovšek, Eleršek, & Filipič, 2014). In addition, when a certain compound is known to be highly potent (e.g. cytostatics), studies regarding the possible mechanism of action, i.e. genotoxicity and/or cytotoxicity are essential. Bioassays, capable of such studies, are e.g. the Ames test and *umu*-Chromotest on various strains of *Salmonella typhimurium* and SOS-chromotest on *Escherichia coli*, both for genotoxicity and cytotoxicity evaluation. In addition, a luminescence inhibition tests using *Vibrio fischeri* for *in vitro* toxicity evaluation based on the effect on metabolic activity can also be applied. Importantly, besides the original testing of pure compounds, modifications allow an evaluation of the overall toxicity of a sample, i.e. WW, SW or sediment containing a naturally present mixture of compounds (Žegura et al., 2009). These types of bioassays can be used also to compare the toxicity of a biologically or chemically treated and non-treated water sample.

A review paper by Toolaram et al. (2014) gathers the data on genotoxic activities of CP, IF and some of their human metabolites/TPs (phosphoramide mustard, 4-OH-peroxy-cyclophosphamide, nor-nitrogen mustard, carboxy-CP and keto-CP). The authors conclude that certain metabolites are even more potent than the parent compounds (Toolaram et al., 2014). Such data clearly show the necessity to include not only parent compounds but also their metabolites/TPs in studies addressing the occurrence and potential effects in the environment.

1.4.3 Toxicity of complex mixtures

The expected environmental concentrations of all APIs (ng L^{-1}) are significantly lower than the concentrations (mg L^{-1}), which may trigger adverse effects on the aquatic

organisms (Grung et al., 2008; Zounkova et al., 2007). Regardless, one must be mindful that exposure to a cocktail of compounds, including API residues, may exhibit additive, synergistic, antagonistic or potentiating effects in the environment. Additive effects of a mixture are the sum of the effects of each individual compound, while synergistic effects of a mixture are greater than the sum. Antagonism occurs, when the effect of one compound in a mixture is reduced by another compound. Finally, a potentiating effect is observed, when the total toxicity of a non-toxic and a toxic compound together is higher than the determined toxicity of the individual toxic compound (Casarett, Doull, & Klaassen, 2001).

It is evident that toxicity of all environmentally present mixtures cannot be evaluated from laboratory tests; instead the prediction of such effects *via* existing data for single compounds can be done. This can be achieved by the application of several models, e.g. concentration addition (CA), independent action (IA) and the combination index (CIn) which represent another more humane alternative for evaluation of compounds toxicity (Hadrup et al., 2013). The concentration addition model, designed for compounds with similar mechanisms of action, is used to determine the presence of synergism or antagonism (Brezovšek et al., 2014; Faust et al., 2001; Loewe, 1927). The IA model is used for predicting the effects of mixtures of compounds with distinct mechanisms of action (Bliss, 1939; Faust et al., 2003). Another model for predicting the toxicity of a mixture is CIn, which can determine whether or not a mixture has an additive (CI = 1), synergistic (CI < 1) or antagonistic (CI > 1) effect (Chou & Talalay, 1984). To date, two studies report the synergistic ecotoxic effects of binary mixtures of cytostatics, namely 5-fluorouracyl, cisplatin, etoposide and imatinib (Brezovšek et al., 2014; Parrella et al., 2014) but no data regarding the toxicity of mixtures of CP, IF and their metabolites/TPs are available.

1.4.4 Toxicity evaluation of various water treatment technologies

Studies on the efficiency of various water treatment technologies can be evaluated in terms of removal efficiency and by the toxicity of the treated effluent, which can be either reduced, increased or remains unchanged compared to the influent (Altmann et al., 2012; Calza et al., 2006; Coelho, 2009). Toxicological studies of CP and IF residues in wastewaters treated biologically, with ozone and with AOPs reported lower, similar or increased toxicity (Table 11). Lai et al. (2015) and Lutterbeck et al. (2015) report lower or no change in toxicity of CP and IF residues in samples treated by UV/TiO_2. Lin et al. (2015) applied the same toxicity test to O_3-treated samples containing CP and IF and reported higher toxicity of effluent *vs.* influent. Delgado et al. (2011) report lower effluent toxicity when WW containing CP at environmental levels was treated by MBR (Table 11).

Table 11: *The toxicity of mixture of CP and/or IF residues (TPs) after investigated water treatments.*

	Toxicity test	Treatment	Initial concentration	Toxicity of treated sample	Reference
CP	biomarker test for cytotoxicity on human liver cells (HepG2)	membrane bioreactor (MBR)	5 µg L^{-1}	lower if compared to influent	(Delgado et al., 2011)
	the combined toxicity test: short-term luminescence inhibition test towards *V. fischeri* according to EN ISO 11348 and the *Photobacterium phosphoreum* growth inhibition test DIN 38412-37	UV/H_2O_2 UV/TiO_2	20 mg L^{-1}	no toxicity of parent compound and no increase in toxicity of treated samples	(Lutterbeck et al., 2015)
	luminescence inhibition test towards *V. fischeri* (Microtox®)	O_3	20 mg L^{-1}	increased toxicity (3-times higher)	(A. Y. H. Lin, J.Hong, P. K., 2015)
	luminescence inhibition test towards *V. fischeri* (Microtox®)	UV/TiO_2	20 mg L^{-1}	increased toxicity which drops to 0 at the end of treatment	(Lai et al., 2015)
IF	luminescence inhibition test towards *V. fischeri* (Microtox®)	O_3	20 mg L^{-1}	increased toxicity (2.7-times higher)	(A. Y. H. Lin, J.Hong, P. K., 2015)
	luminescence inhibition test towards *V. fischeri* (Microtox®)	UV/TiO_2	20 mg L^{-1}	increased toxicity which drops to 0 at the end of treatment	(Lai et al., 2015)

1.5 Methodology

Typically, for quantitative target analysis of cytostatic residues in aqueous samples, analytical methods employ a sample preparation step followed by either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS; Figure 6). When a target analysis is performed for the first time, solid phase extraction (SPE) as a pre-concentration step, the derivatization process, and the instrumental analysis (GC-MS or LC-MS parameters) have to be optimized.

The identification of unknown compounds such as TPs that are formed during water treatment often requires a sample pre-treatment step. If SPE is also performed, it remains unknown, which of the formed TPs are collected and to what degree are they extracted from the sample (Haddad et al., 2015). To avoid potential losses of TPs during this step, high initial concentrations of investigated compounds are typically employed allowing direct chemical analysis for structural elucidation (Figure 6).

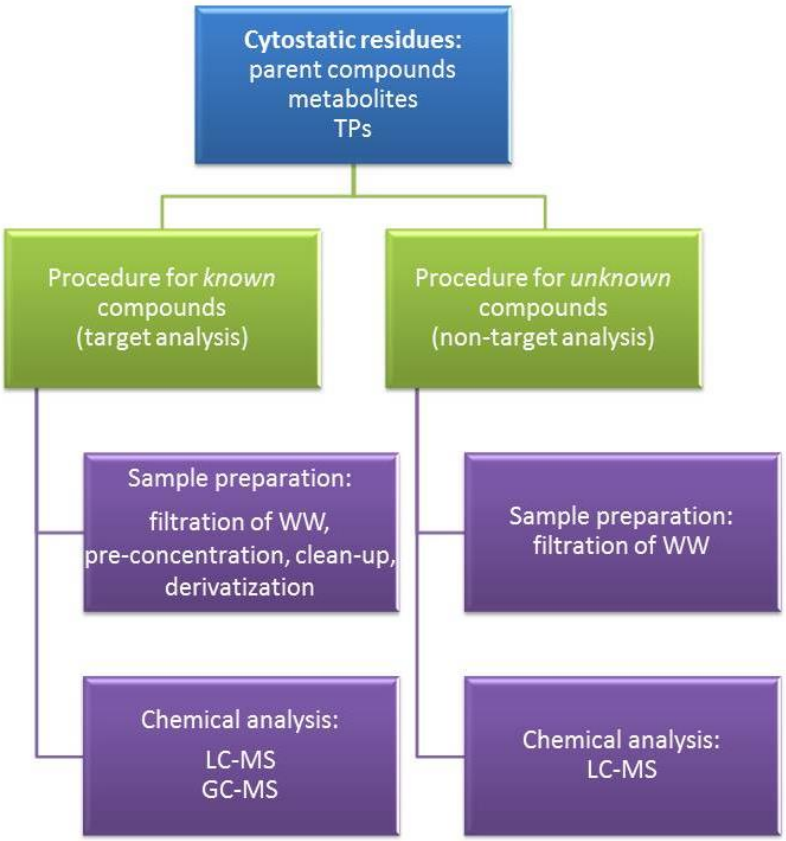


Figure 6: *Typical analytical procedure for target and non-target analysis.*

1.5.1 Sampling and sample pre-treatment

In most cases water sampling consists of a periodic collection of grab samples, which reflects the concentrations at a specific time (Buchberger, 2011). Another approach is composite sampling, which can be either time-proportional or flow-proportional. A composite sample is a sample constructed of multiple individual aliquots taken over a specific period of time or flow volume interval. The composite sampling gives information regarding the average concentrations of analytes in e.g. WWTP effluents, however it must be set up properly to obtain a representative sample (McArdell et al., 2006). A third option is to use the passive sampling, which is based on the free flow of the analyte from the sampled medium to a receiving phase in a sampling device (Vrana et al., 2005). One example is a Polar Organic Chemical Integrative Sampler (POCIS), which gives information about time-weighted average concentrations (Buchberger, 2011).

The transport of collected samples from the sampling site to the laboratory has to be performed appropriately, i.e. on ice and protected from light. Generally, sample filtration through a 0.45 µm filter and acidification or the addition of a disinfectant (e.g. formaldehyde or chlorine) is done to facilitate the SPE procedure and prevent microbial degradation of the analyte, respectively. Finally, samples are usually stored at -20 °C prior to analysis (Buchberger, 2011; McArdell et al., 2006).

The studies on the occurrence of CP and IF (Table 4) performed either grab or composite sampling, whereas the concentrations of metabolites/TPs in aqueous samples are yet to be reported. Interestingly, the majority of studies performed composite sampling, i.e. time-proportional for all matrices with the exception of SW. Filtration with 0.45 µm filters was used for prevention of the clogging of SPE cartridges (Table 4). The reported CP and IF concentrations were in the same concentration ranges regardless of the

sampling type. Since these compounds occur in the environment at very low concentrations (ng L^{-1} or even lower), studies performing passive sampling are necessary to obtain time-weighted average concentrations.

1.5.1.1 Sample extraction

Extraction is a common preparation step for chemical analysis of aqueous samples (WW or SW). Various extraction methods exist such as liquid-liquid extraction (LLE), SPE, solid phase micro extraction (SPME) and ultrasonic solvent extraction (Buchberger, 2011). Solid phase extraction has replaced traditional LLE and is still the most widely used preparation step for the analysis of organic compounds including cytostatic residues from aqueous samples (Hatrík & Tekel, 1996; Santana-Viera, Montesdeoca-Esponda, Sosa-Ferrera, & Santana-Rodríguez, 2015; Zief & Kakodkar, 1994). Besides concentration, SPE simultaneously offers sample clean-up by removing interfering compounds from complex matrices within the washing step (Žvir-Ferenc & Biziuk, 2006).

Figure 7 presents a typical SPE procedure. Extraction is performed by loading the sample onto a pre-conditioned cartridge containing a sorbent material. After percolation and a washing step, the sorbent is dried. The analytes are then eluted using a suitable organic solvent (or their mixtures). Finally, the solvent is removed and/or further preparation (e.g. derivatization in the case of GC-MS) of an extract is performed (McArdell et al., 2006; Zief & Kakodkar, 1994).

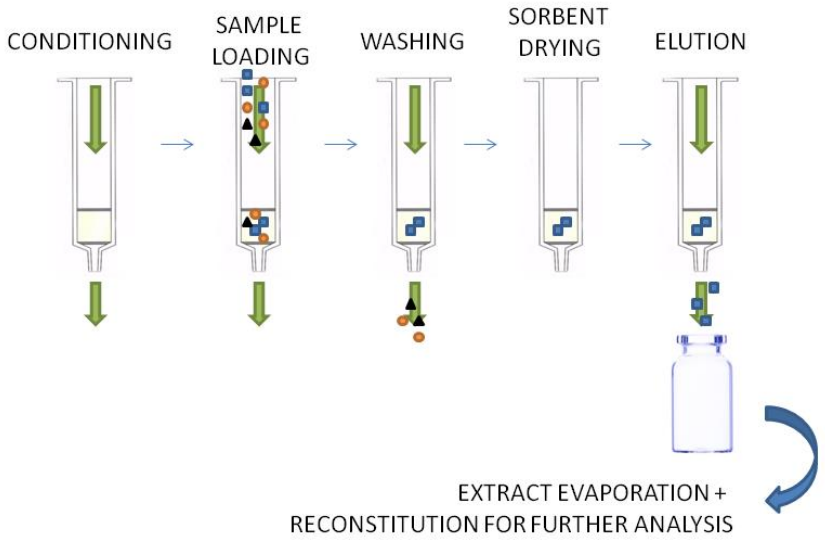


Figure 7: *Schematic presentation of SPE procedure*.

Conventional “off-line” SPE can also be automated, where the instrumentation allowing “on-line” SPE is coupled directly to the LC (or LC-MS) system. This is less time and solvent consuming, requires lower sample volume and minimizes contamination during handling, which results in improved reproducibility of the analysis. Moreover, it increases sensitivity since elution is performed with the mobile phase (MP) used for LC (Santana-Viera et al., 2015).

For successful SPE extraction it is important to use the appropriate sorbent and solvent in the elution step. Interactions between sorbent and analyte can occur *via* van der Waals forces (as “non-polar interactions”), hydrogen bonding, dipole-dipole forces (as “polar” interactions) and cation-anion interactions (as “ionic” interactions), which depend on both the analyte and the type of sorbent material used (Žvir-Ferenc & Biziuk, 2006). The most frequently used sorbents are chemically modified silica with C8 and C18 organic groups,

ion-exchange materials and polymer sorbents, e.g. graphitized or porous carbon and selective sorbents, such as molecularly imprinted polymers and immunosorbents (Žvir-Ferenc & Biziuk, 2006).

Despite the availability of many different types of sorbents, N-vinylpyrrolidone and divinylbenzene (Oasis HLB^{TM}) copolymers and surface modified styrene-divinyl benzene (Strata X^{TM}) sorbents are typically used for the extraction of APIs from aqueous samples (Kosjek & Heath, 2011). For SPE of CP and/or IF either Oasis HLB^{TM} (L. Ferrando-Climent et al., 2015; L. Ferrando-Climent et al., 2013; Gómez-Canela et al., 2012; Kovalova et al., 2012; Köhler et al., 2012; Martín et al., 2011; Moldovan, 2006; Valcárcel et al., 2011; Yin, Shao, et al., 2010; Yin, Yang, Li, Zhang, & Shao, 2010) or Strata X^{TM} SPE cartridges (Buerge et al., 2006; Busetti et al., 2009; Delgado, Schetrite, Gonzalez, & Albasi, 2010; Garcia-Ac et al., 2010; Garcia-Ac et al., 2009; Llewellyn et al., 2011) were used. Several studies have extracted CP and IF using “on-line” SPE also with N-vinylpyrrolidone and divinylbenzene copolymer sorbent proving that this technique is highly applicable for routine analysis of water samples (Garcia-Ac et al., 2009; Kovalova et al., 2012; Negreira, López de Alda, & Barceló, 2013). In all these studies multi-analyte analysis was performed and the optimal conditions including the type of SPE sorbent were determined for all investigated compounds, not only for CP and/or IF. There are no studies describing the preparation of water samples for the analysis of CP and/or IF metabolites/TPs, although published methods for their extraction from human plasma are available. Liquid-liquid extraction was applied for CP and IF metabolites (N-decl-CP, 4-oxocyclophosphamide, carboxy-CP, phosphoramide mustard, hydroxypropylphosphoramide mustard, 2-dechloroethylifosfamide and 4-hydroxyifosfamide) in plasma by Gourmel et al. (1999) and Kalhorn et al. (1999), respectively. Moreover, a successful SPE using C18 sorbent for the analysis of plasma was also reported by Baumann et al. (1999) for CP metabolites (4-hydroxycyclophosphamide, keto-CP, carboxy-CP and 3-dechloroethylifosfamide) and by Momerency et al. (1994) and Wang et al. (1995) for IF metabolites (2- and 3-dechloroethylifosfamide, 4-ketoifosfamide, carboxyifosfamide, ifosfamide mustard, chloroethylamine, 1,3-oxazolidine-2-one and 4-hydroxylifosfamide).

1.5.1.2 Derivatization

Derivatization is a chemical reaction, where compounds are transformed into more volatile and thermally stable forms, rendering them suitable for analysis by GC (King S. & Blau, 1977). In addition, it is also used to increase the amount of information obtained from mass spectral data by e.g. modification of fragmentation (enhancement of molecular weight related ions and enhancement of structurally informative ions) of the sample molecule and for the determination of functional groups (Knapp, 1990).

There are numerous derivatizing agents available for the different functional groups, e.g. alcohols, amines, thiols, carboxylic acids, aldehydes, ketones etc. (Table 12). Various agents can be used (silylating, acylating or alkylating agents) and derivatization “yield” is usually agent-type and agent-dose dependent. In addition, the derivatization can be optimized by testing various experimental conditions such as time and temperature in various solvents. As in the case of SPE, derivatization can be performed either "off-line" or directly in the GC injector as "on-line" process (King S. & Blau, 1977; McArdell et al., 2006).

Table 12: *Chemical agents for derivatization different functional groups*.

Type	Derivatizing agent	"Target" functional groups
silylating agents	N-methyl-N-(trimethylsilyl)trifluoroacetamide) (MSTFA)	-NH -OH -SH -COOH
	N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA)	
	N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide with 1 % tert-butyldimethylchlorosilane (MTBSTFA with 1 % TBDMCS)	
acylating agents	heptafluorobutyric anhydride (HFBA)	
	trifluoroacetic anhydride (TFAA)	
	N-heptafluorobutyrylimidazole (HFBI)	
	N-methyl-bis(trifluoroacetamide) (MBTFA)	
	2,3,4,5,6-pentafluorobenzoyl chloride (PFBCl)	
	acetic anhydride (AC_2O)	
alkylating agents	2,3,4,5,6-pentafluorobenzyl bromide (PFBBr)	
	diazomethane (CH_2N_2)	
other	O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA)	-CO

Although CP and IF have an amine group in their structure, they have only been shown to be successfully derivatized by acylation with TFAA (Momerency et al., 1994; T. Steger-Hartmann et al., 1996). In addition, the alkylation of CP metabolites, namely keto-CP, N-decl-CP and carboxy-CP with diazomethane (CH_2N_2), which is no longer commercially available due to its toxicity and explosiveness, has been reported (Kalhorn et al., 1999; Momerency et al., 1994).

1.5.1.3 Internal standards

The use of internal standard (IS) is essential to obtain reliable results since it compensates for the losses of analytes during sample preparation (surrogate standard, SUR) and/or chemical analysis within the instrument (instrumental standard). An IS is a compound that has similar physico-chemical properties to the analyzed compound; in mass spectrometry ^{13}C, ^{2}H, ^{15}N isotopically labelled compounds are frequently used (Liu, Foster, Cone, & Kumar, 1995). If such isotopically labelled compounds are not available, the use of structurally similar compounds can be applied, but the latter should not be already present in the original samples (McArdell et al., 2006).

For the analysis of CP and/or IF in aqueous samples, isotopically labelled or structurally similar compounds (^{13}C- and/or ^{2}H-cyclophosphamide, ^{13}C- and/or ^{2}H-ifosfamide, ^{13}C-caffeine and trofosfamide) have been used as internal standards (Buerge et al., 2006; Negreira et al., 2014; Sottani et al., 2008). Published multi-analysis studies report also the use of ^{2}H-ibuprofen, ^{13}C-ethyoxyphenacetine and ^{2}H-phenytoin (Busetti et al., 2009; Martín et al., 2011; Zuccato et al., 2005).

1.5.2 Instrumental analysis

Chemical analysis for either qualification or quantification of organic compounds in water is usually based on GC-MS and/or LC-MS techniques. For GC hyphenated techniques, an essential pre-step is derivatization, which enables the detection of environmentally relevant concentration ranges, however this step limits the number of target compounds in the sample. The development of more sophisticated LC-MS instruments, with similar LODs to GC-MS, has led to LC-MS replacing GC-MS for multi-analyte analysis of organic compounds. In addition, LC-MS is also the method of choice for identifying unknowns (Santana-Viera et al., 2015).

1.5.2.1 Separation

1.5.2.1.1 Gas chromatography

Gas chromatograph separates compounds by their varying interactions with stationary phase that can be either a solid or liquid (e.g. polysiloxanes) immobilized on the inner surface of the inert column wall. The MP is an inert carrier gas, e.g. argon, helium or hydrogen (McArdell et al., 2006; Skoog A., Holler F., & Nieman A., 1997a). In GC, the sample must be in volatile form so that when injected into a heated injection port (inlet), the compounds enter the column in the gas phase. The optimization of the oven temperature programme, gas flow, the type and thickness of the stationary phase and the length of the column and diameter govern the separation of the analytes (McArdell et al., 2006; Skoog A. et al., 1997a).

1.5.2.1.2 Liquid chromatography

Liquid chromatography is used for separating polar, non-volatile or thermally labile compounds and is the most appropriate method for most APIs. When analytes in a liquid sample enter the LC column (packed with particles; size: 3 – 5 μm), they partition between the liquid MP and the solid stationary phase. Different stationary phases are available including normal-phase (polar stationary phase, e.g. silica gel) and reversed phase (RP; nonpolar stationary phase, e.g. silica gel alkylated with alkyl groups). Generally, normal-phases are used to separate polar compounds, while RPs are used for the separation of moderately polar compounds. Separation can be improved by modifying the MP, which is typically achieved by adding methanol (MeOH) or other similar water miscible organic solvents (Skoog A., Holler F., & Nieman A., 1997b). Adequate peak shapes and sufficient retention of weakly acidic compounds in RP LC can be obtained by decreasing the pH of the MP by adding an organic acid to transform the analyte into its undissociated state. Alternatively, ammonium formate can be added to form an ion pair with the negatively charged analyte anion (Quintana & Reemtsma, 2004).

In ultra-performance liquid chromatography (UPLC) columns are packed with smaller particles (< 2μm) allowing higher pressures and hence higher flow rates within the columns with no negative influence on the components of the chromatographic system. This results in shorter times of analyses, improved separation and sensitivity and reduced costs in terms of time and solvent consumption (Nováková, Matysová, & Solich, 2006).

1.5.2.2 Mass spectrometry

In both, GC and LC, the mass spectrometer is the detector of choice for analysing water samples containing trace levels of API residues. Common detectors like the flame ionization detector, UV and fluorescence detectors do not share the same performance in terms of selectivity and sensitivity (Skoog A. et al., 1997a, 1997b).

1.5.2.2.1 Principles

The mass spectrometer consists of three main parts: an ion source, a mass analyzer and an ion detector (Dass, 2006a; Skoog A. et al., 1997a). Analytes are firstly ionized in the ion source of which there are two major categories: gas-phase sources and desorption sources. In the former, the analytes are directly converted into gaseous ions, while in the latter the sample is first vaporized and then ionized, which can be achieved using atmospheric pressure ionization (APIon) techniques. Two gas-phase sources, electron-impact ionization (EI) and chemical ionization (CI), are the most widely used ion sources in GC-MS for determining APIs, while electrospray ionization (ESI) as desorption source and atmospheric pressure chemical ionization (APCI) as gas-phase source are applied in LC-MS (Kosjek & Heath, 2008). In EI, the analyte is vaporized and then ionized by bombarding the sample with a beam of electrons, while in CI ions are produced through the collision of the analyte with ions of a reagent gas (e.g. methane) present in the ion source. Electron impact ionization provides more structural information than CI since it is capable of molecular ion fragmentation, while CI as a softer ionization mode provides information regarding the molecular ion (Dass, 2006c). In ESI, the sample is forced through a capillary maintained at several kilovolts with respect to its surroundings (atmospheric pressure). A nebulizer gas flows from outside the capillary to spray the sample. This creates a continuous fine mist of charged droplets as the solvent evaporates, which leads to the formation of ions (Skoog A. et al., 1997b). Another similar APIon technique is APCI, a sub-type of CI. The interface design is similar to ESI, but the principle differs, since APCI vaporizes both the solvent and sample molecules by spraying the sample solution into a heated chamber (400 °C) using an inert gas e.g., nitrogen. The solvent molecules are then ionized by corona discharge to generate stable reaction ions. Both, ESI and APCI result in mostly protonated $[M+H]^+$ or deprotonated $[M-H]^+$ ions, where multiple charged ions occur in ESI and only single charged in APCI. Similarly to CI, APIon techniques are “soft techniques” that provide molecular ion information accompanied by poor fragmentation. Electrospray ionization is a technique often used for identifying TPs (1.5.2.3; Table 15). In addition, APCI can be used in place of ESI due to its lower susceptibility towards matrix effects (Khetan, 2007; Kosjek, Perko, Heath, Kralj, & Žigon, 2011; McArdell et al., 2006).

Once ionized the ions pass into the mass analyzer, where they are separated based on their mass-to-charge (m/z) ratios. Several types of mass analyzers are available, where the most simple is a single quadrupole (Q), which can be employed in both, GS-MS and LC-MS systems. The quadrupole mass analyzer (Figure 8), consists of four parallel rods (two at a negative and two at a positive potential), where ions, depending on their mass-to-charge ratio and the applied voltages on each set of rods, either collide with the rods or pass unhindered into the mass detector (McArdell et al., 2006). The main disadvantage of this technique is its non-selectivity (Kosjek & Heath, 2008). Regardless, it remains one of the most widely used analyzers due to its simplicity, good quantitative analysis, resolution and cost.

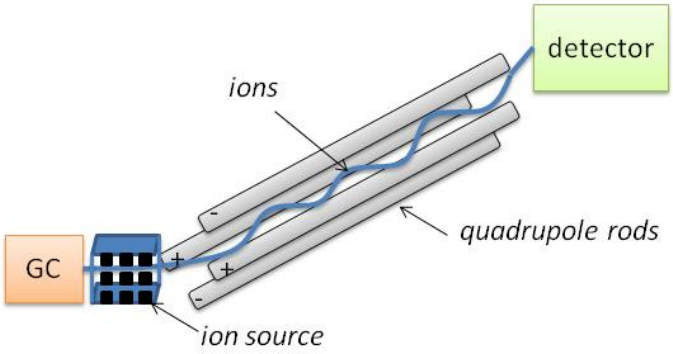


Figure 8: *A schematic presentation of mass spectrometer with ion source and Q as a mass analyzer. The blue curve presents a pathway of ions passing through the quadrupole towards the detector.*

Improved selectivity, higher experimental flexibility and reduced "chemical noise" can be achieved with a triple-quadrupole mass spectrometer (QqQ), which enables MS/MS operation. Tandem mass spectrometry consists of two systems. In the first quadrupole, the target ion is selected from a stream of ions, which then undergoes fragmentation, while in the second, mass analyses of the product ions are performed (Dass, 2006d). In the specific case of QqQ, the first quadrupole preselects the ion of interest, which is then activated in the second quadrupole and finally analyzed in the third quadrupole. This allows some structural characterization, but it is not suitable for complete structural confirmation of unknowns. Since it improves both sensitivity and selectivity of the analytical method compared to a single Q, it is frequently used for quantitative analysis (McArdell et al., 2006). A time-of-flight (TOF) mass detector consists of a long field-free flight tube and separates ions by the differences in their velocity. A short pulse of ions with defined kinetic energy is dispersed in time. The ions with lower m/z values travel faster and reach the detector earlier (Dass, 2006b). The advantages of this analyzer are its high sensitivity and mass accuracy since it belongs to a group of high resolution mass spectrometry (HRMS) instruments with 10,000-20,000 full width at half maximum (FWHM) mass resolving power. High resolution provides important information about an unknown compound since its elemental composition can be calculated by exact mass calculators. Another commonly used mass analyzer for structural elucidation is an ion trap (IT), which is a 3D version of the quadrupole mass analyzer. This analyzer stores ions for a certain length of time in an ion trap device, which consists of two endcap electrodes and one ring electrode between them. Ions are ejected towards the detector by changing the applied potential, which leads to a higher sensitivity if compared to simple Q and the ability to perform multiple stage mass spectrometry (MS^n) experiments, i.e. the generation of spectra of the parent and fragment ions (and their fragment ions etc.). The MS^n spectra provide important information regarding fragmentation patterns of unknown compounds and therefore represent a useful tool for structural elucidation (McArdell et al., 2006). Multiple MS^n experiments accompanied by HRMS can be performed using hybrid mass spectrometers, which couple two different types of mass analyzers, enabling sufficient data for unequivocal structural identification of unknown compounds present in a sample. An example is the Linear Ion Trap-Orbitrap (LTQ-Orbitrap) mass spectrometer, which attains HRMS spectra by Fourier-transformation mass spectrometry. This spectrometer consists of linear IT hyphenated to Orbitrap, which works on the principle of the orbital trapping of ions around an axial central electrode with a pure electrostatic field (Dass, 2006b; Makarov et al., 2006). This technique is sensitive and highly applicable in the analysis of unknown compounds since it gives an accurate mass (spectra can be acquired at high resolution, ca. 100,000 FWMH) that provides elemental

composition and can perform multiple MS^n experiments within the same run. Its use is limited by its price (Dass, 2006b). A schematic representation of the LTQ-Orbitrap is shown in Figure 9. Ions from ESI ion source are admitted into the linear trap of the LTQ, where they are analyzed and further ejected towards the C-trap, a curved quadrupole (Figure 9). After leaving the C-trap, the ions are accelerated with high kinetic energies towards the Orbitrap through a small entrance aperture. The ions are captured in the Orbitrap, where they oscillate along the inner electrode. The ion current is then transformed into a frequency spectra by Fourier transformation.

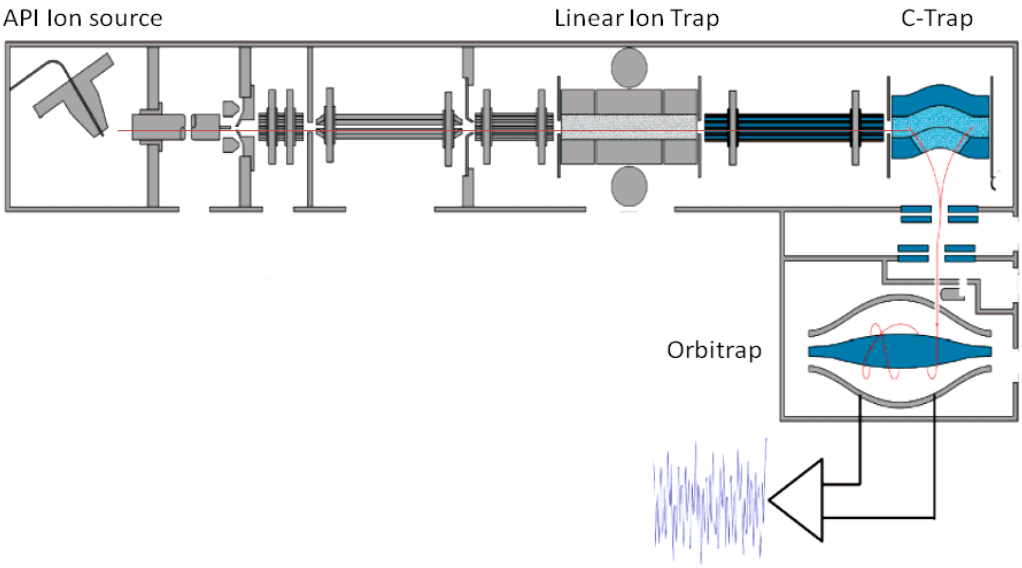


Figure 9: *Schematic presentation of the LTQ Orbitrap mass spectrometer and the ions pathway (marked with red; adapted from Makarov et al., (2006)).*

1.5.2.2.2 Application in qualitative analysis of TPs

The ongoing development of more sophisticated hyphenated LC-MS instruments means that LC-MS remains the instrument of choice for identifying unknown compounds, i.e. TPs. As discussed in Subchapter 1.5.2.2.1, different types of mass analyzers provide either information about fragmentation patterns from MS/MS or MS^n spectra, high accurate masses of TPs or even both (Žonja, Aceña, Pérez, & Barceló, 2013). The performances of different mass analyzers are shown in Table 13.

Table 13: *Performance of various mass analyzers.*

Mass analyzer	Resolution power	Fragmentation	Applicability
Q	4×10^3	MS	quantitative analysis
QqQ	$1\times10^4 - 2\times10^4$	MS/MS	quantitative analysis
TOF	1.5×10^4	MS	partially qualitative for unknown compounds
IT	$10^3 - 10^4$	MS^n	partially qualitative for unknown compounds
LTQ-Orbitrap	10^5	MS^n	qualitative and quantitative analysis

Sometimes additional information is needed for complete structural elucidation of unknown compound. This can be achieved by using other analytical techniques, such as LC with nuclear magnetic resonance (NMR) or hyphenated LC-NMR-MS systems,

derivatization and by matching the obtained spectra against spectra of compounds that are available in mass spectral libraries, such as the National Institute of Standards and Technology (NIST) library (Foti, Alsante, Cheng, Zelesky, & Zell, 2013; Haddad et al., 2015; Kosjek, Andersen, Kompare, Ledin, & Heath, 2009). In addition, several search algorithms can be used for automated TP detection such as MetaboLynx™, Sieve™ and MassHunter™ (Freed, Kale, Ando, Rossi, & Kingsmill, 2004).

1.5.2.3 Chemical analysis of investigated cytostatic residues

Only two studies report the use of GC-MS technique to quantify CP and/or IF in aqueous samples, while all others used LC-MS (Table 14). Despite different instrumentation, the LODs are comparable (all in the low ng L^{-1} range), suggesting the adequate sensitivity of these methods for trace analysis, with the only exception being a study by Kiffmeyer et al. (1998), who used an UV detector (Table 14). In the case of GC-MS analysis, HP-5MS (5 % diphenyl/95 % dimethylpolysiloxane) and Permabond SE-52-DF (5 % phenyl/95 % methylpolysiloxan) columns were used for separation (Moldovan, 2006; T. Steger-Hartmann et al., 1996). In both cases ionization and mass analysis were based on EI and single Q (Table 14). Studies based on LC-MS used mainly RP C18 columns and water in combination with either MeOH or acetonitrile (ACN) as the MPs. In addition, acidification of MP with formic acid was often applied and the ionization was operated in ESI positive mode with QqQ being the most commonly used mass analyzer, followed by either QqQ-LIT (triple quadrupole Linear Ion-Trap) or Orbitrap (Table 14).

Table 14: *Review of literature regarding quantitative analysis of CP and IF*.

Comp.	Matrix	Quantitative Analysis		LOQ/LOD	Reference
		Separation	Detection		
CP, IF	WW	GC: on-column injection (Permabond SE-52-DF); carrier gas: helium	single Q; EI at 70eV; detection mode: selected ion monitoring (SIM)	LODs: 6 ng L^{-1} (CP) 7 ng L^{-1} (IF)	(T. Steger-Hartmann et al., 1996)
CP, IF	river water, WW	RP HPLC: C_{18} column; MP: water/ACN with addition of 10mM ammonium acetate (pH = 5.7)	QqQ-MS; ESI(+); detection mode: multiple reaction monitoring (MRM)	LODs: 10 ng L^{-1} (CP and IF)	(Ternes, 1998)
CP	SW	RP HPLC: C_{18} column; MP: phosphate buffer (pH = 3)/MeOH	UV detector (200 nm)	LOD: 200 µg L^{-1}	(Kiffmeyer et al., 1998)
CP	WW	RP HPLC: C_8 column. MP: 0.1 % formic acid (pH = 2)/ACN	QqQ MS; ESI(+); detection mode: MRM	LOQ: 1.9 ng L^{-1}	(Castiglioni et al., 2005)
CP	WW, SW	RP HPLC: C_8 column MP: 0.1 % formic acid (pH = 2)/ACN	QqQ MS; ESI(+) detection mode: MRM	*n.a.*	(Zuccato et al., 2005)
CP, IF	WW, SW	RP HPLC: C_{18} column. MP: 0.1 % formic acid/0.1 % formic acid in MeOH	QqQ MS; ESI(+); detection mode: MRM	LODs: 0.02 ng L^{-1} (SW; CP and IF) 0.3 ng L^{-1} (WW; CP and IF)	(Buerge et al., 2006)

Comp.	Matrix	Quantitative Analysis		LOQ/LOD	Reference
		Separation	Detection		
CP	SW	GC: HP-5MS column; carrier gas: *n.a.*	Single Q; EI mode at 70 eV; detection mode: SIM	LOQ: 30 ng L^{-1}	(Moldovan, 2006)
CP, IF	WW	RP UPLC: C_{18} column; MP: 0.1 % formic acid/ACN	QqQ MS; ESI(+); detection mode : MRM	LODs: 2 ng L^{-1} (CP and IF)	(Yin, Yang, et al., 2010)
CP, IF	WW	RP HPLC: C_{18} column; MP: 0.1 % formic acid/0.1 % formic acid in MeOH	QqQ MS; ESI(+) and APCI; detection mode: selected reaction monitoring (SRM)	LOQs: 0.11 – 0.4 ng L^{-1} (CP) 0.16 – 0.24 ng L^{-1} (IF)	(Llewellyn et al., 2011)
CP, IF	WW	RP UPLC: C_{18} column; MP: 0.1 % formic acid/ACN	QqQ-LIT; ESI(+); detection mode: 2 SRMs for each compound	LOQs: 3.6 ng L^{-1} (CP) 5.8 ng L^{-1} (IF)	(L. Ferrando-Climent et al., 2013)
CP, IF	WW	RP UPLC: C_{18} column; MP: 0.1 % formic acid/ACN	QqQ-LIT; ESI(+); detection mode: MRM	LOQs: 1.3 ng L^{-1} (CP) 1.7 ng L^{-1} (IF)	(L. Ferrando-Climent et al., 2015)
CP	WW	RP HPLC: C_{18} column; MP: 0.1 % formic acid/ 0.1% (v/v) formic acid in MeOH	Orbitrap ESI(+); detection mode: full scan and HRMS (resolving power = 50,000)	LOQ: 0.35 ng L^{-1}	(Gómez-Canela et al., 2012)
CP, IF	WW	On-line SPE-RP HPLC: C_{18} column; MP: MeOH, ACN, 0.1 % formic acid	QqQ ESI(+); detection mode: SRM	LOQs: 10 – 84 ng L^{-1} (CP) 2 – 17 ng L^{-1} (IF)	(Kovalova et al., 2012)
CP, IF	SW, WW	RP HPLC: C18 column; MP: 0.1 % formic acid in ACN/15 mM ammonium formate containing 0.1 % formic acid	QqQ; ESI(+); detection mode: 2 MRMs	LOQs: 1.7 – 2.3 ng L^{-1} (CP) 1.1 – 1.7 ng L^{-1} (IF)	(Martín et al., 2011)
CP, IF	SW, tap water	RP HPLC: C18 column; MP: ACN/0.1 % formic acid	QqQ-LIT; ESI(+); detection mode: two SRMs	LOQs: 4 ng L^{-1} (CP) 1 ng L^{-1} (IF)	(Valcárcel et al., 2011)
CP, IF	WW	RP HPLC: C18 column; MP: 0.4 % formic acid/1 % formic acid in MeOH	QqQ; ESI(+); detection mode: MRM	LOQs: 310 pg on column (CP) 454 pg on column (IF)	(Busetti et al., 2009)
CP	drinking water	On-line SPE-RP HPLC: C18 column; MP: 0.2 % acetic acid/ACN	QqQ; ESI(+); detection mode: SRM	LOQ: 3.2 ng L^{-1}	(Garcia-Ac et al., 2010)

There are only a few published studies concerning the formation of CP and IF TPs (Chapter 1.3.4.), which are listed in Table 15. Separation of TPs was achieved using an RP C18 column. For ionization, ESI was used, while the applied mass analyzers differed (Table 15). The suitability of these analyzers (QTOF, IT and QqQ) for the identification of unknown TPs is discussed in a review paper by Kosjek et al. (2007). Interestingly, only Fernandez et al. (2010) and Venta et al. (2005) used hyphenated techniques enabling both, MS^n experiments and HRMS.

Table 15: *Qualitative analysis for identification of CP and/or IF TPs.*

Comp.	Qualitative analysis		Reference
	Separation	Detection	
CP	RP LC: C18 column MP: water/ACN	Q-TOF; ESI(+)	(Fernández et al., 2010; Venta et al., 2005)
CP	RP LC: C18 column MP: 0.1 % formic acid/ACN	IT; ESI(+)	(Lutterbeck et al., 2015)
CP, IF	RP LC: C18 column MP: 0.1 % formic acid for positive mode and 5 mM ammonium acetate for negative mode(A); 0.1 % formic acid in MeOH for positive mode and 5 mM ammonium acetate in MeOH for negative mode (B)	QqQ; ESI (+/-)	(Lai et al., 2015)

1.5.3 Validation

The validation of an analytical method is an essential step in analytical chemistry as it ensures that a tested procedure gives reproducible and reliable results. The extent of the validation process and the choice of the parameters to be assessed depend on the type of analysis, customer needs and/or regulations (Taverniers, De Loose, & Van Bockstaele, 2004; Trullols, Ruisánchez, & Rius, 2004). Therefore, it is necessary to define properly the conditions in which the procedure is to be used and the purpose for which it is intended. It is obvious that a validation process is required for any new analytical method and revalidation is necessary when any component of e.g. instrument is replaced or any other changes in the analytical procedure are made. Method validation (Figure 10) typically involves the evaluation of a series of analytical method-performance parameters, i.e. recovery, accuracy (precision and trueness), selectivity, LOQ, LOD, sensitivity, linearity and robustness (Taverniers et al., 2004). This process is an essential step in the case of target analysis of a compound, where its quantification is the main goal. The identification of unknown compounds represents only qualitative analysis, where the validation process is usually omitted due to the absence of commercially available standards (e.g. TPs).

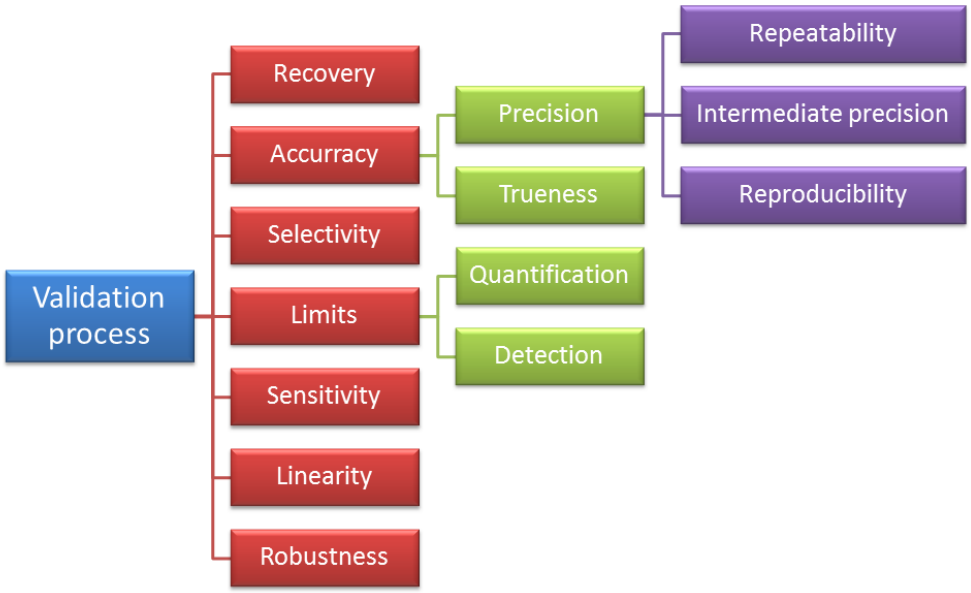


Figure 10: *Parameters for validation of analytical method*.

Recovery is the efficiency of an analytical method, which reveals the amount of detected analyte in a sample by the applied analytical method (Taverniers et al., 2004). It is defined as the ratio between the measured amount of analyte in the spiked sample and the theoretical value of the spike (McArdell et al., 2006).

Accuracy can be determined by precision and trueness. It is defined as the closeness of the agreement between a test result and the accepted reference value. In particular, precision is the agreement between independent test results under stipulated conditions and is reported as SD or relative standard deviation (RSD). Precision measures are divided into three groups:

- repeatability: when the same analyst uses the same equipment in the same laboratory in short intervals
- intermediate precision: when different analysts use the same equipment over an extended period of time
- reproducibility or inter-laboratory precision: when different laboratories with different operators using different equipment perform the analysis (Taverniers et al., 2004; Trullols et al., 2004).

Inter-laboratory precision can be assessed by performing inter-laboratory studies, where different laboratories analyze the same samples using their own in-house analytical methods. They are performed in order to compare the results of the participating laboratories among each other, to assess the proficiency of individual laboratories, to estimate measurement uncertainty and for certifying reference materials (Hund, Massart, & Smeyers-Verbeke, 2000).

Trueness represents the closeness between the mean value determined for the analyte of interest and the accepted true value (or known level actually present in the sample). Reference materials for the determination of trueness are divided into certified reference materials (CRMs) and non-certified (laboratory) materials. Certified reference materials are materials defined by a statement of uncertainty and are traceable to international standards, hence, they are appropriate for validating methods, they allow a comparison of methods and are used to calibrate equipment and methods. When no CRMs are available, a blank sample matrix can be spiked with a known amount of a pure stable in-house

material/standard (Peters, Drummer, & Musshoff, 2007; Taverniers et al., 2004).

Selectivity is the ability of the analytical method to determine specifically the analyte of interest in a sample. It represents a confirmation of the identity of the compound and cannot be calculated but has to be demonstrated. This could be done by using a mass spectrometer to determine if a single peak in a chromatogram belongs either to one compound or represents two different co-eluting compounds (Peters et al., 2007; Taverniers et al., 2004).

Limit of quantification represents the lowest concentration that can be quantified by an analytical method with reasonable statistical certainty. It is recommended that the LOQ lies within the calibration curve. The LOQ is usually calculated as 10-times the SD of the baseline in the blank divided by the slope of the calibration curve. The LOD value represents the lowest concentration that can be reliably distinguished from zero. It can be calculated as 3-times the SD of the baseline in the blank samples divided by the slope of the calibration curve. It is necessary to emphasize that quantification and qualification below the LOQ and LOD, respectively, can be done, but the results are unreliable as the uncertainty associated with levels below LOQ and LOD are higher than the measured value itself (McArdell et al., 2006; Taverniers et al., 2004).

Sensitivity refers to the slope of the calibration curve. A method is sensitive, when a small change in the concentration of the analyte causes a large change in the measured signal (Peters et al., 2007).

Linearity is the ability of the analytical method to obtain responses of analytes proportional to their concentration within a defined concentration range, which lies between the upper and lower linearity limits. The working linear concentration range usually lies between 80 – 150 % of the expected concentration of the analyte in a sample (McArdell et al., 2006; Taverniers et al., 2004).

By determining robustness, the influence of small and deliberate variations on the results can be determined, which provides an indication of its reliability during normal usage (Taverniers et al., 2004; Trullols et al., 2004).

2 Aims and Hypotheses

There are numerous registered APIs for human or veterinary use, which are, depending on their physico-chemical properties, capable of reaching every environmental compartment – air, water, soils and sediments. Over the last few decades greater knowledge of the cycling of APIs in the environment has been gained with little attention being paid to one specific group, cytostatics. Cyclophosphamide and IF, together with their active metabolites/TPs, have a potential to harm aquatic biota once they enter the environment due to their teratogenic, genotoxic, cytotoxic and mutagenic properties. Regardless, little is known about their occurrence, transformations and the risk they pose to the environment. Efficient treatments for these compounds and other cytostatic compounds are also yet to be established. Keeping all that in mind, the aims of this thesis were the following:

- to develop, optimize and validate an analytical method for the trace analysis of CP, IF and their selected metabolites/TPs in various aqueous matrices,
- to organize and participate in an interlaboratory study for determination of trace levels of cytostatic drug residues in waste and natural waters,
- to quantify CP and IF residues in the Slovene aqueous environment,
- to assess the removal efficiency of selected cytostatic residues by biological and abiotic treatments,
- to screen for stable TPs during UV-based water treatments,
- to evaluate the effects of CP, IF, their metabolites/TP and mixtures of these compounds towards aquatic organisms.

Within the present doctoral dissertation, the following hypotheses were tested in order to achieve the proposed aims:

- H1: Cytostatic residues (CP, IF and their metabolites/TPs) are present in Slovene hospital wastewaters, whereas municipal wastewaters contain concentrations below LODs.
- H2: The highest removal of selected cytostatic residues is achieved by AOPs.
- H3: Mixtures of parent compounds and their metabolites/TPs show higher toxicity than the same compounds tested individually.

3 Publications

3.1 Occurrence of Cyclophosphamide and Ifosfamide in Aqueous Environment and Their Removal by Biological and Abiotic Wastewater Treatment Processes

The paper "Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes" by M. Česen, T. Kosjek, M. Laimou-Geraniou, B. Kompare, B. Širok, D. Lambropolou and E. Heath was published in the journal of Science of the Total Environment (I.F. 2014: 4.099) in April 2015. I am solely responsible for method optimization, validation and all the experimental work with the exception of cavitation, which was performed with the help of Maria Laimou-Geraniou and Martin Petkovšek. Prof. Širok provided the cavitation apparatus. I was also responsible for data analysis and drafting the manuscript.

Only few studies address the occurrence of cytostatics in natural and WWs. The removal of such compounds by various biological and/or abiotic treatments is also rarely reported. The occurrence of two commonly prescribed cytostatics: CP and IF, in Slovene WWs is addressed in this study for the first time. Samples were collected from five hospitals in different geographical regions in Slovenia. Where possible, corresponding WWTP influents and effluents were sampled. Cyclophosphamide was detected in 10 and IF in 3 out of the 18 samples analyzed. Hospital WWs contained both compounds in concentrations from below the LODs to 22,000 and to 6,800 ng L^{-1} for CP and IF, respectively. Cyclophosphamide was detected also in two samples of WWTP influent (19 – 27 ng L^{-1}) and one effluent (17 ng L^{-1}), whereas the concentrations of IF in all sampled WWTP influents and effluents were below the LOD.

This study also looked at the removal of CP and IF during advanced biological treatment. Removal efficiency was assessed over a 4-month period for a laboratory scale flow-through bioreactor incorporating attached growth biomass on Mutag Biochip^{TM} carriers. Standard WW parameters were monitored to assess bioreactor performance. The experiments were performed with attached biomass due to i) higher resistance of attached biomass to toxic compounds compared to suspended biomass and ii) due to low or inconsistent removal of CP and IF in experiments with suspended biomass. The average removal efficiency during biological treatment of artificial WW was 42 ± 12 % and 18 ± 11 % for CP and IF, respectively, while in the case of actual hospital WW, the removal was slightly better (CP: 59 ± 15 % IF: 35 ± 9.3 %).

Furthermore, various abiotic treatments of CP and IF containing artificial WW were also studied. Experiments included HC, ozonation and/or UV treatment, either individually or in combination with H_2O_2. Neither HC nor a combination of HC/H_2O_2 successfully removed either CP or IF. Increasing the concentration of H_2O_2 improved the removal of both compounds, when combined with UV, while the opposite effect was observed when the amount of H_2O_2 was increased during ozonation. The highest removal efficiency for CP (99 ± 0.71 %) and IF (94 ± 2.4 %) was achieved using UV/O_3/H_2O_2 (5 g L^{-1} of H_2O_2, 120 minutes). Similar degradation kinetics were observed also when artificial WW was replaced by actual hospital WW treated under the same conditions. Finally, sequential coupling of biological and UV/O_3/H_2O_2 treatment resulted in > 99 % removal for both compounds, with an accompanied high degree of mineralization of organic matter (DOC removal) in the treated sample. The experimental conditions used in the present study were unrealistic regarding the amount of H_2O_2, but were performed with untreated WW, i.e. influent, where the DOC values were comparable to actual DOC values of a typical hospital WW. This represents a first step in the development and implementation of such treatments into real scale applications, i.e. at hospitals, which represent a major source of not only cytostatic compounds, but also other APIs.

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Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes

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HIGHLIGHTS

- Cyclophosphamide (CP) and ifosfamide (IF) were detected in Slovene wastewaters.
- Hydrodynamic cavitation did not result in any significant CP nor IF removal.
- Biological treatment removed 59% and 35% of CP and IF, respectively.
- A combination of selected AOP ($UV/O_3/H_2O_2$ at 5 g L^{-1}) removed 99% (CP) and 94% (IF).
- Coupling biological treatment to a selected AOP removed >99% of CP and IF.

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ABSTRACT

Cytostatic drug residues in the aqueous environment are of concern due to their possible adverse effects on non-target organisms. Here we report the occurrence and removal efficiency of cyclophosphamide (CP) and ifosfamide (IF) by biological and abiotic treatments including advanced oxidation processes (AOPs). Cyclophosphamide was detected in hospital wastewaters (14–22,000 ng L^{-1}), wastewater treatment plant influents (19–27 ng L^{-1}) and effluent (17 ng L^{-1}), whereas IF was detected only in hospital wastewaters (48–6800 ng L^{-1}). The highest removal efficiency during biological treatment (attached growth biomass in a flow through bioreactor) was 59 ± 15% and 35 ± 9.3% for CP and IF, respectively. Also reported are the removal efficiencies of both compounds from wastewater using hydrodynamic cavitation (HC), ozonation (O_3) and/or UV, either individually or in combination with hydrogen peroxide (H_2O_2). Hydrodynamic cavitation did not remove CP and IF to any significant degree. The highest removal efficiencies: 99 ± 0.71% for CP and 94 ± 2.4% for IF, were achieved using $UV/O_3/H_2O_2$ at 5 g L^{-1} for 120 min. When combined with biological treatment, removal efficiencies were >99% for both compounds. This is the first report of combined biological and AOP treatment of CP and IF from wastewater with a removal efficiency >99%.

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1. Introduction

According to the International Agency for Research on Cancer (IARC), there are an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths per year worldwide, which makes cancer the second leading cause of death (IARC, 2013). Amongst the many pharmaceuticals used to treat cancer, cyclophosphamide (CP) and ifosfamide (IF) are two of the oldest and widely prescribed alkylating cytostatic medicines. The current trend in chemotherapy is towards the non-hospitalization of patients i.e., only receiving the chemotherapy

Abbreviations: AOP, advanced oxidation process; COD, chemical oxygen demand; CP, cyclophosphamide; DO, dissolved oxygen; DOC, dissolved organic carbon; GC–MS, gas chromatography coupled to mass spectrometry; HC, hydrodynamic cavitation; HRT, hydraulic retention time; H_2O_2, hydrogen peroxide; IARC, International Agency for Research on Cancer; IF, ifosfamide; NH_4–N, ammonia ion; NO_3–N, nitrate ion; O_3, ozone; OECD, Organisation for Economic Co-operation and Development; SPE, solid-phase extraction; TFAA, trifluoroacetic acid; UV, ultraviolet (irradiation); WHO, World Health Organisation; WWTP, wastewater treatment plant; ε, molar extinction coefficient.

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at the hospital, although hospitals, where chemotherapies are administered daily, remain a significant source of anticancer drug residues. The levels of CP and IF have been reported in hospital wastewaters from the current limits of detection up to 15 μg L^{-1} and 11 μg L^{-1} for CP and IF, respectively (Ferrando-Climent et al., 2013; Gómez-Canela et al., 2012; Kim et al., 2009a; Kümmerer et al., 1997; Negreira et al., 2013, 2014a, 2014b; Steger-Hartmann et al., 1996, 1997; Yin et al., 2010). Hospital wastewaters are usually untreated and discharged directly into the sewerage system, where they eventually arrive at a wastewater treatment facility (Zhang et al., 2013). The concern is that if not completely removed, residues of CP and IF, which are known to have cytotoxic, mutagenic, teratogenic and genotoxic properties, could have adverse effects on aquatic organisms.

Various biological treatment technologies have been applied to reduce the presence of CP and IF in wastewater. Literature review reveals inconsistent removal efficiencies for CP and IF (0–72%) by biological treatment (Buerge et al., 2006; Delgado et al., 2011; Kiffmeyer et al., 1998; Kovalova et al., 2012; Köhler et al., 2012; Kümmerer et al., 1997, 2000; Steger-Hartmann et al., 1997). Amongst them only Kovalova et al. (2012), Köhler et al. (2012) and Delgado et al. (2011) used a non-conventional biological treatment by applying membrane bioreactors. Kovalova et al. (2012) and Köhler et al. (2012), who used hospital wastewater as a matrix, were able to remove 20% and 12% of CP, respectively, while Delgado et al. (2011) removed 80% of CP from an artificial wastewater. To our knowledge there have been no published studies of a non-conventional biological treatment to remove IF. A removal efficiency of 80% for CP using membrane bioreactors suggests that attached growth biomass could be a promising treatment technology for the removal of CP and IF.

The results of several abiotic treatment studies using UV, UV and hydrogen peroxide (UV/H_2O_2), ozone (O_3) and O_3/H_2O_2 to remove CP and studies using UV, UV/H_2O_2 and O_3 to remove IF have been published (Chen et al., 2008; Fernández et al., 2010; Garcia-Ac et al., 2010; Kim and Tanaka, 2009; Kim et al., 2009a, 2009b; Köhler et al., 2012; Lester et al., 2011; Lin et al., 2014, 2015; Lutterbeck et al., 2015; Wols et al., 2013, 2015). The removal of CP and IF by either UV or O_3 alone was disappointing, but by adding H_2O_2 removal efficiency was substantially improved (up to 90%). With the exception of Kim et al. (2009a), Köhler et al. (2012) and Lin et al. (2015) who used UV, UV/H_2O_2 or O_3 to remove CP and/or IF from a wastewater, others used more simple aqueous matrices like deionised and drinking water. In addition, because CP and IF are only partially removed by existing abiotic wastewater treatment technologies, alternative technologies must be investigated. An example is hydrodynamic cavitation (HC), for which promising results for removing pharmaceutical residues from a wastewater have been reported (Zupanc et al., 2014).

The aims of this study were to evaluate for the first time the occurrence of CP and IF in Slovene wastewaters and to investigate their removal from wastewater using biological treatment based on attached-growth biomass, abiotic treatments (HC, UV and/or O_3 with varying initial concentrations of H_2O_2) and combined biological and selected abiotic treatment.

2. Experimental

2.1. Standards, reagents and chemicals

Cyclophosphamide (99%, CAS 50-18-0) and IF (99%, CAS 3778-73-2) were purchased from Sigma Aldrich (Hong Kong, China). The surrogate standard deuterated cyclophosphamide (CP-d_6) was obtained from Niomech — IIT GmbH (Bielefeld, Germany), the derivatizing agent trifluoroacetic anhydride (TFAA, 99%, CAS 407-25-0) was purchased from Fluka (Buchs, Switzerland) and the solvents acetonitrile, ethyl acetate and methanol were purchased from J. T. Baker (Deventer, Netherlands), which were all of analytical grade purity. Hydrogen peroxide (30%; CAS 7722-84-1) was purchased from AppliChem (Darmstadt, Germany). The composition of the artificial wastewater influent used for biological and abiotic treatments is provided in SM-I and described in detail by Kosjek et al. (2007).

2.2. Sample preparation, chemical analysis and method validation

Sample preparation included solid-phase extraction (SPE) and derivatization of previously published methods (Kosjek and Heath, 2011). Briefly, 100 mL of hospital wastewater sample and samples from laboratory experiments and 200 mL of wastewater treatment plant (WWTP) influent and effluent sample were filtered through glass microfiber filters (minimal pore size 0.5 μm, Machery Nagel, Dueren, Germany) to remove solids and through cellulose-nitrate filters (0.45 μm; Sartorius Stedim Biotech, Göttingen, Germany) to remove bacteria. The samples were then pre-concentrated using Oasis HLB (60 mg, 3 cm^3) cartridges (Waters, Massachusetts, USA). Each cartridge was conditioned with 3 mL of ethyl acetate, 3 mL of methanol and equilibrated with 3 mL of water. The samples were then extracted at a flow rate of 3 mL min^{-1}. The sorbents were dried under vacuum before being eluted with 3 mL of ethyl acetate (3 × 1 mL). The remaining organic solvent was removed under a gentle stream of N_2. Finally, 100 μL of TFAA and 100 μL of ethyl acetate were added to the dried extract and left to derivatize for 1 h at 60 °C. The samples were then dried (with N_2) to remove any trifluoroacetic acid formed during derivatization and re-dissolved in 250 μL of ethyl acetate prior to analysis by GC–MS.

The samples were analysed using a HP 6890 series (Hewlett-Packard, Waldbron, Germany) gas chromatograph with a single quadrupole mass selective detector. The capillary column was a DB-5 MS 30 m × 0.25 mm × 0.25 μm (Agilent J&W, CA, US). The carrier gas was He set at a flow rate of 1 mL min^{-1}. One microlitre of sample extract was injected in splitless mode with the injector temperature set to 270 °C. The oven temperature programme was as follows: an initial temperature of 65 °C was ramped at 30 °C min^{-1} to 300 °C, where it was held for 2 min. Total runtime was 9.83 min. The MS was operated in electron impact (EI) mode with an ionization voltage of 70 eV. Selective ion monitoring (SIM) was used for qualitative and quantitative determination by monitoring the following ions: m/z 309, 307 and 150 for CP, m/z 307, 181 and 150 for IF and m/z 313 and 315 for CP-d_6. Instrumental control and data processing were performed using ChemStation software.

The analytical method was validated by determining the following parameters: SPE efficiency, linearity, instrumental and method repeatability, accuracy and sensitivity (LOD and LOQ). Full details are given in SM-II.

2.3. Collection of hospital wastewater and wastewater from WWTPs

Wastewater samples were collected from five Slovene hospitals, labelled as A, B, C, D and E in Table 1, where CP and/or IF are regularly administered to cancer patients. In addition, where possible corresponding WWTP influents and effluents were sampled (hospitals A, B, C and D). Samples were collected by grab and/or 24-h time proportional sampling (Table 1). Hospitals A and B are connected to the same WWTP, while hospital C is connected directly to a WWTP, hence, the hospital C sample corresponds to the WWTP influent. For hospital E, where the wastewater is untreated, only hospital wastewater was collected. Water treatment technologies included mechanical and conventional biological treatment with suspended biomass in case of the WWTP receiving wastewater from hospitals A and B (360,000 PE) and the WWTP receiving wastewater from hospital C (800 PE). The WWTP, which received wastewater from hospital D used mechanical and biological treatment with membrane bioreactors (attached growth biomass on filters; 55,000 PE). Further information about each hospital and WWTP is given in SM-I. All samples were immediately transferred on ice to the laboratory, filtered and stored at −20 °C prior to analysis.

Table 1
The average concentrations of CP and IF and DOC values in Slovene wastewater samples. All analyses were performed in duplicate.

Sample	Sampling date and time	Type of sample	CP (ng L^{-1})	IF (ng L^{-1})	DOC (mg L^{-1})
A: Hospital wastewater	15.4.2013 at 10.00	Grab	750 ± 28	2000 ± 140	n.a.
	5.12.2013 at 11.00	Grab	15 ± 0.4	<LOD	360
	16.1.2014 at 8.30	Grab	1100 ± 200	48 ± 10	350
	9.5.2014 at 10.00	Grab	5300 ± 33	6800 ± 0.6	n.a.
	4.6.2014 at 11.00	Grab	22,000 ± 760	<LOD	n.a.
B: Hospital wastewater	21.11.2013 at 9.30	Grab	130 ± 17	<LOD	200
	21.11.2013	24-h time proportional	130	<LOD	n.a.
A and B: Corresponding WWTP influent	9.12.2013	24-h time proportional	<LOD	<LOD	n.a.
	29.1.2014*	24-h time proportional	27 ± 7.3	<LOD	n.a.
	2.6.2014	24-h time proportional	19 ± 3	<LOD	n.a.
A and B: Corresponding WWTP effluent	10.12.2013	24-h time proportional	<LOD	<LOD	n.a.
	3.6.2014	24-h time proportional	17 ± 5.0	<LOD	n.a.
C: Hospital wastewater/WWTP influent	28.11.2013 at 9.30	Grab	<LOD	<LOD	120
C: Corresponding WWTP effluent	28.11.2013 at 11.00	Grab	<LOD	<LOD	n.a.
D: Hospital wastewater	14.11.2013 at 11.00	Grab	<LOD	<LOD	260
D: Corresponding WWTP influent	14.11.2013	24-h time proportional	<LOD	<LOD	n.a.
D: Corresponding WWTP effluent	15.11.2013	24-h time proportional	<LOD	<LOD	n.a.
E: Hospital wastewater	7.11.2013 at 12.00	Grab	<LOD	<LOD	140

* WWTP effluent was not sampled within this sampling campaign, i.e. on 30.1.2014.

Cyclophosphamide and IF are stable in wastewater samples for up to 3 months if filtered and stored at −20 °C (Negreira et al., 2014b) but despite this all analyses were performed within 2 weeks after sampling. Dissolved organic carbon (DOC) was determined immediately after sampling (Table 1). The procedure is described in SM-II.

2.4. Biological treatment

Experiments were performed in aerated glass bioreactors (4 L) with attached growth biomass on Mutag Biochip™ carriers (Multi Umwelttechnologie AG, Sachsen, Germany). The carriers, made of polyethylene (effective specific surface area of 3000 $m^2\ m^{-3}$), occupied 5% of the bioreactor volume. The total surface area was 150 $m^2\ m^{-3}$. Aeration was achieved using an aquarium pump (Airfizz 259 200, Ferplast, Castelgomberto, Italy, 100 L h^{-1}) fitted with a porous stone aerator and the reactor contents were continuously mixed using a magnetic stirrer (Rotamix SHP 10, Domel, Železniki, Slovenia). The flow through experimental set-up is shown in SM-III. The bioreactors were initially inoculated with 50 mL of biomass from a municipal WWTP and then fed with fresh artificial wastewater (2 L day^{-1}). The biomass incubation period lasted for 3 months, after which a mixture of CP and IF (10 μg L^{-1}) was added continuously to each bioreactor (M1 and M2). A third bioreactor served as the control (M0), in which CP and IF were not added. Reactor M0 was treated under the same conditions as M1 and M2. The hydraulic retention time (HRT) was maintained using a peristaltic dosing pump (Blackstone BL 3, Hanna Instruments, Michigan, USA). The HRT was 48 h to achieve a sufficiently long contact time of CP and IF with the attached-growth biomass. After 1 month of adaptation, a 4-month sampling campaign was performed ($n_{sampling} = 7$). Each sample was filtered, stored at −20 °C and analysed within 2 weeks. The analysis of wastewater parameters was performed within 2 h after sampling. The removal efficiency was calculated as the difference between the influent and effluent concentration divided by the influent concentration (Zupanc et al., 2013). To evaluate removal efficiency from a hospital wastewater, M2 was fed with fresh untreated filtered effluent collected from hospital A.

Nitrification was confirmed by monitoring the NH_4–N and NO_3–N values, while DOC and chemical oxygen demand (COD) were measured to assess the performance of the bioreactors. In addition, dissolved oxygen (DO), T and pH in the bioreactors were determined weekly. All the procedures and results are in the SM-II. The biomass concentration was determined prior to adding CP and IF and both during and at the end of the experiment. The procedure for biomass concentration determination is described in Zupanc et al. (2013).

2.5. Abiotic treatments

2.5.1. UV, UV/H_2O_2, O_3, O_3/H_2O_2 and UV/O_3/H_2O_2 experiments

A laboratory scale water treatment plant, designed at the Institute of Sanitary Engineering (Faculty of Civil and Geodetic Engineering, University of Ljubljana, Ljubljana, Slovenia), was used to evaluate the removal efficiency of the following wastewater treatments: UV, O_3 and AOPs, namely UV/O_3, UV/H_2O_2, O_3/H_2O_2 and UV/O_3/H_2O_2. The experimental design included using different initial concentrations of H_2O_2 (0–5 g L^{-1}) at a fixed treatment time (120 min), whereas for the UV, O_3, UV/O_3 and UV/O_3/H_2O_2 kinetic studies, different sample treatment times were used (Table 2). The apparatus consisted of a polyethylene reactor vessel, a water pump (Iwaki Magnet Pump, Iwaki co. LTD), an air pump (KNF Neuberger, Trenton, US), an air rotameter (Omega Engineering, Conneticut, US), an air dryer module (Lufttrockner module LTM 110–60, AquaCare, Gladbeck, Germany), an O_3 generator (BasiTech III, AquaCare, 500 mg h^{-1}, Gladbeck, Germany), an O_3 reactor (O_3 Reactor OZR 75, AquaCare, Gladbeck, Germany), an O_3 analyser (BMT 964 C, BMT Messetechnik, Maaßstraße, Heidelberg), a water rotameter (GEMÜ 55/21/14, Georgia, US) and an UV lamp (Sterilight copper, Viqua, 12W, Vista, US). The system was controlled using the MicroCleaningPlant computer software (Echo, Slovenske Konjice, Slovenia). Fig. 1 shows a scheme of the experimental set-up, while the actual laboratory scale plant is shown in SM-IV.

Ozone production (500 mg h^{-1}) was achieved by introducing dried ambient air at a flow rate of 0.8 L h^{-1} in an O_3 generator. The O_3 containing gas then passed into the O_3 reactor through a frit. The O_3 concentration was measured using an O_3 analyser (10 mg L^{-1}). The gas and water flow rates served to regulate O_3 dosage, which was approximately 36 mg O_3/g DOC. Irradiation by UV was achieved using a flow through

Table 2
Experimental conditions of the investigated abiotic treatments.

Process	H_2O_2 (g L^{-1})	Time (min)
UV	0	40/80/120
	2.5	120
	5	120
O_3	0	20/40/60/80/120
	2.5	120
	5	120
UV/O_3	0	20/40/60/80/120
	2.5	120
	5	20/40/60/80/120

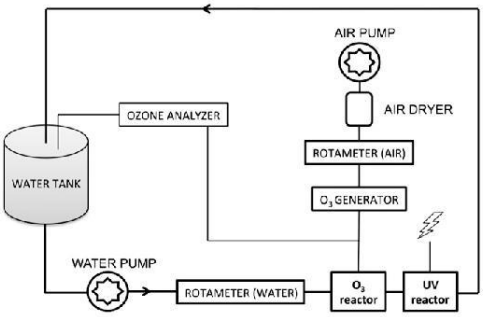


Fig. 1. Schematic of the laboratory scale treatment plant.

low pressure (LP) copper UV lamp (90% irradiation at 254 nm, UV dose 44 mJ cm^{-2} at flow rate 60 L h^{-1}).

The artificial wastewater was filtered through a glass microfiber filter and spiked with 10 μg L^{-1} of either CP or IF. In the case of hospital wastewater, the sample was only filtered since initial analysis revealed measurable quantities of CP and IF. Six litres of sample was then added to the water tank and a subsample (3 L) was used to flush the system. In total 4.8 L of spiked wastewater sample (3.0 L in water tank and 1.8 L in the system) was circulated through the treatment plant (60 L h^{-1}). All experiments were duplicated and the samples were analysed as described in the methodology. The removal efficiency was calculated according to Section 2.4. The DOC values were analysed only for the optimal treatment of artificial and hospital wastewater.

The determination of direct and indirect rate constants, i.e. hydroxyl radical (•OH radical) rate constants for UV and/or O_3 processes was omitted from this study because of the way the equipment was designed (SM-IV) and since only highly complex matrices, i.e. untreated artificial and hospital wastewater were used. Furthermore, the sample treatment time (20–120 min) refers to the time the sample circulates through the system, which is greater than the time the sample is actually exposed to UV and/or O_3. Therefore, taking into account the sample volume (4.8 L), UV lamp and ozone reactors volumes (1.1 L and 1 L, respectively) and sample flow rate (60 L h^{-1}), 20 min of sample treatment corresponds to 2.5 min of exposure to either UV and/or O_3.

2.5.2. Experiments with HC

Cyclophosphamide and IF removal efficiency from artificial wastewater by HC was investigated using two different designs of cavitator: pulsating and shear-induced, both developed at the Faculty of Mechanical Engineering, University of Ljubljana, Ljubljana, Slovenia. All the experiments were carried out in triplicate. The removal efficiency was determined as described in Section 2.4.

The pulsating HC equipment consisted of two reservoirs, connected by a symmetrical Venturi pipe with a 1 mm high and 5 mm wide constriction to create the right conditions for cavitation. The sample was cycled from one reservoir to the other by applying pressure (100 kPa). A scheme of the equipment is given in SM-Va and the details are described elsewhere (Zupanc et al., 2013). Briefly, 1 L of wastewater sample spiked with CP and IF (10 μg L^{-1}) was introduced into the system and the experiment lasted for 15 min, 60 min and 120 min, which is equivalent to 50, 200 and 400 cycles. The experiments were performed both with (3.4 g L^{-1} and 6.8 g L^{-1}) and without the addition of H_2O_2.

In the shear-induced cavitation chamber (SM-Vb), a series of valves were used to regulate sample flow. The static pressure inside the chamber was 100 kPa. A detailed description of the set-up is described elsewhere (Petkovšek et al., 2013; Zupanc et al., 2014). Briefly, 2.5 L of wastewater sample (CP and IF spiked at 10 μg L^{-1}) was introduced into the system. A series of 20 min experiments were performed at both fixed (21 ± 1 °C) and increasing temperatures (20–68 °C). During Experiments 2, 3 and 5 sample temperature rose due to dissipated hydraulic energy as heat (Table 3), while in Exp. 1 and 4 the temperature was maintained at 21 ± 1 °C. Experiments were also performed with and without H_2O_2 (Table 3). Finally, a series of control experiments (Exp. 6, 7 and 8) were performed without cavitation; cooling was not necessary since temperature increases only when the rotors are operating. The experimental set-up is presented in Table 3.

2.6. Data analysis and statistical evaluation

Data analysis and statistical evaluation were performed using Microsoft Excel 2010 and MedCalc (14.8.1.) software. An independent Student t-test ($\alpha = 0.05$) was performed to examine if there were any significant differences in the removal efficiencies of CP and IF between M1 and M2 during biological treatment.

3. Results and discussion

3.1. Determination of CP and IF concentrations and DOC values in wastewaters

Table 1 gives CP and IF concentrations and the DOC values for various wastewater samples (10 hospital wastewaters, 4 WWTP influents and 4 WWTP effluents). Cyclophosphamide was detected in 10 and IF in 3 out of the 18 analysed samples. Hospital wastewaters contained both compounds in concentrations from below the LODs to 22,000 and to 6800 ng L^{-1} for CP and IF, respectively. The concentration of CP (22,000 ng L^{-1}) detected in wastewater from hospital A is the highest concentration so far reported (Kosjek and Heath, 2011; Zhang et al., 2013). The variability in the CP and IF concentrations in hospital wastewaters could be attributed to the size of the hospital, the number of patients receiving chemotherapy on the day of sampling and sampling time (different days and times of sampling). In hospitals C, D and E IF was not administered. Cyclophosphamide was detected in two WWTP influents and one effluent in ng L^{-1} levels, while IF could not be detected in any of the WWTP influent or effluent samples. A comparison of CP concentrations in WWTP influent (19 ng L^{-1}) and effluent (17 ng L^{-1}) samples taken on 2.6.2014 and on 3.6.2014 (in case of WWTP, which receives wastewater from hospitals A and B), suggests poor (if any) removal by conventional biological treatment. The average DOC value for 6 analysed hospital wastewater samples was 240 ± 100 mg L^{-1} (Table 1), which is comparable to the average DOC value of a non-treated artificial wastewater used in the biological and abiotic treatment experiments (280 ± 14 mg L^{-1}).

3.2. Biological treatment of artificial wastewater

The performance of M0, M1 and M2 was assessed by measuring T, DO, pH, NH_4–N, NO_3–N, COD, DOC and biomass concentration. In brief, pH, T and DO were constant and similar in all three bioreactors, the average NH_4–N and COD values in effluents values were in agreement with Slovene guidelines on the quality of WWTP discharge (2012). Nitrification and a satisfactory removal of DOC (over 90%) were observed

Table 3
Experimental design for HC experiments (t = 20 min).

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8
H_2O_2 (g L^{-1})	0	0	3.4	6.8	6.8	0	3.4	6.8
T (°C)	21 ± 1	20–68	20–68	21 ± 1	20–68	21 ± 1	21 ± 1	21 ± 1
Cavitation	yes	yes	yes	yes	yes	no	no	no

in all three bioreactors. The addition of CP and IF to M1 and M2 did not affect the biomass concentration. For detailed results see SM-II.

The low k_{ow} values of CP (0.63) and IF (0.84) mean that CP and IF are highly soluble in water and unlikely to be adsorbed on material surfaces (Llewellyn et al., 2011; Lutterbeck et al., 2015). In addition, according to the literature sorption of CP and IF onto sludge is negligible (Kosjek and Heath, 2011). Regardless, the adsorption of the investigated compounds on glass, carriers, tubes and the porous aeration stones was measured. This was assessed using a control reactor spiked with CP and IF in tap water without biomass. The contents of the reactor were sampled after 12 h, 24 h, 72 h, 96 h and 168 h. After 168 h the concentration of CP and IF had not changed proving that both compounds remain in solution and that their removal efficiency can be attributed to interaction with the biomass.

The removal efficiency of CP and IF during the adaptation period (one month) did not differ significantly from the removal efficiency during the following sampling campaign (data not shown). An independent Student t-test ($\alpha = 0.05$) revealed that differences in removal efficiency in M1 compared to M2 were not statistically significant for either compound. Therefore, the results in Table 4 are presented as the average removal efficiencies of CP and IF in M1 and M2 during 4 months and 7 samplings. The overall average removal efficiency was 42 ± 12% for CP and 18 ± 11% for IF (Table 4). The removal efficiency for CP from an artificial wastewater (80% at 5 μg L^{-1}) using membrane bioreactors reported by Delgado et al. (2010) is approximately twice that obtained using attached biomass, suggesting that their technology represents a better alternative than that used within this study. Only Buerge et al. (2006) conducted a biodegradation study with suspended biomass in artificial wastewater, where IF was spiked at environmentally relevant concentrations (100 and 1000 ng L^{-1}). The authors observed no degradation even after 24 h, whereas the average removal efficiency for IF in our study was 18%.

3.3. Biological treatment of hospital wastewater

To study matrix effects on removal efficiency, a series of experiments using an untreated hospital wastewater (hospital A) were performed. Chemical analysis revealed the presence of CP and IF at 5300 ng L^{-1} and 6800 ng L^{-1}, respectively (Table 1). In addition, the following standard wastewater parameters before and after biological treatment were determined: NH_4–N, NO_3–N, COD and DOC. A decline in the amounts of NH_4–N, COD and DOC was observed, whereas NO_3–N increased, which confirmed that nitrification occurred and that bioreactor performance was adequate when hospital wastewater was introduced into the system (SM-II). The average removal efficiency for hospital wastewater was higher (59 ± 15% for CP and 35 ± 9.3% for IF) than for artificial wastewater (42 ± 12% for CP and 18 ± 11%; Table 4), however these values cannot be compared directly due to the different compositions of artificial wastewater and hospital wastewater. The latter can contain organic matter that may affect the biomass, e.g. other pharmaceuticals and nutrients, which can have an unknown impact on a non-adapted biomass. The removal efficiency of CP from a hospital wastewater (Section 3.2), was on average 3-times higher than that reported by Kovalova et al. (2012), who used a membrane bioreactor system (initial concentration and removal efficiency for CP were 160 ng L^{-1} and 20%, respectively). This suggests that both, experimental design and wastewater composition can affect removal efficiency. Finally, to our knowledge, the removal efficiency for IF obtained within this experiment (35%) is the highest reported for biological treatment of a wastewater containing IF at environmentally relevant concentration.

Table 4
Average removal efficiency of CP and IF during 4-months of biological treatment (M1 and M2).

	CP (%)	IF (%)
1	51 ± 14	26 ± 10
2	22 ± 0.92	7.5 ± 0.61
3	52 ± 3.0	25 ± 2.7
4	35 ± 4.9	36 ± 0.75
5	36 ± 5.1	7.3 ± 2.6
6	46 ± 9.9	7.0 ± 2.8
7	48 ± 2.1	19 ± 0.72
Average	42 ± 12	18 ± 11

3.4. Abiotic treatment of artificial wastewater

3.4.1. UV and/or O_3

The removal efficiency for CP and IF during UV, O_3 and UV/O_3 treatments was investigated at different treatment times (20–120 min). Direct photolysis was not efficient in degrading either CP or IF, which was expected since the chemical structures of CP and IF do not contain any aromatic rings or double C = C bonds, which absorb photons under UV irradiation. The molar extinction coefficient (ε) for CP, reported by Kim and Tanaka (2009), is 9 M^{-1} cm^{-1}, suggesting that CP does not absorb at 254 nm. Furthermore, it was not possible to determine ε for IF, since IF does not absorb light at 254 nm (SM-VI).

Under pseudo first order conditions, the CP and IF degradation can be described by the following Eq. (1):

$$\ln \frac{c(t)}{c(0)} = -k_t \times t \quad (1)$$

where k_t is the first-order rate constant including both direct and indirect oxidation of the investigated compounds (Rosenfeldt and Linden, 2007). The k_t values for CP and IF were 2.7×10^{-5} s^{-1} and 2.4×10^{-5} s^{-1}, respectively. The k_t value for CP is in the same order of magnitude as reported by Kim and Tanaka (2009) in ultrapure water (k_t for CP = 8.3×10^{-5} s^{-1}). This similarity in k_t values suggests that degradation occurs mainly *via* direct photolysis. However, after 120 min of UV treatment only 21% of CP and 16% of IF was removed (Fig. 2).

To increase the removal efficiency for CP and IF, ozonation was trialled as an oxidative treatment. As shown in Fig. 2, after 120 min the removal efficiency for CP and IF improved, with 42% of CP and 36% of IF being removed. It was already demonstrated that direct ozonation, i.e. the absence of •OH radicals, is not efficient in removing CP with a kinetic rate constant of 3.3 ± 0.2 and 2.9 ± 0.3 M^{-1} s^{-1} in ultrapure water and in natural water, respectively (Garcia-Ac et al., 2010; Lin et al., 2015). However, CP and IF can be degraded by •OH radicals, which are secondary oxidants formed during ozone degradation (von Gunten and von Sonntag, 2012). Interestingly, Garcia-Ac et al. (2010) found that when ozonation was performed in natural water without quenching the •OH radicals, removal improved significantly, proving that •OH radicals play a major role in CP removal. In addition, hydroxyl radical formation may be enhanced by complex reactions of O_3 with dissolved organic matter present in the investigated matrix, i.e. with compounds that promote •OH radical formation (Von Gunten and Acero, 2001). The direct ozonation constant for CP and IF was not determined within our study, instead only k_t values during O_3 without any radical scavenger added were determined. The higher k_t values obtained for O_3 (CP = 7.6×10^{-5} s^{-1}; IF = 6.1×10^{-5} s^{-1}) compared to UV confirmed the higher rate of removal for both compounds by ozonation.

In an attempt to increase the formation of •OH radicals, samples were simultaneously treated with UV and O_3. The UV/O_3 treatment is referred to as an AOP since it enhances the formation of •OH radicals (Legrini et al., 1993). The removal efficiency for CP and IF during UV/O_3 increased to 59% and 49% after 120 min of treatment, respectively (Fig. 2). Furthermore, k_t values were slightly higher compared to UV or O_3 (CP = 1.2×10^{-4} s^{-1}; IF = 1.2×10^{-4} s^{-1}). It is interesting that the sum of CP or IF removal efficiency by UV and O_3 is comparable to combined UV/O_3 treatment (Fig. 3). It was already demonstrated that the quantum yield of •OH radical formation from ozone was low, only

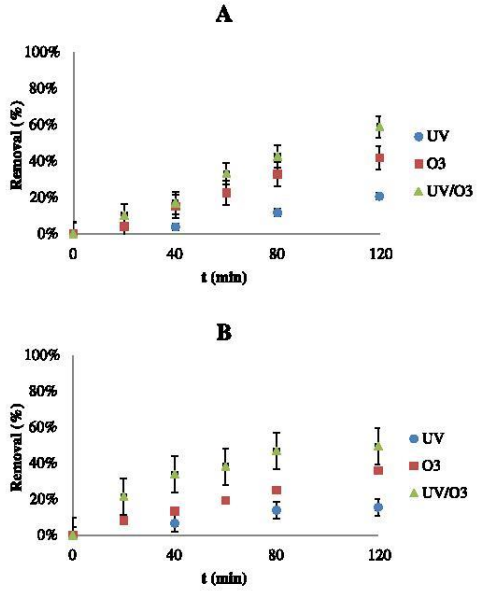


Fig. 2. Cyclophosphamide (A) and IF (B) removal during UV (●), O_3 (■) and UV/O_3 (▲) treatments (20–120 min).

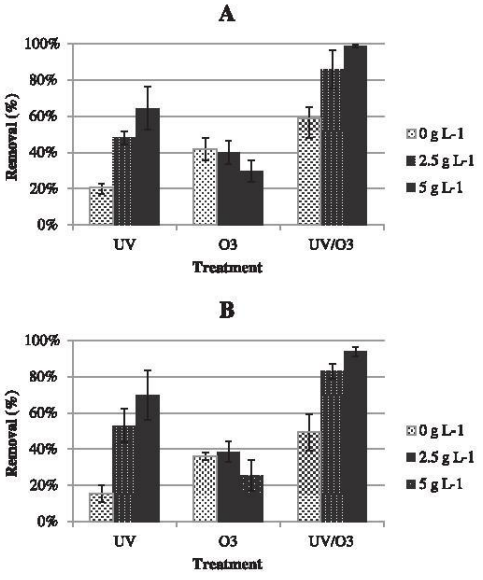


Fig. 3. The average CP (A) and IF (B) removal efficiency ± SD after 120 min treatment by UV, O_3 and UV/O_3 with varying H_2O_2 concentrations (0, 2.5 and 5 g L^{-1}).

0.1 (Reisz et al., 2003). In our equipment the UV lamp sequentially follows the O_3 reactor (Fig. 1) and since it was not possible to measure the O_3 concentration in the UV reactor, it was not possible to determine the residual amount of ozone. It was hypothesized that the photolysis of the residual ozone did not increase the formation of •OH radicals to a significant level, which would result in improved CP and IF removal and this treatment should, therefore, be considered as cumulative and not as an AOP.

Since no significant enhancement in removal efficiency was observed using UV/O_3, another source of •OH radicals i.e. H_2O_2 was trialled. Experiments were also performed where H_2O_2 was added at two concentrations: 2.5 and 5 g L^{-1}, during UV, O_3 and UV/O_3 treatment, since •OH radicals can be formed under the stated treatments (Klavarioti et al., 2009).

Results after 120 min of UV/H_2O_2 at 2.5 g L^{-1} showed 48% and 53% removal of CP and IF, respectively, which confirmed that •OH radicals were the main oxidant involved in their removal (Fig. 3). Several studies carried out in less complex matrices (i.e. miliQ water, river water and treated wastewater) also report higher removal efficiencies when UV/H_2O_2 was applied (Kim et al., 2009a; Köhler et al., 2012; Wols et al., 2013). Their results, however, are not comparable with ours due to the different amounts of H_2O_2 applied, times of UV irradiation/UV doses and the type of aqueous matrix. In addition, CP and IF removal was improved by increasing H_2O_2 concentration, resulting in removal efficiencies of 65% and 70% for CP and IF, respectively, when 5 g L^{-1} of H_2O_2 was used (Fig. 3). This suggests that low or no •OH radical scavenging occurred within the applied H_2O_2 concentrations during UV/H_2O_2 treatment.

Alternatively, the addition of H_2O_2 during ozonation did not improve either CP or IF removal efficiency. Instead, it remained either similar or decreased with increasing H_2O_2 concentration (Fig. 3). This is not in agreement with the literature concerning CP removal with O_3/H_2O_2 (Garcia-Ac et al., 2010). Again, the trend is not directly comparable because of the different H_2O_2 concentrations used (resulting in different molar ratios between O_3 and H_2O_2) and matrix complexity. A similar behaviour was observed in a study by von Gunten and Acero (2001), where O_3 and O_3/H_2O_2 treatment of surface water containing high amounts of natural organic matter was performed. Von Gunten and Acero (2001) demonstrated that the yield of •OH radical formation from O_3 or O_3/H_2O_2 was similar for surface water with a DOC concentration of 3.2 mg L^{-1}. Despite the higher DOC concentration (280 mg L^{-1}) of the wastewater used in our study, a similar behaviour was observed. The removal efficiency was similar for both O_3 and O_3/H_2O_2 treatments with 2.5 g L^{-1} of added H_2O_2 (CP: 42% and 40% and IF: 36% and 39% for O_3 and O_3/H_2O_2, respectively; Fig. 3). In the experiments where the highest amount of H_2O_2 was used, removal efficiencies were even lower, resulting in 30% and 26% removal for CP and IF, respectively (Fig. 3). This is a possible result of competition between organic compounds present in the aqueous matrix and the high concentration of H_2O_2 available to react with •OH radicals. In this case, the •OH radicals formed through ozone decomposition react mainly with the concentrated H_2O_2. The same phenomenon was reported by Glaze et al. (1987), where it was shown that high amounts of H_2O_2 result in the scavenging of •OH radicals during O_3/H_2O_2 treatment (i.e. when mass ratio H_2O_2: O_3 was >0.8) and as a consequence limiting their ability to oxidize the compounds of interest.

Finally, to further improve the degradation of CP and IF, UV/O_3/H_2O_2 treatment was, for the first time, applied for the degradation of CP and IF with the intent to increase the exposure of CP and IF to •OH radicals. The H_2O_2 concentrations were the same as in the UV/H_2O_2 and O_3/H_2O_2 experiments. The removal efficiency for CP and IF increased significantly with increasing H_2O_2 concentration as in the case of UV/H_2O_2 treatment, resulting in removal efficiencies of 86% and 83% (at 2.5 g L^{-1} H_2O_2) and 99% and 94% (at 5 g L^{-1} H_2O_2) for CP and IF, respectively (Fig. 3). We have already shown that neither UV irradiation nor the addition of H_2O_2 significantly affects the formation of •OH radicals during

ozonation. Therefore, all the H_2O_2 was available for •OH radical formation under UV irradiation in the case of $UV/O_3/H_2O_2$ treatment. In contrast to using ozone and 5 g L^{-1} of H_2O_2, where it was hypothesised that the •OH radicals were directly quenched by H_2O_2, in this case UV irradiation breaks down the H_2O_2 leading to the formation of •OH radicals with a quantum yield of 1 (Yu and Barker, 2003). Therefore, the concentration of H_2O_2 decreased during treatment with $UV/O_3/H_2O_2$ and the competition between DOC and H_2O_2 to react with the •OH radicals became negligible. This agrees with the comparable CP or IF removal efficiencies between the sum of UV/H_2O_2 and O_3/H_2O_2 and combined $UV/O_3/H_2O_2$ treatment at both investigated H_2O_2 concentrations (Fig. 3).

With $UV/O_3/H_2O_2$ treatment (5 g L^{-1} of H_2O_2 and 120 min of sample treatment) it was possible to remove 99% of CP and 94% of IF from a highly complex aqueous matrix, i.e. non-treated wastewater with a high initial DOC value.

3.4.2. Hydrodynamic cavitation

Pulsating HC did not remove either CP or IF. The removal efficiency of shear-induced cavitation under various experimental conditions including controls (Exp. 6, 7 and 8) is presented in Table 5. To evaluate the removal efficiency of compounds due to increasing temperature and in the presence of H_2O_2 a series of additional control experiments in a glass beaker (500 ml) were performed. The samples were stirred for 20 min at 21 ± 1 °C and then heated from 21 to 68 °C, both with and without the addition of H_2O_2. The results revealed no removal of CP and IF under the experimental conditions (data not shown). Given the experimental error, it is clear that shear-induced HC, neither alone nor with the addition of H_2O_2 was able to significantly remove CP or IF (Table 5). Only in Exp. 3 (3.4 g L^{-1} H_2O_2) 14% removal of IF was achieved when taking into account the control (Exp. 7). The lower removal efficiency with higher H_2O_2 concentration (6.8 g L^{-1}) could be attributed to the scavenging effect of excess H_2O_2 during treatment, which would have consumed the •OH radicals (Table 5). To prove this phenomenon further studies are necessary using a suitable •OH radical scavenger, such as tert-butanol.

3.5. Comparative kinetic study of selected AOP ($UV/O_3/H_2O_2$): artificial vs. hospital wastewater

After 120 min of $UV/O_3/H_2O_2$ at 5 g L^{-1} treatment, >94% of CP and IF were removed from the non-treated artificial wastewater. Therefore, a comparative kinetic study of CP and IF removal from artificial and hospital wastewater was performed, where k_t values and DOC removal were determined. The hospital wastewater used was the same as that used for biological treatment. After 120 min of treatment, the DOC in the artificial and hospital wastewater had reduced by only 9% and 22%, respectively, suggesting a low degree of mineralization of the compounds, including CP and IF (SM-VII). The concentrations of CP and IF decreased exponentially with time and followed pseudo-first-order kinetics with similar k_t values for artificial (CP: 6.1×10^{-4} s^{-1}; IF: 4.4×10^{-4} s^{-1}) and hospital wastewater (CP: 6.4×10^{-4} s^{-1}; IF: 5.7×10^{-4} s^{-1}; Fig. 4). A recent study by Lin et al. (2015) reports high removal of DOC, CP and IF from hospital wastewater by O_3 (O_3 concentration was 5 mg L^{-1}). Both compounds were completely removed within 20 min of O_3 treatment and 73% of the DOC had been removed after 120 min. It is clear that the method of Lin et al. (2015) was more successful in terms of CP, IF and DOC removal than $UV/O_3/H_2O_2$ treatment presented in this study. The initial concentrations of CP and IF in their sample were 20 mg L^{-1} and the initial DOC of hospital wastewater was only 18 mg L^{-1}. This DOC value is very low for the non-treated hospital wastewater. Furthermore, it does not agree with the DOC values determined in various Slovene hospital wastewaters, which were on average 13-times higher (Table 1). In addition, the removal efficiency depends on the ratio between the amount of compound and the DOC value of the sample and the type of organic matter present. In a study by Lin et al. (2015), CP and IF concentrations represented approximately one third of the total DOC. This ratio and the different wastewater composition could be a reason for the high removal efficiencies obtained for CP and IF under the milder experimental conditions of Lin et al. (2015) compared to those in the present study.

Table 5
CP and IF removal efficiency (% ± SD, n = 3) from wastewater by shear-induced HC.

	CP removal efficiency (%)	IF removal efficiency (%)
Exp. 1	11 ± 7.6	11 ± 17
Exp. 2	15 ± 5.1	25 ± 13.9
Exp. 3	16 ± 6.9	39 ± 6.5
Exp. 4	12 ± 2.6	21 ± 4.1
Exp. 5	15 ± 8.8	22 ± 24
Exp. 6	5.9 ± 5.3	5.0 ± 15
Exp. 7	3.1 ± 7.3	25 ± 4.8
Exp. 8	10 ± 1.9	26 ± 13

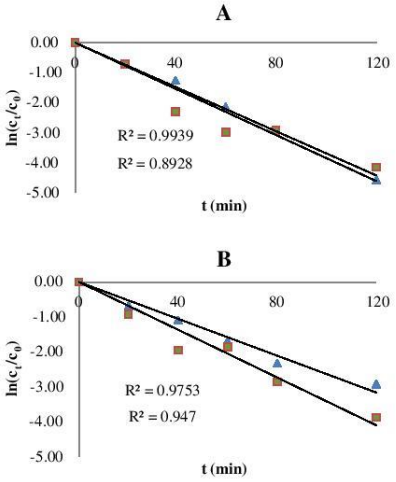


Fig. 4. The removal of CP (A) and IF (B) from artificial (▲) and hospital (■) wastewater during $UV/O_3/H_2O_2$ (CP: A; IF: B).

3.6. Removal efficiency for CP and IF by coupling biological treatment with selected AOP ($UV/O_3/H_2O_2$)

Biological treatment was also coupled to the most efficient AOP: 120 min of $UV/O_3/H_2O_2$ (5 g L^{-1}) and the values of NH_4–N, NO_3–N and DOC were determined at the beginning, after biological treatment and at the end of the experiment. Briefly, NH_4–N and DOC values were reduced and NO_3–N increased after biological treatment confirming nitrification and normal bioreactor performance, whereas no significant differences were observed in the DOC, NH_4–N and NO_3–N after AOP treatment (SM-VIII). Fig. 5 shows the removal efficiencies of CP and IF after biological and abiotic treatment, which resulted 99.7% and 99.8% removal, respectively. Despite a similar amount of compound being removed by AOP alone (99% and 94% for CP and IF, respectively: Fig. 3), it is necessary to emphasize that DOC removal was very high (97%) compared to DOC removal by AOP (9%, SM-VIII) alone. This suggests that the majority of compounds present in the sample were mineralized by combined treatment and only a low amount of transformation products were formed. Only Köhler et al. (2012), who investigated CP removal

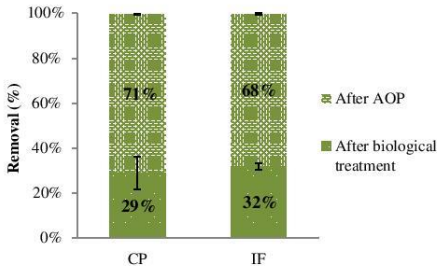


Fig. 5. Average CP and IF removal efficiency ± SD in a coupling experiment: biological treatment followed by AOP: UV/O_3/H_2O_2 at 5 g L^{-1}.

using combined biological treatment and UV/H_2O_2 as an AOP was able to remove between 31% and 77% of CP (initial concentration was 1 μg L^{-1}). To our knowledge, this is the first study that reports an >99% removal of CP and IF from wastewater after coupling biological treatment to a selected AOP (UV/O_3/H_2O_2 at 5 g L^{-1}).

4. Conclusions

Cyclophosphamide and IF were determined in Slovene hospital wastewaters in ng L^{-1} to μg L^{-1} levels and CP was detected also in two WWTP influents and one effluent (ng L^{-1} level). The average removal efficiency for CP and IF during biological treatment with attached biomass on Mutag Biochip™ carriers in flow through bioreactors was lower in artificial wastewater compared to a hospital wastewater. In addition, this study reports the highest IF removal efficiency at environmentally relevant concentrations using this type of biological treatment. Hydrodynamic cavitation did not result in any significant removal of CP and IF and the removal efficiency of the other studied abiotic treatments was dependent on experimental conditions, e.g. the type and duration of treatment and the amount of H_2O_2 added. The average removal efficiency for CP and IF by selected AOP, namely UV/O_3/H_2O_2 (5 g L^{-1} H_2O_2) was higher than HC and biological treatment regardless of the matrix. By coupling biological treatment to UV/O_3 with H_2O_2 a removal efficiency of over 99% for both compounds was achieved, which is the highest removal efficiency so far reported for CP and IF from wastewaters at environmentally relevant concentrations. In addition, a high degree of mineralization of organic matter present in the sample was achieved. These experiments were unrealistic regarding the amount of H_2O_2 and sample treatment time but they were performed using untreated wastewater where the DOC values were comparable to actual DOC values of a hospital wastewater, which represents a first step in the development and implementation of treatments into real world applications, i.e. hospitals. In addition, the investigated compounds are highly recalcitrant to biological degradation and abiotic treatments in comparison to many pharmaceuticals and contaminants, which are expected in hospital wastewaters. This suggests that the majority of pollutants present could be successfully removed under the treatment conditions used within this study.

Future work will include screening for stable transformation products formed during biological and abiotic treatments, especially in the case, where only partial degradation instead of total mineralization was observed (i.e. UV/O_3/H_2O_2 at 5 g L^{-1} treatment). This is important since the transformation products of CP and IF could add to the overall risk posed to the aquatic environment.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.04.109.

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3.2 First Inter-Laboratory Comparison Exercise for the Determination of Anticancer Drugs in Aqueous Samples

The paper “First inter-laboratory comparison exercise for the determination of anticancer drugs in aqueous samples” by E. Heath, M. Česen, N. Negreira, M. López de Alda, L. Ferrando-Climent, L. Bláhová, T. V. Nguyen, M. Adahchour, A. Rübel, N. Llewellyn, J. Ščančar, S. Novaković, V. Mislej, M. Stražar, D. Barceló and T. Kosjek was published in the journal of Environmental Science and Pollution Research (I.F. 2014: 2.828) in June 2015. Prof. Heath and Assist. Prof. Kosjek supervised the work and together we designed the exercise, prepared and shipped the samples. I carried out all the analytical work for CP and IF, data analysis and statistical evaluation as well as drafting the paper. Other co-authors either performed their own analytical work or assisted in the sampling.

Only recently a limited number of laboratories worldwide have begun to analyse cytostatic drug residues in WW. A major problem for such analysis is that there are no CRMs available, therefore, inter-laboratory comparison can be performed to assess the quality of different laboratories results using their own in-house analytical methods (Hund et al., 2000; Yvan Vander Heyden & Smeyers-Verbeke, 2007). A number of inter-laboratory studies addressing emerging contaminants in environmental samples exist in the literature, where only few of them concern APIs, and none have investigated cytostatic drug residues (Dehouck et al., 2003; Farré et al., 2008; Heath et al., 2010; Jones, Betowski, & Lopez-Avila, 1994; Van den Bossche et al., 2010; Y. Vander Heyden et al., 1999).

Laboratories analysing cytostatic compounds in SW and WW were invited to participate in a major inter-laboratory exercise. The aims were to i) promote knowledge exchange, ii) evaluate their performance and iii) elucidate potential errors based on the obtained results. Nine laboratories worldwide confirmed participation. The compounds CP, IF, 5-fluorouracil, gemcitabine, etoposide, methotrexate and cisplatin were selected for analysis. Spiked WW (hospital and WWTP effluent) and SW, and additional non-spiked hospital WW, were distributed to each participating laboratory. Sample preparation included SPE and the use of internal standards. Analyses were performed by either GC-MS or LC-MS spectrometry. Cisplatin was determined using inductively coupled plasma - mass spectrometry (ICP-MS). A required minimum of five participants for statistical analysis were achieved in the case of CP, IF, methotrexate and etoposide, where seven and six participants from different laboratories performed the analysis of CP and IF, respectively. Z-score and Q test revealed 3 outliers using the classical and 4 using robust approach. The smallest absolute differences between the spiked and the measured concentrations were observed in the SW sample. The highest within-laboratory repeatability was observed for one compound, methotrexate, in all matrices (CV $\leq$ 12 %), while overall inter-laboratory reproducibility was poor for all compounds and matrices (CV: 27 – 143 %) with the only exception being methotrexate in the spiked hospital WW (CV: 8 %). The results concerning the analysis for CP and IF revealed adequate repeatability of our analytical method (CP: 1 – 16 % and IF: 1 – 7 %). The overall reproducibility was 29 – 39 % and 27 – 41 % for CP and IF, respectively. Application of the Youden approach to the data revealed three laboratories with total errors for CP and IF and two laboratories with random errors for CP.

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FATE AND EFFECTS OF THE RESIDUES OF ANTICANCER DRUGS IN THE ENVIRONMENT

First inter-laboratory comparison exercise for the determination of anticancer drugs in aqueous samples

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Abstract The results of an inter-laboratory comparison exercise to determine cytostatic anticancer drug residues in surface water, hospital wastewater and wastewater treatment plant effluent are reported. To obtain a critical number of participants, an invitation was sent out to potential laboratories identified to have the necessary knowledge and instrumentation. Nine laboratories worldwide confirmed their participation in the exercise. The compounds selected (based on the extent of use and laboratories capabilities) included cyclophosphamide, ifosfamide, 5-fluorouracil, gemcitabine, etoposide, methotrexate and cisplatinum. Samples of spiked waste (hospital and wastewater treatment plant effluent) and surface water, and additional non-spiked hospital wastewater, were prepared by the organising laboratory (Jožef Stefan Institute) and sent out to each participant partner for analysis. All analytical methods included solid phase extraction (SPE) and the use of surrogate/internal standards for quantification. Chemical analysis was performed using either liquid or gas chromatography mass (MS) or tandem mass (MS/MS) spectrometry. Cisplatinum was determined using inductively coupled plasma mass spectrometry (ICP-MS). A required minimum contribution of five laboratories meant that only cyclophosphamide, ifosfamide, methotrexate and etoposide could be included in the statistical evaluation. *z*-score and *Q* test revealed 3 and 4 outliers using classical and robust approach, respectively. The smallest absolute differences between the spiked values and the measured values were observed in the surface water matrix. The highest within-laboratory repeatability was observed for methotrexate in all three matrices (CV≤12 %). Overall, inter-laboratory reproducibility was poor for all compounds and matrices

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(CV 27–143 %) with the only exception being methotrexate measured in the spiked hospital wastewater (CV=8 %). Random and total errors were identified by means of Youden plots.

Keywords Inter-laboratory study · Cytostatic · Pharmaceutical · Surface water · Wastewater · Hospital effluent

Introduction

The World Cancer Report (2014) published by the World Health Organization (WHO) states that cancer cases are expected to surge 57 % worldwide over the next 20 years, a statistic that is, in the main, attributable to aging global populations (World Cancer Report 2014, IARC, WHO). This is also leading to an increase in the use of chemotherapeutic medicines (CytoThreat Project 2014), which now represents the third largest source of revenue within the pharma industry (Reuters 2008 and Chemoth 2013). These drugs are typically administered to outpatients in oncological units (75 %) using ambulatory infusion, albeit a more recent trend is towards oral administration at home (Kosjek and Heath 2011). After receiving therapy, patients excrete anticancer drugs in both the parent and metabolised forms (Cytotoxic Safety 2013a, b). Excretion rates are drug dependent but, in some cases, can take more than a week, and the compounds can remain in an active stable form for significant periods of time. Illustrative examples are cyclophosphamide and ifosfamide, common anticancer drugs classified as alkylating agents. These two agents under ideal laboratory conditions can remain intact for up to 800 days in the case of cyclophosphamide and 10 to 12 years for ifosfamide, implying that these chemicals could survive under environmental conditions for long periods (Kosjek and Heath 2011). Once excreted from the body, they end up in the sewerage system eventually to arrive at a municipal wastewater treatment plant (WWTP), where studies have demonstrated that they are not completely removed (Kosjek and Heath 2011). Instead, they pass through the treatment process into surface water raising concerns over their presence in the environment and in potable water supplies (Cytotoxic Safety 2013a, b; Crauste-Manciet et al. 2005). This means that although beneficial for the cancer patient, as a consequence of their cytotoxic, genotoxic, mutagenic and/or teratogenic properties cytostatic drug residues may pose a threat to non-target organisms and human health (Cytotoxic Safety 2013a, b).

Until recently, scientists did not have the technology nor the know-how to measure environmental levels of anticancer drugs and only a limited number of laboratories worldwide are analysing them in environmental and wastewater samples (Česen et al. 2015; Ferrando-Climent et al. 2014, 2013; Kosjek and Heath 2011; Kosjek et al. 2015, 2013; Llewellyn et al. 2011; Negreira et al. 2014a, 2013a, b; Parrella et al. 2014). One reason is their low environmental levels (≤ ng L^{-1}), which require the very latest in modern analytical instrumentation, and possibly their toxicity makes laboratories reluctant to analyse these compounds since additional safety protocols and waste collection must be in place. Fortunately, studies are now investigating their presence in environmental and wastewater samples (Česen et al. 2015; Kosjek and Heath 2011; Kosjek et al. 2015, 2013; Negreira et al. 2014a, 2013a, b), and the risks they pose to humans and aquatic organisms have been recently addressed within the EU FP7 CytoThreat project (2014).

In the absence of certified reference materials, inter-laboratory comparisons are necessary to verify analytical results, if laboratories are to have confidence in their analytical abilities. A number of inter-laboratory studies addressing emerging contaminants in environmental samples have been carried out (Farré et al. 2008; Heath et al. 2010a, b; Hund et al. 2000; Jones et al. 1994; Van den Bossche et al. 2010; Vander Heyden and Smeyers-Verbeke 2007), of which only a few address veterinary drug residues (Vander Vander Heyden et al. 1999), nonsteroidal anti-inflammatory drugs and hormones (Farré et al. 2008; Heath et al. 2010a, b) and antibiotics (Dehouck et al. 2003; Van den Bossche et al. 2010). To our knowledge, none included cytostatic drugs. This study was set out to find those laboratories analysing cytostatic drug residues at trace levels in environmental and wastewater samples and to include them in the first inter-laboratory exercise on cytostatic drugs in water to facilitate and promote knowledge exchange between them, evaluate their performance in the analysis of these compounds when using their own in-house analytical methods, and elucidate potential bias and sources of error.

Experimental

Experimental design, sample collection and handling

An invitation to participate in the exercise was offered to those laboratories identified as having the necessary knowledge and instrumentation to determine anticancer residues in environmental and wastewater samples. Participating laboratories were from the following countries: Australia, Czech Republic, Germany, The Netherlands, Singapore, Slovenia, Spain and UK. Each participating laboratory was assigned its own inter-laboratory code (L1 to L9).

Four different samples were included in the exercise: a surface river water spiked with the analytes at a predefined concentration (A), a hospital wastewater (B), the same

hospital wastewater spiked with the target compounds at a known concentration (C) and a spiked municipal WWTP effluent (D). Prior to the exercise, the river water and the WWTP effluent samples had been analysed in the laboratory and found not to contain tested drug residues. These samples were used in the study after spiking them with appropriate environmentally and wastewater relevant concentrations of the target analytes. Analysis of hospital wastewater revealed the presence of several cytostatic residues including CP, IF, 5-FU, GEM, ETO and MTX, but not all those included in this study (cis-Pt). Both samples as collected from the hospital and spiked with the target compounds were included in the trial.

All three matrix samples (the surface water, the WWTP effluent and the hospital effluent) were collected by grab sampling and are actually interrelated since the surface water sample was collected from one of the largest rivers in Slovenia approximately 100 m downstream from the WWTP effluent outflow, and the WWTP receives the effluent wastewater from the hospital. All samples were collected in clean polyethylene containers and transferred on ice immediately to the laboratory, where they were filtered (0.5-μm glass fibre filters) and homogenised.

All the samples, with the exception of sample B, were spiked (Table 1) with the target compounds: cyclophosphamide (CP), ifosfamide (IF), 5-fluorouracil (5-FU), gemcitabine (GEM), etoposide (ETO), methotrexate (MTX) and cisplatinum (Cis-Pt) at environmental and wastewater levels (Kosjek and Heath 2011).

Samples were stored in polyethylene containers at −80 °C. Frozen samples (0.8 L) were shipped on dry ice to each of the participating laboratories. On receipt, the time of arrival and sample condition were recorded. Each laboratory was also asked to process their samples, i.e. filtration and extraction, within 1 week. Finally, the participating laboratories were asked to perform at least two independent analyses per sample. The stability of the compounds CP, IF, 5-FU, GEM, ETO and MTX in HPLC water and in wastewater samples had been studied previously and found to be acceptable under the interlaboratory study conditions (Ferrando-Climent et al. 2013; Negreira et al. 2014b).

Chemicals

The target compounds CP (CAS 50-18-0), IF (CAS 3778-73-2), 5-FU (CAS 51-21-8) and GEM (CAS 95058-81-4) were obtained from Sigma-Aldrich (Steinhem, Germany), MTX (CAS 59-05-2) was obtained from TOCRIS Biosciences (Ellisville, USA) and ETO (CAS 33419-42-0) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Dimethylsulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Luis, USA). Fresh standard solutions of a mixture of the cytostatic compounds were prepared on a weight basis in DMSO.

Analytical methods

Chemical analysis was performed using previously developed analytical methods based on either liquid or gas chromatography (LC or GC) coupled to mass (MS) and most frequently tandem mass (MS/MS) spectrometry (Table 2) (Česen et al. 2015; Ferrando-Climent et al. 2013; Kosjek et al. 2013; Llewellyn et al. 2011; Negreira et al. 2013a, b, 2014b; Odraska et al. 2013; Yin et al. 2010). All analytical methods, with the exception of that based on ICP-MS for Pt determination, included a solid phase extraction (SPE) step, performed either on-line or off-line for sample preconcentration. Internal standards (IS) were used for quantification (Table 2). Laboratory 9, which analysed samples by GC-MS and GC-MS/MS, also included a derivatization step, using trifluoroacetic acid to derivatise CP and IF and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) to derivatise 5-FU.

Statistical parameters

Sample homogeneity was tested for each batch of samples using the chi-square test (Eq. 1):

$$\chi^2 = \sum \frac{(Oi - Ei)^2}{Ei} \quad (1)$$

where *Oi* represents the mean concentration of two parallels of each sample and *Ei* is the mean concentration of each batch containing 10 samples (Heath et al. 2010b). The null hypothesis (H_0) states that homogeneity of samples is achieved, while the alternative hypothesis (H_1) says that homogeneity of samples is not achieved. If χ^2_{tab} (α=0.05) is greater than χ^2_{exp}, then the H_0 is not rejected. To perform the chi-square test, ten random subsamples of each sample (A, C and D) were collected and each sample analysed in duplicate.

Table 1 Spiked matrices and concentrations

Matrix/compound	CP (ng L^{-1})	IF (ng L^{-1})	5FU (ng L^{-1})	GEM (ng L^{-1})	ETO (ng L^{-1})	MTX (ng L^{-1})	Cis-Pt (ng L^{-1})
A: Surface river water	53	33	43	21	52	31	17
C: Hospital wastewater	5239	394	454	397	3420	1141	403
D: WWTP effluent	23	55	66	786	127	432	35

CP cyclophosphamide, *IF* ifosfamide, *5FU* 5-fluorouracil, *GEM* gemcitabine, *ETO* etoposide, *MTX* methotrexate, *Cis-Pt* cisplatinum

Table 2 General information on the applied analytical methods including analytical technique, sample volume, sample pre-treatment and the internal standards used, detection conditions and limits of detection (LOD). A detailed description of the method is in the supplementary material (SM)—Tables S1–S6 and S8–S9 (L7 could not analyse the samples due to their destruction at customs)

Lab code	Instrumentation	Sample volume	SPE	Internal standard (addition before or after extraction)	Detection	LOD[a]
1	LC-MS/MS	5 mL	On-line SPE with PLRP-s (10×2 mm)	IF-d_4 (before)	QTRAP (QqLIT) MS/MS ESI+	IF 0.2 ng L^{-1}
				CP-d_4 (before)		CP 0.1 ng L^{-1}
				ETO-d_3 (before)		ETO 3.0 ng L^{-1}
				GEM-$^{13}C,^{15}N_2$ (before)		GEM 0.3 ng L^{-1}
				MTX-methyl-d_3 (before)		MTX 0.1 ng L^{-1}
2	LC-MS/MS	300 mL	Strata X (200 mg/6 mL)	CP-d_4 (before)	QqQ; MS/MS mode ESI+	CP 0.233 ng L^{-1}
3	LC-MS/MS	250 mL	Double SPE on WAX (6 cc, 150 mg, 30 μm) and HLB (6 cc, 200 mg, 30 μm)	Caffeine d_9 (before) NP (after)	QTRAP ESI+	CP 1.5 ng L^{-1}
						ETO 2 ng L^{-1}
						IF 1.5 ng L^{-1}
						GEM 1.5 ng L^{-1}
4	UPLC-MS/MS	50 mL	HLB (200 mg/6 mL)	CP-$_{d4}$ (after)	QTRAP (QqLIT) MS/MS ESI+	CP 1.1 ng L^{-1}
				ETO-d_3 (after)		ETP 6.4 ng L^{-1}
				MTX-d_3 (after)		IF 1.7 ng L^{-1}
						MTX 1.8 ng L^{-1}
5	HPLC-ICP-MS	15 mL	–	External calibration	ICP-MS	cis-Pt 300 ng L^{-1}
6	LC-MS/MS	4 mL	on-line SPE with HySphere Resin GP (10×2 mm)	Isoproturon-d6 (before) Tribromophenol (after)	QqQ; MS/MS mode (ESI+)	5-FU 100 ng L^{-1}
						CP 1.2 ng L^{-1}
						ETO 3 ng L^{-1}
						IF 1.3 ng L^{-1}
						MTX 50 ng L^{-1}
8	LC-MS/MS	500 mL	Strata X (500 mg/6 mL) Florisil (200 mg/3 mL)	CP-d_4 (before)	QqQ; MS/MS mode (ESI+)	CP 0.03 ng L^{-1}
						IF 0.05 ng L^{-1}
9	GC-MS/MS	100 mL (5-FU)	Isolute ENV+ (500 mg sample A and 1 g for samples B, C, D/6 mL)	5FU-d_6 and 5-chlorouracil (before)	Q; MS-mode (EI): SIM	5FU 0.16 ng L^{-1}
	GC-MS	100/200/400 mL (CP, IF)	Oasis HLB (60 mg/3 mL)	CP-d_6 (for CP and IF, before)	IT; MS-MS mode (EI)	CP 0.6 ng L^{-1} IF 0.3 ng L^{-1}
	ICP-MS	100 mL (cis-Pt)	–	–	ID-ICP-MS	Cis-Pt 0.6 μg Pt L^{-1}

EI electron impact, *ESI* electrospray, *IT* ion trap, *NP* nonylphenol, *QqLIT* quadrupole linear ion trap, *QqQ* triple quadrupole, *SIM* selected ion monitoring, *ID* isotope dilution

[a] Lowest LOD determined (see Supplementary material Table S1–S9)

Outlier detection was performed by calculating *z*-score (Heath et al. 2010a, b) values according to Eq. 2,

$$z = \frac{\chi_{lab} - \chi_0}{\sigma_0} \quad (2)$$

where χ_{lab} is the laboratory mean (classical approach) or median (robust approach), χ_0 is the known spiked concentration or, if unknown, as in sample B, is the average concentration measured by the participating laboratories and σ_0 is the standard deviation. Samples with *z*-values higher than 3 were excluded from further statistical analysis. For suspected outliers, i.e. those whose *z*-score values were between $2.0 \leq |z| \leq 3.0$, a further Dixon test or *Q* test was applied ($\alpha = 0.05$) (Heath et al. 2010b). The equation for the *Q* test (Heath et al. 2010b) is as follows (Eq. 3):

$$Q = \frac{\text{gap}}{\text{range}} \quad (3)$$

Gap is the absolute difference between the suspected outlier and its closest value when arranged in increasing order of concentration, and the range is the difference between highest and lowest value. When Q_{exp} is greater than Q_{tab} (a reference

value corresponding to the sample size and confidence level or α=0.05), then this value is referred as an outlier.

The following statistical parameters were calculated for each series of samples (A, B, C and D) and each compound:

- Mean and median values;
- 95 % confidence intervals;
- Variances (σ^2);
- Standard deviations (σ);
- Relative standard deviation (RSD);
- Standard errors of mean (SEM);
- Minimum and maximum values and first and third quartiles (25P and 75P);
- Number of outliers, upper and lower warning limits (UWL and LWL); and
- Repeatability (r) and reproducibility (R), where both r and R were determined in terms of relative standard deviation or coefficient of variation (CV) and expressed as percentages (%).

The equations used to calculate the UWLs and LWLs are as follows (Eqs. 4 and 5):

$$\mathrm{UWL} = \left(\overline{\chi} + 2\sigma\right) \tag{4}$$

$$\mathrm{LWL} = \left(\overline{\chi} - 2\sigma\right) \tag{5}$$

The repeatability for each sample (A, B, C and D) and selected compound was individually determined for each participating laboratory as the CV corresponding to the ratio between the standard deviation of the laboratory's measurement and its mean concentration value (ISO TC 69/SC 6 N 2011; Eq. 6):

$$\mathrm{CV}(\%) = 100 \times \frac{\sigma_{\mathrm{lab}}}{\chi_{\mathrm{lab}}} \tag{6}$$

The reproducibility was determined as the CV, where the ratio between the standard deviation of all laboratories and the average value of all laboratories were calculated for each compound and sample (ISO TC 69/SC 6 N 2011). All statistical evaluation was performed using MedCalc Software and Excel 2010.

Results and discussion

The stability of CP, IF, 5-FU, GEM, ETO and MTX in HPLC grade water and wastewater samples has been studied by both Ferrando-Climent et al. (2013) and Negreira et al. (2014b)). Their results reveal that these compounds are stable for at least 1 month at −20 °C even in wastewater. Furthermore, the authors (2013) found that CP, IF and MTX were stable for 3 months when stored at −20 °C on SPE Oasis HLB cartridges. Despite this, we requested that the participating laboratories extract their samples within 1 week. Eight laboratories managed to extract the samples within 1–3 days, while laboratory 9 reported a 5-month delay when analysing 5-FU. Unfortunately, samples shipped overseas were destroyed at customs. Sample homogeneity was evaluated using the chi-square test (Eq. 1). The experimental values of χ^2_{exp} were lower than χ^2_{tab} (α=0.05) for each sample type and selected compounds (CP, IF and MTX, (Hund et al. 2000); ISO 13528 2009) proving that the samples were homogeneous (data not shown).

In total, 266 data were received of which 219 were used for statistical evaluation. The low number of laboratories analysing certain compounds (e.g. only two laboratories submitted results for 5-FU and GEM, while for cis-Pt, only one laboratory submitted results that were above the LOD in all matrices) meant that it was not possible to use all the data. Table 3 lists the concentrations reported and the average and standard deviation values for GEM and 5-FU, while the results for CP, IF, MTX and ETO together with their corresponding statistical evaluation by classical and robust approaches are presented in SM Table S10 and Table S11, respectively. To take into account the most probable scenario, when the concentrations of analytes of interest were reported to be below LOQ, a value equivalent to half the reported LOQ was used for the evaluation (European Food Safety Authority 2010).

Laboratory performance regarding GEM (L1 and L3) and 5-FU (L6 and L9) are based on two submitted data sets (Table 3). In the case of GEM, the results showed a fairly good performance by L1 for all 4 samples analysed and by L3 for the surface water sample. However, in the case of the wastewater samples (B, C, D), the values reported by L3 were lower and inconsistent with the spiked values indicating that matrix effects were not sufficiently taken into account with the IS used. In the case of 5-FU, the low number of laboratories providing data (only L6 and L9) may be due to the complex nature (number of steps) of the analytical procedure necessary for determining this compound at trace levels, and because this compound, in contrast to most cytostatics, is more easily ionised in the negative than that in the positive ion mode (Kosjek et al. 2013). This is also the most likely reason behind the high LOD obtained by L6 for 5-FU (100 ng L^{-1}, in the ESI+ mode), which prevented it from being detected at the levels spiked in the surface water (sample A, 43 ng L^{-1}) and in the WWTP effluent (sample D, 66 ng L^{-1}). For L6, the value reported for the spiked hospital wastewater (583 ng L^{-1}) was higher than the spiked concentration (454 ng L^{-1}), but not consistent with the value measured in the non-spiked sample (420 ng L^{-1}) to account for the sum of both concentrations (420 ng L^{-1}+454 ng L^{-1}=874 ng L^{-1}). Interpretation of the possible reasons in this case is difficult. In the case of L9, however, the values measured in the four samples analysed by GC-MS/MS were half to three quarters of the

Table 3 Data reported for GEM and 5-FU by two participating laboratories: raw data from three independent replicate analyses, calculated average and standard deviation and spiked concentration in each sample type

Laboratory code	Sample						
GEM					Average	SD	Spiked conc. (ng L^{-1})
L1	A	19.4	23.4	29.1	24.0	4.9	21
	B	13.6	13.1	–	13.4	0.4	0
	C	402	434	416	417	16	397
	D	704	698	600	667	58	786
L3	A	17	19	23	19.7	3.1	21
	B	231	294	293	273	36	0
	C	191	256	295	247	53	397
	D	170	280	348	266	90	786
5-FU					Average	SD	Spiked conc. (ng L^{-1})
L6	A	150[a]	150[a]	150[a]	150[a]	–	43
	B	440	400	420	420	20	0
	C	610	560	580	583	25	454
	D	150[a]	150[a]	150[a]	150[a]	–	66
L9	A	19	19	19	19	0	43
	B	15	11	8.3	12	3	0
	C	315	364	247	309	59	454
	D	39	41	38	39	5	66

[a] Values reported below LOQ; therefore, half LOQ taken into account for evaluation

spiked value, a discrepancy explained on the basis of the stability results reported by Negreira et al. (2014b)), who found that for wastewater samples stored for 3 months at −20 °C, 25 % of the spiked 5-FU was degraded. In the case of L9, storage time was 5 months.

Classical and robust statistical approaches applied to the data reported for CP, IF, MTX and ETO did not show relevant differences in the number of outliers, which occur only in sample B (non-spiked hospital wastewater): three outliers (for CP) using the classical approach and four outliers (three

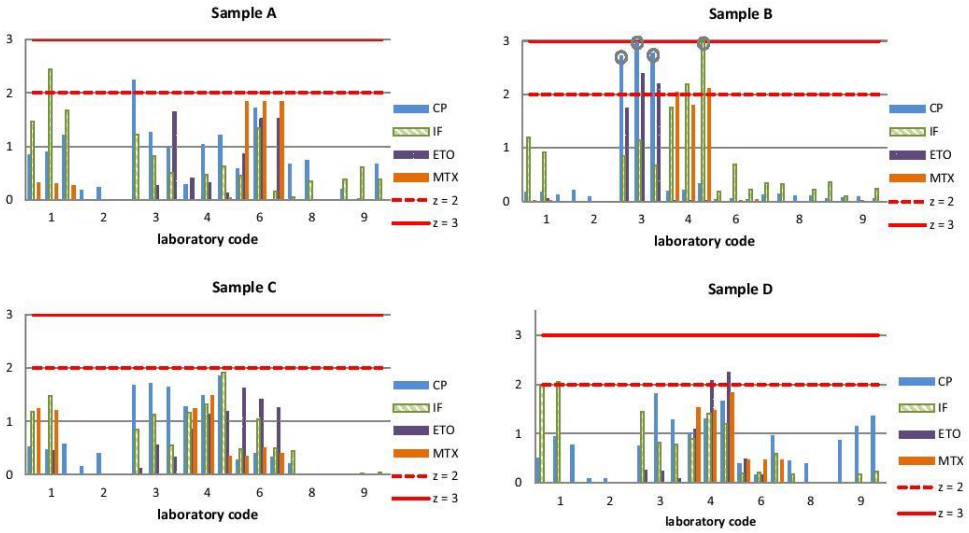


Fig. 1 Graphical presentation of *z*-score values for each compound in the various samples using a robust approach

for CP and one for IF) using the robust approach (SM—Table S12). In total, out of the nine laboratories, L3 and L4 together produced four identified outliers using the robust approach (Fig. 1), while L3 produced three outliers determined using the classical approach (SM—Figure S1). The group L3 experienced difficulties in determining CP, while L4 found determining IF problematic when analysing hospital wastewater. L4, despite extracting only 50 mL of sample, reported acceptable LOD for all tested compounds (low ng L^{-1}, Table 2). The poor analytical performance in determining IF is thought to be a result of not using a deuterated analogue of IF as the internal standard. Similarly, L3 who extracted a larger sample volume (250 mL) compared to L4 (50 mL), also applied structurally different compounds as internal standards and could have improved their method performance by using isotopically labelled analogues as internal standards.

The identification of outliers only in sample B can be attributed to the complexity of the matrix (hospital wastewater) and the comparatively lower concentrations of the analytes when compared to sample C (spiked hospital wastewater), which produced no outliers.

Tables S10 and S11 (SM) show the statistical data determined once the outliers have been removed using both classical and robust approaches, respectively. Again, the only difference between the two data sets occurs in the case of IF in sample B. The data also show an agreement between the values for CP, IF and ETO in samples B and C, where [C]=[B]+[spiked]. In all cases, the values obtained in sample C are slightly lower than those calculated on the basis of the concentration determined in sample B. In the case of MTX, the data values differed significantly from the spiked values in all three matrices, especially in the hospital wastewater, where the measured concentration of MTX was higher in the non-spiked (Sample B; 5832 ng L^{-1}) than that in the spiked sample (sample C; 2271 ng L^{-1}).

Figures 2, 3, 4 and 5 show the concentrations of CP, IF, ETO and MTX, respectively, reported by the various laboratories for all four analysed samples (different characters

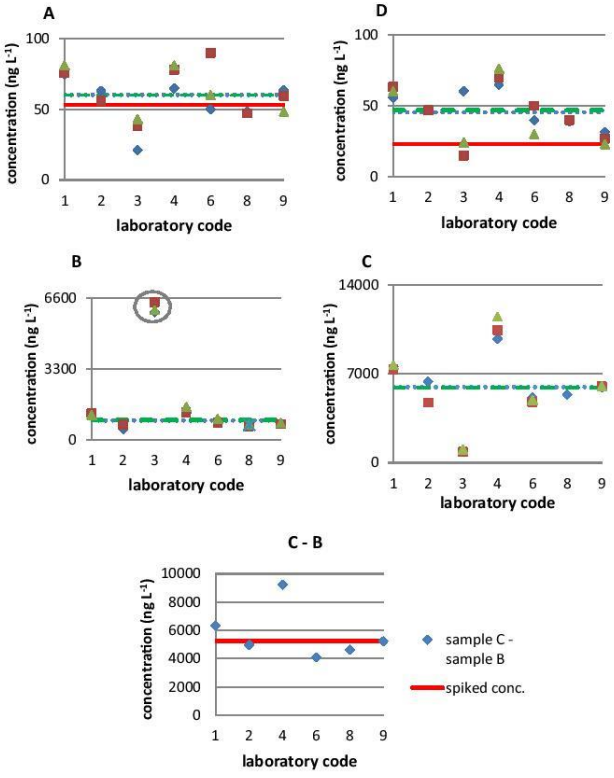


Fig. 2 Reported CP concentrations (*different dots*) in relation to spiked concentrations (*red line*) and inter-lab exercise mean (*dotted blue line*) and median (*dashed green line*) values calculated using robust approach

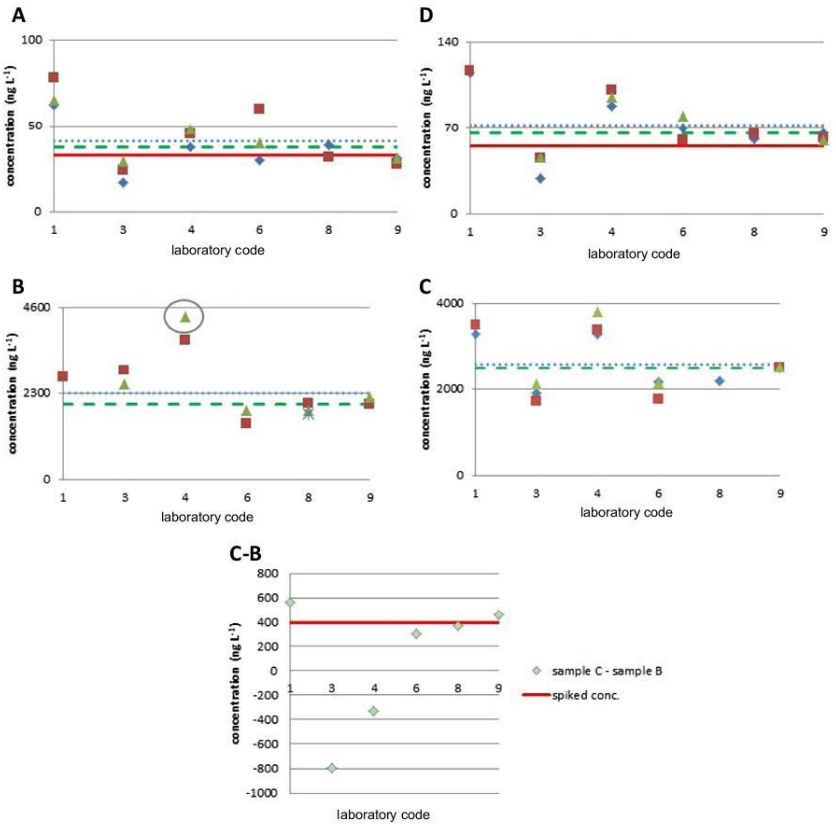


Fig. 3 Reported IF concentrations (*different dots*) in relation to spiked concentrations (*red line*) and inter-lab exercise mean (*dotted blue line*) and median (*dashed green line*) values by calculated using robust approach

representing analytical replicates), the spiked concentrations (red line) and the calculated mean (dotted blue line) and median (dashed green line) values using robust approach. The data for IF using a classical approach is presented in SM—Figure S2 showing the only difference between the two data sets. Outliers (circled) are not included in the calculation of the mean and median values. The final graph for each figure represents the difference between spiked hospital wastewater and non-spiked hospital wastewater (C-B), where the red line represents the spiked value and dots are the average values calculated for each laboratory. Mean and median values of all the laboratories were relatively close to the spiked concentrations in samples A and D for all four compounds. Only in a few cases were negative values observed in the calculation of the difference between samples C and B when determining IF (L3 and L4), ETO (L3) and MTX (L4), i.e. compounds spiked in the hospital water at concentrations comparatively lower than those naturally present in the sample. Negative values were not recorded for CP (and also 5-FU and GEM), where spiked concentrations were higher than naturally occurring levels.

Method repeatability (r), expressed as CV, for each sample (A, B, C and D) and compound of interest is presented in SM—Figure S3. The lowest CVs were observed in the case of MTX (≤12 % for L4, sample C) and the highest in the case of CP (≤72 % for L3, sample D). Empty cells correspond to analyses where at least one of the replicate results was below the LOD or not reported.

Figure 6 shows a plot of the average CV (average of the various laboratory method repeatabilies) vs. the calculated mean concentration (average of the mean concentrations

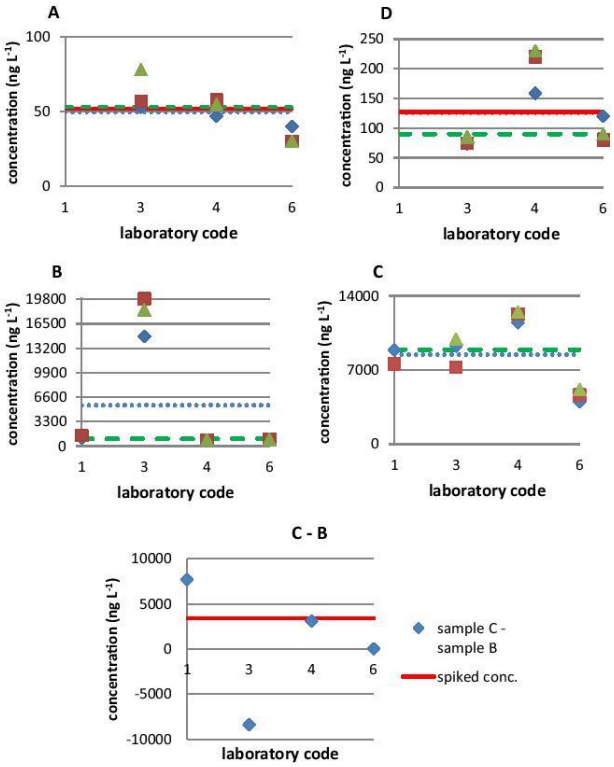


Fig. 4 Reported ETO concentrations (*different dots*) in relation to spiked concentrations (*red line*) and inter-lab exercise mean (*dotted blue line*) and median (*dashed green line*) values calculated using robust approach

reported by the various laboratories) for CP, IF, ETO and MTX, i.e. the compounds determined by most laboratories for the four tested matrices. The CVs were expected to be highest for the lowest concentrations, i.e. in surface water (Heath et al. 2010b), but in reality, the CVs for CP are constant over the concentration range. This is a likely result of using a deuterated CP compound as internal standard (Table 2).

The volume of sample extracted varies between laboratories. Laboratories L2, L3, L8 and L9 extracted 100–500 mL of sample; L4 extracted 50 mL of sample, while L5 extracted 15 mL of sample (Table 2). Both L1 and L6, which perform on-line SPE, extracted the lowest amount of sample: 5 and 4 mL, respectively. A comparison of laboratory performance reveals outliers in the case of L3 and L4 indicating that method performance cannot be directly linked to sample extraction volume.

A comparison of the CV values obtained for the different compounds, matrices and laboratories shows similar CVs between samples B and C, both corresponding to hospital wastewater, even if the CV values were expected to be lower in the spiked sample (C, SM—Figure S4). Likewise, even though high CVs were expected in the WWTP effluent (sample D) because of matrix complexity, no relevant difference in the CVs was observed between the surface water and the treated wastewater (samples A and D). The only exception in this case was the distinctly higher CV obtained by L3 when determining CP in sample D.

The reproducibility (*R*), i.e. the variability in the results reported by the various laboratories, expressed also as CV, for each sample (A, B, C and D) and the compounds CP, IF, ETO and MTX was also calculated (SM—Figure S5). Variability is mostly within 40 %, with the only exceptions being ETO and MTX in sample B. Since variability for these compounds is high in sample B, but not in sample C, it cannot be attributed to distinct matrix effects, since the matrix is the same for both B and C. This high variability derives from the high concentrations reported for ETO and MTX by L3 and L4, respectively, in sample B (>14 μg L^{-1}), concentration values that are difficult to interpret from an analytical perspective, especially considering that L4 used MTX-d_3 as an IS, unless the difference is down to human

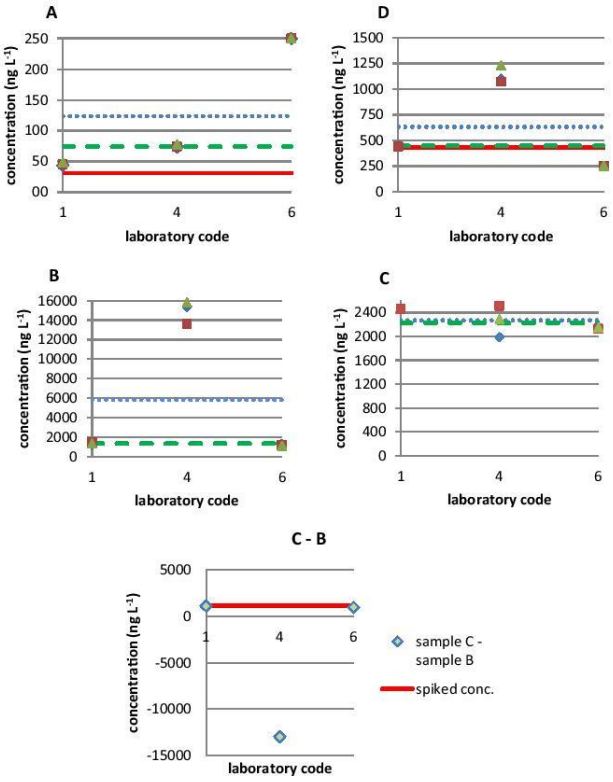


Fig. 5 Reported MTX concentrations (*different dots*) in relation to spiked concentrations (*red line*) and inter-lab exercise mean (*dotted blue line*) and median (*dashed green line*) values calculated using robust approach

error. The inter-laboratory data were also analysed graphically (all laboratories having analysed two samples) using a Youden plot (MedCalc Software 2015), which visualises within-laboratory and between-laboratory variability. In the original Youden plot (Youden 1959), axes are drawn to the same scale

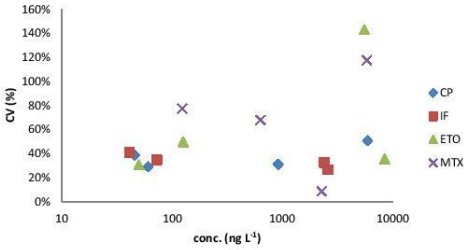


Fig. 6 Mean repeatability (CV) vs. concentration (ng L^{-1}) for various tested compounds

and the two samples must be similar and close in magnitude. Results of individual laboratories are defined by the response variables on the horizontal and vertical axis and are presented as points in the plot. The two median lines, one parallel to *x* and the other parallel to *y* axe, are drawn dividing results evenly. Around their intersection, a Manhattan circle is drawn including 95 % of laboratories and a 45° reference line through the Manhattan median. In the Youden plot, the points that lie near the 45° reference line but far from the Manhattan median indicate large systematic error, points far from the 45° line indicate large random error and points outside the circle indicate a large total error.

Youden plots were calculated for CP and IF in samples A and D due to similar spiked concentrations although the matrix complexity differs greatly. The plots reveal the nature of errors for each laboratory. Points outside the circle indicate large total error, which was observed for L1, L3 and L4 in case of both compounds. Furthermore, random error was observed in case of L8 and L9 for CP, since the data points on the

graph lie far from 45° line (Fig. 7). The points belonging to other participants (L2 and L6 for CP and L6, L8 and L9 for IF), which lie within the 95 % confidence circle and near the Manhattan median, can be labelled as acceptable (Fig. 7). Potential sources of error include the use of large calibration curve ranges (expanding from ng to μg L^{-1}) for analysis of low concentration levels, the existence of matrix interferences and matrix effects, such as signal enhancement or suppression, not corrected for with the internal standards used.

Conclusions

In conclusion, cytostatics are the least researched pharmaceuticals, which is exemplified by the low number of laboratories participating in this study. This is expected to change as awareness grows about their presence in the environment, especially given their cytotoxic, genotoxic, mutagenic and teratogenic properties. Overall, the preparation of the samples for this inter-laboratory study was satisfactory and, with the exception of the one set of samples that was destroyed at customs, all the laboratories received their frozen samples within days. Despite low participation, a minimum amount of data were obtained for statistical analysis for the compounds CP, IF, ETO and MTX. The smallest differences between spiked values and measured values for all compounds were observed for surface water. The highest within-laboratory repeatability was observed in the analysis of the spiked hospital wastewater on account of the higher concentration of the compounds in the sample. Overall, the

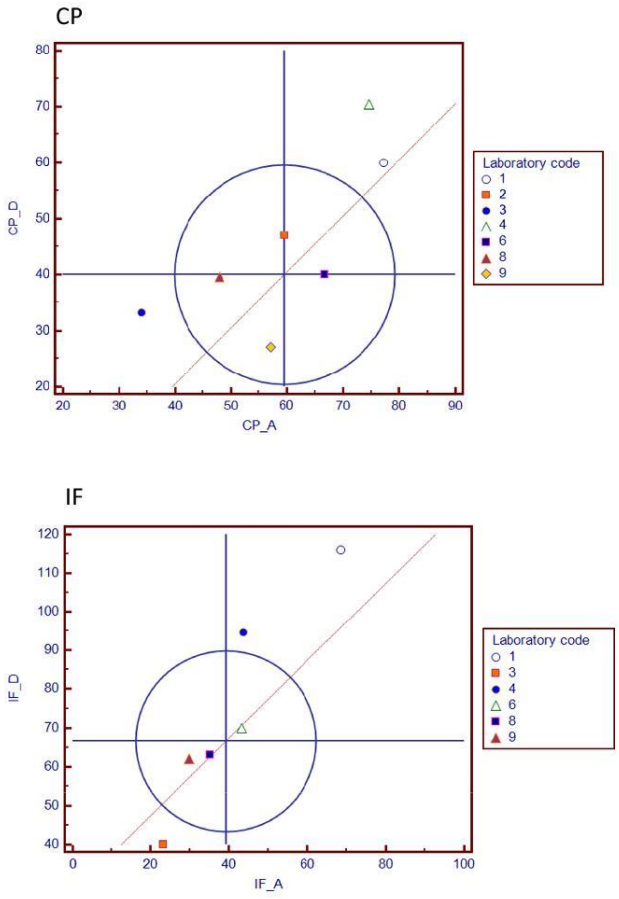


Fig. 7 Youden plots for CP and IF in samples A (spiked river water) and D (spiked WWTP effluent)

reproducibility of results amongst the laboratories was poor for all compounds and matrices, with the exception of MTX in the spiked hospital wastewater. For CP and IF, the Youden test revealed in the case of surface and WWTP effluent samples three laboratories with total errors for both compounds and two laboratories with random errors for CP.

This inter-laboratory study was performed in 2013, when there were only a few laboratories performing trace analysis of cytostatics. Since then, the interest in analysing these compounds has increased significantly and we intend to repeat our inter-laboratory study within the next 2 years with a sufficient number of participant laboratories to obtain a clearer picture of the quality of analytical data obtained for a higher number of diverse cytostatic drugs. For better laboratory performance, application of deuterated internal standards for all tested compounds and MS/MS analysis will be encouraged.

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3.3 Human Metabolites and Transformation Products of Cyclophosphamide and Ifosfamide: Analysis, Occurrence and Formation During Abiotic Treatments

The paper "Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments" by M. Česen, T. Kosjek, F. Busetti, B. Kompare and E. Heath was published in the journal of Environmental Science and Pollution Research (I.F. 2014: 2.828) in February 2016. I performed the experimental work, chemical analyses and data evaluation. I also drafted the manuscript. The identification of TPs was performed under supervision of Dr. Busetti.

This study includes the development of a GC-MS based analytical method for trace level analysis of selected CP and IF metabolites/TPs, namely carboxy-CP, keto-CP and 3-dechloroethyl-ifosfamide or N-decl-CP in various WWs. Among various tested SPE sorbents, keto-CP was successfully extracted along with CP and IF with N-vinylpyrrolidone and divinylbenzene copolymer stationary phase, whereas N-decl-CP and carboxy-CP were extracted using a hydroxylated polystyrene divinylbenzene copolymer. Further optimization included testing various derivatizing agents, solvents and conditions (time and temperature). For optimal derivatization of metabolites/TPs N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide with 1 % tert-butyldimethylchlorosilane was used. Method recoveries were 58 %, 87 % and 103 % for carboxy-CP, keto-CP and N-decl-CP, respectively. The method was assessed in terms of linearity, accuracy, LODs, LOQs and repeatability (method and instrumental) and used to analyse WW from hospital treating oncological patients and corresponding WWTP influent and effluent. A 7-day sampling campaign involved collecting daily grab samples (15^{th} to 21^{st} December 2014). The 24-h time-proportional WWTP influents' and effluents' samples were taken on 18^{th} and 19^{th} December 2014. Levels ≤ 13,200 ng L^{-1}, 2,100 ng L^{-1} and 178 ng L^{-1} of carboxy-CP, N-decl-CP and keto-CP were detected in hospital WW, respectively. Carboxy-CP was found in the highest concentration, whilst IF was the least abundant compound in the majority of samples (25 to 47 ng L^{-1}). The highest concentrations of analytes were determined in samples collected from Tuesday to Friday, whereas samples collected during the weekend and on Monday contained the lowest concentrations. Finally, concentrations of all compounds in samples taken at the WWTP were below the LOD.

The formation of TPs during abiotic treatments (UV, O_3 and H_2O_2) in various aqueous matrices was also studied. Liquid chromatography coupled to high resolution mass spectrometry was used to identify CP and IF TPs in Milli-Q water, formed during UV and UV/H_2O_2 treatment. The following TPs were identified during UV irradiation: N-decl-CP, keto-CP, CP-TP138a and CP-138b (from CP) and N-dechloroethyl-ifosfamide, keto-ifosfamide, imino-ifosfamide and IF-TP138 (from IF). The same TPs were detected within UV/H_2O_2 with an additional CP TP, imino-fosfamide. Three identified TPs were novel: imino-ifosfamide, CP-TP138a and IF-TP138. Additional experiments were performed to investigate the fate of targeted metabolites/TPs in hospital WW during UV/O_3/H_2O_2 treatment. The native concentrations of analytes were in the LOD and LOQ ranges, hence, firm conclusions regarding their fate and kinetic studies were not performed within this part of the study.

This study confirmed the necessity to follow the occurrence of not only parent compounds but also their metabolites/TPs in the aqueous environment. It also shows that various treatments do not lead to mineralization, but rather to the formation of TPs with unknown toxicities towards aqueous biota.

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RESEARCH ARTICLE

Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments

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Abstract This study describes a gas chromatography-mass spectrometry analytical method for the analysis of cytostatic cyclophosphamide (CP), ifosfamide (IF) and their selected metabolites/transformation products (TPs): carboxy-cyclophosphamide (carboxy-CP), keto-cyclophosphamide (keto-CP) and 3-dechloroethyl-ifosfamide/*N*-dechloroethyl-cyclophosphamide (*N*-decl-CP) in wastewater (WW). Keto-cyclophosphamide, CP and IF were extracted with Oasis HLB and *N*-decl-CP and carboxy-CP with Isolute ENV+ cartridges. Analyte derivatization was performed by silylation (metabolites/TPs) and acetylation (CP and IF). The recoveries and LOQs of the developed method were 58, 87 and 103 % and 77.7, 43.7 and 6.7 ng L^{-1} for carboxy-CP, keto-CP and *N*-decl-CP, respectively. After validation, the analytical method was applied to hospital WW and influent and effluent samples of a receiving WW treatment plant. In hospital WW, levels up to 2690, 47.0, 13,200, 2100 and 178 ng L^{-1} were detected for CP, IF, carboxy-CP, *N*-decl-CP and keto-CP, respectively, while in influent and effluent samples concentrations were below LOQs. The formation of TPs during abiotic treatments was also studied. Liquid chromatography-high-resolution mass spectrometry was used to identify CP and IF TPs in ultrapure water, treated with UV and UV/H_2O_2. UV treatment produced four CP TPs and four IF TPs, while UV/H_2O_2 resulted in five CPs and four IF TPs. Besides already known TPs, three novel TPs (CP-TP138a, imino-ifosfamide and IF-TP138) have been tentatively identified. In hospital WW treated by UV/O_3/H_2O_2, none of the target metabolites/TPs resulted above LOQs.

Keywords Cyclophosphamide · Ifosfamide · Cytostatic · Metabolites · Transformation products · Occurrence · Wastewater · Abiotic treatment

Introduction

Cancer is one of the most frequent global diseases (Ferlay et al. 2015). Among many different types of pharmaceuticals used in chemotherapy, cyclophosphamide (CP) and ifosfamide (IF) are two of the oldest yet still frequently used cytostatics (Besse et al. 2012; Booker et al. 2014; Zhang et al. 2013). They belong to a group of alkylating agents, whose mechanism of action is primarily through the alkylation of DNA in cells resulting in repressed cell division (Besse et al. 2012). Once administered, these compounds are partially metabolized and excreted in urine and/or faeces. The pharmacokinetcs, i.e. the excretion rates for both parent compounds and their metabolites, are highly patient-state and administration-type and dose-dependent (Zhang et al. 2006). These residues eventually end up in the aquatic environment via the sewerage system, where they could have effects on non-target organisms due to their known cytotoxic, genotoxic,

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mutagenic and teratogenic properties (Lin et al. 2015; Zhang et al. 2006). Out-patient clinics (i.e. clinics, where patients receive chemotherapy treatment and are dismissed on the same day) are becoming increasingly popular on a global scale (Franquet-Griell et al. 2015; Kosjek and Heath 2011). Regardless, a major source of cytostatic residues represent hospitals, where chemotherapies are performed daily (Zhang et al. 2013). This has been confirmed by studies reporting levels of CP and IF in hospital wastewaters (WWs) in microgram per liter (Gómez-Canela et al. 2012; Kim et al. 2009; Kümmerer et al. 1997; Steger-Hartmann et al. 1996, 1997; Yin et al. 2010; Česen et al. 2015), although their human metabolites/transformation products (TPs) are rarely reported. Only carboxy-cyclophosphamide (carboxy-CP) has been qualitatively determined in a Spanish hospital WW (Ferrando-Climent et al. 2013).

Studies reporting the removal efficiencies for CP and IF during biological treatment, e.g. suspended biomass and membrane bioreactors (Buerge et al. 2006; Delgado et al. 2011; Kiffmeyer et al. 1998; Kovalova et al. 2012; Steger-Hartmann et al. 1997), and abiotic treatments, e.g. UV, ozonation (O_3) and various advanced oxidation processes (AOPs) including UV/H_2O_2, UV/TiO_2 and UV/O_3/H_2O_2, have been published (Chen et al. 2008; Fernández et al. 2010; Garcia-Ac et al. 2010; Köhler et al. 2012; Lai et al. 2015; Lin et al. 2014; Lutterbeck et al. 2015). Their findings show that treatments fail to completely mineralize these compounds leaving behind mixtures of parent compounds and possible TPs of unknown toxicity (Busetti et al. 2015). To date, only a few published studies report the formation of TPs of CP and IF during abiotic treatment. Fernández et al. (2010) and Venta et al. (2005) both observed the formation of keto-cyclophosphamide (keto-CP) during ozonation, while Lutterbeck et al. (2015), investigating the degradation of CP by UV/H_2O_2 and UV/TiO_2, identified five TPs and Lai et al. (2015) investigating UV/TiO_2 treatment identified seven CP TPs and ten IF TPs. These studies were performed in either ultrapure water or buffer solutions at high concentrations (mg L^{-1}).

The aim of this study was to develop an analytical method for quantifying trace levels of selected CP and IF metabolites/TPs: carboxy-CP, keto-CP and either *N*-dechloroethyl-cyclophosphamide or 3-dechloroethyl-ifosfamide (*N*-decl-CP) in WW in order to quantitatively assess their presence in WWs. Furthermore, the formation of CP and IF TPs during UV and UV/H_2O_2 treatment in ultrapure water was examined, which represents a first step in the identification of potential TPs that might occur in real-scale treatment plants. Finally, the degradation and/or formation of the target metabolites/TPs included in this study was monitored during UV/O_3/H_2O_2 treatment of actual hospital WW in order to evaluate the efficiency of this WW treatment, which is capable of nearly complete CP and IF removal from various types of WW (Česen et al. 2015).

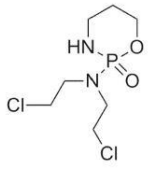
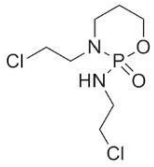
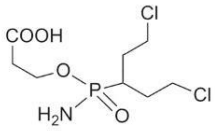
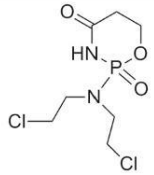
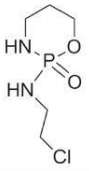
Materials and methods

Caution CP, IF, carboxy-CP, keto-CP and *N*-decl-CP are cytotoxic compounds and must be handled appropriately. Preparation of all stock solutions and samples were performed inside a laminar flow safety hood. A contaminated material was treated as hazardous waste (Eitel et al. 2000).

Standards, solvents and chemicals

Analytical standards of CP (purity 99 %) and IF (purity 99 %) were purchased from Sigma-Aldrich (Hong Kong, China). Carboxy-cyclophosphamide, 4-keto-CP, *N*-decl-CP and the deuterated standard [$^2H_6$] cyclophosphamide (CP-d_6) used as a surrogate standard for CP and IF and analysis by GC-MS were obtained from Niomech—IIT GmbH (Bielefeld, Germany). Cyclophosphamide (CP-d_4) used for LC-MS analysis was obtained from Toronto Research Chemicals (Toronto, Canada). Ibuprofen (IB-d_3), obtained from CDN Isotopes (Quebec, Canada), was used as a surrogate standard for the analysis of metabolites/TPs in WW since their isotopically labelled compounds are not commercially available. Table 1 gives chemical structures and molecular weights (MWs) of the investigated compounds. The derivatizing agents heptafluorobutyric anhydride (HFBA, 99 % purity) and trifluoroacetic anhydride (TFAA, 99 % purity) were purchased from Fluka (Buchs, Switzerland), *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA, 97 % purity) was purchased from Acros Organics (NJ, USA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide) (MSTFA, 98.5 %), acetic anhydride (AC_2O_2, 98 % purity), *N*-methyl-bis(trifluoroacetamide) (MBTFA, 99 % purity), 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr, 99 % purity), 2,3,4,5,6-pentafluorobenzoyl chloride (PFBCl, 99 % purity), heptafluorobutyrylimidazole (HFBI) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1 % *tert*-butyldimethylchlorosilane (MTBSTFA with 1 % TBDMCS, 95 %) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone (ACT), acetonitrile (ACN), cyclohexane (CHX), dichloromethane (DCM), ethyl acetate (EtAc), methanol (MeOH) and iso-propanol (IPA) were purchased from J. T. Baker (Deventer, Netherlands). Toluene (TOL) and acetic acid (AA) were obtained from Merck (Darmstadt, Germany). In case of liquid chromatography-high-resolution mass spectrometry (LC-HRMS), the mobile phases were ultrapure water (Purelab Ultra, Australia) and MeOH (Honeywell Burdick & Johnson, Muskegon, USA). All solvents were of analytical grade purity. Concentrated hydrochloric acid (37 %, HCl) and formic acid (FA) used for optimization of solid-phase extraction (SPE) were purchased from Sigma-Aldrich (St. Luis, USA); ammonia solution (25 % NH_3 sol.) was purchased from Merck (Darmstadt, Germany).

Table 1 Investigated cytostatic compounds and their metabolites/TPs. Elemental composition and exact mass are also reported

Name (acronym)	Structure	Elemental composition	Exact mass (monoisotopic)
Cyclophosphamide (CP)		$C_7H_{16}O_2N_2Cl_2P$	261.0321
Ifosfamide (IF)		$C_7H_{16}O_2N_2Cl_2P$	261.0321
Carboxy-cyclophosphamide (carboxy-CP)		$C_7H_{15}Cl_2N_2O_4P$	292.0141
4-Ketocyclophosphamide (keto-CP)		$C_7H_{14}O_3N_2Cl_2P$	275.0114
***N*-dechloroethyl-cyclophosphamide/ 3-dechloroethylifosfamide (*N*-decl-CP)**		$C_5H_{12}O_2N_2ClP$	198.0319

Formic acid (99 %), also used in the preparation of the liquid mobile phase for LC-HRMS analysis, was purchased from Ajax Finechem Pty Ltd (NSW, Australia). Hydrogen peroxide (30 %) was purchased from AppliChem (Darmstadt, Germany). The stock solutions of CP (108 μg mL^{-1}), IF (99.0 μg mL^{-1}), carboxy-CP (115 μg mL^{-1}), keto-CP (145 μg mL^{-1}), *N*-decl-CP (73.2 μg mL^{-1}) and CP-d_6 (57.1 μg mL^{-1}) were prepared in ACN. Calibration standards were prepared by serial dilution of the stock solutions in ACN, while for the analysis of TPs in ultrapure water, stock solutions of CP, IF and CP-d_4 were prepared in ultrapure water at 27.9, 13.1 and 1.00 mg mL^{-1}, respectively. The composition of artificial WW used for GC-MS method validation is described elsewhere (Kosjek et al. 2007).

Sample collection

The first part of the sampling campaign was performed at a Slovenian hospital specialized in the treatment of cancer patients, where annually, approximately 13,000 admissions (i.e. cancer treatment therapies) are performed. Based on our previous study (Česen et al. 2015), where we evaluated the occurrence of CP and IF residues in this hospital WW at different times, we sampled on seven successive days to address inter-day and intra-day variations (e.g. working days and the weekend) of both parent compounds and selected metabolites/TPs. Accordingly, seven grab samples were taken daily from a WW collection basin at the hospital between 10 a.m. and 12 a.m. over 1 week (15 Dec 2014 to 21 Dec 2014).

The sampled hospital WW is released from the collection basin into the sewerage system (WW load averages approximately 50–60 m^3 day^{-1}), which is connected to the corresponding WWTP (360,000 population equivalents; PE). The time for the hospital WW to reach the WWTP is approximately 2–3 h. Taking into account the average daily WW load of the WWTP (80,000 m^3 day^{-1}), the WW from the hospital represents approximately 0.07 % of the overall WW load. Hence, the concentrations are expected to be 700 times lower in WWTP influent and/or effluent not taking into account WWTP removal of studied compounds. To check whether or not the concentrations of investigated compounds are measurable at the WWTP influent and effluent, additional sampling was performed during mid-week. Two 24-h time-proportional composite samples were collected: WWTP influent was sampled on 18 Dec 2014, while WWTP effluent was collected on 19 Dec 2014 to take into account the hydraulic retention time of the WWTP (15–22 h). Water treatment at the WWTP includes mechanical and conventional biological treatment with suspended biomass. Additional information regarding WW treatment technology is published elsewhere (Česen et al. 2015).

In addition, the hospital WW used in $UV/O_3/H_2O_2$ treatment experiments was sampled by grab sampling at the same hospital as the 1-week sampling campaign for the analysis of metabolites/TPs described above, but on 9 May 2014 at 11 a.m.

All samples were transferred to the laboratory on ice, immediately filtered through glass-microfibre filters (Machery Nagel, Düeren, Germany) and 0.45-μm cellulose nitrate filters from Sartorius Stedim Biotech (Göttingen, Germany) and stored at −20 °C prior to analysis. All analyses were performed within 1 week.

Sample pre-treatment

The first part of this study involved developing and optimizing a gas chromatography-mass spectrometry (GC-MS) analytical method for determining CP, IF and selected metabolites/TPs in various WW samples. To achieve high sensitivity and selectivity, different SPE cartridges and derivatization agents and conditions were tested, whereas the method for parent compounds was adopted from Česen et al. (2015). The optimal conditions of the whole analytical procedure were as follows. For CP, IF and keto-CP, 100 mL of sample was preconcentrated on Oasis HLB (60 mg, 3 mL; Waters, MA, USA). Each cartridge was conditioned with 3 mL of EtAc, 3 mL of MeOH and equilibrated with 3 mL of ultrapure water. The sample was extracted at a flow rate of 3 mL min^{-1} using a Supelco Vacuum Manifold (Bellefonte, USA). After sample loading, the sorbent was dried under vacuum (10 mmHg, 20 min) and the analytes were eluted with 3 mL of EtAc (3 × 1 mL). The solvent was removed under a gentle stream of nitrogen. The SPE procedure for carboxy-CP and *N*-decl-CP was performed using Isolute ENV+ (100 mg, 3 mL) cartridges from Biotage™ (Uppsala, Sweden). Conditioning, equilibration, sample loading and sorbent drying steps were performed as described for CP, IF and keto-CP. Analytes were eluted with 6 mL of 5 % AA in EtAc (3 × 2 mL). The excess solvent was removed under a gentle stream of nitrogen. Derivatization of CP and IF was performed using 100 μL TFAA in 100 μL EtAc at 60 °C for 1 h. The metabolites/TPs were derivatized with 30 μL MTBSTFA with 1 % TBDMCS in 220 μL of ACN. Sample extracts containing metabolites/TPs were derivatized for 20 h at 90 °C.

In the second stage of this study, when LC-HRMS instrumentation became available, identification of unknown TPs formed during UV and UV/H_2O_2 treatment of IF and CP in ultrapure water was performed. For these oxidation experiments, samples were directly injected into the liquid chromatograph-high-resolution mass spectrometer. No additional sample pre-treatment was necessary since the initial spiked concentration of CP and IF was 26.1 mg L^{-1}.

Instrumental analysis and validation

Gas chromatography-mass spectrometry

A HP 6890 series gas chromatograph with a single quadrupole mass spectrometer (Hewlett-Packard, Waldbron, Germany) was used for the analysis of environmental samples and WW samples from abiotic treatment. Chromatographic separation was achieved using a DB-5 MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent, USA) with He as the carrier gas. For optimal chromatographic separation, two different temperature programs were applied, one for CP and IF and one for metabolites/TPs, which was possible since two different derivatization procedures were used. For CP and IF, the conditions and temperature program are presented elsewhere (Česen et al. 2015). For the analysis of metabolites/TPs, 1 μL of the sample extract was injected in splitless mode at 250 °C. The temperature program was as follows: an initial

temperature 100 °C was ramped at 30 °C min^{-1} to 300 °C and held for 1 min. Total GC-MS runtime was 7.17 min. The mass spectrometer was operated in EI mode at 70 eV.

Identification of pharmaceutical derivatives was made in selective ion monitoring (SIM) mode using retention time matching (CP 7.28 min, IF 6.98 min, CP-d_6 7.29 min, IB-d3 5.82 min, *N*-decl-CP 6.13 min, keto-CP 6.67 min, carboxy-CP 6.72 min) and by monitoring the following ions: m/z 309, 307 and 150 for CP; m/z 307, 181 and 150 for IF; m/z 313 and 315 for CP-d_6; m/z 357, 355 and 339 for carboxy-CP; m/z 308, 280 and 195 for *N*-decl-CP; m/z 297, 295 and 242 for keto-CP; and m/z 266 and 205 for IB-d_3. The TIC and mass spectra of the investigated metabolites/TPs are presented in the Supplementary Material (SM) 1. For instrumental control and data processing, Chemstation software (version A.03.00) was used.

For method validation, the linearity, accuracy, instrumental detection limits (IDLs), limits of detection (LOD), limit of quantification (LOQ) and repeatability (method and instrumental) were determined. The calibration range was 100–10, 100 ng $L^{-1}$and the coefficient of determination (R^2) was used to express calibration curve linearity. The method accuracy was expressed as [(experimental value − spiked value)/spiked value] (n = 3). Instrumental detection limits were calculated as three times the standard deviation of the baseline of the blank divided by the slope of their calibration curve using standards. The LODs and LOQs were calculated as three times and ten times the standard deviation of the baseline of the blank, respectively, divided by the slope of their calibration curve (n = 3) using WW as matrix. Repeatability was taken as the relative standard deviation (RSD) of three replicates (n = 3, 5000 ng L^{-1}), while instrumental repeatability was taken as the RSD of three consecutive injections of the same sample (5000 ng L^{-1}). Potential losses of analytes from filtration, i.e. the adsorption of analytes onto the glass-microfibre filters (approx. 0.5 μm) and cellulose nitrate filters (0.45 μm), were assessed by comparing filtered and non-filtered samples spiked at 5000 ng L^{-1} in tap water (n = 3).

Liquid chromatography-high-resolution mass spectrometry

The degradation pattern of parent compounds CP and IF as well as the identification of TPs formed during UV and UV/H_2O_2 treatment was performed using LC-HRMS using a Thermo Accela 600 LC system coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Corporation, Waltham, MA, USA). The system was equipped with an electrospray ion source (ESI) operated in positive ionization mode. Separation was achieved using a Kinetex C18 column (100 × 2.1 mm, 3 μm, 100 Å) from Phenomenex (Sydney, Australia) at a flow rate of 100 μL min^{-1}. The injection volume was 10 μL. The mobile phase comprised ultrapure water (A) and MeOH (B) both containing 0.1 % of formic acid. Initial conditions were 70 % A for 3 min, followed by a linear gradient to 5 % A in 15 min and held for 10 min. Afterwards, the initial conditions were re-established within 0.1 min and the column was re-equilibrated for 10 min before injecting the next sample. Total run time was 35 min. To minimize carryover, the needle was rinsed in the injection port with 1 mL of pure MeOH before and after each injection. The following ESI settings were applied: spray and capillary voltages were set at +3.5 kV and +35 V, respectively; capillary temperature was 275 °C; sheath gas flow rate was 18 arbitrary units, whereas auxiliary and sweep gas flow were off. Full calibration of the mass spectrometer was performed weekly with the positive ion calibration solution provided by Thermo Scientific. Optical lenses were optimized with a standard solution of caffeine and/or CP and IF prior to each measurement. For increased mass accuracy on the LTQ Orbitrap XL, a plasticizer interfering peak present in LC mobile phase at m/z 214.0887 (*n*-butyl benzenesulfonamide, $C_6H_5SO_2NH(CH_2)_3CH_3$, $[M+H]^+$ = 214.0896 m/z) was used for the lock mass function.

To evaluate CP and IF degradation by UV irradiation and UV/H_2O_2 treatment, reaction mixtures were analysed using selected reaction monitoring (SRM). The following SRM transitions and collision-induced dissociation (CID) energies were applied: CP (261 → 233 m/z; CID = 25); IF (261 → 154 m/z; CID = 25); CP-d_4 (265 → 235 m/z; CID = 23). The calibration curves for CP (R^2 = 0.9930) and IF (R^2 = 0.9940) were performed at 0.250–26.1 mg L^{-1} to check linearity. Samples showing approximately >40 % degradation of either CP or IF were further analysed using LC-HRMS to identify possible TPs. The analysis was conducted using the LTQ Orbitrap-XL mass spectrometer in data-dependent mode (full-scan mode from 70 to 450 m/z with a mass resolution of 60,000 (at 400 m/z) followed by HRMS-MS on the top five most intense peaks from the MS scan at a mass resolution of 30,000 (at 400 m/z). To proceed with the structural elucidation of TPs and fragments, samples were also infused into the mass spectrometer for analysis of ions in high-resolution product ions scan mode (MS^2 to MS^4) with a mass resolution of 30,000 (at 400 m/z). To identify unknown compounds, both chromatographic retention time and the deviation of the measured mass (i.e. parent compound and fragments) relative to the theoretical mass (<3 ppm, relative error) were used. Data was processed using Xcalibur QualBrowser 2.0.7 SP1, TraceFinder 3.1 and MassFrontier 7.0, where TraceFinder 3.1 and XCalibur QualBrowser 2.0.7 SP1 were used for qualitative/quantitative analysis of CP, IF and TPs, while MassFrontier 7.0 was used for spectral interpretation and structural elucidation of TPs.

Water treatment set-up

UV/O_3/H_2O_2 experiments in hospital WW

Initial chemical analysis of the hospital WW used for this set of experiments revealed measurable concentrations of the investigated compounds, and additional spiking of hospital WW

with the analytes was deemed not necessary. The degradation experiment was conducted on a laboratory-scale water treatment plant, where the removal and transformations of oxazaphosphorine-based compounds (CP, IF, *N*-decl-CP, keto-CP and carboxy-CP) present in hospital WW were studied during UV/O_3/H_2O_2 (H_2O_2 at 5000 mg L^{-1}) treatment (20–120 min). A detailed description of the equipment and experimental conditions are reported elsewhere (Česen et al. 2015). Briefly, an LP Copper UV lamp (12 W) was used for UV irradiation (UV dose = 44 mJ cm^{-2}). The equipment is designed for the simultaneous ozonation of a sample using an O_3 generator (500 mg h^{-1}) producing O_3 at 10 mg L^{-1}. The sample (4.8 L) was circulated through the system at 60 L h^{-1}.

UV and UV/H_2O_2 experiments in ultrapure water

Transformation product formation during UV and UV/H_2O_2 treatment was studied using a cylindrical glass reactor containing 500 mL of ultrapure water spiked with either CP or IF at 26.1 mg L^{-1} (10 mM). The reactor contents were stirred continuously with a magnetic stirrer (500 rpm), and a low-pressure Mercury (LP Hg) lamp (Heraeus Noblelight model TNN 15/32, 15 W) was introduced into the reactor and kept isolated from the testing solution by a quartz cooling well. The reaction solution was kept at a constant T = 20 °C throughout the experiment. The fluence rate values were determined by chemical actinometry using a 250 μM solution of H_2O_2 (Nicole et al. 1990). The measured UV intensity was 4.5×10^{-6} Ein sec^{-1}. For the UV/H_2O_2 experiments, the H_2O_2 concentration was 8.5 mg L^{-1}. For UV experiments, samples were taken at 0, 5, 10, 15, 20, 40 and 60 min, while for UV/H_2O_2 experiments, sampling times were 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, 20, 24, 28, 32 and 36 min.

Results and discussion

GC-MS method for determining CP and IF metabolites/TPs in WW

Selection of derivatization agent and conditions

To increase the sensitivity of the analytical method and achieve simultaneous determination of all investigated compounds, different derivatization agents, solvents, their mixtures and reaction times and temperatures were tested. Keto-CP and *N*-decl-CP contain oxazaphosphinane moieties in their structures, which include a secondary amine group, while carboxy-CP has a free carboxylic group and a primary amine in the phosphordiamide group (Table 1). Based on the compounds' structural characteristics, various derivatizing agents were investigated. Preliminary experiments were performed using silylating (MSTFA, MTBSTFA, MTBSTFA with 1 % TBDMCS), acylating (HFBA, TFAA, HFBI, MBTFA, PFBCl, Ac_2O_2) and alkylating (PFBBr) agents with 1 mL of EtAc at 60 °C. Derivatization time varied from 1 h to 16 h. Most of the acylating agents have a fluorine atom in their structure and are usually determined using an electron capture detector (ECD), since an ECD is extremely sensitive for halogenated compounds. Regardless, selected acylating agents for derivatization of investigated metabolites/TPs were examined since parent compounds were successfully derivatized with TFAA and analysed using a mass selective detector (MSD) that gave LODs at nanogram per liter levels (Česen et al. 2015). In addition, there are two studies, in which *N*-decl-CP, keto-CP and carboxy-CP were derivatized with TFAA and keto-CP with diazomethane (no longer commercially available) and analysed by GC-MS (Kalhorn et al. 1999; Momerency et al. 1994). Unfortunately, the derivatization of these compounds with TFAA (2 h at 70 °C) resulted in a low chromatographic response (Momerency et al. 1994). Increasing time and temperature of derivatization did not improve sensitivity. Derivatization with HFBA produced similar results to TFAA, while other acylating and alkylating agents failed to sufficiently derivatize the investigated compounds. Derivatization with silylating agents revealed that the most effective derivatization agent was MTBSTFA with 1 % TBDMCS, where the addition of TBDMCS catalyzes the reaction of the hindered amines resulting in an increased MS response compared to MTBSTFA alone.

Different solvents were also tested: ACT, ACN, CHX, DCM, EtAc and TOL, with MTBSTFA containing 1 % TBDMCS. Reactions were conducted at 75 °C for 16 h, where ACN was shown to be the most appropriate solvent (SM 2). Further step involved the optimization of reaction time (16–32 h) and temperature (60–90 °C), where 90 °C for 20 h was chosen as the optimal conditions (SM 3). Finally, to further increase the sensitivity of the analytical method, various ratios between derivatizing agent (5–30 μL) and solvent (220–1000 μL) were examined. A lower solvent volume (220 μL), i.e. increased concentration, resulted in increased response for keto-CP and carboxy-CP. For carboxy-CP, there was a tenfold increase in response. The response of *N*-decl-CP remained unchanged when the amount of solvent was reduced (500 and 220 μL). Derivatization of compounds with lower volumes of derivatizing agent was also examined (5, 10 and 15 μL). The responses were lower, so 30 and 220 μL were taken as the optimal volumes of MTBSTFA with 1 % TBDMCS and ACN, respectively.

The deuterated standard, IB-d_3, was added prior to the SPE procedure and was successfully derivatized with MTBSTFA with 1 % TBDMCS and produced similar responses under tested experimental conditions. Finally, the stability of the derivatized compounds was also assessed. The analytes of interest were stable for up to 24 h at room temperature and

for up to 5 days at −20 °C resulting in less than a 10 % degradation under both storage conditions ($n=3$).

Optimization of the SPE procedure

Solid-phase extraction was studied separately for tap water and WW. The SPE recovery was determined as a quotient of peak area of analyte spiked prior to SPE and that obtained by spiking the same amount of analyte added post SPE. Initially, 200 mL of tap water spiked with 5000 ng L^{-1} of metabolites/TPs were extracted using various types of reversed phase and mixed mode (i.e. reversed phase and strong anion exchange) SPE stationary phases along with the use of different solvents or solutions adopted in the SPE elution step (SM 4). In the case of Supelco DPA6S and Supelco EnviCarb SPE stationary phases, a comparison between non-acidified (pH = 7) and acidified samples (pH = 2) was made (SM 4). The final extracts were blown down under a gentle stream of nitrogen and further derivatized as described in "Sample pre-treatment".

Results revealed variations in SPE efficiencies when using different types of sorbents and eluents for compounds of interest (SM 4). Except for Oasis MAX, which has both reversed-phase and ion-exchange retention modes, all others contained only a reversed-phase sorbent. Recoveries >50 % were only observed for ABN Evolute, Strata C18, Supelco EnviCarb and Strata X for keto-CP. Using Oasis HLB and Isolute ENV+, a recovery >50 % was obtained for *N*-decl-CP and keto-CP. Supelco DPA6S generally resulted in the poor recovery (0–4 %) of all compounds regardless of the eluting solvents and sample pH. Based on these preliminary tests, Oasis HLB and Isolute ENV+ cartridges were favored for further optimization of the SPE procedure for keto-CP and *N*-decl-CP in WW. Carboxy-CP was successfully extracted when an Oasis MAX phase was used and eluted with 2 % FA in MeOH (recovery >90 %; SM 4). This can be explained by the ionic exchange retention mechanism between the anionic form of carboxy-CP (i.e. containing a free carboxylic group) and the positively charged quaternary amine on the Oasis MAX sorbent. After this initial screening step, selected SPE procedures were examined in terms of repeatability (SM 4). The described SPE procedure for tap water was used to evaluate analyte losses during filtration step ("Gas chromatography-mass spectrometry").

Recovery was also determined for WW, a more complex matrix. Carboxy-CP was not extracted successfully using the same method as for tap water even when the amount of sorbent was increased to 150 mg and the elution volume to 6 mL of 2 % FA in MeOH (recovery was 28.8 ± 0.4 %, $n=3$). It is assumed that matrix complexity influences the retention of carboxy-CP resulting in lower recoveries. Therefore, carboxy-CP was included in the SPE optimization procedure in WW for keto-CP and *N*-decl-CP with Oasis HLB (elution: EtAc) and Isolute ENV+ (elution, 5 % AA in EtAc). Furthermore, SPE recoveries from WW for all three compounds were determined for ABN Evolute, Strata C18, Strata X and Supelco EnviCarb eluted with MeOH, for which determined recoveries were all below 30 % (SM 4).

As expected, SPE recoveries of metabolites/TPs in WW were found to be consistently lower than for tap water also when Oasis HLB and Isolute ENV+ were used as the stationary phases (SM 4, SM 5). This can be explained by the high affinity of matrix interferences present in WW for the SPE sorbents causing 'saturation' of the sorbent beds and the consequent breakthrough of analytes during the loading step (Busetti et al. 2006). Breakthrough is more pronounced for polar analytes since reverse-phase interactions with the stationary phase are weaker. Overall, this problem can be overcome by using higher amounts of sorbent and/or by loading smaller volumes of sample. Therefore, in order to improve the recovery of metabolites/TPs in WW, the sample volume was reduced to 100 mL and the mass of stationary phase was increased. For this purpose, the following cartridges were tested: 60 and 500 mg Oasis HLB and 100 and 500 mg Isolute ENV+.

The results revealed an improved SPE recovery for carboxy-CP (recovery 58.1 ± 6.0 %) when a lower volume of sample (100 mL) was used for extraction with Isolute ENV+ cartridges regardless of the mass of sorbent (Table 2). *N*-Decl-CP was also efficiently extracted with ENV+ (recovery 102.9 ± 2.9 %) using either 100 or 200 mL; hence, to unify the procedure for *N*-decl-CP and carboxy-CP, 100 mL of sample was chosen as optimum. Finally, keto-CP was successfully extracted with HLB Oasis cartridges using either 60 or 500 mg of sorbent. Therefore, in order to unify the SPE procedure for keto-CP with CP and IF, 60-mg cartridges were used (Česen et al. 2015). Table 2 presents the optimal conditions for SPE and average ($n=3$) SPE recoveries of the investigated metabolites/TPs.

Validation

Table 3 reports the linearity, slope, accuracy, repeatability (instrumental and method), IDLs, LODs and LOQs of the analytical method as well as analyte recovery after a filtration step.

The R^2 values were above 0.97 for all investigated compounds showing good linearity over the working concentration range. Method accuracy was below 25 % in all cases, whereas method and instrumental repeatability were below 13 and 8.5 % for all investigated compounds, respectively. The LODs varied between 2 and 23 ng L^{-1}, while the LOQs were between 6.7 and 77.7 ng L^{-1}. The results confirmed that filtration did not affect the recoveries (Table 3).

Table 2 Optimized SPE procedure and average SPE recoveries ± RSD for metabolites/TPs in WW

Compound	SPE cartridge	SPE conditioning	Sample volume	SPE elution	Recovery % ± RSD; ($n = 3$)
N-decl-CP	Isolute ENV+ (100 mg/3 mL)	3 mL EtAc 3 mL MeOH 3 mL water	100 mL	(3 × 2 mL) 5 % AA in EtAc	102.9 ± 2.8
Carboxy-CP					58.1 ± 10.3
Keto-CP	Oasis HLB (60 mg/3 mL)	3 mL EtAc 3 mL MeOH 3 mL water		(3 × 1 mL) EtAc	86.7 ± 16.9

Cytostatic residues: CP, IF, *N*-decl-CP, keto-CP and carboxy-CP in hospital WW

Occurrence of CP, IF and metabolites/TPs in hospital and municipal WWs

Chemical analysis of grab samples from hospital WW revealed concentrations of investigated compounds in the nanogram-per-liter to microgram-per-liter range, except for the sample taken on Sunday (21 Dec 2014), where keto-CP was below the LOD (Fig. 1, SM 6).

Apart from IF and to a lesser extent also keto-CP, the other compounds all show a similar trend, i.e. the highest concentrations were detected in samples taken from Tuesday to Friday, whereas samples collected at the weekend and on Monday contained the lowest concentrations of the investigated compounds. This is expected since a lower number of patients receive chemotherapy over the weekend. A high inter-patient variability in pharmacokinetics among patients receiving chemotherapy with CP or IF is known; therefore, it is hard to correlate the excretion of parent compounds and metabolites to their presence in hospital WW (Gilard et al. 1993; Kaijser et al. 1994; Tasso et al. 1992; Zhang et al. 2006). However, the ratio of the excreted compounds (of the doses applied) reported in the literature (Table 4) was partially in an agreement with the results obtained within this study (Fig. 1, SM 6). For example, high excretion of CP metabolite, carboxy-CP, correlates with the highest concentrations (>10, 000 ng L^{-1}; SM 6) observed for this metabolite in WW. Its highest concentration was detected in a sample collected on Tuesday (Fig. 1, SM 6). This compound has been qualitatively determined in a hospital WW by Ferrando-Climent et al. (2013), whereas all other metabolites/TPs are detected in WW for the first time, and therefore, a comparison with other data in the literature is not possible. Moreover, CP and *N*-decl-CP (up to 2680 and 2100 ng L^{-1}, respectively; SM 6) were present in higher amounts than keto-CP (up to 178 ng L^{-1}; SM 6), agreeing with the higher excretion for these two compounds compared to keto-CP (Table 4). Cyclophosphamide is prescribed more often than IF due to fewer side effects, which explains why IF was present in lower amounts (up to 47 ng L^{-1}; Fig. 1, SM 6; (Weiss 1991)). In addition, since IF was the least abundant, we also assume that *N*-decl-CP that was detected in hospital WW samples originated mainly from CP therapy.

From Table 5, it is apparent that concentrations of CP and IF in hospital WWs span three orders of magnitude, from below LOQ/few nanogram per liter up to thousands of nanogram per liter. The results of our study are in agreement with the literature as the detected concentrations of CP and IF in hospital WW samples are also within this range. Although an in-depth study with time-proportional or flow-proportional sampling and intra-day variations would be necessary to confirm the findings of this study, these preliminary results

Table 3 Validation parameters for the GC-MS method used to analyse parent compounds and selected metabolites/TPs in WW samples

R^2 value	CP 0.9959	IF 0.9991	Carboxy-CP 0.9800	Keto-CP 0.9796	*N*-decl-CP 0.9903
Accuracy (%)	3.7	2.4	25.0	2.1	9.1
Slope of the calibration curve	0.0025	0.0016	0.00091	0.0013	0.0021
IDL (ng L^{-1})	2.7	1.9	7.7	4.5	1.2
LOD (ng L^{-1})	2.3	4.8	23.0	13.1	2.0
LOQ (ng L^{-1})	7.7	16.0	77.7	43.7	6.7
Method repeatability (RSD; %)	2.9	7.1	7.5	10.1	13.0
Instrumental repeatability (RSD; %)	4.3	8.5	4.5	5.2	7.0
Filtration step recovery (%)	99.3	100.3	95.1	100.8	97.7

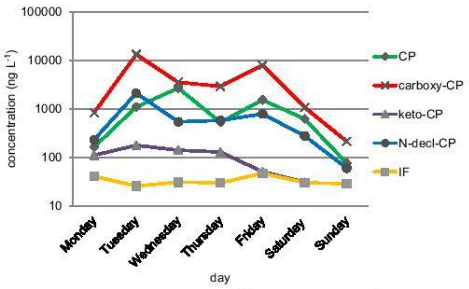


Fig. 1 Daily concentrations (ng L^{-1}) of CP, IF and selected human metabolites/TPs in hospital WW ($n=2$; RSD was below 15.9 % for all compounds); sampling campaign: 15 Dec 2014 (Monday) to 21 Dec 2014 (Sunday)

suggest that structural analogues (e.g. human metabolites) should also be included in studies since these compounds can occur at even higher concentrations than the parent compounds, and once in the environment, they also have the potential to harm aquatic biota to a similar or even higher degree (Farré et al. 2008).

None of the investigated compounds were detected in the 24-h time-proportional samples taken at the WWTP (influent and effluent). This is probably due to the high dilution factor of the hospital WW into the municipal WW. Because hospital WW represents only approximately 0.07 % of the overall WW load at this WWTP, concentrations below the LOD were expected. For example, 13,202 ng L^{-1} was the highest concentration in hospital WW (for carboxy-CP, Fig. 1, SM 6); the theoretical value detected in the influent should be 9.2 ng L^{-1}, which is approximately eight times lower than LOD determined for carboxy-CP (Table 3). This agrees with the majority of the published studies that report concentrations of the parent compounds in influents and effluents below LOQs (Table 5).

A study addressing the OECD-based bioassays for ecotoxicity and genotoxicity assessment of the compounds investigated within this paper has been recently published

Table 4 The excretion of CP, IF and their metabolites reported in literature

Compound	Excretion as % of the applied dose	Reference
CP	16–17 %	(Joqueviel et al. 1998; Zhang et al. 2006)
IF	18 %	(Gilard et al. 1993)
Carboxy-CP	11–23 %	(Joqueviel et al. 1998)
keto-CP	0.3–0.6 %	(Joqueviel et al. 1998)
N-Decl-CP	2–3 % (CP) 11 % (IF)	(Gilard et al. 1993; Joqueviel et al. 1998)

(Česen et al. 2016). Interestingly, the most ecotoxic ($EC_{50}=17.1$ mg L^{-1}) and genotoxic (significant genotoxic activity at 320 mg L^{-1}) compound was carboxy-CP, which was also the most abundant compound in the WW samples. In addition, the authors investigated the ecotoxicity and genotoxicity of a mixture of CP + IF + carboxy-CP + keto-CP + *N*-decl-CP. The effects of the mixture on the tested organisms in both bioassays were higher than expected compared to that of the compounds tested individually; however, the concentrations producing a significant effect were still in a milligram-per-liter range (Česen et al. 2016). Based on the concentrations of CP, IF and metabolites/TPs recorded in this study (Fig. 1, SM 6) and reported in the literature (Table 4), the risk associated with the presence of these chemicals in the environmental is minimal. Regardless, further studies addressing the actual environmental concentrations of the investigated metabolites/TPs are needed to make any firm conclusions regarding their possible threat to the environment.

Fate of investigated compounds during UV/O_3/H_2O_2 treatment

Native concentrations of CP and IF in hospital WW were 5340 and 6830 ng L^{-1}, respectively (Česen et al. 2015). Both CP and IF were effectively removed by UV/O_3/H_2O_2 treatment (CP removal >97.9 % and IF removal >98.4 % at $t=120$ min). In addition, carboxy-CP (26.2 ng L^{-1}) and keto-CP (152.0 ng L^{-1}) were also detected in the same hospital WW sample (Fig. 2). The detected concentrations of metabolites/TPs were significantly lower in these samples than in a series of samples taken during the sampling campaign in December 2014 (Fig. 1, SM 6). This is due to the nature of sampling (i.e. grab sampling), expectable. Therefore, unfortunately, the uncertainty of the obtained results for carboxy-CP is too high to draw any firm conclusions regarding the formation/degradation of this metabolite/TP. As regards keto-CP, the concentration was initially below the LOQ, but then increased after 60 min of treatment, which can be attributed to the degradation of CP. This is the first study reporting the degradation/formation of targeted metabolites/TPs during UV/O_3/H_2O_2 treatment of a hospital WW. The data suggests that the decrease in CP and IF results in the formation of unknown TPs during investigated treatment; hence, further studies are needed to identify them. In addition, due to the described behaviour of the targeted metabolites/TPs and very low concentrations (i.e. ng L^{-1}), the kinetic studies were omitted from this part of our work.

Transformation product formation during UV and UV/H_2O_2 treatment

Degradation rate constants for CP and IF during UV and UV/H_2O_2 treatment in ultrapure water were 0.012 and

Table 5 Concentration ranges (ng L^{-1}) of CP and IF in hospital WW, WWTP influents and effluents as well as surface water from literature

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min-max; ng L^{-1})	Reference
CP	Hospital WW	24-h time proportional $n = 7$	19–4500	(Steger-Hartmann et al. 1997)
		24-h time proportional $n = 1$	146	(Steger-Hartmann et al. 1996)
		Grab $n = 12$	<LOD (2)–21	(Thomas et al. 2007)
		Grab $n = 65$ (21 hospitals)	6–2000	(Yin et al. 2010)
		24-h time proportional $n = 1$	5730	(Gómez-Canela et al. 2012)
		Grab $n = 1$ (4 hospitals)	<LOQ (3.6)–200.7	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 7$	<LOQ (3.0)–100.0	(Negreira et al. 2014)
		Grab $n = 30$ (2 hospitals)	<LOQ (4.0)–4720	(Gomez-Canela et al. 2014)
		Grab $n = 10$ (5 hospitals)	14–22000	(Česen et al. 2015)
	WWTP influent	8-h time proportional n = 2	<LOD (6)–143 <LOD (6)–8	(Steger-Hartmann et al. 1997)
		Flow proportional (24 h) $n = 5$ (3 WWTPs)	2–11	(Buerge et al. 2006)
		24-h time proportional $n = 2$	<LOD (2)	(Thomas et al. 2007)
		24-h time proportional $n = 3$	<LOQ (7.1)	(Martín et al. 2011)
		24-h time proportional $n = 2$ (3 WWTPs)	<LOD (0.35)–13100	(Gómez-Canela et al. 2012)
		Grab $n = 2$ (3 WWTPs)	<LOQ (3.6)–25.5	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 1$ (12 WWTPs)	<LOQ (3.0)–43.8	(Negreira et al. 2014)
		24-h time proportional $n = 15$ (2 WWTPs)	<LOQ (4.0)–10	(Gomez-Canela et al. 2014)
		8-h time proportional $n = 2$	6–17 8–15	(Steger-Hartmann et al. 1997)
		24-h time proportional or grab $n = 4$ (4 WWTPs)	<LOD (0.55)–27	(Česen et al. 2015)
	WWTP effluent	Grab $n = 1$	<LOD (10)–20	(Ternes 1998)
		24-h time proportional $n = 9$ (different WWTPs)	<LOQ (1.9)–9	(Castiglioni et al. 2005)
		Flow proportional (24 h) $n = 5$ (3 WWTPs)	2–10	(Buerge et al. 2006)
		24-h time proportional $n = 1$ (8 WWTPs)	Median 0.6	(Zuccato et al. 2005)
		24-h time proportional $n = 2$	<LOD (2)	(Thomas et al. 2007)
		24-h time proportional and grab $n = 3$ (2 WWTPs)	<LOQ (100)	(Busetti et al. 2009)
		24-h time proportional $n = 3$	<LOQ (7.7)	(Martín et al. 2011)
		Grab $n = 3$ (2 WWTPs)	0.19–3.7	(Llewellyn et al. 2011)
		24-h time proportional $n = 2$ (3 WWTPs)	<LOD (0.35)	(Gómez-Canela et al. 2012)
		24-h time proportional $n = 1$ (12 WWTPs)	<LOQ (SM)-25.0	(Negreira et al. 2014)
		24-h time proportional $n = 15$ (2 WWTPs)	<LOQ (4.0)–5	(Gomez-Canela et al. 2014)

Table 5 (continued)

	Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min-max; ng L^{-1})	Reference
		Grab $n = 1$	<LOD (10)	(Ternes 1998)
		24-h time proportional or grab $n = 4$ (4 WWTPs)	<LOD (0.55)–17	(Česen et al. 2015)
	Surface water	Grab $n = 5$ (3 SWs)	0.05–0.17	(Buerge et al. 2006)
		2.5-h time proportional $n = 1$ (2 SWs)	<LOD (data not available)	(Zuccato et al. 2005)
		Grab $n = 2$ (4 SWs)	<LOQ (30)–65	(Moldovan 2006)
		Grab $n = 3$	<LOQ (5.5)	(Martín et al. 2011)
		Grab $n = 5$ (5 rivers)	<LOD (3)	(Valcárcel et al. 2011)
		Grab	<LOD (6)–1914	(Kümmerer et al. 1997)
IF	Hospital WW	24-h time proportional $n = 1$	24	(Steger-Hartmann et al. 1996)
		Grab $n = 12$	<LOD (2)–338	(Thomas et al. 2007)
		Grab $n = 65$ (21 hospitals)	4–10647	(Yin et al. 2010)
		Grab $n = 1$ (4 hospitals)	<LOQ (5.8)–227.9	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 7$	<LOQ (2.0)–19.4	(Negreira et al. 2014)
		Grab $n = 30$ (2 hospitals)	<LOQ (4.0)–86200	(Gomez-Canela et al. 2014)
		6-h time proportional $n = 2$	7–29 <LOD (6)–29	(Kümmerer et al. 1997)
		Grab $n = 10$ (5 hospitals)		(Česen et al. 2015)
	WWTP influent	Flow proportional (24 h) $n = 5$ (3 WWTPs)	<LOD (0.3)–15	(Buerge et al. 2006)
		24-h time proportional $n = 2$	<LOD (2)	(Thomas et al. 2007)
		24-h time proportional $n = 3$	3.5 ± 0.1 (mean ± SD)	(Martín et al. 2011)
		Grab $n = 2$ (3 WWTPs)	<LOQ (5.8)–130.1	(Ferrando-Climent et al. 2013)
		24-h time proportional $n = 1$ (12 WWTPs)	<LOQ (2.0)–27.9	(Negreira et al. 2014)
		24-h time proportional $n = 15$ (2 WWTPs)	<LOQ (6.0)	(Gomez-Canela et al. 2014)
		6-h time proportional $n = 2$	10–40 <LOD (6)–43	(Kümmerer et al. 1997)
		Grab $n = 1$	<LOD (10)–2900	(Ternes 1998)
		24-h time proportional or grab $n = 4$ (4 WWTPs)	<LOD (0.36)	(Česen et al. 2015)
	WWTP effluent	Flow proportional (24 h) $n = 5$ (3 WWTPs)	1.7–6	(Buerge et al. 2006)
		24-h time proportional $n = 2$	<LOD (2)–71	(Thomas et al. 2007)
		24-h time proportional and grab $n = 3$ (2 WWTPs)	<LOQ (100)	(Busetti et al. 2009)
		24-h time proportional $n = 3$	1.2 ± 0.1 (mean ± SD)	(Martín et al. 2011)
		Grab $n = 3$ (2 WWTPs)	<LOQ (0.24)	(Llewellyn et al. 2011)

Table 5 (continued)

Type of water	Sampling (flow-proportional, time-proportional or grab and number of samplings)	Conc. range (min-max; ng L^{-1})	Reference
	24-h time proportional $n=1$ (12 WWTPs)	<LOQ (2.0)–15.9	(Negreira et al. 2014)
	Grab $n=1$	<LOD (10)	(Ternes 1998)
	Grab $n=5$ (3 SWs)	0.05–0.14	(Buerge et al. 2006)
	24-h time proportional or grab $n=4$ (4 WWTPs)	<LOD (0.36)	(Česen et al. 2015)
Surface water	Grab $n=3$	<LOQ (4.4)	(Martín et al. 2011)
	Grab $n=5$ (5 rivers)	<LOD (1)–41	(Valcárcel et al. 2011)

0.010 min^{-1}, respectively. These values agree with the literature (Kim and Tanaka 2009; Lai et al. 2015; Lutterbeck et al. 2015) and detailed results are presented in SM 7. To identify TPs formed during selected oxidative treatments, samples showing parent compound removal >40 % (SM 7) were analysed by LC-HRMS.

When compared to UV/H_2O_2, UV treatment generally produced fewer and less abundant TPs (SM 8a and b). For example, for CP, four TPs were formed during UV irradiation (N-decl-CP, keto-CP, CP-TP138a and CP-TP138b), while five TPs were formed by UV/H_2O_2 (N-decl-CP, keto-CP, CP-TP138a, CP-TP138b and CP-TP259). With the exception of CP-TP138a, all CP TPs identified have been reported elsewhere during various advanced abiotic treatments, including UV/H_2O_2, UV/TiO_2, O_3 and O_3/H_2O_2 (Fernández et al. 2010; Lai et al. 2015; Lutterbeck et al. 2015; Venta et al. 2005), but not for UV irradiation alone. Lutterbeck et al. (2015) also detected the same TPs during UV/H_2O_2 treatment using a Hg medium-pressure UV lamp. Interestingly, Lutterbeck et al. (2015) and Lai et al. (2015), using UV/TiO_2 treatment, do not report the presence of the same TPs despite the similar experimental conditions (SM 8a and b). In addition, other TPs reported by Lai et al. (2015) for CP, corresponding to m/z 249, 227 and 142, were not detected within the present study, whereas keto-CP, identified within this study, is the only reported TP, which was formed during ozonation treatment (Fernández et al. 2010; Venta et al. 2005). Interestingly, CP-TP138a has the same elemental formula and fragmentation pathway as CP-TP138b, a CP TP previously reported by Lutterbeck et al. (2015), but with a significantly different chromatographic retention time (i.e. CP-TP138a, Rt=2.35 min; CP-TP138b, Rt=4.71 min). This suggests that CP-TP138a is more polar than CP-TP138b. However, it retains a very similar structure to CP-TP138b as the fragmentation spectra of the two TPs are very similar, apart from the relative abundance of the parent ion and fragments under the same collision energy conditions. A possible chemical structure taking into account elemental composition, increased polarity and fragmentation spectra has been tentatively assigned to CP-TP138a (Table SM 4).

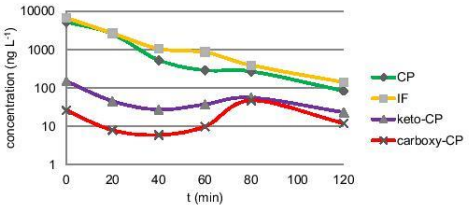


Fig. 2 Average concentrations (ng L^{-1}) of CP, IF, keto-CP and carboxy-CP during $UV/O_3/H_2O_2$ (5 g L^{-1}) treatment of hospital WW ($n=2$; RSD was below 10.5 % for all compounds). *N*-decl-CP was below LOD within this experiment

For IF, the TPs are structurally similar to those of CP, the only difference being the position of the CH_2CH_2-Cl group in the molecules. UV irradiation as well as UV/H_2O_2 treatments produced four main TPs: IF-TP199, IF-TP275, IF-TP138 and IF-TP259. So far, only Lai et al. (2015) have identified TPs of IF formed during abiotic treatment (i.e. UV/TiO_2). Two TPs, IF-TP199 (*N*-deschloroethyl-ifosfamide) and IF-TP275 (keto-ifosfamide), which were reported by Lai et al. (2015), who also investigated the formation of TPs from the degradation of IF by UV/H_2O_2 and UV/TiO_2, were identified within this study. A novel TP, IF-TP259 (imino-ifosfamide), during UV and UV/H_2O_2 treatment was also observed. This TP has been previously reported as a metabolite of IF by Li et al. (2010), who investigated the metabolism of IF in mice. Finally, IF-TP138 was also detected as a novel TP formed during UV and UV/H_2O_2 treatment. IF-TP138 is likely to be identical to CP-TP138a, having the same elemental formula, fragmentation spectra and very similar chromatographic retention time.

The identification of TPs of CP and IF under abiotic conditions partially confirmed published findings and with the exception of CP-TP138a, CP-TP138b and IF-TP138

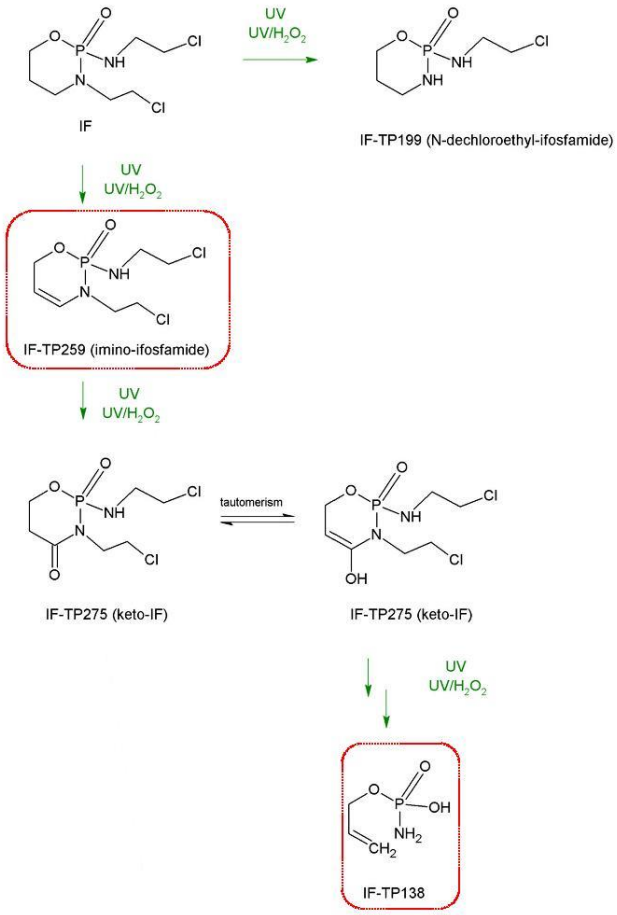


Fig. 3 Pathway for IF TPs during UV and UV/H_2O_2 treatments

(identified in this study), all TPs are also known metabolites (Li et al. 2010). The chromatographic retention times of the TPs were consistent with proposed structures, where the increased polarity of the TPs leads to lower retention times relative to CP and IF. Furthermore, the mass accuracy relative error of the parent compounds and fragments was below 2 ppm indicating a good agreement between the experimental and theoretical data (SM 8a and b).

The proposed formations of CP and IF TPs during UV and UV/H_2O_2 treatment in ultrapure water are presented in SM 8a and Fig. 3, respectively. The pathway for CP TP formation during UV/H_2O_2 is adopted from Lutterbeck et al. (2015), whereas the pathway for IF is proposed for the first time (novel TPs are circled). Figure 3 shows the proposed three-step formation of IF TPs during UV/H_2O_2 treatment. In the first step, *N*-dechloroethyl-ifosfamide and IF-TP259 are formed by dechloroethylation and dehydrogenation, respectively. In the following step, the oxidation of IF-TP259 leads to the formation of IF-TP275. In the last step, the tautomeric form of IF-TP275 is further oxidized to form IF-TP138.

Conclusions

This study describes the development of a GC-MS method for determining human metabolites/TPs of CP and IF (carboxy-CP, keto-CP and *N*-decl-CP) in hospital and municipal WWs at trace levels (low ng L^{-1}). Application of the method to

hospital WW revealed levels of the investigated metabolites/TPs and parent compounds from <LOD to 13,000 ng L^{-1}, whereas in WWTP influent and effluent were all below the LOD. When hospital WW samples were treated using $UV/O_3/H_2O_2$, the levels of CP and IF decreased with time, while after an initial drop in concentration, keto-CP increased slightly as a product of the degradation of CP. The other targeted metabolites/TPs were all below the LOQ. LC-HRMS not only confirmed the presence of already known TPs but also revealed three novel TPs: imino-ifosfamide, IF-TP138 and CP-TP138a. Future work will include the screening for additional TPs and their occurrence in surface waters.

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3.4 Ecotoxicity and Genotoxicity of Cyclophosphamide, Ifosfamide, Their Metabolites/Transformation Products and Their Mixtures

The paper “Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures” by M. Česen, T. Eleršek, M. Novak, B. Žegura, T. Kosjek, M. Filipič and E. Heath was published in the journal of Environmental Pollution (I.F. 2014: 4.143) in December 2015. The study was performed in collaboration with Dr. Eleršek and Assist. Prof. Žegura and under supervision of Prof. Dr. Filipič. I performed the ecotoxicity assays and data evaluation, while genotoxicity tests were performed with the help of Matjaž Novak. I also drafted the manuscript.

Environmental studies concerning cytostatics report the occurrence and effects in the aquatic environment, while their metabolites/TPs are poorly investigated. The aim of this study was to examine the ecotoxicity of CP, IF and their metabolites/TPs: keto-CP, N-decl-CP and carboxy-CP towards algae *P. subcapitata* and cyanobacteria *S. leopoliensis*. The tested organisms were chosen because they are the main primary producers at the bottom of the food chain in the aquatic ecosystems and any disturbance in their communities’ can affect higher trophic levels . Moreover, SOS/*umu*C response on *Salmonella typhimurium* TA1535/pSK1002 in the presence and absence of metabolic activation (S9) was studied to assess the genotoxic activity. Cyclophosphamide and IF were proven to be non-toxic towards both phytoplankton species in concentrations < 320 mg L^{-1}. Among all examined metabolites/TPs, only carboxy-CP (EC_{50} = 17.1 mg L^{-1}) was toxic towards *S. leopoliensis*. Based on the EU Directive 93/67/EEC (1996) this compound is classified as "harmful" to aquatic organisms. The SOS/*umu*C assay (the same concentration range as in the ecotoxicity assays), revealed genotoxic activity of CP and carboxy-CP in the presence of S9 at 320 mg L^{-1}. Only carboxy-CP was genotoxic also in the absence of S9, indicating that this compound is a direct acting genotoxin.

The cytostatic residues occur in the environment as complex mixtures. Although the environmental concentrations of individual compounds are low (ng L^{-1}), the effects of mixtures might be of ecotoxicological importance (Brain et al., 2004). Therefore, the ecotoxicity of a mixture of CP, IF and metabolites/TPs was determined experimentally and compared to that predicted by the concentration addition (CA) model. Moreover, a comparison between the genotoxicity of single compounds and the complex mixture was also performed. The toxicity determined experimentally was higher (EC_{50} = 11.5 mg L^{-1}) than predicted (EC_{50} = 21.1 mg L^{-1}). These results indicated a potentiation effect of the mixture. The complex mixture also revealed genotoxic activity in the presence of metabolic activation at approximately 10-times lower concentration (37.2 mg L^{-1}) if compared to the genotoxicity of CP and carboxy-CP. This suggests that in the metabolically activated mixture, the inactive compounds were either transformed to genotoxic metabolites and/or they potentiated the genotoxicity of CP and carboxy-CP.

Another aim of this study was to evaluate the removal efficiency of CP and IF mixture during UV irradiation and to simultaneously follow the ecotoxicity of potential stable TPs formed during this treatment. UV irradiation of water samples resulted in efficient removal of CP (69 %) and IF (63 %) after 48 h with degradation rate constants 0.024 h^{-1} and 0.020 h^{-1} for CP and IF, respectively. The UV degradation failed to mineralize the treated compounds. Instead, the formation of TPs was assumed, where the target GC/MS analysis revealed that investigated human metabolites/TPs within this study were not formed during UV irradiation or that their presence was below the LODs. Finally, the UV irradiated mixture did not inhibit the growth of *S. leopoliensis* confirming that abiotic treatment of binary mixture of CP and IF has no influence on overall toxicity.

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Ecotoxicity and genotoxicity of cyclophospamide, ifosfamide, their metabolites/transformation products and their mixtures

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ABSTRACT

Cyclophosphamide (CP) and ifosfamide (IF) are commonly used cytostatic drugs that repress cell division by interaction with DNA. The present study investigates the ecotoxicity and genotoxicity of CP, IF, their human metabolites/transformation products (TPs) carboxy-cyclophosphamide (CPCOOH), keto-cyclophosphamide (ketoCP) and N-dechloroethyl-cyclophosphamide (NdCP) as individual compounds and as mixture. The two parent compounds (CP and IF), at concentrations up to 320 mg L^{-1}, were non-toxic towards the alga *Pseudokirchneriella subcapitata* and cyanobacterium *Synecococcus leopoliensis*. Further ecotoxicity studies of metabolites/TPs and a mixture of parent compounds and metabolites/TPs performed in cyanobacteria *S. leopoliensis*, showed that only CPCOOH (EC_{50} = 17.1 mg L^{-1}) was toxic. The measured toxicity (EC_{50} = 11.5 mg L^{-1}) of the mixture was lower from the toxicity predicted by concentration addition model (EC_{50} = 21.1 mg L^{-1}) indicating potentiating effects of the CPCOOH toxicity. The SOS/*umuC* assay with *Salmonella typhimurium* revealed genotoxic activity of CP, CPCOOH and the mixture in the presence of S9 metabolic activation. Only CPCOOH was genotoxic also in the absence of metabolic activation indicating that this compound is a direct acting genotoxin. This finding is of particular importance as in the environment such compounds can directly affect DNA of non-target organisms and also explains toxicity of CPCOOH against cyanobacteria *S. leopoliensis*. The degradation study with UV irradiation of samples containing CP and IF showed efficient degradation of both compounds and remained non-toxic towards *S. leopoliensis*, suggesting that no stable TPs with adverse effects were formed. To our knowledge, this is the first study describing the ecotoxicity and genotoxicity of the commonly used cytostatics CP and IF, their known metabolites/TPs and their mixture. The results indicate the importance of toxicological evaluation and monitoring of drug metabolites as they may be for certain aquatic species more hazardous than parent compounds.

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1. Introduction

Cyclophosphamide (CP) and ifosfamide (IF) are two commonly prescribed chemotherapeutic drugs. They belong to a group of alkylating agents and their primary mode of action is based on their interaction with DNA resulting in repressed cell division (Besse et al., 2012). After the excretion from the human body the unaltered parent compounds and their human metabolites end up in the sewerage system where they eventually reach a municipal wastewater treatment plant, where according to several published studies they are not completely removed (Buerge et al., 2006; Delgado et al., 2011; Kiffmeyer et al., 1998; Kovalova et al., 2012; Köhler et al., 2012; Kümmerer et al., 2000, 1997; Steger-

Abbreviations: AFLB1, aflatoxin B1; CP, cyclophosphamide; CPCOOH, carboxy-cyclophosphamide; DOC, dissolved organic carbon; EC_{50}, the concentration that gives half-maximal response (50%); HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; H_2O_2, hydrogen peroxide; IF, ifosfamide; ketoCP, keto-cyclophosphamide; NdCP, N-dechloroethyl-cyclophosphamide; NADP, nicotinamide adenine dinucleotide phosphate; NOEC, no observed effect concentration; NQNO, 4-nitroquinoline-N-oxide; ONPG, O-nitrophenyl-ß-D-galactopyranoside; S9, rat liver post-mitochondrial fraction; TP, transformation product.

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Hartmann et al., 1997). Once released into the aqueous environment, the parent compounds and their pharmacologically active metabolites and/or transformation products (TPs), formed during water treatment or under environmental conditions, might have a negative impact on aquatic biota (Kümmerer et al., 2000; Lenz et al., 2007). Cyclophosphamide and IF have been determined in ng L^{-1} to μg L^{-1} levels in hospital wastewaters (Ferrando-Climent et al., 2013; Gómez-Canela et al., 2012; Kim et al., 2009a; Negreira et al., 2014, 2013a, 2013b; Steger-Hartmann et al., 1996; Yin et al., 2010) and in ng L^{-1} quantities in wastewater treatment plant influents and effluents (Buerge et al., 2006; Kümmerer et al., 1997; Ternes, 1998; Zuccato et al., 2005) and surface waters (Buerge et al., 2006; Moldovan, 2006; Valcárcel et al., 2011). Although several CP and IF human metabolites are known to be excreted by patients receiving chemotherapy, their presence in the environment is rarely reported. Only one CP metabolite/TP, namely carboxy-cyclophosphamide (CPCOOH), has been qualitatively determined in a Spanish hospital wastewater (Ferrando-Climent et al., 2013).

To reduce the levels of CP and IF in wastewaters, various wastewater treatment technologies can be applied including biological treatment, ozonation, UV irradiation and advanced oxidation processes such as ozonation/H_2O_2, UV/H_2O_2 and UV/TiO_2 (Chen et al., 2008; Delgado et al., 2011; Fernández et al., 2010; Kim et al., 2009b; Kovalova et al., 2012; Kümmerer et al., 1997; Lester et al.; Lester et al., 2011; Lin et al., 2014; Steger-Hartmann et al., 1997; Wols et al., 2013), where parent compound removal was used as a parameter for evaluating treatment efficiency. However, during water treatment (biological and abiotic), compounds are exposed to several degradation factors, e.g. microbes, reactive oxygen species, sunlight, UV light, ozone and chemical oxidative agents that may generate potentially toxic TPs. Identification of these structurally diverse compounds is a challenging task, where TPs may have a different toxic potential from that of the parent compound, which must be taken into account. In addition, the toxicity studies of TPs require authentic standards that are often not commercially available and have to be custom synthesized. Therefore, a more rational approach is to evaluate the changes in the toxicity of sample before and after treatment (influent and effluent), which considers the change in the whole toxicity of the sample. Studies describing degradation of CP and IF reported either a lower or similar toxicity of treated samples in comparison to the untreated control (Delgado et al., 2011, 2010; Hansel et al., 1996; Lin et al., 2015; Lutterbeck et al., 2015) with the exception of a recent study by (Lin et al., 2015b), who reported a slight increase in the acute toxicity of ozone treated samples containing either CP or IF.

When evaluating adverse effects of compounds in the environment it is important to study their toxicity on organisms from different trophic levels: phytoplankton, invertebrate and vertebrate species (Zounkova et al., 2010). In the aquatic ecosystems phytoplankton species are the main primary producers at the bottom of the food chain and any disturbance of these phytoplankton communities' can affect communities at higher trophic levels (Geis et al., 2000; Pfleeger et al., 1990). To assess the toxicity of environmental pollutants towards aquatic primary producers, tests based on the OECD Guidelines for Testing of Chemicals (i.e. Freshwater Alga and Cyanobacteria, Growth Inhibition Test) are commonly performed (OECD TG 201, 2011). A literature overview revealed that only two CP growth inhibition studies have been performed using the alga *Pseudokirchneriella subcapitata*, both showing low toxicity with no observed effect concentration (NOEC) > 100 mg L^{-1} (Grung et al., 2008; Zounkova et al., 2007). No published ecotoxicity studies, to the authors knowledge, have included IF and the human metabolites/TPs of CP and IF. On the other hand, the review paper by Toolaram et al. (2014) reveals genotoxic activity of CP, IF and some of their human metabolites (phosphoramide mustard, 4-OH-peroxy-cyclophosphamide, nor-nitrogen mustard, CPCOOH and keto-cyclophosphamide; ketoCP), with certain metabolites being more potent than the parent compounds (Toolaram et al., 2014). In the aquatic environment, organisms are exposed to a complex mixture of numerous compounds including cytostatic drugs and their metabolites, which may exert higher adverse effects on non-target organisms than expected based on the measured effect of the exposure to a single compound. Therefore it is important to study both, the effects of pure compounds and even more the effects of their mixtures (Brain et al., 2004). Recently published studies describe toxic activities of mixtures of several cytostatic drugs (Brezovšek et al., 2013, 2014; Parrella et al., 2014), however, to the best of our knowledge, the toxic potential of mixture of CP, IF and their metabolites/TPs has not been investigated yet.

The aim of this study was to evaluate ecotoxicity and genotoxicity of the parent cytostatics CP and IF and their commercially available human metabolites/TPs: CPCOOH, ketoCP and N-dechloroethyl-cyclophosphamide and its structural analogue 3-dechloroethyl-ifosfamide (NdCP) and their mixture by determining the effect on growth of primary producers (alga *P. subcapitata* and cyanobacteria *Synecococcus leopoliensis*) and SOS/*umuC* response of *Salmonella typhimurium* TA1535/pSK1002, respectively. Furthermore, the measured influence of the mixture of CP, IF and the three metabolites/TPs on growth of *S. leopoliensis* was compared to the predicted effect determined with the concentration addition (CA) model (Loewe, 1927; Loewe and Muischnek, 1926). Finally, to evaluate the treatment efficiency of CP and IF by UV irradiation, the degradation along with ecotoxicity of potential stable TPs formed during this treatment was assessed on *S. leopoliensis*.

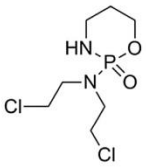
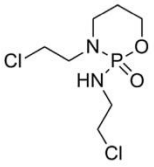
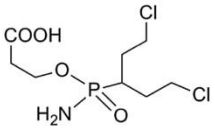
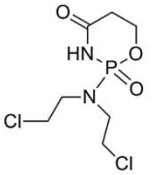
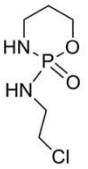
2. Materials and methods

2.1. Standards, reagents and chemicals

Cyclophosphamide, IF, CPCOOH, ketoCP and NdCP were handled in compliance with the internal safety standards for working with anti-neoplastic drugs (Eitel et al., 2000). All wastes were disposed of as hazardous waste. The operator protected themselves against UV irradiation during experiments by wearing protective clothes and UV-protective glasses.

Cyclophosphamide (99%) and IF (99%) were purchased from Sigma Aldrich (Hong Kong, China). Carboxy-cyclophosphamide, ketoCP and NdCP (a structural analogue of CP and IF metabolites/TPs) were obtained from Niomech - IIT GmbH (Bielefeld, Germany). Chemical structures, CAS numbers and the molecular weights of the investigated compounds are presented in Table 1. Stock solutions for ecotoxicity bioassays were prepared in OECD medium and filtered through 0.2 μm cellulose-nitrate filters (Sartorius Stedim Biotech, Göttingen, Germany). The concentrations in the stock solutions were as follows: CP: 3.06 mg mL^{-1}; IF 1.01 mg mL^{-1}; CPCOOH: 5.43 mg mL^{-1}; ketoCP: 0.55 mg mL^{-1} and NdCP: 5.56 mg mL^{-1}. For the UV irradiation experiments, stock solutions of CP and IF were prepared in MilliQ water (Millipore Ltd, Darmstadt, Germany) at 16.05 and 16.16 mg mL^{-1}, respectively. The solutions were sterilized by filtration (0.2 μm cellulose-nitrate filters). The stock solutions used for genotoxicity testing were prepared either in dimethylsulfoxide (DMSO; metabolites/TPs) or in sterile bidistilled water (CP and IF). The concentrations of stock solutions were 20 mg mL^{-1} and 50 mg mL^{-1} for parent compounds and metabolites/TPs, respectively. The solvent DMSO was purchased from Sigma–Aldrich (Steinheim, Germany) and was of analytical grade purity.

Table 1
Names, abbreviations, structures, CAS numbers and molecular weights (MW) of investigated compounds.

Name and abbreviation	Structure	CAS	MW
cyclophosphamide CP		50-18-0	198.59
ifosfamide IF		3778-73-2	198.59
carboxycyclophosphamide CPCOOH		22788-18-7	293.08
4-ketocyclophosphamide ketoCP		27046-19-1	275.07
N-dechloroethylcyclophosphamide or 3-dechloroethylifosfamide NdCP		36761-83-8	198.59

The chemicals used for SOS/*umu*C bioassay, i.e. ampicillin, glucose-6-phosphate (disodium salt), D-(+)-glucose (anhydrous), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium dodecyl sulphate, Na_2CO_3, $MgCl_2 \cdot H_2O$, Na_2HPO_4, $Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, aflatoxin B1 (AFLB1) and 4-nitroquinoline-N-oxide (NQNO) were obtained from Sigma–Aldrich (St. Louis, USA), nicotinamide adenine dinucleotide phosphate (NADP) and NaCl from Merck (Darmstadt, Germany), while O-nitrophenyl-ß-D-galactopyranoside (ONPG), β-mercaptoetanol, KCl and $MgSO_4 \cdot 7H_2O$ were purchased from Fluka (Buchs, Switzerland). Lyophilized Aroclor 1254 induced male rat liver post-mitochondrial fraction (S9) was obtained from Moltox (Boone, USA) and trypton from BD Biosciences (Sparks, USA).

We assessed the stability of all investigated compounds under the experimental conditions. Detailed descriptions and results are presented in SM-1a. The stability of the parent compounds in HPLC water (J. T. Baker, Deventer, Netherlands) at 4 °C was confirmed by Negreira et al. (2013b), whereas the stability of metabolites/TPs was investigated as part of this study (Supplementary Material; SM-1b).

2.2. Bioassays

2.2.1. Ecotoxicity on green alga *P. subcapitata* and the cyanobacterium *S. leopoliensis*

Green alga *P. subcapitata* SAG 61.81 and the cyanobacterium *S. leopoliensis* SAG 1402-1 were obtained from the algae collection SAG - Sammlung von Algenkulturen Univerzität Göttingen (SM-2). The organisms were cultivated in an OECD medium (pH = 8.1, composition of the medium is described in the Annex of OECD TG 201 (2011)), according to the Guidelines in OECD TG 201 (2011). The pH of the medium was measured at the beginning and at the end of experiments and did not change significantly during the

performance of the experiments. The bioassays were conducted in glass Erlenmeyer flasks (V_{final} = 20 mL) under 24 h day^{-1} light irradiation (cooled lamps Sylvania GRO-Lux F 18W/GRO-T8; intensity: 80–120 μE m^{-2} s^{-1}), with constant shaking at 100 rpm (GFL 3017, Burgwedel, Germany) and kept at 24 ± 2 °C. The V_{final} consisted of the OECD medium, tested organism and a solution of either the five investigated compounds, a mixture of all five compounds or the UV-irradiated samples. According to OECD TG 201, the initial cell densities were 5×10^3 - 5×10^4 cells mL^{-1} and 5×10^4 - 5×10^5 cells mL^{-1} for the algae and cyanobacteria, respectively (OECD TG 201, 2011). Cell growth was determined after 24, 48 and 72 h of exposure to the parent compounds (CP and IF), their metabolites/TPs and their mixture using a flow cytometer (BD FACSCalibur, Sparks, MD, USA), whereas in the case of UV-irradiated samples it was determined by cell counting under an inverted light microscope (Nikon Eclipse 80i, Nikon, Japan) as described elsewhere (Elersek, 2012). The algal/cyanobacterial growth rate, expressed as the logarithmic increase in cell number within 72 h of exposure to the test compounds, a mixture of compounds or the UV-irradiated samples was compared to the control cultures exposed under the same experimental conditions. The parent compounds' ecotoxicity was assessed for both species, whereas metabolites/TPs, mixtures and UV-irradiated samples were tested only towards *S. leopoliensis*. The experiments for determining the ecotoxicity of individual compounds and their mixture were performed three times independently, each time in triplicates, while, the preliminary experiments for determining the ecotoxicity of control and UV-irradiated samples were performed twice in duplicate. The experiment for determining the ecotoxicity of a CP and IF mixture (UV experiments) was performed once in triplicate. According to the OECD guidelines, the ecotoxicity of each compound was determined at five concentrations as geometric series with a factor 3.2 (3.05, 9.77, 31.25, 100 and 320 mg L^{-1}). Standard solutions were prepared in sterilized MilliQ water. The toxicity of the UV-irradiated samples was evaluated as follows; 10 mL of UV-irradiated sample (medium was sterilized MilliQ water) was added to 10 ml of 2 M OECD medium (concentrated by factor 2) with preculture of *S. leopoliensis* to give a final volume of 20 mL with 1 M OECD medium (as used within all the performed experiments). The initial amount of CP and IF during the UV experiments (10 mg L^{-1}) was diluted at 1:1 ratio for the bioassays to give a final concentration of CP and IF at 5 mg L^{-1}.

2.2.2. Genotoxicity on S. typhimurium TA1535/pSK1002

The evaluation of genotoxic activity of the investigated compounds and their mixture was assessed by SOS/*umuC* assay according to Reifferscheid et al. (1991). The genotoxicity of individual compounds and their mixture was evaluated using the same concentrations as in ecotoxicity bioassays (3.05, 9.77, 31.25, 100 and 320 mg L^{-1} for individual compounds and 6, 7.9, 10.5, 17.1 and 37.2 mg L^{-1} for the mixture). The genotoxicity tests were conducted with (+S9) and without (-S9) metabolic activation in a triplicate on a microtiter plates, where a genetically modified *S. typhimurium* TA1535, carrying a multicopy plasmid pSK1002 with fused *umu*C-lacZ genes, was used as the tested organism. Briefly, the overnight bacterial culture was diluted in a ratio 1:10 with fresh TGA medium and agitated (300 rpm) at 37 °C for 1.5 h until the bacteria reached the exponential growth phase. The bioassay was performed with the following mixture: 10 μL of solution containing compound/mixture in DMSO or bidistilled water and 90 μL of bacterial culture. In the case of metabolic activation, 10 μL of the tested compound was incubated with 90 μL of the S9 bacterial culture mixture, containing 10% of the S9 extract. The microtiter plates were further incubated at 37 °C for 2 h with shaking. Afterwards, the incubation mixture was diluted with fresh TGA medium (1:10) and incubated at 37 °C for additional 2 h with shaking. The bacterial growth rate was determined by measuring absorbance at 600 nm. The β-galactosidase activity (stopped with Na_2CO_3 after 30 min of incubation at 25 °C and shaking) was assessed using ONPG as a substrate, where the absorbance of the o-nitrophenol was measured (at 420 nm).

4-nitroquinoline-N-oxide (0.25 mg L^{-1}) and AFB1 (5 mg L^{-1}) were used as a positive controls without and with metabolic activation, respectively. In addition, distilled water and DMSO (the same amount as in tested samples) were used as the negative and solvent control.

2.3. Experimental and predicted ecotoxicity of the mixture

The ecotoxic activity of the mixture of CP, IF, ketoCP, NdCP and CPCOOH was tested at concentration in the mass ratio 1:1:1:1:1. The concentrations were based on $EC_{5\text{-}90}$ values calculated for CPCOOH (see Chapter 3.1, Fig. 2, Table 2), namely 6, 7.9, 10.5, 17.1 and 37.2 mg L^{-1} since CPCOOH was the only compound that significantly inhibited the growth of *S. leopoliensis* within the working concentration range, while other tested compounds did not have any effect at concentrations up to 320 mg L^{-1}. Moreover, by applying the same concentration of each compound in the mixture it was possible to compare the effect of individual compound (i.e. CPCOOH) to the effect of the mixture (see Chapter 3.1, Fig. 2, Table 2). The obtained results were compared to the CA model as described by Loewe (1927), originally by Loewe and Muischnek (1926). The CA model is based on the assumption that when the investigated compounds share similar sites and modes of action, the effect of a mixture remains constant if one compound is replaced by another (Faust et al., 2003). The model was used to predict ecotoxicity since the investigated parent compounds and their human metabolites/TPs share similar structures and modes of action, i.e. interaction with DNA (alkylation of DNA).

2.4. UV irradiation of samples

Experiments were performed in a cylindrical glass reactor containing 760 mL of sterilized aqueous solution (MilliQ) spiked with the mixture of CP and IF (10 mg L^{-1} each). The solution was stirred continuously in the dark using a magnetic stirrer (200 rpm). A monochromatic low pressure (LP) mercury lamp (Photochemical reactors LTD, Berkshire, UK) was introduced into the reactor and kept separate from the test solution using a quartz cooling well. Sample temperature was maintained at 20 °C. The LP lamp (6 W) as an irradiation source emits 90% of its radiation at 254 nm. The UV intensity, measured using ferrioxalate actinometry as described in manufacturers' instructions, was 0.78 μE m^{-2} s^{-1}.

The removal of CP and IF during UV irradiation was assessed by GC/MS as described elsewhere (Česen et al., 2015). Briefly, samples (0.1 mL) were dried under nitrogen at 35 °C to dryness and further derivatised with trifluoroacetic anhydride in ethyl acetate (for 1 h at 60 °C) for further chemical analysis. The removal was calculated as the ratio between CP and IF concentrations in UV irradiated *vs.* non-irradiated samples at the following specified times: 6, 12, 24, 36 and 48 h. The DOC (dissolved organic carbon) values were analysed using the Hach Lange cuvette method (LCK385) in combination with a DR/2800 spectrophotometer (Hach Lange, Düsseldorf, Germany) in order to assess mineralization degree of CP and IF.

2.5. Data evaluation

Data analysis and statistical evaluation of the toxicities were performed using Microsoft Excel 2010 and Prism 5 (GraphPad Inc.,

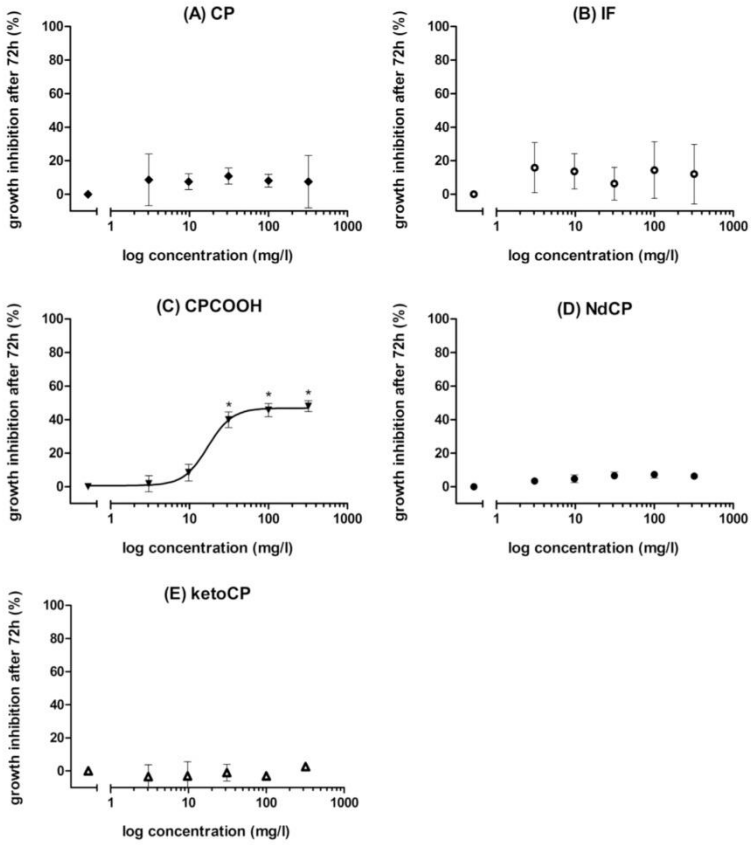


Fig. 1. The effect of the investigated compounds (A–E) on the growth of *S. leopoliensis*. Growth inhibition (as the mean ± 95% confidence interval) is presented as a function of log concentration after 72 h of exposure. * indicates statistically significant difference in comparison to the control sample.

La Jolla, USA) software. In the case of ecotoxicity bioassays, the measurements from each individual flask (pooled together) were used for the analysis instead of the means of replicates as described in OECD TG 201 (2011). For the Figures presenting the % growth inhibition (Y) *vs*. log concentration of the tested compound and the EC_X values, a nonlinear regression model was applied, using the following Eqs. (1–3):

$$\log EC_F = \log EC_{50} + \left(\frac{1}{hill\ slope}\right) \times \log\left(\frac{F\%}{(100 - F\%)}\right) \quad (1)$$

$$F\% = \frac{(Y - Bottom)}{(Top - Bottom)} \times 100 \quad (2)$$

$$Y = Bottom + \frac{(Top - Bottom)}{\left(1 + 10^{(\log EC_{50} - X) \times slope)}\right)} \quad (3)$$

where EC_F represents the concentration of the investigated compound which gives a response of F% of the way between Bottom and Top (e.g., EC_{10}, EC_{20}, EC_{50}), where Bottom and Top are the plateaus in the units of the response (% growth inhibition). In addition, the slope describes the steepness of the family of curves. The important parameters in Prism 5 were set as: constraint for the bottom of the nonlinear curve to "shared value for all datasets". In addition, the outliers were identified by the Prism 5 software (based on ROUT method) and were excluded from further calculations. Non-parametric ANOVA (Kruskal–Wallis test; $\alpha = 0.05$) and Dunn's post-test ($p < 0.05$) were used to determine if any significant differences exist between the controls and the effect of the investigated compound or mixtures of compounds towards the test organisms.

The calculation of CA model was performed using the Eq. (4):

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x,i}} = 1 \quad (4)$$

where c_i represents the concentration of the i-th compound in a

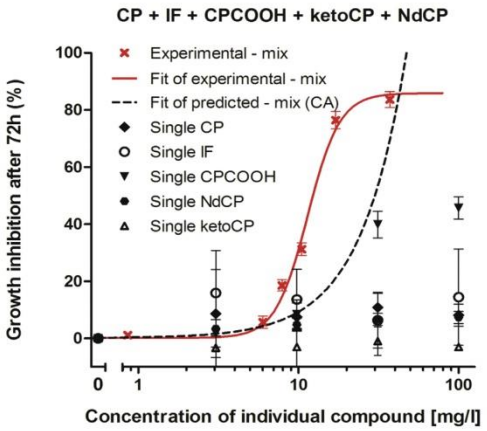


Fig. 2. Growth inhibition (mean ± 95% confidence interval) based on data from individual compounds (different symbols), experiments with a mixture (mix) of compounds at the same concentration as in case of experiments with individual compounds (full line) and curve of predicted data with CA model (dashed line).

tested mixture that exhibits x% of the total effect, whereas $EC_{x,i}$ is the concentration of the compound *i* which provokes the same effect (x%) if applied individually.

The bacterial growth rate (G) for determination of cytotoxic activity was calculated as the ratio of $OD600_{sample}$ to $OD600_{control}$ (the blank sample was subtracted in both cases). The calculated growth ratio for a compound/mixture less than 0.75 (i.e. 25% inhibition of biomass growth) was considered as cytotoxic. The β-galactosidase activity (U) in relative units was calculated as the ratio of $OD420_{sample}$ to $OD600_{sample}$ (the blank sample was subtracted in both cases). The induction ratio (IR) was determined as the ratio of $OD420_{sample}$ to $OD420_{control}$ (the blank sample was subtracted in both cases), divided by G. The IR $\geq$ 2 was considered as the threshold at which the compound/mixture was considered as genotoxic.

3. Results and discussion

In the present study the ecotoxic and genotoxic effects of the investigated cytostatics, their metabolites/TPs and their mixture were evaluated on primary producers (green algae *P. subcapitata* and cyanobacterium *S. leopoliensis*) and *S.typhimurium* TA1535/pSK1002, respectively.

3.1. Ecotoxicity studies

3.1.1. Individual compounds

Toxicity was assessed in terms of growth inhibition, expressed as EC_x and NOEC values (Table 2). Growth inhibition experiments revealed that, within the tested concentration ranges, neither CP or IF inhibited the growth of *P. subcapitata* or *S. leopoliensis* (Fig. 1A, B and SM-3). The observed results for CP on *P. subcapitata* (SM-3) are in the agreement with literature (Grung et al., 2008; Zounkova et al., 2007), whereas to our knowledge the effects of IF towards these species are yet to be reported. Since prokaryotic organisms (cyanobacteria belongs to prokaryotes) are more abundant in biological water treatment than eukaryotic organisms (Wagner and Loy, 2002) and because the alga and cyanobacteria did not differ in the susceptibility towards exposure to CP and IF, all following experiments involving metabolites/TPs, mixtures of compounds and toxicity of the CP and IF after UV irradiation were performed using only *S. leopoliensis*. Growth inhibition of *S. leopoliensis* by investigated human metabolites/TPs is shown in Fig. 1C, D and E. Only CPCOOH significantly inhibited growth of cyanobacteria.

The EU Directive 93/67/EEC (1996) classifies substances according to their EC_{50} values towards aquatic organisms as very toxic (EC_{50} < 1 mg L^{-1}), toxic (EC_{50} 1–10 mg L^{-1}) or harmful (EC_{50} 10–100 mg L^{-1}). Substances with EC_{50} above 100 mg L^{-1} are not classified. The EC_{50} value for the metabolite/TP CPCOOH was 17.1 mg/L (Table 2). Therefore, according to the EU Directive 93/67/EEC (1996), CPCOOH is classified as harmful to aquatic organisms. Interestingly, CPCOOH was the only compound that had a significant effect compared to other compounds, despite their similarities in chemical structure and potential mode of action. So far, CPCOOH is the only CP metabolite/TP, which has been detected in hospital wastewater in Spain, whereas environmental concentrations in e.g. surface waters have not been reported yet. Unfortunately, due to the chemical instrumentation used and analysis, which allowed only the confirmation of its presence in the hospital wastewater, the concentration of CPCOOH was not determined in the study. Therefore, any environmental and human risk assessment for CPCOOH is not possible at this stage (Ferrando-Climent et al., 2013).

3.1.2. Mixture of CP, IF and their metabolites/TPs

Exposure to the mixture of CP, IF and their metabolites/TPs revealed higher growth inhibition of *S. leopoliensis* than that predicted by the CA model, which is especially noticeable within EC_{20-70} effect concentration range (Fig. 2). The experimentally determined EC_{50} was 11.5 mg L^{-1} (95% CI: 10.3–12.9 mg L^{-1}), whereas the predicted EC_{50} based on the CA model was 21.1 mg L^{-1}. It is also apparent that the dynamics of growth inhibition based on experimental data differ from predicted assumptions for concentrations >5 mg L^{-1}, suggesting the potentiation effect of a mixture containing parent compounds and their human metabolites/TPs. A potentiation effect is observed when the toxicity of a mixture of a

Table 2
EC_x, NOEC values and growth inhibition of *S. leopoliensis* for each test compounds; n.c. = not converged (Prism 5).

Compound	$EC_{5\text{-}90}$ and 95% CI (mg L^{-1})	NOEC (mg L^{-1})	Measured maximal growth inhibition in tested concentration range
CP	n.c.	Above 320	0–20%
IF	n.c.	Above 320	0–39%
CPCOOH	EC_5: 6.0 (4.4–8.3) EC_{10}: 7.9 (6.0–10.2) EC_{20}: 10.5 (8.5–12.9) EC_{50}: 17.1(14.4–20.3) EC_{90}: 37.2 (27.8–49.7)	9.8	0–54%
ketoCP	n.c.	Above 320	0–3%
NdCP	n.c	Above 320	0–7%

non-toxic compound and a toxic compound results in an effect, higher than the determined toxicity of an individual toxic compound (Li et al., 2015). Such phenomenon, shown in Fig. 2, was observed within this experiment, where the sum of a mixture's toxicity, i.e. CP + IF + CPCOOH + ketoCP + NdCP was higher than the toxicity of CPCOOH alone. Although there are different ways of performing such mixture experiments, e.g. by using mass ratio, molar ratio and ratio based on pharmacokinetics, which could lead to different outcomes, we have chosen mass ratio due to the observed effect of only CPCOOH and since all the other work presented in this study is based on mass concentration. Moreover, the EC_{50} values of individual CPCOOH (17.1 mg L^{-1}) and mixture (11.5 mg L^{-1}) were in the same concentration range, indicating that only mild potentiation effect occurred by the tested mixture. Our results revealed similar "something from nothing" effect, a phenomenon that was already described in literature for e.g. weak estrogenic chemicals (Silva et al., 2002).

3.2. Genotoxicity of individual compounds and the mixture

CP and IF are prodrugs that require cytochrome P450 mediated transformation to nucleophylic intermediates that alkylate DNA molecule (Sladek, 1988). Therefore genotoxic properties of CP, IF, their metabolites/TPs and the mixture were assessed using SOS/*umu*C assay (Reifferscheid et al., 1991; Schmid et al., 1997; Yasunaga et al., 2006) in the absence (-S9) and presence (+S9) of metabolic activation. The results are presented in Table 3. Within the selected concentration range, none of the tested compounds/mixture showed cytotoxic effects (G values were >0.75; Table 3). Of the two parent compounds, only CP exhibited significant genotoxic activity in the presence of S9 at the highest concentration tested (IR = 2.72 ± 0.55), which indicates that metabolic activation led to the formation of genotoxic metabolites. This is in accordance with the published studies addressing CP genotoxicity using Ames test, where CP was mutagenic only in the presence of S9 metabolic activation (Benedict et al., 1977; Hales, 1982; Winckler et al., 1984; Yasunaga et al., 2006). On the contrary to our results, Benedict et al. (1977) reported mutagenic activity also for IF in the presence of S9, however compared to CP it was significantly less potent. Of the three investigated metabolites/TPs, only CPCOOH exhibited significant genotoxic activity in the absence (2.75 ± 0.12) and the presence (2.04 ± 0.47) of metabolic activation (Table 3). This partially agrees with the study by Winckler et al. (1984), who investigated mutagenic activities of CP and its main metabolites (including ketoCP and CPCOOH) with the Ames test. The authors report higher genotoxic potential of CPCOOH and similar genotoxic potential for ketoCP, if compared to CP, regardless of the metabolic activation. In our study, higher genotoxic potential was observed when CPCOOH was tested in the absence of metabolic activation (2.75 *vs.* 2.04; Table 3), whereas, Winckler et al. (1984) reported that all CP metabolites tested in the Ames assay, including CPCOOH, were activated by S9 mix to a higher mutagenic potential.

The finding that CPCOOH was active also without metabolic activation in the SOS/*umu*C test may explain the ecotoxic activity of CPCOOH towards *S. leopoliensis*. Cyanobacteria do not express cytochrome P450 enzymes. Consequently CP and IF were not transformed to nucleophylic intermediates that react with DNA and suppress cell division, which rendered cynobacteria unsusceptible to the exposure to the parent compounds.

The mixture of the two parent compounds and their three metabolites at the concentrations tested without metabolic activation revealed no significant genotoxic activity, which is in agreement with the negative results of individual compounds. Only CPCOOH exerted genotoxic effects in the absence of S9; however at much higher concentrations. On the other hand in the presence of the metabolic activation the mixture revealed significant genotoxic activity at the highest concentration tested (the concentration of each compound in the mixture was 37.2 mg L^{-1}). This result can be correlated with the outcomes obtained with individual compounds, where CP and CPCOOH had genotoxic activity. However, the concentrations of individual CP and CPCOOH with IR factor >2 were 320 mg L^{-1}, while the highest concentration of each individual compound in the mixture was 37.2 mg L^{-1}, hence, nearly 10-fold lower. This suggests that in the metabolically activated mixture the inactive components were either transformed to genotoxic metabolites or they potentiated genotoxicity of CP and CPCOOH.

Table 3
The genotoxicity of the investigated compounds and their mixture *via* the induction of SOS response in *S. typhimurium* TA 1535/pSK1002 in the presence (+S9) and in the absence (S-9) of metabolic activation.

Comp.	Conc. (mg L^{-1})	- S9		+ S9	
		G^a ± SD	IR^b ± SD	G^a ± SD	IR^b ± SD
C1[c]	0	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.03
C2[d]	0	1.00 ± 0.04	1.00 ± 0.13	1.00 ± 0.05	1.00 ± 0.14
AFB1	5	/	/	0.73 ± 0.13	**2.57 ± 0.33**
NQNO	1.14	0.91 ± 0.03	**3.95 ± 0.11**	/	/
CP	3.05	1.09 ± 0.02	1.01 ± 0.04	1.07 ± 0.02	0.90 ± 0.10
	9.7	1.03 ± 0.02	1.00 ± 0.05	1.01 ± 0.03	1.04 ± 0.03
	31.2	1.00 ± 0.02	0.97 ± 0.06	1.03 ± 0.07	0.88 ± 0.21
	100	1.02 ± 0.05	0.95 ± 0.06	0.98 ± 0.03	0.94 ± 0.03
	320	1.01 ± 0.24	1.01 ± 0.04	0.80 ± 0.03	**2.72 ± 0.55**
IF	3.05	1.01 ± 0.03	1.02 ± 0.07	1.09 ± 0.12	1.04 ± 0.13
	9.77	1.02 ± 0.07	0.95 ± 0.12	1.02 ± 0.05	1.10 ± 0.33
	31.25	1.02 ± 0.07	0.99 ± 0.02	0.97 ± 0.07	1.57 ± 0.59
	100	0.98 ± 0.03	1.03 ± 0.03	0.97 ± 0.04	1.42 ± 0.62
	320	0.98 ± 0.02	1.04 ± 0.07	0.93 ± 0.05	1.33 ± 0.36
CPCOOH	3.05	0.91 ± 0.03	1.09 ± 0.05	1.08 ± 0.08	1.01 ± 0.24
	9.77	0.89 ± 0.04	1.10 ± 0.09	1.02 ± 0.02	1.05 ± 0.18
	31.25	0.87 ± 0.01	1.26 ± 0.01	1.00 ± 0.02	1.08 ± 0.19
	100	0.87 ± 0.01	1.56 ± 0.05	0.94 ± 0.05	1.15 ± 0.24
	320	0.86 ± 0.01	**2.75 ± 0.12**	0.82 ± 0.04	**2.04 ± 0.47**
ketoCP	3.05	0.93 ± 0.02	1.17 ± 0.04	1.11 ± 0.06	0.73 ± 0.19
	9.77	0.94 ± 0.02	1.05 ± 0.08	1.10 ± 0.04	0.72 ± 0.33
	31.25	0.93 ± 0.02	1.04 ± 0.06	0.96 ± 0.00	0.96 ± 0.08
	100	0.96 ± 0.01	0.96 ± 0.08	1.01 ± 0.02	0.78 ± 0.05
	320	0.94 ± 0.06	1.04 ± 0.15	0.93 ± 0.04	0.89 ± 0.18
NdCP	3.05	0.96 ± 0.04	1.08 ± 0.03	1.02 ± 0.04	1.08 ± 0.31
	9.77	0.95 ± 0.04	1.17 ± 0.05	1.02 ± 0.06	0.96 ± 0.33
	31.25	0.92 ± 0.04	1.23 ± 0.06	1.07 ± 0.03	1.03 ± 0.13
	100	0.94 ± 0.01	1.13 ± 0.02	1.11 ± 0.04	0.87 ± 0.25
	320	0.91 ± 0.02	1.12 ± 0.03	1.07 ± 0.04	1.03 ± 0.32
mixture[e]	6.0	1.05 ± 0.04	1.04 ± 0.04	1.01 ± 0.08	1.54 ± 0.54
	7.9	1.07 ± 0.05	1.17 ± 0.09	0.98 ± 0.05	1.75 ± 0.13
	10.5	1.08 ± 0.04	1.15 ± 0.06	1.05 ± 0.06	1.72 ± 0.21
	17.1	1.00 ± 0.02	1.23 ± 0.11	1.03 ± 0.04	1.70 ± 0.57
	37.2	0.99 ± 0.03	1.35 ± 0.08	1.00 ± 0.07	**2.18 ± 0.49**

Note a: G = bacterial growth rate; G < 0.75 is considered as toxic for bacteria.
Note b: IR = induction ratio; IR > 2 is considered as genotoxic response.
Note c: control used for the calculation of genotoxicity of the metabolites/TPs (NdCP, CPCOOH, ketoCP).
Note d: control used for calculation of the genotoxicity of the CP, IF and mixture.
Note e: the indicated concentrations are referred to the concentration of each of each of the individual compound in the mixture.

3.3. Degradation and ecotoxicity of CP and IF during UV treatment

This study also investigated the ecotoxicity of CP and IF mixture during UV irradiation. Prior to UV irradiation of CP and IF, to determine whether or not the degradation products exert a different toxicity from that of the parent compounds, the toxicities of both compounds individually and in a mixture were compared. Since there were no statistical differences in growth inhibition when testing compounds either individually or as a mixture (SM-4), both compounds were UV-irradiated simultaneously. In

addition, in the control experiment (48 h, no UV irradiation) degradation of CP and IF was negligible and there was no significant change in ecotoxicity (SM-5).

The results of the degradation study of CP and IF mixture showed that both compounds were degraded during UV irradiation, resulting in 69.4 ± 3.6% and 62.9 ± 2.3% final removal of CP and IF, respectively (Fig. 3).

When exposed to UV irradiation, CP and IF concentration decreased exponentially with time (0–48 h) and followed pseudo-first-order kinetics. The degradation rate constants, half-lives ($t_{1/2}$) and the R^2 of the degradation curves for CP were 0.024 h^{-1}, 28.8 h and 0.975, respectively. Similarly, for IF, the degradation rate constants, $t_{1/2}$ and the R^2 were 0.020 h^{-1}, 34.66 h and 0.937, respectively.

The DOC analysis of UV-irradiated samples revealed no significant decrease in DOC over the 48 h. This suggests that UV degradation failed to mineralize both compounds to CO_2 and H_2O. Instead, it was assumed that only partial degradation was achieved, resulting in the formation of TPs. The GC/MS analysis revealed that no investigated human metabolites/TPs within this study were formed upon UV irradiation experiment or their presence was below LODs (SM-6). Similar recent studies by Lutterbeck et al. (2015) and Lai et al. (2015) report the identification of CP and IF TPs during similar photo-assisted advanced oxidation processes, namely UV/H_2O_2 and UV/TiO_2, respectively, with the accompanied toxicity evaluation of the treated samples. Both studies proposed TPs formation pathways, where among all identified TPs, ketoCP and NdCP are reported, whereas CPCOOH was not detected/identified as a TP. Due to the similarity of these treatments with the UV irradiation performed within this study, we assume that ketoCP and NdCP were formed also during UV irradiation, but were below the detection limit of the method.

The ecotoxicity evaluation of the UV irradiated CP and IF mixture samples towards *S. leopoliensis* showed no growth inhibition (Fig. 3). This agrees with the findings of a recent study by Lutterbeck et al. (2015), who also report no significant toxicity of treated samples containing CP towards *Vibrio fischeri*, where CP removals were up to 100%. Similarly, the study by Lai et al. (2015) reported no final toxicity (after the initial increase) towards *V. fischeri* of the samples containing either CP or IF, treated with UV/TiO_2. This is in agreement with the results of this study regarding the toxicity of individual compounds, where parent compounds as well as metabolites/TPs ketoCP and NdCP were not toxic towards the test organism. In a study where ozonation was used to degrade CP and IF, the authors report high removal of both compounds (up to 100% at pH = 11), accompanied by a significant increase in toxicity towards *V. fischeri* (Lin et al., 2015). This study did not identify the TPs that were formed during ozonation. Since it was shown that CPCOOH is capable of growth inhibition of the test organism in the present study, it is possible that this TP was formed during the ozonation treatment and contributed to the overall toxicity as Lin et al. (2015) reported. However, further studies are necessary to confirm these assumptions.

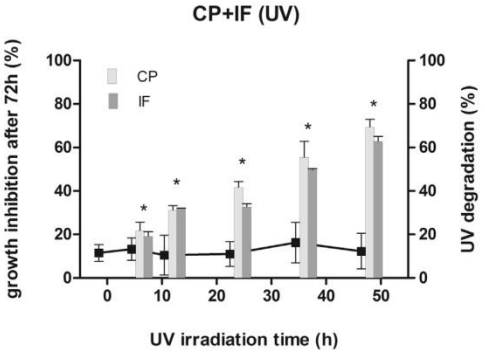


Fig. 3. Growth inhibition (black line with ■, representing the mean ± 95% confidence interval) and degradation of CP and IF (mean ± SD, both at 10 mg L^{-1}) during UV treatment. * indicates statistically significant difference of CP and IF degradation at determined sampling time compared to control (0 h).

4. Conclusions

Within the present study, we investigated the ecotoxicity and genotoxicity of CP, IF, their metabolites/TPs as individual compounds and as mixture. With the exception of human metabolite/TP, CPCOOH, which revealed significant ecotoxic activity towards *S. leopoliensis*, all other compounds, namely CP, IF, ketoCP and NdCP, were non-toxic at the investigated concentrations. In accordance with the EU Directive 93/67/EEC (1996) CPCOOH can be classified as harmful to aquatic organisms. These findings underline the importance of ecotoxicity evaluation, not only for the parent compounds, but also for their structurally related human metabolites/TPs. Furthermore, the toxicity of investigated parent compounds and metabolites/TPs as a mixture suggests potentiation ecotoxic effects. The CA model predicted lower ecotoxicity of the mixture than observed within the performed experiments, which shows the importance of toxicity assessment of the mixture. The genotoxicity evaluation of the tested compounds and their mixtures with the SOS/*umu*C assay confirmed genotoxic activity of CP in the presence of the metabolic activation, whereas CPCOOH was active also without metabolic activation. Considering the mechanism of toxicity of CP and IF the latter finding indicate that the metabolite CPCOOH can directly interact with DNA and consequently in *S. leopoliensis* only CPCOOH caused growth inhibition. For the environmental risk assessment this is particularly important finding which indicates that for the phytoplankton this metabolite is more hazardous than parent compounds. Finally, despite significant degradation of CP and IF, the UV irradiated samples were not toxic towards investigated organism suggesting that unknown TPs formed during UV irradiation are non-ecotoxic and therefore further structural elucidation of these species is of minor importance.

The findings of the present study indicate that to carry out a proper environmental risk assessment further studies, especially on the occurrence of CPCOOH in the environment, are needed. Current pesticides regulations for risk assessment regarding soil already take into account metabolites/TPs, since certain TPs are more toxic than the parent compounds (European Food Safety Authority, 2014). Based on the results of this study, the same guidance for evaluating risk should be established for the cytostatic and other pharmaceutical residues that might occur in the aquatic environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.12.017.

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4 Conclusions

This thesis has contributed to our understanding of the occurrence, treatment technologies and effects of two important cytostatic drugs: cyclophosphamide and ifosfamide together with their human metabolites and TPs. The work is reported in four scientific papers, published in SCI international journals, and was presented at 11 national and international conferences, either as oral (5) or poster (9) presentations (see Appendix). The major conclusions of this work are the following:

☑ Analytical methods for trace analysis of CP, IF and their selected human metabolites/TPs, namely carboxy-CP, keto-CP and N-decl-CP, in WW and/or natural water were developed, optimized and validated:

Although one of the main goals was to develop a single analytical method for all target compounds in WW, optimization was achieved using different SPE sorbents: one for CP, IF and keto-CP and the other for carboxy-CP and N-decl-CP. Similarly, two different derivatization processes were applied. Cyclophosphamide and IF were derivatized by acetylation and metabolites/TPs with silylation. The analytical procedure for quantitative determination of metabolites/TPs in WW was reported for the first time.

☑ The performance of the analytical method for the analysis of CP and IF in various aqueous matrices (i.e. hospital WWs, WWTP influents and effluents and SW) was evaluated within an interlaboratory comparison exercise:

This was the first such interlaboratory comparison to include cytostatic compounds. The repeatability of developed method for CP and IF determination was satisfactory, whereas the reproducibility of participants was poor for all tested matrices. Application of the Youden approach to the data reported for CP and IF in the surface and WWTP effluent samples revealed three laboratories with total errors for both compounds and two laboratories with random errors for CP.

☑ The methods were successfully applied to the analysis of WW from five Slovene hospitals and municipal WWTPs influents and effluents:

CP and IF were detected in measurable concentrations in two out of five hospital effluents. Cyclophosphamide was also detected in samples of WWTP influent and effluent, whereas the concentrations of IF in all sampled WWTP influents and effluents were below the LODs. Hospital WW sampled daily for a period of one week revealed that the compound present in the highest concentrations was metabolite/TP carboxy-CP and in the lowest IF. In addition, the concentrations of all analytes were the highest from Tuesday until Friday in hospital WW and were below

LODs in samples taken at WWTP. These outcomes partially confirmed my first hypothesis since CP was detected also in municipal WW (*H1)*.

☑ Cyclophosphamide and IF removal efficiency of an advanced biological treatment incorporating attached-growth biomass on Mutag^{TM} carriers and various abiotic treatments including UV, ozone and HC with/without the addition of H_2O_2 was assessed. Finally, the removal by sequentially coupled biological and selected abiotic treatment was evaluated:

Advanced biological treatment and HC alone and in combination with H_2O_2 only partially removed CP and IF from WW. Increasing the amount of H_2O_2 did improve the removal efficiency in case of UV/H_2O_2 compared to UV treatment, while the opposite phenomenon was observed for ozonation. The highest removal efficiency was obtained by UV/O_3/H_2O_2 (at 5 g L^{-1}) treatment (> 94 % for CP and IF). When this treatment was sequentially coupled to biological treatment, removal efficiency was even higher, > 99 % for both compounds. These results confirmed my second hypothesis (*H2)*.

☑ Transformation products formed during UV and UV/H_2O_2 treatment were studied:

Within this study, several TPs were identified during UV and UV/H_2O_2 treatment, among which three are new to science: CP-TP138a, imino-ifosfamide and IF-TP138. It was confirmed that TPs formed under abiotic treatment can be structural analogues to human metabolites. The formation pathways for IF TPs during investigated treatments were proposed for the first time.

☑ The ecotoxicity and genotoxicity of CP, IF, studied human metabolites/TPs, (carboxy-CP, keto-CP and N-decl-CP), their mixtures and UV-treated samples containing parent compounds were evaluated:

Only carboxy-CP was found to be harmful to aquatic organisms at the concentrations tested. Furthermore, the investigated mixture was ecotoxic and genotoxic with an observed potentiation effect confirming the third hypothesis (*H3)*. UV treatment had no effect on overall ecotoxicity.

Future work should include the optimization of the most promising treatment technology in terms of CP and IF removal, i.e. UV/O_3/H_2O_2, where the main focus represents the scale-up and cost-efficiency evaluation of this treatment. Both compounds are recalcitrant to biological degradation and abiotic treatments when compared to other APIs in hospital WW suggesting that other organic micropollutants could be successfully removed under the applied treatment conditions. Based on the toxicity of carboxy-CP, a CP metabolite/TP, this compound should be monitored in SWs and considered to be included in an ERA.

The work presented within this thesis contributes to a better understanding of the cycling and effects of CP and IF residues in the environment. However, to obtain a broader picture of the environmental issues related to other antineoplastics, other representative compounds should be similarly investigated.

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7 Appendix

International and national working experiences, learning courses and achievements:

- Seminar: Practical Essentials of Gas Chromatography & GC-MS (Anthias Consulting Ltd, Ljubljana, Slovenia, 28th – 30th November 2011)
- Seminar: Vodenje tehnološkega procesa čiščenja odpadne vode (WWTP Domžale-Kamnik, Domžale, Slovenia, 19th – 20th September 2012)
- Summer school: 9th Summer School of Environmental Chemistry and Ecotoxicology (RECETOX, Masaryk University, Brno, Czech Republic, 24th – 29th June 2013)
- Seminar: Interpretation of MS and MS-MS Mass Spectra (Instrumentaila d.d., Zagreb, Croatia, 19th – 20th November 2013)
- Seminar: Method Development in Quantitative Analysis using LC-MS (Instrumentalia d.d., Zagreb, Croatia, 21st – 22nd November 2013)
- Finalist in competition for European Young Chemist Award 2014 at the EuCheMS Chemistry Congress 2014, where oral presentation entitled "The occurrence and removal of cytostatics cyclophosphamide and ifosfamide in wastewaters" was given (Istanbul, Turkey, 3rd September 2014)
- Training at CWQRC (Curtin Water Quality Research Centre, Curtin University, Perth, Australia, January 2015 – April 2015, 3 months)

Personal Bibliography:

1.01 Original scientific article

1. Heath, E., Česen, M., Negreira, N., de Alda, M. L., Ferrando-Climent, L., Blahova, L., et al. (2015). First inter-laboratory comparison exercise for the determination of anticancer drugs in aqueous samples. *Environmental Science and Pollution Research*, 1-13.

2. Česen, M., Eleršek, T., Novak, M., Žegura, B., Kosjek, T., Filipič, M., et al. (2016). Ecotoxicity and genotoxicity of cyclophosphamide, ifosfamide, their metabolites/transformation products and their mixtures. *Environmental Pollution*, 210, 192-201.

3. Česen, M., Kosjek, T., Busetti, F., Kompare, B., & Heath, E. (2016). Human metabolites and transformation products of cyclophosphamide and ifosfamide: analysis, occurrence and formation during abiotic treatments. *Environmental Science and Pollution Research International*, 1-15.

4. Česen, M., Kosjek, T., Laimou-Geraniou, M., Kompare, B., Širok, B., Lambropolou, D., et al. (2015). Occurrence of cyclophosphamide and ifosfamide in aqueous environment and their removal by biological and abiotic wastewater treatment processes. *Science of The Total Environment*, 527–528(0), 465-473.

1.08 Published scientific conference contribution

5. ČESEN, Marjeta, KOSJEK, Tina, KOMPARE, Boris, HEATH, Ester. Removal of the cytostatics cyclophosphamide and ifosfamide by advanced biological and oxidation processes. V: Slovenski kemijski dnevi 2014, Maribor, 11.-12. september 2014. KRAVANJA, Zdravko (ur.), BOGATAJ, Miloš (ur.), NOVAK-PINTARIČ, Zorka (ur.). *Slovenski kemijski dnevi 2014, Maribor, 11. - 12. september 2014*. Maribor: Fakulteta za kemijo in kemijsko tehnologijo, 2014, 10 str. [COBISS.SI-ID 27926567]

6. ČESEN, Marjeta, KOSJEK, Tina, HEATH, Ester. First worldwide interlaboratory study on cytostatic compounds in aqueous samples. V: 6. študentska konferenca Mednarodne podiplomske šole Jožefa Stefana = 6th Jožef Stefan International Postgraduate School Students' Conference, 20.-22. 05. 2014, Ljubljana. TRDIN, Nejc (ur.), et al. *Zbornik : 1. del = Proceedings : part 1*. Ljubljana: Mednarodna podiplomska šola Jožefa Stefana, 2014, str. 13-22. http://ipssc.mps.si/2014/proceedingsIPSSC2014Papers.pdf. [COBISS.SI-ID 27732519]

7. ČESEN, Marjeta, KOSJEK, Tina, HEATH, Ester. Degradation of cytostatics cyclophosphamide, ifosfamide and their human metabolites by UV irradiation. V: KRAVANJA, Zdravko (ur.), BRODNJAK-VONČINA, Darinka (ur.), BOGATAJ, Miloš (ur.). *Slovenski kemijski dnevi 2013, Maribor, 10.-12. september 2013*. Maribor: Fakulteta za kemijo in kemijsko tehnologijo, 2013, 10 str. [COBISS.SI-ID 27380775]

8. ČESEN, Marjeta, KOSJEK, Tina, HEATH, Ester. Optimization of derivatization process for human metabolites of common cytostatics cyclophosphamide and ifosfamide. V: 5. študentska konferenca Mednarodne podiplomske šole Jožefa Stefana = 5th Jožef Stefan International Postgraduate School Students Conference, 23. maj 2013, Ljubljana, Slovenija. TRDIN, Nejc (ur.), et al. *Zbornik = Proceedings*. Ljubljana: Mednarodna podiplomska šola Jožefa Stefana, 2013, str. 4-14. [COBISS.SI-ID 26776103]

9. ČESEN, Marjeta, KOSJEK, Tina, HEATH, Ester. Cytostatics cyclophosphamide and ifosfamide - do they occur in Slovene wastewaters and surface waters?. V: 4. študentska konferenca Mednarodne podiplomske šole Jožefa Stefana = 4th Jožef Stefan International Postgraduate School Students Conference, 25. maj 2012, Ljubljana, Slovenija. PETELIN, Dejan (ur.), TAVČAR, Aleš (ur.), KALUŽA, Boštjan (ur.). *Zbornik = Proceedings*. Ljubljana: Mednarodna podiplomska šola Jožefa Stefana, 2012, str. 9-14. [COBISS.SI-ID 25832743]

1.10 Published scientific conference contribution abstract (invited lecture)

10. HEATH, Ester, KOSJEK, Tina, ČESEN, Marjeta, ŠČANČAR, Janez, ŽONJA, Božo, NEGREIRA, Noelia, LOPEZ DE ALDA, Miren, BARCELÓ, Damià. CytoThreat and its contribution to our understanding of the fate of cytostatic pharmaceuticals in the

environment. V: SABATER, Laila (ur.), BARCELÓ, Damià (ur.). *Pharmaceuticals in wastewaters and surface waters under multistressors situation : fate, adverse effects, risks and removal technologies : [book of abstracts of the] Globaqua-Cytothreat-Endetech-Scarce Workshop, 2nd-3rd December 2014, Barcelona, Spain*. [S. l.: s. n.], 2014, str. 26. [COBISS.SI-ID 28194087]

1.12 Published scientific conference contribution abstract

11. ČESEN, Marjeta, KOSJEK, Tina, BUSETTI, Francesco, HEATH, Ester. Cyclophosphamide and ifosfamide residues in aqueous environment : occurrence, treatment and toxicity. V: 15th EuCheMS International Congress of Chemistry and Environment, ICCE 2015, 20-24 September 2015, Leipzig, Germany. *Book of abstracts*. [S. l.: s. n.], 2015, str. 143-144. [COBISS.SI-ID 28899623]

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MONOGRAPHS AND OTHER COMPLETED WORKS

2.11 Undergraduate thesis

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