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Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain☆,☆☆

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ABSTRACT

Anticancer drugs are continuously released into hospital and urban wastewaters, where they, most commonly, undergo conventional treatment in wastewater treatment plants (WWTPs). Wastewaters contain complex mixtures of substances including parent compounds, their metabolites and transformation products (TPs). In this study, samples of hospital effluents and WWTP influents and effluents from Slovenia and Spain were analyzed for twenty-two selected anticancer drugs, their metabolites and transformation products. Acute and chronic toxicity tests were performed on the crustacean *Ceriodaphnia dubia*, genotoxicity was determined with *Tradescantia* and *Allium cepa* micronucleus (MN) assays and *in vitro* comet assay in zebrafish (*Danio rerio*) liver cell line (ZFL cells). Sixty of the two hundred-twenty determinations revealed detectable levels of anticancer drug residues. Among the targeted compounds, platinum based were most frequently detected (90%). Furthermore, erlotinib was detected in 80%, cyclophosphamide and tamoxifen in 70% and methotrexate in 60% of the samples. Seven of ten samples were toxic to *C. dubia* after acute exposure, whereas after chronic exposure all samples reduced reproduction of *C. dubia* at high sample dilutions. *Allium cepa* proved insensitive to the potential genotoxicity of the tested samples, while in *Tradescantia* increased MN frequencies were induced by a hospital effluent and WWTP influents. In ZFL comet assay all but one sample induced a significant increase of DNA strand breaks. Correlations of chemotherapeutics or their TPs were detected for all bioassays except for *Allium cepa* genotoxicity test, however for each test the highest correlations were found for different substances indicating differential sensitivities of the test organisms.

1. Introduction

The presence of anticancer drugs in the aquatic environment has prompted significant interest concerning their potential adverse ecological effects. After administration to patients the drugs are excreted through faeces and urine as mixtures of unchanged parent compounds and their metabolites and can enter the aquatic environment predominantly *via* treated and untreated

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hospital and municipal wastewaters. These excreted mixtures of parent compounds and metabolites may undergo further abiotic and/or biotic transformation, either during wastewater treatment or in the environment. Recent scientific interest has focused especially on occurrence and fate of anticancer drugs, their metabolites and transformation products (TPs) in aquatic systems (Kosjek et al., 2013; Martín et al., 2014; Negreira et al., 2014a; Česen et al., 2015, 2016a).

Anticancer drug residues occur in the aquatic environment at sub-ng L^{-1} levels (Xie, 2012), which are too low to pose an immediate threat to aquatic organisms, but can cause long-term delayed toxic effects since they interfere directly or indirectly with DNA. Recently, Brezovšek et al. (2014) showed that 5-fluorouracil and cisplatin cause chronic effects in green algae (*Pseudokirchneriella subcapitata*), resulting in growth inhibition at concentrations equivalent to those found in hospital effluents. Chronic exposure to these drugs was also shown to inhibit reproduction in crustaceans (Parrella et al., 2014a). At chronic exposure, 5-fluorouracil produced in zebrafish histopathological changes, and genotoxic effects at environmental concentrations (Kovacs et al., 2015). It is crucial, therefore, to consider the possible toxic/genotoxic effects in organisms exposed over their life span to the continuous presence and possible accumulation of not just the parent anticancer drugs, but also their metabolites and transformation products (Toolaram et al., 2014; Česen et al., 2016b).

Hospital wastewaters are a major source of anticancer drugs. Usually these waters are not treated at source, but are discharged directly into the sewerage system, finally arriving at a wastewater treatment plant (WWTP) (Ferk et al., 2009; Verlicchi et al., 2012; Zhang et al., 2013; Česen et al., 2015). In addition, urban wastewaters receive a substantial contribution of excreted anticancer drugs as the result of outpatient treatment (Ferrando-Climent et al., 2013). Studies reveal that conventional treatments do not achieve high removal efficiencies for these compounds, which are in many cases resistant to biodegradation (Zhang et al., 2013; Ferrando-Climent et al., 2014; Martín et al., 2014; Orias and Perrodin, 2014). Thus, the likelihood of pharmaceuticals and their residues remaining active after release from WWTPs, and reaching surface waters is high (Rowney et al., 2009; Besse et al., 2012; Johnson et al., 2013).

The aims of this study were to evaluate, in two sampling campaigns, the occurrence of twenty-two selected anticancer drug residues including their metabolites and transformation products (from this point onwards collectively named as TPs) in hospital effluents (a Slovenian oncological clinic and a Spanish general hospital) and municipal WWTP influents and effluents from the same two countries that differ in terms of water resources, WWTP technology and water reuse. Moreover, to investigate the relationship between the occurrence of anticancer drugs in wastewater samples and their possible biological and ecological effects, a multispecies toxicological evaluation was performed on some indicators, followed by a correlation analysis. Chemical characterisation was performed using chromatography coupled to mass spectrometry (GC-MS and LC-MS/MS), while the toxicological evaluation was performed using the following test systems: acute and chronic aquatic toxicity tests in the crustacean *Ceriodaphnia dubia*, a very sensitive primary consumer of the freshwater aquatic chain; micronucleus (MN) assays in Tradescantia and *Allium cepa* as representatives of higher plants and an *in vitro* cytotoxicity test and a comet assay for genotoxicity using zebrafish (*Danio rerio*) liver cell line (ZFL cells) as a model for vertebrates. These bioindicators are extensively used to investigate the whole toxicity of chemicals in the environment. These organisms are sensitive to a wide range of aquatic contaminants and allow to address the biological effects of chemicals on different organizational structures.

2. Materials and methods

2.1. Chemicals and standards

The following compounds were determined in the wastewater samples: cisplatin (*cis*-Pt, as total Pt), 5-fluorouracil (5-FU, CAS 51-21-8), cyclophosphamide (CP, CAS 50-18-0), ifosfamide (IF, CAS 3778-73-2), keto-cyclophosphamide (keto-CP, CAS 27046-19-1), 2-dechloroethylifosfamide or *N*-dechloroethylcyclophosphamide (*N*-decl-CP, CAS 36761-83-8), carboxy-cyclophosphamide (carboxy-CP, CAS 22788-18-7), capecitabine (CAP, CAS 154361-50-9), doxorubicin (DOX, CAS 23214-92-8), erlotinib (ERL, CAS 183321-74-6), etoposide (ETP, CAS 33419-42-0), gemcitabine (GEM, CAS 95058-81-4), imatinib mesylate (IMA, CAS 220127-57-1), irinotecan (IRI, CAS 97682-44-5), methotrexate (MET, CAS 59-05-2), hydroxymethotrexate (OH-MET, CAS 5939-37-7), paclitaxel (PAC, CAS 33069-62-4), 6(α)-hydroxypaclitaxel (OH-PAC, CAS 153212-75-0), tamoxifen (TAM, CAS 10540-29-1), endoxifen or 4-hydroxy-*N*-desmethyl-tamoxifen (OH-D-TAM, CAS 112093-28-4), (Z)-4-hydroxytamoxifen (OH-TAM, CAS 68047-06-3) and temozolomide (TMZ, CAS 85622-93-1). Limits of detection (LOD) and quantification (LOQ) are shown in Table 1.

2.1.1. Cyclophosphamide, ifosfamide and their TPs

Cyclophosphamide (99%) and IF (99%) were purchased from Sigma Aldrich (Hong Kong, China). Carboxy-CP, 4-keto-CP, *N*-decl-CP and deuterated cyclophosphamide (CP-d_6,; CAS 951173-63-0) used as internal standard for CP and IF analysis were obtained from Niomech - IIT GmbH (Bielefeld, Germany). The deuterated ibuprofen (IB-d_3, CAS 121662-14-4), obtained from CDN Isotopes (Quebec, Canada), was used as internal standard for the analysis of TPs. The derivatizing agent trifluoroacetic anhydride (TFAA, 99%) was purchased from Fluka (Buchs, Switzerland) and *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA with 1% TBDMCS, 95%) was purchased from Sigma Aldrich (Steinheim, Germany). All solvents were of analytical grade purity.

2.1.2. Fluorouracil

Fluorouracil (CAS 51-21-8; ≥99%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Internal standard 5-FU-d_6 (CAS 90344-84-6; 98%) was purchased from LGC Standards GmbH (Wesel, Germany) while 5-Chlorouracil (5-CU, CAS 1820-81-1; 98%) was obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). The agent used for derivatization, MTBSTFA was purchased from Acros Organics (Geel, Belgium). All solvents were of analytical grade purity.

2.1.3. Pt

Merck stock Pt solution (1000 μg Pt mL^{-1} in 8% hydrochloric acid; HCl) was diluted daily with water for the preparation of fresh calibration standard solutions that were used for the determination of the total concentrations of Pt in the samples. All chemicals were of analytical reagent grade and acids of suprapure quality (Merck, Darmstadt, Germany). All water used was of ultrapure quality (18.2 MΩ cm, Direct-Q 5 Ultrapure water system, Millipore Watertown, MA, USA).

2.1.4. Multi-target analysis of 15 anticancer drugs and TPs

Capecitabine, DOX hydrochloride, OH-D-TAM, ERL hydrochloride, ETP, GEM hydrochloride, IRI hydrochloride trihydrate, OH-MET, OH-PAC, OH-TAM, IMA mesylate, MET, TAM citrate and TMZ were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and PAC was supplied by Aldrich (Milwaukee, WI, USA) at the highest available purity (>99%). The isotopically labeled

Table 1
Abbreviations for substances analyzed in wastewater samples (H from hospital, Wi WWTP influent, We WWTP effluent) and the limits of detection (LOD) and quantification (LOQ).

Abbreviation	Substance	LOD (ng/L)		LOQ (ng/L)	
		We	H/Wi	We	H/Wi
5-FU	5-fluorouracil	0.5	0.5	1.6	1.6
CAP	capecitabine	0.5	0.7	3.5	5.0
Carboxy-CP	carboxy-cyclophosphamide	23	23	78	78
CP	cyclophosphamide	2.3	2.3	7.7	7.7
DOX	doxorubicin	0.7	0.8	2.4	2.5
ERL	erlotinib	0.9	0.5	3.0	1.7
ETP	etoposide	12	20	40	65
GEM	gemcitabine	0.7	0.7	9.3	9.3
IF	ifosfamide	4.8	4.8	16	16
IMA	imatinib	36	54	120	180
IRI	irinotecan	0.4	1.4	1.2	4.5
Keto-CP	keto-cyclophosphamide	13	13	44	44
MET	methotrexate	0.5	0.6	1.8	2.0
N-decl-CP	2-dechloroethylifosfamide or *N*-dechloroethylcyclophosphamide	2.0	2.0	6.7	6.7
OH-D-TAM	endoxifen or 4-hydroxy-*N*-desmethyl-tamoxifen	1.5	1.5	5.0	5.0
OH-MET	hydroxymethotrexate	1.3	1.6	4.3	5.2
OH-PAC	6(α)-hydroxypaclitaxel	1.1	1.1	3.6	3.6
OH-TAM	(Z)-4-hydroxytamoxifen	0.3	0.7	1.1	5.0
PAC	paclitaxel	1.2	1.3	4.0	4.4
Pt	platinum	1.0	1.0	2.0	2.0
TAM	tamoxifen	0.9	1.0	3.0	3.4
TMZ	temozolomide	1.0	1.1	9.3	9.3

standards capecitabine-d_{11}, gemcitabine-$^{13}C^{15}N_2$ hydrochloride, erlotinib-d_6 hydrochloride, etoposide-d_3, 7-hydroxymethotrexate-d_3, 4-hydroxy-ethyl-tamoxifen-d_5, 4-hydroxy-*N*-desmethyl-tamoxifen-d_5, 6α-hydroxypaclitaxel-d_5, irinotecan-d_{10} hydrochloride, methotrexate-methyl-d_3, *N*-desmethyl-imatinib-d_8, paclitaxel-d_5 and temozolomide-d_3 were purchased from Santa Cruz Biotechnology.

2.2. Sampling

For the purpose of the present study, ten wastewater samples were collected in total, five in Ljubljana (Slovenia) and five in Barcelona (Spain). Two types of samples were collected in January 2014 in Ljubljana (L) and Barcelona (B), namely, hospital effluents (H) and wastewater treatment plant influents (Wi) (Table 2). The next sampling campaign, performed in both countries in June 2014, included three sites: H, Wi, and wastewater treatment plant effluents (We, Table 2). The coding and sampling details are presented in Table 2. The Slovenian hospital under investigation is a medium-sized specialised oncological clinic with 290 beds, 13,000 admissions and 110,000 outpatients per year, with a hydraulic load of 50–60 m^3/day. The Spanish hospital is a large general hospital including cancer treatment ward with 850 beds, 41,000 admissions and 465,000 outpatients per year, with a hydraulic load of 400 m^3/day. Water treatment technologies receiving wastewater from hospitals in Slovenia and Spain included mechanical and conventional biological treatment with suspended biomass. The Slovenian wastewater treatment plant is designed for 360,000 population equivalents (PE) with an average load/inflow of 80,000 m^3 wastewater entering the WWTP per day. The sludge retention time is 7 days, while the hydraulic retention time is 21 h. The average biomass concentration in the biological tank is 3.2 g L^{-1}. The Spanish wastewater treatment plant is designed for 1,700,000 PE with an average load/inflow of 234,000 m^3 of wastewater entering the WWTP on a daily basis. The sludge retention time is 15–20 days, while the hydraulic retention time is 8–12 h. The average biomass concentration in the biological tank is 3.5–4.0 g L^{-1}. All collected samples (between 5 and 10 L) were filtered (0.5 μm glass fibre filters) and frozen immediately after collection. Shipment took place 2 days after sample collection. All samples were received at final destination frozen and were either defrosted and analyzed or stored frozen for no longer than 2 months prior to analysis, period

Table 2
Coding of collected wastewater samples within sampling campaigns in Slovenia and Spain in January and June 2014.

Code	Country	City	Wastewater type	Type of sampling	Date	T (°C)	Flow (m^3/day)ow
L-1H	Slovenia	Ljubljana	Hospital	Grab[a]	16.1.2014	n.a.	[c]
L-1Wi	Slovenia	Ljubljana	WWTP influent	24-h time proportional	29.1.2014	14.0	82,500
L-2H	Slovenia	Ljubljana	Hospital	Grab[a]	4.6.2014	n.a.	[c]
L-2Wi	Slovenia	Ljubljana	WWTP influent	24-h time proportional	2.6.2014	19.6	59,400
L-2We	Slovenia	Ljubljana	WWTP effluent	24-h time proportional	3.6.2014	21	59,700
B-1H	Spain	Barcelona	Hospital	Grab[b]	21.1.2014	12.4	334
B-1Wi	Spain	Barcelona	WWTP influent	24-h time proportional	21.1.2014	18.0	177,262
B-2H	Spain	Barcelona	Hospital	Grab[b]	3.6.2014	23.1	341
B-2Wi	Spain	Barcelona	WWTP influent	24-h time proportional	3.6.2014	24.0	235,670
B-2We	Spain	Barcelona	WWTP effluent	24-h time proportional	3.6.2014	24.0	235,670

n.a. not available.
[a] Snap samples collected at approximate 5 min intervals between 9:30 and 10:30.
[b] Snap samples collected at approximate 5 min intervals between 12:00 and 13:00.
[c] ww collected in collection tank (flow not applicable).

during which most compounds are stable (Negreira et al., 2014b).

2.3. Chemical analysis

All the samples were filtered through 0.45 μm-filters. The Slovene laboratory used Sartorius Stedim Biotech, Göttingen, Germany and the Spanish laboratory used Whatman (Fairfield, Connecticut, USA) prior to chemical analyses.

2.3.1. Cyclophosphamide, ifosfamide and TPs

Cyclophosphamide, IF and keto-CP were extracted from 100 mL sample with Oasis HLB (60 mg, 3 cc) cartridges (Waters, Massachusetts, USA). Cartridges were conditioned with ethyl acetate, methanol and water. The sorbent was dried before elution with ethyl acetate. Carboxy-CP and *N*-decl-CP were extracted with Isolute ENV + (100 mg, 3 cc) cartridges. Conditioning and sorbent drying were performed as for CP, IF and keto-CP, while 5% acetic acid/ethyl acetate was used for elution. CP and IF were derivatized with TFAA and TPs with MTBSTFA with 1% TBDMCS. The details are described elsewhere (Česen et al., 2015, 2016a).

2.3.2. Fluorouracil

One hundred millilitre samples were concentrated using SPE with Isolute ENV + cartridges (1 g, 6 mL, Biotage AB, Uppsala, Sweden). The cartridges were conditioned with 6 mL of methanol, equilibrated with 6 mL of deionised water and enriched with 100 mL of wastewater samples (pH = 6). These were then vacuum-dried and compounds of interest eluted with 3 × 2 mL of methanol. The extracts were dried under nitrogen and dissolved in 150 μL ethyl acetate. Derivatization (1h at 80 °C) was performed using 30 μL of MTBSTFA. All details are described elsewhere (Kosjek et al., 2013).

2.3.3. Pt

10 mL samples were acidified with 0.1 mL of HNO_3 (CAS 7697-37-2, Merck, Darmstadt, Germany) per 100 mL of sample for total Pt determination (Vidmar et al., 2015).

2.3.4. Fifteen anticancer drugs and TPs

A previously published multi-residue method was used for determination of fifteen selected cytostatic compounds and metabolites (Negreira et al., 2013). In brief, 5 mL of samples acidified to pH 2 containing a mixture of the isotopically labeled standards at 100 ng L^{-1} were filtered and preconcentrated (5 mL) using on-line SPE with an automated Symbiosis™ Pico system from Spark Holland (Emmen, The Netherlands) with PLRP-s (crosslinked styrene-divinylbenzene polymer, 10 mm × 2 mm i.d., 15–25 μm particle size) cartridges.

2.4. Instrumental analysis

2.4.1. Cyclophosphamide, ifosfamide and TPs

An HP 6890 series (Hewlett-Packard, Waldbron, Germany) gas chromatograph with a single quadrupole mass selective detector was used (Česen et al., 2015, 2016a). Separation was performed with two different temperature programmes, one for parent compounds and the other for their TPs. The MS was operated in the EI ionisation mode at 70 eV. The presence of investigated compounds in samples was qualitatively and quantitatively confirmed by retention time and by selective ion monitoring (SIM mode) as described in Česen et al. (2015, 2016a).

2.4.2. Fluorouracil

An Agilent 450-GC hyphenated with an ion trap 240-MS mass spectrometer was employed to determine 5-FU. The details regarding instrumental conditions are given in Kosjek et al. (2013). The ion trap MS was operated in electron impact (EI) ionisation mode. To confirm the identity of 5-FU in actual water samples the following criteria were applied: retention time matching; MSMS spectrum match and product ion ratios.

2.4.3. Pt

The content of *cis*-Pt in all the samples analyzed was determined by measuring total Pt using inductively coupled plasma mass spectrometry (Agilent Technologies 7700× ICP-MS, Tokyo, Japan). The ^{195}Pt isotope was monitored. At optimized instrumental parameters, instrumental LOD and LOQ were 0.005 ng Pt mL^{-1} and 0.017 ng Pt mL^{-1} (3 σ and 10 of the blanks). The linearity of the signal was confirmed from LOD to 10 μg Pt mL^{-1}. Repeatability of the measurements was >3%. The following ICP-MS operating parameters were employed: forward power of 1500 W, plasma gas flow of 15.0 L min^{-1}, carrier gas flow of 0.25 L min^{-1} and a dilution gas flow of 0.92 L min^{-1}.

2.4.4. Fifteen anticancer drugs and TPs

As reported previously by Negreira et al. (2013), on-line SPE was coupled to a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source (Applied Biosystems-Sciex, Foster City, California, USA). The mass spectrometer was operated in the positive ion mode. Data acquisition was performed in the selected reaction monitoring (SRM) mode. Quantification was performed by stable isotope dilution method. For positive confirmation of the presence of a compound in a sample, the LC retention of the compound in the sample must match that of the standard with a margin of ± 2%, and its SRM1/SRM2 ratio cannot deviate by > 20–50% (depending on the SRM1/SRM2 value) from the ratio in the standard (Council of the European Communities, 2002).

2.5. Toxicity tests

2.5.1. Acute and chronic toxicity in Ceriodaphnia dubia

The *C. dubia* acute toxicity test was performed on neonates hatched from ephippia (MicroBioTest) after 3–4 days of incubation under a light source of 6000 lux at 25 ± 1 °C in synthetic ISO medium (hardness of 250 mg L^{-1} expressed as $CaCO_3$) according to EPA-600-4-90 procedure (US EPA, 1993). At least four sample dilutions were prepared starting from each raw sample in three replicates. Ten organisms were exposed for 24h to 1.0 mL of sample dilutions. Organisms were incubated in the dark at 25 °C in multiwell plates. The dilution causing 50% lethality after 24h was indicated as LC50.

The *C. dubia* population growth inhibition test (ISO 20665, 2008) was performed over 7 days on neonates (less than 24h old) of at least the third generation of females coming from a healthy mass culture (starting organisms were purchased from Aquatic Research Organisms, Inc., Hampton, NH, USA). Organisms were individually exposed in glass beakers with 25 mL of sample (ten replicates for each sample dilutions). In both, acute and chronic tests, the highest dilution was ≤90% (v/v) of the raw sample, on account of the 10% of test medium, which is required for a sufficiently high concentration of dissolved oxygen and, in case of chronic test, food supply. Beakers with daphnids were incubated at 25 °C with a 16:8 h light: dark cycle (600 lux). Daily, at the renewal time, the offspring produced by each parent organism, was counted and removed (starting from the fourth day of exposure). The organisms were fed daily on 200 μL of an YCT/algae (1/1) suspension. YCT is a mix of 5 g L^{-1} each of food fish, *Saccharomyces cerevisiae* and Alfalfa (*Medicago sativa*). The algal cells were from cultures of *Pseudokirchneriella subcapitata* (10^8 cells mL^{-1}). The reproductive output of females

exposed to the samples was compared to that of the negative control to calculate the dilution able to determine the median reproduction inhibition effect in 7 days (EC50).

Two independent experiments were performed for each sample. Computations of EC50 values were performed by estimating the number of offspring from each sample by negative binomial regression with a log link. EC50 values were then computed as ln(2) divided by the slope parameter. No Observed Effect Concentration (NOEC) for each wastewater sample was determined based on the regression equation by calculating the dose that corresponds to the lower 95% confidence limit of the number of offspring in the control sample.

2.5.2. Micronucleus (MN) assay with Tradescantia

Tradescantia MN (Trad MN) assays were performed according to the protocol of Ma et al. (1994) with clone #4430. Per concentration, 15 cuttings were exposed to the water samples for 24h followed by a 24h recovery time. After the treatment, the inflorescences were fixed in a mix of ethanol/acetic acid (3:1) for 24h and stored in 70% ethanol. Per experimental point, tetrad preparations of at least five buds were made and stained with 2% acetocarmine (Sigma St. Louis, USA). 1500 early phase tetrads were scored in each experimental group. Tap water was used as a negative control. Maleic hydrazide (MH, Sigma St. Louis, USA) (20 mg L^{-1}) was used in all experimental series as a positive control. The results were analyzed by generalized linear model with Poisson counts and a log link. Comparisons to negative control were done by simple contrasts and p-values were corrected according to Bonferroni-Holm; p-values $\leq$0.05 were considered as significant.

2.5.3. Micronucleus assay in Allium cepa

Allium MN assays were performed according to the standard protocol published by Ma et al. (1995). Young onion bulbs (diameter 12–21 mm, Schneeball Weiss, Austrosaat, Vienna, Austria) were placed in 13 mL glass tubes filled with tap water in the dark for 24h. Subsequently, the roots (length $\approx$ 1 cm) were exposed to test samples and their dilutions in the dark for 24h and then transferred to fresh tap water for an additional 24h. At the end of the recovery period, the roots were fixed in a mix of ethanol and glacial acetic acid (3:1) for 24h and stored in 70% ethanol. Tap water was used in all experiments as a negative control. Maleic hydrazide (MH) 10 mg L^{-1} was used in all experimental series as a positive control.

Micronuclei were scored according to the criteria described by Ma et al. (1995) using 2% acetocarmine for staining. For each experimental point, the MN frequencies were determined in five plants. From each bulb, two slides were made and 500 cells were evaluated per slide (5000 cells per dose) after staining. Furthermore, also the mitotic indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The results were analyzed by a generalized linear model with Poisson counts and a log link. Comparisons to negative control were done by simple contrasts and p-values were corrected according to Bonferroni-Holm; p-values $\leq$0.05 were considered as significant.

2.5.4. In vitro comet assay in zebrafish (Danio rerio) liver cell line

The zebrafish (*Danio rerio*) liver cell line (ZFL) is derived from adult zebrafish (Ghosh et al., 1994) and was obtained from the American Type Culture Collection (ATCC number: CRL-2634). Cells were cultured under a humidified air atmosphere at 28 °C in a medium containing 50% Leibovitz L-15 (ATTC), 35% DMEM (Gibco), and 15% Ham F-12 (Gibco), supplemented with 15 mM HEPES (Invitrogen, Paisley, UK), 0.15 g L^{-1} NaHCO3, 0.01 mg mL^{-1} insulin, 50 ng mL^{-1} epidermal growth factor (EGF; Invitrogen, Paisley, UK), 0.1% penicillin/streptomycin, and 5% heat inactivated fetal bovine serum (FBS; ATTC).

Prior to the genotoxicity testing the cytotoxicity of the samples was determined with the MTS (CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA) according to the manufacturers' protocol. To ensure sterile conditions the samples were filtered through 0.22 μm-filters (Corning Costar Corporation, Corning, NY, USA). ZFL cells (8000 cells/well) were seeded into 96-well microtiter plates (Nunc, Naperville, IL, USA) and incubated 24 h at 28 °C to attach. The growth medium was then replaced by fresh medium containing 0, 10, 20 and 30 v/v % of the sample and incubated for a further 72h. Subsequently, the MTS/PMS mixture was added to each well and incubated at 28 °C for additional 3h. The optical density (OD) was measured at 490 nm with a microplate spectrofluorimeter (Synergy MX, BioTek, Winooski, USA). Cell viability was determined by comparing absorbance values of control cells with absorbance values of treated cells. The viability was measured in three independent experiments with five replicates per treatment point. Etoposide (1 μg mL^{-1}) was used as a positive control. The statistical significance between treated groups and the control was determined with one-way ANOVA followed by Dunnett's multiple comparison test and $p < 0.05$ was considered as statistically significant.

For the comet assay ZFL cells (50,000 cells/well) were seeded into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA) and were incubated at 28 °C for 24h to attach. The growth medium was then replaced with fresh medium containing non-cytotoxic concentrations of the samples and incubated for 72 h at 28 °C. At the end of the exposure, the comet assay was performed as described by Tice et al. (2000) with minor modifications (Štraser et al., 2011). The slides were stained with ethidium bromide (5 mg mL^{-1}) and images of 50 randomly selected nuclei per experimental point were analyzed with image analysis software Comet Assay IV (Perceptive Instruments, UK). The results were obtained from three independent experiments. Benzo[a]pyrene (BaP; 50 μM) and etoposide (ETP; 100 ng mL^{-1}) were used as positive controls. Statistical significance between the control and the treated groups was determined by one-way analysis of variance (ANOVA; Kruskal–Wallis), and Dunnett's multiple comparison for multiple comparison versus the control. DNA damage was expressed as percentages of tail DNA.

2.6. Analysis of the relationship between concentration of chemicals and eco-toxicity/genotoxicity data

For the statistical analysis, only those substances that were detected in at least 7 of the 10 samples were included. This was the case for CP, ERL, MET, OH-TAM, Pt and TAM.

No correlation analyses were performed with the data from Allium MN assay as consistently negative results were obtained with this model.

The concentration-response functions for the *C. dubia* acute and chronic toxicity assays were used to calculate the 10, 20, 30, and 100% wastewater responses to match the concentrations applied in the ZFL comet assay and in the Tradescantia MN assays. Results of the chemical analysis were used to calculate the concentrations of chemotherapeutics and TPs in the diluted samples. If the dilution would result in a concentration less than half the LOD, the data were omitted. Spearman correlation coefficients were computed for data from the *C. dubia* chronic and acute toxicity tests and the ZFL comet assay and Tradescantia MN assay.

To explore the possible effects of a mixture of substances present in wastewater samples, the data were assessed by multivariate stepwise linear regression of the toxicity test results using the log concentrations of the selected compounds. As p-value for inclusion 5% was chosen and for removal a p-value of 10%.

Table 3
Concentration (average ± SD of three replicates in ng L^{-1}) of analyzed anticancer drugs, metabolites or transformation products detected in wastewater samples from Slovenia and Spain (January and June 2014). Confident limits are in parentheses.

Substance (ng/L)	L-1H[a]	L-1Wi	L-2H	L-2Wi	L-2We	B-1H	B-1Wi	B-2H	B-2Wi	B-2We
Pt[b]	226 ± 4	27 ± 3	352 ± 8	23 ± 1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CP	1080 ± 200	27 ± 7	22100 ± 800	19 ± 3	17 ± 5	32 ± 1	6.0 ± 2.5	<LOD	<LOD	<LOD
IF	48 ± 10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Keto-CP	270 ± 4	<LOD	1340 ± 10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
N-decl-CP	847 ± 58	<LOD	5520 ± 110	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Carboxy-CP	17700 ± 400	<LOD	60600 ± 1000	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
5-FU	6.9 ± 1.0	3.1 ± 0.4	<LOQ	<LOD	<LOD	2.1 ± 0.3	<LOQ	<LOD	3.5 ± 0.5	<LOQ
GEM	<LOD	61 ± 1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
MET	19 ± 2	303 ± 5	3920 ± 70	29 ± 1	<LOD	29 ± 7	29 ± 2	<LOD	8.3 ± 1.3	<LOD
OH-MET	<LOD	366 ± 35	490 ± 49	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
IRI	9.2 ± 2.4	49 ± 10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
ERL	4.0 ± 0.2	8.1 ± 2.2	2.0 ± 0.1	3.5 ± 0.4	3.8 ± 0.3	5.5 ± 0.3	7.2 ± 0.6	2.4 ± 0.1	6.1 ± 0.2	3.3 ± 0.1
CAP	<LOD	<LOD	106 ± 6	158 ± 13	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
OH-D-TAM	<LOD	66 ± 25	<LOD	<LOD	<LOD	11 ± 0	75 ± 8	<LOD	<LOD	14 ± 0
OH-TAM	<LOQ	35 ± 14	10 ± 0	<LOQ	<LOD	<LOQ	7.7 ± 0.9	<LOD	<LOQ	<LOQ
TAM	<LOQ	61 ± 13	10 ± 0	11 ± 1	7.1 ± 0.4	<LOD	15 ± 4	7.4 ± 0.1	6.7 ± 0.0	<LOD

TMZ, DOX, PAC and OH-PAC are not shown in the table since they were <LOD in all samples.
IMA and ETP, not shown since they were detected only in L-1Wi and L-2H, respectively, above LOD but below LOQ.
The abbreviations of the substances are given in Table 1.
[a] For codes of the sample sites see Table 2.
[b] Cisplatin and all metabolites were below LOD, therefore only total platinum concentrations are given.

During all laboratory work, safety precautions, using personal protective equipment, were taken and proper disposal procedures for hazardous wastes were followed.

3. Results and discussion

3.1. Results of chemical analysis

Table 3 shows concentrations of analyzed anticancer drugs, metabolites or transformation products detected in wastewater samples from Slovenia and Spain. Even though it is difficult, if not impossible, to compare both hospitals and WWTPs since they differ in size and application of tested compounds (Table 2), we still noticed that concentrations of the measured compounds were in general higher in Slovenian than in Spanish wastewaters in both campaigns and at all sampling points. When examining results for Slovene wastewaters, we notice a decrease of the total concentrations of the studied compounds in the following order H > Wi > We, while in Spain, levels were generally similar in all three matrices (Fig. 1). Higher levels of total concentrations in Slovene hospital wastewater, when compared to WWTP samples, were expected since hospital wastewater, which was collected from an oncological ward, represents only 0.07% of the total load at the corresponding WWTP (Fig. 1). On the contrary, Spanish samples were collected at a general hospital, which is approximately 4-times bigger and includes besides cancer treatment ward also other wards diluting anticancer drug concentrations; accordingly the differences between the detected concentrations of analytes in hospital effluents and WWTP influents were less significant.

There was no systematic difference between detected concentrations of analytes in samples collected in January and June in Ljubljana, Slovenia. This agrees with the fact that hospital WW was sampled at the collection basin and that flow at the corresponding WWTP was similar at both sampling campaigns (Table 2). On the contrary, we did find some differences between the two sampling campaigns in the Spanish samples where, with the exception of 5-FU and TAM, the levels of drugs were lower in summer in both the hospital and the WWTP influent samples despite the flows being in

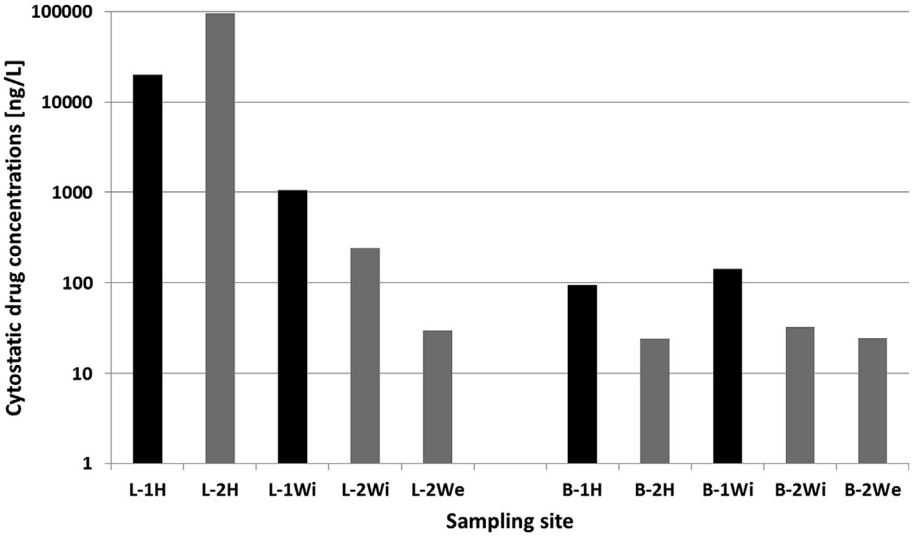

Fig. 1. Cytostatic compounds concentrations in various water samples (January: black bars, June: grey bars, H: hospital wastewater; Wi: WWTP influent; and We: WWTP effluent, L: collected in Ljubljana, Slovenia and B: Barcelona, Spain).

the same range (Table 2). This might be attributed to a higher degradation activity in summer because of the effect of the temperature (12–18 °C in winter *vs* 23–24 °C).

CP and IF share similar chemical structures and both act as alklyating cytostatic agents. Regardless, CP was detected in all Slovene samples and in two Spanish samples (B-1H and B-1Wi) sampled in January, while IF was above LOD only in Slovene hospital sample (L-1H). This agrees with the fact that CP is administered more often than IF due to less severe side effects after being administered (Česen et al., 2016a). CP, and especially its TPs, were also major contributors to the total load of anticancer drugs in the Slovenian hospital effluent (Fig. 2). For example, the sample taken in June at Slovene hospital (L-2H) contained the highest concentration of CP among all analyzed parent compounds (22.0 μg L^{-1}). This level was approximately 20-times higher than the one reported in the sample taken in January at the same location (1.08 μg L^{-1}). It is difficult to provide an explanation for this difference since many factors can affect the amount of a certain compound, for example hospital water consumption at the time of sampling, sampling method (grab sampling or time/flow proportional sampling), time of sampling, number of hospitalized patients, number of patients receiving chemotherapy with CP and the pharmacokinetics of these patients.

The most abundant TP/metabolite in this study was a CP TP, namely carboxy-CP in L-2H (60.6 μg L^{-1}). Moreover, the concentrations of all CP TPs were higher in the hospital sample taken in June (L-2H) and correspond to a higher CP concentration in the same sample if compared to the sample taken in January (L-1H). Regardless, the ratios between the measured concentrations of CP and its TPs are not comparable in samples from both campaigns. The reason for this discrepancy could also be the varying pharmacokinetics of patients receiving the therapy at the time of sampling as all targeted TPs are also known human metabolites (Česen et al., 2016a). With the exception of CP, all the samples taken from WWTPs in Ljubljana and Barcelona contained other CP and IF residues (carboxy-CP, keto-CP and *N*-decl-CP) < LODs. The reason is most likely the dilution of hospital wastewater in the wastewater that enters the WWTPs. In addition, among all the detected compounds in WWTP effluents (CP, ERL, TAM and OH-D-TAM), CP was the most abundant (17 ng L^{-1} in L-2We sample). Generally, CP dominated in all Slovene samples including its metabolites in hospital wastewaters, while in Spanish samples, the predominant compounds were MET, ERL and TAM without a clear pattern observed for each matrix.

CAP, which is a prodrug of 5-FU, was found at concentrations above 100 ng L^{-1} in the Slovenian hospital and wastewater influent samples L-2H and L-2Wi, respectively, while 5-FU was either not detected or detected at concentrations in the low ng L^{-1} range. This unexpected finding might be explained by the higher biodegradability of 5-FU compared to CAP. In addition, the global trend is towards the prescription of CAP over 5-FU since CAP allows oral administration and has less severe side-effects, while 5-FU is still intravenously administered (Kosjek et al., 2013).

Total platinum was detected in higher concentrations in all samples from Slovenia compared to Spain. The highest concentrations of total platinum were detected in Slovene hospital wastewater (L-1H and L-2H), where a slightly higher concentration was reported for the sample taken in June (352 ng L^{-1}) than in January (226 ng L^{-1}) (Table 3).

Between all tested compounds, only ERL was detected in all the tested samples. In all cases, ERL concentrations were lower in the hospital WWs than in the corresponding WWTP influents and effluents. One reason could be that sources other than hospitals contribute towards the input of ERL in wastewater, i.e. out-patients who receive chemotherapy at the hospital, but excrete cytostatic residues *via* urine at home. Another explanation is that the difference derives from different sampling approaches. Hospital wastewaters were representative of a short time interval (1h, Table 2), while time-proportional sampling was performed at the corresponding WWTPs (Table 2).

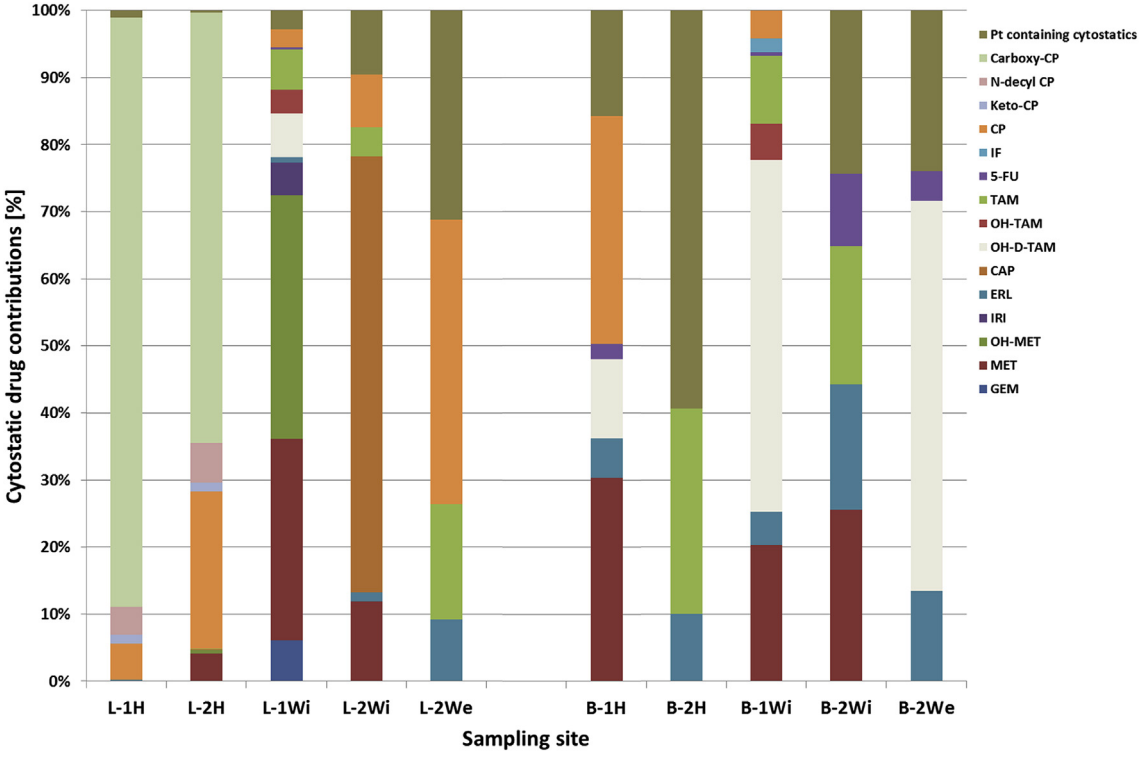

Fig. 2. Relative contribution (% of total concentration) of the various cytostatic compounds measured in each of the samples analyzed.

In contrast to ERL, several other targeted analytes within this study showed concentrations in the hospital wastewater always higher than in the corresponding WWTP influent, i.e. Pt, IF and the already mentioned CP residues. Overall, 11 out of the 16 compounds detected were at least in one occasion in higher concentrations in the WWTP influent than in the corresponding hospital effluent (Table 3). While for 5-FU, GEM, MET, IRI, TAM and especially CAP the explanation can be the same as for ERL, the TP, namely OH-MET, could be present in higher concentrations in WWTP influent than in hospital effluent due to their transformation from the parent compounds on their way from the hospital to the WWTP (e.g. hydrolysis, biodegradation; Kosjek and Heath, 2011; Kosjek et al., 2015). Similarly, OH-D-TAM was detected at higher concentration in the effluent than in the influent of the Spanish WWTP in June, most likely due to (bio)transformation from TAM during wastewater treatment via benzene hydroxylation and *N*-demethylation, since they are common reaction pathways (Klein et al., 2013). Besides OH-D-TAM and ERL, also CP and TAM were detected in Slovene WWTP effluent at second sampling campaign at concentrations 17 ng L^{-1} and 7.1 ng L^{-1}, respectively (Table 3). Their calculated removal (8.5% for CP and 35.5% for TAM) was expected as these compounds are known to be poorly biodegradable during the conventional biological treatment (Česen et al., 2015). On the contrary, several compounds were removed during wastewater treatment and their concentrations were <LOD in the WWTP effluent, while they were detected in WWTP influents in June in Slovene samples, i.e. Pt, MET, CAP and, surprisingly, TAM in Spanish samples since this compound is known to be poorly biodegradable (Table 3). Based on the results of this study and others like that conducted by Negreira et al. (2014a), we can conclude that the compounds CP, ERL, TAM and OH-D-TAM are among the most recalcitrant and their occurrence in wastewaters and surface waters should be studied further as well as their potential to be degraded by alternative treatment processes like advanced oxidation process.

3.2. Results of toxicity tests

3.2.1. Acute and chronic toxicity in Ceriodaphnia dubia

When anticancer drugs were tested as single compounds, they induced acute toxic effects in daphnids at concentrations in the order of mg L^{-1} (Parrella et al., 2014a). These levels were much higher than those found by chemical analysis in the present study (Table 3) where the concentrations of the anticancer residues were generally in the range of ng L^{-1}. Only the concentrations of CP and its TPs were in the order of μg L^{-1} in Slovenian hospital wastewaters (L-1H and L-2H), although CP occurrence is not of particular concern as it induced acute toxicity in daphnids at concentrations of g L^{-1} (Sanderson et al., 2003). Of the wastewaters examined, three samples (L-2H, L-2We and B-1H) did not induce acute effect in *C. dubia*, while the LC50 values of other samples ranged from 28.9 to 77.5% (v/v) of the raw sample (Table 4).

The effects of the tested samples on the reproduction of *C. dubia* after 7-day chronic exposure were observed at one-two orders of magnitude higher dilutions than acute toxicity. The most pronounced long-term toxic effect was observed for B-2H, with a median inhibition of reproduction at 1.4% (v/v) of the raw sample and a NOEC value equal to 0.1% (v/v). According to Table 3 and Fig. 2, B-2H toxicity could be due to the presence of TAM, ERL and to a lesser degree to Pt compounds. Indeed, tamoxifen is able to cause chronic toxicity in *C. dubia* at ng L^{-1} (DellaGreca et al., 2007) and Pt compounds (e.g. Cisplatin) and kinase inhibitors (e.g. Erlotinib or Imatinib) can inhibit daphnid reproduction at μg L^{-1} (Besse et al., 2012; Parrella et al., 2014a). On the other hand, according to Parrella et al. (2014b), when Pt compounds and kinase inhibitors are in mixtures, they can have additive or synergistic effects in

Table 4
Acute and chronic toxicity of Slovenian and Spanish wastewater samples collected in January and June 2014 in *C. dubia*.

Assay	Effect indicator	L-1H[a]	L-1Wi	L-2H	L-2Wi	L-2We	B-1H	B-1Wi	B-2H	B-2Wi	B-2We
C.dubia acute	LC50[b]	64.9 (58.9–70.8)	68.6 (65.9–71.3)	>100	44.3 (40.7–47.8)	>100	>100	64.0 (59.4–68.6)	77.5 (70.9–84.1)	28.9 (25.8–32.0)	51.9 (47.1–56.6)
C.dubia chronic	EC50[b]	3.7 (2.8–5.3)	3.0 (2.4–4.1)	7.4 (5.2–12.7)	3.6 (2.7–5.4)	7.0 (5.0–11.4)	5.6 (4.0–8.9)	3.5 (2.5–6.0)	1.4 (1.0–2.1)	15.9 (11.2–27.8)	11.7 (8.4–19.2)
	NOEC[b]	2.6	2.1	4.1	1.8	4.7	3.1	2.4	0.1	6.7	6.3

[a] For abbreviation of samples sites see Table 2.
[b] Median effective concentrations and No Observed Effect Concentration (NOEC) expressed as the percentage (v/v).

C. dubia causing the same effect on reproduction at much lower concentrations than each compound individually tested. Since the same anticancer drugs were found also in the other samples, the observed chronic toxicity could either be due to synergistic effects of these compounds in mixtures or to the presence of other active compounds not identified in this study that might contribute to the overall toxicity of the samples.

3.2.2. Micronucleus assay with Tradescantia and Allium cepa

Tradescantia MN assay results for June samples are presented in Fig. 3. It can be seen that positive results were obtained with undiluted samples from the Slovenian oncological clinic (L-2H), the corresponding influent from the WWTP (L-2Wi) and from the Spanish B-2Wi. In the Allium MN assay no significant increase of MN frequencies or decrease of mitotic indices were detected (Fig. S1 of supplementary materials, ESI). In previous experiments with Tradescantia MN assays, the NOEC values were for CP 1.0 μM (279 μg L^{-1}), for Pt compounds 0.1 μM (30 μg L^{-1}) and for 5-FU 30 μM (3.9 mg L^{-1}) (Mišík et al., 2014), which are several orders higher than the concentration determined in the positive samples. To our knowledge, no data is available concerning the effects of MET, ERL and CAP that were also detected in the positive samples, in higher plants. It is unlikely that at low doses, which were detected in the wastewater samples, these residues of anticancer drugs induced genotoxic effect. However, previous investigations with Tradescantia demonstrated that certain genotoxic agents act synergistically (Gill and Sandhu, 1992; Knasmueller et al., 1992; Ma et al., 1992). Hence, it cannot be excluded that such an effect (i.e. synergism) also accounts for the present findings. Nevertheless, wastewater effluents that are finally released into surface water were not genotoxic and therefore it is unlikely that they represent significant threat for higher plants.

3.2.3. In vitro comet assay in zebrafish (Danio rerio) liver cell line

None of the tested wastewater samples at 10, 20, 30% v/v significantly reduced the viability of ZFL cells (Fig. S2). Therefore, the comet assay was performed at the same sample dilutions. All samples caused significant dose dependent increase in DNA damage except L-2We (Fig. 4). The most potent was the sample of oncological clinic wastewater collected in June (L-2H) that induced the highest increase in DNA damage and it was the only sample inducing significant increase in DNA damage at the lowest tested concentration. The sample L-2H had the highest burden with the residues of anticancer drugs (Fig. 1) and showed the highest genotoxic potential also in Tradescantia MN assay (Fig. 3). The corresponding WWTP influent (L-2Wi) exerted much lower genotoxic potential and the corresponding effluent (L-2We) was not genotoxic indicating removal or inactivation of genotoxic pollutants. The samples from Spain had similar potential regardless of sampling point. The genotoxicity of January samples was higher than of June samples (Fig. 4). The genotoxic potential of B-2We was comparable to that of the corresponding influent (B-2Wi). Previously, it has been shown that under the same exposure conditions as used in the present study in ZFL cells, 5-FU and Pt induced significant increase in DNA damage at concentrations ≥10 and 100 μg L^{-1}, respectively (Gajski et al., 2015), while CP and IF were inactive at concentrations up to 1 mg L^{-1} (personal M. Novak, 16. 7. 2016). These concentrations are several orders higher than the concentrations detected in the samples from the present study. However, potential threat for aquatic organisms cannot be excluded because, based on the results of *in vitro* genotoxicity assays, it is not possible quantitatively predict hazard for exposed organisms.

3.3. Correlation between chemical and toxicological characteristics of the samples

The results of correlation analysis are shown in Tables 5 and 6. In Table 5 the concentrations of chemicals in wastewater samples were correlated to each toxicity/genotoxicity test, while in Table 6 standardized regression coefficients and p-values as well as adjusted multiple correlation coefficients were reported (p-values were: 5% for inclusion and 10% for removal). Nevertheless, because of the restricted number of samples, a more comprehensive multivariate analysis including interaction terms was not possible.

The results of *C. dubia* acute toxicity tests correlated significantly only with the concentrations of ERL (the higher the concentration, the lower the survival), which is also the only chemical that was significant in the stepwise regression analysis (Tables 5 and 6). As reported in different studies, many anticancer drugs belonging to the tyrosine kinase inhibitor family, as ERL, are able to affect the crustacean survival at high concentrations (mg L^{-1} or hundreds of μg L^{-1}) (Constantine and Huggett, 2010; Parrella et al., 2014a; Franquet-Griell et al., 2015). Nevertheless, when these chemicals are in mixtures with other anticancer drugs such as Pt compounds and antimetabolites, they are able to cause additive or synergistic toxic effects at lower concentrations (Parrella et al., 2014b). This phenomenon could also occur in wastewaters because organisms, by different pharmacokinetic mechanisms, are able to absorb, accumulate and eliminate drugs. *C. dubia* reproductive toxicity test results correlated well with the levels of all chemicals except for CP; OH-TAM picked up by stepwise regression as the only independent predictor (Table 6). Borgatta et al. (2015) showed that OH-TAM

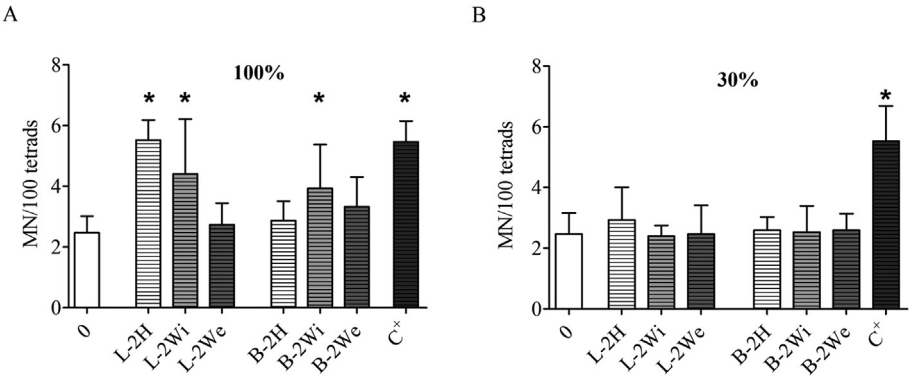

Fig. 3. Results of Tradescantia MN assays with undiluted (A, 100%) and with diluted (B, to 30% of original sample) hospital and WWTP wastewater samples collected in June 2014 (for sample codes see Table 2). Bars indicate mean ± S.D. obtained with 5 inflorescences per experimental point. From each inflorescence, 300 tetrads were analyzed. The results were analyzed by generalized linear model with Poisson counts and a log link. Comparisons to negative control were done by simple contrasts and p-values were corrected according to Bonferroni-Holm; *p-values ≤0.05 were considered as significant. Tap water was used as negative control and solvent, MH (20 mg L^{-1}) was used as positive control (C^+).

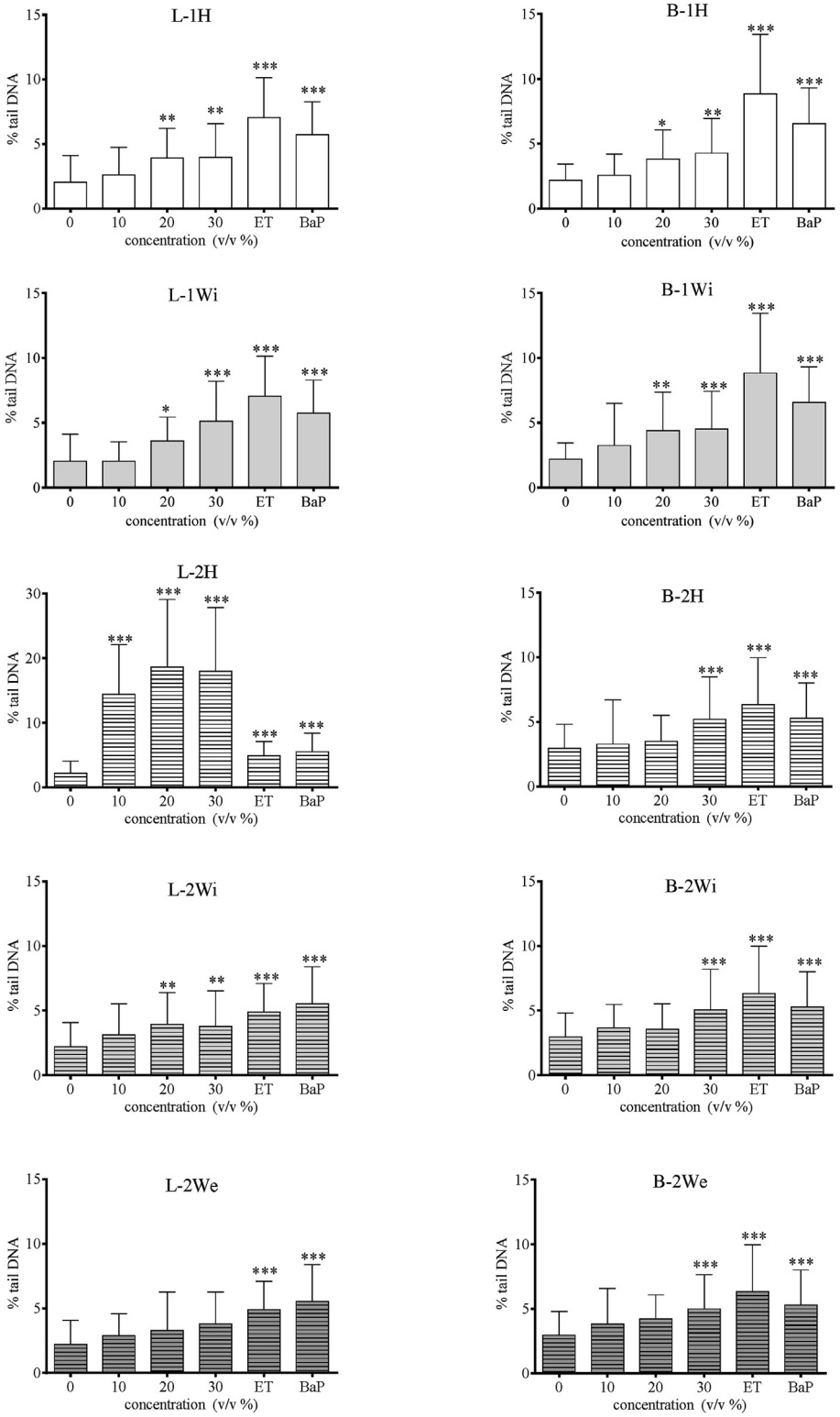

Fig. 4. DNA damage induced by hospital and WWTP wastewater samples (for sample codes see Table 2). The ZFL cells were exposed to 10, 20 or 30% (v/v) of the wastewater sample for 72 h. DNA damage was assessed with the comet assay and is expressed as percent of tail DNA. Fifty nuclei were analyzed per experimental point in each of the three independent experiments. Benzo[a]pyrene (BaP; 50 μM) and Etoposide (ETP; 100 ng mL^{-1}) were used as the positive controls. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between exposed and the control cells (0) is indicated by $^{*}p < 0.05$, $^{***}p < 0.001$, $^{***}p < 0.0001$.

induced toxic effects on non-target aquatic species as the cladoceran crustacean *Daphnia pulex* in a two-generation study starting from units of μg L^{-1}. These authors hypothesize that the decrease of the daphnids fitness could be due to the interaction of endocrine disruptors such as OH-TAM with oestrogen-related nuclear receptors in many organisms, including invertebrates (Thomson et al., 2009).

In order to better understand the toxic potential of the samples

Table 5
Spearman's rank correlation coefficients of concentrations of chemicals in wastewater samples with results of toxicity/genotoxicity tests (type of effect indicator in second line). p-values in parentheses.

Substances[a]	*C. dubia* chronic[b]	*C. dubia* acute	ZFL comet	Tradescantia MN
ng L^{-1}	Log offspring	Logit vital	Beta	Log OR
Pt	−0.61 (<0.001)	−0.15 (0.365)	0.42 (0.022)	0.72 (0.008)
CP	−0.31 (0.055)	0.06 (0.720)	0.28 (0.140)	0.33 (0.290)
MET	−0.54 (0.003)	0.16 (0.421)	0.47 (0.031)	0.60 (0.208)
ERL	−0.56 (<0.001)	−0.35 (0.025)	0.17 (0.364)	0.59 (0.045)
OH TAM	−0.62 (<0.001)	0.10 (0.570)	0.20 (0.359)	0.41 (0.314)
TAM	−0.49 (0.004)	−0.15 (0.409)	0.34 (0.100)	0.79 (0.007)

[a] For abbreviations see Table 1.
[b] *C. dubia* acute and chronic toxicity test results correlate negatively and ZFL comet and Tradescantia MN assay correlate positively if increasing concentrations are related to increased toxicity.

Table 6
Results of stepwise multivariate linear regression of toxicity/genotoxicity test results on log concentration of anti-cancer drugs and metabolites in wastewater samples. Standardized regression coefficients (Beta) and p-values as well as adjusted multiple correlation coefficients are shown.

Assay	Endpoint	Substance[a]	Beta[b]	*p*-value	adj.R[b]
C. dubia acute	Logit vital	ERL	−0.675	<0.001	0.431
C. dubia chronic	Log offspring	OH-TAM	−0.568	0.004	0.292
ZFL comet	Beta	MET	0.896	<0.001	0.803
Tradescantia MN	Log OR	TAM	0.836	0.039	0.625

[a] For abbreviations see Table 1.
[b] Negative coefficient for *C. dubia* acute and chronic assays indicates increased toxicity with increasing concentration, while for Tradescantia and Zebrafish assays a positive coefficient indicates increased genotoxicity.

investigated, Acute LC50/Chronic EC50 (AC) ratios were calculated. They ranged from 12.3 (L-2Wi) to 22.9 (L-1Wi) for Slovenian wastewaters and from 1.8 (B-2Wi) to 55.4 (B-2H) for Spanish samples. Interestingly, AC ratios were higher than 10 in all instances except for Spanish summer Wi and We with 1.8 and 4.4 values, respectively, showing a more evident lethal than chronic effect without a remarkable occurrence of the anticancer drugs detected (Tables 3 and 4) and finally suggesting that other uninvestigated substances might have affected the observed toxic effects. Another unexpected finding was observed in the case of the Spanish summer sampling characterized by an increase in chronic toxicity from Wi to We with a corresponding increase in the concentration of OH-D-TAM (from < LOD in the influent to 14.4 ng L^{-1} in the effluent, Table 3).

Increase in MN frequencies in Tradescantia correlated with Pt, ERL and TAM, the latter was also entered in a stepwise regression. Pt compounds and kinase inhibitors can cause genotoxic effects and induce the formation of micronuclei in Tradescantia at concentrations in the order from hundreds of μg L^{-1} to mg L^{-1}. However, in mixtures, anticancer drugs are able to cause additive or synergistic effects at low concentrations (μg L^{-1}) (Parrella et al., 2014b; Mišík et al., 2016). Ko et al. (2014) showed how in other eukaryotic models tamoxifen could enhance erlotinib-induced cytotoxicity through down-regulating AKT-mediated thymidine phosphorylase expression increasing the genotoxicity of the real samples. Similar results were obtained in the comet assay with ZFL cells, but in this case, significant correlations were obtained only for Pt and MET with MET included in the regression model. Pt compounds are able to cause DNA strand breaks in ZFL after 24 and 72h starting from 100 μg L^{-1} (Gajski et al., 2015), but it was demonstrated that these drugs in mixture with MET can exert in *in vitro* experimental models a moderate synergism at ng L^{-1} levels (Chou et al., 1993).

Correlation analyses revealed that only 10–50% of the differences in toxicity of wastewater samples are explained by the concentration of the substances measured. Indeed, even if MET, ERL and TAM were found at concentrations lower than the other anticancer drugs, they contribute to the results of stepwise multivariate linear regression of toxicity tests. Furthermore, while an additional impact could be exerted by an interaction between these substances, it cannot be excluded that substances in wastewaters not determined in this study contribute to these effects. Indeed, in Spain, Gracia-Lor et al. (2012) who monitored the presence of several pharmaceuticals (analgesics and anti-inflammatories, lipid regulators, antibiotics) in urban influent WW and effluent WW from three different WWTPs found that conventional treatment processes do not completely remove these persistent micropollutants. Furthermore, in Spanish general hospital wastewaters, different compounds (analgesics and anti-inflammatories, antibiotics, β-blockers, diuretics, iodinated contrast media) were found (ng-mg L^{-1}) that pose a risk to aquatic organisms belonging to different trophic levels (Mendoza et al., 2015). In the Slovenian influent and effluent samples, from different wastewater treatment plants, other emerging contaminants such as Endocrine Disruptor Compounds (EDCs) including estrone, 17β-estradiol and estriol were present in ng L^{-1} levels as shown by Avberšek et al. (2013). These compounds are able to affect organisms (gonadal abnormalities, reproductive deficiencies, egg and offspring development deficiencies, and vitellogenin induction alterations) at trace concentrations (ng L^{-1}) (Campbell et al., 2006; Isidori et al., 2010; Bistan et al., 2011). Bistan et al. (2011) underline the presence of other EDCs such as nonylphenols and octylphenols in Slovenian surface waters, suggesting that the Slovenian treatment processes are not efficient in removing persistent endocrine disrupting xenobiotics from wastewater. This is not surprising since WWTPs are not designed to remove micro organic pollutants. Considering that hormones and related anti-hormones are used in the endocrine anticancer therapy (Besse et al., 2012) and are discharged in the oncological hospital wastewaters, probably other hormonally active drugs could explain the present findings. The endocrine disruptors, as well as other emerging pollutants, as reported by Kasprzyk-Horderna et al., 2009 and Schug et al., 2011, acting through nonsteroid receptors, transcriptional coactivators and several enzymatic pathways, could represent a risk for different organisms, up to be capable of affecting human population when these xenobiotics are found in wastewaters, especially when they represent the main contributors to river flows involved in the human water recycling and reuse. Also it should be noted that according UNEP (http://www.unep.org/pdf/SickWater_screen.pdf) "A staggering 80–90 per cent of all wastewater generated in developing countries is discharged directly into surface water bodies". Similar data concerning presence of pharmaceutical in these regions are available in work of the Rehman et al. (2015) and indicate elevated health risk for human population in these areas. In this context, our data also underline importance of proper waste water treatment procedures.

4. Conclusions

Hospital wastewater can contain high levels of anticancer drug residues that vary with the therapeutic regimen. In our study, twelve out of the fifteen investigated parent compounds were present in Slovenian and Spanish hospital and municipal wastewaters while six on seven TPs have also been identified. Interestingly, a statistical correlation between the results of the toxicity tests and the drugs identified was found, so that the aim of this study was reached. Nevertheless, several unknown compounds surely might contribute to the observed effects. The applied eco/genotoxicity tests demonstrated that hospital effluents and WWTP

influents and effluents could be harmful for the aquatic environment at concentrations of ng L^{-1}. It may be desirable that measured concentrations as well as toxicity/genotoxicity results should be considered for the environmental risk assessment in order to encourage the environmental agencies to enforce regulations that these emerging pollutants do not have yet.

Seasonal fluctuations were observed for hospital wastewater regarding both acute and chronic toxicity. However, the toxicity does not correlate with the total burden of the samples and the residues of anticancer drugs indicating that other unidentified pollutants contributed to these differences. Basically, the effluents from the WWTPs investigated should be diluted by more than 90% to obtain no observed toxic effects as shown by NOEC values.

The possible presence of different pollutants in complex mixtures makes it difficult to comprehend the actual ecological risk posed by the targeted compounds. Thus, a chemical characterisation closely linked to an eco/genotoxicological approach as used in this study, can lead to a better understanding of the environmental toxic/genotoxic potential of xenobiotic mixtures.

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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.2016.10.039.

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