Science of the Total Environment 466–467 (2014) 421–438

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Investigation of PPCPs in wastewater treatment plants in Greece: Occurrence, removal and environmental risk assessment

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HIGHLIGHTS

- Comprehensive study of 18 PPCPs in eight WWTPs in Greece
- Further confirmation was accomplished by LC–MS/LTQ Orbitrap.
- Report on the occurrence of trimethoprim transformation products in wastewaters
- Occurrence of budesonide, a glucocorticoid steroid, is extensively studied in WWTPs.
- Triclosan was the most critical compound in terms of contribution and environmental risk.

GRAPHICAL ABSTRACT

ARTICLE INFO

Article history:
Received 15 May 2013
Received in revised form 13 July 2013
Accepted 13 July 2013
Available online 7 August 2013

Editor: D. Barcelo

Keywords:
Hospital wastewaters
LC–MS
LC–MS/LTQ–Orbitrap
Municipal wastewaters
PPCPs
Environmental risk assessment

ABSTRACT

In the present work, an extensive study on the presence of eighteen pharmaceuticals and personal care products (PPCPs) in eight wastewater treatment plants (WWTPs) of Greece has been conducted. The study covered four sampling periods over 1-year, where samples (influents; effluents) from eight WWTPs of various cities in Greece were taken. All WWTPs investigated are equipped with conventional activated sludge treatment. A common preconcentration step based on SPE was applied, followed by LC–UV/Vis–ESI–MS. Further confirmation of positive findings was accomplished by using LC coupled to a high resolution Orbitrap mass spectrometer. The results showed the occurrence of all target compounds in the wastewater samples with concentrations up to 96.65 μg/L. Paracetamol, caffeine, trimethoprim, sulfamethoxazole, carbamazepine, diclofenac and salicylic acid were the dominant compounds, while tolfenamic acid, fenofibrate and simvastatin were the less frequently detected compounds with concentrations in effluents below the LOQ. The removal efficiencies showed that many WWTPs were unable to effectively remove most of the PPCPs investigated. Finally, the study provides an assessment of the environmental risk posed by their presence in wastewaters by means of the risk quotient (RQ). RQs were more than unity for various compounds in the effluents expressing possible threat for the aquatic environment. Triclosan was found to be the most critical compound in terms of contribution and environmental risk, concluding that it should be seriously considered as a candidate for regulatory monitoring and prioritization on a European scale on the basis of realistic PNECs. The results of the extensive monitoring study contributed to a better insight on PPCPs in Greece and their presence in influent and effluent wastewaters. Furthermore, the

Abbreviations: PPCPs, Pharmaceuticals and personal care products; WWTPs, Wastewater treatment plants; WW, Wastewaters; LC–MS, Liquid chromatography–mass spectrometry; ESI, Electron spray ionization; LTQ, Linear trap quadrupole; USD, US dollar; OECD, Organization for Economic Co-operation and Development; SPE, Solid phase extraction; PI, Positive ionization; NI, Negative ionization; SIM, Selected ion monitoring; LOD, Limit of detection; LOQ, Limit of quantification; RQ, Risk quotient; EMEA, European Medicines Agency; MEC, Measured environmental concentration; PNEC, Predicted no-effect concentration; AF, Assessment factor; WFD, Water Framework Directive; LC_{50}, Lethal concentration 50; EC_{50}, Effective concentration 50; NOEC, No observed effect concentration; HRT, Hydraulic retention time; SRT, Sludge retention time.

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http://dx.doi.org/10.1016/j.scitotenv.2013.07.044

unequivocal identification of two transformation products of trimethoprim in real wastewaters by using the advantages of the LTQ Orbitrap capabilities provides information that should be taken into consideration in future PPCP monitoring studies in wastewaters.

1. Introduction

In recent years, presence of pharmaceuticals and personal care products (PPCPs) in the aquatic environment has been referred as one of the most urgent environmental concerns (Al-Odaini et al., 2010). These compounds are released mostly through urban wastewater and many of them can further spread through the water cycle, even reaching drinking water, due to their hydrophilic character and low removal at wastewater treatment plants (WWTPs). This fact has initiated a huge scientific effort to better understand the occurrence and fate of most commonly administered PPCP compounds in urban and hospital wastewaters and assess their potential environmental effects. In this light, several of investigations have been conducted in various types of wastewater samples in different areas around the World (Gracia-Lor et al., 2011, 2012a; Gros et al., 2010; Kosma et al., 2010; López-Serna et al., 2011; Stamatis and Konstantinou, 2013; Verlicchi et al., 2012a).

Very little data are currently available in Greece on the occurrence and fate of PPCPs in wastewaters. Until recently, research and monitoring data on the environmental occurrence of PPCPs in wastewaters of Greece have been limited to studies focused on a small number of targeted compounds in localized areas, with no considerations on the WWTP efficiency (Botitsi et al., 2007; Samaras et al., 2010). To the author's knowledge, only two specialized reports have been published (Kosma et al., 2010; Stamatis and Konstantinou, 2013) dealing with the monitoring of a number of PPCPs during one whole year and their treatment removal in wastewaters.

Despite its small size relative to other European countries, Greece is one of the big pharmaceutical per capita spenders after the United States and Canada, with expenditure of USD 677, much higher than the OECD average of USD 487 (OECD, 2011). In 2001 Greece was the second and fifth European country in total antibiotic use of ambulatory and hospital care, respectively (Botitsi et al., 2007). In addition, according to data collected by the OECD (OECD, 2003), pharmaceutical expenditure in Greece, ranged around 14% of total health care expenditure in 2001, approximately equal to Germany's (14.3%), Sweden's (13.5%) and the UK's (15.8%) but lower than those of other Mediterranean countries (Portugal 22.8%, Italy 22.3%, Spain 22%) and the OECD countries' average of 16.9%. Nevertheless, the real amount of applied drugs is uncertain, but significantly higher for some pharmaceuticals, taking into account that the annual consumption of a certain drug is difficult and often based on estimates (Thacker, 2005).

Current information thus suggests that the high pharmaceutical consumption in Greece will reflect high PPCP inputs of local WWTPs. Therefore, it is important to study more extensively and comprehensively the occurrence and fate of the most widespread PPCPs in Greek conventional WWTPs.

In order to address this, the present study constitutes an attempt to accurately measure the concentrations of eighteen PPCPs in untreated and final effluent of eight contrasting WWTPs located in various provinces of North West (N.W.) Greece. These pharmaceuticals were the analgesic/anti-inflammatory drugs salicylic acid, ibuprofen, paracetamol, naproxen, diclofenac, tolfenamic acid and phenazone, the lipid regulators gemfibrozil, fenofibrate, bezafibrate and clofibric acid, the antibiotics trimethoprim and sulfamethoxazole, the antiepileptic carbamazepine, the psychomotor stimulant caffeine, the glucocorticoid steroid budesonide, the disinfectant triclosan and the hypolipidemic statin simvastatin. They were mainly chosen according to their high annual consumption, previous studies about their occurrence and removal in wastewaters and surface waters, their stability and poor elimination during WWTPs as well as the concern about their possible effects on human and aquatic organisms (Gracia-Lor et al., 2012a; Gros et al., 2010; Kosma et al., 2010; Verlicchi et al., 2012a). Although the present study was limited to target compounds, further investigations shall be carried out to increase the number of measured analytes and to elucidate levels of conjugated or metabolic forms of the active compounds.

The compared WWTPs are equipped with conventional activated sludge secondary treatment and nitrogen and phosphate removal. A snapshot of the ability of these systems to remove such compounds is provided by comparing their global removal efficiencies for each substance. The biodegradability of the target PPCPs in different operational systems was overviewed and the seasonal variation in the elimination of PPCPs was also assessed. To the best of our knowledge this is the first report referring to such a big monitoring plan in wastewaters in Greece at the same period of time, which would provide a more comprehensive snapshot of the studied area. In addition, there is a lack of data on the removal efficiency across the varying configurations of WWTPs and only very few works (Kosma et al., 2010) consider a relatively broad set of PPCPs in several types of wastewaters in N.W. Greece (Epirus, Macedonia, Aitoloakarnania), an area characterized by an important rural population, humid climate and the operation of small WWTPs. Finally, a risk analysis is provided in order to assess and compare the potential environmental risk of various types of wastewaters (hospital and municipal effluents) by evaluating the ratio between the measured environmental concentration (MEC) and the predicted no-effect concentration (PNEC) for these wastewaters (Gros et al., 2010; Valcárcel et al., 2011; Zhao et al., 2010).

2. Experimental materials and methods

2.1. Chemicals

Pharmaceutical analytical standards were purchased from Promochem (Wesel, Germany). Simvastatin and Trimethoprim (vetranal) were supplied from Sigma-Aldrich (Steinheim, Germany). Solvents such as methanol and acetone were obtained from Pestiscan (Labscan, Ltd., Dublin, Ireland) and anhydrous sodium sulfate from Merck (Darmstadt, Germany). Acetonitrile (ACN) and water (for chromatographic analysis, LC–MS grade) were received from Fisher Scientific (Leicestershire, UK). Formic acid (purity, 98–100%), was obtained from Merck KGaA (Darmstadt, Germany). Oasis HLB (200 mg, 6 cm^3) and Oasis MCX (150 mg, 6 cm^3) cartridges were purchased from Waters Corporation (Milford, MA, U.S.A.). Stock 1000 mg/L solutions of each pharmaceutical were prepared in methanol and stored at −20 °C. A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in methanol–water (50–50 v/v). Table 1 lists the physicochemical properties of pharmaceuticals investigated.

2.2. Sampling sites and sample collection

Samples were collected from eight WWTPs located in various cities in Greece. Table 2 shows the characteristics of the WWTPs studied.

As can be seen from Table 2, all WWTPs investigated are equipped with conventional activated sludge secondary treatment and nitrogen and phosphate removal. Main differences among them refer to their water treatment capacity, the hydraulic retention times and solid retention times. Hospital WWTP is the smaller unit from all the units investigated as it receives lower loads than the other bigger municipal

Table 1
Physicochemical properties of the target compounds.

Therapeutic groups	Compounds	Molecular formula	MW	pKa[a]	LogK_{ow}[a]	Pv (mm Hg)[ab]	LogK_d	K_{biol} (L gSS^{-1} d^{-1})
Analgesics/ anti-inflammatory	Salicylic acid	$C_7H_6O_3$	138.12	2.3/3.5[c]	1.13/2.26[c]/−2.42[c]	8.20E−05	1.36[b]	
	Ibuprofen	$C_{13}H_{18}O_2$	206.28	4.9	3.97/0.45[c]	1.86E−04	1.84[b]/0.9[c]	1.5–20[c] 21–35[bc] 9–22[c] 1.33–>3[c]
	Paracetamol	$C_8H_9NO_2$	151.17	9.4	0.46	7.00E−06	0.4[b]/3.06[c]	58–80[bc] 106–240[c]
	Naproxen	$C_{14}H_{14}O_3$	230.26	4.2	3.50	1.89E−06	1.1[bc]	<0.2-c 1–1.9[bc] 0.4–0.8[c] 0.08–0.4[c]
	Diclofenac	$C_{14}H_{11}Cl_2NO_2$	296.15	4.2	4.51/0.7[c]	6.14E−08	1.2[bc]	<0.04–1.2[c] ≤0.1[bc] < 0.002–<0.1[c]
	Tolfenamic acid	$C_{14}H_{12}ClNO_2$	261.71	4.3	5.38[c]	2.59E−07		
	Phenazone	$C_{11}H_{12}N_2O$	188.23	1.5	0.38	3.06E−05		
Lipid regulators	Gemfibrozil	$C_{15}H_{22}O_3$	250.34	4.7	4.77	3.05E−05	1.87[b]/1.28[c]	6.4–9.6[bc] 0.5–1.8[c]
	Fenofibrate	$C_{20}H_{21}ClO_4$	360.83	4.5	5.19	5.35E−09		
	Bezafibrate	$C_{19}H_{20}ClNO_4$	361.82	3.6[c]	4.25[c]	6.29E−14		2.1–3.0[c] 3.4–4.5[c] 0.77–> 2.9[c]
	Clofibric acid	$C_{10}H_{11}ClO_3$	214.65	3.2[b]/–3.18[c]	2.57[bc]	1.13E−04	0.7[b]	0.3–0.8[bc] 0.1–0.23[c] 0.09–0.1[c]
Antibiotics	Trimethoprim	$C_{14}H_{18}N_4O_3$	290.32	6.6/7.2[c]	1.33/0.91[c]	9.88E−09	2.2–2.6[c] 2.3[c]	0.15[c]
	Sulfamethoxazole	$C_{10}H_{10}N_3O_3S$	253.28	5.7[c]	0.89[c]	6.93E−08	2.1–2.7[c] 2.3–2.6[c]	0.3[c]
Phsychiatrics-antiepileptics	Carbamazepine	$C_{15}H_{12}N_2O$	236.27	7/13.9[c]	2.47	1.84E-07	1.82[b]/0.1[c]	≤0.1[c] < 0.03–<0.06[c] < 0.005–<0.008[c]
Psychomotor stimulants	Caffeine	$C_8H_{10}N_4O_2$	194.20	10.4	−0.007	15	2.30	
Glucocorticoid steroids	Budesonide	$C_{25}H_{34}O_6$	430.53	7.9	2.18	2.73		
Disinfectants	Triclosan	$C_{12}H_7Cl_3O_2$	289.54	4.5/8.1[c]	4.80/5.34[c]	6.45E−07	4.3[b]	
Hypolipidemic statins	Simvastatin	$C_{25}H_{38}O_5$	418.57	13.2	4.68/5.19[c]	n.d.		

Literature data from: [a]Kosma et al. (2010), [b]Stamatis and Konstantinou (2013), and [c]Verlicchi et al. (2012a).

WWTPs. However, this fact does not indicate that the hospital WWTP is an efficient unit since even though lower loads reach the unit, their variety and complexity makes them a major source of contamination that substantially contributes to the total wastewater load of the municipal WWTP. For example a big number of undesirable organic xeniobiotic compounds such as pharmaceutical residues, radionuclides, antibiotic resistant bacteria, solvents and disinfectants as well as laboratories and surgeries materials are included in the burden of the hospital wastewaters making the loads that result into the urban network even more complex (Fatta-Kassinos et al., 2011; Ort et al., 2010).

During the study, a total number of 64 influent and effluent samples were collected from the eight WWTPs, covering a monitoring program for the four seasons over 1-year monitoring period (2010–2011). All samples were taken over a 24-hour period (composite samples). Weeks without significant rainfall were chosen in order to avoid dilution effects. The pH on the sampling dates ranged from 6.87 to 8.39, with the average being 7.57.

Description of hospital and municipal WWTPs of Ioannina City as well as details on sample collection can be found elsewhere (Kosma et al., 2010). A description for the rest of WWTPs is given in the Supplementary Data.

2.3. Sample preparation and solid phase extraction procedure

Wastewater samples were collected in amber glass bottles pre-rinsed with deionized water. The samples were immediately transported to the laboratory and filtered through 1 μm glass fiber filters GF/B (Whatman, UK) prior to analysis, in order to eliminate suspended solid matter. Afterwards, the samples were stored in the dark at 4 °C and extracted within 48 h in all the cases.

Table 2
Characteristics of the WWTPs studied.

WWTP	Population served	Average flow (m^3/d)	Treated wastewater	SRT (d)	HRT (h)	Primary treatment	Secondary treatment	Final receiver
Ioannina City	100,000	25,276	Urban and industrial	11	1.5–4	Grit removal-primary settling	Activated sludge	Kalamas River
Ioannina hospital	800	550	Hospital	1.5	6	Grit removal	Activated sludge	Urban network
Arta	38,000	11,500	Urban	18	4	Grit removal	Activated sludge	Arachthos River
Preveza	25,000	7000	Urban	28	20	Grit removal	Activated sludge	Ionian Sea
Agrinio	90,000	14,000	Urban	1–3	24	Grit removal	Activated sludge	Acheloos River
Grevena	20,000	4000	Urban	20	16	Grit removal	Activated sludge	Grevenitis River
Kozani	70,400	10,000	Urban	25	39	Grit removal	Activated sludge	Polifitos Lake
Veroia	45,000	9800	Urban	20	22	Grit removal-primary settling	Activated sludge	Stank 66-Aliakmonas River

Solid retention time (SRT); Hydraulic retention time (HRT).

Analytes were extracted using off line SPE system connected to a vacuum pump. In order to optimize the extraction method, two cartridges were tested, Oasis HLB (divinylbenzene/N-vinylpyrrolidone copolymer −200 mg, 6 cm^3) and Oasis MCX (mixed polymeric-cation exchange −150 mg, 6 cm^3) in different pH values (pH 2, 4, 7 and 8.5). The results showed that most of the compounds exhibited higher recoveries using HLB cartridges at pH 7. Similar recoveries were also observed in spiked real WW samples without pH adjustment. So, taking into consideration that the mean pH value of the WW samples was 7.57 and in order to be able to extract all the target compounds in one single step, finally no pH adjustment of the samples was chosen (Buchberger, 2011; Gracia-Lor et al., 2010; Gros et al., 2006).

The cartridges were preconditioned with 5 mL of methanol and 5 mL of HPLC-grade water. After the conditioning step sample aliquots of 400 mL were loaded into the cartridge at a flow rate of 10 mL/min and finally washed with 5 mL of HPLC-grade water prior to the elution, in order to remove interfering compounds. Next the cartridges were dried under vacuum for 10 min. The analytes were eluted with 2 × 5 mL of methanol at 1 mL/min. The extracts were dried over anhydrous sodium sulfate and then under a gentle stream of nitrogen until dryness. The samples were then reconstituted with 0.5 mL of methanol:water, 50:50 (v/v) and stored at −20 °C until being analyzed.

2.4. LC–UV/Vis–ESI-MS analysis

The HPLC system consisted of a SIL 20A autosampler with the volume injection set to 20 μL and LC-20AB pump both from Shimadzu (Kyoto, Japan). The analytes were separated using a C_{18} (Restek) analytical column 150 × 4.6 mm with 5 μm particle size (Restek, USA). Detection was carried out using a SPD 20A UV–vis detector coupled in series with the LC–MS 2010EV mass selective detector, equipped with an atmospheric pressure ionization source electrospray (ESI) interface. The samples were analyzed using the ESI interface in positive (PI) and negative (NI) ionization mode. For the analysis in PI mode a gradient elution was performed by a binary gradient composed of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) according to the following program: Initial conditions 90% A, decreased to 20% in 30 min, decreased to 10% in 5 min, returns to the initial conditions after 2 min and re-equilibration time was set at 3 min. The total run analysis lasted 40 min. The analysis in NI mode was performed by using solvent A (water) and solvent B (acetonitrile) according to the following program: Initial conditions 90% A, decreased to 65% in 5 min, decreased to 60% in 7 min, decreased to 50% in 3 min, decreased to 20% in 5 min, decreased to 10% in 5 min, returns to the initial conditions after 2 min and re-equilibration time was set at 3 min. The total run analysis lasted 30 min. Column temperature was set at 40 °C and the flow rate was 0.5 mL/min. The drying gas was operated at flow 10 L/min at 200 °C. The nebulizing pressure was 100 psi, capillary voltage was 4500 V for positive ionization and −3500 V for negative ionization and the fragmentation voltage was set at 5 V (for PI and NI ionization, respectively). The compounds that were analyzed under positive ionization mode were paracetamol, trimethoprim, caffeine, sulfamethoxazole, phenazone, carbamazepine, bezafibrate, budesonide, fenofibrate and simvastatin, while the compounds that were analyzed under negative ionization mode were salicylic acid, clofibric acid, naproxen, ibuprofen, diclofenac, tolfenamic acid, gemfibrozil and triclosan. For each compound the precursor molecular ion ($[M + H]$ or $[M - H]$) and at least one confirming ion in the selected-ion monitoring (SIM) mode was acquired except for fenofibrate and simvastatin (Table 3). Fenofibrate did not show any precursor molecular ion response, the most intense signal was at m/z 319, suggesting the loss of one methylene and one carbon monoxide group $[M - CH_2 - CO + H]^+$. For simvastatin two distinct ions were present, corresponding to $[M + H]^+$ molecular ion at m/z 419 and to $[M + Na]^+$ ion at m/z 441, with the second presenting higher intensity and therefore chosen as suitable ion for confirmation (Martín et al., 2011). The identification of target compounds was performed by matching the retention time (within 2.5%) and mass spectrum with standards.

2.5. LC–MS/LTQ ORBITRAP XL analysis

In recent years, liquid chromatography coupled to a high resolution Orbitrap mass spectrometer (LC–LTQ-Orbitrap) has proven to be a powerful and reliable analytical tool for identifying a lot of organic pollutants (Chitescu et al., 2012; Cortés-Francisco et al., 2011; Hogenboom et al., 2009; Pinhancos et al., 2011; Wille et al., 2011). Nevertheless, its applicability for the identification of PPCPs in wastewaters has not been very prevalent yet. In the present study, further confirmation of positive findings was accomplished by LC–LTQ-Orbitrap mass spectrometer. It is a new type of a mass analyzer that allows wide mass range detection with high resolving power, mass accuracy and dynamic range. Using Orbitrap MS, a maximum mass deviation of 5 ppm is allowed giving a high reliability in identification.

An LC–MS using LTQ Orbitrap XL from Thermo Fischer Scientific (Bremen, Germany) was used. The LC system was equipped with an Accela AS autosampler and Accela Pump (Ser. No 750157) pump. The analytes were separated using a Thermo hypersil gold column 150 × 2.1 mm with 5 μm particle size. Full scan acquisition over a mass range of 120–500 Da was performed at spray voltage 3.2 kV and a resolution of 60,000 was applied. Capillary voltage at 40 V, tube lens voltage at 110 V, sheath gas flow rate at 60 arbitrary units (au), auxiliary gas flow rate at 20 au and capillary temperature at 350 °C were chosen. The data were acquired and processed using the Thermo Xcalibur 2.1 software package.

Identification of compounds was based on both their retention time, relative to that of the standards and their accurate mass, i.e. by matching the theoretical mass with the observed mass (four decimals). A comparison of calculated exact mass values and measured accurate mass values are listed in Table 4.

For the analysis in PI mode the separation conditions were as follows: solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid) at a flow rate of 0.45 mL/min according to the following program: Initial conditions 100% A, decreased to 90% in 2 min, decreased to 60% in 10 min, then decreased to 0% in 13 min, remain 0% for 3 min and finally returns to the initial conditions after 2 min with the re-equilibration of the column set at 3 min. The analysis in NI mode was performed by using solvent A (water with 0.1% ammonia) and solvent B (methanol with 0.1% ammonia) at a flow rate of 0.2 mL/min according to 2.4.

Table 3
Instrumental parameters for target compounds using LC–MS in SIM mode.

PPCPs	Polarity (ESI)	Time (min)	m/z ions	Relative ion intensity %
Paracetamol	+	6.267	**152**, 110	100, 40.09
Caffeine	+	8.425	**195**, 138	100, 60.16
Trimethoprim	+	11.517	**291**, 261, 230	100, 51.38, 36.18
Phenazone	+	12.192	**189**, 147, 56	100, 86.52, 12.29
Sulfamethoxazole	+	14.235	**254**, 156, 92	100, 40.23, 12.31
Carbamazepine	+	18.692	**237**, 194, 192	100, 80.39, 48.59
Bezafibrate	+	22.975	**362**, 276, 316	100, 25.93, 14.86
Budesonide	+	23.333	**431**, 413, 323	100, 98.28, 29.81
Fenofibrate	+	27.025	**319**	100
Simvastatin	+	33.650	**441**, 419, 267	100, 98.27, 97.64
Naproxen	−	12.158	**229**, 185	100, 80.20
Salicylic acid	−	12.397	**137**, 93	100, 97.8
Ibuprofen	−	12.437	**205**, 160,161	100, 90.21, 71.12
Clofibric acid	−	19.788	**213**, 127	100, 12.63
Diclofenac	−	22.331	**294**, 295, 250	100, 40.91, 12.72
Gemfibrozil	−	24.040	**249**, 121	100, 82.63
Tolfenamic acid	−	24.318	**260**, 396	100, 10.12
Triclosan	−	24.715	**287**, 289	100, 90.86

Quantitation ions in bold.

In Fig. SD1, extracted chromatograms (positive ionization mode) of PPCPs standard (500 ppb) and PPCPs found in the influent of Grevena WWTP in spring, based on accurate mass with a mass window of below 5 ppm are shown.

2.6. Analytical performance

Identification and confirmation of the target compounds was based on the quality control procedures established by the European Union (EU) regulations (EU Commission Decision, 2002). Thus, identifications of pharmaceuticals in wastewater samples were made by comparing the retention time, identifying the target and qualifier ions, and determining the qualifier-to-target ratios of the peak in the wastewaters with that of a pharmaceutical standard. Acceptance criteria for positive identification consisted of retention times within (0.50 min of the expected) value and % qualifier-to-target ratios within 20% of the standard (0.1 mg/L) for qualifier-to-target abundance percentages greater than 50%. For less than 50%, the criterion for the qualifier-to-target ratios was set at 30% of the calibration standard.

Internal quality control was applied in every batch of samples in order to check if the system is under control. This quality control implies a matrix-matched calibration, a reagent blank, a matrix blank and a spiked blank sample at 0.5 μg/L in order to evaluate stability of the proposed method with time.

Quantification was performed with matrix matched calibration curves, by using influent and effluent SPE extracts, respectively. With each batch of 12 samples, a five-point calibration curve was prepared for analytes concentrations between the limit of quantifications (LOQs) and 10 LOQs by injections before and after those of the sample extracts. In addition, two quality control (QC) samples were injected in every batch of samples. The QC samples were blank wastewater sample fortified at LOQ level and 10 times the LOQ level. Blanks were subtracted and recoveries taken into account for concentration calculations.

The limit of detection (LOD) and LOQ of the LC–MS methods were determined by the injection of spiked water samples ($n = 3$) and calculated as the minimum detectable amount of analyte with a signal-to-noise ratio of 3:1 and 10:1, respectively. Any peak above the LOQ was quantified (Table SD1) (Kosma et al., 2010). LODs in distilled water ranged between 1.8 and 70.7 ng/L, in influent wastewaters between 2.9 and 112.9 ng/L and in effluent wastewaters between 2.0 and 78.3 ng/L.

Precision of the LC–MS chromatographic method, determined as relative standard deviation (RSD), was obtained from the repeated injections ($n = 5$) of a spiked extract during the same day (repeatability) and in different days (reproducibility) (Table SD1). Recovery studies ($n = 3$) were carried out in LC–MS method by spiking samples at two concentration levels of 0.2 and 2 μg/L. Recoveries were determined for distilled water and wastewaters (Table SD2). The recoveries were calculated by using influent and effluent wastewaters spiked with the analytes at concentrations of 0.2 and 2 μg/L. Due to the fact that unspiked WWTP influents and effluents already contained some of the compounds, the concentration of the respective unspiked sample was subtracted from the concentration in the spiked sample and then divided by the spiked level. Mean recoveries in distilled water ranged from 49.4 to 101.4% in 0.2 μg/L and from 58.6 to 100.8% in 2 μg/L. In the influents, recoveries varied from 43.3 to 99.3% and from 52.7 to 112.1%, in 0.2 and 2 μg/L, respectively. In the effluents, recoveries varied from 45.5 to 121.4% and from 49.8 to 126.5%, in 0.2 and 2 μg/L, respectively.

Matrix effects such as signal suppression/enhancement are a substantial concern in LC/MS studies, especially in electrospray mass spectrometry due to the fact that ESI is very liable to other components present in the sample which may lead to inaccurate results (Botitsi et al., 2007; Gómez et al., 2007; Gros et al., 2006; Botitsi et al., 2007; Nödler et al., 2010). Ion suppression or enhancement were evaluated by comparing the peak areas of spiked influent and effluent wastewater extracts (area matrix), after subtracting the peak areas corresponding to native analytes present in the sample (area blank), with the peak areas from solvent (methanol–water 50:50, v/v) spiked at the same level (area solvent) (Eq. (1)) (Gros et al., 2009, 2012).

$$\%\ \text{suppression/enhancement} = 100 - (((\text{area matrix} - \text{area blank}) * 100)/\text{area solvent}). \quad (1)$$

When signal suppression or enhancement occurs the signal intensity of the analytes decreases or increases, respectively (Gros et al., 2009; Kasprzyk-Hordern et al., 2008; López-Serna et al., 2011). The percentage of signal suppression/enhancement in wastewaters is depicted in Table SD1 in the Supplementary data.

2.7. Risk quotients (RQ) and ecotoxicological risk assessment

The risk of quotient (RQ) comprises a useful tool in order to characterize potential ecological risk of many contaminants in aquatic ecosystems (Gros et al., 2010). Based on EMEA guidelines, RQ was calculated as the ratio between Measured Environmental Concentration (MEC) and

Table 4
PPCPs and corresponding retention times, exact and accurate mass information, mass error deviation and double bond and ring equivalent number (DBE).

PPCPs	Elemental composition	RT (min)	Exact mass (theoretical)	Accurate/nominal mass (detected)	Error (ppm)	DBE	Ionization mode
Paracetamol	$C_8H_9NO_2$	2.59	152.0706	152.0712	3.452	4.5	+
Trimethoprim	$C_{14}H_{18}N_4O_3$	5.52	291.1452	291.1462	3.548	7.5	+
Caffeine	$C_8H_{10}N_4O_2$	5.82	195.0877	195.0883	3.321	5.5	+
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	6.28	254.0594	254.0600	2.407	6.5	+
Phenazone	$C_{11}H_{12}N_2O$	7.11	189.1022	189.1027	2.435	6.5	+
Carbamazepine	$C_{15}H_{12}N_2O$	14.08	237.1022	237.1030	3.207	10.5	+
Bezafibrate	$C_{19}H_{20}ClNO_4$	17.13	362.1154	362.1170	4.522	9.5	+
Budesonide	$C_{25}H_{34}O_6$	18.34	431.2428	431.2440	2.747	8.5	+
Fenofibrate	$C_{17}H_{16}ClO_4$	18.84	319.0737	319.0742	3.249	9.5	+
Simvastatin	$C_{25}H_{38}O_5$	21.54	419.2792	419.2809	4.065	6.5	+
Salicylic acid	$C_7H_6O_3$	1.35	137.0244	137.0249	3.620	5.0	−
Clofibric acid	$C_{10}H_{11}ClO_3$	2.31	213.0324	213.0332	3.778	5.5	−
Naproxen	$C_{14}H_{14}O_3$	2.34	229.0870	229.0879	3.852	8.5	−
Ibuprofen	$C_{13}H_{18}O_2$	9.89	205.1234	205.1234	−0.015	5.5	−
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	10.67	294.0094	294.0101	2.356	9.5	−
Tolfenamic acid	$C_{14}H_{12}ClNO_2$	12.68	260.0484	260.0492	3.155	9.5	−
Gemfibrozil	$C_{15}H_{22}O_3$	12.97	249.1496	249.1502	2.337	5.5	−
Triclosan	$C_{12}H_7Cl_3O_2$	20.76	286.9439	286.9442	1.095	8.5	−

Predicted No-Effect Concentration (PNEC) using worst case assumptions (Eq. (2)). Worst case assumption refers to the case where the highest concentration of the target compound was detected. So, MEC corresponded to the highest measured concentration detected in wastewater samples (Gros et al., 2010; Santos et al., 2007) and PNEC was estimated using the lowest values of acute EC_{50} or LC_{50} or the chronic NOEC, divided by a default assessment factor (AF) (Damásio et al., 2011; Ginebreda et al., 2010; Gros et al., 2010; Hernando et al., 2006; Ramaswamy et al., 2011; Stuer-Lauridsen et al., 2000). According to Water Framework Directive (WFD), for each pharmaceutical compound, two estimations were made with the toxicity data obtained from the literature for three different representative trophic levels of the ecosystem, such as fish, invertebrates and algae (Tables SD3 and SD4). The first, was the PNEC estimated from the acute toxicity test results (Eq. (3)) and the second was the PNEC estimated from the chronic toxicity test results (Eq. (4)) (Ferrari et al., 2004; Ginebreda et al., 2010).

$$RQ = exposure/toxicity = \text{water or sediment concentration (MEC)}/LC_{50}\text{or } EC_{50}\text{or NOEC (PNEC)} \quad (2)$$

where,

$$PNEC_{acute} = (EC_{50}\text{or } LC_{50})/1000 \quad (3)$$

$$PNEC_{chronic} = NOEC/AF. \quad (4)$$

Assessment factors where applied following the guidelines of the Technical Guidance Document on Risk Assessment of the European Union EC (1996). An AF of 1000 was applied when at least one short-term L(E)C_{50} from each of the three evaluated trophic levels was available, an AF of 100 was applied when one long-term NOEC was available, an AF of 50 was applied when two-long term NOECs were available for species in two different trophic levels and finally, an AF of 10 was applied when long-term NOECs from at least three species were available (European Commission, 2003; Zhao et al., 2010).

For risk analysis a commonly used risk ranking criterion was applied. When the ratio between the exposure concentration and predicted no effect concentration equals or exceeds to 1, then an ecological "high risk" is suspected ($RQ \geq 1$). Similar criteria that construe this ratio (RQ) proposed by Hernando et al. (2006) refer that when $0.01 < RQ < 0.1$ "low risk" is suspected and when $0.1 < RQ < 1$ "medium risk" is suspected.

3. Results and discussion

3.1. Occurrence of PPCPs in WWTPs

The method developed in the present study was applied for the determination of 64 influent and effluent wastewaters samples. The samples were collected over the four seasons of a year: autumn 2010 (October), winter 2011 (February), spring 2011 (may) and summer 2011 (July) in eight WWTPs. Tables 5 and 6 show the percentage of positive findings of the target compounds, as well as the range and mean concentrations found in each WWTP in influent and effluent wastewater samples, respectively.

All compounds found to be present in the influents. The majority of PPCPs were detected in more than 50% of the samples, except for tolfenamic acid, fenofibrate, clofibric acid and simvastatin. Tolfenamic acid was present only at concentrations below the limit of quantification (LOQ), while clofibric acid was quantified in only two samples (Veroia and Grevena in July) at concentrations above the LOQ. The most abundant compounds were carbamazepine (100%), salicylic acid (96.9%), trimethoprim (96.9%), diclofenac (93.8%) and sulfamethoxazole (90.6%). Paracetamol and caffeine were also detected in more than 80% of the samples. The highest concentration in the influents was observed for caffeine (Arta in July), while the lowest was observed for phenazone (Grevena in February) in concentrations of 96648.3 ng/L and 9.3 n/L, respectively.

Among the analgesic/ant-inflammatory drugs the highest average concentrations were found for salicylic acid in Ioannina City (30353.6 ng/L) and hospital (32064.6 ng/L) and for paracetamol in Veroia (40451.7 ng/L) and Arta (8313.2 ng/L). High concentrations presented also ibuprofen in Ioannina City, ibuprofen in hospital, naproxen in Preveza, naproxen in Veroia and diclofenac in Preveza with maximum levels of 8890.1, 6023.9, 5899.9, 3119.0 and 5164.0 ng/L and mean concentrations of 2633.4, 2048.5, 1814.0, 1583.7 and 1346.0 ng/L, respectively.

Among the lipid regulators, the most detected compounds were gemfibrozil and bezafibrate. Gemfibrozil presented high concentrations in Ioannina City, hospital, Grevena and Veroia with mean values of 347.1, 515.2, 312.0 and 215.1 ng/L, respectively, while bezafibrate presented high concentrations in Ioannina City, hospital and Preveza with mean concentrations of 429.8, 315.4 and 210.4 ng/L, respectively.

The antibiotics were present in a high percentage in the samples analyzed. Both, trimethoprim and sulfamethoxazole, presented their highest concentrations in Ioannina hospital with mean levels of 621.8 and 1464.5 ng/L, respectively. It is worth to point out that accurate mass screening of LTQ Orbitrap analyzer confirmed the presence of two transformation products (TPs) of trimethoprim in the effluents of two municipal WWTPs, (Ioannina and Veroia), during the summer sampling. Trimethoprim is known to undergo cleavage and oxidation on multiple function groups and forms a variety of oxidative TPs through the biotransformation and phototransformation reactions (Sirtori et al., 2010; Kosma et al., 2011). Taking this into consideration, orbitrap and multiple data mining techniques have been used for predicted trimethoprim TP detection and structural characterization. To search for TP ions that were not visible in the total ion chromatograms (TIC) post-acquisition data mining approach, using extracted-ion chromatography (EIC), was performed. The detected trimethoprim TPs were elucidated on the basis of the mass shifts from the parent molecule, molecular formulae derived from the accurate mass measurements, and the interpretation of accurate MS/MS spectra (Table 7). Two compounds (TP1 and TP2) were detected and identified. The first one (TP1) with theoretical exact mass measurement of m/z 305.1244 indicated a protonated molecule of $C_{14}H_{17}N_4O_4$, showing that one oxygen atom had been introduced into trimethoprim molecule ($C_{14}H_{19}O_3N_4$, protonated molecular ion) by forming a keto-derivative. The identity of this TP ((2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl) methanone) was further confirmed by the appearance of the characteristic fragment at m/z 137.0458 ($C_5H_5N_4O$, protonated molecule) with −2.826 ppm mass accuracy. DBE data (see Table 7) is also consistent with the proposed structure since the formation of a C═O bond increase the DBE of the keto-derivative by one (8.5) in relation to the parent molecule of trimethoptim (7.5). The second one (TP2, m/z 139; $C_5H_7N_4O$, protonated molecule) is derived from the cleavage of trimethoprim molecule at the C–C bond between the trimethoxy aromatic ring and the dibenzylic carbon with sequential addition of a hydroxyl group (Sirtori et al., 2010; Kosma et al., 2011). Based on the best fit formula and MS/MS data, this TP would be assigned as 2,4-diaminopyrimidine-5-carbaldehyde. The experimental findings were compared with the results obtained from the solar photolysis of trimethoprim in natural waters (Kosma et al., 2011), where e.g. a very good correlation for the accurate mass measurements and the interpretation of accurate MS/MS spectra of the detected TPs was confirmed. Here we report on the first evidence of the presence of TPs of trimethoprim on real wastewaters. This new record confirms the transformation of trimethoprim in biological treatment facilities and emphasize the urgent need for further work in order to improve our knowledge and explore the impact of different factors on the transformation pattern and distribution

Table 5
Ranges of concentrations and corresponding mean values in brackets of PPCPs found in the influents of the eight WWTPs.

PPCPs	Influent wastewaters (n = 32) (ng/L)								
	%. P.F.	Ioannina City	Ioannina hospital	Arta	Preveza	Agrinio	Grevena	Kozani	Veroia
Analgesics/anti-inflammatory									
SA	96.9	4138.4–89133.5 (42348.1)	5290.6–75847.4 (32064.6)	bql–4970.7 (1464.0)	bql–453.0 (348.1)	n.d.–697.8 (362.7)	bql–988.3 (619.4)	377.6–419.1 (395.9)	349.6–3875.3 (1261.6)
IBU	59.4	418.2–8890.1 (2633.4)	378.9–6023.9 (2048.5)	n.d.–425.6 (177.0)	n.d.–524.2 (279.9)	n.d.–412.1 (56.5)	bql–612.2 (494.6)	n.d.	n.d.
PAR	87.5	120.5–6421.6 (2872.5)	1139.6–4574.7 (2519.6)	126.3–24148.8 (8313.2)	bql–847.1 (293.0)	n.d.–358.0 (139.9)	n.d.–5663.5 (3047.2)	n.d.–7552.6 (4184.9)	bql–65402.8 (30353.6)
NPX	71.9	n.d.–726.8 (230.3)	994.7–1902.9 (1469.8)	n.d.–1839.5 (544.4)	n.d.–5899.9 (1814.0)	n.d.–951.3 (324.6)	n.d.–983.3 (306.6)	n.d.–992.1 (574.5)	n.d.–3119.0 (1583.7)
DCF	93.8	81.1–143.0 (100.8)	n.d.–530.3 (180.7)	bql–216.5 (106.9)	bql–5164.0 (1346.0)	bql–238.1 (98.8)	bql–120.2 (78.3)	n.d.–142.0 (83.1)	bql–84.0 (bql)
TA	43.8	n.d.–bql	n.d.–bql	n.d.–bql	n.d.–bql	n.d.–bql	n.d.	n.d.–bql	n.d.–bql
PNZ	56.3	60.3–155.2 (102.1)	n.d.–190.2 (66.0)	n.d.–22.3 (7.4)	n.d.–bql	n.d.–13.0 (5.1)	n.d.–10.4 (5.7)	n.d.–18.2 (6.4)	n.d.–23.5 (7.7)
Lipid regulators									
GMF	65.6	n.d.–733.2 (347.1)	n.d.–899.3 (515.2)	n.d.–190.9 (bql)	n.d.–243.7 (bql)	n.d.–329.0 (bql)	n.d.–566.8 (312.0)	n.d.–382.0 (bql)	n.d.–512.1 (215.1)
FNB	43.8	n.d.–269.6 (93.0)	n.d.–76.1 (bql)	n.d.–69.1 (bql)	n.d.–61.0 (31.2)	n.d.–40.2 (bql)	n.d.–29.7 (bql)	n.d.–62.3 (33.4)	n.d.–71.2 (bql)
BZF	65.6	245.2–755.9 (429.8)	n.d.–945.9 (315.4)	n.d.–144.8 (60.8)	bql–769.5 (210.4)	n.d.–25.7 (bql)	32.5–105.7 (45.9)	n.d.–26.8 (bql)	n.d.–77.4 (35.8)
CA	15.6	n.d.–bql	n.d.	n.d.	n.d.–bql	n.d.	n.d.–265.9 (bql)	n.d.	n.d.–119.2 (bql)
Antibiotics									
TMP	96.9	36.7–180.3 (132.1)	31.7–1866.2 (621.8)	bql–54.5 (23.1)	bql–42.1 (16.2)	bql–39.1 (16.7)	n.d.–19.6 (7.9)	bql–72.9 (33.7)	bql–39.4 (22.9)
SMX	90.6	367.9–2170.4 (904.2)	122.2–2626.3 (1464.5)	12.7–224.1 (112.2)	36.3–213.3 (119.5)	n.d.–41.3 (16.4)	n.d.–323.3 (132.8)	90.6–532.5 (227.2)	38.9–280.9 (140.9)
Antiepileptics									
CBZ	100	29.8–221.6 (98.8)	22.4–129.1. (77.7)	36.9–74.9 (59.7)	bql–46.6 (21.8)	20.6–194.7 (95.4)	17.5–354.7 (133.2)	31.9–215.5 (83.8)	16.7–54.0 (38.9)
Psychomotor stimulants									
CAF	84.4	17047.1–36238.0 (26107.5)	10532.8–55698.1 (28214.1)	212.9–96648.3 (58087.5)	n.d.–883.2 (361.7)	n.d.–1849.1 (615.0)	n.d.–1927.2 (642.6)	n.d.–64501.1 (24556.5)	1659.8–53498.5 (14928.7)
Glucocorticoid steroids									
BUD	59.4	bql–170.2 (70.5)	bql–420.8 (114.7)	n.d.–85.4 (26.6)	n.d.–125.2 (59.1)	n.d.–75.7 (26.3)	n.d.–80.3 (27.5)	n.d.	n.d.
Disinfectants									
TCS	68.8	n.d.–527.9 (202.7)	n.d.–988.9 (452.6)	n.d.–196.5 (bql)	n.d.–bql	n.d.–233.2 (bql)	n.d.–201.7 (113.2)	blq–149.6 (bql)	149.9–1742.5 (681.2)
Hypolipidemic statins									
SIM	34.4	n.d.–52.7 (24.9)	n.d.–62.0 (26.9)	n.d.–53.3 (25.7)	n.d.–39.8 (17.9)	n.d.	n.d.–59.4 (26.2)	n.d.–91.2 (39.6)	n.d.

P.F.: Positive findings, SA: Salicylic acid, IBU: Ibuprofen, PAR: Paracetamol, NPX: Naproxen, DCF: Diclofenac, TA: Tolfenamic acid, PNZ: Phenazone, GMF: Gemfibrozil, FNB: Fenofibrate, BZF: Bezafibrate, CA: Clofibric acid, TMP: Trimethoprim, SMX: Sulfamethoxazole, CBZ: Carbamazepine, CAF: Caffeine, BUD: Budesonide, TCS: Triclosan, SIM: Simvastatin.

potential of this pharmaceutical in engineering systems and the environment. Fig. 1 demonstrates a chromatogram of a wastewater sample from WWTP (Ioannina, Summer) with high resolution spectra showing accurate masses and mass errors of trimethoprim and its detected TPs.

The antiepileptic carbamazepine was present at 100% of the analyzed samples presenting the highest concentration in Grevena (354.7 ng/L). The psychomotor stimulant caffeine showed very high concentrations at all sampling points, reaching concentrations up to 96648.3 ng/L.

The disinfectant triclosan which was present in all WWTPs reached concentrations up to 1742.5 ng/L, in Veroia. Triclosan, which was registered as High Production Volume Compound (OECD, 2004), is commonly used as synthetic preservative and antimicrobial agent in wide range of PCPs of daily use, such as hand soaps, skin creams, toothpaste or deodorants but it is also used in a number of household items, such as plastic cutting boards, sport equipment, shoes or furniture (von der Ohe et al., 2012).

Budesonide, which belongs to the class of corticosteroids, was present at 59.4% of the samples. In spite of the fact that corticosteroids are consumed in large quantities worldwide (Al-Odaini et al., 2010) and have been highlighted to pose a potential environmental risk (Kugathas et al., 2012), their occurrence in environmental matrices has not gained significant attention until recently and information in the scientific literature is scant and not systematic (Chang et al., 2007; Hou et al., 2005; Kugathas et al., 2012; Piram et al., 2008). Piram et al. (2008) reported the occurrence of budesonide in the effluent of a WWTP in France but not in the influent. To the authors knowledge this is the first study in which the occurrence of budesonide is studied so extensively in WWTPs. According to our findings, it was present in six of the eight WWTPs studied, with most of the concentrations being below the LOQ. Its highest concentration was obtained in the hospital

Table 6
Ranges of concentrations and corresponding mean values in brackets of PPCPs found in the effluents of the eight WWTPs.

PPCPs	Effluent wastewaters (n = 3) (ng/L)								
	% P.F.	Ioannina City	Ioannina hospital	Arta	Preveza	Agrinio	Grevena	Kozani	Veroia
Analgesics/anti-inflammatory									
SA	87.5	229.0–431.9 (327.7)	bql–682.5 (332.6)	n.d.–240.3 (bql)	bql	n.d.–bql	blq–211.8 (bql)	bql	bql
IBU	25.0	n.d.–301.2 (bql)	n.d.–289.0 (bql)	n.d.	n.d.–bql	n.d.–bql	n.d.–bql	n.d.	n.d.
PAR	78.2	bql–207.2 (96.1)	n.d.–93.3 (bql)	n.d.–532.5 (195.7)	blq–192.3 (192.3)	n.d.–93.6 (bql)	n.d.–315.7 (166.0)	n.d.–444.0 (209.7)	bql–1060.3 (368.7)
NPX	50.0	n.d.	bql–406.0 (216.8)	n.d.–483.5 (128.0)	n.d.–331.9 (170.7)	n.d.–183.3 (58.8)	n.d.–431.7 (153.4)	n.d.–58.2 (bql)	1040.0–1076.0 (534.0)
DCF	71.9	blq–170.0 (98.0)	n.d.–188.1 (63.2)	n.d.–382.5 (162.5)	n.d.–121.5 (56.1)	n.d.–325.5 (154.9)	n.d.–69.2 (bql)	n.d.–250.7 (97.1)	n.d.–336.8 (145.6)
TA	37.5	n.d.–bql	n.d.–bql	n.d.–bql	n.d.–bql	n.d.	n.d.	n.d.–bql	n.d.–bql
PNZ	21.9	n.d.–7.0 (bql)	n.d.	n.d.–bql	n.d.	n.d.–bql	n.d.–bql	n.d.–bql	n.d.
Lipid regulators									
GMF	50.0	n.d.–230.9 (bql)	n.d.–355.6 (168.4)	n.d.–bql	n.d.–bql	n.d.–bql	n.d.–192.8 (bql)	n.d.–bql	n.d.–127.6 (bql)
FNB	11.1	n.d.–bql	n.d.	n.d.	n.d.	n.d.–bql	n.d.–bql	n.d.–bql	n.d.
BZF	46.9	27.4–233.8 (128.0)	n.d.–344.2 (111.8)	n.d.–20.0 (bql)	n.d.–278.2 (71.8)	n.d.–bql	n.d.–bql	n.d.–bql	n.d.–bql
CA	9.4	n.d.	n.d.	n.d.	n.d.	n.d.–bql	n.d.	n.d.	n.d.–70.8 (25.8)
Antibiotics									
TMP	84.4	bql–111.2 (59.8)	bql–533.2 (186.7)	bql–27.4 (11.6)	bql–23.1 (8.0)	n.d.–20.0 (8.3)	n.d.–bql	bql–7.0 (bql)	n.d.–18.6 (10.0)
SMX	81.3	n.d.–55.9 (23.7)	37.0–481.3 (205.7)	bql–34.4 (21.8)	bql–27.9 (15.9)	n.d.–16.1 (bql)	n.d.–28.4 (11.0)	12.1–25.9 (21.8)	bql–72.9 (30.6)
Antiepileptics									
CBZ	93.8	85.0–133.3 (119.9)	42.3–202.6 (147.4)	170.6–292.6 (211.9)	31.9–101.6 (57.7)	n.d.–148.6 (61.7)	51.7–416.8 (224.8)	n.d.–126.2 (76.6)	28.0–241.7 (110.5)
Psychomotor stimulants									
CAF	56.3	99.2–587.2 (335.2)	54.0–670.6 (348.9)	n.d.–1180.5 (415.8)	n.d.–34.0 (bql)	n.d.–29.3 (bql)	n.d.–188.2 (50.3)	n.d.–939.9 (440.2)	n.d.–523.1 (189.9)
Glucocorticoid steroids									
BUD	43.8	bql–454.7 (288.6)	n.d.–610.8 (254.7)	n.d.–bql	nd.–195.5 (90.4)	n.d.–61.4 (bql)	n.d.–70.2 (bql)	n.d.	n.d.
Disinfectants									
TCS	34.4	n.d.–130.9 (bql)	n.d.–288.0 (133.6)	n.d.–bql	n.d.–bql	n.d.	n.d.–bql	n.d.	n.d.–452.1 (139.2)
Hypolipidemic statins									
SIM	9.4	n.d.–bql	n.d.–bql	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

P.F.: Positive findings, SA: Salicylic acid, IBU: Ibuprofen, PAR: Paracetamol, NPX: Naproxen, DCF: Diclofenac, TA: Tolfenamic acid, PNZ: Phenazone, GMF: Gemfibrozil, FNB: Fenofibrate, BZF: Bezafibrate, CA: Clofibric acid, TMP: Trimethoprim, SMX: Sulfamethoxazole, CBZ: Carbamazepine, CAF: Caffeine, BUD: Budesonide, TCS: Triclosan, SIM: Simvastatin.

influent (420.8 ng/L). As a consequence, its occurrence in WWTPs may be attributed to the fact that it is used consecutively and in large quantities or it might be disposed as unused or expired pharmaceutical in toilets. The occurrence of budesonide in wastewaters was also confirmed by the Orbitrap mass analyzer by using its screening and MS/MS capabilities (Fig. 2).

Regarding the effluents, all compounds were also found to be present. Salicylic acid (87.5%), paracetamol (78.2%), trimethoprim (84.4%), sulfamethoxazole (81.3%) and carbamazepine (93.8%) were the most abundant compounds. The highest concentration in the effluents was observed for naproxen (Veroiain July), while the lowest was observed for trimethoprim (Kozani in October) in concentrations of 1076.0 ng/L and 6.6 ng/L, respectively.

Tolfenamic acid, fenofibrate and simvastatin were found only in concentrations below the LOQ. Clofibric acid was found only in three samples, but only in one sample at concentrations above the LOQ (70.8 ng/L, Veroia in July). Phenazone was also present only in one sample above the LOQ in Ioannina City in May (7.0 ng/L). Ibuprofen was present in eight samples with maximum concentration of 301.2 ng/L in Ioannina City. Caffeine was present in 56% of the samples with maximum concentration of 1180.5 ng/L in Arta. Naproxen and gemfibrozil were present in 50% of the samples with maximum concentrations of 1076.0 and 355.6 ng/L in Veroia and Ioannina hospital, respectively. Bezafibrate which was present in 46.9% of the samples showed the highest concentrations in Ioannina hospital (344.2 ng/L).

Table 7
MS/MS data of trimethoprim and its detected transformation products.

Compounds	RT (min)	Elemental composition	Exact mass (theoretical)	Accurate/ nominal mass (detected)	Error (ppm)	DBE
TMP	5.52	$C_{14}H_{19}O_3N_4$	291.1452	291.1462	3.548	7.5
		$C_{13}H_{15}O_3N_4$	275.1139	275.1138	−0.243	8.5
		$C_{12}H_{13}O_3N_4$	261.0982	261.0979	−1.213	8.5
		$C_{12}H_{14}ON_4$	230.1162	230.1161	−0.489	8.0
		$C_5H_7N_4$	123.0665	123.0663	−1.810	4.5
TP1	6.74	$C_{14}H_{17}O_4N_4$	305.1244	305.1237	−2.397	8.5
		$C_{13}H_{13}O_4N_4$	289.0931	289.0929	−0.800	9.5
		$C_{12}H_{11}O_4N_4$	275.0775	275.0771	−1.386	9.5
		$C_{12}H_{11}O_3N_4$	259.0826	259.0823	−1.030	9.5
		$C_5H_5ON_4$	137.0458	137.0454	−2.826	5.5
TP2	3.02	$C_5H_7ON_4$	139.0614	139.0610	−3.145	4.5

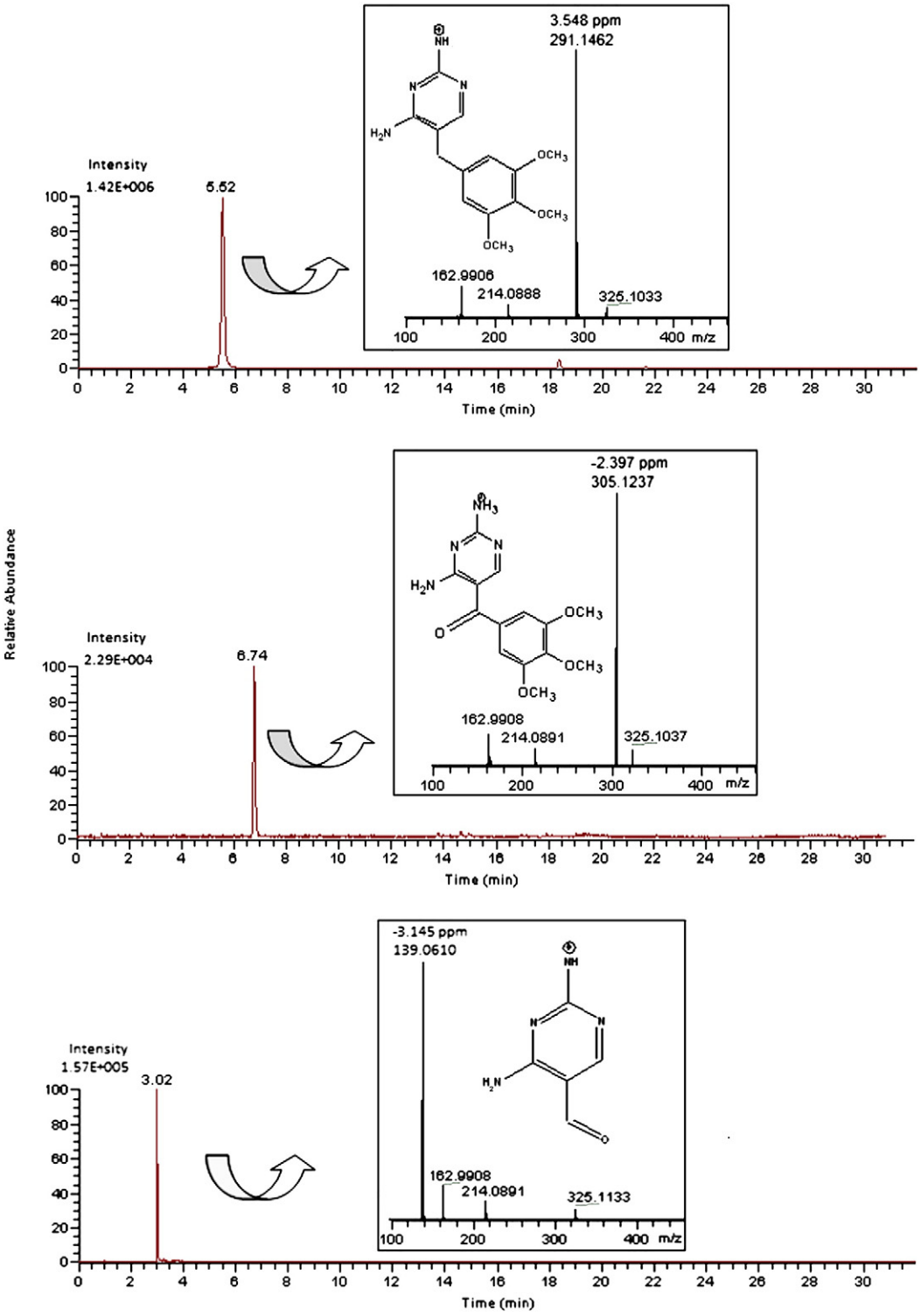

Fig. 1. Chromatogram from the effluent of Ioannina WWTP (Summer) with high resolution spectra showing accurate masses and mass errors of trimethoprim and its detected transformation products.

Finally, budesonide and triclosan were present in lower than 45% of the samples analyzed.

Until now, there are limited literature referring to the comparison of PPCPs occurrence in influents and effluents of different WWTPs in Greece. However, the results obtained in the present study showed that they are in agreement with other findings (Gracia-Lor et al., 2011, 2012a, 2012b; Gros et al., 2009, 2010, 2012; Kosma et al., 2010; López-Serna et al., 2011; Pedrouzo et al., 2011; Samaras et al., 2010, 2013; Verlicchi et al., 2012a, 2012b). For instance, Gracia-Lor et al. (2012a) found that in the influents of three WWTPs of Castellon province in Spain, the concentrations of naproxen, gemfibrozil, diclofenac and bezafibrate were ranged between 270 and 3580 ng/L, 160–2120 ng/L, 260–1490 ng/L and 20–460 ng/L, respectively, while their concentrations in the effluents were lower, ranged from the LOQ up to 740 ng/L. In another interesting study (Verlicchi et al., 2012b) conducted in the area of North Italy (three hospital and one municipal

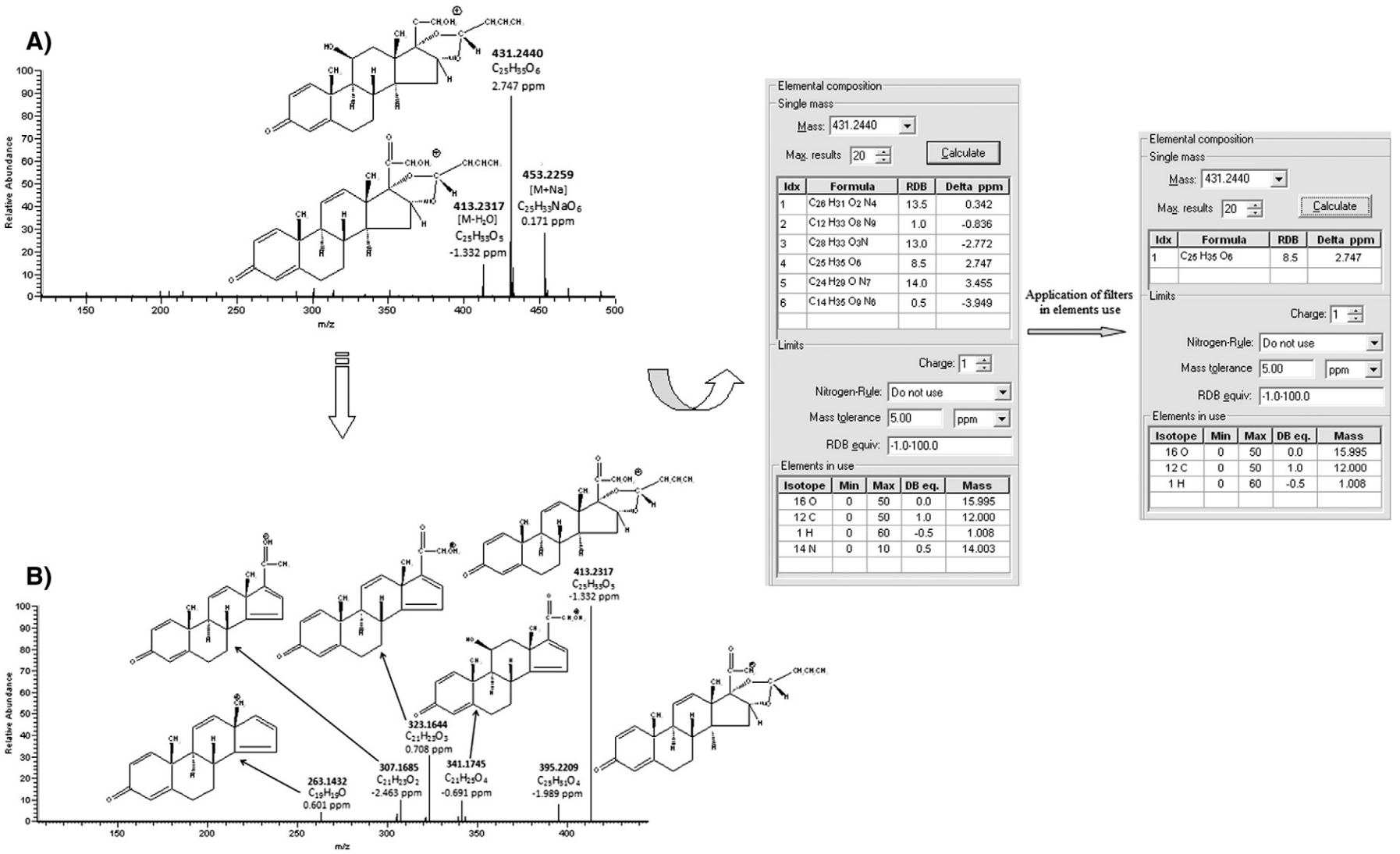

Fig. 2. Full scan accurate mass product ion spectrum of Budesonide identified in influent wastewater sample of hospital WWTP of Ioannina City (Spring); (B) MS/MS data obtained using HCD Orbitrap MS targeting the ion, m/z 431.2440, at a resolution setting of 60,000 FWHM, and the proposed fragmentation pathway of budesonide in positive ESI mode.

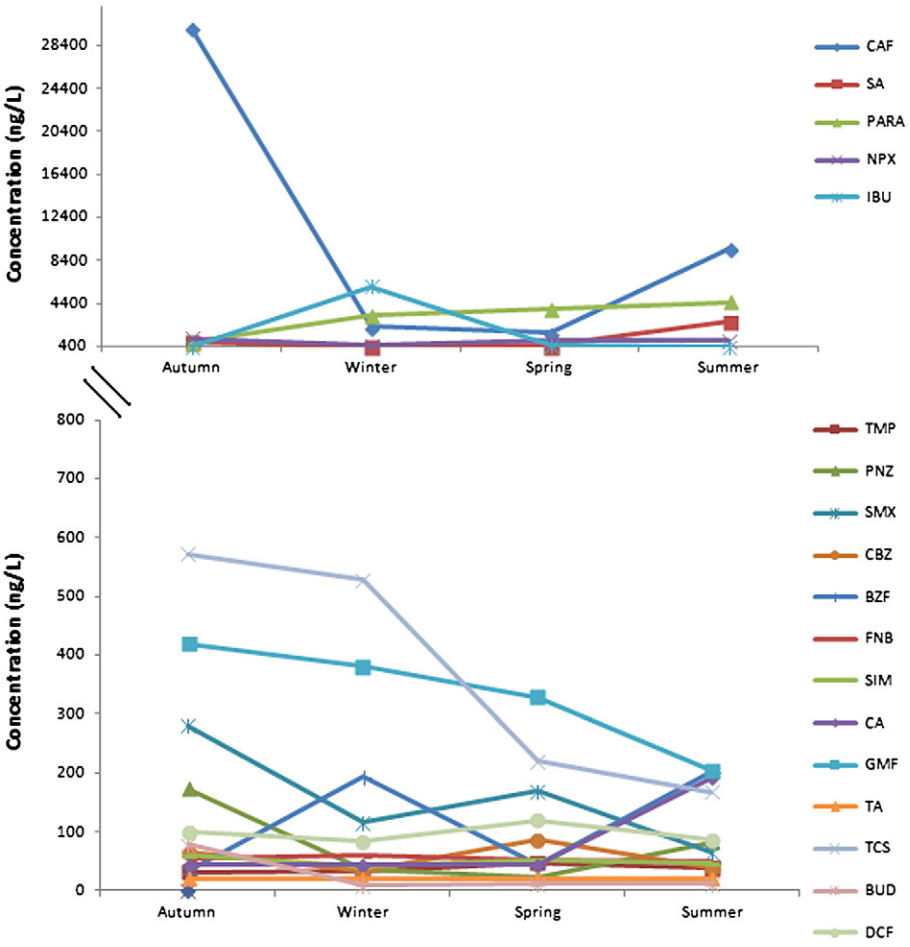

Fig. 3. Median concentrations of PPCPs in the influents of the WWTPs.

WWTPs), concentrations of paracetamol in municipal influents were ranged between 500 and 1200 ng/L, while in the effluents ranged between 12 and 58 ng/L. Phenazone was not detected in any sample whereas the concentration levels of clofibric acid were ranged at low ng/L levels in both municipal influents (<LOD-0.012 ng/L) and effluents (<LOD-0.0060 ng/L). Sulfamethoxazole was among the most prevalent antibiotics with concentrations up to 740 ng/L and 240 ng/L in municipal influents and effluents, respectively.

Based on the outcomes of our investigation, it can be concluded that hospital was a significant contributor of PPCPs in the wastewaters. In general, a comparison between hospital and urban wastewaters showed that in terms of individual compounds, phenazone, gemfibrozil,

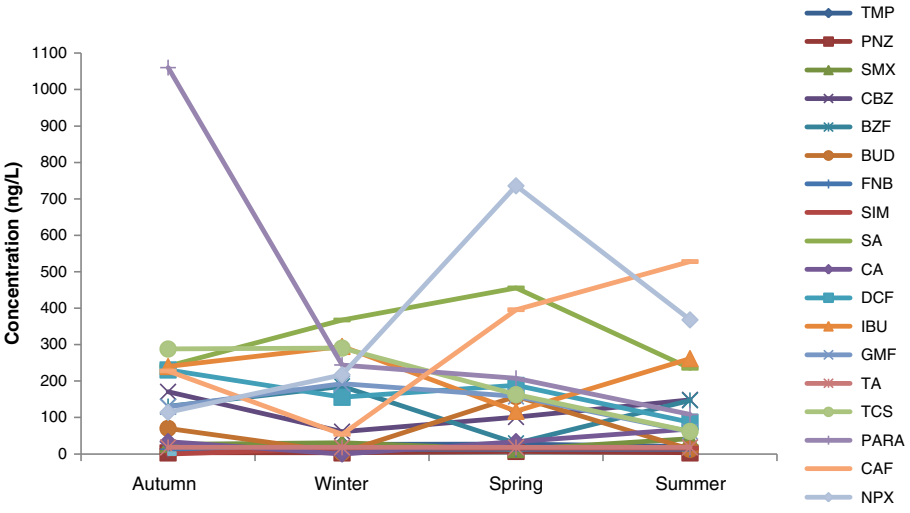

Fig. 4. Median concentrations of PPCPs in the effluents of the WWTPs.

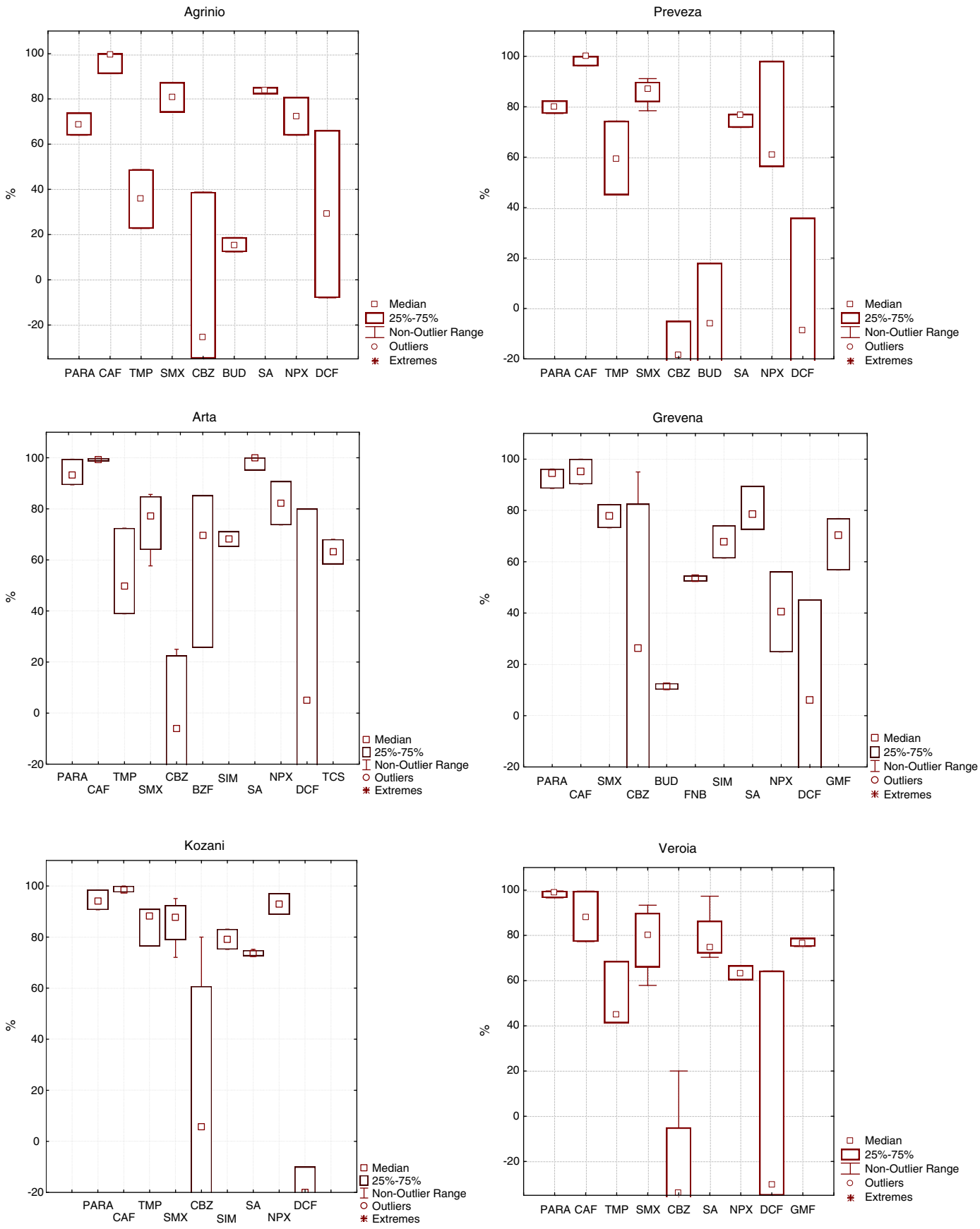

Fig. 5. Box and Whisker graphs indicating the removal efficiencies of PPCs in the eight WWTPs (PARA: Paracetamol, CAF: Caffeine, TMP: Trimethoprim, PNZ: Phenazone, SMX: Sulfamethoxazole, CBZ: Carbamazepine, BZF: Bezafibrate, BUD: Budesonide, FNB: Fenofibrate, SIM: Simvastatin, SA: Salicylic acid, NPX: Naproxen, DCF: Diclofenac, GMF: Gemfibrozil, TCS: Triclosan).

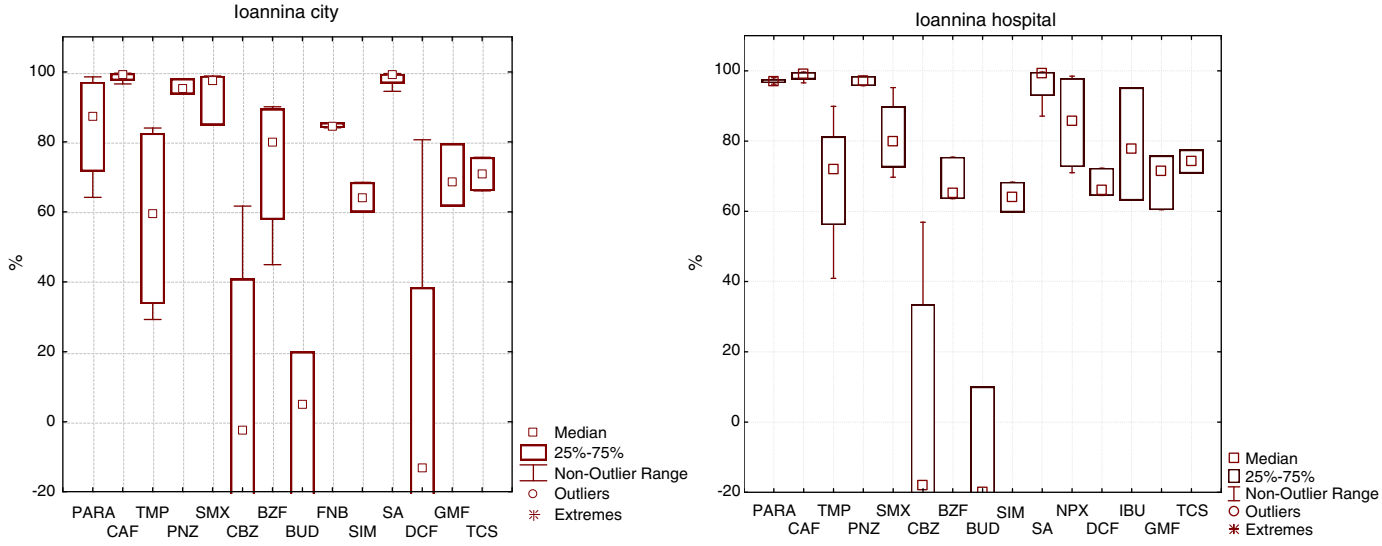

Fig. 5 (*continued*).

trimethoprim, sulfamethoxazole, budesonide and triclosan presented higher concentrations in influent hospital wastewaters than in the influent urban wastewaters, while salicylic acid, gemfibrozil, bezafibrate, trimethoprim and sulfamethoxazole presented higher concentrations in effluent hospital wastewaters than in effluent urban wastewaters.

3.2. Seasonal variation analysis of the PPCPs in WWTPs

According to the results obtained in the present study, a seasonal variation analysis is feasible for the eighteen PPCPs. As it can be seen in Figs. 3 and 4 most of the compounds did not show any significant variation between the sampling months, due to the fact that they belong to those therapeutic categories that are used for therapeutic reasons all over the year. As it can be seen in Fig. 3 caffeine presented by far the highest loads in autumn (29969.6 ng/L) maybe due to the fact that in this season coffees and beverages are consumed even cold or hot. Also in the summer (9478.7 ng/L) the consumptions are much higher because the daylight conditions in Greece are elevated (Kosma et al., 2010).

Seasonal variation was also observed for ibuprofen and bezafibrate whose concentrations were higher in the winter period (6023.9 and 195.0 ng/L, respectively) and for triclosan which showed higher concentrations in autumn (571.9 ng/L) and winter (527.9 ng/L) than in spring (220.6 ng/L) and summer (167.8 ng/L), maybe due to its antimicrobial and antibacterial usage against contagious and infectious viruses.

3.3. Removal of PPCPs in WWTPs

The occurrence of pharmaceuticals in the effluents and consequently their removal efficiency from WWTPs, depends mainly on their biodegradability and their physiochemical properties such as water solubility, tendency or not getting adsorbed by activated sludge and tendency to volatilize. Furthermore, the unit treatment processes employed in each WWTP can affect the elimination of pharmaceuticals. Some of the factors that can influence the elimination of PPCPs are found to be (a) the temperature of the function, where lower efficiencies have been reported during winter periods in colder climates (Gros et al., 2010; Verlicchi et al., 2012a; Vieno et al., 2005), (b) redox conditions (different removal efficiencies have been observed for anaerobic, anoxic and aerobic conditions), (c) pH conditions effecting the kinetic behavior of the compounds (neutral, cationic, anionic or zwitterionic condition of the molecule may change), (d) different kinetic behavior (K_{biol}), and (e) the hydraulic retention time (HRT) and sludge retention time (SRT) (Gros et al., 2010; Suárez et al., 2008; Verlicchi et al., 2012a). In the present study, the removal efficiencies of pharmaceuticals were determined as the percentage of reduction between the dissolved aqueous phase concentrations of the pharmaceuticals in the influent and the dissolved aqueous phase concentration of the pharmaceuticals in the effluent. However, it should be noted that because the concentrations of PPCPs in the sludge were not determined due to the difficulty to sample and analyze such complex matrices, the contribution of sludge adsorption or biodegradation could not be distinguished. Therefore, it would be advantageous to determine levels of parent compounds present in sewage sludge.

Box plots indicating the removal efficiencies of the PPCPs in the eight WWTPs during the monitoring period are shown in Fig. 5. Lines in each box show the lower quartile (25%) and upper quartile (75%) of the concentration values of each pharmaceutical compound. The whiskers or lines extending from each box show the extent of the data up to 1.5 times the interquartile range (IQR). The point inside each box shows the median concentration. Outlier values are marked with o symbols. From the results derived, it is not feasible to reach a general conclusion for each group of the PPCPs studied because each compound behaves differently. It was noticed that the elimination of many compounds was incomplete (Gracia-Lor et al., 2012a; Gros et al., 2010). In the cases that some compounds were not detected in the effluents, LOQ/2 was used to estimate their removal efficiencies. Also, in many cases some pharmaceuticals were detected in the effluents but not in the influents e.g. phenazone in Agrinio in February, and budesonide in Arta, Ioannina hospital and city, where they were detected at concentrations below the LOQ. These compounds might also be present in the influents, in low levels, but they could not be detected due to the complexity of the wastewater samples analyzed (ion suppression/enhancement) (Gracia-Lor et al., 2012a; Gros et al., 2010; Jelic et al., 2011; Lacey et al., 2008). Furthermore, in many WWTPs, the compounds were detected only in one sampling period, so there was not possible to estimate the median removal efficiencies in box plots (e.g. bezafibrate removal in Kozani was estimated only in Autumn). Those compounds that in any case presented the above discrepancies were omitted from Fig. 5.

Many compounds presented high removal efficiencies during the wastewater treatment processes, such as the analgesics and anti-inflammatory drugs salicylic acid (70–100%), ibuprofen (63–97%), paracetamol (64–100%) and phenazone (66–98%). These results are in agreement with those reported elsewhere (Gracia-Lor et al., 2012a;

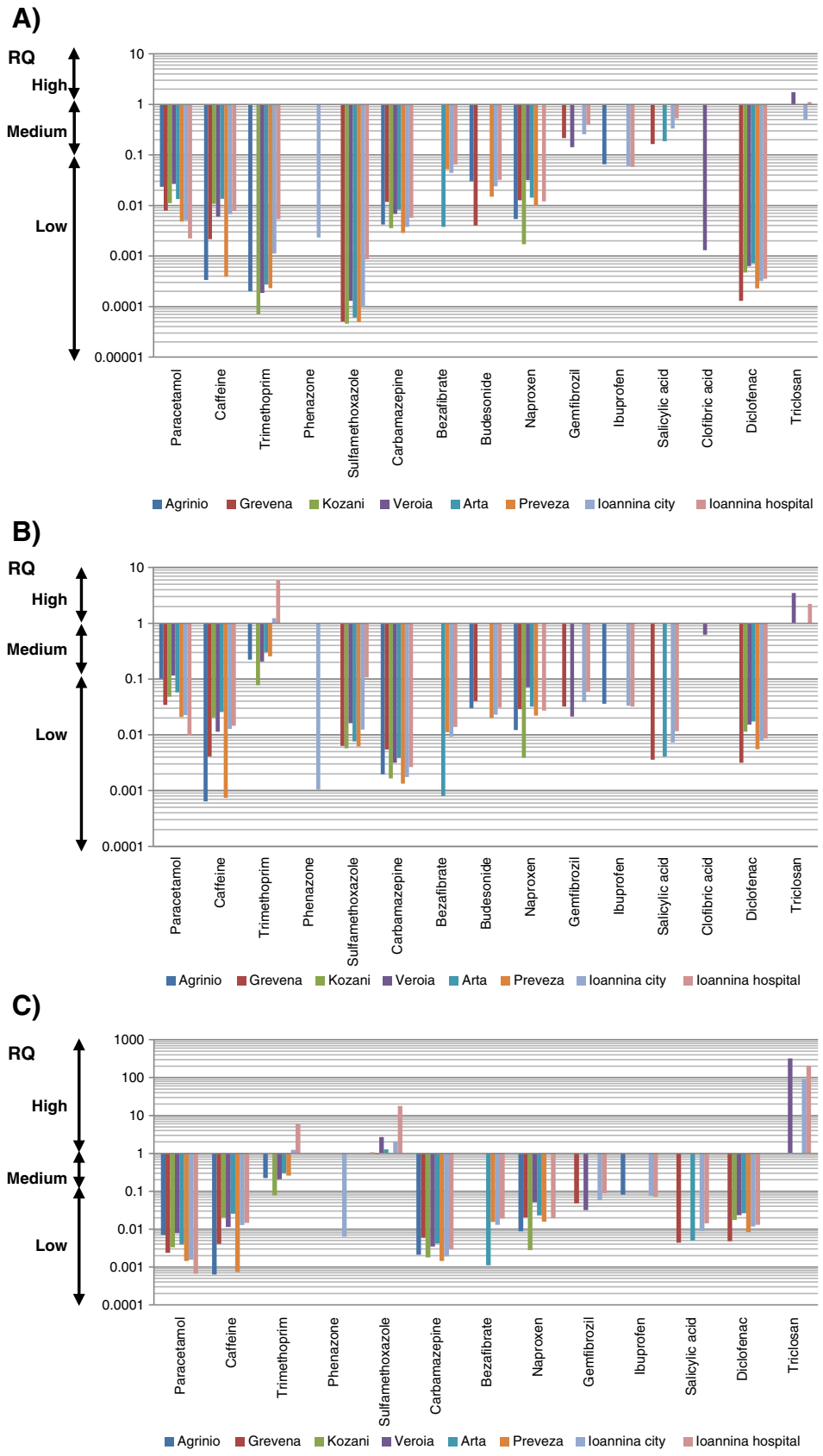

Fig. 6. Risk quotients for pharmaceuticals in effluent wastewaters were estimated for fish (a), invertebrates (b) and algae (c) for acute toxicity.

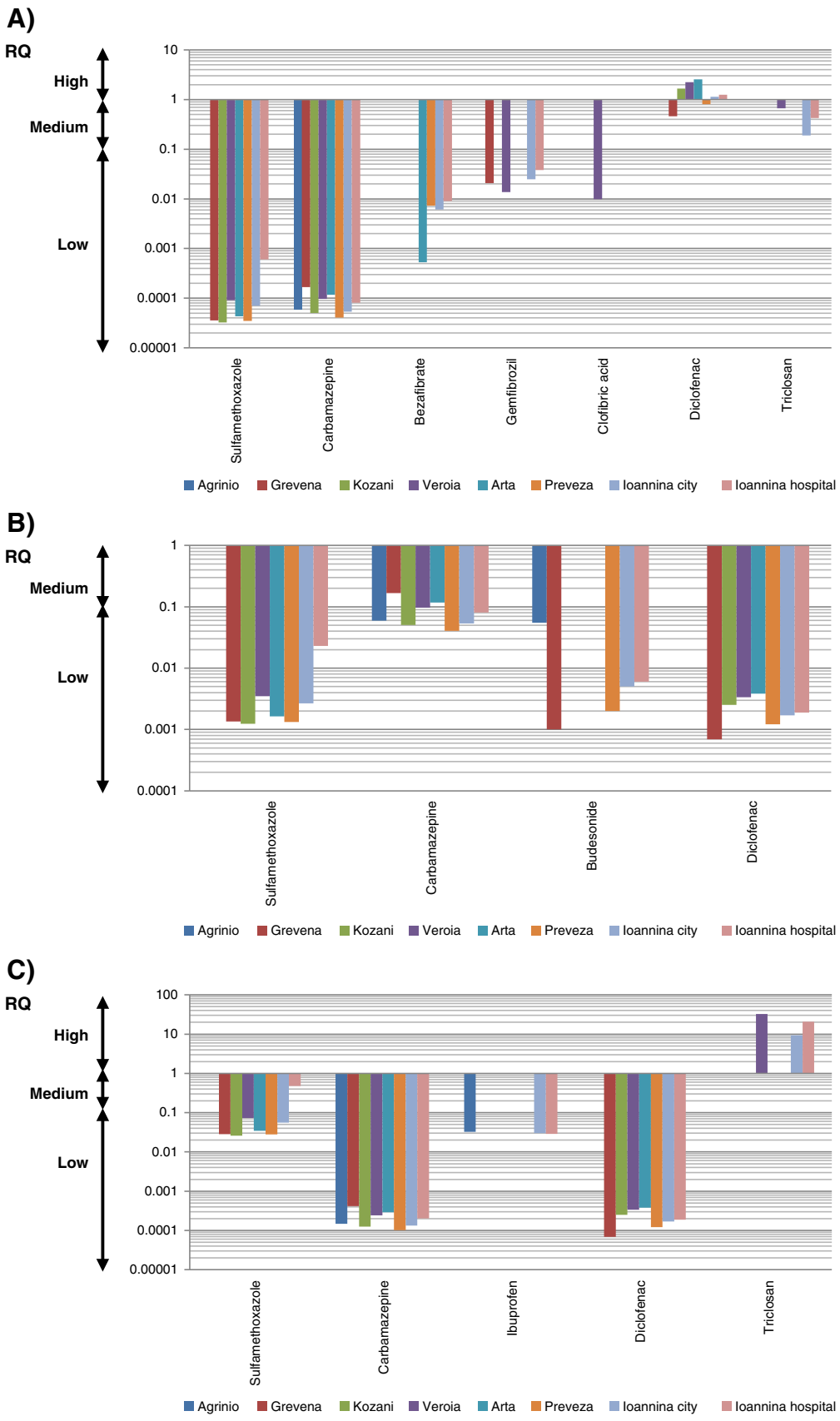

Fig. 7. Risk quotients for pharmaceuticals in effluent wastewaters were estimated for fish (a), invertebrates (b) and algae (c) for chronic toxicity.

Gros et al., 2010; Kosma et al., 2010). It has been reported that analgesics and anti-inflammatory drugs can present higher removal efficiencies in WWTPs with longer HRT and SRT, in reactors including nitrification and denitrification steps (Kosma et al., 2010; Ziylan and Ince, 2011). However, according to Gros et al. (2010), there are substances which show high K_{biol} and K_d, like ibuprofen and other analgesics and anti-inflammatories, that are very well removed independently of SRT and HRT. In the present study, most of the NSAIDs did not seem to present any significant variations in the removal efficiencies between different operational systems with different HRTs.

Caffeine which is marked as the number one drug worldwide found commonly in many beverages and sweets and is known as over-the counter-drug, presented high removal efficiencies in all WWTPs (77–100%).

Removal efficiency of trimethoprim fluctuated in low levels (23–91%) while the removal of sulfamethoxazole presented medium removal efficiencies (58–99%) (Gracia-Lor et al., 2010; Gros et al., 2012; Verlicchi et al., 2012a). These two drugs are known as resistant compounds to biological transformation (Verlicchi et al., 2012a) and trimethoprim was found to present only partial removal in processes with higher HRT (Gros et al., 2010). In addition, the main metabolites of sulfamethoxazole, N4-acetylated products when entering the WWTPs are biologically inactive and may retransform back to the initial parent compound (Göbel et al., 2007). Bezafibrate (26–90%) and naproxen (25–98%) presented removals that ranged between very low and very high values.

Carbamazepine, diclofenac and budesonide presented medium, low or negative removal efficiencies since in many cases their concentrations were higher in the effluents than in the influents (Göbel et al., 2007; Gracia-Lor et al., 2012a; Gros et al., 2010; Verlicchi et al., 2012a; Vieno et al., 2007). High effluent concentrations of these compounds may appear due to the conversion of their glucuronides and other conjugated metabolites to their parent compound through enzymatic processes in the WWTPs (Kimura et al., 2005; Piram et al., 2008; Vieno et al., 2007; Verlicchi et al., 2012a). In addition negative removals of PPCPs may occur due to their release from particles (Verlicchi et al., 2012a, 2012b; Zorita et al., 2009). Furthermore, long HRTs of some WWTPs may lead to sampling variations since the collection of the samples in the effluents might not take place according to the HRTs. In this case, collection of composite samples seems to be a reliable way to practice (Clara et al., 2005; Roberts and Thomas, 2006). Next, it should be noticed that lower effluent concentrations may occur due to some instrumental errors that may result in “apparent” releases of the compounds investigated.

As it can be seen in Fig. 5, the rest of the compounds showed medium removal efficiencies. Fenofibrate showed mean removal efficiencies around 68%, while simvastatin and triclosan showed mean removal efficiencies of 65% and 67%, respectively. These findings are in good agreement with previous studies (Beausse, 2004; Petrović et al., 2003; Ternes, 1998; Von der Ohe et al., 2012; Yu et al., 2006).

The majority of the studied compounds did not show any variance on the removal efficiencies throughout the four seasonal periods during the monitoring study. Paracetamol and bezafibrate showed only slight increment in mean removals in summer (98% for paracetamol and 71% for bezafibrate) and spring (96% for paracetamol and 75% for bezafibrate) than in winter (85% for paracetamol and 59% for bezafibrate) and autumn (88% for paracetamol and 70% for bezafibrate). In addition, trimethoprim presented its highest mean removals in summer (76%). On the other hand, ibuprofen which was not detected at any sample in summer, presented its highest mean removals in winter period (95%). However, it seems that it is not clear yet whether meteorological variables (e.g. temperature, rainfall etc.) can affect the transformation of pharmaceuticals (Göbel et al., 2007; Tauxe-Wuersch et al., 2005; Ternes, 1998; Verlicchi et al., 2012a).

Another issue that is reported to play a significant role in PPCPs removal, is the solid–liquid partition coefficient K_d which according to Ternes et al. (2004), compounds with $K_d < 500$ L kg^{-1} or $\log K_d < 2.7$ provide poor sorption onto sludge. According to the simple criteria proposed by Verlicchi et al. (2012a) ($K_{biol} < 0.1$ L/(gSS d) → poor degradability/$0.1 < K_{biol} < 10$ L/(gSS d) →→ quite good biodegradability/$K_{biol} > 10$ L/(gSS d) →→→ very good degradability) as well as the constants K_d and K_{biol} that are listed in Table 1, adsorption onto sludge seemed to play a significant role only in the removal of triclosan and paracetamol (Stamatis and Konstantinou, 2013; Verlicchi et al., 2012a). For example, about 30% of triclosan is absorbed to sludge due to its hydrophobic nature ($\log K_{ow} = 4.8/5.34$) and thus usually achieves removal efficiencies between 58 and 99% (von der Ohe et al., 2012).

The rest of the compounds exhibited low K_d values, showing that there is a negligible sorption onto sludge and suggesting that removals observed in WWTPs are mainly the result of their biodegradation (Ternes et al., 2004).

Generally, there weren't noticed any big differences in removal efficiencies over the eight WWTPs. Maybe this is due to the fact that all of them utilize activated sludge systems which apply also the removal of phosphorous, denitrification and nitrification treatments. Even in the hospital WWTP which was the smallest treatment of all plants, the elimination of PPCPs was adequate enough because the only source of sewage that reaches this plant is the hospital with also lower volumes than the other plants. On the other hand, big WWTPs have to deal with complex wastewaters urban and industrial, storm waters and sink waters.

3.4. Ecological risk assessment of pharmaceuticals

In the present study, RQs were determined in effluent wastewaters, since they are the ones that are being discharged into the environment. Results for the RQ values for fish, invertebrates and algae calculated from acute and chronic toxicity data are reported in Figs. 6 and 7.

As it can be seen in Fig. 7, there is a lack of chronic toxicity data for many PPCPs. This is due to the fact that PPCPs have been recently referred as emerging compounds and are used in large quantities worldwide. Because of the low but adherence occurrence of PPCPs in the environment the lack of chronic data comprises a big disadvantage to the determination of their effective risk assessment, as they are most likely to induce chronic rather than acute toxic effects. Chronic data were available only for eleven of the compounds, whereas, no acute toxicological data were available for simvastatin (Grung et al., 2008).

The results derived from risk quotient approach in three trophic levels showed that three of the analyzed compounds (triclosan, trimethroprim and sulfamethoxazole) pose high acute risk and two (diclofenac and triclosan) high chronic risk (RQ > 1), respectively. Algae seemed to be the most sensitive species, since PPCPs posed high acute and chronic ecotoxicological risk to them.

Triclosan was in both cases the most critical compound in terms of contribution and environmental risk. Von der Ohe et al. (2012) reported that triclosan can enter the environment, disperse and persist to a greater extent than the expected. The occurrence of triclosan, its tendency to bioaccumulate (even in human milk) and its dioxin-like TPs pose a great concern about its potential impact on human and ecosystem health. Also, Brausch and Rand (2011), performed a hazard assessment, which revealed that triclosan was the PCP that might represent the greatest hazard for aquatic environment. Hence, triclosan should be seriously considered as a candidate for regulatory monitoring and prioritization on a European scale on the basis of realistic PNECs (Von der Ohe et al., 2012).

This estimation of RQs however, is made for each compound separately but it must be taken into consideration the fact that in the aquatic environment PPCPs are present in a big variety of various

therapeutic classes which may lead to toxicity risks that did not result in single compounds (Cleuvers, 2003, 2004; European Commission, 2003; Gros et al., 2010; Pomati et al., 2008).

4. Conclusions

A simple analytical methodology based on the use of LC–MS has been applied in this work for the simultaneous quantification and confirmation of 18 target PPCPs in influent and effluent wastewater samples from eight different WWTPs in Greece. This extensive screening campaign confirms the presence of almost all the target compounds, contributed to a better insight on PPCPs in Greece and their presence in Greek influent and effluent wastewaters.

Concentration levels ranged from 9.3 to 96648.3 ng/L in the influents and from 6.6 to 1076.0 ng/L in the effluents, indicating that conventional wastewater treatment plants cannot efficiently remove most of the PPCPs. The most abundant compounds were carbamazepine, salicylic acid, paracetamol, trimethoprim and sulfamethoxazole. For most of the compounds there was not clearly observed any seasonal variation between the sampling months, due to the fact that they belong to those therapeutic categories that are used for therapeutic reasons throughout the year. Accurate mass screening by LTQ orbitrap analyzer which enables the identification of non-target compounds, such as the TPs of trimethoprim, demonstrated that this approach is a valuable tool to unequivocally identify PPCPs and their TPs in wastewaters. Although the present study is focused on the identification of trimethoprim TPs, much more research efforts should be made to develop better knowledge on the fate, behavior and structure of PPCP TPs that have not been extensively studied up to now.

Generally, the results indicated that the studied WWTPs cannot efficiently remove most of the target PPCPs. Removal efficiencies of the analytes varied from relatively high rates (e.g. salicylic acid ibuprofen, paracetamol, phenazone) to medium (e.g. sulfamethoxazole and fenofibrate) or low ones (e.g. trimethoprim), whereas negative rates were observed in some cases (e.g. carbamazepine, diclofenac and budesonide).

Results from screening level risk characterization in the effluents showed high acute and chronic risk for some of the investigated compounds, implying that significant impact on freshwater communities is likely and so is the associated risk. Hence, further PPCP occurrence, exposure and toxicological input (especially for long-term (chronic) effects on organisms and possible effects of combined exposure to multiple compounds) is required for better understanding of their possible adverse effects to non-target organisms and in order to provide a more comprehensive picture of its impact in the environment.

Acknowledgments

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek National Funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund.

The authors would like to thank the Unit of Environmental, Organic and Biochemical high resolution analysis-ORBITRAP-LC–MS of the University of Ioannina for providing access to the facilities. We also want to acknowledge the staff and the colleagues from the WWTPs for supplying us the wastewater samples and for their cooperation during the sampling campaign.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.07.044.

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