Environmental Research 191 (2020) 110152

Contents lists available at ScienceDirect

Environmental Research

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

Assessment of multiclass pharmaceutical active compounds (PhACs) in hospital WWTP influent and effluent samples by UHPLC-Orbitrap MS: Temporal variation, removals and environmental risk assessment

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ARTICLE INFO

Keywords:
Pharmaceuticals
Hospital wastewaters
Occurrence
Elimination
Risk characterization
Metabolites

ABSTRACT

Nowadays the occurrence and associated risks of Pharmaceutical Active Compounds (PhACs) in the aquatic environment comprises a major issue. In the present study, a comprehensive survey on contamination profiles, occurrence, removals, temporal variation and ecological risk of multiclass multiresidue PhACs, such as antibiotics, non-steroidal anti-inflammatories, lipid regulators and phsychiatrics, (including past and newly monitored PhACs as well as some of their metabolites) was performed in wastewaters from the WWTP of Ioannina University hospital along one year period on a monthly sampling basis. WWTP influent and effluent samples were analyzed for physicochemical quality parameters and PhACs concentration levels using Ultra High Performance Liquid Chromatography-Orbitrap-Mass Spectrometry (UHPLC-Orbitrap-MS), after Solid Phase Extraction (SPE) through Oasis HLB cartridges. Influent concentrations ranged between < LOQ (Limit of Quantification) for diclofenac and tolfenamic acid and 48586 ng/L for caffeine, while effluent concentrations between < LOQ for tolfenamic acid and simvastatin and 3361 ng/L for caffeine. Removal efficiencies ranged between −132.6% for venlafaxine and 100% for caffeine. Environmental risk assessment by means of Risk Quotient (RQ) for maximum and minimum concentration levels as well as optimized by the frequency of exceeding toxicity threshold values, RQ_f, was applied revealing that up to 12 PhACs posed acute toxicity (clofibric acid, fenofibrate, sulfadiazine, sulfamethoxazole, trimethoprim, amitryptiline, fluoxetine, fluvoxamine, norfluoxetine, sertraline, venlafaxine, caffeine) while up to 4 compounds exerted long-term toxicity (sulfamethoxazole, fluoxetine, sertraline, caffeine) at least for one of the studied organisms. Furthermore, mixture $RQ_{MEC/PNEC}$ and RQ_{STU} effect of multiple compounds showed high potential risks of the target groups in some cases, although some contaminants were not included due to lack of available data. Results can be used to prioritization of PhACs and their metabolites for surveillance in receiving water bodies as well as development of knowledge on toxicity and mechanism(s) of action.

1. Introduction

The last years numerous studies have been published concerning the presence of pharmaceutical active compounds (PhACs) in the aquatic environment. The extensive and long-term use of PhACs in human and veterinary medicine, due to the population growth and the development of medical techniques, has rendered them as contaminants of emerging concern (ECs) since they pose a potentially threat to the ecology of the receiving environment (Letsinger et al., 2019; Pereira et al., 2015; Wang et al., 2018). The occurrence of PhACs in the environment becomes more concerning considering the fact that they do not exist alone but in mixtures which might lead into undesirable additive or synergistic effects (Kosma et al., 2014; Petrie et al., 2016). The Institute for Health Informatics (IMS), reported that global medicine spending exhibits an

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https://doi.org/10.1016/j.envres.2020.110152
Received 11 June 2020; Received in revised form 30 July 2020; Accepted 24 August 2020
Available online 30 August 2020

increase of 4–7% in the past five years, depending on the country (Aitken, 2021). In addition, more than 50% of all medicines are not prescribed, dispensed or sold in a proper way while many people cannot take them correctly (Fekadu et al., 2019).

The main source of pharmaceutical pollution is highly associated with urban and hospital Wastewater Treatment Plant (WWTP) effluents (Krakkó et al., 2019). Due to the numerous care activities performed in hospitals, hospital wastewater (HWW) constitutes a complex matrix with high loads of PhACs and metabolites, disinfectants, heavy metals, reagents etc. (Frédéric and Yves, 2014; Konstas et al., 2019). These intensive and continuous activities lead to high concentrations of PhACs in HWW effluents, which sometimes are higher than in urban wastewaters (Frédéric and Yves, 2014).

Conventional WWTP technologies are not designed and constructed to efficient remove PhACs at trace levels and therefore, treated effluents still contain pharmaceuticals in considerable concentrations. In most of the cases, PhACs are not metabolized in a great extent in the human body and conclude in unchanged form in the sewage systems (Krakkó et al., 2019). Depending on the compound, excretion rate from the human body as well as removal from WWTPs, can vary from 0.04 to 100% and from 0 to 100%, respectively (Fekadu et al., 2019). In a recent review study, Fekadu et al. (2019) reported that the top ten most frequently detected pharmaceuticals in African and European studies, concerning freshwater aquatic environments, were sulfamethoxazole, carbamazepine, diclofenac, trimethoprim, ibuprofen, naproxen, paracetamol (acetaminophen), ketoprofen, venlafaxine and clarithromycin. Moreover, in Europe, compounds such as diclofenac, ibuprofen, triclosan, sulfadimidine, carbamazepine and fluoxetine were reported in concentrations higher than the available ecotoxicity endpoints (Fekadu et al., 2019). Being aware of the fact that information about the long-term and low-level exposure of PhACs is limited, potential threat to the aquatic environment and to humans is probable (Konstas et al., 2019; Krakkó et al., 2019).

Different studies have shown that concentrations of some PhACs can vary in influent and effluent wastewaters along the year (Fekadu et al., 2019). Monitoring campaigns is the most useful tool in order to gain information about the variation of PhACs concentration, mainly in cases where there are not enough data regarding the consumption of them, as in the case of Greece (Papageorgiou et al., 2016a,b). Therefore, monitoring data concerning the occurrence and fate of PhACs during wastewater treatment processes is of crucial importance in order to evaluate the efficiency of the plants and PhACs contribution in the final receiving water body (Kosma et al., 2019).

In the present work, a comprehensive monitoring study was performed in order to determine and evaluate the levels of a wide multiclass of PhACs in the University hospital WWTP of Ioannina city, located in northwestern Greece. In particular, a one-year study took place in order to: (1) characterize the occurrence (levels, temporal variation and correlations) of 35 multiresidue multiclass PhACs in influent and effluent samples, by means of UHPLC–LTQ/Orbitrap HRMS. The selection of PhACs was based on their consumption, previous studies conducted by the same research group (Kosma et al., 2010, 2014; 2019) and was enriched by newly emerging PhACs as well as metabolites; (2) to estimate the removal efficiencies and seasonal variation of past and newly emerging compounds and metabolites in the WWTP; (3) to provide an updated and completed environmental risk assessment of the target compounds in the aquatic environment. The hospital WWTP effluents were channeled into the municipal WWTP, which discharges its treated effluents into Kalamas River (the largest river of the region), being a considerable inflow to the River's flow, and eventually to its estuaries and the Ionian Sea (Kosma et al., 2019).

2. Materials and methods

2.1. Materials, sampling campaign and sample preparation

Pharmaceutical standards, reagents, solvents and materials are presented in the supplementary material section. Table S1 lists the physicochemical properties of the studied compounds as well as their therapeutic use. A one year sampling campaign was carried out in the WWTP of the University Hospital of Ioannina city (Epirus region, Greece). The hospital is an academic medical centre which interrelates with Ioannina University's School of Medicine and the Ioannina School of Nursing. There are nearly 45,000 people treated annually in the Hospitals Care Clinics and almost 130,000 people use hospitals Casualty Department every year. The hospital has a capacity of 800 beds while the staff consists of 1800 employees. The WWTP applies a pretreatment (grit-removal), a flow equilibration tank, and a biological secondary treatment concluding with disinfection (addition of 15% solution NaClO). The Hydraulic RetentionTime (HRT) of the WWTP is 6 h, while the Solid Retention Time (SRT) in 1.5 day (Konstas et al., 2019; Kosma et al., 2019). The plant discharges its effluent wastewater into the urban network which results into the municipal WWTP, thus the evaluation of its efficiency in order to predict final environmental loads has substantial interest. Influent and effluent samples were collected monthly from October 2018 to September 2019. Raw influents and final effluents were collected as grab samples taking into account the hydraulic retention time of the WWTP. Amber glass bottles were used for the sampling, which were transported immediately to the laboratory. The samples were filtered (0.45 μm) and preserved at 4 °C until the treatments' application within 24 h.

Physicochemical parameters of influent and effluent wastewater samples during the sampling campaign are determined by standard methods, as described in supporting information section, and summarized in Table 1 (min, max, standard deviation (SD) and median values), Table S2 and Table S3. With the exception of NO_3^-, being higher in the present study, the ranges of physicochemical parameters values are within those reported by Verlicchi (2018) for hospital effluents collected in different countries over a 20-year span.

Extraction of PhACs in the influents and the effluents was carried out with Solid Phase Extraction (SPE), by using Oasis HLB (200 mg, 6 cm^3) cartridges according to the methodology described in supporting information section.

2.2. UHPLC-LTQ/Orbitrap MS conditions

A UHPLC Accela LC system, connected with a hybrid LTQ-FT Orbitrap XL 2.5.5 SP1 mass spectrometer equipped with an electrospray ionization source (ESI) (Thermo Fisher Scientific, Inc., GmbH, Bremen, Germany) was used for the analysis of the samples. Full scan in positive (PI) and negative (NI) ionization mode was applied with mass resolving power of 60,000 FWHM and mass range of 100–500 Da and extracted ion chromatograms were used for identification and quantification purposes. In addition, a data-dependent acquisition (full MS/dd-MS^2) based on Collision Induced Dissociation (CID), was performed (Table S4). Detailed operational parameters of LTQ-FT Orbitrap instrument was reported in supporting information section. Chromatographic separation was performed on a reversed phase SpeedCore C18 analytical column with 50 mm × 2.1 mm, 2.6 μm particle size (Fortis Technologies, Cheshire, UK). In PI 0.1% formic acid in water and 0.1% formic acid in methanol, used as mobile phases A and B, respectively. The elution gradient started at 95% A (initial conditions), remained 95% for 1 min, progressed to 30% in 3 min, then progressed to 0% in 6 min, and returned to the initial conditions after 3 min with re-equilibration of the column set at 1 min. Total run time was 10 min. The flow rate was 0.4 mL/min with an injection volume of 5 μL. In NI water and methanol, used as mobile phases A and B, respectively. The elution gradient started at 90% A (initial conditions), remained 90% for 0.5 min, progressed to

Table 1
Min, max, mean, standard deviation (S.D.) and median values of physicochemical parameters measured in influent (Infl.) and effluent (Effl.) of hospital WWTP during the one-year monitoring study as well as typical range of physicochemical parameters.

Parameters	Min		Max		Mean		S.D.		Median		Typical range of Hospital WW[a]
	Infl.	Effl.	Inf.	Effl.	Infl.	Effl.	Infl.	Effl.	Infl.	Effl.	
Conductivity (μS/cm)	201	289	2120	1978	748	725	662	667	390	382	300–2700
TDS (mg/L)	202	308	2146	1951	750	731	653	647	389	390	116–3260
Temperature °C	12.3	12.2	25.4	25.7	22.1	21.4	3.4	3.8	22.7	21.8	–
Turbidity NTU	27	4	113	17	62	10	27	4	63	10	–
Salinity ‰	0	0	0.9	0.8	0	0	0	0	0	0	–
pH	7.1	5.5	8.9	7.3	7.7	6.5	0.6	0.6	7.6	6.7	6–9
COD (mg/L)	179	<10	545	119	340	43	102	40	343	27	39–7764
BOD_5 (mg/L)	96	1	293	16	166	7	63	4	156	8	16–2575
PO_4^{3-} (mg/L)	5.7	3.0	21.1	50.6	11.6	12.2	4.2	12.6	10.8	10.0	6–19
NO_3^- (mg/L)	<2.2	31.9	7.3	134.1	5.4	101.4	1.7	28.4	5.7	105.2	1–2

[a] Verlicchi (2018).

30% in 2 min, reaching 10% in 3 min, decreased in 5% at 3.9 min, decreased in 0% at 4.5 min, remained 0% for 0.5 min, and returned to the initial conditions after 1 min with re-equilibration of the column set at 2 min. Total run time was 8 min. The flow rate was 0.4 mL/min with an injection volume of 20 μL. In both cases the analytes were separated at 27 °C.

2.3. Quality assurance and quality control (QA/QC)

Quality assurance and quality control (QA/QC), as well as validation studies in wastewater samples were based on the guidelines set by the EU Commission (European Commission Decision, 2002). Quantification of PhACs was carried out by using matrix matched standard calibration curves (influent and effluent) prepared in 20:80 (v/v) methanol: water with 0.1% formic acid. In the case of psychiatric drugs matrix matched standard calibration curves contained the appropriate amount of each isotopically labeled standard (ILIS), as it was the only group of analytes for which ILIS were available (see Table S5). Since it was not possible to use the corresponding ^{2}H-labeled compound as an internal standard for all the target psychiatrics, every ILIS was chosen for more than one psychiatric. Wastewater samples already contained target analytes so, blank samples were analyzed in order to determine their concentrations, which were afterwards subtracted from the spiked samples. With every batch of twelve samples, a five-point calibration curve was performed. In addition, quality control (QC) samples, which were blank wastewater samples spiked at LOQ and 10 times the LOQ level, respectively were added.

The validation parameters studied in influents and effluents were: limits of detection (LODs) and quantification (LOQs), linearity, precision as repeatability and reproducibility, recovery and matrix effects (see Tables S5 and S6). LODs and LOQs of the target compounds in wastewater ranged in the influents between 0.9 ng/L for CBZ and 45.6 ng/L for DMS and in the effluents between 0.5 ng/L for CBZ and 32.3 ng/L for DMS, respectively (Table S5). The linearity of the proposed method was investigated by constructing a 12-point calibration curve from LOQ to approximately 100 LOQ for each compound, applying correlation coefficient of ≥0.99, for all PhACs. The precision of the method was determined by the repeatability and reproducibility studies, and expressed as the relative standard deviation RSD (%). The RSD (n = 5) values for intra-day ranged between 2.8 and 12.0% and the RSD for inter-day (n = 5) ranged between 3.9 and 15.6% (Table S5). Recoveries (n = 3) were carried out in three spiking levels; 50, 250 and 500 ng/L (Table S6). Matrix effect (signal suppression or enhancement) was calculated by applying the equation reported in our previous works (Kosma et al., 2014, 2015; 2019) and is given in Table S5.

2.4. Removal efficiencies and environmental risk characterization

Removal efficiencies of PhACs were calculated as percentages of reduction between the concentration of PhACs in influent and effluent (Kosma et al., 2014, 2015; 2019). Obtained results are represented in Box and Whisker graphs where; lines in each box show the lower quartile (25%) and upper quartile (75%) of the determined values of each PhAC. The whiskers or lines extending from each box represent the extent of the data up to 1.5 times the interquartile range (IQR). Median concentration is represented by the point inside each box while outlier values are marked with o symbols.

The risk assessment of the target PhACs was calculated in terms of risk quotient (RQ), according to EMEA guidelines (EMEA, 2006). RQs based on the ratio between Measured Environmental Concentration (MEC) and Predicted No-Effect Concentration (PNEC), for three aquatic organisms; fish, invertebrates and algae. Concerning MEC, two different scenarios were applied: (a) the worst case scenario by using the maximum concentration of each PhAC and (b) using the average concentration of each PhAC (Palma et al., 2020), as a less conservative scenario. PNEC has been determined: (i) for acute toxicity, using the lowest values of EC50 or LC50 divided by an assessment factor (AF) of 1000 and (ii) for chronic toxicity, using NOEC divided by an AF of 100, 50 and 10 when one long-term NOEC, two-long term NOECs or three long-term NOECs, from the three aquatic organisms are available, respectively (European Commission, 2003; Kosma et al., 2014, 2019). In the present study, an extensive literature review concerning ecotoxicological information for fish, invertebrates and algae was conducted, for acute and chronic toxicity, and the data are given in Tables S7 and S8. Levels of concern taken into consideration for RQ were as follows; high ecological risk for RQ ≥ 1, medium risk for 0.1<RQ<1 and low or negligible risk for 0.01<RQ<0.1 (Hernando et al., 2006; Kosma et al., 2014).

Furthermore, according to Zhou et al. (2019) the optimized risk quotient value (RQ_f) was determined based on mean RQ value and the frequency of MECs exceeding PNEC. Estimation of RQ_f by considering the variability of concentration above PNEC favors to screen the pollutants that are widely distributed and frequently detected. The RQ_f was calculated according to the following equation (Eq. (1)):

$$RQ_f = RQ \times F = \frac{MEC}{PNEC} \times F \quad (1)$$

where, $F = \frac{NO1}{NO2}$

RQ represents the ratio of the mean concentration and PNEC; F represents the frequency of MECs exceeding PNEC; NO_1 represents the number of samples with concentrations higher than PNECs; NO_2 represents the total number of samples. RQ_f is classified into 5 groups: when $RQ_f \geq 1$, high environmental risk is expected (high); when $0.1 \leq RQ_f < 1$, moderate environmental risk is expected (moderate); when $0.01 < RQ_f < 0.1$, small-scale adverse effect is expected (endurable); when $0 < RQ_f < 0.01$ quite limited environmental risk is expected (negligible) and when $RQ_f = 0$ no environmental risk is expected (safe) (Zhou et al., 2019).

Finally, taking into consideration the fact that PhACs do not occur in the environment as isolated single substances, but as multi-component mixtures, the joint ecotoxicity of those chemical cocktails was also estimated in the present study. Therefore, two possible approaches based on the sum of Toxic Units (STU) (with a toxic unit being TU = MEC/EC50 (or NOEC)) for each of the trophic levels (fish, daphnids, algae), were applied in order to assess the risk of chemical mixtures of each therapeutic group for acute and chronic toxicity: (i) the MEC/PNEC ratios of the components are summed up to a final Risk Quotient corresponding to the mixture of each therapeutic group (termed $RQ_{MEC/PNEC}$) (Eq. (2)):

$$RQ_{MEC/PNEC} = \sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} = \sum_{i=1}^{n} \frac{PEC_i}{\min(EC50_{fish}, EC50_{invertebrates}, EC50_{algae})\, i \times 1/AF} \quad (2)$$

(ii) RQ for the mixture equals to the sum of toxic units of the trophic level with the highest predicted sensitivity to the mixture (maximum STU of all analyzed trophic levels) multiplied with the corresponding AF (e.g. 1000 for acute toxicity) (termed RQ_{STU}) (Eq. (3)):

$$RQ_{STU} = \max(STU_{fish}, STU_{invertebrates}, STU_{algae}) \times AF = \max\left(\sum_{i=1}^{n} \frac{PEC_i}{PNEC_{i,fish}}, \sum_{i=1}^{n} \frac{PEC_i}{PNEC_{i,invertebrates}}, \sum_{i=1}^{n} \frac{PEC_i}{PNEC_{i,algae}}\right) \times AF \quad (3)$$

MEC in both cases was applied for maximum and for mean concentration. The ratio between $RQ_{PEC/PNEC}$ and RQ_{STU} ranges between 1–3 (Backhaus and Faust, 2012; Backhaus and Karlsson, 2014).

2.5. Statistical analysis

Descriptive statistics (range: minimum-maximum, mean, and median) were calculated by using the Microsoft® Excel® Windows program. Spearman correlation coefficient was used to assess the relationship of PhACs to physicochemical parameters in influents and effluents. A Wilcoxon matched pairs test was used to determine the differences between the sampling seasons, applying a $p<0.05$ significance level. Box and whisker graphs were applied for presenting removal efficiencies. Separate Principal Component Analysis (PCA) was applied to explore the variability of i) physicochemical parameters and

Table 2
Minimum, maximum and mean (n = 12) concentrations (ng/L) as well as detection frequency (%) for each pharmaceutical compound in hospital WWTP influent and effluent samples during one year period.

Therapeutic groups	Compounds	Hospital WWTP influent				Hospital WWTP effluent			
		Minimum (ng/L)	Maximum (ng/L)	Mean (ng/L)	Detection frequency (%)	Minimum (ng/L)	Maximum (ng/L)	Mean (ng/L)	Detection frequency (%)
Analgesics/ NSAIDs	Diclofenac (DCF)	n.d.	<LOQ	<LOQ	16.7	n.d.	n.d.	–	0.0
	Salicylic acid (SA)	24.9	3603	1310	100.0	n.d.	15.2	64.8	66.7
	Tolfenamic acid (TA)	n.d.	<LOQ	<LOQ	33.3	n.d.	<LOQ	<LOQ	25.0
Lipid regulators	Bezafibrate (BZF)	n.d.	178.8	178.8	25.0	n.d.	n.d.	–	0.0
	Clofibric acid (CA)	n.d.	925.4	925.4	25.0	n.d.	1517	799.0	16.7
	Fenofibrate (FNB)	114.1	1185	426.8	100.0	n.d.	298.3	176.5	41.7
	Gemfibrozil (GMF)	n.d.	354.2	354.2	25.0	n.d.	331.1	331.1	25.0
	Simvastatin (SIM)	n.d.	379.6	277.2	16.7	n.d.	<LOQ	<LOQ	8.3
Antibiotics	Sulfadiazine (SDZ)	n.d.	565.3	347.6	66.7	n.d.	538.2	251.8	33.3
	Sulfamethoxazole (SMX)	<LOQ	2369	524.8	100.0	96.5	1624	383.4	100.0
	Sulfapyridine (SPY)	n.d.	451.5	262.1	41.7	n.d.	120.9	116.9	16.7
	Sulfathiazole (STZ)	n.d.	109.9	109.9	8.3	n.d.	113.4	113.4	8.3
	Trimethoprim (TMP)	n.d.	858.3	294.5	91.7	n.d.	611.5	94.2	91.7
Psychiatrics	Amisulpride (AMS)	n.d.	888.9	319.7	83.3	n.d.	1709	602.7	75.0
	Amitryptiline (AMT)	n.d.	93.2	38.8	58.3	n.d.	161.7	57.2	66.7
	Bupropion (BUP)	n.d.	50.4	29.9	50.0	n.d.	98.2	39.9	75.0
	Carbamazepine (CBZ)	41.1	720.7	274.0	100.0	173.2	1426	643.8	100.0
	Citalopram (CIT)	n.d.	715.6	211.9	91.7	n.d.	172.6	83.1	91.7
	Clozapine (CZP)	n.d.	551.9	213.4	66.7	n.d.	113.4	64.3	66.7
	Fluoxetine (FLX)	n.d.	486.0	212.5	75.0	n.d.	252.5	74.8	66.7
	Fluvoxamine (FXM)	n.d.	511.5	203.0	58.3	n.d.	617.2	324.9	33.3
	Haloperidol (HAL)	n.d.	147.3	134.2	16.7	n.d.	133.3	98.0	50.0
	Mirtazapine (MTZ)	n.d.	220.6	79.9	66.7	n.d.	200.9	76.2	75.0
	N-desmethyl olanzapine (DMO)	n.d.	<LOQ	<LOQ	8.3	n.d.	93.8	93.8	8.3
	N-desmethyl sertraline (DMS)	n.d.	127.5	127.5	8.3	n.d.	171.1	171.1	8.3
	Norfluoxetine (NFX)	n.d.	415.4	227.0	66.7	n.d.	112.1	67.8	66.7
	O-desmethyl venlafaxine (ODV)	12.5	2852	1006	100.0	110.4	2845	1383.4	100.0
	Olanzapine (OLN)	n.d.	121.3	68.4	16.7	n.d.	97.6	97.6	8.3
	Paroxetine (PRX)	n.d.	201.0	89.3	33.3	n.d.	44.5	40.5	41.7
	Quetiapine (QTP)	n.d.	58.9	37.1	33.3	n.d.	55.9	41.8	25.0
	Risperidone (RIS)	n.d.	1059	316.5	91.7	n.d.	189.0	67.0	66.7
	Sertraline (STR)	n.d.	102.6	79.5	41.7	n.d.	218.3	94.3	58.3
	Venlafaxine (VNX)	n.d.	582.4	140.2	83.3	n.d.	1071	550.3	91.7
Stimulant	Caffeine (CAF)	9272	48583	31513	100.0	n.d.	3361.3	677.7	66.7
Corticosteroid	Budesonide (BUD)	n.d.	676.8	300.1	100.0	n.d.	154.2	92.1	41.7

n.d.: not detected.
<LOQ: below the limit of quantification.
Pharmaceuticals are presented by alphabetical order for the different therapeutic groups.

ii) environmental concentrations of each family of PhACs. All analyses were performed with Statistica (Trial version 13.0.0.17, TIBCO software Inc.). The Heat map-hierarchical cluster analysis was carried out by the web tool ClustVis (Metsalu and Vilo, 2015) which uses internally several R packages. Input datasets were standardized, namely they have been transformed by subtracting some reference value (typically a sample mean) from each value and dividing it by the standard deviation (typically a sample SD). Standardization makes the results of a variety of statistical techniques entirely independent of the ranges of values or the units of measurements. Pearson's moment correlation factor (r) was performed between the variables, and those strongly correlated were unselected from further statistical analysis to avoid multicollinearity. For data analysis, concentration values were set to half of LOD for not detected (below LOD) cases and half of the LOQ if they were between LOD and LOQ.

3. Results and discussion

3.1. Occurrence of pharmaceuticals in hospital WWTP influent and effluent

Table 2 summarizes the occurrence and concentration levels of the target pharmaceuticals in influent and effluent of the hospital WWTP during the one year sampling period. Results showed that the compounds were determined at levels from a few ng/L to a few of μg/L. Concerning the influents, all compounds have been detected at least in one sample, even at concentrations < LOQ. More specifically, DCF, TA and DMO were detected only in <LOQ concentrations with a frequency detection of 16.7, 33.3 and 8.3%, respectively. The most frequently detected compounds, in 100% of the samples, were SA, FNB, SMX, CBZ, ODV, CAF and BUD. The highest average concentration was observed for CAF (31513 ng/L) while the lowest average concentration was observed for BUP (29.9 ng/L). Similar results, e.g. CAF being the most frequently detected compounds in higher concentrations and SMX, CBZ, FNB being among the most frequently detected compounds, have been reported also for hospital wastewaters elsewhere (Couto et al., 2019; Paíga et al., 2019; Verlicchi et al., 2012) as well as in our previous studies (Kosma et al., 2014, 2019; Papageorgiou et al., 2016a,b).

As for the effluents concern, 33 out of 35 target PhACs were detected in all the samples. DCF and BZF were not detected at any sample, while TA and SIM were present in the 25% and 8.3% of the samples respectively, both only in concentrations < LOQ. The most dominant compounds identified in over 90% of the samples were SMX (100%), TMP (91.7%), CBZ (100%), CIT (91.7%), ODV (100%) and VNX (91.7%). The highest average concentration was observed for the psychiatric drug ODV (1383 ng/L) while the lowest also for another psychiatric drug BUP (39.9 ng/L). Similar results to our finding are presented in previous works (Kosma et al., 2014, 2019; Santos et al., 2013; Verlicchi, 2018; Verlicchi et al., 2012; Yuan et al., 2013).

In many cases, the presence of PhACs in WWTPs might differ along the year depending on various reasons. For instance, a major fact is the usage rate of each PhAC depending on the disease (e.g. allergies, influenza, respiratory infections etc.). Other factors might be the seasonal consumption or seasonal conditions such as the influence of sunlight exposure which might induce the photodegradation of some compounds or the rain amounts by a diluting effect on PhACs concentration (Paíga et al., 2019; Reis et al., 2019). Furthermore, in warmer seasons the higher temperature of the water can contribute to the enhancement of biodegrading activity. Regarding seasonal consumption rates, there are several PhACs such as antiepiliptics or lipid regulators that are similarly consumed during the whole year and thus they might not show any seasonal variation (Couto et al., 2019).

Fig. 1 and Fig. 2 represent concentrations of PhACs during the monthly sampling campaigns, while their seasonal variation is shown in Fig. S1 and Table S9. Further discussion for each therapeutic category is given in the following subsections.

3.1.1. Analgesics/NSAIDs

Among the analgesics/NSAIDs, the metabolite SA was detected in 100% and 66.7% of influent and effluent samples, respectively. In

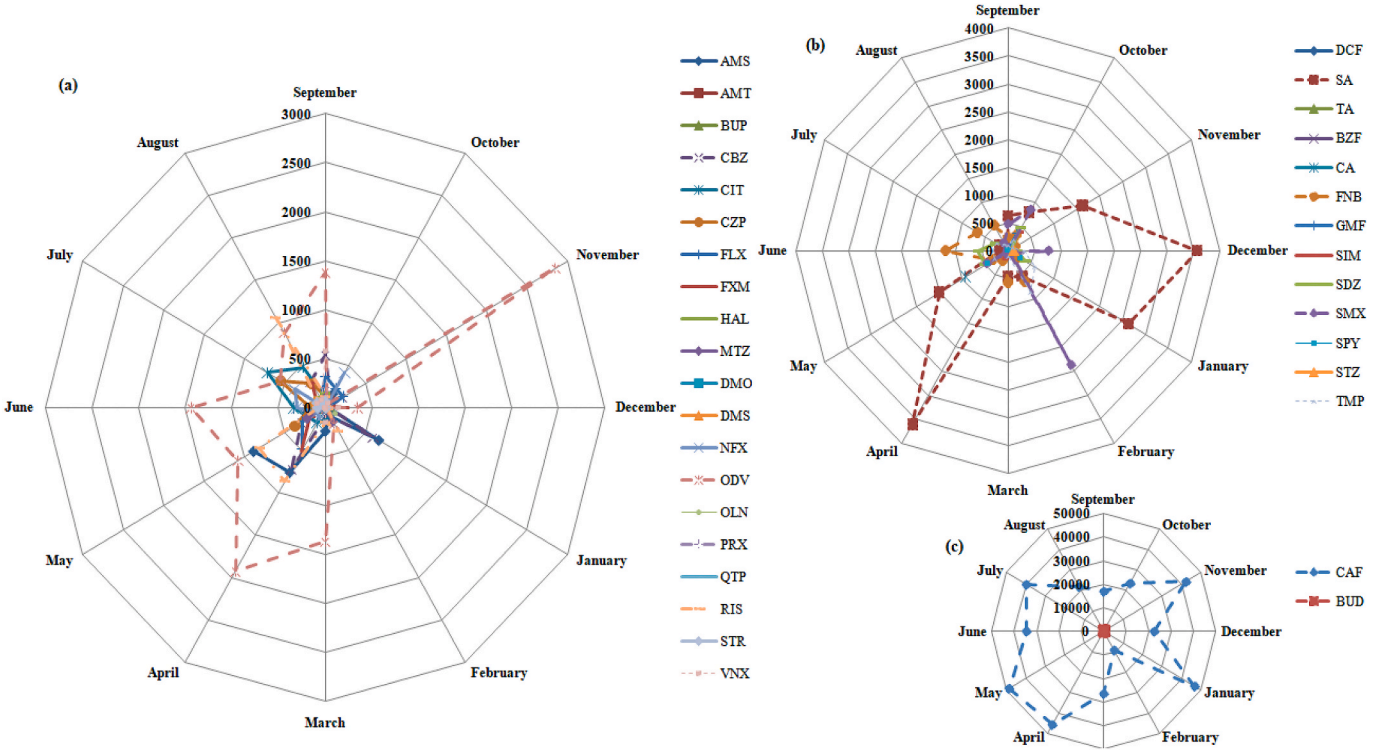

Fig. 1. Concentrations (ng/L) of PhACs in hospital WWTP influent during one year period: (a) Psychiatrics; (b) Analgesics/NSAIDs, lipid regulators, antibiotics and (c) Stimulant and Corticoid.

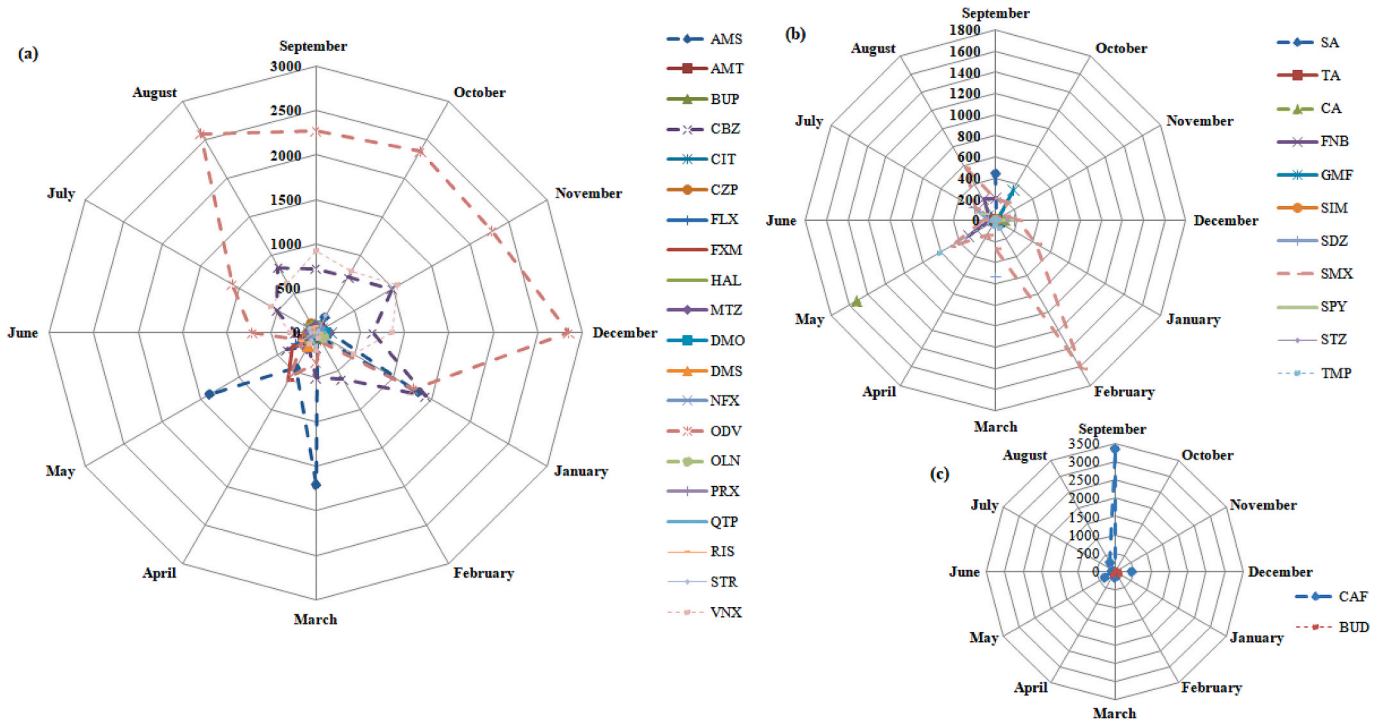

Fig. 2. Concentrations (ng/L) of PhACs in hospital WWTP effluent during one year period: (a) Psychiatrics; (b) Analgesics/NSAIDs, lipid regulators, antibiotics and (c) Stimulant and Corticoid.

influent samples concentration levels ranged from 24.9 ng/L in August to 3603 ng/L in April while in the effluents from 15.2 ng/L in September to 64.8 ng/L in January. SA is the major metabolite of acetylsalicylic acid (aspirin®) but there are several other sources from which it can derive, since it is also used as an additive in some skin-care products, in toothpastes and as a food preservative (Konstas et al., 2019). In Greece, aspirin is one of the most popular first line analgesics while it is also a freely available over-the-counter drug (Papageorgiou et al., 2016a,b). SA was detected during the whole year in the influents with higher mean concentrations in winter (2245.3 ng/L) and lower in summer (122.9 ng/L), although with no significant differences ($p>0.05$; $p = 0.108$), since it constitutes an NSAID which is consumed also to cure inflammation caused by influenza, which occurs more frequently during colder months (Tang et al., 2019). As for the other two analgesics/anti-inflamatories, DCF and TA concern, their detected concentrations were only at levels < LOQ. TA is excreted unchanged in 8.8%, therefore, low concentrations might enter the influent, while for DCF excretion rates vary from <1 to 90% (Krakkó et al., 2019). Similar results for the concentration levels of SA in hospital wastewaters have been observed in the literature (Kosma et al., 2014; Verlicchi et al., 2012), while for DCF and TA similar and/or higher concentrations have been found (Couto et al., 2019; Paíga et al., 2019; Verlicchi et al., 2012).

3.1.2. Lipid regulators

Regarding lipid regulators, FNB was the most frequently detected compound, presented in 100% of influent samples with concentration levels ranging between 114.1 ng/L in December and 1186 ng/L in June, while in the effluents it was present in 41.7% of the samples with an average concentration of 176.5 ng/L. BZF, CA and GMF were all found in the influents in 25% of the analyzed samples, with just one case for each compound in levels above the LOQ. More specifically, they were detected at concentrations of 178.8 ng/L, 925.4 ng/L and 354.2 ng/L in September, May and October, respectively. The hypolipidemic statin, SIM was present only in two samples in average concentrations of 272.2 ng/L. In the effluents, BZF was not detected at any sample and SIM was detected only in one sample in concentration < LOQ. CA was found in two samples, in concentrations ranging from 81.1 ng/L in December to 15717 ng/L in May and GMF was found only in one sample above the LOQ (354.2 ng/L in October). Similar results on the concentration levels for the above lipid regulators have been noticed in previous studies (Kosma et al., 2014; Santos et al., 2013; Verlicchi et al., 2012). Generally, no significant difference was observed concerning concentrations of lipid regulators during the one year sampling period. This group of PhACs is similarly consumed during the whole year and thus they might not show any seasonal variation ($p>0.05$) (Couto et al., 2019).

3.1.3. Antibiotics

Antibiotics are one of the most frequently detected therapeutic groups in hospital effluents, mainly due to their excretion in urine (up to 63%) and, in a less extent, in faeces, as metabolites or in the unchanged form. Sulfonamides (SAs) are among the most ubiquitous classes of antibiotics found in hospital effluents due to their characteristics (broad-spectrum, low price and stable properties) together with other antibiotics like TMP. Usually, they are found at higher concentrations than those reported in urban wastewaters, reaching a few hundreds of μg/L (Verlicchi, 2018). According to Zhang et al. (2020) the overuse and abuse of antibiotics have promoted the development and spread of antibiotic resistance genes, originating from antibiotic resistant bacteria, which pose potential risks to human health and eco-environment. In the present study, detection of four SAs and TMP was investigated. In the influents, SDZ, SMX, SPY and STZ were detected in 66.7, 91.7, 41.7 and 8.3% of the samples. The highest concentration was found for SMX (2368 ng/L in February), while the lowest was detected for STZ which was present only in one casee in December at the concentration of 109.9 ng/L. SDZ and STZ were found in average concentrations of 347.6 and 262.1 ng/L, respectively. In the effluents, SMX was again the most frequently detected compound (100%) with concentrations ranging between 96.5 ng/L in November and 1624 ng/L in February. SDZ, SPY and STZ presented average concentrations of 251.8, 116.9 and 113.4 ng/L, respectively. SMX was found in higher mean concentrations in winter (1073.9 ng/L) although with no significant differences ($p>0.05$). Usually, the occurrence of antibiotics in hospital effluents follows a

seasonal trend, being detected in higher concentrations in the winter than in the summer (Verlicchi, 2018). Another reason for the higher concentrations in winter is the fact that antibiotics might be bio- and photodegradated in higher rates during summer months (Pan et al., 2020). Similar or higher concentrations of SAs in hospital wastewaters have been found in previous studies (Kosma et al., 2014; Verlicchi, 2018), with SMX being the most frequently detected compound in European, American and Asian hospital effluents at concentrations ranging from <4 ng/L to 37.3 μg/L. SDZ was the second most detected antibiotic in the effluents with concentrations ranging from 9 to 2330 ng/L (Verlicchi, 2018).

The antibiotic TMP was first consumed in association with SMX in a ratio of 5:1 (SMX:TMP). At the moment, the use of SAs and TMP in jointed formulations (i.e. labeled J01E according to WHO) is ranked in the fourth place globally, since it is used in the treatment of various infections such as pneumonia and urinary tract infections (Thiebault, 2020). In the present study, TMP was found in the influents in concentrations ranging from not detected to 858.3 ng/L, presenting slightly higher concentrations in winter while in the effluents from not detected to 611.5 ng/L, in spring. Our findings are in accordance with previous investigations were, TMP was found in effluent wastewaters in Europe, North and South America and Asia in concentrations ranging from <2 ng/L to around 15 μg/L (Verlicchi, 2018). Furthermore, it was noticed that effluent concentrations of antibiotics in some cases were higher than the influents, which could be ascribed to the conversion of the conjugated compounds to their parent during the treatment process (Cui et al., 2020).

3.1.4. Psychiatrics

Prescription of psychiatric drugs during the last years have been increased, therefore their detection in WWTPs is gaining interest (Kosma et al., 2019; Silva et al., 2015; Subedi and Kannan, 2015). Concerning the present study, the University hospital holds a psychiatry clinic consequently, the evaluation of psychiatric drugs' loads into the effluents seems of substantial interest. After their intake, psychiatrics undergo metabolism in the human body and are excreted in urine and faces either unchanged or in form of biologically active metabolites. However, these metabolites may deconjugate back to the parent compound when reaching WWTPs (Kosma et al., 2014).

In the influents, the most frequently detected compounds, with over 80% detection, were CBZ (100%), ODV (100%), CIT (91.7%), RIS (91.7%), AMS (83.3%) and VNF (83.3%). The highest concentration levels were found for ODV, the principle metabolite of VNF, determined in higher mean concentrations in autumn (1425 ng/L) and spring (1458 ng/L) ($p>0.05$). Higher mean concentrations were also observed for AMS, RIS and CBZ in spring (629.9, 608.2 ng/L and 371.0 respectively) ($p>0.05$). AMS is slightly metabolized with the most of the drug excreted unchanged in faeces (64%) and in urine (33%), while for RIS up to 30% can be excreted unchanged. Low detection frequency was observed for DMO and DMS, the metabolite of STR, which were found only in one sample at concentrations < LOQ and 127.5 ng/L, respectively. Furthermore, AMT with a detection frequency of 58.3% presented the lowest concentration (3.8 ng/L in December). In addition, FLX as well as its metabolite NFX, presented average concentrations of 212.5 and 227.0 ng/L, respectively. The rest of the psychiatrics were found in lower concentrations.

In the effluents, CBZ (100%), ODV (100%), CIT (91.7%) and VNX (91.7%), were the most frequently detected compounds. The highest average concentrations were found for ODV (1383 ng/L), AMS (602.7 ng/L), CBZ (643.8 ng/L) and VNX (550.3 ng/L). OLN and its metabolite DMO as well as DMS were found only in one sample in concentrations of 93.8, 171.1 and 97.6 ng/L, respectively. Concentration levels of psychiatrics in influents and effluents within the same ranges were found in previous studies in Europe and China (Kosma et al., 2019; Santos et al., 2013; Silva et al., 2012; Verlicchi, 2018; Verlicchi et al., 2012; Wu et al., 2015; Yuan et al., 2013). Santos et al. (2013) have detected in hospital effluents concentrations of CBZ, VNX and CIT up to 2040 ng/L (pediatric hospital), 1914 ng/L (maternity hospital) and 888 ng/L (pediatric hospital), respectively. However, higher concentrations (up to 14,400 ng/L) have been detected for CBZ in the effluents of four general hospitals in Korea (Sim et al., 2011).

Many psychiatric compounds have been detected at similar or higher concentrations in the effluents than in the influents. This is might be due to the microbial transformation of parent or conjugated forms of these drugs through enzymatic processes that take place in the WWTP or changes in the adsorption behavior to particles during the treatment processes (Kosma et al., 2019).

3.1.5. Caffeine and other therapeutic classes

CAF is a natural alkaloid present in coffee and other beverages, products containing cocoa or chocolate, dietary supplements, and in some medications such as some analgesic formulations (Wielens Becker et al., 2020). Thus, unknown loadings of CAF through WWTP effluents reach final receivers, being therefore one of the most frequently detected compound in EU freshwater bodies, found in concentrations up to 50, 000 ng/L (Di Lorenzo et al., 2019). Furthermore, in previous studies, caffeine has been used as a tracer of anthropogenic contamination (Buerge et al., 2003; Daneshvar et al., 2012; Paíga and Delerue-matos, 2017). CAF was found in the 100% of the samples in the influent with the highest concentrations ranging from 9271.6 in February to 48583 ng/L in May. As for the effluents, CAF was detected in 66.7% of the samples with an average concentration of 677.7 ng/L in winter ($p>0.05$). Similar or higher concentrations were found for CAF in previous works (Kosma et al., 2014; Paíga et al., 2019; Papageorgiou et al., 2016a,b; Petrie et al., 2016).

BUD belongs to glucocorticoids (GCs) which are one of the most prescribed pharmaceutical classes worldwide that might induce potential risks in the aquatic environment even at low concentrations (Weizel et al., 2020). In the influents, BUD was detected in 100% of the samples with concentrations ranging from not detected to 676.8 ng/L, while in the effluents it was found in 41% of the samples with concentrations ranging from not detected to 154.2 ng/L. BUD presented higher concentrations in the influents during spring ($p>0.05$), that maybe due to its use as a nasal spray for the symptomatic management of seasonal or perennial allergic rhinitis.

Potential associations between physicochemical parameters and concentration levels of PhACs, categorized in groups, have been calculated by applying the Spearman correlation coefficients and were shown in Tables S10 and S11. A correlation coefficient near −1 or 1 means a strong negative or positive linear relationship between two variables, while one closes to 0 demonstrates a weak relationship.

In the influent, as expected conductivity and TDS showed strong positive correlation ($r = 0.979$, $p<0.01$), since their relationship is expressed by a simple equation TDS = k conductivity (in 25 °C) (Rusydi, 2018). Nitrates and phosphates are positively correlated ($r = 0.615$, $p<0.01$) as they both come from in-hospital sources. Nitrates and COD are also well associated ($r = 0.832$, $p<0.01$), while temperature was positively correlated with lipid regulators ($r = 0.637$, $p<0.01$) and psychiatrics ($r = 0.697$, $p<0.01$), revealing that higher temperatures may influence their residual concentrations. On the contrary, the corticoid BUD exhibited negative correlation with temperature ($r = -0.787$, $p<0.01$). The higher water temperatures in combination with long daylight periods may be important factors for the increment of biodegradation rates and phototransformation mechanisms of some PhACs (Palma et al., 2020). In another aspect, temperature may affect the usage pattern of some PhACs, since some diseases (e.g. colds, influenza, respiratory infections etc.) occur more frequently in winter or spring (Lin et al., 2018). Correlation values in the rest of the parameters reflect the usage trends of PhACs in hospital. In the effluents, similar correlation patterns were also observed.

3.1.6. Principal component analysis (PCA)

Table S12summarizes the eighen values and the selected PCs with their explained variance (%) for each group (influent-effluent) of both compound families and physicochemical parameters. Merged PCA plots are depicted in Fig. 3 after analyzing the main compound's families as variables while the sampling months are illustrated as the active cases. The vector length and direction reflects the importance of each variable's contribution to each of the two axes. The percentage of variance is explained by each PC (Factor) in parenthesis. According to the Kaiser criterion those PCs with eigenvalues >1 is sufficiently explaining most of the variance and those PCs were kept to analysis (Table S12).

Analysis of the principal components in influent and effluent for the main families of the selected emerging contaminants reveals certain differences. In influent, PC1, which is a linear combination of the sum of means of the psychiatrics, stimulants and antibiotics, accounted for the 38.92% of the total variability (Table S12). Psychiatrics and stimulants are loosely and negatively correlated with antibiotics. The second principal component (PC2) was the combination of lipid regulators and analgesics/NSAIDs negatively correlated with, explaining 26.52% of the total variance. The quality of representation with two PCs reaches a cumulative variance of 65.45%.

On the other hand, in effluent wastewater analysis, PC1 comprises of a linear combination of analgesics and stimulants (37.8% explained variance) which are also correlated, while PC2 explained a variance of 30.8% with corticosteroids being the main contributors. These could be attributed to concurrent use and the similar high removal rates of analgesics and stimulants exhibiting lower concentration levels in effluent. In addition, even if antibiotics and psychiatrics contribute mainly in the PC1 of either influent or effluent analysis, an opposite trend of them was distinguished, probably due to the incomplete removal of psychiatrics in contrary to antibiotics that are readily degraded in general.

In both plots (Fig. 3), red dots are the twelve months (cases) of sampling during the one-year campaign. Concerning influent, similarities between cases according to their point markers distances reveal common characteristics for months starting at the end of spring till the beginning of autumn including May, June, July, August and September. This is consistent taking into consideration the concentrations detected in influent during that time period and the common weather characteristics exhibited in Greece. On effluent PCA plot, one could assume that September is quietly in distance with almost all other months. It has to be noted that in both plots, September's contribution to PC1 variance is executive and that is due to the higher concentrations detected in influent and effluent that month of year.

In Fig. S2 (A,B) the merged plots of active variables and cases for the PCA analysis of physicochemical parameters measured during the one-year sampling campaign, are depicted. In Table S12 the eigenvalues are depicted together with the selected PCs and their explained variance. Influent wastewater PCA analysis revealed three PCs accounted for 82.6% of the total variability, with PO_4^{3-} and COD being the variables that contributed most in PC1, conductivity in PC2 and pH in PC3. Conductivity and turbidity are negatively correlated with each other. In the effluent, two PCs were selected; the PCA explained 39.4% of variability in the first axis (BOD and pH are the main contributors) and 27.0% of variability in the second axis (main contributors: COD and PO_4^{3-}).

Concerning the cases (temporal) PCA plot, at influent, no clear similarities are shown between months but the main contributors in the three PCs are February, July and May, respectively. On the other hand, effluent PCA analysis revealed a small clustering of the winter and spring months with the exception of March. The main contributors for PC1 are September and November and for PC2 is July.

3.1.7. Heat map-hierarchical cluster analysis (HM-HCA)

Concentration heat map (Fig. 4) shows temporal (monthly) variations of target PhACs. In the influent, the higher concentrations variation (red color) of individual drugs is mainly located in months September, October and July. Taking into consideration the fact that in July, the presence of two main psychiatric drugs' metabolites is ubiquitous, one could assume also that the exceptional weather conditions contributed to their parent compounds' degradation in a greater extent. In the effluents, high concentrations are observed in all autumn months as well as in spring.

The dendrograms shown in Fig. 4 revealed three distinct clusters of individually sampling months (horizontal axis) in influent being July, then September and October while in effluent being May, then December and April. Thus, similarities spread in influent from the middle of summer to the middle of autumn while in effluent spreads from the middle of winter to the end of spring, suggesting that different temporal use of PhACs occurs but also their removal is greatly affected

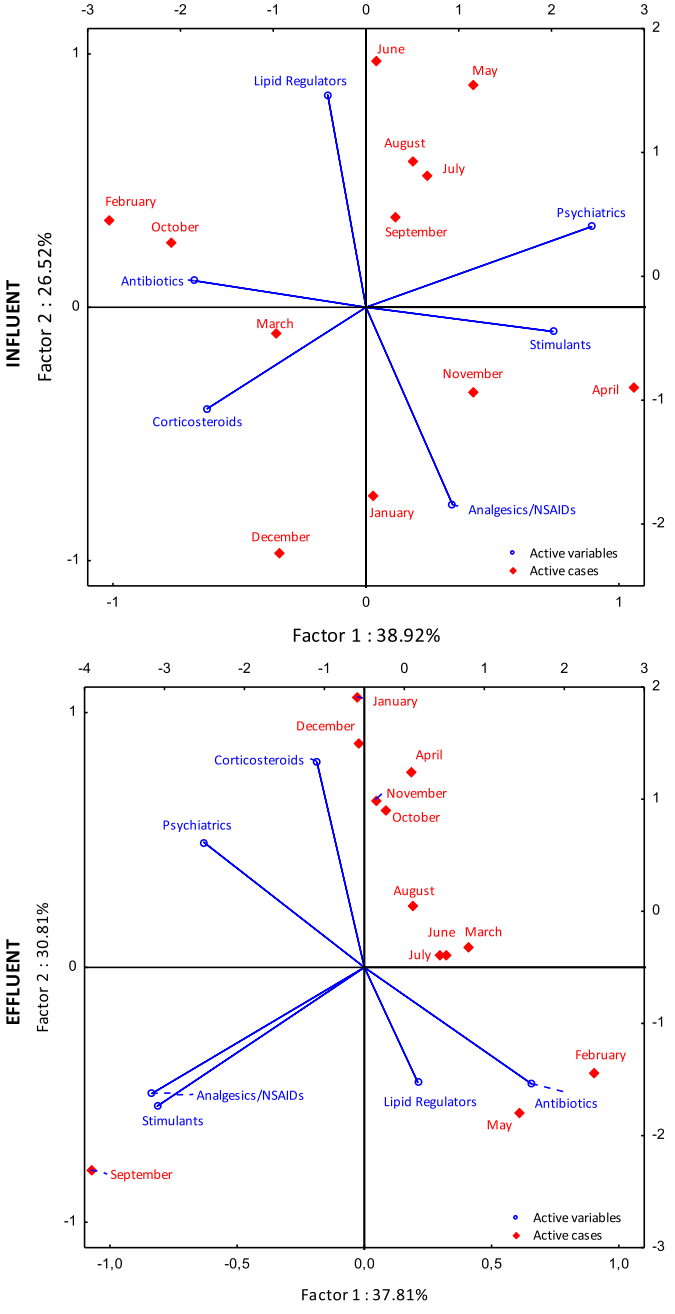

Fig. 3. Principal component analysis (PCA) graph combining cases plot (monthly sampling-12 samples) and variables (vectors or concentration levels found for ECs families) for influent (A) and effluent (B) wastewater from the University hospital of Ioannina.

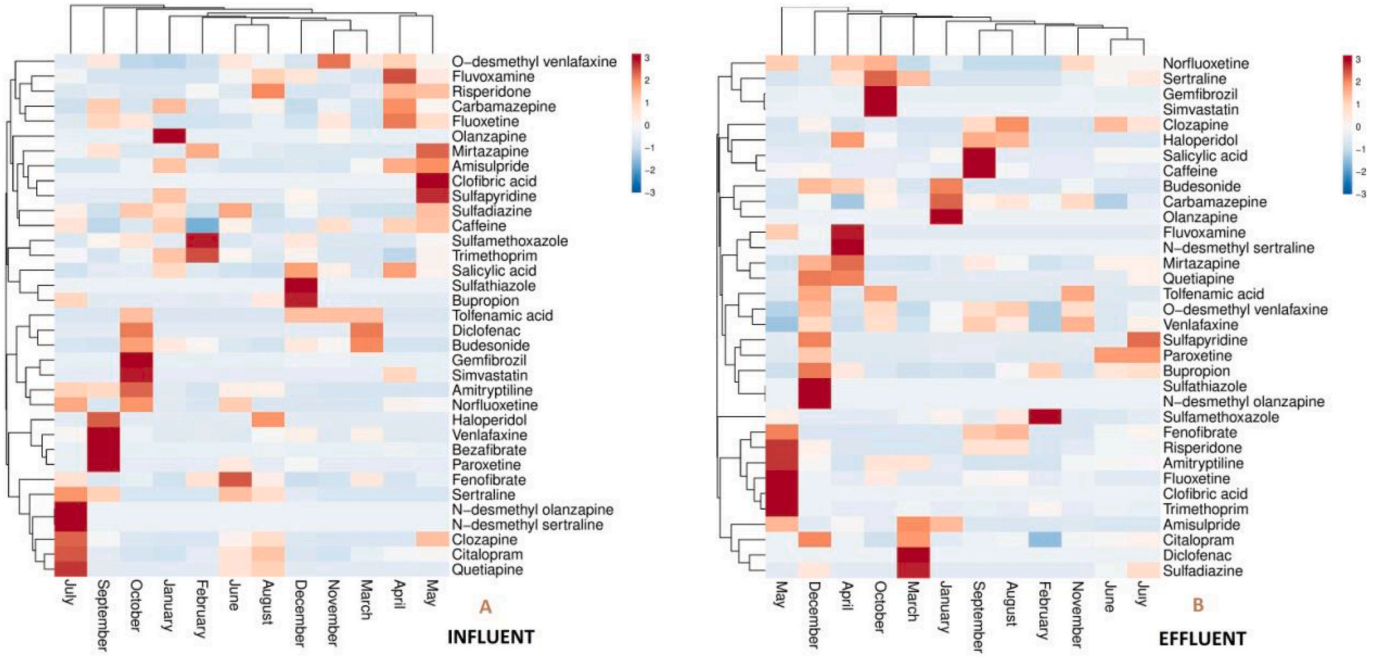

Fig. 4. Heat map and cluster analysis of 35 PhACs detected during the one year sampling campaign in WWTP of the University hospital of Ioannina; A) influent and B) effluent. The color of each cell represents the detected concentrations at different months (temporal variation) (ng/L). Concentrations of individual compound were normalized (by row). The color gradient represents the relative concentration from the lowest (−3) to highest (3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by weather conditions.

Clustering of the 35 PhACs (vertical axis, Fig. 4), on the other hand, is more complicated in both influent and effluent. In effluent, a distinct cluster at first, separates almost half of the detected analytes but the next grouping consists mainly of psychiatric drugs, two of their metabolites and FNB along with BZF. Other distinct clusters exist as for example, SMX and TMP grouping, which are known to be used together. In effluent, the first distinct cluster contains psychiatric drugs, as RIS, AMT, FLX, AMS and CIT as well as antibiotics such as SMX, SDZ and TMP; FNB and CA (lipid regulators) and DCF (analgesics).

3.2. Removal efficiencies

Removal efficiencies were evaluated according to our previous works (Kosma et al., 2014, 2015; 2019) and are shown in Fig. 5. Negative, low, medium and high removals were found depending on the pharmaceutical compound. Alike removals for the studied PhACs were reported in previous works (Evgenidou et al., 2015; Kosma et al., 2014; Paíga et al., 2019; Papageorgiou et al., 2019; Stamatis and Konstantinou, 2013). Variation in the removals of PhACs was frequently observed since there are diverse factors that can affect the processes, such as the temperature, configuration of the plant (C, N and P removal, biological reactor shape) and operating conditions (SRT, HRT, pH etc.) (Kosma et al., 2019; Verlicchi et al., 2012). Removal efficiencies ranged between −132.6% for VNX and 100% for CAF. High removal rates were also observed for SA, ranging from 72.7 to 99.9%. Concerning lipid regulators, CA and GMF presented negative and low removal efficiencies while BZF, FNB and SIM presented generally high removals (in most of the cases >70%). Couto et al.(2019) reported that one major mechanism which is associated with the biodegradation of PhACs in the WWTP is the co-metabolism, where the compound is degraded through the enzymes secreted by the microorganisms of the biological sludge. It was found that for some PhACs, including BZF, the co-metabolic biodegradation process was the main mechanism involved in its removal process

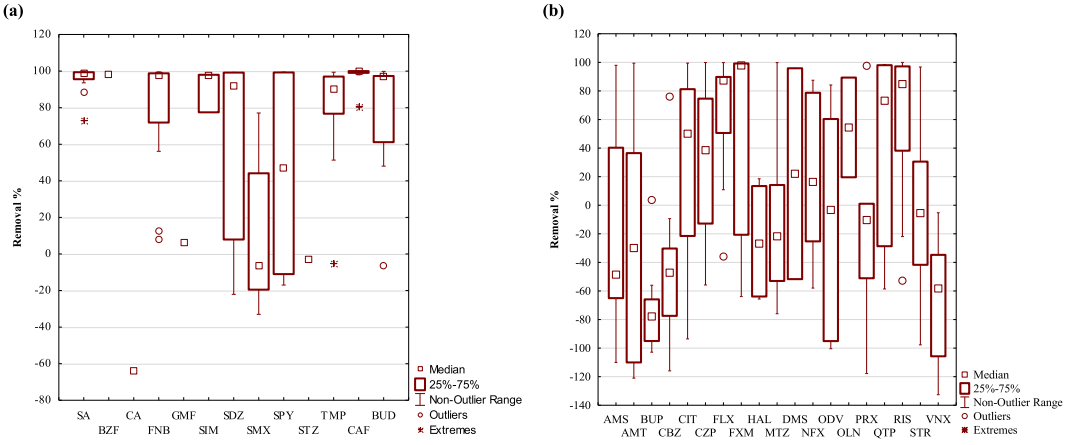

Fig. 5. Box and Whiskers plot for removal efficiencies (%) of: (a) Analgesics/NSAIDs, Lipid regulators, Antibiotics, Stimulant and Corticoid and (b) psychiatrics during one year period.

(Couto et al., 2019).

The removals of antibiotics varied also from negative in many cases (−33% for SMX) to high values (99.5% for SPY). SAs and particularly SMX presented in most of the cases negative removals. It was found that aromatic compounds bearing sulphate or halogen functional group are not easily degraded by biological treatments (Couto et al., 2019). Furthermore, it has been found that degradation of ionizable compounds such as SMX is highly depended on the pH. In acidic media SMX is in a hydrophobic form which results in higher elimination. Negative removals of SMX, in sewage treatment plants treating hospital effluents in South India were also observed (Verlicchi, 2018). TPM's removal was in most of the cases >80%. TMP was found to be removed under both anoxic and aerobic conditions (Couto et al., 2019). Similar removal efficiencies (>80%) were also observed for TMP in hospital effluents using a full scale CAS system operating in different countries (Vietnam, India, South Korea and China) (Verlicchi, 2018). Concerning CAF, its elimination was in almost all the cases 100% during the year, while for BUD, removals ranged between negative and high values.

As it was reported in the previous section many psychiatrics detected in similar or higher concentrations in the effluents than in the influents resulting in low or negative removals in many cases. Microbial transformation of parent or conjugated forms of these drugs through enzymatic processes in the WWTP or changes in the adsorption behavior to particles during the treatment, are the main reasons (Kosma et al., 2019). VNX presented only negative removal efficiencies (between −132.6% and −5.2%) while CBZ, BUP, MTZ and STR, in most of the cases presented also negative eliminations. Similar results have been reported elsewhere where negative removal of a wide range of PhACs in conventional WWTPs has been described (Couto et al., 2019; Evgenidou et al., 2015; Kosma et al., 2014; Papageorgiou et al., 2019; Pereira et al., 2015; Yuan et al., 2013). FLX, FVX and RIS presented generally medium to high removal efficiencies, >70% in most of the cases. The rest of the

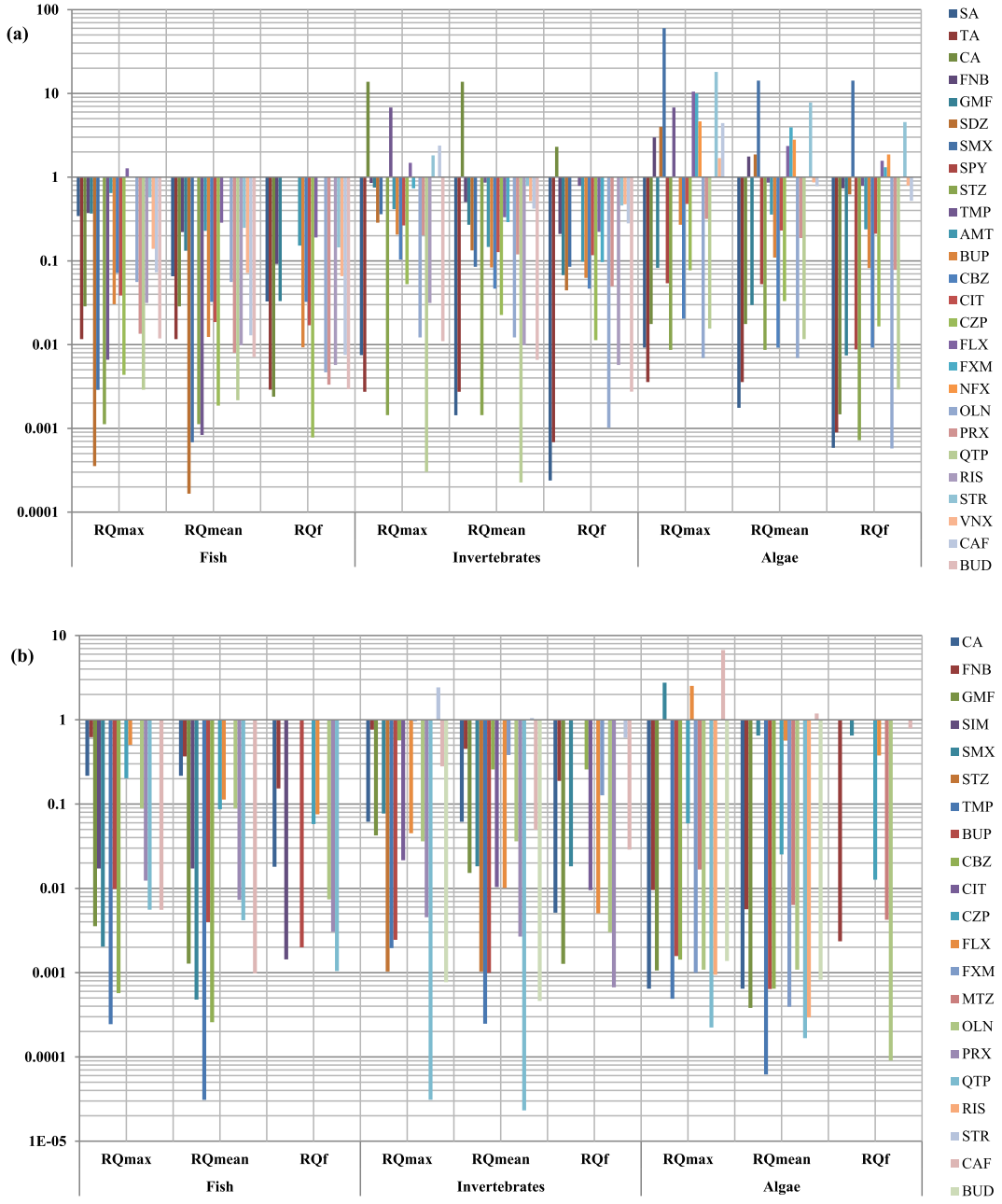

Fig. 6. Risk quotients (RQs) based on maximum and mean concentrations and RQ_f values using mean concentrations in hospital WWTP effluents, for three taxonomic classes; algae, invertebrates and fish and (a) acute, (b) chronic toxicity levels.

psychiatrics were eliminated with values varying from negative to high percentages (e.g. OLN and QTP). The ranges are in accordance with previous works in hospital WWTPs (Kosma et al., 2014, 2019; Yuan et al., 2013). For example, Yuan et al. (2013), found that a secondary treatment of a psychiatric hospital wastewater was effective in removing the majority of the compounds e.g. OLN (93–98%), RIS (72–95%) and QTP (>73%) (Verlicchi, 2018).

Removals' seasonal variation was also estimated and is depicted in Fig. S4 and Fig. S5. Removals vary across the seasons depending on various parameters. It was reported that, the efficiency of a WWTP might be affected by seasonal variation leading to increase of some PhACs in the effluents in rainy and winter periods since due to low temperatures and reduced HRTs, the microbial activity and biological reactions are reduced (Kosma et al., 2019). Overall, investigation concerning significant differences between the removals of PhACs during the seasonal sampling, revealed no significance ($p<0.05$), since in most of the cases removals of the compounds ranged between positive to negative values during the whole year.

3.3. Environmental risk assessment

RQs corresponding to maximum and mean concentrations as well as optimized RQ_f values corresponding to the mean concentrations of the target PhACs in the effluent wastewaters, for acute and chronic toxicity, in three trophic levels (fish invertebrates and algae) are depicted in Fig. 6. The non-availability of data concerning acute or chronic toxicity for some compounds (e.g. HAL, DMO, DMS, NFX, ODV) hampered the estimation of their RQs and RQ_f values. This fact designates the necessity of conducting more extensive studies concerning the ecotoxicological data not only of the parent compounds but for their metabolites also, since they seem to be more persistent and abundant in some cases (Kosma et al., 2019).

Concerning acute toxicity, high risks ($RQ_{max}>1$) were posed in invertebrates by CA, TMP, FLX, STR, VNX and CAF. CA presented also high RQ_{mean} and RQ_f in invertebrates ($RQ_{fCAF} = 2.303$, F = 16.7%). This fact, indicates that there is a potential high risk of CA, but the possibility for the organisms to be exposed to unsafe levels is lower than 16.7%. Compared with the original RQ value, generally, RQ_f showed greater difference in potential environmental risks of PhACs after taking into consideration the frequency of MECs exceeding PNECs. Only FLX presented $RQ_{max}>1$ in fish, being the only compound which was harmful for all three levels. The most sensitive species seemed to be algae, where 11 compounds showed $RQ_{max}>1$ with SMX, STR, FLX and FMX reaching a value of RQ_{max} = 60.2, 18.0, 10.5 and 9.9, respectively. Seven compounds exhibited high $RQ_{mean}>1$ in algae, such as SMX (RQ_{mean} = 14.202), STR (RQ_{mean}=7.796) and FXM (RQ_{mean} = 3.937). For three compounds, the antibiotic SMX, and the psychiatrics NFX and STR, RQ_f values for algae were above 1 ($RQ_{fSMX} = 14.202$, F = 100%); RQ_{fNFX} = 1.869, F = 66.7%; $RQ_{fSTR} = 4.584$, F = 58.3%) highlighting the danger of these compounds in the receiving aquatic ecosystems. For the compounds yielded RQ_f values between 0.1 and 1.0, a moderate environmental risk was probable.

Verlicchi (2018) reported that antibiotics were identified as the therapeutic group present in hospital effluents that cause the greatest concern and they were pointed out as the main contributors for the high environmental risk of these effluents. In addition, algae showed to be the most sensitive species to the toxic effect of antibiotics, nevertheless antibiotics might pose risk to all the trophic levels, representing a threat for the entire aquatic ecosystem. In the same study, it was referred that RQs calculated for hospital wastewaters varied widely from 10^{-3} to 10^{3}. Frédéric and Yves (2014) reported RQ >1 for TMP, and SMX, while Mendoza et al. (2015) found RQ>1 for TMP in a hospital from Valencia (Spain). Furthermore, it was found that measured RQ was higher than 1.0 for SMX taking into consideration urban and hospital consumptions. As a result, SMX was identified as high priority compound for the water cycle (Verlicchi, 2018). Felis et al. (2020) reported that harmful effects of antibiotics in algae might cause disorders to the organisms of higher trophic levels. Since algae are at the bottom of the food chain, even small changes in the algal population may dis-equilibrate the aquatic system (Lin et al., 2018).

Furthermore, Wee et al. (2019), stated the synergistic effect in fish mortality which was observed as a result of exposure to the pharmaceutical mixture, including CAF, SMX, ciprofloxacine (CIP), DCF and propanolol (PRN). In addition, oxidative stress was caused by sub-lethal concentrations of PRN and CAF in the marine amphipod *Ampelisca brevicornis* (Di Lorenzo et al., 2019). Some other studies concerning psychiatrics reported that concentrations in ng/L of BUP, FLX, STR and VNX have shown to alter the behavior of fathead minnows (*Pimephales promelas*) and amphipods (*Echinogammarus marinus*), increase mortality and alter tissue morphology of fathead minnows (Guler and Ford, 2010; Painter et al., 2009; Schultz et al., 2010). Schultz et al. (2010) found also that metabolites of phsychiatrics have shown to accumulate in the brain tissues of white suckers (*Catostomus commerson)*.

Chronic toxicity study in the present work revealed that only STR presented high RQ_{max} (RQ = 2.43) and RQ_{mean} (RQ = 1.05) in invertebrates, while in algae SMX, FLX and CAF induced $RQ_{mean}>1$. It was found that the effect of chronic exposure to sub-lethal concentrations of CAF increases the activity and alertness, disrupt sleeping patterns and drop learning attitudes and memory in invertebrates (Di Lorenzo et al., 2019). Furthermore, CAF showed also high $RQ_{max} = 1.19$ in algae. None of the compounds presented $RQ_f>1$. In addition, no toxicity was observed for fish. The RQ_f values of four (FNB, CBZ, FXM, STR) and three (SMX, FLX, CAF) compounds in invertebrates and algae respectively, were found to obtain values between 0.1 and 1.0, which indicated moderate risk.

Concerning RQs for the mixture of PhACs, Table 3 represents the $RQ_{MEC/PNEC}$, RQ_{STU} and their ratio, for acute and chronic toxicity, taking into consideration maximum and mean concentrations, respectively. It is worth noticing that in some cases available data concerning acute and chronic toxicity were missing, thus the corresponding RQs of individual compounds were not included in the mixtures. $RQ_{MEC/PNEC}$ and RQ_{STU} values in all cases ranged between 0.08 and 70.94 indicating high risk for some mixtures. The ratio of $RQ_{MEC/PNEC}$ and RQ_{STU} based on acute toxicity reached values up to 1.27. Similar results were found in seven European STP effluents where an RQ_{STU} of 16–48 was reported and the ratio was <1.3 in all cases (Backhaus and Karlsson, 2014). On the other hand, the ratio based on chronic toxicity reached values up to 1.62, showing the complexity of long term exposure (Liu et al., 2018).

Long-term exposure of PhACs and combined effects of PhACs' mixtures could lead to significant risks, which vary depending on the exposure, and dose. Furthermore, the co-presence of PhACs with other chemical pollutants such as heavy metals or organic pollutants could contribute to additive, synergistic and antagonistic effects on the endocrine system (Wee et al., 2019). Therefore, further research on toxic effects should be done, by undergoing the test organisms to PhACs levels found in the environment not only individually but simultaneously to other pollutants (Paíga et al., 2019).

4. Conclusions and future perspectives

The occurrence, removal, temporal variation and ecological risk of 35 PhACs, belonging to various therapeutic categories, in a University hospital WWTP were investigated monthly, along one year. Concentrations found at levels from a few ng/L to a few μg/L. In the influents all monitored compounds were detected at concentrations ranging from <LOQ (for DCF and TA) to 48582.8 ng/L (for CAF), while in the effluents, 33 out of 35 PhACs were detected at least once with CAF presenting the highest concentrations (3361.3 ng/L). Analysis of the principal components in influent and effluent for the main groups of the selected compounds between the sampling months revealed certain differences mainly explained by in-hospital uses and removal efficiencies as influenced by in-processes physicochemical factors. Specific temporal

Table 3
Calculated $RQ_{MEC/PNEC}$, RQ_{STU} and their ratio, based on acute and chronic toxicity for mean and maximum concentrations.

Therapeutic group	$Acute_{max}$			$Chronic_{max}$			$Acute_{mean}$			$Chronic_{mean}$		
	$RQ_{MEC/PNEC}$	RQ_{STU}	$RQ_{MEC/PNEC}/RQ_{STU}$	$RQ_{MEC/PNEC}$	RQ_{STU}	$RQ_{MEC/PNEC}/RQ_{STU}$	$RQ_{MEC/PNEC}$	RQ_{STU}	$RQ_{MEC/PNEC}/RQ_{STU}$	$RQ_{MEC/PNEC}$	RQ_{STU}	$RQ_{MEC/PNEC}/RQ_{STU}$
Analgesics/ NSAIDs	0.35	0.35	1.00	–	–	–	0.08	0.08	1.00	–	–	–
Lipid regulators	17.53	15.40	1.14	0.34	0.28	1.19	15.83	14.57	1.09	0.28	0.25	1.09
Antibiotics	70.94	70.94	1.00	2.75	2.75	1.00	16.94	16.94	1.00	0.65	0.65	1.00
Psychiatrics	32.58	32.45	1.00	3.20	2.53	1.27	12.05	11.97	1.00	0.92	0.57	1.62
Stimulant	4.42	4.42	1.00	6.72	6.72	1.00	0.78	0.78	1.00	1.19	1.19	1.00

variations of the target PhACs were depicted using hierarchical cluster analysis.

Removal efficiencies of PhACs varied from negative (e.g. VNX, ODV and CBZ), low (e.g. HAL and GMF), medium to high (e.g. CAF, SA, TMP and SIM) levels since there are various factors that can impact each one of them such as temperature or plant configuration. Pharmaceutical metabolites demonstrated also low or negative removals, highlighting the importance of including metabolites in analytical methods and considering their contribution to pharmacological/toxicological activity. Temporal variation of PhACs might differ depending on various factors such as their usage rage, sunlight exposure inducing their photodegradation.

Environmental risk assessment corresponding to maximum and mean concentrations as well as RQ_f values corresponding to the mean concentrations of the target PhACs revealed that up to 12 PhACs have the potency to pose acute toxicity while up to 4 compounds presented high risk for long-term toxicity, with algae being the most sensitive species. Furthermore, the toxic effects of multiple compounds showed high potential risks of the target groups in some cases. These risks should be taken into future prioritization of PhACs for monitoring in receiving water bodies and for performing further toxicity studies in order to evaluate environmental impact of mixtures of PhACs and to evaluate the optimum technology guaranteeing the highest removal of the most persistent and toxic compounds. In addition, since numerous of PhACs can be found in the environment in their parent form, as conjugates or transformed to structurally related products, further prioritization should take into consideration, not only (eco) toxicity, but different criteria such as consumption/sales, physico-chemical properties, degradability/persistence, resistance to treatment and degradation products. Furthermore, in pandemic or outbreak cases, like the current Covid-19 issue, large amounts of antimicrobials/antiseptics are consumed but their occurrence has not been studied so far and their release into environment is unknown. Therefore, study on the presence of these compounds in wastewaters is of crucial importance and could induce relevant epidemiological information about lifestyle habits and public health issues, as well as possible effects in the receiving environment.

Credit author statement

Christina Kosma: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. Margarita Kapsi: Investigation. Panagiotis-Spyridon Konstas: Investigation. Epameinondas Trantopoulos: Investigation. Vasiliki Boti: Methodology, Formal analysis, Validation, Visualization, Writing - original draft. Ioannis Konstantinou: Conceptualization, Methodology, Visualization, Writing - original draftWriting - Writing - review & editing, Supervision, Finding acquisition. Triantafyllos Albanis: Conceptualization, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was co-funded by the European Union and National Funds of the participating countries (Interreg-IPA CBC, Greece-Albania, “PhaRem”). 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.110152.

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