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Exploring the correlations between *epi* indicators of COVID-19 and the concentration of pharmaceutical compounds in wastewater treatment plants in Northern Portugal

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ABSTRACT

The COVID-19 pandemic caused by the SARS-CoV-2 virus led to changes in the lifestyle and human behaviour, which resulted in different consumption patterns of some classes of pharmaceuticals including curative, symptom-relieving, and psychotropic drugs. The trends in the consumption of these compounds are related to their concentrations in wastewater systems, since incompletely metabolised drugs (or their metabolites back transformed into the parental form) may be detected and quantified by analytical methods.

Pharmaceuticals are highly recalcitrant compounds and conventional activated sludge processes implemented in wastewater treatment plants (WWTP) are ineffective at degrading these substances. As a results, these compounds end up in waterways or accumulate in the sludge, being a serious concern given their potential effects on ecosystems and public health. Therefore, it is crucial to evaluate the presence of pharmaceuticals in water and sludge to assist in the search for more effective processes. In this work, eight pharmaceuticals from five therapeutic classes were analysed in wastewater and sludge samples collected in two WWTP located in the Northern Portugal, during the third COVID-19 epidemic wave in Portugal. The two WWTP demonstrated a similar pattern with respect to the concentration levels in that period. However, the drugs loads reaching each WWTP were dissimilar when normalising the concentrations to the inlet flow rate. Acetaminophen (ACET) was the compound detected at highest concentrations in aqueous samples of both WWTP (98. 516 $\mu g\ L^{-1}$ in WWTP2 and 123. 506 $\mu g\ L^{-1}$in WWTP1), indicating that this drug is extensively used without the need of a prescription, known of general public knowledge as an antipyretic and analgesic agent to treat pain and fever. The concentrations determined in the sludge samples were below 1.65 μg g^{-1} in both WWTP, the highest value being found for azithromycin (AZT). This result may be justified by the physico-chemical characteristics of the compound that favour its adsorption to the sludge surface through ionic interactions. It was not possible to establish a clear relationship between the incidence of COVID-19 cases in the sewer catchment and the concentration of drugs detected in the same period. However, looking at the data obtained, the high incidence of COVID-19 in January 2021 is in line with the high concentration of drugs detected in the aqueous and sludge samples but prediction of drug load from viral load data was unfeasible.

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1. Introduction

During the last decades, the production and consumption of pharmaceuticals has increased rapidly with the development of medical and pharmaceuticals sciences (Sim et al., 2011). Moreover, the global pandemic situation caused coronavirus 2 (SARS-CoV-2), has led to an increased consumption of some medicines, as reported in recent studies (Di Marcantonio et al., 2022; Galani et al., 2021; WHO, 2021). In Portugal, the COVID-19 pandemic officially started on the 2nd March 2020.

The coronavirus disease caused by the SARS-CoV-2 virus is mostly characterized in healthy individuals by mild to moderate respiratory illness. However, some individuals might become seriously ill (e.g., severe respiratory infection and multiorgan dysfunction) (WHO, 2022). Moreover, the mental health of the general population due to lockdown policies was also affected. All these events may have had implications in the use of certain pharmaceuticals, including antibiotics, analgesics, antidepressants, corticosteroids, amongst others (Koster et al., 2021; Rabeea et al., 2021).

The higher consumption of some classes of pharmaceuticals (or their metabolites) during the pandemic period, excreted through faeces and urine (Heberer, 2002), may have resulted in an increase of their concentration at WWTPs as well as in the treated effluents, considering the relatively low efficiency of conventional WWTP to remove some of these compounds (Kim et al., 2005; Kümmerer, 2009; Pereira et al., 2015). Additionally, less polar compounds, even when resistant to (bio)degradation, can be removed in biological treatment due to adsorption on sludge (Peng et al., 2012; Zhang et al., 2019). Overall, the occurrence of these type of substances in these compartments may result in the contamination of surface, ground and drinking waters, and also bioaccumulation and amplification in the food web when contaminated sludge is used as fertilizer, impacting negatively the ecosystems and human health (Sharma et al., 2022; Zenker et al., 2014). Therefore, identifying the most relevant ones is of paramount importance so that efficient, and more targeted, remediation measures can be pursued.

This work aimed to assess the presence and load of a set of pharmaceuticals resulting either from the implementation of therapeutic actions specifically prescribed for patients infected with SARS-CoV-2, or from changes in their consumption patterns, in wastewater and sludge samples from two WWTP located in the North of Portugal. Five pharmaceutical compounds used in the treatment of COVID-19, or mitigation of the associated symptoms, were assessed, namely the analgesic (Acetaminophen) ACET, the nonsteroidal anti-inflammatory drugs (NSAIDs) Ibuprofen (IBU) and diclofenac (DCF), the antibiotic azithromycin (AZT), and the corticosteroid dexamethasone (DEXA). Three pharmaceuticals that may be used to cope with psychological problems and stress caused by the situation were also evaluated: the antidepressants venlafaxine (VFX) and fluoxetine (FLX), belonging to the selective serotonin reuptake inhibitors, and the anticonvulsant carbamazepine (CBZ) that is occasionally used to treat bipolar disorders. By analysing these drugs, it was not only intended to assess the situation at the entrance of the WWTP, and their treatment capacity, and to evaluate a possible correlation of the selected drugs with the incidence of COVID-19 cases as well, in order to assess some of the effects of this pandemic also in the environment.

2. Materials and methods

2.1. Water and sludge samples

The aqueous samples, i.e. influent, effluent and water for reuse (WfR), which is a recycled treated wastewater used for beneficial purposes, such as landscape irrigation and agricultural, industrial processes, amongst others (Mo and Zhang, 2013), were collected from WWTP1 and WWTP2 weekly, between January and April 2021, and then further spaced out until September 2021 (Table 1). In the period of this study (January – September 2021), Portugal was going through its third wave of the pandemic (SNS - Serviço Nacional de Saúde, 2022; Zanin and Papo, 2020). Until September 16, 2021, there were 1 059 409 confirmed cases, with a total of 17 888 deaths (Direção- Geral da Saúde, 2021; SNS - Serviço Nacional de Saúde, 2022). Secondary and tertiary effluents were analysed in the WWTP1 and WWTP2, respectively (Figs. S1, S2) (SimDouro, 2023, 2017; Tratave, 2023). The collection and transportation of composite samples, at the different sampling points, was carried out in sterile amber borosilicate glass bottles. The samples were shipped to the laboratory in less than 8 h under refrigeration (3 to 5 °C).

Table 1
Sampling dates in 2021 for each matrix: influent, effluent water for reuse (WfR), and sludge.

Collected samples	Influent (INF)	Effluent (EFF)	WfR	Sludge
18th of January	X	X	X	X
25th of January	X	X	X	–
1st of February	X	X	X	X
8th of February	X	X	X	–
15th of February	X	X	X	–
22nd of February	X	X	X	–
1st of March	X	X	X	X
8th of March	X	X	X	–
15th of March	X	X	X	–
22nd of March	X	X	X	–
29th of March	X	X	X	–
5th of April	X	X	X	X
19th of April	X	X	X	–
3rd of May	–	–	–	X
17th of May	X	X	X	–
14th of June	X	X	X	–
12th of July	X	X	X	–
3rd of August	X	X	X	–
6th of September	X	X	X	–
14th of September	X	X	X	–

All the aqueous samples were filtered (0.45 μm PVDF filters, Whatman, U.K.) and kept at −20 °C until further processing. The preparation of aqueous samples and the quantification of the pharmaceutical compounds were carried out at the Catalan Institute for Water Research (ICRA) following an established method (Gros et al., 2012).

Sludge samples were analysed monthly between January and May 2021 (Table 1). Digested sludge samples and sewage sludge, were analysed for WWTP1 and WWTP2, respectively (Figs. S1, S2) (SimDouro, 2023, 2017; Tratave, 2023). All the sludge samples were kept at −20 °C until further processing.

2.2. Chemicals

All reference standards (> 98% purity): azithromycin dehydrate (AZT), carbamazepine (CBZ), diclofenac sodium (DCF), fluoxetine hydrochloride (FLX), venlafaxine hydrochloride (VFX), acetaminophen (ACET), dexamethasone crystalline (DEXA), and ibuprofen (IBU), were purchased from Sigma-Aldrich (Steinhein, Germany). The respective deuterated compounds used as internal standards (azithromycin-d3, diclofenac-d4, fluoxetine-d5, ibuprofen-d3, carbamazepine-d10, venlafaxine-d6; >98% purity) were also obtained from Sigma-Aldrich (Steinhein, Germany). Stock solutions of each reference and internal standard (ca. 1000 μg mL^{-1}) were prepared in methanol. A working solution containing all reference analytes (1.0 μg mL^{-1}), and another containing all the internal standards (5.0 μg mL^{-1}), were prepared by dilution of the individual stock solutions in methanol.

Methanol (MeOH, ≥ 99.9% purity), ethyl acetate (EtAc, ≥ 99.9% purity) and formic acid (≥ 96% purity) were purchased from Merck (Kenilworth, New Jersey, USA). Acetonitrile MS grade was acquired from VWR International (Oregon, USA). Ultrapure water (resistivity > 18.2 MΩ cm at 25 °C) was supplied by a Milli-Q water system from Millipore (Massachusetts, USA).

2.3. Extraction of pharmaceuticals

2.3.1. Aqueous samples

The preparation of aqueous samples followed the established method described by Gros et al. (2012). Concisely, preconcentration of samples (25 and 50 mL for influent and effluent, respectively) was conducted by Solid Phase Extraction (SPE). Samples were spiked with a standard solution containing surrogate standards and an appropriate volume of a Na_2EDTA solution to achieve a final concentration of 0.1% (m/m). Cartridges were conditioned with 5 mL of methanol followed by 5 mL of HPLC-grade water, at a flow rate of 2 mL min^{-1}. After conditioning, 25 mL of influent or 50 mL effluent samples were loaded onto the cartridges at a flow rate of 1 mL min^{-1}. Analytes were eluted at a flow rate of 2 mL min^{-1}, using 6 mL of methanol. Eluate was evaporated under gentle nitrogen stream and reconstituted in 1 mL of methanol water (10:90, v/v). Finally, 10 μL of 10 ng μL^{-1} internal standard mix was added in all samples.

Calibration curves were prepared in the same methanol water mixture adding different volumes of a standard solution containing all the target analytes. All calibration points were spiked with the internal standard mix as in the case of real water samples.

2.3.2. Sludge samples

The extraction of pharmaceuticals from sludge was performed according to Gallardo-Altamirano et al. (2021). Briefly, sludge samples were lyophilized until constant weight (about 5 days). Freeze-dried sludge samples were crushed and sieved, and aliquots of 0.5 g were placed in a 50-mL falcon tube. For quantification purposes, a matrix-matched calibration including blanks was performed in duplicate for each matrix, using 0.5 g aliquots of a pool containing freeze-dried and sieved sludge from all days, which were spiked with 1 mL of working solutions containing all the analytes at different concentrations (2.5, 10, 25, 50, 100, 250, 500 ng mL^{-1}), except the blanks that were spiked with 1 mL of MeOH. Each 0.5 g aliquot was then spiked with 100 μL of a solution containing the internal standards and 2 mL of acetone was added to all samples to promote the interaction between analytes and internal standards with sludge. The samples were kept overnight in a fume hood. For extraction, 5 mL of the extraction solvent (MeOH/HPLC water 1:2 (v/v)) were added to the tubes that were vortexed for 1 min, followed by 10 min in an ultrasonic bath. After the first extraction step, the tubes were centrifuged for 10 min (4000 rpm, 4 °C) and the supernatants collected in 16-mL glass test tubes. This extraction procedure was repeated twice, but the extraction solvent used in the third extraction step was 5 mL of 0.1% formic acid in MeOH/water 1:1 (v/v). The total volume of supernatant (ca. 15 mL) was evaporated to less than 10 mL under a gentle stream of nitrogen at 24 °C using a TurboVap® to reduce the amount of MeOH for the further SPE step. The methanolic extract was diluted in ultrapure water in volumetric flasks of 100 mL to ensure a solvent concentration of less than 10%. These aqueous extracts were filtered through a 0.45-μm pore size nylon membrane (47 mm diameter).

For SPE of the resulting aqueous extracts, Oasis® HLB cartridges (60 mg, 3 mL, Waters Corporation - Milford, MA, USA) were preconditioned with 5 mL of ethyl acetate and 5 mL of MeOH and equilibrated with 5 mL of ultrapure water. The filtered extract samples were loaded onto the cartridges at a flow rate of 1 mL min^{-1}. A washing step was conducted twice with 3 mL of ultrapure water and the cartridges were then dried under vacuum aspiration for 30 min. Analytes were eluted with 3 × 3 mL of EtAc:MeOH (1:1, v/v), and the resulting extracts were evaporated to dryness under a gentle stream of nitrogen. The dried extracts were reconstituted with 1 mL of H_2O/MeOH (90:10) and filtered to 2 mL vials using 0.22 μm polytetrafluoroethylene (PTFE) syringe filters for further UHPLC-MS/MS analysis (Gallardo-Altamirano et al., 2021).

The pharmaceuticals selected for quantification in sludge samples included 3 that were also analysed in aqueous samples (VFX, CBZ, and AZT,), and 3 additional compounds were targeted (DEXA, DCF, and FLX). IBU and ACET were not evaluated due to the very high matrix effect that originated very low sensitivity for these compounds in this sample matrix.

2.4. Analytic methods

2.4.1. Determination of pharmaceuticals in aqueous samples

Chromatographic separation was carried out in an Ultra-Performance liquid chromatography system (Waters Corp. Milford, MA, USA) coupled to a 5500 QqLit, triple quadrupole-linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA). For chromatographic separation, an Acquity HSS T3 column (50 × 2.1 mm i. d., 1.7 μm particle size) and an Acquity BEH C18 column (50 × 2.1 mm i. d., 1.7 μm particle size) were used for the compounds analysed under positive and negative electrospray ionization, respectively, both purchased from Waters Corporation. For the analysis in positive ionization mode, methanol and 10 mM formic acid/ammonium formate (pH 3.2) were used as eluents, at a flow rate of 0.5 mL min^{-1}, whereas for the analyses in negative ion mode, acetonitrile and 5 mM ammonium acetate/ammonia (pH 8) were used as eluents at a flow rate of 0.6 mL min^{-1}. The injection volume was 5 μL. MS/MS parameters, ionization mode, and retention times of analytes and internal standard, are presented in Table S1. Limits of detection (LOD) and of quantification (LOQ), and recovery values of the 5 analysed pharmaceuticals are presented in Table S2.

2.4.2. Sludge samples

A Shimadzu Corporation apparatus (Tokyo, Japan) consisting of a LC (UHPLC, Nexera) and a triple quadrupole mass spectrometer detector (LCMS-8040) was used. For chromatographic separation, a Kinetex™ 1.7 μm XB-C18 100 Å column (100 × 2.1 mm i.d.) supplied by Phenomenex, Inc. (California, USA) was used and the mobile phase (at a flow rate of 0.25 mL min^{-1}) consisted of ultrapure water and acetonitrile acidified with 0.1% formic acid, operating at gradient mode (15% of organic phase during 2 min, a linear gradient during 8 min up to 95%, which was kept during 2.5 min, after which the initial conditions were set again in 1 min to condition the column during 4.5 min). Column oven and autosampler temperatures were set at 35 °C and 15 °C, respectively. The volume of injection was 10 μL. Capillary voltage, drying gas flow, nebulizing gas flow, desolvation and source temperature of the mass spectrometer were 4.5 kV, 14.0 dm^3 min^{-1}, 2.8 dm^3 min^{-1}, 250 °C and 400 °C, respectively. The duplicate matrix-matched calibrations solutions were analysed in duplicate injections. Retention time and selected reaction monitoring (SRM) instrument parameters for detection of each analyte by tandem mass spectrometry are provided in Table S3.

2.5. Multivariate statistical analysis

A principal component analysis (PCA) was performed using the loads of VFX, ACET, AZT and IBU obtained from both WWTP. The main goal was to establish relationships between the pharmaceutical compounds load during the period of analysis, by plotting the two most important principal components (PC).

Partial least square (PLS) was performed using data from the COVID-19 incidence cases in the region served by the respective WWTP and viral load (matrix X) to assess VFX, ACET, AZT, CBZ, and IBU loads (matrix Y). The samples were randomly divided into a training set and a validation set. For PLS model accuracy the correlation coefficient of the different datasets (training, validation and overall - training + validation), the root mean squared error of prediction (RMSEP) and residual prediction deviation (RPD) of the predictive model for the overall dataset were considered. PCA and PLS were performed in Matlab 9.2 (The Mathworks, Natick MA, USA).

2.6. SARS-CoV-2 viral load analysis

The eco-epidemiological modelling activities of the data generated at the WWTP and in the receiving environment, the identification of new biomarkers to assess the presence of SARS-CoV-2 in the various matrices of the wastewater, and the analysis of the correlation of viral load with microbiological indicators, were carried out by the Faculty of Sciences of the University of Lisbon in conjunction with the Laboratory of Analysis of the *Instituto Superior Técnico* (LAIST), of the University of Lisbon. LAIST was also responsible for the development of molecular diagnostic methods and microbiological analysis of wastewater and for the detection and quantification of the virus in wastewater, based on the relative quantification of the N_Sarbecco, E_Sarbecco and RdRp genes, as described in (Monteiro et al., 2022).

3. Results

3.1. Pharmaceuticals detected in aqueous samples

The minimal, maximal, and median concentration values of the pharmaceuticals found in the aqueous samples, specifically in the influent, effluent, and water for reuse (WfR), are depicted in Table 2, which also includes published data obtained for other Portuguese WWTP in years before the pandemic situation caused by SARS-CoV-2. The results of each type of sample are discussed individually in the following sections.

3.1.1. Influent

The ranges of pharmaceuticals concentration evaluated in the influents of both WWTP under study over time are presented in Figs. 1 and 2. A similar pattern of drug concentrations at the two WWTP can be observed. The pharmaceutical detected at the highest concentration was ACET, reaching a maximum of ca. 123.5 μg L^{-1} in the WWTP1 (Fig. 1, Table 1), a value 5 times lower than the values reported in pre-pandemic periods in Olhavas WWTP influents, and 2 times lower than in Coimbrão WWTP influents (Paíga et al., 2019; P. 2016). The other pharmaceuticals were detected at concentrations significantly lower than ACET, IBU reaching ca. 20.6 μg L^{-1} in both WWTP. The loads of VFX, CBZ, AZT, and DEXA did not exceed 1.09 μg L^{-1}.

In the tributary of WWTP1, VFX oscillated from 717 ng L^{-1}, on 18th January, to 412 ng L^{-1}, on 22nd February, and then increased to 1088 ng L^{-1}, on 19th April, and in the following months it remained at 700–800 ng L^{-1} (Fig. S3, A), while in the tributary of WWTP2 the values oscillated between 381 and 1081 ng L^{-1} from January to April, and then remained in the range 600–900 ng L^{-1} (Fig. S3 B). The amount of CBZ in the tributary of WWTP1 varied from 176 to 69 ng L^{-1} in January and February, respectively, and increased from 150 ng L^{-1}, in March, to 549 ng L^{-1}, in September (Fig. S3, C). Samples from WWTP2 displayed a decrease in CBZ from 220 ng L^{-1} to 85 ng L^{-1}, from 18th to 25th January. Thereafter, the concentration increased to 683 ng L^{-1} by September 6 and decreased again, to 363 ng L^{-1}, by September 14 (Fig. S3, D).

Regarding the evolution of concentrations over time, CBZ had an increasing trend throughout the study period in both WWTP and VFX increased until April and then remained constant (Fig. 3A, B). This result suggests an increase in the consumption of these psychiatric drugs as the pandemic extended. ACET kept an increasing trend until April in WWTP1, and March in WWTP2, then it remained nearly constant until July, after which a slight decrease was observed in WWTP1 (Fig. 3A), while in WWTP2 (Fig. 3B) it increased. The concentration of IBU remained constant throughout the study period in both WWTP, which may be explained by the fact that this is a pharmaceutical widely used for pain relief, colds, flu, and other respiratory illnesses, and does not require medical prescription nor is a specific medication for COVID-19.

The concentration values of the pharmaceuticals at the entrance of WWTP were normalised to the entrance flow rate (Fig. 3C, D), which allows a better understanding of the consumption patterns. Although

Table 2
Concentration of pharmaceuticals (ng L^{-1}) detected in aqueous samples in this study, and comparison with the literature values reported in Portugal.

Year	WWTP		Population equivalent (inhabitants)	VFX (ng L^{-1})		CBZ (ng L^{-1})		AZT (ng L^{-1})		ACET (ng L^{-1})		IBU (ng L^{-1})		DEXA (ng L^{-1})		Reference
				Med.	Min-Max	Med.	Min-Max	Med.	Min-Max	Med.	Min-Max	Med.	Min-Max	Med.	Min-Max	
2021	WWTP1	INF	30,000	717	412–1088	216	69–339	235	<MDL-480	78,896	28,128–123,506	15,040	12,011–20,579	–	<MDL-275	This study
		EFF		804	318–999	582	264–813	80	1,2–152	301	74–622	618	<MDL-3700	–	< MDL	
		WfR		450	12–907	446	<MDL-784	88	71–133	334	71–133	1181	132–3447	–	< MDL	
	WWTP2	INF	315,548	751	327–1087	277	63–683	233	<MDL-333	67,550	21,239–98,516	12,696	8099–18,470	–	<MDL-283	
		EFF		459	223–1023	473	318–690	72	<MDL-177	164	<MDL-255	1815	<MDL-3494	–	<MDL	
2013 - 2014	North, center, Lisbon and Tagus Valley, Alentejo, Algarve	INF	10,457,300	–	–	–	–	21	n.d. - 719	–	–	5508	n.d. - 28,900	–	–	(Pereira et al., 2015; A.M.P.T. 2016)
		EFF		–	–	–	–	3	n.d. - 200	–	–	950	n.d. - 6200	–	–	
Oct 2013–Jun 2014	Olhalvas	INF	21,726	12	n.d. - 39	90	51 - 226	38	n.d. - 67	159,225	2024 - 615,135	7628	3877 - 19,118	–	–	(P. Paíga et al., 2016)
		EFF		166	64 - 327	128	84 - 245	<MDL	n.d. - 22	1723	46 - 2463	539	<MDL - 1097	–	–	
	Coimbrão	INF	110,131	50	n.d. - 67	100	47 - 120	n.d	< MDL	119,560	32,610 - 287,801	16,361	12,557 - 24,505	–	–	
		EFF		198	87 - 374	106	63 - 242	n.d	< MDL	2309	313 - 4909	2273	1418 - 3304	–	–	
June 2017	Coimbrão	INF	248,685	275	271 - 279	689	462 - 1339	283	n.d. - 453	477	n.d. - 728	689	127 - 7681	–	–	(Paíga et al., 2019)
		EFF		484	453 - 515	1107	790 - 1427	257	207 - 316	n.d.	n.d.	196	80 - 358	–	–	
Sept 2016-Jan 2019	Beirolas	INF	213,500	–	–	590	350 - 803	–	–	–	–	11,416	5762 - 16,746	–	–	(Silva et al., 2021)
	Faro	INF	44,530	–	–	594	374 - 858	–	–	–	–	13,259	6948 - 20,859	–	–	

n.d. – not determined; INF – influent; EFF – effluent; WfR – water for reuse; Med – median; Min – minimum; Max – maximum; MDL – method detection limit.

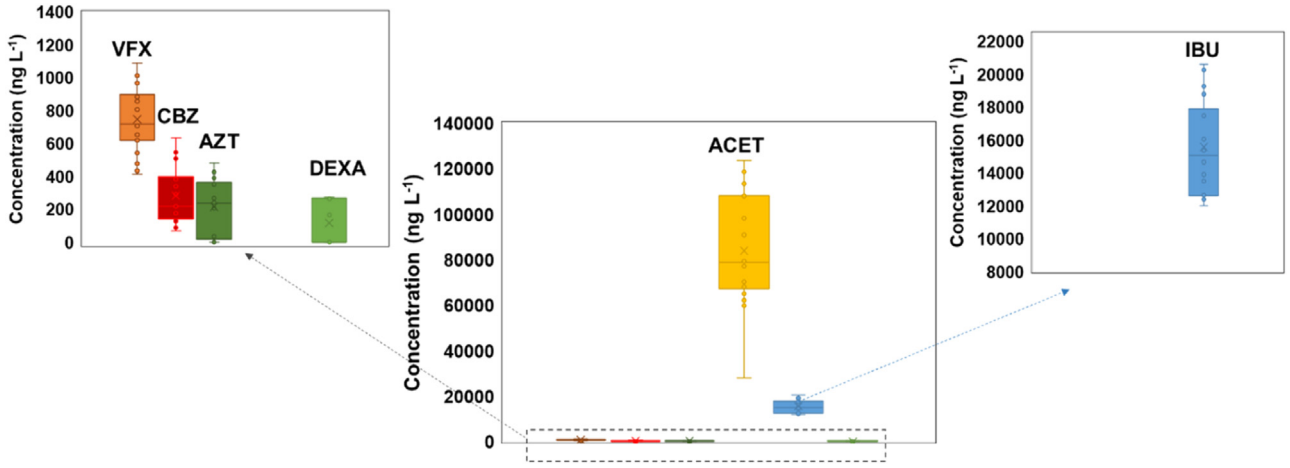

Fig. 1. Concentration range (ng L^{-1}) of the pharmaceuticals ACET (yellow), IBU (blue), VFX (orange), CBZ (red), AZT (dark green) and DEXA (light green), in the influent of the WWTP1 in the January-September 2021 period.

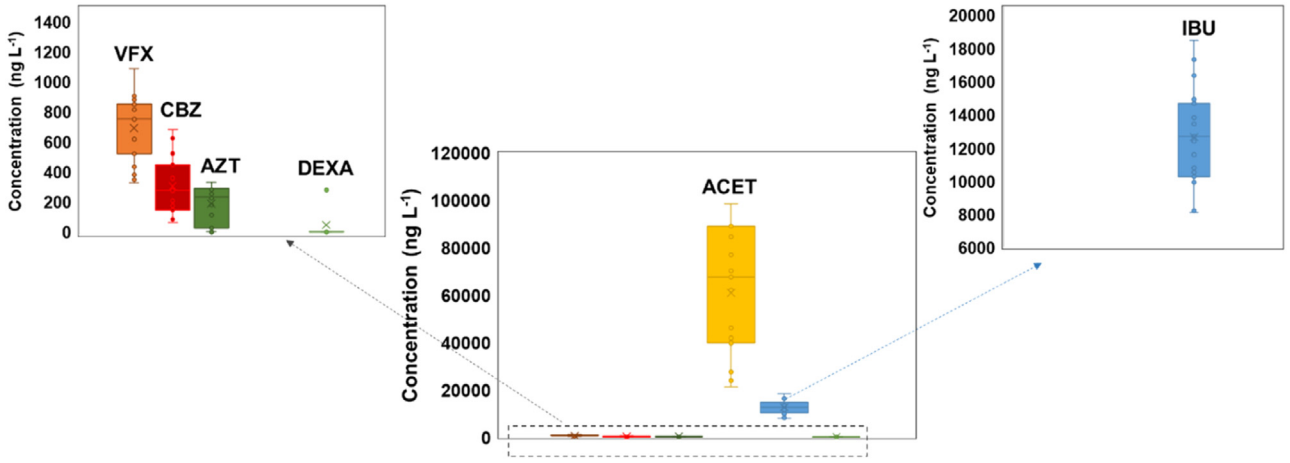

Fig. 2. Concentration range (ng L^{-1}) of the pharmaceuticals ACET (yellow), IBU (blue), VFX (orange), CBZ (red), AZT (dark green) and DEXA (light green), in the influent of the WWTP2, in the January-September 2021 period.

concentrations of pharmaceuticals in both WWTP had a very similar profile (Fig. 3A, B), the pharmaceuticals load reaching both WWTP had a different pattern (Fig. 3C, D). At WWTP1 (Fig. 3C), the ACET and VFX load decreased from April onwards, once the tributary flow rate also decreased significantly from April 2021 until the end of the study period. This pattern is expected since ACET consumption is generally much higher during the winter due to the superior prevalence of fever episodes resulting from seasonal infections (e.g., flu, COVID-19, other virus and also bacterial infections). The influent loads of IBU decreased over time and that of AZT decreased after an increase from January to February. This may be related to the fact that these drugs are more commonly used in winter, and also due to the higher incidence of COVID-19 seen in those months. CBZ increases until April and then suffers a slight decrease and one possibility for this decrease could be the fact that from April onwards the temperatures are milder, and the days longer, which is proven to contribute to an improvement in symptoms linked to psychiatric pathologies, especially depression and low mood (Sansone and Sansone, 2013; Zhang et al., 2021). At WWTP2, the tributary flow rate was constant over time and thus, the pharmaceuticals load had similar profiles to the concentrations detected in the influent (Fig. 3B, D).

Comparing to the values reported in Portugal before the pandemic situation (Table 1), in the present study VFX was detected at higher concentrations than in the WWTP of Olhavas (≈ 60-fold) and Coimbrão (≈ 14-fold) in the period of 2013 and 2014 (P. Paíga et al., 2016), being also ca. 3-fold superior to the reported values in the WWTP of Coimbrão, where sampling was performed hourly over a 24-hour period in June 2017 (Paíga et al., 2019). This suggests that the prescription and consumption of this antidepressant may have increased due to the pandemic situation (Campitelli et al., 2021; Hirschtritt et al., 2021). However, it is important to highlight that these values reported in the literature do not refer to the same regions under focus in this study. Regarding CBZ, the obtained concentrations were approximately 2-fold higher than those reported in 2013–2014 by P. Paíga et al. (2016), but 3-fold lower than the obtained in Coimbrão in 2017 (Paíga et al., 2019), and lower than the concentrations obtained in Beirolas and Faro between 2016 and 2019 (Silva et al., 2021). Therefore, the correlation with the period of COVID-19 is difficult, besides also being different sampling sites. The levels of AZT detected in both WWTP are circa 6 times higher than those reported for Olhavas WWTP in 2013–2014 (P. Paíga et al., 2016) and circa 11 times higher than the obtained in the study of Pereira et al. (2015, A.M.P.T. 2016) also in 2013–2014, which evaluated several regions: North, Lisbon and Tagus Valey, Alentejo and Algarve. This indicative superior consumption pattern of AZT was expected due to its use for respiratory infections resulting from COVID-19, which may be potentiated by the incorrect usage by COVID-19 positive patients with mild symptoms. Nevertheless, AZT showed concentrations quite similar to those previously obtained in Coimbrão (Paíga et al., 2019). ACET was detected at higher values than the previously reported: approxi-

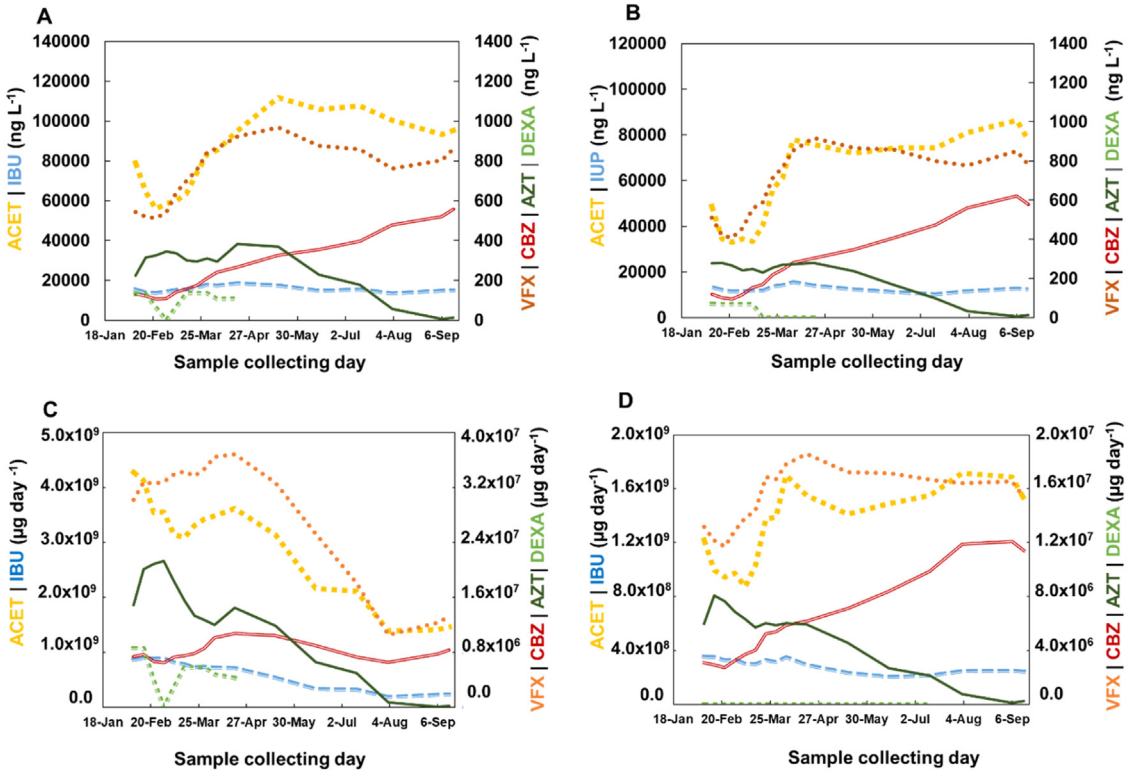

Fig. 3. Moving averages of the influent concentrations (ng L^{-1}) (A, B) and load (ng day^{-1}) (C, D) of the pharmaceuticals ACET (■), IBU (=), VFX (●), CBZ (=), AZT (▬) and DEXA (▬), in the influent of the WWTP1 (A, C) and WWTP2 (B, D).

mately 1.5–2-fold than the amount detected in 2013–2014 in Olhavas and Coimbrão, respectively (P. Paíga et al., 2016), and 165-fold higher than in Coimbrão, 2017 (Paíga et al., 2019). As explained above, this was expected for paracetamol as it is the first line pharmaceutical used for fever conditions. The amounts of IBU were similar to the reported in Coimbrão in 2013–2014 (P. Paíga et al., 2016), and in Beirolas and Faro, in 2016–2019 (Silva et al., 2021), but superior to those reported by Pereira et al. (2015, A.M.P.T. 2016) and P. Paíga et al. (2016). Although very useful for fever and pain, there was some speculation about the role of IBU in COVID-19 patients that was not proved generating some social concerns regarding its use (Poutoglidou et al., 2021).

3.1.2. Pharmaceuticals detected in effluents and WFR

The results showed a similar concentration of VFX in the influent, treated effluent streams, and WfR of WWTP1 (Fig. S3 A). However, VFX concentration was found higher in the effluent of WWTP2 than in the influent in January and February (≈650–1000 ng L^{-1}), and then lowered to values around 200–500 ng L^{-1} (Fig. S3 B). This phenomenon was previously reported for this antidepressant and the authors ascribed it to the possible dissolution of compounds accumulated in aggregates and/or to the back-transformation or de-conjugation of metabolites into the original drugs (Styszko et al., 2021). It is well known that some pharmaceuticals excreted in their conjugated forms can be hydrolysed back to the parent compound by enzymes in the secondary treatment (López-Serna et al., 2012). Such negative removals of certain compounds can be also related to the sampling of grab samples instead of composite samples and not considering the residence time, so that the water body entering the WWTP is not the same as the one being released. Higher concentrations in the effluent and in WfR, in comparison to the raw effluent, were also observed for CBZ at both WWTP under study (Fig. S3 C and D). The observed increase of CBZ concentration in the effluent may be caused by the same reasons described for VFX and also due to the presence of amide in the CBZ structure and the electron deficiency, which makes this compound less susceptible to biodegradation under aerobic conditions (López-Serna et al., 2012; Tiwari et al., 2021).

On contrary, the antibiotic AZT was detected at lower concentrations in the treated effluents and WfR than in the raw influents, with concentrations ranging from 70 to 100 ng L^{-1} in the secondary effluents and WfR of WWTP1 (Table 1, Fig. S3 E), and mostly below the method detection limit (MDL) in the tertiary effluents of WWTP2 (Table 1, Fig. S3 F), showing the high efficiency of both WWTP on removing this medicine from wastewater.

Similarly, to our findings in the influent, ACET was the pharmaceutical detected at the highest levels in the effluent of both WWTP. In the samples collected at WWTP1, the values ranged from ≈70 to ≈620 ng L^{-1} in the period from January to March but decreased afterwards (Fig. S3 G). A similar pattern was registered for the WFR samples. Interestingly, these values are about 200 times lower than those determined in the respective influent samples. In the samples collected at WWTP2, ACET levels ranged from ≈255 to ≈ 148 ng L^{-1}, in January, and then dropped to levels below the MDL (Fig. S3 H).

This decreasing trend of ACET from winter to summer was also observed for IBU at the outlet of WWTP. This anti-inflammatory was detected in the effluent and WfR samples of WWTP1, in January and February, with concentrations in the order of ≈300 – 4000 ng L^{-1} (the highest value being registered on 8th February), dropping to ≈350 ng L^{-1} in March, and to values bellow the MDL from May to September (Fig. S3). In the effluent of WWTP2 (Fig. S3 J), from January to April, IBU was detected at concentrations in the range of 2000 - 3500 ng L^{-1}, similarly to the values at the inlet, but afterwards the concentration was < MDL.

Some authors have been reporting higher concentrations for most drugs during winter, attributed to increasing human consumption in colder months (Golovko et al., 2014; Sun et al., 2016), or slower degradation (Mu et al., 2017), while others have demonstrated higher levels in summer, due to the lower precipitation and river flow

Table 3
Concentration of pharmaceuticals (ng g^{-1}) detected in sludge samples in this study (January-September 2021), and values reported in the literature before COVID-19 pandemic.

WWTP		Sampling	Population equivalent (inhabitants)	VFX (ng g^{-1})	CBZ (ng g^{-1})	AZT (ng g^{-1})	ACET (ng g^{-1})	IBU (ng g^{-1})	DEXA (ng g^{-1})	FLX (ng g^{-1})	DCF (ng g^{-1})	References
WWTP1		Jan 2021	30.000	211.8	29.7	1652.2	-	-	<LOD	635.9	207.8	This study
		Fev 2021		319.4	65.9	n.d.	-	-	<LOD	437.3	134.1	
		Mar 2021		44.5	45.2	772.1	-	-	<LOD	457.8	123.7	
		April 2021		639.8	206.9	632.5	-	-	<LOD	470.4	205.7	
		May 2021		146.4	36.4	1086.1	-	-	<LOD	726.2	267.1	
WWTP2		Jan 2021	315.548	902.8	87.5	214.2	-	-	<LOD	176.1	83	
		Fev 2021		461.9	27.3	220.8	-	-	<LOD	111.3	92.3	
		Mar 2021		312.1	35.3	151	-	-	<LOD	76.5	70.5	
		April 2021		475.2	62.1	138.5	-	-	<LOD	145.2	75.6	
		May 2021		264.9	21.7	167.7	-	-	<LOD	145.7	79.4	
Granada, Huelva, Malaga and Seville (South of Spain)		n.d.	–	–	<MDL- 5.9	–	–	<MDL-33.8	–	–	0.7 - 7.2	(Luis Malvar et al., 2020)
Senec (Slovakia)		n.d.	–	44	86	153	–	1274	–	–	330	(Ivanová et al., 2018)
Athens (Greece)		2006–2013	3.700.000	79.7	71.4	122	–	–	–	46.6	27.5	(Thomaidi et al., 2016)
Seville (Spain)	North	January 2008 to January 2009	350.000	-	0.259	-	-	1.889	-	-	<LOD	(Martín et al., 2012)
	South		950.000	-	0.262	-	-	0.687	-	-	<LOD	
	East		200.000	-	0.231	-	-	3.237	-	-	<LOD	
	West		200.000	-	0.460	-	-	0.524	-	-	<LOD	
Fernão Ferro (Seixal, Portugal)		n.d.	32,700	-	-	-	-	Low[a]	-	Low[a]	Medium[a]	(Salgado et al., 2012)
Setúbal, Cussena, Valdeão, Quinta da Bomba, Fernão Ferro (Portugal)		23 May - 7 July; 2 - 25 October	n.d.	-	-	-	-	550 –3398	-	77 - 77	2259 –17,785	(Salgado et al., 2010)
Tarragona (Catalonia, Spain).		March 2007 - March 2008	n.d.	-	0.012–0.042	-	0.064–0.419	0.044–0.114	-		<LOD – 0.083	(Nieto et al., 2010)
Reus (Catalonia, Spain)			n.d.	-	0.011–0.042	-	0.013–0.153	0.024–0.076	-		<LOD – 0.087	

n.d. – Not determined; MDL – method limit detection; LOD – Limit of detection.

[a] Removal by adsorption in sludge. Low removal represents <25% removal from the average influent load, medium represents >25% and <75% removal, while high removal represents >75% removal.

(Pereira et al., 2015; Silva et al., 2021; Tiwari et al., 2017). According to Lindqvist et al. (2005), the lower removal of IBU during the winter results from the lower residence time of the treated water in the WWTP due to the higher flow rate in the period of greater precipitation. In the period of analysis, higher pluviosity was observed in January and February (Fig. S4), which corresponds to the months when IBU was detected at the highest concentrations in the effluents of both WWTP studied (Fig. S3, I and J).

3.2. Pharmaceuticals detected in sludge samples

Table 3 details the concentrations of pharmaceuticals found in the sewage sludge samples.

Except for 2 pharmaceuticals, DEXA that was not detected in any sludge sample, and AZT that was not detected in February in the sample collected at WWTP1, the other targeted pharmaceuticals were detected in all sludge samples in both WWTP (Table 3, Fig. S5).

In WWTP1, the antibiotic AZT and the antidepressant FLX were the compounds detected at the highest levels, particularly in the samples collected in January and May (Fig. S5 A, B). On contrary, CBZ was the compound detected at the lowest concentrations (from 29.7 to 65.9 ng g^{-1}), except in April when it reached 206.9 ng g^{-1} (Fig. S5 C). In the case of the antidepressant VFX, although no trend could be observed, the values quantified in the sludge samples of WWTP1 fluctuated between 44.5 ng g^{-1} in March and 639.8 ng g^{-1} in April (Fig. S5 D). The concentration of DCF in WWTP1 sludge ranged from 134.1 ng g^{-1} in February to 267.1 ng g^{-1} in May.

Compared to the samples collected at WWTP1, those collected at WWTP2 had higher amounts of the antidepressant VFX (902.8 ng g^{-1} in January, decreasing to 264.9 ng g^{-1} in May) (Fig. S5 D), and of the antibiotic AZT (> 200 ng g^{-1} in January and February) (Fig. S5 A). CBZ was the compound quantified at the lowest concentration in sludge samples (ranging from 87.5 ng g^{-1} in January and 21.7 ng g^{-1} in May) (Fig. S5 C), which may be explained by the low adsorption coefficient of this compound (Tiwari et al., 2021). Accordingly, in the pre-pandemic situation, Greece and Slovakia registered similar values of CBZ to our findings (Ivanová et al., 2018; Thomaidi et al., 2016), while in Spain, it was found at significantly lower amounts (Luis Malvar et al., 2020; Martín et al., 2012; Nieto et al., 2010). Sludge collected at WWTP2 displayed similar values of DCF in all months under analysis (≈70–90 ng g^{-1}) (Fig. S5 E) and the concentration of DEXA in sludge was always below the LOD (Table 3).

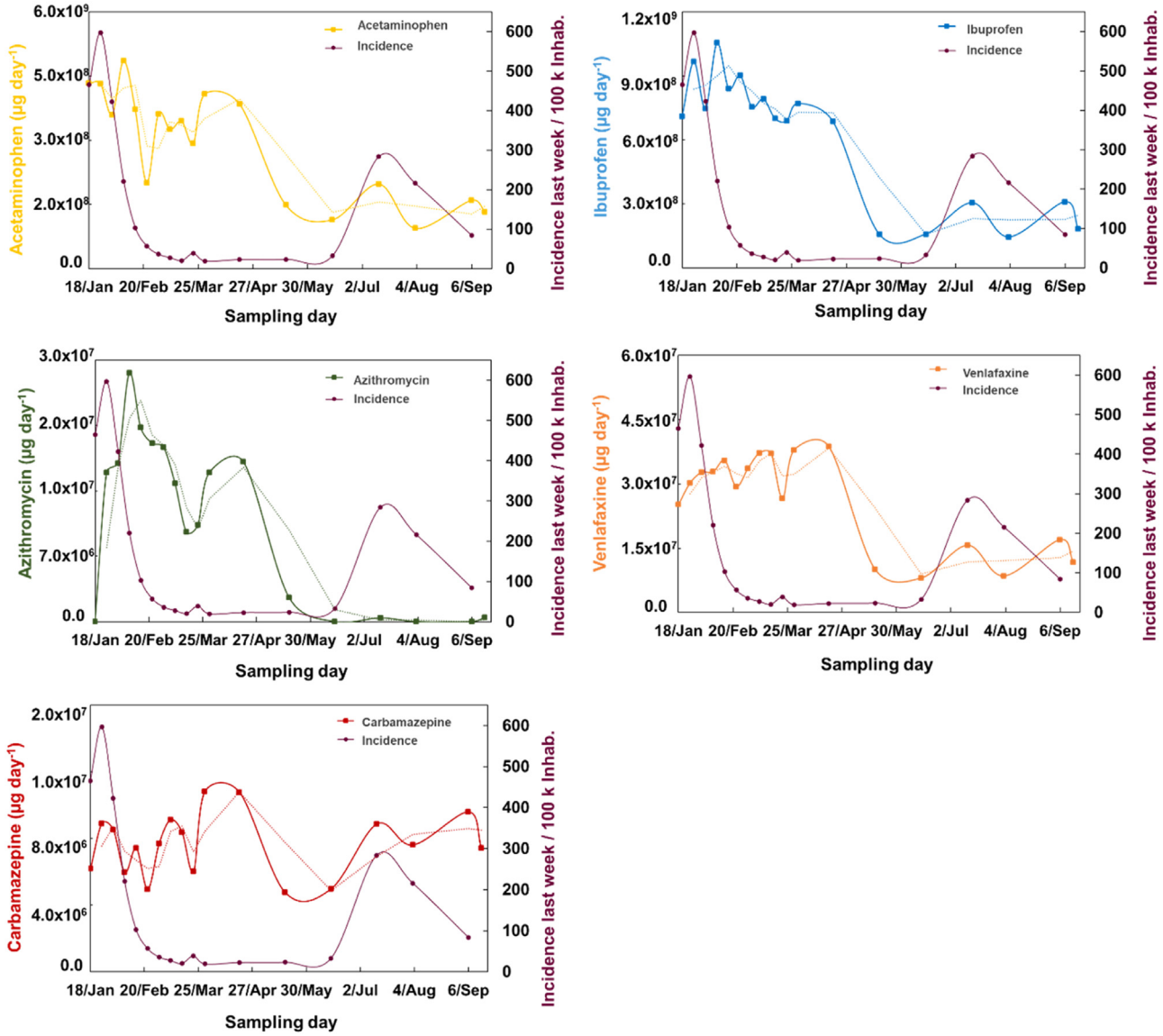

Fig. 4. Pharmaceuticals load in the WWTP1 and COVID-19 incidence per 100 000 inhabitants in the region served by this plant.

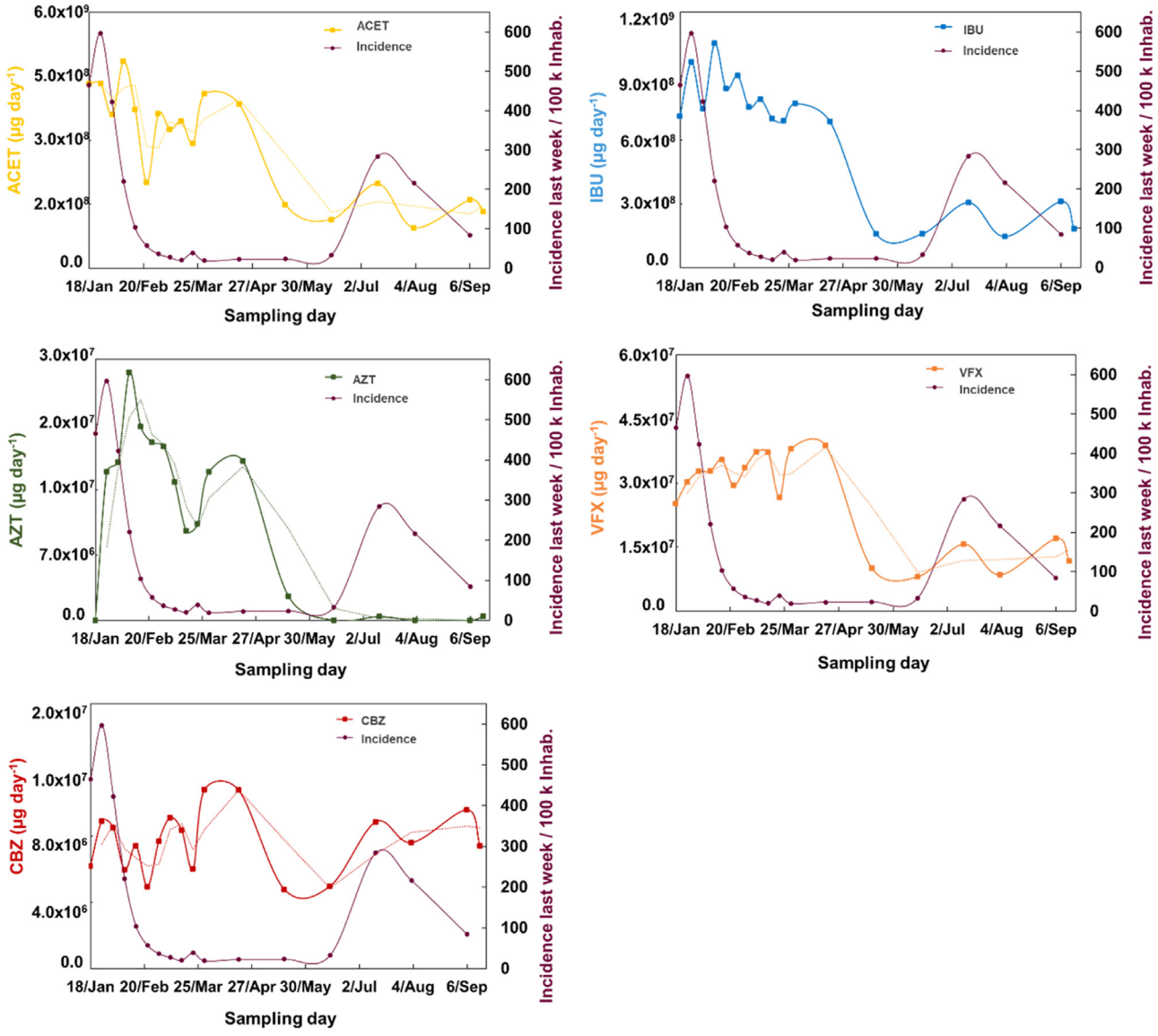

Fig. 4. Continued

The comparative study of the concentration of pharmaceuticals detected in the aqueous samples (influent, effluent, WfR), and sludge samples from the WWTP1 and WWTP2 is present in Figs. S3, S6 and S7. Due to the physicochemical characteristics of VFX, such as the high water solubility, low n-octanol/water partition coefficient, and low Henry coefficient, it is expected to be found in the aqueous phase rather than being volatilized or retained in the activated sludge, which may also explain the poor removal efficiency of this compound, since, for many pharmaceuticals, removal is mainly due to adsorption on sludge (Gómez et al., 2007; Jones et al., 2007; Rúa-Gómez et al., 2012).

Regarding AZT, the high concentration values found in sludge samples may explain the removal efficiency observed for this compound, probably due to adsorption of the pharmaceutical onto sewage sludge. Some studies reported that antibiotics, for instance AZT, can be adsorbed on the surface of sludge with negative charge through ionic interactions (Radjenović et al., 2009).

Previously, IBU, FLX and DCF had been detected in sludge samples of the Portuguese WWTP of Setúbal, Cussena, Valdeão, Quinta da Bomba, and Fernão Ferro (Table 2). The antidepressant FLX was found at lower concentrations than those detected in this study, probably because the consumption of antidepressant increased during the pandemic (Campitelli et al., 2021; Hirschtritt et al., 2021). On the other hand, the concentrations of DCF reported by Salgado et al. (2010) were considerably higher than those detected in the present study. A similar pattern was reported by Salgado et al. (2012), where the concentration of FLX adsorbed onto sludge was considered low (removal by adsorption <25%), while DCF was high (adsorption represents >75% removal). On the other hand, before COVID-19 pandemic, lower values were found in south Spain (Luis Malvar et al., 2020; Martín et al., 2012), as well as in Catalonia (Nieto et al., 2010).

3.3. Pharmaceuticals detected in water and sludge during the third epidemic wave in Portugal versus the incidence of COVID-19 cases

The incidence of COVID-19 cases per 100 000 inhabitants, per week, in the region served by the respective WWTP, for the period in which this study was conducted (January - September 2021) is shown in Figs. 4 and 5. In January 2021, the SARS-CoV-2 variant of concern Alpha, which was considered of easier contagion and higher transmissibility comparatively to the previous variants under circulation, placed Portugal as one of the countries with the highest incidence rate (new daily cases per 100 000 inhabitants) (Chen et al., 2021).

Figs. 4 and 5 demonstrate the results of the back-calculation of drug load per day in relation to the incidence rate. The high concentration of AZT detected in aqueous samples of both WWTP is in accordance with the high incidence of COVID-19 in January. From 25th of January to the end of February, the incidence of COVID-19 decreased significantly from 597 cases to 57 cases per 100 000 inhabitants in the region served by WWTP1, and from 1911 cases to 75 cases per 100 000 inhabitants in the region served by the WWTP2. The quantified concentrations of AZT also decreased accordingly, slowly until March and then more steeply, reaching values below the MDL.

From February to June, the incidence was kept nearly constant and then a new peak surged in July in the region served by WWTP1. A slight increase in incidence occurred from June to September in the region of WWTP2 (Figs. 4 and 5). Except for AZT, there was also a slight increase in the concentration of all other drugs in this period.

In the initial period under study, it was possible to correlate the incidence of COVID-19 with the concentration of the pharmaceuticals determined in the WWTP, since the huge number of cases was accompanied by an increase of some of the detected drugs. With the decrease in the number of COVID-19 new cases, a decrease in the pharmaceuticals in WWTP1 was also observed, but it was much less pronounced. ACET and IBU are analgesic/antipyretic highly consumed in Portugal, so high concentrations, particularly in winter, are expected (Adeleye et al., 2022; Monteiro et al., 2017). On the other hand, the pandemic situation is known to have affected mental health and increased stress in the population (Campitelli et al., 2021; Hirschtritt et al., 2021), which may explain the higher concentrations of VFX and CBZ registered.

3.4. Pharmaceuticals detected in water and sludge in the pandemic context versus SARS-CoV-2 viral load in the same infrastructures

As shown in Fig. 6, considering the results obtained for the different drugs, there are differences between the two WWTP under study, with formation of two clusters. The PCA was performed with the load data of pharmaceutical compounds (VFX, ACET, AZT and IBU) detected from both WWTP. A reasonable cluster separation between each WWTP was obtained as only three data values from the WWTP1 seem to be similar to those from the WWTP2.

It is also set up that PC1 is positively influenced by the data collected from all drugs, mainly ACET and VFX, leading to discrimination of the two WWTP under study (Fig. 7A), and that PC2 is negatively influenced by AZT and IBU data, and positively influenced by CBZ data (Fig. 7B).

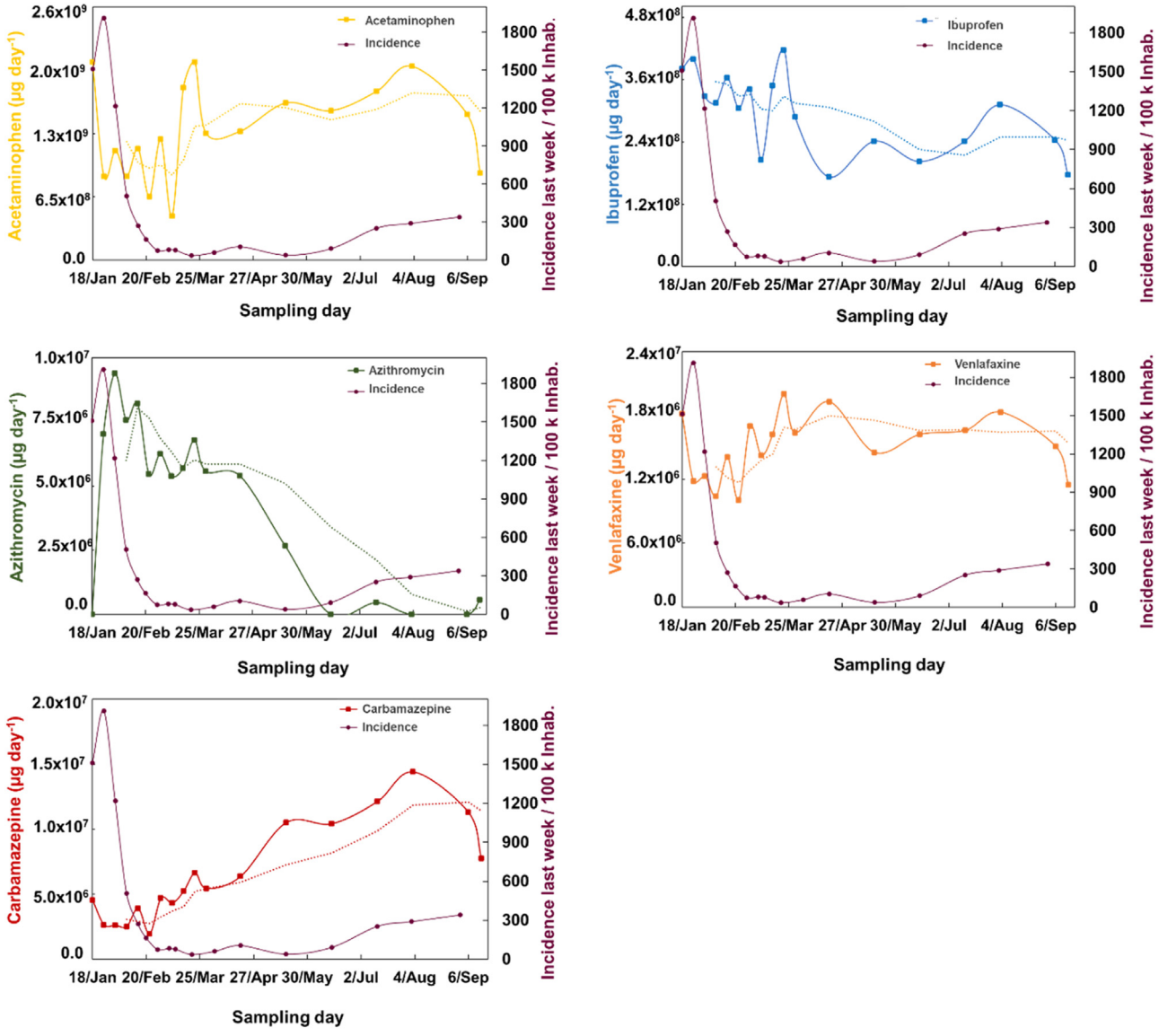

Fig. 5. Pharmaceuticals load in the WWTP2 and COVID-19 incidence per 100 000 inhabitants in the region served by this plant.

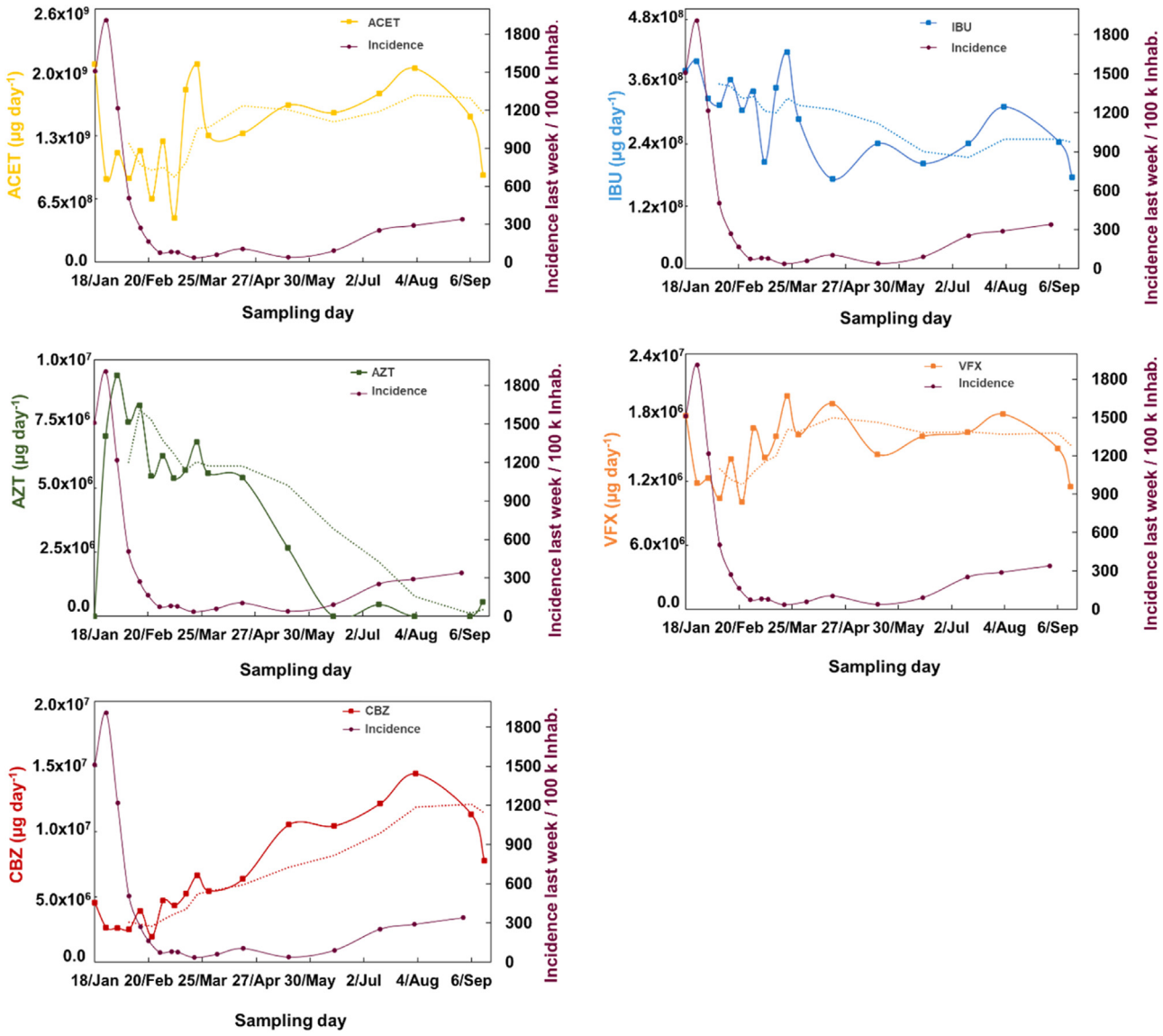

Fig. 5. Continued

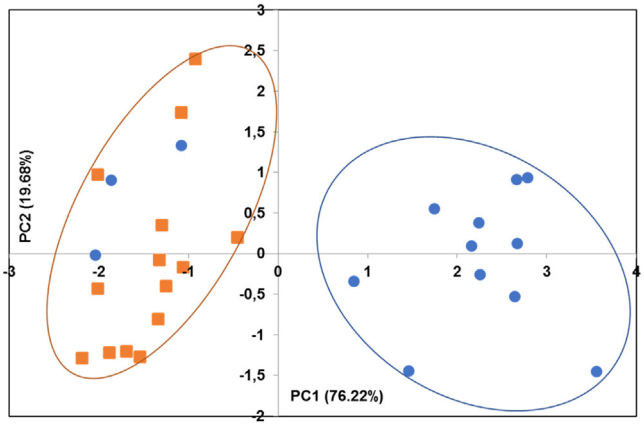

Fig. 6. PCA with the load data of pharmaceutical compounds detected in the two WWTP. PC1 and PC2 explain 76.22 and 19.68% of the dataset variance, for WWTP1 (■) and WWTP2 (●), respectively.

According to PLS analysis, it is not possible to find a correlation for VFX and ACET loads based on the data obtained (Fig. 8A, B). For the remaining drugs (AZT, CBZ, and IBU loads), despite correlation coefficients of 0.625, 0.575 and 0.427, respectively (Fig. 8C, D, E), the RMSEP is extensively high when compared with the standard deviation of the data obtained, resulting in RPD lower than 3, thus configuring a low robust model and its unsuitability towards the pharmaceutical compounds assessment. Concluding, with the incidence and viral load data (Fig. S8), it was not possible to assess the pharmaceutical compounds load. A larger dataset with wider incidence and viral loads ranges would be required, as well as other variables from the process which were not considered in this study.

4 Conclusions

No clear relationship could be established between the incidence of COVID-19 cases and the concentration of the target drugs. However, the high incidence of COVID-19 in January 2021 is in line with the high concentration of pharmaceuticals detected in that month in both aqueous and sludge samples. Accordingly, there was a peak of incidence in the region of WWTP1 between June and July, which was indeed accompanied

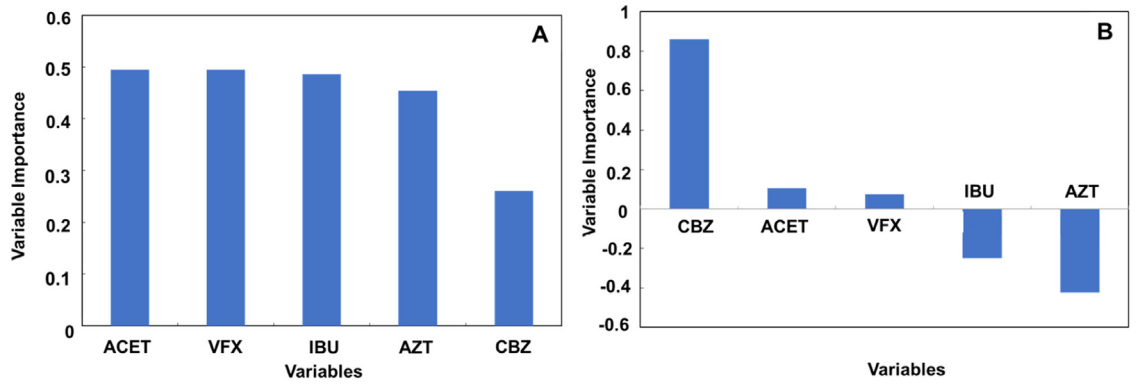

Fig. 7. Loadings representing the influence of the variables in PC1 (A) and in PC2 (B).

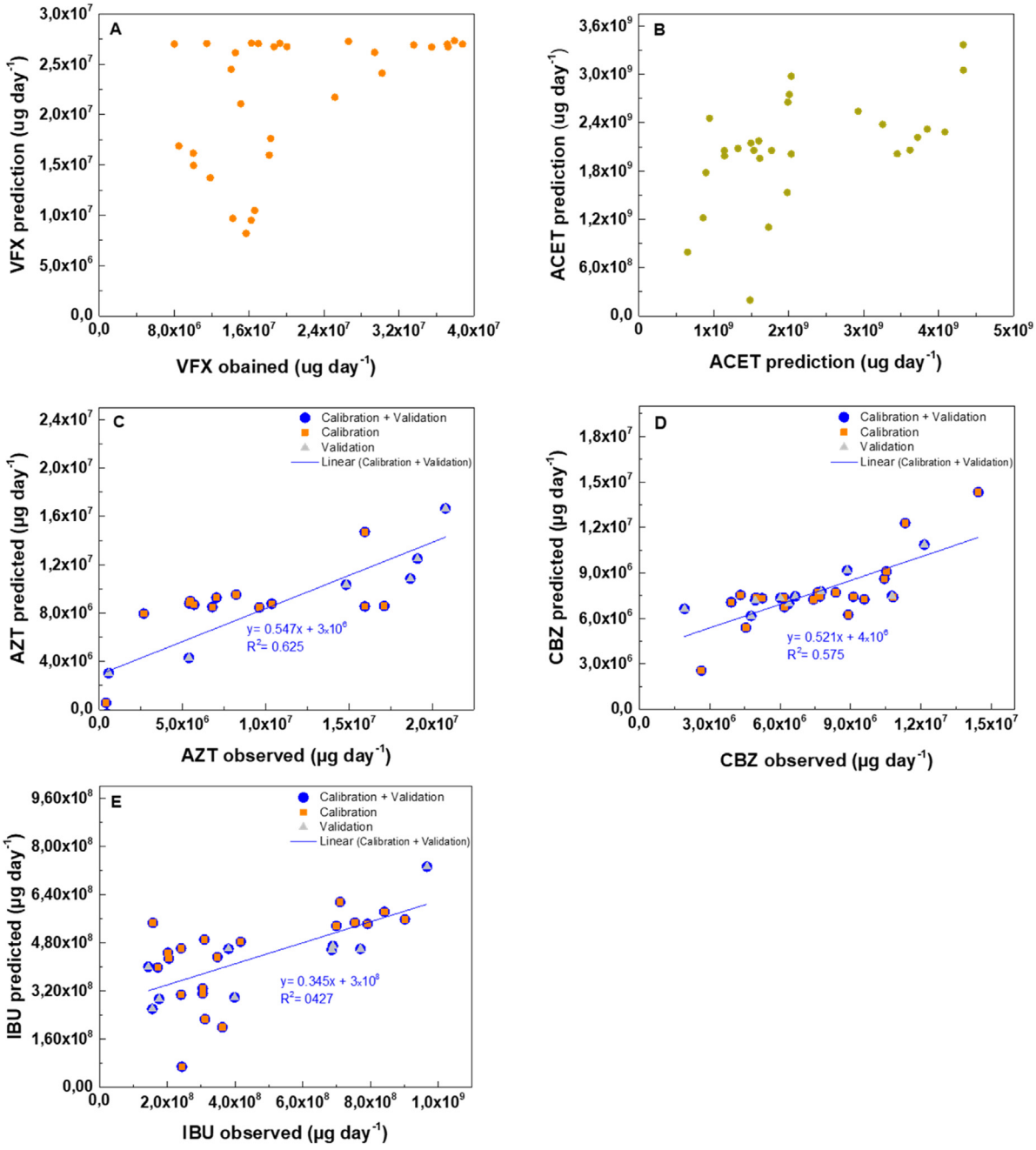

Fig. 8. Relationship between the predicted and observed VFX (A), ACET (B), AZT (C), CBZ (D), IBU (E) for the PLS regression with incidence cases and viral load in the WWTP1 and WWTP2.

by a concentration rising of VFX, CBZ, IBU and ACET at the entrance of the WWTP. Since there is no pre-pandemic occurrence data of the target drugs in these two WWTP under study, it is not possible to ascertain whether the values found were higher than before the pandemics. However, based on information from other WWTP located in Portugal, VFX, CBZ, AZT and ACET were detected at higher concentrations than previously registered, whereas IBU levels were similar to those reported in the pre-pandemic period.

The possible correlation between the COVID-19 incidence, the viral load (i.e. SARS-CoV-2 RNA) measured in the regions served by both WWTP, and the quantified drug loads, was studied. However, with the variables considered, the prediction models were not able to forecast drug loads based on incidence data and viral load information.

In spite of this, the study here presented of real cases of pharmaceuticals distribution, is very important so that the development of effective processes can be done taking into account the real scenario. It is also important to evaluate whether the conventional treatment processes that we currently have are being effective or not, especially at times when certain pollutants may have an increase as it was the case with pharmaceuticals during the COVID-19 pandemic.

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.

CRediT authorship contribution statement

Ana R. Silva: Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. **Daniela P. Mesquita:** Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **M. Salomé Duarte:** Formal analysis, Investigation. **Ana R. Lado Ribeiro:** Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **M.Fernando R. Pereira:** Funding acquisition, Supervision, Resources, Writing – review & editing. **M. Madalena Alves:** Funding acquisition, Project administration, Supervision, Validation, Resources, Writing – review & editing. **Sílvia Monteiro:** Formal analysis, Investigation, Writing – review & editing. **Ricardo Santos:** Validation, Writing – review & editing. **Mónica V. Cunha:** Data curation, Validation, Writing – review & editing. **Sandra Jorge:** Project administration. **Joana Vieira:** Project administration. **João Vilaça:** Funding acquisition, Project administration. **Luísa C. Lopes:** Project administration. **Marta Carvalho:** Funding acquisition, Project administration. **Carlos Brito:** Funding acquisition, Project administration, Validation, Resources. **António Martins:** Funding acquisition, Project administration. **Luciana Pereira:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hazadv.2023.100315.

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