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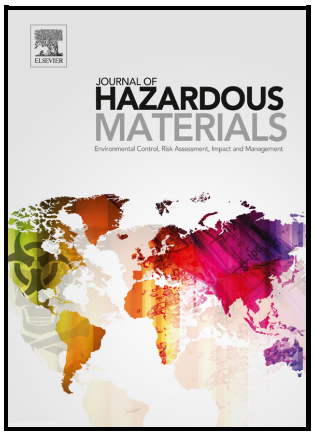
Multi-target analysis of cytostatics in hospital effluents over a 9-month period



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Multi-target analysis of cytostatics in hospital effluents over a 9-month period

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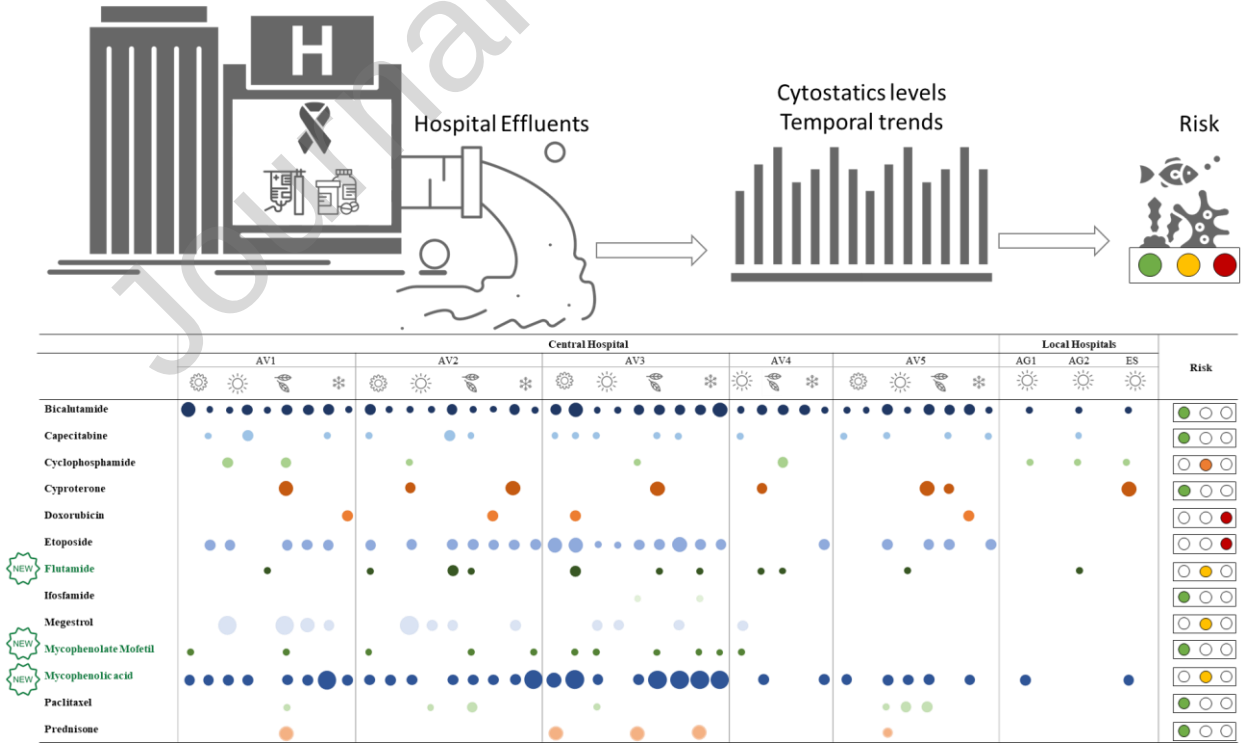
Graphical abstract



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Highlights:

- 13 pharmaceuticals were monitored and detected in hospital effluents
- Temporal evolution of cytostatics in hospital wastewaters was evaluated
- Bicalutamide and mycophenolic acid were the cytostatics found at higher frequency
- The highest concentration was recorded for mycophenolic acid (5340±211 ng/L)
- 6 out of 11 cytostatics are suspected to induce risk to aquatic organisms

Abstract

The consumption of cytostatics, pharmaceuticals prescribed in chemotherapy, is increasing every year and worldwide, along with the incidence of cancer. The presence and the temporal evolution of cytostatics in wastewaters from a Portuguese hospital center was evaluated through a 9-month sampling campaign, comprising a total of one hundred and twenty-nine samples, collected from May 2019 to February 2020. Eleven cytostatics out of thirteen pharmaceuticals were studied, including flutamide, mycophenolate mofetil and mycophenolic acid, which have never been monitored before. Target analytes were extracted and quantified by solid-phase extraction coupled to liquid-chromatography-tandem mass spectrometry analysis; the method was fully validated. All pharmaceuticals were detected in at least one sample, bicalutamide being the one found with higher frequency (detected in all samples), followed by mycophenolic acid, which was also the compound detected at higher concentrations (up to 5340±211 ng/L). Etoposide, classified as carcinogenic to humans, was detected in 60% of the samples at concentrations up to 142±15 ng/L. The risk from exposure to cytostatics was estimated for aquatic organisms living in receiving bodies. Cyclophosphamide, doxorubicin, etoposide, flutamide, megestrol and mycophenolic acid are

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suspected to induce risk. Long-term and synergic effects should not be neglected, even for the cytostatics for which no risk was estimated.

Keywords

anticancer drugs, hospital wastewaters, solid-phase extraction, mass spectrometry, occurrence, toxicity, risk, hazardous medicinal products, temporal trend.

Environmental Implication

Cytostatics are pharmaceuticals used in chemotherapy, whose consumption is expected to increase by 70% over the next two decades, together with increasing cancer incidences. Cytostatics have strong mechanisms of action, along with carcinogenic, mutagenic and teratogenic properties. The continuous release of cytostatics into sewer system and the lack of adequate treatments have risen concerns about environmental integrity/sustainability. Thus, the temporal analysis of the loads of cytostatics in hospital effluents and the risks for aquatic organisms are extremely important for understanding and tackling the problem at the source (i.e. before reaching the sewage sludge and then the water bodies).

1. Introduction

Cancer is one of the most mortal diseases worldwide and its incidence is increasing every year. In Europe, this disease causes about 20% of the total deaths, with around 3 million new cases and 1.7 million deaths per year due to cancer (WHO, 2021). In Portugal, 60,467 new cancer cases were confirmed in 2020, colorectum cancer being the one with the highest incidence, followed by breast and prostate cancers (IARC, 2021a). To treat this disease, many procedures are available: surgery, chemotherapy, radiation therapy, targeted therapy, immunotherapy, stem cell/bone marrow transplant and hormone therapy. The best treatment

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approach is chosen depending on the type/stage of the cancer and the availability of the treatment. Among these, chemotherapy is the second most applied treatment (ACS, 2021).

Chemotherapy consists in the use of medicines to kill cancerous cells. These medicines, called cytostatics, anticancer drugs or cytotoxics (class L of the Anatomical Therapeutic Chemical classification, i.e. antineoplastic and immunomodulating agents), can be administered in the oral form (most of the times at home, with frequent visits to the hospital) or in the intravenous form (usually performed in hospitals or health care facilities) (NHS, 2020). Although they are very effective in the treatment of cancer, cytostatics also affect healthy tissues, especially if they replicate fast such as blood cells, skin cells, stomach cells, etc. According to the International Agency for Research on Cancer (IARC, 2021b), some cytostatics were already identified as carcinogenic to humans, such as etoposide, cyclophosphamide, tamoxifen, azathioprine, busulfan and chlorambucil. Others, as doxorubicin, cisplatin, dacarbazine and mitoxantrone have been classified as probably or possibly carcinogenic to humans. Still, most of cytostatics were not classified yet since there is a lack of toxicological studies.

After administration, the human body is not capable of metabolizing all the medicines, part of them being excreted through urine and feces. Thus, cytostatics, as well as other medicines, bacteria and viruses, are constantly released from hospitals to the water cycle system. Around the globe, there are hospitals which already have a wastewater treatment plant (WWTP) to provide the local elimination of micropollutants prior to the discharge of the effluents into the urban sewer (Majumder *et al.*, 2021; Sim *et al.*, 2013). Still, sometimes, these treatments are not enough for the removal of the most recalcitrant pharmaceutical substances and most of the worldwide hospitals do not have remediation technologies for their wastewaters.

The main objective of this study is to evaluate the presence of thirteen pharmaceuticals of concern (bicalutamide, capecitabine, cyclophosphamide, cyproterone, doxorubicin,

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etoposide, flutamide, ifosfamide, megestrol, mycophenolate mofetil, mycophenolic acid, paclitaxel and prednisone) in Portuguese hospital effluents during a 9-month campaign, comprising a total of one hundred and twenty-nine samples. Solid-phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC–MS/MS) were used for the extraction and quantification of the target cytostatics, respectively. Up to the authors' knowledge, this is the first time flutamide, mycophenolate mofetil and mycophenolic acid are monitored in hospital effluents worldwide.

Data on the levels of cytostatics in Portugal are scarce, with only one published study regarding the monitorization of cytostatics in Portuguese wastewaters, from a Northern urban WWTP (Gouveia *et al.*, 2020). Still, there is a complete lack of information about the occurrence of cytostatics in Portuguese hospital wastewaters, which openly highpoints the novelty of the present work. Another novelty of the current work relies on the fact that, this is one of the very few monitoring studies for which the temporal trends of cytostatics levels in effluents was performed.

2. Experimental

2.1. Chemicals and reagents

Bicalutamide, capecitabine, cyclophosphamide, cyproterone, doxorubicin, etoposide, flutamide, ifosfamide, megestrol, mycophenolate mofetil, mycophenolic acid, paclitaxel and prednisone analytical standards of 98–99% purity were acquired from Sigma-Aldrich (St. Louis, USA) and Cayman Chemical Company (Ann Arbor, USA). Although prednisone and cyproterone are not considered cytostatic drugs, they are administered in combination with several cytostatics during cancer treatment, mainly as anti-inflammatories or immunosuppressants. Methanol (MeOH), acetonitrile (ACN) and Milli-Q water were supplied by Merck (Darmstadt, Germany). All solvents used were of LC–MS grade.

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Mycophenolic acid-d3 (MPA-d3) and cyclophosphamide-d4 (CYC-d4) were used as internal standards (IS); both were acquired from Sigma-Aldrich (St. Louis, USA). Stock standard solutions were prepared at a concentration of 1000 mg/L in MeOH, except for paclitaxel that was prepared in ACN. Working solutions were prepared at 10 mg/L in MeOH, except paclitaxel that was prepared in ACN. Formic acid (HCOOH) and HCl, used for pH adjustment, were purchased from Sigma-Aldrich (St. Louis, USA). SPE cartridges, Oasis HLB (6 cc, 200 mg) were purchased from Waters (Milford, MA, USA). Nylon membrane filters (Whatman 0.8 μm and 0.45 μm), used for sample filtration, were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Safety considerations on cytostatic drugs handling

During the cytostatics' manipulation, a meticulous control on handling procedures, storage conditions and safety guidelines were followed. The procedures which involve these compounds were accomplished in a safety hood with vertical laminar airflow. An absorbent paper made of polyethylene was used to protect the work surfaces and isopropanol was used for cleaning all the materials in contact with the cytostatics; the dischargeable ones were treated as hazardous waste. After each procedure, UV radiation was applied in the safety hood for 15 minutes.

2.3. Sampling scheme

Spot wastewater samples were grabbed in triplicate (1.5 L each) from a central hospital - Aveiro hospital (AV), in five sampling points: AV1 (surgery), AV2 (administrative part of the hospital), AV3 (anatomy), AV4 (pathology) and AV5 (emergency room). Aiming at studying the temporal variation in the pharmaceuticals levels, a monthly sampling from May 2019 to February 2020, except August 2019, was defined. The samplings were performed

always at around the same hours each month. Two local hospitals belonging to the same hospital centre were also included in this survey, with one sample from a local hospital with a palliative care unit, where cancer patients are hospitalized (Estarreja, ES), and another sample from another local hospital in the same geographical region (Águeda, AG, with two sampling points, AG1 and AG2). These two local hospitals (AG and ES) were sampled only one time, in July 2019. A total of one hundred and twenty-nine samples were collected, being one hundred and twenty from the AV hospital, three from the ES hospital and six from the AG hospital (forty sampling months from AV hospital and one month from three sites in AG and ES, collected in triplicate). After being collected, the samples were stored at -18 °C for a maximum of 48 h, until being processed for analysis.

The sampling scheme and the available consumption data of cytostatics in the target hospitals gently provided by the hospital administration are presented in Table SI-1 and Table SI-2 in the Supplementary Information, respectively.

2.4. Sample preparation and extraction

Firstly, the samples were centrifugated at 2665 g for 10 min to remove suspended particles. After that, two filtration steps were applied: the first one with 0.8 μm nylon membrane filters and the second one with 0.45 μm nylon membrane filters. Then, samples were acidified at pH 2 with HCl 1 M, before the SPE procedure. The SPE procedure applied to extract the pharmaceuticals from wastewaters was based on the methodology published by Gómez-Canela *et al.* (2014). The methodology was applied and validated for all target pharmaceuticals, including some cytostatics not considered before in the study of Gómez-Canela *et al.*, namely bicalutamide, flutamide, mycophenolate mofetil and mycophenolic acid.

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SPE cartridges were conditioned using 6 mL MeOH and 6 mL H_2O with 100 mmol/L NH_4OAc. Then, 100 mL of sample (pH = 2) was loaded through the cartridge at a flow rate of approximately 1 mL/min. The cartridges were further dried for about 30-45 min and the elution was performed with 6 mL MeOH and 6 mL MeOH:HCOOH (95:5). The internal standards were added in this step of the process to a final concentration of 20 μg/L. The eluate was slowly evaporated to dryness and reconstituted in 200 μL ACN for further analysis in the LC-MS/MS. Each sample was processed in triplicate.

2.5. Instrumental analysis

The analyses of the extracts were carried out in a liquid chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with an Autosampler SIL-30 AC, an Oven CTO-20 AC, two Pumps LC-30AD, a Degasser DGU-20A5, a System Controller CBM-20A, a LC Solution Version 5.41SP1 and a triple quadrupole mass spectrometer detector Shimadzu LCMS-8040. Data was acquired and processed using the LabSolutions software package.

The stationary phase in the chromatography was a Luna C18 column (150×2.1 mm ID, particle size 5 μm; Phenomenex) and the mobile phase consisted of a binary mixture of water (A) and methanol (B), both acidified with 0.1% HCOOH, in a flow rate of 0.2 mL/min. Gradient elution started at 5% B, increased to 20% B in 15 min, with a further increase up to 45% B in 15 min and up to 100% in 9 min. After 2 min at 100% B, the initial conditions were regained (4 min) and the system was stabilized for 5 min (total running time: 50 minutes). The injection volume was of 5 μL. An electrospray ionization source was operated in positive and negative modes. The precursor ions $[M + H]^+$/$[M - H]^-$ and the two most abundant fragments were used for the identification (transition 2) and quantification (transition 1) of the target analytes (detailed information in Table 1). Optimized parameters were cone voltage (4.5 V for positive and -3.5 V for negative ionized compounds), collision energy (from 10 to

50 eV), 3.0 dm^3/min for nebulizing gas flow, 7.5 dm^3/min for drying gas flow, 400 °C for heat block temperature and 250 °C for desolvation line temperature (Gouveia *et al.*, 2020).

2.6. Validation of the method

The calibration curves were performed in a concentration range of 1 - 250 μg/L, using nine calibration standards in ACN, and the internal standard quantification was accomplished using MPA-d3 as surrogate for etoposide, capecitabine, mycophenolic acid, mycophenolate mofetil and prednisone, and CYC-d4 for the remaining pharmaceuticals. Both were added before the evaporation, which is the extraction step more prone to losses.

The instrumental detection limits (IDLs) were determined for a Signal-to-Noise ratio of 3, considering the average of the values obtained for all calibration points. The method detection limits (MDLs) were further obtained from IDLs, considering the concentration factor of the extraction process.

Recovery assays were performed using different hospital wastewaters spiked with known amounts of the target pharmaceuticals, according to the information provided in the Supplementary Information (section SI-B).

Intra-day and inter-day precisions were obtained by measuring the analytical response for two analytical standards in six consecutive injections through six different days. More detailed information is provided in the same section of the Supplementary Information.

The global uncertainty associated to the quantification of the thirteen selected cytostatics by the proposed methodology was estimated by the bottom-up approach of the EURACHEM-CITAC Guide (Ellison and Williams, 2012). Detailed equations and results can be found in the Supplementary Information (section SI-C).

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2.7. Predicted concentration in surface waters and estimation of the risk to aquatic organisms

To evaluate if the concentrations measured for each pharmaceutical in hospital effluents could represent a threat to aquatic biota, upon their discharge into urban sewer and then into water bodies, the risk quotient was estimated. Risk was estimated from the quotient between the predicted concentration of each compound in surface waters and the PNEC (Predicted no effect concentration), a value obtained from published toxicological data by applying an assessment factor, which depends on the available information (e.g., chronic or acute toxicity, number of trophic levels for which the data is available) (Gouveia *et al.*, 2019).

The concentrations of target pharmaceuticals in surface waters were predicted from the measured concentrations in hospital effluents. According to Verlicchi and co-workers, there is an average dilution of 4-10 times for cytostatic drugs' concentrations from hospital effluents to WWTP influents (Verlicchi *et al.*, 2010). Furthermore, a 10-fold dilution factor from WWTPs to surface waters was considered as recommended in the Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use (EMEA, 2006). Assuming the worst-case scenario, it was then decided to apply a dilution factor of 4 from the hospitals to the urban WWTP, no degradation in WWTPs and a dilution factor of 10 from WWTP effluents to surface waters. For the compounds for which risk was predicted using these assumptions, a dilution factor of 10 was then considered from the hospital effluents to urban WWTPs, to refine the results and provide insightful information on the risk-factors variability. The toxicological information and the PNEC values used to estimate the risk are compiled in the Supplementary Information (Section SI-D).

3. Results and discussion

3.1. Validation of the method

Good linearity was achieved for all compounds using the optimized parameters for LC-MS/MS analysis, in a range of 1–250 μg/L (corresponding to 2–500 ng/L in the real samples, i.e. before extraction), with correlation coefficients higher than 0.998 (Table 1). The MDL values obtained were relatively low, varying from 0.08 ng/L for flutamide to 1.85 ng/L for cyproterone. These limits are suitable for the detection of the target cytostatics in hospital effluents as their concentrations in these matrices ranged from tens ng/L to mg/L (11 ng/L for capecitabine - 6820 μg/L for ifosfamide) according to the literature (Azuma *et al.*, 2016; Hamon *et al.*, 2018). The results of intra and inter-day precisions were satisfactory, varying between 1% for megestrol and 9% for cyproterone (intra-day precision), and between 3% for mycophenolate mofetil and 15% for flutamide (inter-day precision) - Table 1. An average recovery of 83±23% was achieved for the target pharmaceuticals, using different hospital wastewaters (Table 1), with individual recoveries ranging from 43±6% for doxorubicin and 120±4% for cyclophosphamide. Similar recoveries have been reported in the literature for capecitabine, cyclophosphamide, cyproterone, doxorubicin, etoposide, ifosfamide, megestrol, paclitaxel and prednisone (Table SI-4 in the Supplementary Information). Up to the authors' knowledge, this is the first analytical methodology for the identification and quantification of flutamide, mycophenolic acid and mycophenolate mofetil in hospital effluents. A graphical representation of the global uncertainty of the method is given in Figure SI-1 in the Supplementary Information. It is possible to conclude that the global uncertainty is approximately 15% for all pharmaceuticals at concentrations above 30 ng/L, with the exception of paclitaxel, whose uncertainty is 22%.

The concentrations of the target pharmaceuticals in hospital effluents presented in this work do not contemplate recovery correction since other sources of uncertainty may also affect the final result (e.g., standard preparation, linear regression and precision). Therefore, global

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uncertainties should be considered when evaluating the concentrations of pharmaceuticals presented from now on.

Table 1. Chromatographic/ MS information and validation parameters obtained for the analysis of thirteen pharmaceuticals in hospital effluents by SPE-LC-MS/MS.

Pharmaceutical	rt (min)	Molecular ion (m/z) (CV, V)	Transition 1 (CE, eV)	Transition 2 (CE, eV)	Linearity (µg/L)	R	MDL (ng/L)	%Rec	Intra-day precision, RSD (%)	Inter-day precision, RSD (%)
Bicalutamide	37.66±0.04	429.0 $[M-H]^-$ (-3.5)	429.00→255.05 (16)	429.00→184.95 (39)	1-150	0.9986	0.10	96±9	8	10
Capecitabine	36.38±0.02	360.2 $[M+H]^+$ (4.5)	360.20→244.00 (-13)	360.20→174.00 (-23)	1-250	0.9999	0.11	108±4	7	8
Cyclophosphamide	32.26±0.05	260.9 $[M+H]^+$ (4.5)	260.90→139.95 (-23)	260.90→106.05 (-19)	1-75	0.9999	1.08	120±4	3	6
Cyproterone	39.46±0.06	417.2 $[M-H]^-$ (-3.5)	417.20→357.15 (-18)	417.20→279.00 (-25)	5-75	0.9981	1.85	82±3	9	5
Doxorubicin	34.88±0.33	544.0 $[M+H]^+$ (4.5)	544.00→397.00 (-13)	544.00→381.00 (-28)	15-250	0.9996	0.48	43±6	6	12
Etoposide	35.07±0.03	589.2 $[M+H]^+$ (4.5)	589.20→228.95 (-20)	598.20→185.10 (-37)	1-50	0.9980	0.53	54±13	5	8
Flutamide	38.64±0.04	275.0 $[M-H]^-$ (-3.5)	275.00→201.95 (24)	275.00→205.05 (21)	1-75	0.9995	0.08	77±2	5	15
Ifosfamide	30.52±0.04	260.9 $[M+H]^+$ (4.5)	260.90→92.05 (-26)	260.90→153.95 (-23)	1-75	0.9998	0.41	105±10	3	10
Megestrol	39.71±0.06	385.1 $[M+H]^+$ (4.5)	385.10→267.10 (-20)	385.10→325.15 (-15)	1-75	0.9999	0.40	58±6	1	5
Mycophenolate mofetil	33.50±0.54	434.1 $[M+H]^+$ (4.5)	434.10→114.05 (-27)	434.10→194.95 (-36)	1-250	0.9988	0.13	64±12	2	3
Mycophenolic acid	37.89±0.03	321.0 $[M+H]^+$ (4.5)	321.00→207.0 (-23)	321.00→303.10 (-10)	5-75	0.9983	0.47	90±9	5	8
Paclitaxel	38.97±0.22	876.2 $[M+H]^+$ (4.5)	876.20→308.0 (-30)	876.20→591.15 (-28)	1-250	0.9995	0.37	80±10	4	11
Prednisone	35.89±0.02	359.1 $[M+H]^+$ (4.5)	359.10→146.95 (-26)	359.10→341.15 (-13)	5-150	0.9989	1.69	102±14	3	4

MDL – Method Detection Limit; Rec – recoveries (%); RSD – relative standard deviation; rt – retention time

3.2. Presence of cytostatics in hospital effluents

Among the thirteen pharmaceuticals studied, all of them were detected at least once in the 129 samples analyzed. Considering the AV hospital, bicalutamide was the most frequently detected cytostatic (present in all samples analyzed), followed by mycophenolic acid (present in 96 samples, 80% frequency) and etoposide (78 samples, 65% frequency) (Figure 1). Ifosfamide was the less frequently detected cytostatic, being identified in two months. Regarding the AG and ES hospitals, bicalutamide was also found in all samples, as well as cyclophosphamide. On the other side, doxorubicin, etoposide, ifosfamide, megestrol, mycophenolate mofetil, paclitaxel and prednisone were not detected in these two hospitals. Figure 1 shows the frequency of detection of each compound in all the samples analyzed, considering the three studied hospitals.

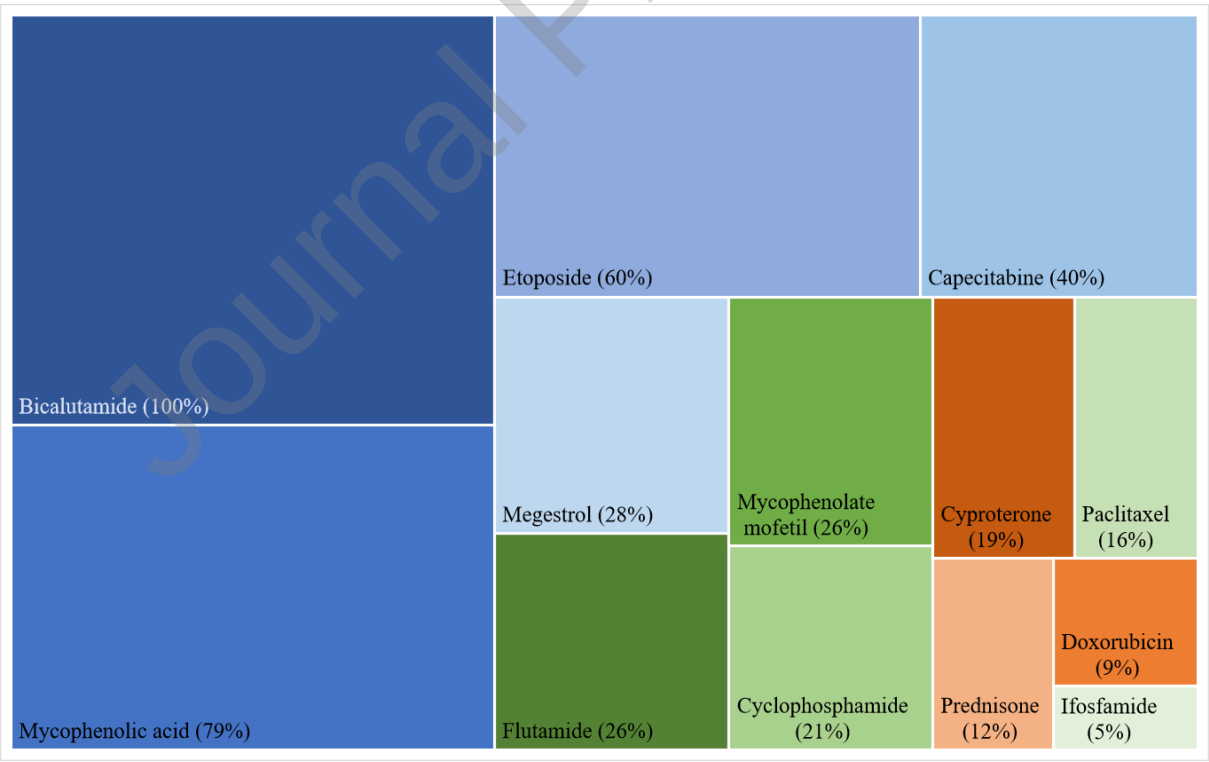

Figure 1. Frequency of detection (relative frequency) of the selected pharmaceuticals in all the samples analyzed. Green colors correspond to the pharmaceuticals included in group A

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(see text for explanation), orange colors to those in group B and blue colors to those in group C.

The concentrations found for the studied pharmaceuticals (Table SI-5) widely varied between 0.11 ng/L for capecitabine in July (AV3) and 5340 ng/L for mycophenolic acid in February (AV2). Figure 2 shows a schematic representation of the concentrations found for each pharmaceutical in every month and hospital unit.

Compiling the results obtained (in terms of detection frequency and concentrations found), three groups were defined: (**group A**; green in Figure 1) cytostatics with low frequency of detection and found at concentrations < 50 ng/L, which comprises cyclophosphamide, flutamide, ifosfamide, mycophenolate mofetil and paclitaxel; (**group B**; orange in Figure 1) pharmaceuticals with low frequency of detection and found at concentrations 50–250 ng/L, which comprises cyproterone, doxorubicin and prednisone; and (**group C**; blue in Figure 1) pharmaceuticals with high frequency of detection (≥ 28%).

Among the cytostatics considered in **group A**, cyclophosphamide, flutamide, ifosfamide, mycophenolate mofetil and paclitaxel were detected in 21%, 26%, 5%, 26% and 16% of the samples, respectively (marked in green colors in Figure 1), at concentrations up to 22±3 ng/L for cyclophosphamide, 6.2±0.3 ng/L for flutamide, 3.0±0.1 ng/L for ifosfamide, 5±1 ng/L for mycophenolate mofetil and 9.4±0.3 ng/L for paclitaxel (Figure 3 and Table SI-5 in the Supplementary Information, which describes all the concentrations found). Ifosfamide, mycophenolate mofetil and paclitaxel were not detected neither in AG nor ES hospitals.

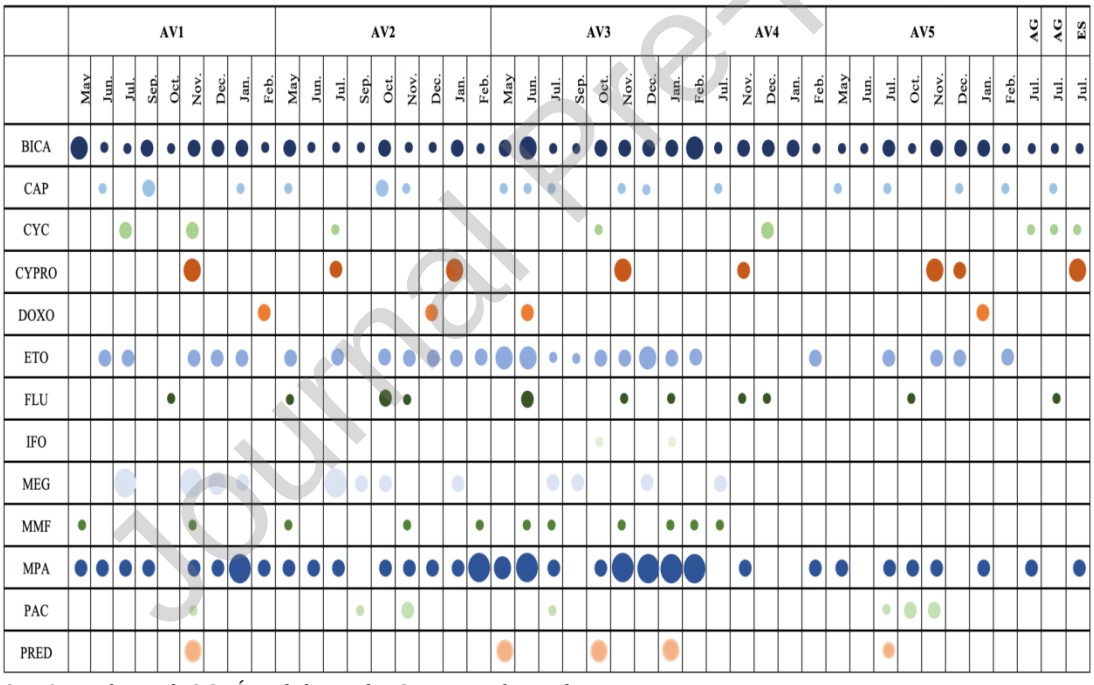
Regarding mycophenolate mofetil, a cytostatic included in the basic immunosuppression procedures (National Library of Medicine, 2021b; Sánchez-Lázaro *et al.*, 2010), it is excreted in the urine in an extend of about 87% as an inactive form of mycophenolic acid (Drugbank, 2021d). This justifies its low concentrations (up to 5±1 ng/L) and frequency of detection

(26%), despite being the most consumed cytostatic among the studied ones (average consumption of 2 kg/month – Table SI-2 in the Supplementary Information). It should be emphasized that, up to the authors' knowledge, this is the first monitoring study of mycophenolate mofetil in hospital effluents and thus comparisons with other studies/locations are not possible to conduct.

For cyclophosphamide, our results (21% detection frequency and concentrations up to 22±3 ng/L) display a high degree of variability between sampling sites and months, which are in accordance with the literature (Azuma *et al.*, 2016; Česen *et al.*, 2015; Ferrando-Climent *et al.*, 2013; Isidori *et al.*, 2016; Kovalova *et al.*, 2012). In fact, the available studies regarding the presence of cyclophosphamide in hospital effluents disclosed very distinct concentrations. Hamon *et al.* (2018) reported the highest concentration of cyclophosphamide in wastewaters, from a French hospital, going up to 687 µg/L, followed by 29.1 µg/L detected by Oliveira Klein and co-workers in a Brazilian hospital (Oliveira Klein *et al.*, 2021). On the other side, there are some studies which did not detect cyclophosphamide at all in hospital wastewaters (in Japan, Saudi Arabia and Australia) (Al Qarni *et al.*, 2016; Busetti *et al.*, 2009; Thomas *et al.*, 2007). Cyclophosphamide has a low biological half-life (3-12 hours), being excreted in the parent form at relatively low percentages (10-20% in urine) (Drugbank, 2022). Most of chemotherapy treatments can last from 5 minutes to 8 hours, meaning that cyclophosphamide would be likely excreted at home by ambulatory patients. This may justify the low frequency of detection and the low concentrations of cyclophosphamide in hospital wastewaters. It is important to emphasize that cyclophosphamide was identified by the International Agency for Research on Cancer as carcinogenic to humans (IARC, 2021b).

For ifosfamide, our results (5% detection frequency and concentrations up to 3.0±0.1 ng/L) show a low frequency of detection at extremely low concentrations. These results were expected since ifosfamide was not administered in the hospitals. This compound is widely monitored in hospital effluents worldwide, the maximum concentration also being measured

in France by the same group mentioned before for cyclophosphamide (682 µg/L; (Hamon *et al.*, 2018)). As reported for cyclophosphamide, opposite findings can also be found in the literature, e.g., ifosfamide was never detected in Spanish hospital wastewaters (Ferrando-Climent *et al.*, 2014). Actually, such a high variability of cytostatic loads among hospitals could be attributed to: (i) the different chemotherapy approaches prescribed at the hospitals (e.g., prescription of different cytostatics to treat the same cancer pathology); (ii) the different cancer incidences in the country where the hospital is located, which would increase the consumption of certain cytostatics over others (i.e., each country has its own identity in terms of the most common cancer among the population); (iii) the specialization in the treatment of certain types of cancer (there are reference hospitals in oncology).



Note:
AV- Aveiro hospital; **AG**- Águeda hospital; **ES**- Estarreja hospital;
BICA- bicalutamide; **CAP**- capecitabine; **CYC**- cyclophosphamide; **CYPRO**- cyproterone; **DOXO**- doxorubicin; **ETO**- etoposide; **FLU**- flutamide; **IFO**- ifosfamide; **MEG**- megestrol; **MMF**- mycophenolate mofetil; **MPA**- mycophenolic acid; **PAC**- paclitaxel; **PRED**- prednisone.

- concentration ≤ 5 ng/L
- 5 ng/L < concentration ≤ 50 ng/L
- 50 ng/L < concentration ≤ 300 ng/L
- concentration > 300 ng/L

Figure 2. Schematic representation of the concentrations found for each pharmaceutical in every month/box studied.

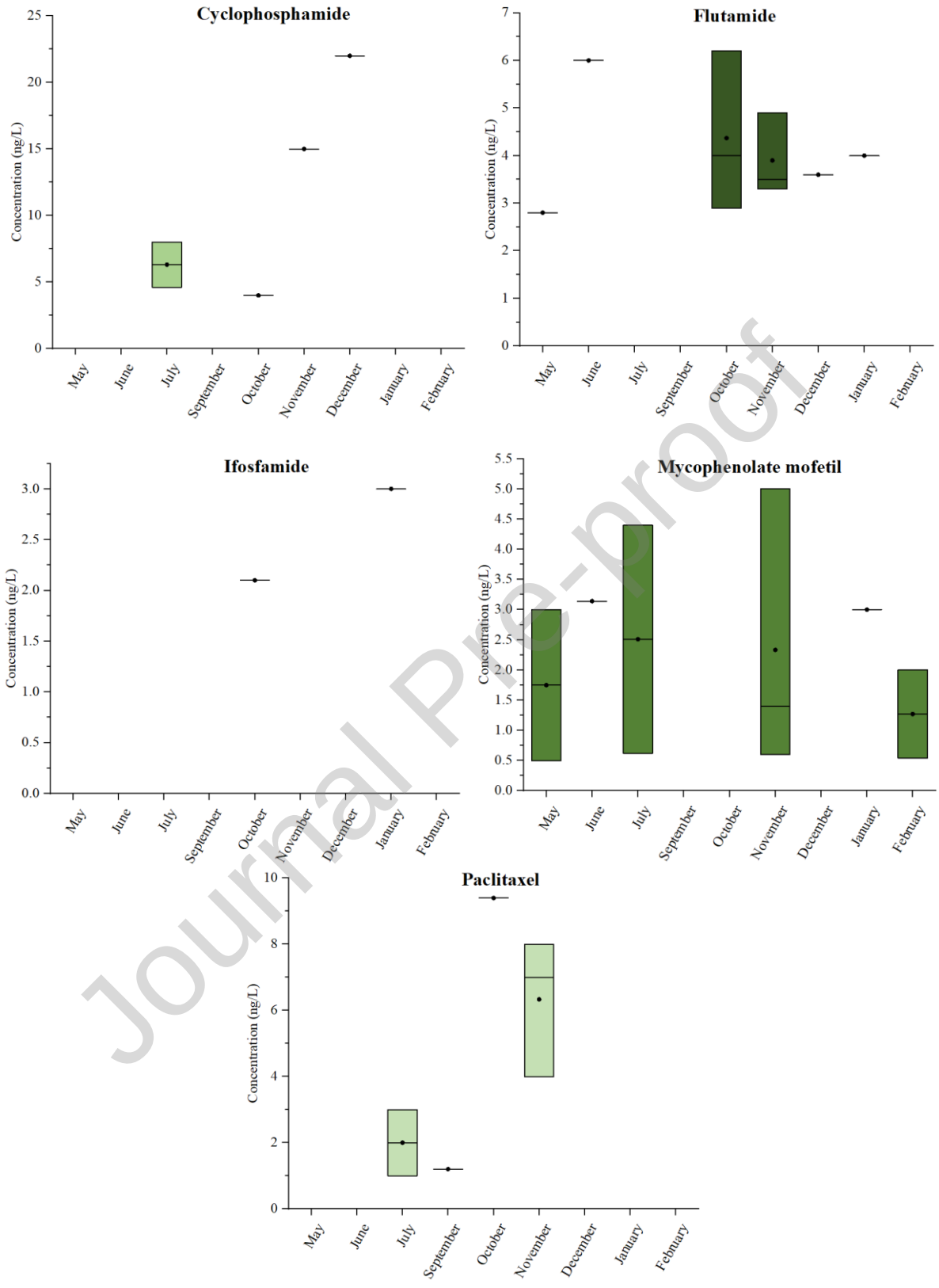


Figure 3. Box and Whisker diagrams with monthly representation of the concentrations found for cyclophosphamide, flutamide, ifosfamide, mycophenolate mofetil and paclitaxel in AV hospital.

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Flutamide, with 26% detection frequency, shows a high variability in terms of sampling months, with similar concentrations between them (varying from 2.07±0.04 to 6.2±0.3 ng/L). To the best of the authors' knowledge, there are no studies regarding flutamide's presence neither in hospital wastewaters nor in any other aqueous matrix. Flutamide was not administered in the studied hospitals, which corroborates its low concentrations in the wastewaters analyzed.

For paclitaxel, our results (low frequency of detection and concentrations: 16% detection and concentrations up to 9.4±0.3 ng/L) can be justified by the consumption data, which is relatively low (average of 2 g/month), as well as by its average biological half-life of 52.7 hours (Drugbank, 2021e), especially if administered to outpatients. There are four papers that monitored paclitaxel in hospital wastewaters: it was not detected in Spain and China (Isidori *et al.*, 2016; Negreira *et al.*, 2014) and detected at concentrations up to 100±14 ng/L also in Spain (Ferrando-Climent *et al.*, 2013; 2014).

Among the cytostatics considered in **group B** (orange color in Figure 1), cyproterone was detected in 19% of the total samples, 21 from the AV and 3 from the AG and ES hospitals. The highest concentration found was 85±23 ng/L, in November (AV5), and the lowest concentration 24±10 ng/L, in December (AV5) – Figure 4. The administration of cyproterone is quite stable between months, with an average of 84 g/month. Its low frequency of detection can be justified by its biological half-life of 38-96 hours (Drugbank, 2007). The relatively high concentrations of cyproterone in the wastewater samples could possibly be justified by its high excretion rate (60% in the bile and 33% through the kidneys) (Drugbank, 2007). Up to the authors' best knowledge, there is only one paper regarding the monitoring of cyproterone in hospital wastewaters, and it was not detected in any of the analyzed samples (Gómez-Canela *et al.*, 2014). As detailed above, there are many factors that could support such variability on cytostatics concentrations between countries.

Doxorubicin was found in 12 of the 129 samples analyzed, which corresponds to 9% detection (Figure 1), at concentrations up to 46±14 ng/L in December (AV2) – Figure 4. According to the data provided, doxorubicin has low consumption records (average of 2 g/month). This, combined with its high tendency to adsorb on particulate matter (Wu *et al.*, 2013) and the low percentage of doxorubicin eliminated by urine in a relatively long time (5-12% within 5 days) (Drugbank, 2021c) may justify being the second pharmaceutical less detected in the wastewater samples. When comparing to the literature, there are some studies where doxorubicin was not detected in effluents from hospitals located in Spain, Slovenia and China (Gómez-Canela *et al.*, 2014; Isidori *et al.*, 2016; Negreira *et al.*, 2014; Yin *et al.*, 2010). Another study, performed by Mahnik *et al.* (2007), quantified doxorubicin in concentrations up to 1350 ng/L in Vienna, Austria, a concentration much higher than the maximum concentration measured in the present work. Still, this compound was identified by the International Agency for Research on Cancer as probably carcinogenic to humans, meaning that there is strong evidence that it may cause cancer in humans (IARC, 2021b).

Prednisone was found in 12% of the samples, at concentrations varying from 17±2 ng/L to 221±34 ng/L, the highest one being detected in May (AV3). These concentrations are of the same order of those reported in the two monitoring studies available in the literature concerning this matter (up to 545 ng/L) (Gómez-Canela *et al.*, 2014; Schriks *et al.*, 2010). Regarding its administration in the studied hospitals, no information was provided since prednisone is used in several therapeutic indications, and thus its administration is not controlled. Prednisone half-life in human organism is around 2-3 hours, being excreted in urine mainly as sulfate and glucuronide conjugates. This may justify its low frequency of detection in its original form.

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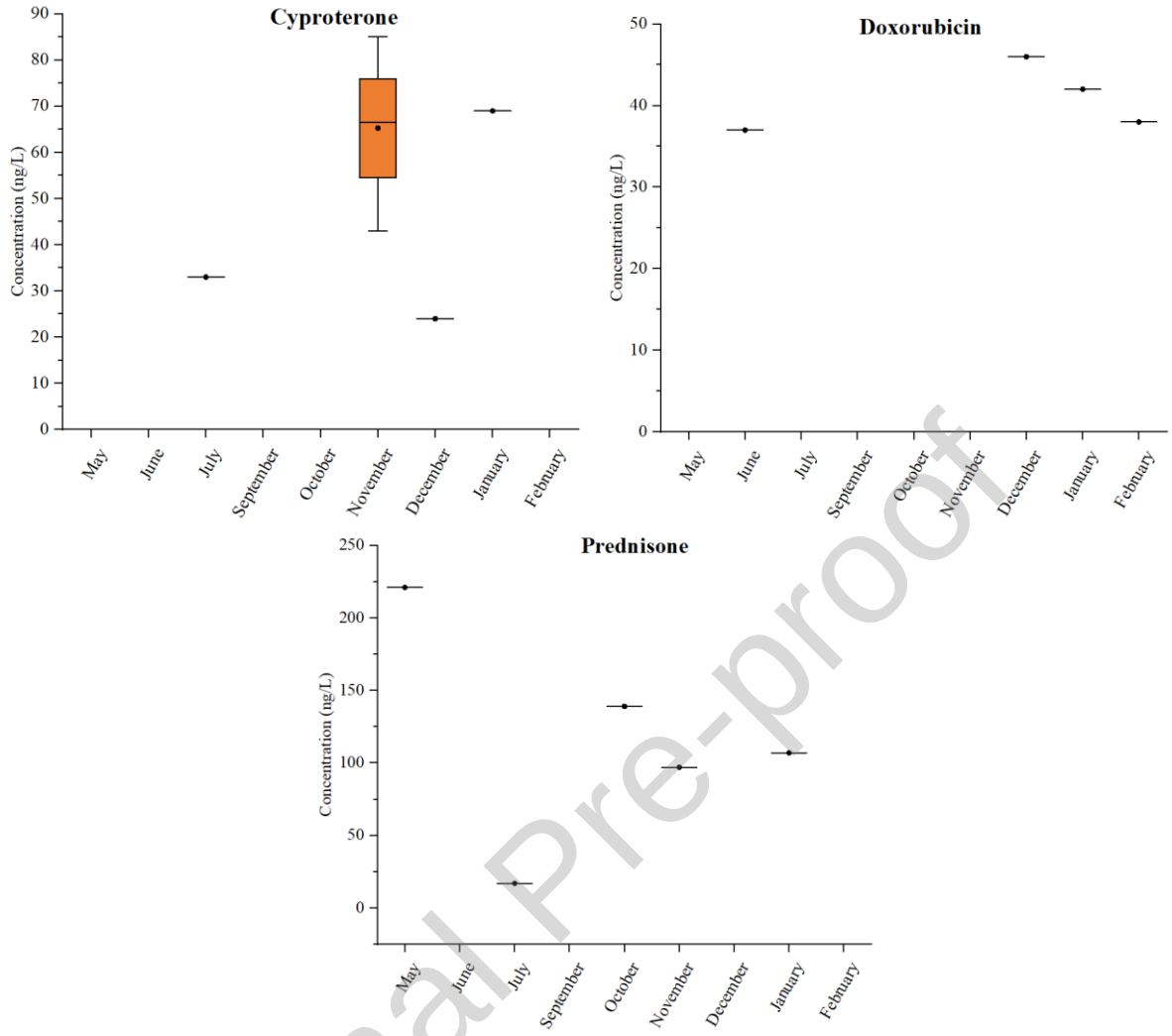

Figure 4. Box and Whisker diagrams with monthly representation of the concentrations found for cyproterone, doxorubicin and prednisone in AV hospital.

The cytostatics considered in **group C** (blue color in Figure 1) were detected at higher frequencies and include bicalutamide, mycophenolic acid, etoposide, megestrol and capecitabine. Bicalutamide was detected in all samples analyzed (120 samples from the AV hospital, 6 samples from the AG hospital and 3 sample from the ES hospital), with concentrations varying from 0.193±0.001 ng/L in June (AV2) to 94±8 ng/L in February (AV3) (Figure 5 and Table SI-5 in the Supplementary Information). Bicalutamide is used in the treatment of prostatic cancer and ca. 31% of the drug is excreted unchanged either by urine or feces, with a biological half-life of 5.9 days (Drugbank, 2021a). This long biological

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half-life may lead to a gradual release of bicalutamide into the sewer system, and consequently to its frequent detection. A very consistent administration of bicalutamide among the studied months also corroborates its high detection (being May, November and December the months with higher consumption of bicalutamide: > 150 g/month *versus* 85 g/month average in the remaining months). Nevertheless, the higher concentrations of bicalutamide were measured in May (51±1 ng/L), June (57±7 ng/L) and February (94±8 ng/L). The reason for the non-coincidence of the months of highest consumption records with the months of highest concentrations of bicalutamide may be related to the date and time of sampling. Up to the authors' best knowledge, there is only one study regarding bicalutamide's concentration in hospital effluents, with a maximum of 77 ng/L, in Japan, which is within the concentrations reported in this paper (Azuma *et al.*, 2016) – Figure 6.

Mycophenolic acid was detected in 96 of the 120 samples from AV hospital (80% frequency) and in 6 of the 9 samples from the AG and ES hospitals. This was the compound found at higher concentrations, the highest one being 5340±211 ng/L in the month of February in AV2 (Table SI-5 and Figure 5). Mycophenolic acid is an active metabolite of the prodrug mycophenolate mofetil, being synthetized by liver enzymes. The fact that mycophenolic acid was the compound detected at higher concentrations is expected, since mycophenolate mofetil is largely used in the prevention of tissue rejection following organ transplantation, and Portugal has registered one of the highest numbers of transplantation surgeries across Europe (Statista, 2021). Therefore, mycophenolic acid is detected at higher concentrations, despite of being mycophenolate mofetil the pharmaceutical mostly administered in the treatments, as stated before (average of 2 kg/month, with similar administration patterns between months). As stated before, 87% of the administered dose of mycophenolate mofetil was found to be excreted in the urine as an inactive form of mycophenolic acid in the first days after administration (Drugbank, 2021d). Differences in the concentrations found can

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rely in different water consumption records and place of excretion of this compound by the patients.

Etoposide was also among the most frequently detected cytostatics, being found in 60% of the samples analyzed (78 out of 129, all of them from AV hospital - Figure 1). The higher concentration of etoposide was detected in the month of May in AV3 (142±15 ng/L) (Figure 5 and Table SI-5). Etoposide is used in the treatment of several cancers (e.g., lung cancer, ovarian cancer, leukemia, etc.) and its consumption in AV hospital was of around 1 g/month. Although it was very similar between the studied months, May, October and February had the highest administration amount, reaching about 2 g. About 56% and 44% of etoposide is excreted by the patients through urine and feces, respectively (FDA, 2010). Since the February and October samplings occurred in the middle of the month and full excretion can take up to 120 hours, the effects of the higher consumption in these months could not be fully confirmed. Among the reported information in the literature, there are five papers reporting the occurrence of etoposide in hospital effluents: two of them did not detect it (from Slovenia and Spain); the others reported concentrations between 42 ng/L and 714 ng/L in Spain and China, which is within the range of the present findings – Figure 6 (Ferrando-Climent *et al.*, 2013; 2014; Isidori *et al.*, 2016; Negreira *et al.*, 2014; Yin *et al.*, 2010). It is important to remember that etoposide was already classified by IARC as carcinogenic for humans (Group 1) and its presence in hospital effluents can represent a threat to humans and the overall environment.

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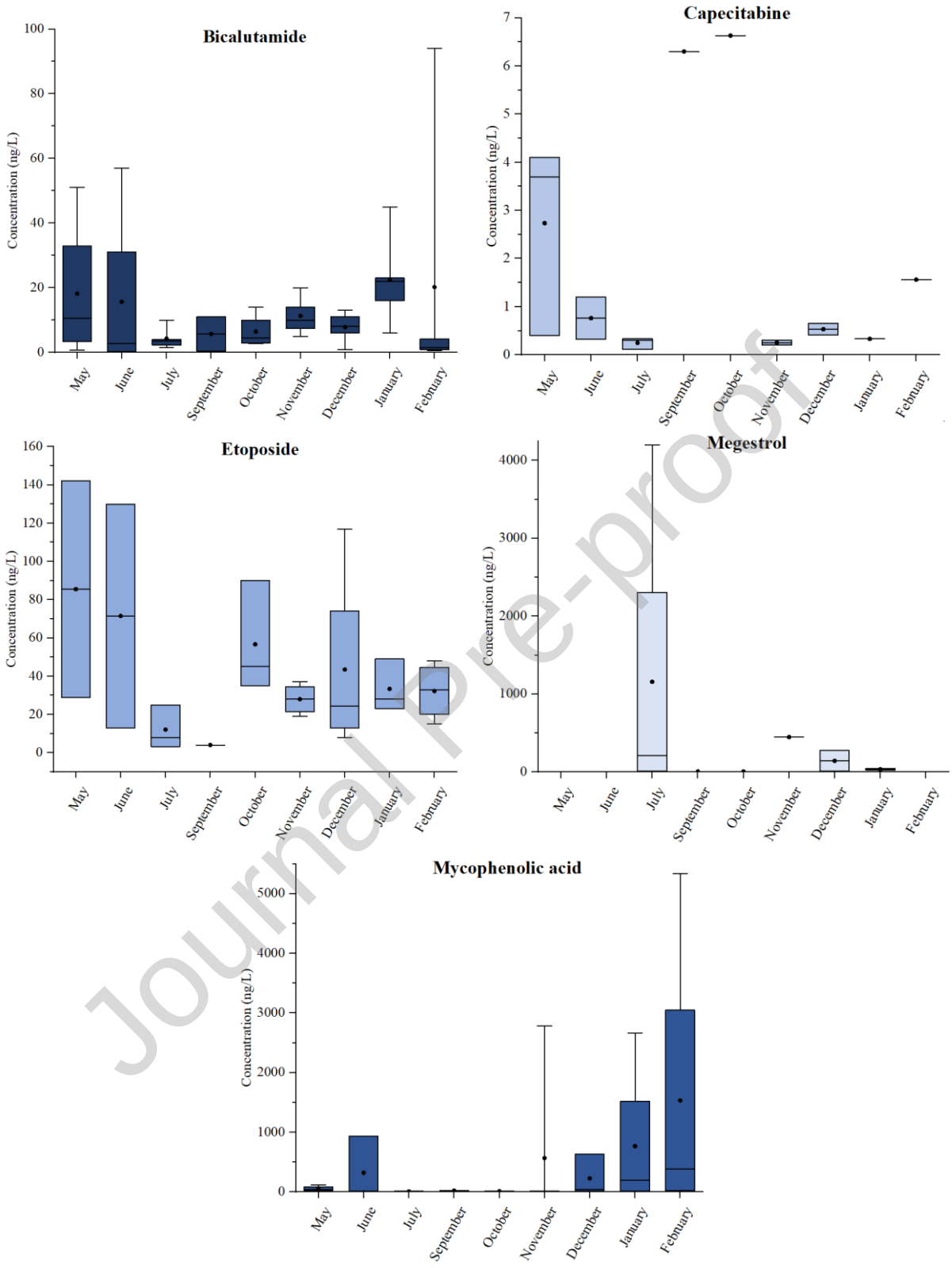

Figure 5. Box and Whisker diagrams with monthly representation of the concentrations found for bicalutamide, capecitabine, etoposide, megestrol and mycophenolic acid in AV hospital.

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Following etoposide, capecitabine was detected in 40% of the analyzed samples (48 out of the 120 in the AV hospital, and 3 samples from the AG hospital). The highest concentration of capecitabine was found in October in AV2 (6.63±0.03 ng/L) – Figure 5 and Table SI-5 in the Supplementary Information. All the samples analyzed showed the presence of capecitabine in at least one month, with the exceptions of AG2, in the AG hospital and ES, which were only sampled in July. Regarding the consumption data, an average of 617 g was administered per month, October, December and February being the months with the highest consumption (679-735 g/month). However, the administration rates did not show a high variation between months, being difficult to notice its differences in the results reported, especially considering such low concentrations. Although capecitabine is a frequently administered cytostatic, essentially used in the treatment of breast and colon cancers, this medicine is administered orally and usually to outpatients (National Library of Medicine, 2021a). Although about 96% of capecitabine is excreted within one hour after administration, only 3% of the administered dose is excreted unmetabolized (Drugbank, 2021b). This justifies its high frequency of detection at low concentrations, in the present study. Capecitabine was monitored in three studies regarding hospital effluents, being not detected in one of them, and detected in concentrations up to 490 ng/L in Spain, much higher concentrations than those found in this work - Figure 6 (Azuma *et al.*, 2016; Gómez-Canela *et al.*, 2014; Negreira *et al.*, 2014).

Megestrol was detected in 28% of the total samples (36 samples from the AV hospital), and it was not found neither in AG nor ES hospitals (Figure 1). Megestrol was the second compound detected at higher concentrations, being found at a maximum of 4200±704 ng/L in July (AV1) – Figure 5 and Table SI-5 in the Supplementary Information. This compound was detected in highly variable concentrations, starting from 5.2±0.4 ng/L in October (AV2). When consumption data is analyzed, it can be confirmed that its administration is variable,

ranging from 40 g in November to 124 g in September. This clearly demonstrates there are punctual administrations of this compound in the AV hospital. Megestrol is used in breast and endometrial cancers, loss of appetite, muscle wasting, and weight loss associated with cancer and/or AIDS (Chemocare, 2021). Its biological half-life elimination by the patients lasts about 1-3.5 days and excretion ranges from 83.1% and 94.7% (Medsafe, 2019). The high excretion rates corroborate the high detection of the drug in the wastewater samples. Considering the highest concentration found in July as a punctual exceptional administration of this compound, the remaining samples showed an average concentration of 114±175 ng/L, which also accentuates the variability of the concentrations detected (which may be related to the biological half-life and the fact of the drug being administered to in- or outpatients). Megestrol was only monitored and detected once in hospital effluents, in concentrations up to 1260 ng/L in Spain (Gómez-Canela *et al.*, 2014) – Figure 6.

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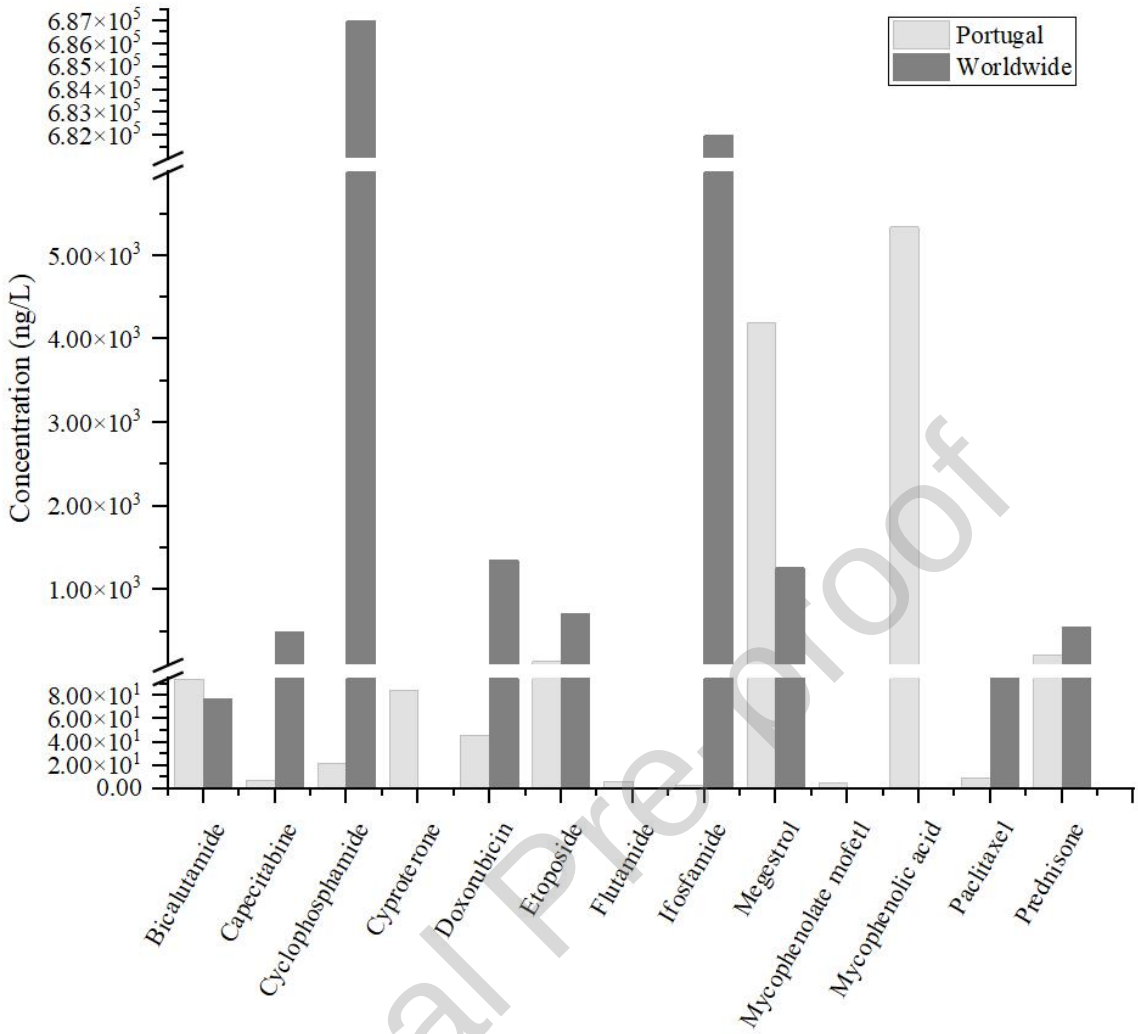

Figure 6. Comparison between the concentrations of pharmaceuticals (ng/L) detected in this work with those reported in the literature.

Regarding the temporal trends (Figures 2 and 6), a high variability within locations and between months can be observed. Several points were considered for sampling throughout the hospital's sewage system and, for confidential reasons, the access to the design/plant of the network was restricted. This information is crucial to analyse the sampling points' interdependencies and to estimate the flow rate in the system. In any case, the high dependence of water consumption with the time would not allow to accurately determine the flow rate of the wastewater in the system, at the specific time (and location) of the sampling, every single month. Such high variability in the concentrations and the absence of a clear

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temporal trend is likely attributed to the type of sampling (spot samples), but it is also a consequence of the high number of at play, including not only the consumption of each cytostatic, but also the consumption of water, the pharmacodynamics and pharmacokinetics of each compound (pharmaceuticals with completely different biological half-lives and excretion rates), the number of inpatients/ outpatients, the stability of the pharmaceuticals in wastewater, among other factors. Looking at the concentrations found, doxorubicin is the cytostatic with a lower variation in its concentrations between months (10% variation) and megestrol is the compound with the highest variation (262%), with an average of 98% variation for all the compounds. Aiming to understand if the consumption of pharmaceuticals and the consumption of water over the 9-month period are determining factors for the high variability in the concentrations measured in hospital wastewaters and the absence of a clear temporal trend as it was hypothesized, the data related to the three variables was plotted along the time (sampling months) for each pharmaceutical. As an example, the plots for capecitabine and mycophenolate mofetil are presented in Figure SI-2 of the Supplementary Information (similar trends were observed for the remaining pharmaceuticals). As shown, it is difficult to establish a trend between the consumption of cytostatics and water with the concentrations of cytostatics measured in hospital wastewaters. In some cases, it was found that the highest measured concentration is associated to the lowest administration doses or the highest water consumption records. This suggests that these two parameters may not be the main driven factors for the high variability in the concentrations.Therefore, the present findings highlight the importance of conducting long-time sampling schemes (preferentially comprising composite samples instead of grab ones) over predicting cytostatics load into water system based on consumption records and other theoretical data.

3.3. Predicted concentration in surface waters and determination of the risk quotient

As stated in section 2.7, it was estimated the concentration of all the target pharmaceuticals in receiving bodies/surface waters from the measured concentrations in the hospitals' wastewaters, assuming a dilution factor of 4 from hospital effluents to the urban WWTPs and 10 from the WWTP to surface waters, and no degradation in the WWTPs (worst-case scenario). Thus, maximum concentrations varying from 0.075 ng/L (ifosfamide) to 134 ng/L (mycophenolic acid) were predicted. Comparing these findings with the concentrations of cytostatics in surface waters, predicted from the number of medicine units consumed by patient under chemotherapy, both in specialized hospitals and pharmacies from the Center Portugal (the same geographical region under analysis in the present work) between 2007 and 2015, it can be concluded that the predicted concentrations are of the same order of magnitude (ranging between 0.02 ng/L for flutamide and 155.81 ng/L for mycophenolate mofetil) (Santos *et al.*, 2017).When comparing these values with measured concentrations of cytostatic drugs in surface waters worldwide, it can be seen that predicted concentrations in this study are of the same order (or even lower) than the ones actually measured (Franquet-Griell *et al.*, 2017; Gouveia *et al.*, 2022; Valcárcel *et al.*, 2011).

When the risk quotient was predicted, flutamide, megestrol and mycophenolic acid showed possibly a low risk to aquatic life; cyclophosphamide showed a potential moderate risk to aquatic organisms and for doxorubicin and etoposide a high risk was estimated, with a risk quotient higher than 1 (these results are represented in Table SI-3 in the Supplementary Information). The risk quotient was also determined for the above-mentioned compounds, using a 10-fold dilution factor from hospital wastewaters to urban WWTPs. In this case, no risk would be expected for flutamide and megestrol, but the same conclusions are obtained for the remaining drugs.

It is important to highlight that although the worst-case scenario was assumed, there are some compounds that may be metabolized, degraded or even diluted during the sewage passage, which would lead to lower predicted concentrations in surface waters. However, it is known

that most of these pharmaceuticals are excreted at patients' homes (NHS, 2020), meaning that the contribution of the hospitals sewage to the overall cytostatic load in the water system is lower than domestics' sewage. This thus mean that the real risks posed to aquatic organisms might be even underestimated, under the conditions assumed in this study. Indeed, it was found that the risks estimated for aquatic organisms in this study are generally lower than in our previous work (Santos *et al.*, 2017), even when the same toxicological data is used in the risk determination and considering the same geographical region (Portugal – Center region). It is noteworthy that only the contribution of one main hospital (plus two secondary ones) from the Center region in Portugal was considered as source of cytostatics' contamination in the present work, whereas the data related to all cytostatics' medicines prescribed/consumed under chemotherapy in the region (both in hospitals and pharmacies) were used in our previous estimation (Santos *et al.*, 2017). Furthermore, it should be kept in mind that the period of analysis is different, and this parameter should not be ruled out since the increased incidence of cancer among population demands a higher prescription of cytostatics' medicines, which contributes to increased cytostatics' loads in water system (both in amount and type of cytostatics due to the medical advances). Another factor that is important to take into consideration in the risk analysis is the geographical region since the cytostatics consumption pattern is highly region-dependent (González Peña *et al.*, 2021; Santos *et al.*, 2017). Our research team has previously estimated the risks for aquatic organisms based on concentrations of cytostatics measured in WWTP's effluents from the Northern Portugal and different results from the present study (Center Portugal) were attained (high risk versus low risk for mycophenolic acid in this study). Nevertheless, the same risk (high risk) was predicted for mycophenolic acid in different studies for the same geographical region – northern Portugal (Gouveia *et al.*, 2020; Santos *et al.*, 2017).

In any case, cyclophosphamide and etoposide, which are suspected to induce risk in aquatic organisms, according to the present findings, were already identified as carcinogenic

compounds by the International Agency for Research on Cancer (IARC, 2021b) and, therefore, the ALARA (As Low As Reasonably Achievable) principle remains the best standard to reduce the risks from exposure to these drugs. Moreover, long-term and synergetic effects should not be neglected, highlighting the need for more efficient removal and/or degradation strategies to be applied not only in urban WWTPs but also in hospitals and oncology centers.

4. Conclusions

Wastewaters from three Portuguese hospitals were collected to analyze the presence of thirteen pharmaceuticals. AV hospital' effluents were monthly collected in five sampling points for 9 months, between May 2019 and February 2020. Two local hospitals belonging to the same hospital centre (AG and ES) were included in this survey in the month of July. Pharmaceuticals were extracted and quantified by SPE and LC-MS/MS, respectively. The methodology was successfully validated, being achieved a good linearity in the range of 1-250 µg/L (2-500 ng/L in the final effluent) with high correlation coefficients (> 0.998), with good intra- and inter-day precisions (up to 9% for cyproterone and up to 15% for flutamide, respectively) and good recoveries (83±23 % average). The MDL values obtained were relatively low, varying from 0.08 ng/L for flutamide to 1.85 ng/L for cyproterone. The global uncertainty of the method has an average of 14% for concentrations above 30 ng/L, with the exception of paclitaxel, for which the global uncertainty stabilizes at around 22%.

The presence of cytostatics in hospital effluents was confirmed, being all the target compounds detected in at least one sample. Bicalutamide was the cytostatic detected with highest frequency, being present in all samples analyzed, followed by mycophenolic acid (79% of frequency of detection). Mycophenolic acid was the cytostatic detected at higher concentrations (up to 5340±211 ng/L), followed by megestrol (up to 4200±704 ng/L).

Bicalutamide, capecitabine and etoposide are also among the most frequently detected cytostatics, with a maximum concentration of 142±15 ng/L found for etoposide. It is highly important to emphasize that this compound is classified as carcinogenic by the IARC and its presence in wastewater effluents should not be ignored.

Other important conclusions from this work are related to the high variability of concentrations found between months (up to 262% variation), and the fact that it was not possible to design a trend between the consumption data and the concentrations found of each compound; this highlights the importance of long-time sampling campaigns instead of punctual samplings.

The risk these cytostatics are potentially posing to aquatic organisms was estimated by predicting pharmaceuticals' concentrations in surface waters. It was verified that flutamide, megestrol and mycophenolic acid showed possibly a low risk to aquatic life; cyclophosphamide showed a potential moderate risk to aquatic organisms; and for doxorubicin and etoposide a high risk was estimated. However, the fact that no risk was achieved for the other cytostatics does not mean the risks associated to long-term and synergic exposure should be disregarded.

Author Contributions:

Sample collection, A.C.A.S.; Conceptualization, T.I.A.G., A.C.A.S. and M.S.F.S.; methodology, T.I.A.G.; validation of the method, T.I.A.G.; formal analysis, T.I.A.G., A.C.A.S. and M.S.F.S.; resources, M.S.F.S., A.A., A.M.T.S. and M.G.F.; data curation, T.I.A.G.; writing—original draft preparation, T.I.A.G.; writing—review and editing, T.I.A.G., A.C.A.S., M.G.F., M.S.F.S., A.M.T.S. and A.A.; visualization, T.I.A.G. and A.C.A.S.; supervision, M.S.F.S. and A.A.; project administration, M.S.F.S., A.A., A.C.A.S.

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and M.G.F.; funding acquisition, M.S.F.S., A.A. and M.G.F. All authors have read and agreed to the published version of the manuscript.

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CRediT authorship contribution statement

Sample collection, A.C.A.S.; Conceptualization, T.I.A.G., A.C.A.S. and M.S.F.S.; methodology, T.I.A.G.; validation of the method, T.I.A.G.; formal analysis, T.I.A.G., A.C.A.S. and M.S.F.S.; resources, M.S.F.S., A.A., A.M.T.S. and M.G.F.; data curation, T.I.A.G.; writing—original draft preparation, T.I.A.G.; writing—review and editing, T.I.A.G., A.C.A.S., M.G.F., M.S.F.S., A.M.T.S. and A.A.; visualization, T.I.A.G. and A.C.A.S.; supervision, M.S.F.S. and A.A.; project administration, M.S.F.S., A.A., A.C.A.S. and M.G.F.; funding acquisition, M.S.F.S., A.A. and M.G.F. All authors have read and agreed to the published version of the manuscript.

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.

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