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

Teresa I.A. Gouveia[1,2], Adrián M.T. Silva[2,3], Mara G. Freire[4], Ana C.A. Sousa[5]*, Arminda Alves[1,2], Mónica S.F. Santos[1,2]*

[1] LEPABE—Laboratory for Process Engineering, Environment, Biotechnology and Energy, Faculty of Engineering, University of Porto, R. Dr. Roberto Frias, 4200-465 Porto, Portugal

[2] ALiCE - Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

[3] LSRE-LCM - Laboratory of Separation and Reaction Engineering – Laboratory of Catalysis and Materials, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465, Porto, Portugal

[4] CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[5] Comprehensive Health Research Centre (CHRC) and Department of Biology, School of Science and Technology, University of Évora, 7006-554 Évora, Portugal.

* Corresponding authors: mssantos@fe.up.pt; Tel.: +351-2-2508-4854 and acsousa@uevora.pt; Tel.: +351-266- 760881

SI-A. Sampling scheme

Table SI-1. Sampling scheme of the hospital wastewaters.

	Sampling site	Code
May 2019	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV5
June 2019	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV5
July 2019	Águeda Hospital	AG1
	Águeda Hospital	AG2
	Estarreja Hospital	ES
	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV4
	Aveiro Hospital	AV5

	Sampling site	Code
September 2019	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
October 2019	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV5
November 2019	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV4
	Aveiro Hospital	AV5
December 2019	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV4
	Aveiro Hospital	AV5
January 2020	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV4
	Aveiro Hospital	AV5
February 2020	Aveiro Hospital	AV1
	Aveiro Hospital	AV2
	Aveiro Hospital	AV3
	Aveiro Hospital	AV4
	Aveiro Hospital	AV5

Table SI-2. Consumption data of pharmaceuticals provided by the hospitals.

AVEIRO (AV)	**May-19**	**Jun-19**	**Jul-19**	**Sep-19**	**Oct-19**	**Nov-19**	**Dec-19**	**Jan-20**	**Feb-20**	**AVERAGE (g)**
Bicalutamide (g)	161	72	84	81	86	150	156	94	99	109
Capecitabine (g)	545	569	661	449	679	568	714	630	735	617
Cyclophosphamide (g)	30	24	31	20	29	28	22	40	47	30
Cyproterone (g)	113	60	91	63	80	95	75	78	102	84
Doxorubicin (g)	1	1	2	1	2	2	2	2	3	2
Etoposide (g)	2	0.6	1	1	2	0.2	0.4	0.9	2	1
Megestrol (g)	104	69	76	124	63	40	78	56	92	78
Mycophenolate mofetil (g)	1,618	1,798	2,506	2,375	2,485	1,260	2,690	2,580	2,413	2,192
Paclitaxel (g)	1	2	3	1	2	0.9	2	3	6	2
ESTARREJA (ES)	**May-19**	**Jun-19**	**Jul-19**	**Sep-19**	**Oct-19**	**Nov-19**	**Dec-19**	**Jan-20**	**Feb-20**	**AVERAGE (g)**
Bicalutamide (g)			0.4							0.4
Megestrol (g)			2							2

Note: Ifosfamide and flutamide were not administered in the studied hospitals; information regarding prednisone administration was not controlled by the Aveiro hospital due to its prescription in several treatments; mycophenolic acid consumption' was not provided.

SI-B. Validation of the method

- Recovery assays were performed using different hospital wastewaters spiked at a concentration level of 0.1 µg/L of each target pharmaceutical. The spiked and non-spiked hospital wastewaters were processed and extracted by SPE, being the two internal standards added before the evaporation step (section 2.4 of the manuscript). Afterwards, the final extracts of both spiked and non-spiked samples were analysed by LC-MS/MS (section 2.5. of the manuscript). The difference between the mass of each pharmaceutical in the SPE extracts from the spiked hospital wastewater (*Ms*) and non-spiked hospital wastewater (*M0*) was then compared to the mass of pharmaceutical added (*Ss*). This *Ss* was determined by preparing a standard in acetonitrile (the final solvent of SPE extracts), with the same spike used to fortify the hospital wastewater plus the same spike of internal standards added to the SPE extracts, and by direct injecting it in the LC-MS/MS. Recoveries were then calculated according to Equation 1:

$$\%R = \frac{(Ms - M0)}{Ss} \times 100 \qquad \text{Eq. 1}$$

  where *Ms* is the mass of pharmaceuticals in the extract from the spiked wastewater, *M0* the mass of pharmaceuticals in the extract from the original wastewater (without spike) and *Ss* the mass of pharmaceuticals in the spike.
- Intra-day precision was obtained from the coefficient of variation of six consecutive injections of two standard solutions (7.5 µg/L and 50 µg/L).
- Inter-day precision was determined by measuring the analytical response of the same two analytical standards in six different days.

SI-C. Estimation of the Global Uncertainty Associated to the Analytical Results

The approach proposed by the International Organization for Standardization and adopted by EURACHEM-CITAC Guide was applied to estimate the global uncertainty (U) associated to the results (Ellison and Williams, 2012). Four sources of uncertainty were considered: (i) U1: the one associated with the preparation of standards (estimated using the error propagation law for the different dilution steps from the stock standard solution); (ii) U2: the one associated with the calibration curve (calculated for the different concentration levels of the standards);

(iii) U3: the one associated with the precision of the chromatographic method (estimated as the average result of relative standard deviation for the different concentration levels of intermediate precision assays); and (iv) U4: the uncertainty associated with the accuracy (calculated as the average percent recovery obtained within all the experiments). Global uncertainty is expressed as:

$$U = \sqrt{U1^2 + U2^2 + U3^2 + U4^2} \quad \text{(B1)}$$

where:

U1 is the uncertainty associated with the preparation of standards (estimated using the error propagation law for the different dilution steps from the stock standard solution):

$$U1 = \sqrt{\sum_i \left(\frac{\Delta mi}{mi}\right)} \quad \text{(B2)}$$

Δmi—error associated with the measurement equipment; *mi*—measured value.

U2 is the uncertainty associated with the calibration curve (calculated for the different mass levels of the standards):

$$U2 = \frac{sx_0}{x_0} = \frac{1}{x_0}\frac{s_{y/x}}{a}\sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{a^2 \sum_i (x_i - \bar{x})^2}} = \frac{1}{x_0}\frac{1}{a}\sqrt{\frac{\sum_i (y_i - y_0)^2}{n-2}} \quad \text{(B3)}$$

sx0—standard deviation of the concentration; *x0*—mass; *a*—slope of the calibration curve (y = ax + b); m—number of replicates performed; *n*—number of standards to build the calibration curve; *y0*—*y* values calculated by the calibration curve from x values; $\bar{y}$—average of yi (experimental) values of the calibration curve standards; *xi*—concentration of each standard used in the calibration curve; $\bar{x}$—average of xi values; *yi*—experimental values of the calibration curve standards.

U3 is the uncertainty associated with the precision of the chromatographic method (estimated as the average result of relative standard deviation for the different concentration levels of intermediate precision assays):

$$U_3 = \frac{s}{\bar{y}\sqrt{n}} \quad \text{(B4)}$$

s—standard deviation of experimental y values in precision assays; $\bar{y}$—average of experimental y values in precision assays; *n*—number of precision assays.

U4 is the uncertainty associated with the accuracy (calculated as the average percent recovery obtained within all the experiments):

$$U_4 = \frac{s(\eta)}{\sqrt{n}} \quad \text{(B5)}$$

s(η)—relative standard deviation of the average percent recovery; *n*—number of accuracy assays.

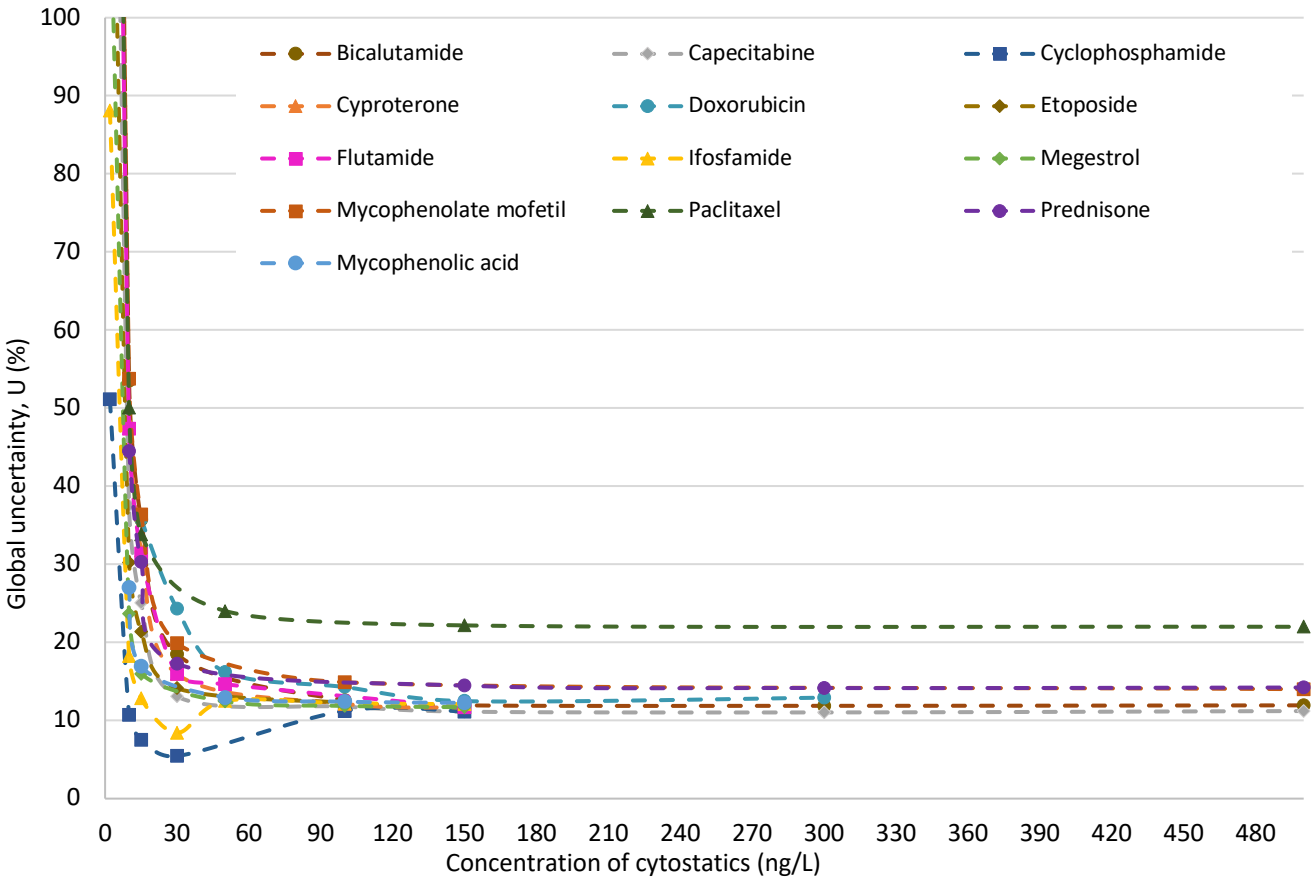

Figure SI-1. Global uncertainty of the analytical methodology for the identification and quantification of the 13 pharmaceuticals in hospital wastewaters by SPE-LC–MS/MS.

SI-D. Predicted concentrations in surface Waters, toxicological data and risk quotients obtained for the studied pharmaceuticals

The predicted no-effect concentrations (PNECs) were defined as the lowest values obtained from the quotient between the toxicity values found for each cytostatic and an assessment factor (AF). Depending on the toxicological dose descriptor (e.g., EC_{50}, LC_{50}, NOEC, and LOEC), the nature of the toxicity value (i.e., acute or chronic toxicity), and the number of known trophic toxicological levels, the following AF values were assumed: AF=1000, when at least one short-term toxicological value was available for each trophic level; AF=100, when at least one long-term result was available for one trophic level; AF=50, when two long-term results from species representing two trophic levels were available; AF=10, when long-term results from at least three species representing three trophic levels existed. Whenever available, long-term toxicity values were used instead of short-term values, even though short-term values may result in lower PNECs. In the absence of experimental toxicity data, ECOSAR models were used for PNEC calculations. The analysis of the risk quotient was estimated according to the following indicators: $RQ \geq 1$ indicates high risk, $0.1 \leq RQ < 1$ suggests moderate risk, $0.01 \leq RQ < 0.1$ indicates low risk, and $RQ < 0.01$ denotes no risk to aquatic biota.

Table SI-3. Ecotoxicological information, calculated PNECs, PECs and risk quotient for the studied pharmaceuticals.

	Ecotoxicity data, AF	**Organism**	**PNEC (ng/L)**	**PEC SW (ng/L)**	**Risk quotient**	**Bibliography**
Bicalutamide	NOEC 21 days, 100	*D. magna*	5600	2.4	<0.01	Santa-Cruz-Biotechnology (2010)
Capecitabine	NOAEC 24 h, 50	*D. magna*	45	0.2	<0.01	Parrella *et al.* (2015)
Cyclophosphamide	NOEC 168 h, 10	*D. rerio*	1.3	0.6	0.42	Li *et al.* (2022)
Cyproterone	EC50 48 h, 1000	*D. magna*	2400	2.1	<0.01	Franquet-Griell *et al.* (2015)
Doxorubicin	NOEC 24 h, 10	*D. magna*	0.2	1.2	5.75	Parrella *et al.* (2015)
Etoposide	NOAEC 24 h, 10	*C. dubia*	1	3.6	3.55	Parrella *et al.* (2015)
Flutamide	NOEC 96 h, 10	*Rotifer*	10	0.2	0.02	Preston *et al.* (2000)
Ifosfamide	NOEC 7 days, 50	*C. dubia*	317000	0.1	<0.01	Russo *et al.* (2018)
Megestrol	LC50 48 h, 1000	*D. magna*	5000	105	0.02	FDA (1996)
Mycophenolate mofetil	NOEC 72 h, 50	*Algae*	2000	0.1	<0.01	GPL (2022)
Mycophenolic acid	NOAEL, 70 h, 100	*D. rerio*	1600	134	0.08	Jiang *et al.* (2016)
Paclitaxel	LC50 48 h, 1000	*D. magna*	740	0.2	<0.01	FDA-CDER (1996)
Prednisone	NOEC	*Daphnid*	248000	5.5	<0.01	ECOSAR estimation

PNEC- Predicted no effect concentration; PEC SW- predicted environmental concentration in surface waters; AF- assessment factor.

SI-E. Literature review: method detection limits, recoveries and concentrations for the studied pharmaceuticals in hospital effluents.

Table SI-4. Literature review: MDLs, recoveries and concentrations for the studied pharmaceuticals in hospital effluents.

Pharmaceutical	Analytical method		Concentration(ng/L)	Reference
	MDL (ng/L)	Recovery (%)	Hospital Effluent	
Bicalutamide			<MDL-77 (average 22)	Azuma *et al.* (2016)
Capecitabine	15.0	92±3	<MDL-490	Gómez-Canela *et al.* (2014)
	5.0	88-119	<MDL	Negreira *et al.* (2014)
	0.7		<MDL-112	Isidori *et al.* (2016)
			11	Azuma *et al.* (2016)
Cyclophosphamide	6.0	30	146	Steger-Hartmann *et al.* (1996)
	6.0	86	19-4486	Steger-Hartmann *et al.* (1997)
			<2-21	Thomas *et al.* (2007)
		115	<100	Busetti *et al.* (2009)
	2.0		100	Yin *et al.* (2010)
	3.1	56-113	<MDL-5730	Gómez-Canela *et al.* (2012)
	9.0-110.0	103-134	<MDL-161±26	Kovalova *et al.* (2012)
	1.1	88±13	<MDL-200.7	Ferrando-Climent *et al.* (2013)
	1.1		3089-14826	Ferrando-Climent *et al.* (2014)
	4.4	79±3	<MDL-4720	Gómez-Canela *et al.* (2014)
	3.0	99-102	<MDL-100	Negreira *et al.* (2014)
	0.78	98.58-99.42	<14-22000	Česen *et al.* (2015)
	2.3		<MDL-22900	Isidori *et al.* (2016)
	1000		<MDL	Al Qarni *et al.* (2016)
			384	Azuma et al. (2016)
			687000	Hamon *et al.* (2018)
	300		<MDL-29100	Oliveira Klein *et al.* (2021)
Cyproterone	4.1	92±2	<MDL	Gómez-Canela et al. (2014)

Pharmaceutical	Analytical method		Concentration (ng/L)	Reference
	MDL (ng/L)	Recovery (%)	Hospital Effluent	
Doxorubicin	50.0	79.8-89.9	100-500	Mahnik *et al.* (2006)
	60.0		<260-1350	Mahnik *et al.* (2007)
	10.0		<MDL	Yin *et al.* (2010)
	54.0	100±11	<MDL	Gómez-Canela *et al*. (2014)
	2.5	71-76	<MDL	Negreira *et al*. (2014)
	0.8		<MDL	Isidori *et al.* (2016)
Etoposide	5.0		42	Yin *et al.* (2010)
	24.0	47±4	<MDL-406	Ferrando-Climent *et al*. (2013)
	24.0		<80-714	Ferrando-Climent *et al*. (2014)
	12.0-20.0	81-106	<MDL	Negreira *et al*. (2014)
	20.0		<65	Isidori *et al*. (2016)
Ifosfamide	7.0	39	24	Steger-Hartmann *et al.* (1996)
	6.0		<MDL-1914	Kümmerer *et al.* (1997)
			<2-338	Thomas *et al.* (2007)
		86-97	<100	Busetti *et al*. (2009)
	2.0		151	Yin *et al.* (2010)
	25	101-152	<MDL-895±293	Kovalova *et al*. (2012)
	1.7	83±7	<MDL-227.9	Ferrando-Climent *et al.* (2013)
	1.7		<MDL	Ferrando-Climent *et al.* (2014)
	5.7	76±10	<MDL-86200	Gómez-Canela *et al.* (2014)
	2.0	92-107	<MDL-19.4	Negreira et al. (2014)
	2.8	92-94	48–6800	Česen *et al*. (2015)
	4.8		<MDL-58	Isidori *et al.* (2016)
			6820000	Hamon *et al.* (2018)

Pharmaceutical	Analytical method		Concentration (ng/L)	Reference
	MDL (ng/L)	Recovery (%)	Hospital Effluent	
Megestrol	3.5	81±4	<MDL-1260	Gómez-Canela *et al.* (2014)
Paclitaxel	5.5	59±8	<MDL-99.7	Ferrando-Climent *et al.* (2013)
	5.5		<18-100	Ferrando-Climent *et al*. (2014)
	4.4	101-113	<MDL	Negreira *et al*. (2014)
	1.3		<MDL	Isidori *et al*. (2016)
Prednisone	20.0	80-90	117-545	Schriks *et al.* (2010)
	12.0	59±4	<MDL-210	Gómez-Canela *et al.* (2014)

MDL – Method detection limit

SI-E. Concentrations measured for each pharmaceutical in hospital effluents

Table SI-5. Concentrations, in ng/L, found for each pharmaceutical studied (bicalutamide, capecitabine, cyclophosphamide, cyproterone, doxorubicin, etoposide, flutamide, ifosfamide, megestrol, mycophenolate mofetil, acid mycophenolic paclitaxel and prednisone) in all the samples analysed.

Cyclophosphamide	**Capecitabine**	**Bicalutamide**	**Frequency of detection**
27/120 (21%)	51/129 (40%)	129/129 (100%)	
		51±1	May
	< 0.37	5±2	Jun.
8±2		1.5±0.2	Jul.
	6.3±0.1	11±2	Sep.
		2.8±0.4	Oct.
15±2		10±5	Nov.
		11±4	Dec.
	< 0.37	22±2	Jan.
		1.5±0.4	Feb.
	0.4±0.1	6±1	May
		< 0.33	Jun.
4.6±0.5		4±1	Jul.
		0.5±0.1	Sep.
	6.63±0.03	6±2	Oct.
	< 0.37	5±1	Nov.
		0.9±0.4	Dec.
		23±2	Jan.
		0.5±0.1	Feb.
	3.7±0.5	15±3	May
	1.2±0.6	57±7	Jun.
	< 0.37	3.5±0.3	Jul.
		0.4±0.1	Sep.
4±1		14±4	Oct.
	< 0.37	20±5	Nov.
	0.41±0.1	13±3	Dec.
		45±7	Jan.
		94±8	Feb.
	< 0.37	2.3±0.1	Jul.
		7.4±0.5	Nov.
22±3		6±1	Dec.
		16±3	Jan.
		4.1±0.3	Feb.
	4.1±0.2	0.74±0.02	May
		0.4±0.2	Jun.
	< 0.37	10±1	Jul.
		3±1	Oct.
		14±3	Nov.
	0.65±0.03	8±1	Dec.
		6±4	Jan.
	1.56±0.3	0.9±0.5	Feb.
< 2.33		0.64±0.03	Jul.
< 2.33	2.3±0.1	< 0.33	Jul.
< 2.33		0.5±0.1	Jul.

AV1
AV2
AV3
AV4
AV5
AG1
AG2
ES

Ifosfamide	Flutamide	Etoposide	Doxorubicin	Cyproterone		
6/129 (5%)	34/129 (26%)	77/129 (60%)	12/129 (9%)	25/129 (19%)	**Frequency of detection**	
					May	**AV1**
		13±1			Jun.	
		25±1			Jul.	
					Sep.	
	2.9±0.1				Oct.	
		37±5		67±11	Nov.	
		18±3			Dec.	
		23±3			Jan.	
			38±2		Feb.	
	2.8±0.1	29±2			May	**AV2**
					Jun.	
		8±2		33±3	Jul.	
					Sep.	
	6.2±0.3	35±4			Oct.	
	3.5±0.4	32±2			Nov.	
		31±5	46±14		Dec.	
		28±1		69±6	Jan.	
		15±4			Feb.	
		142±15			May	**AV3**
	6±1	130±45	37±1		Jun.	
		3.2±0.1			Jul.	
		4.0±0.3			Sep.	
2.1±0.2		45±10			Oct.	
	3.3±0.2	19±1		66±16	Nov.	
		117±7			Dec.	
3.0±0.1	4.0±0.1	49±11			Jan.	
		25±3			Feb.	
					Jul.	**AV4**
	4.9±0.4			43±1	Nov.	
	3.6±0.4				Dec.	
					Jan.	
		41±19			Feb.	
					May	**AV5**
					Jun.	
		8±2			Jul.	
	4±1	90±6			Oct.	
		24±1		85±23	Nov.	
		8±2		24±10	Dec.	
			42±4		Jan.	
		48±10			Feb.	
					Jul.	**AG1**
	2.07±0.04				Jul.	**AG2**
				62±7	Jul.	**ES**

Mycophenolic acid	Mycophenolate mofetil	Megestrol		
102/129 (79%)	34/129 (26%)	36/129 (28%)	**Frequency of detection**	
37±8	3±2		May	
16±1			Jun.	
13±1		4200±704	Jul.	
22±1			Sep.	
			Oct.	**AV1**
15±3	0.6±0.1	449±92	Nov.	
41±4		278±60	Dec.	
376±151		46±16	Jan.	
16±3			Feb.	
12±1	0.5±0.1		May	
16±1			Jun.	
8±1		410±20	Jul.	
		5.7±0.3	Sep.	
11±1		5.2±0.4	Oct.	**AV2**
10.7±0.5	1.4±0.1		Nov.	
12±1			Dec.	
10±1		24±2	Jan.	
5340±211	0.54±0.03		Feb.	
116.4±0.2			May	
932±151	3.14±0.01		Jun.	
6±1	4.4±0.2	8±3	Jul.	
		6±1	Sep.	
8±1			Oct.	**AV3**
2787±212	5±1		Nov.	
630±48		9±2	Dec.	
2665±241	3±1		Jan.	
752±208	2±1		Feb.	
	0.62±0.03	13±1	Jul.	
10.0±0.3			Nov.	
			Dec.	**AV4**
			Jan.	
22±4			Feb.	
41±1			May	
			Jun.	
7.2±0.3			Jul.	
14±1			Oct.	**AV5**
9.5±1.7			Nov.	
			Dec.	
11±2			Jan.	
291±13			Feb.	
18±2			Jul.	**AG1**
			Jul.	**AG2**
12.7±0.3			Jul.	**ES**

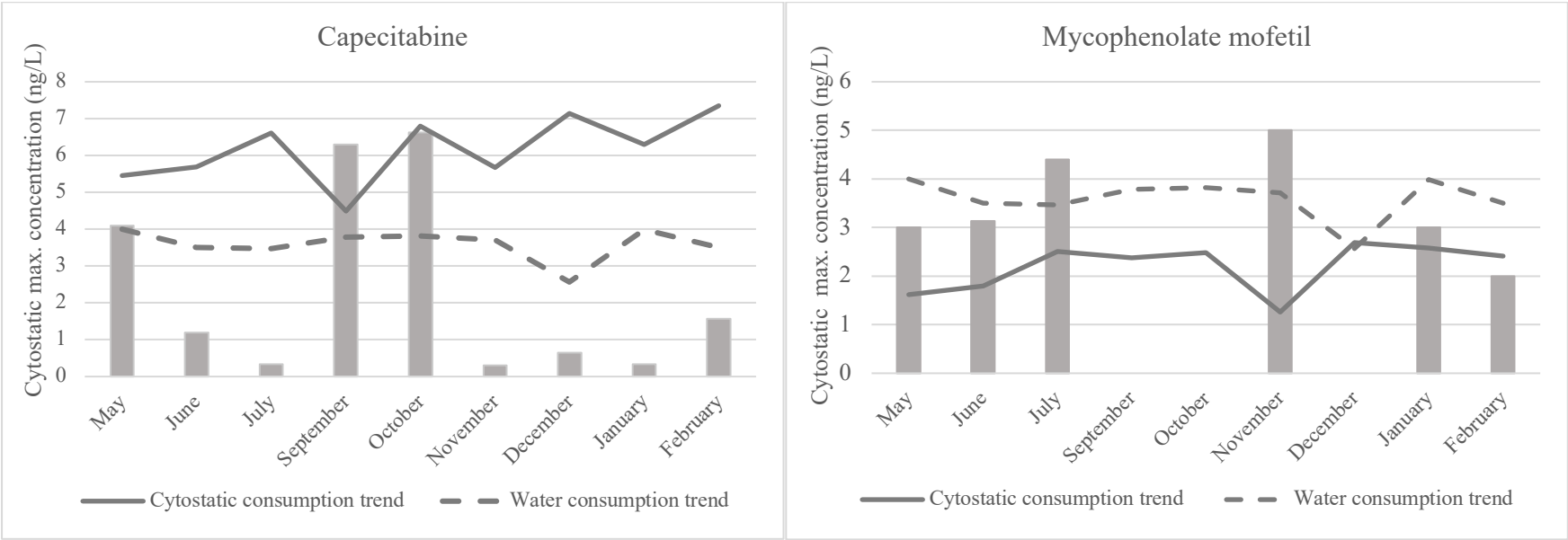

Figure SI-2. Relation between the consumption trends and the maximum concentrations of capecitabine and mycophenolate mofetil measured in hospital wastewaters.

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