Treatment of anticancer drugs in hospital and wastewater effluents using nanofiltration

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Abstract

Anticancer drugs are currently widely used for the treatment of cancer and have been detected in hospital effluents, wastewater treatment plant effluents and river water samples in concentrations up to the $\mu g.L^{-1}$ range. Within the next two decades, the annual number of cancer cases is expected to rise, which will lead to an increase in the consumption of anticancer drugs. Therefore, the development of effective treatment options for their removal from wastewater is crucial to avoid the release of these emerging contaminants in the aquatic environment. The aim

of this study was to assess the viability of nanofiltration for remediation, using as benchmark two representative membranes (Desal 5DK and NF270) to remove four widely consumed anticancer drugs (paclitaxel, etoposide, cyclophosphamide and ifosfamide) from different matrices (laboratory grade water, synthetic urine and real secondary effluent). The filtration of synthetic urine spiked with the target compounds was tested to explore the possibility of the treatment of source-separated urine in hospitals whereas real secondary effluent collected at a wastewater treatment plant was tested to explore the possibility of adding nanofiltration as tertiary treatment to efficiently remove the target contaminants and avoid their release into the environment. Experimental results showed that the Desal 5DK membrane is more effective than the NF270 membrane for the rejection of these compounds. It presented average rejections higher than 89% for all the target anticancer drugs spiked in synthetic urine and real secondary effluent, showing no significant matrix influence on the rejection results. *Daphnia magna* toxicity tests showed that the immobilization effect observed in the permeate samples was lower than the feed samples. The concentrated retentate samples may be toxic to freshwater crustaceans and should therefore be subject to further treatment.

1 Introduction

Pharmaceuticals were first detected in surface waters during the 1970s and since then, there has been a worldwide growing concern about their presence in the aquatic environment (Ebele et al., 2017). Most of the pharmaceutical compounds are incompletely assimilated and metabolized by the human body and are thus excreted via urine and feces and released into wastewater treatment plants (WWTPs). Generally, since many pharmaceuticals present low removal efficiencies in conventional WWTPs, these emerging contaminants are discharged into the aquatic environment (Roberts et al., 2006; Verlicchi et al., 2010 ; Jelic et al., 2011).

Many pharmaceutical groups have been detected in the aquatic environment including anticancer drugs, antidepressants, antibiotics, hormones, antiepileptics, β blockers, analgesics and anti-inflammatories (Tiwari et al., 2017), being anticancer drugs one of the most concerning

contaminants in our water system (Kümmerer, 2001; Rowney et al., 2009). Anticancer drugs, also named antineoplastic drugs, are a group of pharmaceuticals used in the treatment of cancer, which are designed to disrupt or prevent cellular proliferation usually by interaction with DNA function and cell signaling (Novak et al., 2017). Although antineoplastic drugs are designed to kill rapidly growing cells such as those found in cancer tumours, since many of these drugs present lack of selectivity (Chari, 2007), in addition to the tumour cells they can attack healthy cells and cause cytotoxic, genotoxic, mutagenic as well as teratogenic effects, leading to adverse effects in any eukaryotic living organism (Kümmerer et al., 2000; Johnson et al., 2008). Anticancer drugs are thus considered of great environmental concern in terms of their potential risk to healthy individuals, in particular to children, pregnant women and elderly people (Rowney et al., 2009).

A considerable number of studies have focused on the occurrence and fate of anticancer drugs in the environment. The occurrence of anticancer drugs in hospital effluents has been reported in concentrations up to hundreds of micrograms per liter (Steger-Hartmann et al., 1996; Mahnik et al., 2007; Yin et al., 2010; Gómez-Canela et al., 2014). Since hospital effluents are usually discharged into the WWTP without any preliminary treatment, hospitals are one of the input sources of these drugs in the environment (Verlicchi et al., 2010). Furthermore, nowadays, many anticancer drugs are consumed by out-patients rather than hospitalized patients and consequently, these drugs are also directly discharged in the WWTPs (Weissbrodt et al., 2009; Besse et al., 2012). Several anticancer drugs have been detected in wastewater effluents as well as in river water samples. Among these drugs, cyclophosphamide and ifosfamide are two of the most reported antineoplastic agents found in wastewater effluent and river water samples (Steger-Hartmann et al., 1997; Ternes, 1998; Metcalfe et al., 2003; Zuccato et al., 2005; Buerge et al., 2006; Negreira et al., 2013). The detection of anticancer drugs in wastewater effluents and surface waters indicates that these drugs are incompletely removed by conventional WWTPs. Buerge et al. (2006) reported the occurrence of cyclophosphamide and ifosfamide in the effluents of three WWTPs located in Switzerland at concentrations up to 10 $ng.L^{-1}$ and found that those concentrations were similar to those in the influent. This study also detected the

presence of both compounds in the river Limmat, downstream from the Zurich WWTP effluent discharge, cyclophosphamide up to 0.17 $ng.L^{-1}$ and ifosfamide up to 0.14 $ng.L^{-1}$. Cyclophosphamide and tamoxifen were also found in effluent from a WWTP located in Girona (Spain) and in the receiving river at concentrations up to 25 $ng.L^{-1}$ and 42 $ng.L^{-1}$, respectively (Ferrando-Climent et al., 2014). In the same way, Martín et al. (2011) studied the occurrence of numerous widely used anticancer drugs and six of the studied drugs (cytarabine, doxorubicin, etoposide, gemcitabine, ifosfamide and vinorelbine) were found at concentrations levels up to 14 $ng.L^{-1}$ in influent and effluent of a traditional activated sludge WWTP, showing an insignificant degradation during the wastewater treatment.

Within the next two decades, the annual cancer cases are expected to rise to 22 million (Ferlay et al., 2013), which means that the consumption of anticancer drugs and their consequent release will drastically increase. Since most anticancer drugs have limited biological degradability, the development of an effective treatment option as an alternative to the conventional methods is crucial to avoid the release of these drugs in the aquatic environment (Kosjek and Heath, 2011).

During the last two decades, the interest in the use of nanofiltration (NF) for water treatment has widely increased due to the complete or nearly complete removal of organic micropollutants as well as the ability to integrate it with other systems (Taheran et al., 2016). Several nanofiltration studies have been focused on the different mechanisms and efficiencies involved in the removal of pharmaceuticals (e.g., Nghiem et al., 2005; Yoon et al., 2006) but only few were focused on the removal of anticancer drugs (e.g., Wang *et al.*, 2009).

The rejection of organic solutes by nanofiltration membranes is strongly dependent on solute physico-chemical properties, membrane properties, operating conditions and feed water characteristics (e.g., organic matter, ions content) (Bellona et al., 2004; Verliefde et al., 2009). Indeed, several studies have reported the influence of natural organic matter (NOM) on the rejection of organic contaminants; however, most of these studies were carried out in synthetic model waters (Hu et al., 2007; Zazouli et al., 2009; Lin et al., 2014) and few were performed in real natural water matrices (Van der Bruggen et al., 2001; Comerton et al., 2008; Sanches et al.,

2011; Azaïs et al., 2016; Garcia-Ivars et al., 2017). Moreover, conflicting results about the influence of organic matter were reported. For example, Kimura et al. (2009) found that the rejection of six pharmaceuticals by an NF membrane was enhanced in the presence of NOM. On the other hand, Bellona et al. (2010) investigated the removal of eight non-ionic trace organic contaminants using different NF membranes and found that the rejection of some of these organic compounds was negatively influenced by the presence of NOM.

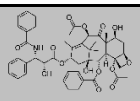
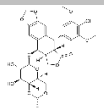
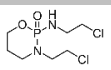
The aim of this study was to evaluate and compare the effectiveness of two NF membranes to remove four widely used anticancer drugs with diverse chemical structures spiked in matrices with very different compositions (laboratory grade water, synthetic urine and real secondary effluent from a wastewater treatment plant). The filtration of synthetic urine spiked with the target compounds was tested because a large proportion of pharmaceuticals is excreted via urine (Dodd et al., 2008) and the treatment of source-separated urine in hospitals could prove to be an attractive alternative to minimize the release of these compounds into the WWTPs. Real secondary effluent collected at a WWTP was also tested to explore the possibility of adding NF as tertiary treatment, to efficiently remove the target contaminants and avoid their release into the environment.

2 Materials and Methods

2.1 Anticancer drugs selection and properties

Based on the consumption data of the Portuguese Oncology Institute Francisco Gentil (Lisbon) and reported occurrence levels in different wastewaters (e.g., Martín et al., 2011; Ferrando-Climent et al., 2014), four anticancer drugs were selected as target drugs for the present study: paclitaxel, etoposide, cyclophosphamide and ifosfamide. The selected anticancer drugs have different chemical structures as well as molecular weight (ranging from 261.1 to 853.9 Da) and thus different physico-chemical properties (Table 1).

120 Table 1. Main physico-chemical properties and structures of the selected anticancer drugs

Compound	Therapeutic group[a]	Chemical structure	Molecular weight (Da)	Log Kow[b]	pK_a
Paclitaxel	Plant alkaloid		853.93	3	10.36
Etoposide	Plant alkaloid		588.57	0.6	9.33
Cyclophosphamide	Alkylating agent		261.09	0.8	2.3 11.1
Ifosfamide	Alkylating agent		261.09	0.86	2.5 9.01

121 [a]Classified by the World Health Organization (WHO) - https://www.whocc.no/atc_ddd_index/

122 [b]Drug bank database - https://www.drugbank.ca/

123

124 As described in Table 1, paclitaxel is the most hydrophobic compound. Etoposide and
125 paclitaxel have a much higher molecular weight than cyclophosphamide and ifosfamide.

126 Chemical standards for paclitaxel, etoposide, cyclophosphamide and ifosfamide were
127 purchased as solids of the highest purity grade commercially available (≥98%, Sigma Aldrich).
128 Since these compounds are highly toxic, their handling requires strict safety precautions in order
129 to guarantee the protection of research workers as described by Negreira et al (2013).

130 Stock solutions of each anticancer drug were prepared in methanol (1.2-9.4 $g.L^{-1}$) and
131 stored at -20°C.

132

133 2.2 Different matrix selection and characterization

134 Laboratory grade water, synthetic urine and real secondary effluent were used as matrices
135 in the nanofiltration experiments.

The laboratory grade water was produced by a MilliQ water system (Millipore, CA, USA; Resistivity: 18.2 MΩ at 25 °C; TOC < 5μg.L^{-1}; bacteria < 1 CFU.mL^{-1}; particulates < 1 particulate larger than 0.22 μm.mL^{-1}).

Synthetic urine was prepared by dissolving 3.8 g.L^{-1} potassium chloride, 8.5 g.L^{-1} sodium chloride, 24.5 g.L^{-1} urea, 1.03 g.L^{-1} citric acid, 0.34 g.L^{-1} ascorbic acid, 1.18 g.L^{-1} potassium phosphate, 1.4 g.L^{-1} creatinine, 0.64 g.L^{-1} sodium hydroxide, 0.47 g.L^{-1} sodium bicarbonate and 0.28 mL sulfuric acid in laboratory grade water (CDC, 2010). All reagents used were of the highest purity grade.

The real secondary effluent was supplied by a wastewater treatment utility located in Lisbon, Portugal. The water matrix was collected after the biological treatment and prior to the final disinfection step. The matrix was stored in glass bottles, transported to the laboratory and kept at 4 °C. Table 2 represents the main characteristics of the wastewater effluent collected in the month of January and used in the NF experiments. The target anticancer drugs were not detected in the real secondary effluent samples collected.

Table 2. Characterization of the real secondary effluent used in the nanofiltration experiments

Physico-chemical properties	**Secondary effluent**
Nitrogen (ammonia) (mg.L^{-1} NH_4)	17
Biochemical oxygen demand (mg.L^{-1} O_2)	10
Chemical oxygen demand (mg.L^{-1} O_2)	50
pH (20 °C)	7.1
Total suspended solids (mg.L^{-1})	8

2.3 Nanofiltration experimental assays

Nanofiltration experiments were carried out in a laboratory scale stainless steel dead-end stirred cell (Membrane Extraction Technology Ltd., UK) with an effective membrane area of 54 cm^2.

Two different nanofiltration membranes were used to test the removal of the target anticancer drugs: Desal 5 DK (GE Osmonics, USA) and NF270 (DOW FILMTEC, USA). These membranes are thin composite membranes with a polysulphone support layer and are negatively charged at neutral pH. The Desal 5DK membrane has a molecular weight cut off of 150-300 Da, whereas NF270 has a molecular weight cut off of 300 Da (information provided by the manufacturers).

Before use, the nanofiltration membranes were cleaned with laboratory grade water to remove any impurities left over from the manufacturing process or preservatives. Prior to each experiment, laboratory grade water was filtered at 10 bar until a constant flux was achieved. After 3 hours of compaction, the flux was measured at different pressures and the membrane permeance was determined from the slope of flux against pressure. The permeate volume was continuously measured using a KERN PFB balance connected to a computer. The mean clean water permeance values ($L.\ h^{-1}.\ m^{-2}.bar^{-1}$) determined for the Desal 5DK and NF270 were 2.8 ± 0.3 and 11.05 ± 0.5, respectively.

For each type of membrane, two different assays were performed (with a new piece of membrane in each assay) in laboratory grade water, synthetic urine and real secondary effluent to test the reproducibility of the proposed process.

Before each experiment, 250 mL of fresh feed solution was prepared and the anticancer drugs were spiked at a concentration of 500 $\mu g.L^{-1}$ in the different matrices. Anticancer drugs have been detected in hospital wastewater effluents at lower levels (up to 124 $ug.L^{-1}$) (Mahnik et al., 2007). The higher spiked concentration in this study (500 $\mu g.L^{-1}$) was chosen based on the expected rejections and taking into account the detection limits of the analytical method by direct injection. Furthermore, Wang et al. (2009) reported that the percent rejection of cyclophosphamide was independent of the spiked concentration (in the range of 200-600 $\mu g.L^{-1}$) (Wang et al., 2009).

The fresh solutions were fed to the cell and stirred during 30 minutes at 300 rpm to promote the contact of the target compounds with the membrane. Then, pressure was applied in the feed solution by compressed high purity nitrogen gas and the experiments were conducted

until 200 mL of permeate was collected. All experiments with laboratory grade water and real secondary effluent were performed at a constant pressure of 10 bar whereas synthetic urine experiments were carried out at 15 bar due to the higher osmotic pressure.

According to equation 1, the osmotic pressure difference ($\Delta\pi$) between the retentate and the permeate side of the membrane can have a significant impact in the permeate flux profile since it may cause a decrease in the driving force.

$$J_V = L_p(\Delta P - \Delta\pi) \quad (1)$$

where L_p is the membrane permeance and ΔP is the transmembrane pressure. Hence, the impact of the partial retention of low molecular weight molecules, mainly dissolved salts, on the decrease of permeate flux was evaluated for the synthetic urine experiments as well as for the secondary effluent experiments. The osmotic pressure difference between the retentate and the permeate side was calculated by using the Van´t Hoff equation (equation 2):

$$\Delta\pi = RT(C_{M,r} - C_{M,p}) \quad (2)$$

where R is the gas constant (0.082 atm.L.mol^{-1}.K^{-1}), T is the absolute temperature, $C_{M,r}$ is the molar concentration of the solute in the retentate stream and $C_{M,p}$ is the molar concentration of the solute in the permeate side. Regarding the synthetic urine experiments, the osmotic pressure was calculated considering the dissolved salts and urea since they are present in higher concentrations when compared to the other compounds and have a lower molecular weight, making them more relevant in terms of osmotic pressure, due to their high molar concentration. As for the secondary effluent experiments, the concentration of reference ions was used to estimate the osmotic pressure difference.

208 The osmotic pressure was estimated throughout the nanofiltration experiments by using
209 a mass balance to determine the concentration of the low molecular weight compounds in the
210 retentate and permeate side of the membrane during time. It was assumed that the rejections of
211 these compounds were constant during the experiments (the rejection values used are
212 represented later in Figure 3) and that there was no significant concentration polarization of
213 these compounds. The equations used in the mass balance are described in the Supporting
214 Information section.

215 With the aim of analyzing if there was any degradation of the target compounds in the
216 feed solution during the filtration experiments, 50 mL of fresh solution was placed in a glass
217 bottle and used as a control. The control was kept during the nanofiltration experiment under the
218 same conditions as the feed solution in the Met cell, with no light exposure and at room
219 temperature. The initial and the final concentration of the target drugs in the control were
220 analyzed and no significant differences in the concentrations were detected (lower than 13%),
221 showing that there was no degradation of the target drugs during the nanofiltration experiments.

222 After each nanofiltration experiment, samples from the feed, permeate and retentate
223 were stored at -20 °C to avoid the degradation of target compounds before their analysis. All the
224 samples obtained from the filtration experiments using the secondary effluent were filtered with
225 0.2 µm filters (GE Healthcare, UK Limited) to avoid the damage of the LC-MS/MS equipment
226 due to the particulate matter. The filter retention of the different target compounds was analyzed
227 and varied between 0 and 4%.

228 The apparent rejection for each target drug was calculated using equation 3:

229

$$Rejection\ (\%) = \left(1 - \frac{C_p}{C_f}\right) \times 100 \qquad (3)$$

230 where C_p and C_f are the concentration of a target drug in the permeate and feed,
231 respectively.

232 Furthermore, the mass of the target drugs adsorbed on the membrane was determined by
233 a mass balance using equation 4:
234

$$Adsorption\ (\mu g) = C_f \times V_f - C_p \times V_p - C_r \times V_r \quad (4)$$

235 where C_p, C_f and C_r are the concentration of the target drug in the permeate, feed and
236 retentate, respectively. V_p, V_r and V_f are the volume of permeate, retentate and feed,
237 respectively.
238

239 ## 2.4 Analytical Methods

240 Solid phase extraction (SPE) was performed in the permeate samples obtained from the
241 filtration experiments using the real secondary effluent. The SPE procedure was conducted
242 using Oasis hydrophilic-lipophilic balance (HLB) cartridges (3cc; 60mg) placed on an SPE
243 Extraction Manifold with 20 positions (Waters, Milford, MA). The SPE cartridges were
244 sequentially preconditioned with 6 mL of methanol and 6mL of Milli-Q water at a flux of
245 approximately 5mL.min^{-1}. Each permeate sample (140 mL) was loaded onto a cartridge at
246 approximately 1 mL.min^{-1} after which the cartridges were rinsed with 1 mL of Milli-Q water
247 and then dried with a stream of nitrogen for 15 min or until cartridges were visibly dry. Next,
248 the cartridges were eluted with 6mL of methanol. The resulting extract was dried with a gentle
249 stream of nitrogen until complete evaporation and reconstituted with 200µL of methanol.
250 All the other feed, permeate and retentate samples were analysed by direct injection in
251 the LC-MS/MS system.
252 The selected anticancer drugs were quantified by LC-MS/MS analysis using a Waters
253 Alliance HPLC system (Waters 2695 separation module, Ireland) comprising a quaternary
254 pump, an on-line solvent degasser, auto sampler and column oven. An analytical method
255 proposed by Negreira et al. (2013) was modified to detect the target compounds. The separation
256 of antineoplastic agents was done on a reversed-phase column (Luna 5µm C18(2) 100A, 150 x

2.0 mm) at 35°C using an injection volume of 10 µL. The mobile phase consisted of 0.5% formic acid in Milli-Q water (A) and acetonitrile (B). A flow rate of 0.30 $mL.min^{-1}$ was used, and the gradient conditions applied consisted of a linear increase from 5% to 100% B in 7 min; 100% B was maintained for 3 minutes and a linear decrease to 5% B was conducted over the following 2 minutes and kept steady for 3 minutes.

Tandem mass spectrometry (MS/MS) detection was performed on a Micromass Quattro Micro triple quadrupole (Waters, Ireland) using an electrospray ionization (ESI) source operating at 120°C and applying a capillary voltage of 3.0 kV. High purity nitrogen (N_2) was used both as drying gas and as a nebulizing gas. Ultra-high-purity argon (Ar) was used as collision gas. The optimization of the MS/MS conditions and product ions monitored for each compound is presented in the Supporting Information section (Table SI). The data was acquired and processed using the MassLynx software (version 4.1).

Calcium, potassium, sodium, aluminum, chromium, iron and zinc were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES Ultima model, Horiba Jobin-Yvon, France). The characteristics of the equipment and operating conditions are specified in the Supporting Information section (Table SII).

The anions, including chloride, sulfate, nitrate and phosphate were determined by ion chromatography (IC, Dionex ICS3000) using a Thermo Ionpac AS9-HC column (250 x 4.0 mm). The mobile phase used was a solution with 8.0 mM sodium carbonate prepared in HPLC grade water. The flow rate of the mobile phase was 1.0 $mL.min^{-1}$. The temperature was set at 25°C and the injection volume was 10 µL. The reference ions and metals present in the real secondary effluent were analyzed prior to each nanofiltration experiment (Table 3).

Table 3. Average and standard deviation values of the reference ions and metals measured in the real secondary effluent samples prior to each nanofiltration experiment

Ions & Metals	Secondary effluent (mg.L^{-1})
Calcium	18.5 ± 1.45
Magnesium	11.9 ± 3.85
Potassium	1606.4 ± 595.12
Sodium	31.6 ± 12.2
Chloride	53.3 ± 5.55
Nitrate	21.0 ± 0.51
Sulfate	18.2 ± 1.02
Phosphate	nd*
Aluminium	nd*
Chromium	nd*
Iron	0.07± 0.003
Zinc	0.01± 0.0001

*nd- means not detected in the real secondary effluent samples

2.5 Toxicity

A crustacean toxicity test for freshwater (DaphToxKIT F magna, MicroBioTestsInc, Belgium), developed based on the European Standard EN ISO 6341(1996) and OECD 202 (2004) guidelines, was used to test the acute immobilization of *Daphnia magna* when exposed to different concentrations of a mixture of the cytotoxic drugs that mimic the concentrations in the feed, and the highest possible concentrations that could be expected in the membrane filtration assays, in the permeate and retentate samples.

Newly hatched neonates (less than 24 hours old) were therefore exposed to a mixture of the four anticancer drugs (cyclophosphamide, ifosfamide, etoposide and paclitaxel) spiked in real wastewater effluent at three different concentrations: 5, 500 and 5000 µg.L^{-1}. Five neonates per well and four replicates for each concentration (with a total of 20 neonates for each tested

297 concentration and controls) were used in the assay. The negative control was synthetic
298 freshwater (ISO medium) provided in the kit. Two additional controls were performed, the
299 unspiked matrix (wastewater without the compounds) and sterile distilled deionized water
300 (blank). For the blank test, only three replicates were performed and therefore 15 neonates were
301 treated. Total duration of the exposure was 48 hours at 20 ± 1 °C in the dark. The test endpoint
302 is the inhibition of mobility. At 24 hours and 48 hours, immobilized organisms were counted,
303 and the results expressed as the percentage of neonates immobilized.
304

305 # 3 Results and discussion

306 ## 3.1 Influence of different matrices on permeate flux

307 The effectiveness of nanofiltration to remove anticancer drugs was tested using two
308 nanofiltration membranes (Desal 5DK and NF270) and by spiking the target compounds in
309 different matrices. Figure 1 represents the variation of the normalized permeate flux (J_v/J_{v0}) for
310 each nanofiltration membrane during the filtration of laboratory grade water, synthetic urine and
311 real secondary effluent. The flux ratio decline was obtained by considering J_{v0} as the permeate
312 flux of fresh membrane, which was measured with laboratory grade water prior to each
313 experiment at the same pressure of the experiment. As plotted in Figure 1, some J_v/J_{v0} values
314 were slightly higher than 1 due to inherent analytical errors involved in the measurement of the
315 permeate volume.
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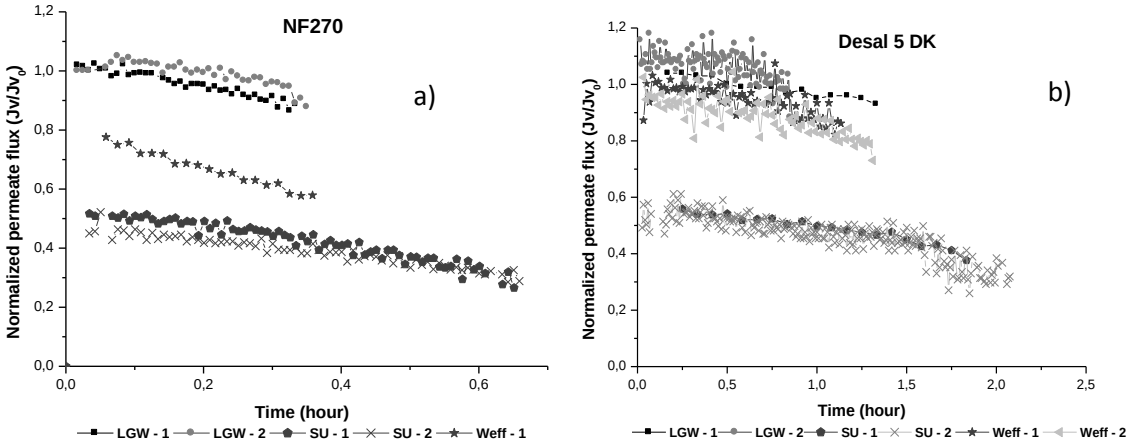


Figure 1 Normalized permeate flux (Jv/Jv0) during the filtration of the target anticancer drugs spiked in laboratory grade water (LGW), synthetic urine (SU) and real secondary effluent (Weff) using the membranes a) NF270 and b) Desal5DK

Comparing the J_v/J_{v0} results obtained for each experiment, it can be observed that the presence of anticancer drugs does not significantly affect the flux of the membranes since the laboratory grade water experiments appear to have a relatively mild flux decline and close to the pure water flux. The total flux decline was 10% and 5% for NF270 and Desal 5 DK, respectively.

On the other hand, the experiments performed with synthetic urine and real secondary effluent presented lower fluxes when compared to the laboratory grade water assays. Regarding the synthetic urine filtration experiments, there was an immediate flux decline of 50% and 45% for NF270 and Desal 5DK, respectively, which was much higher than the initial flux decline obtained with the real secondary effluent. The final flux decline in synthetic urine was 75% and 70% for NF270 and Desal 5DK, respectively. This permeate flux decline could be an indication of a high fouling, however, since synthetic urine presents a high content of dissolved salts and urea, and the nanofiltration processes partially reject dissolved salts as well as small organic molecules, the resulting osmotic pressure difference, which acts against the transport of water from the feed to the permeate side of the membrane, must also be considered.

Even though it was not as sharp as the synthetic urine flux decline, both membranes showed a total permeate flux decrease during the filtration of the real secondary effluent of 42%

and 20%, for NF270 and Desal 5DK, respectively. In the same way, the osmotic pressure was estimated using the concentration of reference ions.

The extent to which the osmotic pressure affected the nanofiltration performance was evaluated by estimating this parameter throughout the nanofiltration experiments of synthetic urine and real secondary effluent. For this, it was assumed that the rejection of ions was constant during the all process and subsequently, the molar concentration of the ions in the retentate and permeate was calculated over time (the rejection values used are represented in Figure 3). Figure 2 represents the permeance of the membranes calculated taking into account the osmotic pressure difference evolution.

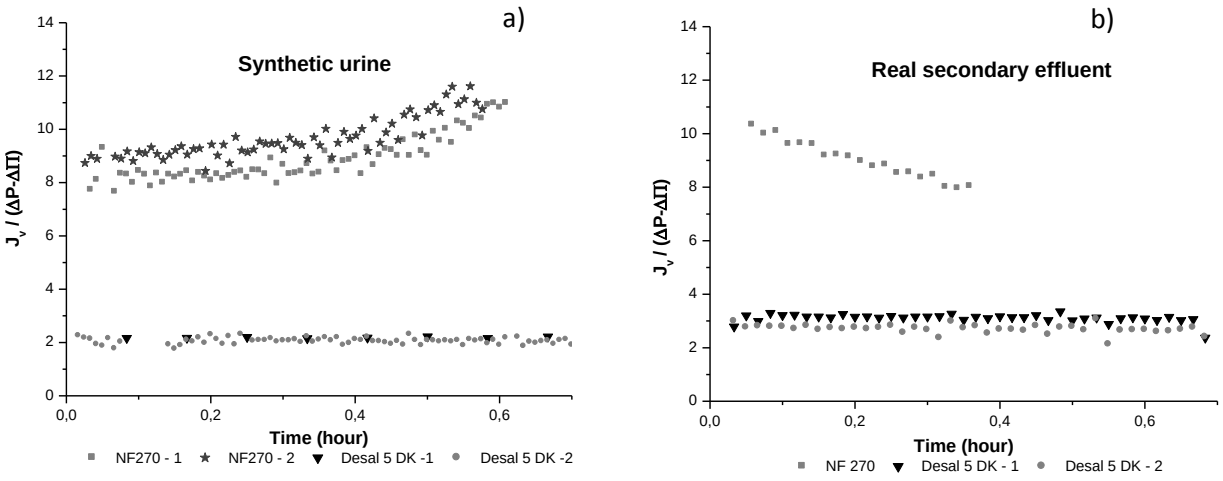


Figure 2 Membrane permeance considering the osmotic pressure difference ($\Delta\pi$) evolution during the nanofiltration experiment with a) synthetic urine and b) real secondary effluent using the membranes NF270 and Desal5DK

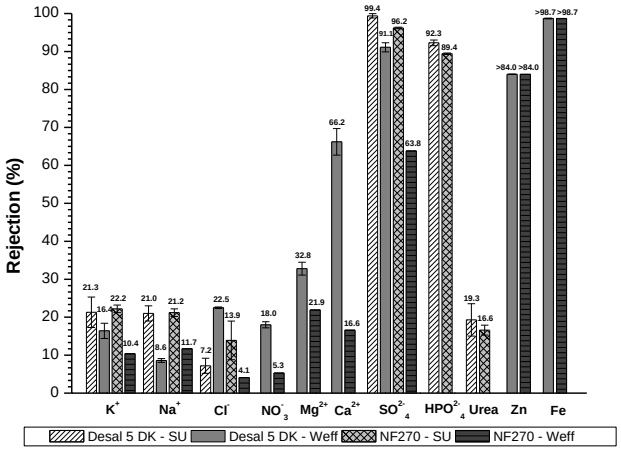
As can be observed in Figure 2a, in the case of all synthetic urine experiments, when the osmotic pressure difference was considered, the membrane permeance was constant throughout the experiment and quite similar to the pure water permeance, which suggests that there was no fouling of the membrane. As expected, the osmotic pressure difference between the retentate and the permeate side of the membrane increased during the course of these assays, achieving a

final estimated value of 9.90 and 10.3 for NF270 and Desal 5DK, respectively. This osmotic pressure increase leads to a severe decrease of the driving force (ΔP-$\Delta\pi$), which explains the significant decline of the flux in the synthetic urine experiments (shown in Figure 1).

Regarding the real secondary effluent experiments, the osmotic pressure difference was not significant, with a final estimated value of 0.6 and 0.9 for NF270 and Desal 5Dk, respectively. These values are relatively low, particularly when compared to the osmotic pressure determined for the synthetic urine experiments. Hence, the flux decline observed in the real secondary effluent for the NF270 membrane (Figure 1 and Figure 2b) can be attributed to the presence of natural organic matter which causes fouling of the membrane due to the organic matter retention and adsorption. On the other hand, Desal 5DK did not show a significant flux decline in the experiments with real secondary effluent. It has been reported in the literature that the susceptibility of membranes to fouling is dependent on different membrane properties (e.g., surface hydrophobicity, zeta potential and surface roughness) (Boussu et al., 2007) . With regard to the degree of hydrophobicity, similar values of contact angles measurements with pure water have been found in the literature for both membranes, such as 30° for NF270 (Mänttäri et al., 2006) and 31° for Desal 5DK (Tanninen et al., 2004). In the same way, similar zeta potential values at pH 7 have been reported for these membranes (-15 mV and -5 mV for NF270 and Desal 5DK, respectively) (Tanninen et al., 2004). Additionally, according to Norberg et al. (2007) NF270 and Desal 5DK present similar surface roughness values. Consequently, the higher flux decline of NF270 may be explained by the fact that this membrane has a larger MWCO and thus, is more susceptible to fouling.

3.2 Ions and urea rejection

The rejection of ions, metals and urea present in synthetic urine as well as in secondary effluent is represented in Figure 3.



404

405 Figure 3 Rejection of ions, metals and urea present in synthetic urine (SU) and real secondary effluent (Weff) using
406 different nanofiltration membranes (Desal 5 DK and NF270)[1]

407

408 As expected for nanofiltration membranes, the results present in Figure 3 show that the
409 rejection of monovalent ions (Na^+, K^+, Cl^- and NO_3^-) is lower than the rejection of multivalent
410 ions (Mg^{2+}, Ca^{2+}, SO^{2-}_4 and HPO^{2-}_4). The rejection of dissolved ions using a nanofiltration
411 membrane is strongly dependent on the membrane charge and thus, on the feed water chemistry
412 (Bellona et al., 2004). The membranes used in this study (NF270 and Desal 5 DK) are
413 negatively charged and therefore, the cations present in the different matrices (e.g., sodium,
414 potassium, magnesium, calcium) were attracted to the membrane whereas the anions (e.g.,
415 chloride, nitrate, sulphate and phosphate) were repelled by the membranes. Consequently, the
416 transport of the cations through the membrane is facilitated (cations with higher mobility are
417 more prone to pass through the membrane) and in order to preserve the electroneutrality
418 condition, the anions are also transported. However, as expected, the permeation of divalent
419 anions (sulphate and phosphate) was lower since they have a higher ionic charge and a higher
420 ionic radius. Furthermore, it was noticed that in general, the rejection of ions was higher in the
421 synthetic urine experiments than in the real secondary effluent assays.

422 As represented in Figure 3, the rejection of urea was low for both membranes (up to
423 19%), which was expected since this compound has a low molecular weight (60 Da) and is

[1] Zn and Fe were not detected in the Weff permeate. The rejection was calculated using the method detection limit (1ppb).

uncharged. Similar rejections of urea were obtained in other studies using nanofiltration membranes (Yoon et al., 2005; Pronk et al., 2006).

3.3 Removal of the selected anticancer drugs by NF membranes

As stated by Bellona et al. (2004), different parameters influence the rejection of organic compounds on NF/RO membranes, such as solute properties (e.g., molecular size, hydrophobicity), membrane properties (e.g., molecular weight cut-off, surface charge), feed composition (e.g., NOM, ionic strength, pH) and operating conditions (e.g., pressure, temperature). The rejection of a solute by a nanofiltration membrane can be due to several mechanisms, such as, size exclusion (sieving, steric effect), electrostatic interactions and hydrophobic interactions which can lead to the adsorption of the target compounds onto the membrane.

The four selected anticancer drugs present different structures as well as physico-chemical properties (e.g., molecular weight, hydrophobicity, pKa) which may lead to a different NF rejection performance. The rejection results obtained for the four anticancer drugs using Desal 5 DK and NF270 membranes for the three different matrices are represented in Figure 4.

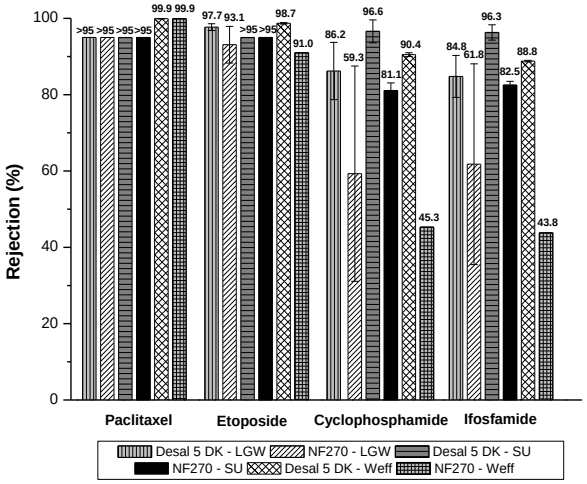


Figure 4 Rejection of the target anticancer drugs spiked in different matrices - Laboratory grade water (LGW), synthetic urine (SU) and real secondary effluent (Weff) using different nanofiltration membranes (Desal 5DK and NF270)[2,3]

As expected, the target drugs with the highest molecular weight, namely etoposide and paclitaxel, were more efficiently removed than cyclophosphamide and ifosfamide, with rejections higher than 90% for all the performed experiments. The concentration of etoposide in the permeate was lower than the direct injection detection limit in the filtration of synthetic urine whereas the concentration of paclitaxel was lower than the direct injection detection limit for all the conducted filtration experiments. Solid phase extraction was performed in the samples obtained from the filtration experiments using the real secondary effluent. Paclitaxel was detected in these permeates at a concentration of 35 ng.L^{-1} and 104 ng.L^{-1} , for Desal 5DK and NF270, respectively.

Since etoposide has a low log K_{ow} coefficient and is in its uncharged form at the experimental pH conditions, weak interactions with NF270 and Desal 5DK membranes are expected, and therefore, the rejection of this compound is mainly due to size exclusion. On the other hand, paclitaxel is uncharged at the experimental pH conditions but is hydrophobic, thus,

[2] Paclitaxel was not detected in the permeate of LGW and SU experiments. The minimum estimated rejection was calculated using the direct injection method detection limit (25 µg.L^{-1}). SPE was performed to quantify the permeate obtained in the Weff experiments. The concentration detected in the permeate was 35 ng.L^{-1} and 104 ng.L^{-1} for Desal 5DK and NF270, respectively.

[3] Etoposide was not detected in the permeate of SU experiments. The minimum estimated rejection was calculated using the direct injection method detection limit (25 µg.L^{-1}).

466 this drug can potentially adsorb onto the membrane surface. Indeed, in most of the experiments,
467 paclitaxel was the compound with the highest observed adsorption (Table 4).

468 Cyclophosphamide and ifosfamide are hydrophilic drugs and different pKa values are
469 reported in the literature for these two compounds. According to Mioduszewska *et al.* (2017),
470 cyclophosphamide is uncharged in the pH range from 4 to 10.5 while ifosfamide is neutral up to
471 a pH of 7. Consequently, in the filtration experiments performed at neutral pH, the rejection of
472 cyclophosphamide and ifosfamide was mainly governed by a steric hindrance mechanism.

473 As previously mentioned, for each type of membrane, two different experiments were
474 performed with laboratory grade water, synthetic urine and real secondary effluent, thus, the
475 error bars present in Figure 4 represent the average of two rejection values obtained in different
476 nanofiltration assays. In general, there was no significant variation of rejection in the duplicate
477 assays conducted for each target drug spiked in the different matrices. The exception was the
478 rejection of cyclophosphamide and ifosfamide obtained in the assay with the NF270 membrane
479 for the laboratory grade water matrix that was quite different between the two different assays,
480 ranging from 31% to 87% and from 36% to 88%, for cyclophosphamide and ifosfamide,
481 respectively. These significant variations may be explained because cyclophosphamide and
482 ifosfamide have a lower molecular weight (261.09) when compared to the other target drugs and
483 a molecular weight closer to the molecular weight cut off of the NF270 membrane (300 Da). In
484 fact, the rejections obtained for these two drugs in the assays performed with real effluent were
485 quite low and in the range of the rejections obtained in the laboratory grade water experiments,
486 being 45% for cyclophosphamide and 44% for ifosfamide. However, in the case of the synthetic
487 urine assays, the rejections obtained in the duplicate assays had no significant difference and
488 were slightly higher than those from the other matrices.

489 Regardless of the target anticancer drug, the Desal 5DK membrane presented higher and
490 more consistent rejections as well as no significant matrix influence on the rejection of the
491 contaminants. For example, the rejections obtained in laboratory grade water duplicate assays
492 ranged from 79% to 94% for cyclophosphamide and from 79% to 90% for ifosfamide, which
493 could be due to the use of different pieces of membrane as well as to the inherent analytical

494 method error. Consequently, when comparing these results with the rejections obtained in the
495 assays performed with synthetic urine and real secondary effluents (higher than 89%), it is
496 possible to conclude that the variation was lower; therefore, there was no significant matrix
497 effect.

498 Although the selected anticancer drugs presented different physico-chemical properties
499 and were spiked in very different matrices, Desal 5DK proved to be more effective than NF270
500 in removing these drugs.

501

502 Table 4. Rejection and adsorption values of the target anticancer drugs on the two membranes (Desal 5DK and NF270) in the
503 different nanofiltration experiments

		Laboratory grade water		**Synthetic urine**		**Real secondary effluent**	
		Desal 5 DK	**NF 270**	**Desal 5 DK**	**NF 270**	**Desal 5DK**	**NF270****
Paclitaxel	Rejection (%)	nd	nd	nd	nd	99.9 ±0.005	99.9
	Adsorption* (µg)	1.79±1.79 - 6.79±6.79	32.5±14.4 - 37.9±14.4	19.0±10.2 - 26.3±7.80	46.94±10.6 - 51.9±10.6	55.09±29.1	22.9
Etoposide	Rejection (%)	97.7 ± 0.9	93.1 ± 4. 8	nd	nd	98.7 ± 0.2	91.0
	Adsorption (µg)	32.4±10.7	0.47 ± 0.47	4.85±4.85 - 7.28±7.28	3.73±1.29 - 8.73±1.29	10.7±10.7	0
Cyclophosphamide	Rejection (%)	86.2 ± 7.5	59.3 ± 28.2	96.6 ± 2.7	81.1 ± 2.0	90.4 ± 0.5	45.3
	Adsorption (µg)	6.01±6.01	0	0	0	0	0
Ifosfamide	Rejection (%)	84.8 ± 5.5	61.8 ± 26.3	96.3 ± 2.2	82.5 ± 0.7	88.8 ± 0.2	43.8
	Adsorption (µg)	6.63±6.63	0	0	0	0	0

504 "nd" means not detected in the permeate using the analytical methods described*;* * When the target compound was not detected in
505 the permeate, a range of adsorption values was calculated considering concentrations in the permeate ranging between the method
506 limit detection and zero; **due to the higher variability and lower effectiveness obtained in all matrices using the NF270 membrane,
507 the last assay in secondary wastewater effluent was not repeated and thus error values are not reported

3.4 Toxicity

Different concentrations of the mixture of the cytotoxic drugs were spiked in the real secondary effluent to mimic the concentrations of feed, permeate and retentate and tested using the *Daphnia magna* toxicity assay. The results obtained are summarized in Table 5. To validate the *Daphnia magna* toxicity test, the number of dead plus immobile organisms should not exceed 10 % in the controls (Table 5). This condition was verified in all the control samples. The results obtained were statistically analyzed by the means of One-Way ANOVA (Post-Hoc Tukey HSD) using IBM SPSS Statistics 20.0 software. The level of significance was set at p-value < 0.05 for all statistical analysis.

Table 5. Average percentage of immobilization effect and standard deviation (± SD) caused by the different mixtures of cyclophosphamide, ifosfamide, paclitaxel and etoposide (0, 5, 500 and 5000 µg.L^{-1}) on Daphnia magna neonates.

	24 hours	**48 hours**
Concentration (µg.L^{-1})	**Immobilization Effect (%) ± SD**	**Immobilization Effect (%) ± SD**
0	0 ± 0	0 ± 0
5	5 ± 8,66	15 ± 8,66
500	10 ± 10	20 ± 14,14
5000	20 ± 14,1	45*,** ± 8,66
Negative Control	0 ± 0	0 ± 0
Blank	6,7 ± 9,43	6,7 ± 9,43

* Significantly different from the negative control (p-value < 0.05, One-Way ANOVA, Post-Hoc Tukey HSD); ** Significantly different compared to the same sample analyzed at 24 hours (p-value < 0.05, One-Way ANOVA).

After 24 hours of exposure, the mixture at the higher concentration tested (5000 µg.L^{-1}) immobilized 20% ± 14.1 of *Daphnia* neonates. Despite the increase of immobilized neonates induced by this concentration of the mixture, compared to the controls, the value is not statistically relevant. As expected, at 48 hours the immobilization effect induced by this mixture increased, achieving a significant value when compared to the negative control (p-value =

0,000). When comparing the results of the same concentration for different exposure times (24 and 48 hours), only the higher concentration presented differences (p-value = 0.040). This information shows a significant increase of toxicity between the two exposures times tested at 5000 $\mu g.L^{-1}$, indicating that time is an important factor in ecotoxicology assessment of these drugs.

Toxicity data concerning the individual interaction of these compounds in *Daphnia magna* have already been reported (Sanderson et al., 2003; Zounková et al., 2007; Česen et al., 2016; Białk-Bielińska et al., 2017) but information regarding the interaction of these four compounds as a mixture was not yet described to the best of our knowledge. One of the parameters used for toxicity evaluation parameter is the calculation of EC50, the concentration of the drug which results in a 50 % reduction of immobilization. In this particular case, it was not possible to calculate this factor, because none of the concentrations tested induces an immobilization effect higher than 50% (Sebaugh, 2011). However, it was determined that 48 hours of exposure to the 5000 $\mu g.L^{-1}$ concentration induces 45% ± 8.66 immobilization effect on *Daphnia magna*, very close to 50 %. This concentration is lower than most of the EC50 values reported for each individual compound (Table 6).

Table 6. Available ecotoxicological data (EC50 $\mu g.L^{-1}$; 48 hours exposure) for the investigated compounds in our study in Daphnia magna neonates.

Anticancer drugs	**EC50 ($\mu g.L^{-1}$)**	**References**
Cyclophosphamide	100000	(Białk-Bielińska et al., 2017)
	1000000	(Zounková et al., 2007)
	1750000*	(Sanderson et al., 2003)
Ifosfamide	100000	(Białk-Bielińska et al., 2017)
	1750000*	(Sanderson et al., 2003)
Etoposide	30000	(Zounková et al., 2007)
Paclitaxel	740	(Zounková et al., 2007)

* Predicted concentrations required to induce toxic hazard to aquatic organism (ECOSAR).

549 According to the classification of EC-Directive 93/67/EEC (1996), regarding aquatic
550 organisms, compounds with EC50 < 1000 $\mu g.L^{-1}$ are considered very toxic; EC50 between 1000
551 – 10 000 $\mu g.L^{-1}$ are considered toxic; EC50 between 10 000 - 100 000 $\mu g.L^{-1}$ are considered
552 harmful and EC50 > 100 000 $\mu g.L^{-1}$ are not harmful (Česen et al., 2016).
553 Taking into account this information, the results of this paper suggest that if
554 nanofiltration is applied, effective treatment of the retentate produced will be required.
555

556 ## 4 Conclusions

557 Anticancer drugs present a highly potent mechanism of action, which makes these drugs
558 of a great environmental concern. Hence, the development of effective treatment options is
559 crucial to avoid the release of these emerging contaminants in the aquatic environment.
560 Within the present study**,** the potential of two nanofiltration membranes (Desal 5DK and
561 NF270) to remove four widely used anticancer drugs from different matrices was assessed.
562 Even though the selected anticancer drugs presented different physico-chemical properties and
563 were spiked in matrices with very different compositions, Desal 5DK proved to be more
564 effective than NF270 in removing these drugs, showing no significant matrix influence on the
565 rejection results. Additionally, this membrane proved to be less susceptible to fouling in the
566 experiments performed with real secondary effluent. For these reasons, Desal 5DK membrane
567 could potentially be used to ensure the removal of anticancer drugs in hospital or wastewater
568 treatment facilities.
569 An acute ecotoxicological test was performed to evaluate the immobilization effect of
570 *Daphnia magna* when exposed to different concentrations of a mixture of the anticancer drugs
571 that mimic the concentrations in the feed, permeate and retentate samples obtained from the
572 nanofiltration experiments. The retentate samples were found to induce acute toxicity to
573 freshwater crustaceans and should therefore be subject to further treatment.
574

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