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Removal of a cytostatic drug by a membrane bioreactor

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ABSTRACT

The application of membrane bioreactor process is investigated in the aim of evaluating the potential for removal of cyclophosphamide (CP). Two laboratory-scale membrane bioreactors (MBR) were run in parallel, one with CP and its principal metabolites (MBR1-CPs), and one without (MBR2-control). Removal of CP in an MBR and its effects on the membrane performance, chemical oxygen demand (COD) and total nitrogen (TN) removal efficiency were studied. Removals of 80% were achieved for CP and the metabolite 4-ketocyclophosphamide under the operating conditions studied. Both adsorption and degradation affect the overall removal. The toxicity of CP and its metabolites does not alter the COD or TN removal efficiency of MBR. However, it induces a modification of the biological suspended solids and so a modification of the membrane fouling.

Keywords: Cyclophosphamide; Membrane bioreactor; Micropollutants; Wastewater

1. Introduction

The occurrence and fate of pharmaceutically active compounds (PhACs) in the natural environment has been recognized as one of the emerging issues in environmental chemistry [1, 2]. Pharmaceuticals are designed to have biological activity in humans and may, in principle, have adverse effects on aquatic organisms. Compounds with a very potent mechanisms of action, such as cytostatic drugs (one of the most toxic pharmaceuticals in common use), are of particular environmental concern even though consumption rates and expected concentrations in the environment may be comparatively low [3, 4, 5]. Their mechanism of action, involving metabolic activation and unspecific alkylation of nucleophilic compounds, accounts for genotoxic effects described in the literature [3]. Due to their mode of action, practically all eukaryotic organisms are vulnerable to damage, with teratogenicity being the greatest concern at such levels [5].

The increasing use of anticancer drugs and their presence in wastewater is a relatively new issue and few studies have been published [3–10]. They usually enter hospital effluents partially transformed or even unchanged via the urine and faeces of patients under medical treatment. Therefore, they are assumed to be environmentally relevant compounds. As hospital effluents generally reach the municipal sewer network without any preliminary treatment, hospitals are an undeniable release source of anticancer agents [9]. The compounds reach the aquatic environment via hospital or domestic wastewater and wastewater treatment plants (WWTPs) [3]. As cytostatic agents are known to be carcinogenic, mutagenic and toxic for reproduction, they should be removed from wastewater at their source [8].

The cytotstatic drug cyclophosphamide (CP) is one of the oldest known cytostatics and is one of the most

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frequently used in cancer chemotherapy [11]. CP is a prodrug that requires biotransformation to become cytotoxic [12, 13]. It is transformed by hepatic and intracellular enzymes to active alkylating metabolites [14]. Besides its cytotoxic effects, CP possesses teratogenic and mutagenic properties and is a known human carcinogen [3, 10].

CP has been detected in hospital sewage in concentrations ranging from 20 ng/L to 4.5 µg/L [10]. Buerge et al. (2006) [3] found no difference in CP concentrations between influent and effluent in two Swiss sewage treatment plants (STPs). They detected 0.15–0.17 ng/L CP in receiving waters associated with a Swiss STP. Using lake water from Lake Zurich, they reported half-lives of 80 days in the dark and 44 days under simulated sunlight conditions for CP at 20°C. The recalcitrance of many cytotoxic drugs in the highly biologically active environment of activated sludge indicates that they will be extremely persistent in river water [5].

The removal mechanisms of the micropollutants (i.e. PhACs) during biological wastewater treatment include, with different relevance for the different substances, adsorption on sludge flocs, biodegradation, volatilisation, and stripping [15]. Moreover, stripping is not a relevant process for pharmaceuticals since they have a fairly good solubility and therefore a low gas-water-partitioning coefficient [16].

Many of the cytotoxic drugs tested so far appear to be poorly biodegradable, if at all, when incubated with activated sludge [3, 10, 17, 18]. CP appears to be poorly biodegradable. For example, in a 39-day continuous dosing experiment, an average of 83% CP was recovered from the waste stream of a laboratory scale activated sludge plant [10]. Thus, little CP was removed by the activated sludge particles. No degradation was observed in activated sludge incubation experiments within 24 h at a concentration of ~ 100 ng CP/L [3].

The majority of cytotoxic drugs are highly soluble (10–50 g/L at 20°C–25°C), with predicted log KowS between –1 and 3 [19]. From the medical viewpoint, it is clearly desirable for drugs to exert their effect and then be rapidly removed from the body either through excretion aided by their hydrophilic properties, or by metabolism [20]. A preliminary examination of the properties of this cytotoxic drug shows that it has low-vapour pressure, and so is unlikely to volatilise under normal conditions, and that it is hydrophilic [14]. Some authors conclude that the removal of pharmaceuticals and personal care products by sorption onto suspended solids is an important mechanism for hydrophobic compounds and compounds with positively charged functional groups (e.g. amines) [16].

Theoretically, several operational conditions exist in membrane bioreactors (MBRs), which favour enhanced biotransformation and mineralization of PhACs [21, 22]. The membrane bioreactor is the result of combining biological treatment and a liquid/solid membrane. The hydraulic residence time and sludge age are completely decoupled and MBRs offer flexibility of operation. Membrane bioreactors usually operate at high sludge ages and high concentrations of biomass. This allows an intensification of biological processes, which may increase the elimination of contaminants, like PhACs, that have special characteristics such as low bio-degradability and low concentration. In recent years, several studies have been conducted on biological wastewater treatment in order to compare the elimination rates of pharmaceuticals and personal care products in conventional activated sludge treatment (CAS) and membrane bioreactors [21–28]. MBRs showed significantly better removal of persistent pharmaceuticals in some cases [23, 26, 27, 28].

The aim of this work is to evaluate the cyclophosphamide (CP) removal (at concentrations close to those detected in hospital sewage) in a membrane bioreactor system (MBR) and the consequences for conventional pollution removal (COD, chemical oxygen demand, and TN, total nitrogen). Two laboratory-scale membrane bioreactors (MBR) were run in parallel, one with the cytostatic drugs (MBR1-CPs), and one without (MBR2-control). In order to check whether the addition of cyclophosphamide and its principal metabolites could affect the treatment performance, the COD and TN removal efficiencies were compared between the control and the CP reactor.

2. Materials and methods

The schematic diagram of the crossflow MBR pilot system is shown in fig 1. The reactor consisted of a bioreactor with a working volume of 20L and a membrane module. The membrane module was a ceramic tubular Membralox® (MF) membrane with 0.0055 m^2 of surface area and pore size of 0.1 µm (Pall Exekia, France). In order to keep the bioreactor completely mixed, a Ruston turbine was installed (260 rpm).

Two identical lab-scale crossflow MBRs were run in parallel. Each reactor was inoculated with the same activated sludge from a municipal wastewater treatment plant (dry weight, 3g/L). Raw water was composed of domestic water (average flux 9.75 L/day, from the same wastewater treatment plants, Brax, France, 2000 person-equivalent) pre-screened to 200µm and completed with Viandox® (average flux 0.25 L/day, commercial product, soya bean extract) so as to reach the chemical oxygen demand (COD) required to achieve the high volumetric loading rate of 1.1 $KgCOD.m^{-3}.d^{-1}$. (Average inlet COD, 2300 mg/L; average inlet TN soluble, 175 mg/L). One of the MBRs was used as a control **(MBR2-Control)**, while cyclophosphamide (5µg/L) and its principal metabolites (acrolein 2.25 µg/L, phosphoramide mustard 8.88 µg/L, 4-ketocyclophosphamide

0.58 µg/L, and nitrogen mustard 0.517 µg/L) were continuously added to the other **(MBR1-CPs)**.

Chemicals were supplied by NIOMECH, part of IIT GmbH (University of Bielefeld, Universitäts str. 25, DE-33615 Bielefeld): D-18845— 4-keto-cyclophosphamide ; D-18846—phosphoramid mustard ; D—19990-nitrogen mustard hydrochloride, and by SIGMA (St Quentin Fallavier, France): 01680 Acrolein; C0768 cyclophosphamide.

The hydraulic retention time (HRT) was 48 h, temperature was 25°C–32°C and pH 7–8. The sludge retention time (SRT) was around 50 days, which led to a low food to micro-organisms (F/M) ratio. The F/M in the MBR1-CP was 0.14 (kg COD/kg MLSS.d) and 0.11 in the MBR2-Control at steady-state. The resulting biomass concentrations were 8.89 in MBR1-CP and 10.84 in MBR2-Control. Treatment was operated in aerobic/anoxic conditions to allow nitrification and denitrification of the influent. Dissolved oxygen levels between 0–4.5 mg O_2/L were maintained. The aeration cycle was: 2 minaeration/23 min without aeration.

Pressures were measured at the inlet (Fig 1. P1), outlet (Fig 1. P2), and permeate side of the membrane (Fig 1. P3) in order to determine the transmembrane pressure (TMP). At constant permeate flux, TMP indicates the extent of membrane fouling and it was calculated as follows:

$$TMP = \left(\frac{P1 + P2}{2} - P3 \right)$$

2.3. Batch experiments on sludge adsorption of CP

The different equilibrium concentrations of CP were achieved by spiking various concentrations of CP (1, 10, 20 and 50 mg/L) into the same mass of sludge (7.5 g/L). The sludge, taken from MBR2-Control, was centrifuged and then washed with distilled water and dried at 60°C to sterilise the sludge. The mixture (50 mL of solution) was placed in conical flasks (250 mL) and shaken at 25°C for 48 h, which was considered more than sufficient time for adsorption equilibrium to be reached. Finally, the supernatant was used for analysis of CP. The control samples without sludge were simultaneously processed under the same conditions. The results showed that CP was steady during the entire process without sludge.

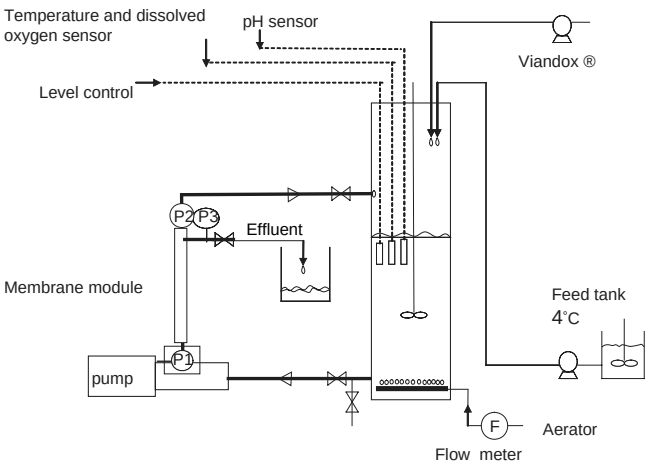

Fig. 1. Schematic diagram of MBR pilot.

2.4. Analytical methods

Mixed liquor suspended solids (MLSS) were measured according to standard methods (APHA, 2005). Chemical oxygen demand (COD) and total nitrogen (TN) were measured by spectrometric methods with reagent kits (HACH). The transmembrane pressure (TMP), which indicated the extent of membrane fouling, was monitored regularly.

2.5. Sample extraction and methods of CP and 4-ketoCP analysis

The analysis of CP and 4-ketoCP was performed by LC-MS-MS after lyophilisation and extraction with dichloromethane. The analysis of other metabolites is still under investigation.

2.5.1. Extraction

All CP samples were concentrated by a lyophilisation-extraction procedure. Briefly, 200 µL isophosphamide (0.1 mg/mL) was added into 100 mL CP sample as an internal standard. The 100 mL sample was frozen in the 500 mL glass bottles (Quickfit, England) in a liquid nitrogen bath in a rotation evaporator (Phenomenex, France) for about 12 min. Then the frozen sample bottle was connected with the lyophiliser (CARLO ERBA, France) for one night under vacuum conditions. After lyophilisation, the sample powder obtained was carefully transferred into a 30 mL glass tube (Scientific, France). 10 mL dichloromethane was then added into the bottle. The bottle was shaken manually for 10 min to completely dissolve the remaining powder. This operation was repeated two more times with 5 mL dichloromethane and all the dichloromethane fractions were brought together in the 30 mL tube. The sample tube was shaken gently in the shaking bed (Stuart, France) for 30 min to further dissolve CP in the dichloromethane. The sample was centrifuged for 10 min at 2000 rpm. The dichloromethane phase was carefully transferred into a 20 mL glass tube with a pipette and the tube was placed in the evaporator (PIERCE 18780, France) to be completely dried under a gentle nitrogen stream. These operations were repeated twice with 5 mL dichloromethane. Finally,

for LC-MS-MS analysis: 100 µL methanol/ammonium formate buffer (50/ 50) was added, pH 5.7.

CP and 4-keto CP recoveries in different water matrixes were mostly greater than 75% and the overall variability of the method was below 8%. The extracted samples were stored at –80°C for further analysis

2.5.2. LC-MS-MS

The LC-MS-MS method was applied for CP confirmation and quantification at lower CP concentration and in a complex water matrix. The injection volume was 20 µL. The mobile phase consisted of a gradient of methanol-ammonium formate buffer CH_5NO_2 2mM (pH 5.7) (Fluka) circulated at an isocratic flow rate of 0.20 mL/min (see Table 1). The column used was C18 125 mm/2mm Nucléosil 100Å-5µm HD maintained at a temperature 30°C. A guard column was also used: Frit SS Blk 0.5µm, 0.094 × 0.065 × 0.250 (Cil Cluzeau Info Labo).

The MS was operated in the positive electrospray ionisation (ESI^+) mode using multiple reaction monitoring (MRM). The scan range was m/z [70–290] in the MS/MS mode, at a scan rate of 3 µscans and 200 ms. Under ESI^+ conditions, an abundant protonated molecule $[M+H]^+$ at m/z 233 and the fragment ions at m/z 239, loss of chlorine, were observed. The cone voltage and collision energy for each transition were programmed through the Excalibur acquisition software. The detection limit of the method (LC-MS-MS) for CP and 4-keto CP was 10 ng/mL.

All solvents (methanol, dichloromethane) were of HPLC grade from Sigma, France. Ultrapure water was used as the eluent in liquid chromatography tandem mass spectrometry (LC/MS/MS).

3. Results and discussion

The experiments were performed for 160 days. The three major changes were: Day 21, First day of addition of cyclophosphamide and its principal metabolites to MBR1-CP; Day 65, increase of crossflow velocity from 4 to 5 m/s in both reactors; Day 114, change of membranes for two new membranes with similar initial permeability.

Table 1
Methanol-ammonium formate buffer gradient.

Time (min)	Mobile phase
0	80% buffer/20% methanol
9	80% buffer/20% methanol
9.5	55% buffer/45% methanol
25	55% buffer/45% methanol

3.1. Removal of Cyclophosphamide (CP) in MBR

Figure 2 illustrates the CP and 4-ketoCP removal efficiency in MBRs. Removal of CP started from the beginning of experiment. At steady-state conditions, the pharmaceutical removal efficiencies remained quite stable, 80% for CP and for 4-ketoCP.

Preliminary investigations in a simplified test system indicated a low degradability of CP [3, 10, 29]. Even though other studies indicate that CP is not biodegradable, or only poorly, we observed a removal of this molecule in our study. Because CP and metabolite molecular dimensions were well below the pore size of the microfiltration membranes used in the MBR, physical retention by the membrane was negligible.

CP biodegradation by sludge in the MBR was supported by the transformation of CP via its metabolite 4-ketoCP, which was visible from changes in the

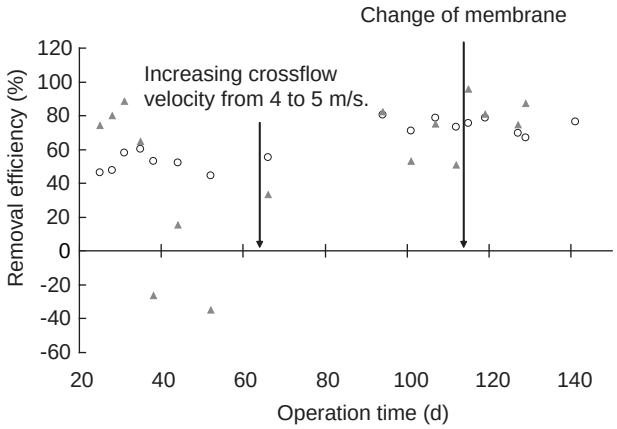

Fig. 2. Removal of Cyclophosphamide (0) and 4-ketoCyclophosphamide (Δ).

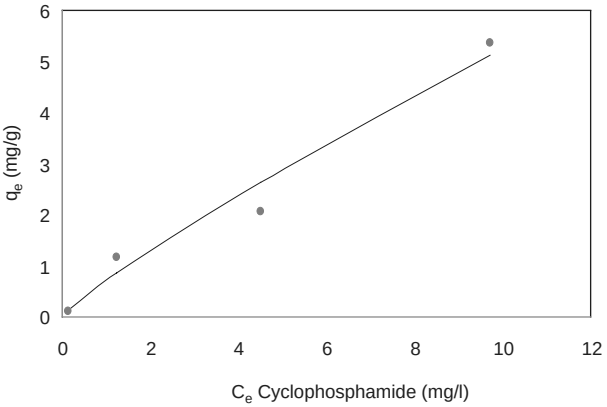

Fig. 3. The adsorption isothermal curve of cyclophosphamide (CP) adsorbed by sludge in the MBR. C_e represents the equilibrium concentration of CP in liquor. q_e represents the adsorbed CP in sludge when in equilibrium. The fitting equation was $q_e = 0.7185\ C_e^{0.8637}$ ($R^2 = 0.9794$). Freundlich constants: $K_f = 718.5$ L/kg and $1/n = 0.8637$.

4-ketoCP concentrations: concentrations in the reactor were higher that in the feed. The removal efficiency of 4-ketoCP decreased from day 35 to day 66 (Fig. 2).

The adsorption isothermal curve of cyclophosphamide (CP) adsorbed by sludge in the MBR is shown in Fig 3.

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The fitting results showed that the sludge adsorption of CP followed the Freundlich adsorption. The range of log KD was from 2.66 to 2.97. According to these results, sludge adsorption might play an important role in the process of CP removal. We conclude that both adsorption and degradation affect the overall removal. Furthermore, sorption may also influence the rate of other processes such as biodegradation [30]. As CP was present at low concentrations, CP could not be used as the primary source of energy/carbon, so it could be suggested that CP was cometabolically degraded. In previous studies, some authors indicate that cometabolic transformation may be the major removal mechanism of some PhAC compounds in activated sludge treatment of municipal wastewater [21, 25].

3.2. Removal efficiencies COD and TN

In order to check whether the addition of cyclophosphamide and its principal metabolites could affect the treatment performance, the removal efficiencies of COD and TN were compared between MBR2-control and MBR1-CP (Figs. 4 and 5).

The removal efficiencies were almost identical in both MBRs, indicating that addition of pharmaceuticals had a negligible effect on the efficiency of the treatment. COD removal in the system was attributable to two factors,

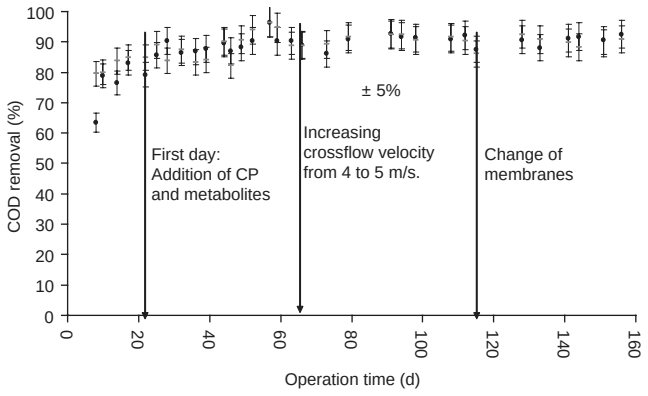

Fig. 4. COD removal during the operation. MBR1-CP (✹) and MBR2-Control (–).

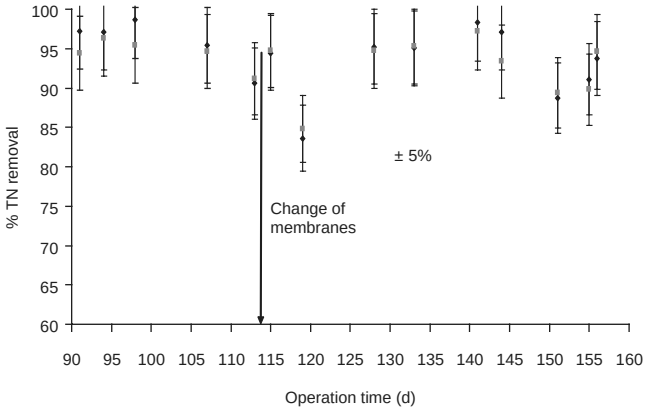

Fig. 5. TN removal variation for both MBRs. MBR1-CP (✹) and MBR2-Control(■).

one was biological removal by micro-organisms and the other was physical retention by the membrane. Due to the complete retention of biomass, MBRs can be operated at much higher sludge concentrations. The high sludge retention time allows for adaptation of the microorganisms in general, and of potentially slow-growing specialist bacteria in particular, and establishes a more diverse microbial community with broader physiological capabilities. Thus, the effect of CPs could be offset by the high concentration of biomass (due to complete retention of biomass and high sludge age) in the bioreactor, thus maintaining high overall performance in the removal of COD (Fig. 4) and TN (Fig. 5).

3.3. Membrane performance

Membrane performance was tested by measuring transmembrane pressure (TPM). Figure 6 shows the variation of transmembrane pressure (TMP) for both bioreactors.

TMP showed the same behaviour in both bioreactors until day 65, even after the addition of Cps. The increase in TMP from day 45 to day 65 was similar for both reactors, indicating that membrane fouling was rather related to operating conditions. To reduce membrane fouling, on day 65, the crossflow velocity was increased from 4 to 5 m/s. In MBR2-control, this increase resulted in a reduction of membrane fouling (the pressure was stabilised around 0.60 bars). In MBR1-CPs, TMP decreased from day 66 to day 75, and then TMP returned to the value (1.3 bars) it had before the increase in crossflow velocity. On day 114, we changed the membranes of both MBRs for two new membranes with the same initial permeability. In this way, in addition to reducing the transmembrane pressure, we wanted to determine whether the increase in TMP in MBR1-CPs was related to irreversible membrane fouling or related to the physicochemical properties of the mixed liquor. After this change, the TMP MBR2-control

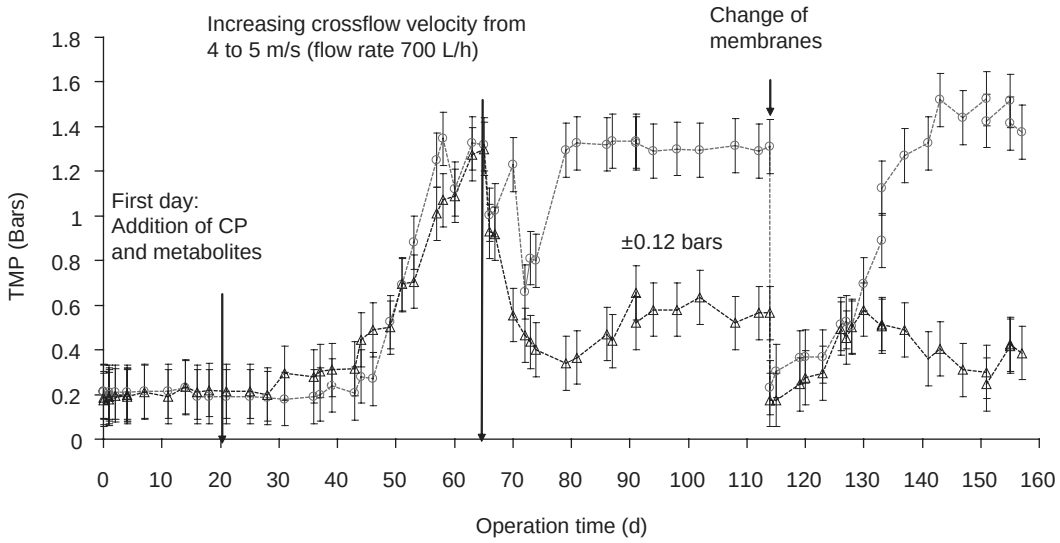

Fig. 6. Transmembrane pressure (TMP) variation. MBR1-CP (O) and MBR2-Control (Δ).

increased, reaching the same value as before the change of membrane (0.6 bar) and then TMP decreased to 0.4 bars. Regarding MBR1-CPs, TMP increased significantly up to 1.5 bar (higher than before the change, 1.3 bar).

This result shows that the response of activated sludge to imposed mechanical shear differed markedly according to the presence or absence of CPs. Activated sludge in MBR1-CPs showed less capacity to adapt to mechanical stress than that in MBR2-control. Cps toxicity on activated sludge modified the characteristics of the biological matrices and weakened their resistance to mechanical stress. It could be suggested that the toxic level of CP and metabolites in the influent could have induced the micro-organisms to secrete more EPS for their protection. Increasing crossflow velocity resulted in the release of biopolymers from the EPS floc-matrix into the bulk liquid. The change of membrane (day 114) did not change anything in the pressure. Thus, that the quality of the sludge, its mechanical resistance, which was weakened by the cytostatic, governed the fouling phenomena in MBR1-CP.

4. Conclusion

In this work, two MBRs were operated in order to evaluate MBR potential for cytostatic drug bioremoval and to study the effects of such drugs on the membrane performance, COD and total nitrogen (TN) removal efficiency. Cyclophosphamide and 4-ketocyclophosphamide removals of 80% were achieved under a hydraulic retention time of 48 h, a solid retention time of 50 days and a mixed liquor suspended solids concentration of 8.89 g/L. Thus CP concentration in MBR effluent was about 1 µg/L. Sludge adsorption might play an important role in the process of CP removal. Moreover, the detection of 4-ketoCP as an intermediate product of CP biodegradation could demonstrate the importance of biodegradation. Both adsorption and degradation affect the overall removal. Removal rates observed for COD and TN were above 90% and 93% respectively. The toxicity of cyclophosphamide and its metabolites does not alter the COD and total nitrogen removal efficiency of MBRs. However, it induces a modification of the biological suspended solids and so a modification of the membrane fouling. The results of this study prove that advances in wastewater treatment using an MBR provide a suitable process for lowering CP concentrations before discharge into the aqueous environment. Despite this clear benefit of MBR, removal is only partially achieved and a tertiary treatment is necessary for the complete elimination of cytostatic compounds' toxicity. In addition, if the sludge becomes toxic when treating this kind of pollution, incineration would become relevant.

Acknowledgements

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Symbols

Ce — Equilibrium concentration of adsorbed in solution, mg/L
Kf — Freundlich constant, L/kg
qe — Mass adsorbed/mass adsorbant, mg/g
$1/n$ — Freundlich constant

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