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Cytotoxicity micropollutant removal in a crossflow membrane bioreactor

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A B S T R A C T

The application of membrane bioreactor (MBR) technology was investigated with the aim of evaluating its potential for cytostatic drug and cytotoxicity bioremoval. The toxicity removal was assessed from bio marker test. CP removal of up to 80\% was achieved under the operating conditions studied (HRT of 48 h and a SRT of 50 days). The increase of TMP was associated with an increase of supernatant toxicity as if fouling led to retention of the toxicity. Peaks of supernatant cytotoxicity were correlated with peaks in supernatant humic acid contents. It may suggest that molecules with a toxic effect may be adsorbed or entrapped in humic acids substances. Our study then points out that advances in wastewater treatment using an MBR can provide a suitable process for lowering CP concentrations before discharge into the aqueous environment. However, a tertiary treatment is necessary if complete elimination of toxicity is targeted.

1. Introduction

Pharmaceuticals are designed to have biological activity in humans and may have adverse effects on aquatic organisms. Pharmaceuticals and other micropollutants in wastewater pose a new challenge to both wastewater professionals and the pharmaceutical industry (Larsen et al., 2004). Compounds with a very potent mechanism of action, such as cytostatic drugs, are of particular environmental concern, even though consumption rates and expected concentrations in the environment may be comparatively low (Buerge et al., 2006; Kümmerer, 2001). The demand for chemotherapy treatment is increasing by about 10\% per year in developed countries. In addition, cancer incidence is rising and this is not simply due to a greater proportion of elderly people in the population (Johnson et al., 2008).

The alkylating antineoplastic drug cyclophosphamide (CP) is one of the oldest known cytostatics and is one of the most frequently used agents in cancer chemotherapy (Gilard et al., 1994). After application to patients, the agent is excreted renally and up to 20\% of the dose may leave the body unmetabolized. Besides its cytotoxic effects, CP possesses teratogenic and mutagenic properties and is a known human carcinogen (Steger-Hartmann et al., 1997). CP is a prodrug that requires biotransformation to become cytotoxic (Moore, 1991; Sladek, 1994). It is transformed, via hepatic and intracellular enzymes, into active alkylating metabolites, 4-hydroxycyclophosphamide, aldophosphamide, acrolein and phosphoramide mustard (Joqueviel et al., 1998). Such drugs, partially transformed or even unchanged, usually enter hospital effluents 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 sewage network without any preliminary treatment, hospitals are an undeniable release source of anticancer agents (Mahnik et al., 2007). The compounds finally reach the aquatic environment via hospital or domestic wastewater and wastewater treatment plants (WWTPs) (Buerge et al., 2006).

CP has been detected in concentrations ranging from 20 ng/L to 4.5 \mu g/L in hospital sewage and concentrations ranging from 7 to 143 ng/L have also been found in samples from the influent and the effluent of the communal sewage treatment plant into which the hospital’s sewage water is discharged (Steger-Hartmann et al., 1997). CP has been detected in surface waters in Switzerland. Concentrations ranged from 50 to 170 pg/L and were thus several orders of magnitude lower than the levels at which acute ecotoxicological effects have been reported in the literature (mg/L range). However, due to a lack of studies on chronic effects on aquatic organisms and data on the occurrence and effects of metabolites, a final risk assessment cannot be made (Buerge et al., 2006).

Theoretically, there are several operational conditions in MBRs which favour enhanced biotransformation and mineralization of pharmaceutically active compounds (PhACs) (Clara et al., 2005;
De Wever et al., 2007). Membrane bioreactors (MBR) usually operate at high sludge retention times and high concentrations of biomass, allowing an intensification of the biological process by the implementation of resistant and low-growth biomass (De Wever et al., 2007). These elements may increase the elimination of pollutants with special characteristics, such as low bio-degradability and low concentration, like PhACs. Although much research is being directed towards the study of the removal mechanisms of micropollutants (Urase and Kikuta, 2005), little attention is being paid to toxicity removal. In previous papers (Avella et al., 2010; Delgado Luis et al., 2010b), the influence of CP and its principal metabolites on microbial behavior as well as on extracellular polymeric substances formation in a MBR system were reported. In this work, the application of membrane bioreactor (MBR) technology is investigated here with the aim of evaluating its potential for cytotoxicity and cytostatic drug bioremoval.

2. Methods

2.1. Pilot-scale experiments

The schematic diagram of a crossflow MBR pilot system is shown in Fig. 1. The MBR pilot was inoculated with activated sludge from a municipal sewage treatment plant (initial dry weight, 3 g/L). Raw water was composed of domestic water (wastewater treatment plants, Brax, France, 2000 equivalent-inhabitant) pre-screened to 200 μm and completed with Viandox® (commercial product, soya bean extract) so as to reach the chemical oxygen demand (COD) required to achieve high volumetric loading rates (Table 1). Treatment was operated in aerobic/anoxic conditions. Cyclophosphamide (5 μg/L) and its main metabolites (acrolein 2250 ng/L, phosphoramido mustard 8880 ng/L, 4-ketocyclophosphamide (Keto CP) 580 ng/L, nitrogen mustard 517 ng/L) were continuously added to the pilot. Chemicals were supplied by NIOMECH, part of (Keto CP) 580 ng/L, nitrogen mustard 517 ng/L) were continuously added to the pilot. Chemicals were supplied by NIOMECH, part of IIT GmbH (University of Bielefeld, Universitäts str., 25, DE-33615 (Keto CP) 580 ng/L, nitrogen mustard 517 ng/L) were continuously added to the pilot. Chemicals were supplied by NIOMECH, part of IIT GmbH (University of Bielefeld, Universitäts str., 25, DE-33615 Bielefeld); D-18845 – 4-ketocyclophosphamide; D-18846 – phosphoramido mustard; and D-19990 – nitrogen mustard hydrochloride, and by SIGMA (St. Quentin Fallavier, France): 01680 acrolein; C0768 cyclophosphamide.

The membrane module was a ceramic tubular Membralox® (MF) with 0.0055 m² surface area and pore size 0.1 μm (Pall Exekia, France). In order to keep complete mixing in the bioreactor, a Ruston turbine was installed (260 rpm). Dissolved oxygen and pH in the bioreactor were monitored. The operating conditions of the MBR during the experiments are given in Table 1. 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 (Eq. (1)):

\[ \text{TMP} = \left( \frac{P_1 + P_3}{2} - P_2 \right) \]

2.2. Analytical methods

The chemical composition of soluble extracellular polymeric substances (EPS) was analysed for proteins, humic substances and polysaccharides. Proteins and humic substances were measured by the modified Lowry method with bovine serum albumin and humic acid as standard (Frolund et al., 1995). Polysaccharides were determined according to the anthrone method with glucose as standard (Dreywood, 1946).

2.3. Sample extraction and method of CP analysis

The analysis of CP was performed by LC–MS–MS after lyophilisation and extraction with dichloromethane.

2.3.1. Extraction

All CP samples were concentrated by a lyophilisation–extraction procedure. Briefly, 200 μL isophosphamide (0.1 mg/mL) was added into a 100-mL CP sample as an internal standard. The 100-mL sample was frozen in 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) over night under vacuum conditions. After lyophilisation, the sample powder obtained was carefully transferred into a 30-mL glass tube (Scientific, France). The 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 transferred into a 20-mL glass tube with a pipette and the tube was placed in the evaporator (PIERCE 18780, France) over night under vacuum conditions. After lyophilisation, the sample powder obtained was carefully transferred into a 30-mL glass tube (Scientific, France). The 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 simple was centrifuged for 10 min at 2000 rpm. The dichloromethane phase was 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), pH 5.7, was added.

CP recoveries in different water matrices 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.

2.3.2. LC–MS–MS

The LC–MS–MS (LCQ Advantage, Thermo Finnigan) 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 CH₃NO₂; 2 mM (pH 5.7) (Fluka) circulated at an isocratic flow rate of 0.20 mL/min (time/methanol/ammonium formate buffer = 0/20/80 – 9.5/45/55). The column used was a C18 125 mm/2 mm Nucleosil 100 A – 5 μm HD maintained at a temperature 30 °C. The column was protected by a filter of 0.5 μm (Frit SS Blk – Cluzeau France). The MS was operated in the positive electrospray ionisation (ESI+) mode, using multiple reactions monitoring (MRM). Under ESI+ conditions, acquisition
was performed by monitoring the following transition 261 > 233 for CP and isophosphamide. 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 was 10 ng/mL. All solvents (methanol and dichloromethane) were of HPLC grade from Sigma, France. Ultrapure water was used as the eluent in liquid chromatography tandem mass spectrometry (LC/MS/MS).

2.4. Cytotoxicity analyses

Cytotoxicity tests were performed periodically on influent, supernatant and permeate MBR samples. Fig. 2 shows an example of this test where the cytotoxicity was evaluated by measuring either the proliferation (>100% in Fig. 2) or viability (death, <100% in Fig. 2) of treated cells (in contact with MBR samples, e.g., in Fig. 2 cells were treated with MBR supernatant samples) compared to controls (untreated cells without MBR samples = 100% in Fig. 2) as described previously (Faucet-Marquis et al., 2008). Tests of cell proliferation or viability were made on human liver cells (HepG2) because this cell line was most sensitive to the effects of the products tested. Proliferation or viability was measured quantitatively by the colorimetric tetrazolium (MTT) assay and based on the metabolic activity of viable cells.

3. Results and discussion

The experiments were performed over 160 days in the first experimental campaign. The three major changes were: day 21, first day of addition of cyclophosphamide and its principal metabolites (CPs) to MBR; 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. This was done not only to reduce the transmembrane pressure but also to determine whether the increase in TMP in MBR was related to irreversible membrane fouling or to the physicochemical properties of the mixed liquor (Delgado Luis et al., 2010a).

The experiments were performed over 223 days in the second experimental campaign. Day 108 was the first day of addition of pharmaceutical compounds (CPs) into the MBR. The activated sludge was exposed over a long period (107 days) (more than 1.5 times the sludge retention time) before the addition of the cocktail. Thus, we can also assume that the biomass was completely adapted to the imposed operating conditions before the addition of pharmaceutical compounds (CPs).

3.1. Cyclophosphamide removal efficiency in MBR

Fig. 3(a) and (b) show CP concentration variations in the influent, supernatant and permeate during experimental campaign I and experimental campaign II, respectively. For both campaigns, it was observed that the CP concentrations in the supernatant and permeate of the bioreactor were very similar. It can thus be supposed that there is no retention by the membrane during filtration, whatever its degree of fouling (Fig. 3). Therefore, it could be assumed that CP retention by the membrane + gel layer developed on the surface of the membrane was negligible.

The CP removal efficiencies during both campaigns are shown in Fig. 4(a). The time axis has been recalculated so that day 0 is the first day of the cocktail addition. Preliminary investigations in a simplified test system indicated a low degradability of CP (Buerge et al., 2006; Kümmnerer et al., 1996). Based on the degradation studies in a laboratory-scale sewage treatment plant and the analytical findings in sewage water. Steger-Hartmann et al. (1997) concluded that excreted CP was only poorly degraded during its passage through the sewage treatment plant. Even though some studies (Buerge et al., 2006; Kümmnerer et al., 1996) indicate that CP is not or is only poorly biodegradable, a removal of CP was observed in our study. Removal of CP started from the beginning of the experiment (Fig. 4(a)). After 70 days of continuous addition of the cocktail (CP and its principal metabolites), the CP removal efficiency was up to 80% during both campaigns (Fig. 4(a)) and proved the ability of the MBR to partially remove cytotoxic compounds. However, at the end of experimental campaign I, values greater than 80% removal were achieved, possibly due to a longer period of adaptation of the micro-organisms to the cocktail of pharmaceutical compounds than in the second campaign (treatment with the

![Fig. 2. Cell proliferation or viability variation in cells treated with MBR supernatant samples compared to controls. Experimental campaign I.](Image)
cocktail for 2.7 times the sludge retention time during experimental campaign I and 1.6 times the sludge retention time during experimental campaign II). The CP removal mechanisms have been reported previously. Adsorption onto sludge flocs and biodegradation both affect the overall removal (Delgado et al., 2009). As CP was present at low concentrations, it could not be used as the primary source of energy/carbon, so it could be suggested that CP was cometabolically degraded. In previous studies, other authors have indicated that cometabolic transformation may be the major removal mechanism of some PhAC compounds in activated sludge treatment of municipal wastewater (De Wever et al., 2007; Joss et al., 2005).

Even if a comparison between the results of the two campaigns is difficult because several variables were changed, not only the sludge retention time, it appears that a sludge age of 50 days, a hydraulic retention time of 48 h was sufficient to achieve a removal efficiency of 80%. Nevertheless, the effect of sludge age on the elimination of some micropolutants has already been studied (Clara et al., 2005; De Wever et al., 2007; Weiss and Reemtsma, 2008). Weiss and Reemtsma (2008) studied the influence of sludge age on the elimination of polar micropolutants. In this study, a slight trend towards improvement of the elimination was observed by increasing the sludge age from 26 to 37 days, but it remained statistically insignificant. In addition, they observed that increasing the sludge age to 102 days seemed adverse to a compound that was supposed to be degraded by cometabolism. On the other hand, concerning hydraulic retention time (HRT), previous studies suggest that a minimum HRT is needed for optimal degradation, which seems to be situated above 5 h (De Wever et al., 2007). Weiss and Reemtsma (2008) anticipated that the lowest HRT of 7 h was long enough for trace pollutant removal by MBR and that a further extension would have only marginal effects on polar pollutant removal. On the other hand, Joss et al. (2005) noted that HRT had only a minor influence on the removal of pharmaceuticals and fragrances, when considering HRTs of 0.7 h for a fixed bed system, 13 h for an MBR and up to 17 h for a CAS. In our studies, the HRTs were greater than 32 h. Based on previously studies, it could thus be assumed that a further extension would have only marginal effects on cyclophosphamide removal.

3.2. Cytotoxic removal efficiency in MBR

In previous work (Delgado Luis et al., 2010b), it was observed a chemical stress caused by CPs on micro-organism in the MBR. CPs toxicity could caused a diversion of carbon and/or energy from growth to adaptive responses and protection (i.e., an increase in extracellular polymeric substances (EPS) concentration in the biological sludge, especially of soluble substances, mainly polysaccharides and proteins was observed. The formation of EPS seemed to be a protection mechanism (Avella et al., 2010)), and

![Fig. 3.](image-url)
an inhibition of catabolism and anabolism that can be offset by the biomass still active under low substrate/biomass condition in the bioreactor which helped to maintain high overall performance in the removal of conventional pollution.

As a further investigation to the earlier work, in this work it was evaluated the MBR potential for CP cytotoxicity bioremoval. Cytotoxicity tests were performed on influent, supernatant and permeate MBR samples. Cytotoxicity was evaluated using hepatic cell culture in optimised conditions, by measurement of either cell proliferation or death as explained in Section 2.4.

Fig. 4(b) shows the cytotoxicity variations in comparison to control (100% viability) during experimental campaign II. A large decrease was observed in permeate toxicity compared to the influent toxicity. Thus, this result proved the ability of the MBR to partially remove the cytotoxicity of CPs. Some small differences were observed between the supernatant and permeate toxicities in the MBR during this experimental campaign. It could be assumed that, during experimental campaign II, the cytotoxicity retention by the membrane + the gel layer developed on the surface of the membrane was negligible. During this experimental campaign II, the activated sludge was exposed over a long period (107 days, more than 1.5 times the sludge retention time) before the addition of pharmaceutical compounds (CPs). Therefore, the biomass was completely adapted to the imposed operating conditions before the addition of pharmaceutical compounds (CPs) and only a few changes were observed in the evolution of the TMP (figure) and the soluble extracellular polymeric substance (EPS) concentration (data no shown) in the MBR. On the other hand, during experimental campaign I, more changes were observed in the variation of EPS and the variation of TMP in the MBR. The cytotoxicity removal was also observed (data not shown), but differences were found between the supernatant and permeate cytotoxicity measured (Fig. 5).

In order to establish a link between the cytotoxicity (death or proliferation) observed in the supernatant and permeate of the MBR pilot and bioreactor behavior, the cytotoxicity tests compared to 100% viability of the control in the permeate and in the supernatant, are represented in Fig. 5(a) and (b), respectively for experimental campaign I. The variation of transmembrane pressure during experimental campaign I is also presented. It was observed (Fig. 5(a)) that the increase in cytotoxicity in the supernatant of the MBR pilot occurred around the days when transmembrane pressure increased: in day 64 TMP was 1.35 bars and viability (proliferation) was above 120%, in day 108 TMP was 1.35 bars and viability (proliferation) was above 120% and in day 1.45 TMP was 1.5 and viability (death) was below 80%. Variation in permeate toxicity (Fig. 5(b), ranged from 92% to 105%) was less pronounced than in supernatant samples. This means that the fouled state of the membrane seems to be responsible for the toxicity retention; it participates in the toxicity concentration in the supernatant when the membrane is fouled and the concentration decreases when the membrane is clean or only slightly fouled.

Fig. 6 shows supernatant and permeate MBR pilot cytotoxicity and the variation of the humic acid concentration during experimental campaign I. The same comparison was attempted with protein and polysaccharides but without any significant results (results not shown). Increased cytotoxicity was observed in the supernatant on days when the concentration of humic acid also increased (Fig. 6(a)). Once again, because the variation in permeate toxicity (Fig. 6(b), ranged from 92% to 105%) was less pronounced than in supernatant samples, it was not possible to draw any conclusions. Nevertheless, Fig. 6(a) strongly suggested that these toxic compounds could be adsorbed on humic substances, which are themselves retained by the membrane and are known to contribute strongly to membrane fouling in MBR. Previously sorption

Fig. 4. (a) CP removal efficiencies: ●, experimental campaign I; and □, experimental campaign II. (b) Cytotoxicity variation compared to control (100% viability). Experimental campaign II: ■, influent; ○, supernatant; and ●, permeate.

Fig. 5. (a) Supernatant cytotoxicity and transmembrane pressure variation compared to control (100% viability) during experimental campaign I: □, TMP; ●, cytotoxicity. (b) Permeate cytotoxicity and transmembrane pressure variation compared to control (100% viability) during experimental campaign I: □, TMP; ●, cytotoxicity.
studies (Ra et al., 2008) revealed that some micropolllutants (i.e., diclofenac) could be sorbed onto suspended particles coated by humic acid and the corresponding reduction in their aquatic toxicity (Borcherding and Wolf, 2001). Moreover, in our study, it was previously observed that, during both campaigns, there was no retention of the CP alone by the membrane during filtration, whatever its degree of fouling (Fig. 3). It can be assumed that the toxicity retention observed was principally due not to CP but to CP metabolites, which may be adsorbed on the colloidal phase or flocs. This is in agreement with previous work reporting that CP is a produg that requires biotransformation to become cytotoxic (Sladek, 1994).

The results of this study prove that advanced wastewater treatment using an MBR provides a suitable process for lowering CP concentrations before discharge into the aqueous environment. However, residual toxicity was measured at permeate. Therefore, a tertiary treatment step is needed. In this way, Wang et al. (2009) studied the rejection of CP by nanofiltration (NF) and reverse osmosis (RO) membranes from ultrapure (Milli-Q) water and membrane bioreactor (MBR) effluent. The authors showed that the RO membrane provided excellent rejection (>90%) under all operating conditions. Conversely, efficiency of CP rejection by NF membrane was poor: in the range of 20–40% from Milli-Q water and around 60% from MBR effluent (i.e., membrane fouling and interactions between the CP and water matrix appeared to contribute to the higher rejection of CP).

Finally, our study points out that detailed understanding of the behavior of pharmaceuticals in the wastewater treatment requires consideration of their microbial metabolites, i.e., the removal of both parent drug and metabolites toxicity. Moreover, it is also important to evaluate the toxicity in the total bioreactor matrix (liquid-soluble phase, colloidal phases and solid phase) to determine if toxicity is completely eliminated, or just transferred to another phase.

The long-term evaluation of membrane bioreactor technology on an industrial scale for hospital effluent, preferably at the outlet of an oncology department, or on wastewater from the pharmaceutical industry will ultimately clarify industrial-scale membrane bioreactor potential in the chemical removal of cytotoxic compounds and their associated toxicity. However, membrane bioreactors offer two important advantages for the elimination of pharmaceutical compounds from hospital wastewater: (1) the compact design allows implementation at the hospital site and (2) the possibility of operation at high sludge ages to adapt the biomass to the micropollutant. In these conditions, the amount of sludge produced is reduced and the simultaneous elimination of the COD and nitrogen can be conducted under selected operating conditions. In addition, if the sludge became toxic when treating hospital wastewater, treatment by incineration would become relevant. The decrease in the quantity of sludge to be incinerated would entail a reduction in operating costs.

4. Conclusion

The analyses performed allow us to draw the following conclusions:

- CP removal of up to 80% was achieved under a HRT of 48 h and a SRT of 50 days.
- During experimental campaign I, the increase of TMP was associated with an increase of supernatant toxicity. Peaks of supernatant cytotoxicity corresponded with peaks in supernatant humic acid contents. It could be suggested that molecules with a toxic effect may be adsorbed on humic acids substance.
- Despite advanced MBR wastewater treatment for CP and cytotoxic removal, a tertiary treatment is necessary for the complete elimination of cytostatic compound toxicity.

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