Effect of cytostatic drugs on microbial behaviour in membrane bioreactor system

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A B S T R A C T

The aim of this work is to evaluate the influence of cyclophosphamide and its principal metabolites (CPs) on microbial behaviour in a membrane bioreactor system. Two laboratory-scale membrane bioreactors (MBR) were run in parallel with a sludge retention time of 70 days (one with the cytostatic drugs, MBR-CPs, the second without, MBR-control). The microbial activity was measured by respirometric analysis. The endogenous and exogenous respirations of heterotrophic micro-organisms were evaluated. Micro-organisms exposed to CPs showed higher endogenous respiration rates and lower exogenous respiration rates than micro-organisms present in MBR-control. The effects were observed several days after adding the cocktail. Reduced sludge production was observed in MBR-CPs compared to MBR-control. This reduction of sludge production and the increase in the endogenous respiration rate in relation to MBR-control suggest that the chemical stress caused by CPs led to a diversion of carbon and/or energy from growth to adaptive responses and protection. In addition, the inhibitory effect on the assimilation of exogenous substrate (reduced exogenous respiration rate) suggests an inhibition of catabolism and anabolism despite the low CPs concentration studied (μg/L). However, this inhibitory effect can be offset by the biomass still active under low ratio (substrate/biomass) conditions in the bioreactor (due to complete retention of biomass and high sludge age), which helped to maintain high overall performance in the removal of conventional pollution.

1. Introduction

The increasing use of anticancer drugs and their presence in wastewater is a relatively new issue and few studies have been published (Buerge et al., 2006; Johnson et al., 2008; Kümmerer, 2001; Lenz et al., 2005, 2007; Mahnik et al., 2007; Steger-Hartmann et al., 1997). 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). They usually enter the 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 (Mahnik et al., 2007). The compounds reach the aquatic environment via hospital or domestic wastewater and wastewater treatment plants (WWTPs) (Buerge et al., 2006).

The alkylating antineoplastic drug cyclophosphamide (CP) is one of the oldest known cytostatics and is one of the most frequently used in cancer chemotherapy (Huitema Alwin et al., 2001). CP is a prodrug that requires biotransformation to become cytotoxic (Moore, 1991; Sladek, 1994). It is transformed by hepatic and intracellular enzymes to active alkylating metabolites, 4-hydroxycyclophophosphamide, aldophosphamide, acrolein and phosphoramide mustard (Joqueviel et al., 1998). CP has been detected in concentrations ranging from 20 ng/L to 4.5 μg/L in hospital sewage. The presence of CP has also been proved in samples from the influent and the effluent of the communal sewage treatment plant into which a hospital's sewage water is discarded. Concentrations ranged from 7 to 143 ng/l (Steger-Hartmann et al., 1997). Cyclophosphamide has been detected in surface waters in Switzerland, where 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 the 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).

The occurrence and fate of pharmaceutically active compounds in the natural environment has been recognized as one of the emerging issues in environmental chemistry (Halling-Sorensen et al., 1998; Sarmah et al., 2006). Although much research is being directed toward the study of micropollutant removal mechanisms (Byrns, 2001; Dionisi et al., 2006; Urase and Kikuta, 2005) little attention is being paid to the effect of micropollutants on the

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performance of treatment plants. In such plants, which rely on robust microbial activity, a chemical perturbation may cause toxicity, deflocculation, disrupted nitrification and incomplete treatment (Love and Bott, 2002; Paxeus, 1996).

In the activated sludge process, oxygen consumption is directly associated with both substrate removal and biomass growth. Therefore, the oxygen uptake rate per unit volume per unit time (OUR) is widely recognized as an important parameter for monitoring the biomass viability (Spanjers et al., 1998).

Oxygen consuming reactions correspond to: (1) oxidation of organic substances; (2) synthesis of organic biomass; (3) auto-oxidation of organic biomass. The first two reactions correspond to exogenous substrate oxidation: a part of the organic compounds is oxidized to CO_2 and H_2O (catabolic processes), and a part is utilized for the synthesis of reserve material and new cells (anabolic processes) (Rodde-Pellegrin et al., 2002).

The aim of this work is to evaluate the influence cyclophosphamide and its principal metabolites (all called CPs in what follows) on microbial behaviour 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 (MBR-CPs), and one without (MBR-control). The microbial activity of activated sludge from the two MBRs was measured by respirometric analyses. In order to verify whether the addition of cyclophosphamide and its principal metabolites could affect the biological activity, the endogenous and exogenous respirations of heterotrophic micro-organisms from two activated sludges were measured and a comparison was made between the activated sludge from the control (without drugs, MBR-control) and CPs membrane bioreactor (presence of cytostatic drugs, MBR-CPs).

2. Methods

2.1. Pilot scale experiments

Two identical lab-scale crossflow MBR were run in parallel. One of them was used as a control (MBR-control), while cyclophosphamide (5 μg/L) and its principal metabolites (acrolein 2250 ng/l, phosphoramide mustard 8880 ng/L, 4-ketocyclophosphamide (Keto CP) 580 ng/L, and nitrogen mustard 517 ng/L) were continuously added to the other (MBR-CPs). Each reactor was run for 223 days.

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

The membrane modules were ceramic tubular Membralox® (MF) 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). Dissolved oxygen and pH were monitored in the bioreactor. The operating conditions of the membrane bioreactors (MBRs) during the experimentation are given in Table 1. Treatment was operated in aerobic/anoxic conditions to allow nitrification and denitrification of the influent.

2.2. Wastewater and micro-organisms

Each reactor was inoculated with the same activated sludge from a municipal wastewater treatment plant (dry weight, 3 g/L). Raw water was composed of domestic water (14.89 L/day, from the same wastewater treatment plants, Brax, France, 2000 person-equivalent) pre-screened to 200 μm and completed with Viandox® (0.11 L/day, commercial product, soya bean extract) so as to reach the chemical oxygen demand (COD) required to achieve high volumetric loading rates of 1.27 Kg COD m^{-3} d^{-1}.

Table 1
Operating conditions of the membrane bioreactors (MBRs) during the experimentation.

Parameter	
Working volume (L)	20
Temperature (°C)	25–32
pH	7–8
Inlet COD (mg DCO/L)	1694.7 ± 629.6
Average volumetric organic load (kg COD m^{-3} d^{-1})	1.27 ± 0.42
Average F/M ratio (kg CODinlet/KgMLSS/d) at steady state	0.11
Solids retention time (SRT) (d)	70
Hydraulic retention time (HRT) (h)	32
Aeration cycle	2 min aeration/17 min without aeration
Dissolved oxygen concentration (mgO_2/L)	0–4.5
Crossflow velocity (m/s)	4–5

2.3. 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). Particle size distributions of the activated sludge were measured by the light scattering method (Malvern MasterSizer/E, UK).

2.4. Biological activity measurement

In order to determine microbial activity, representative samples were taken within the MBRs at various times, and endogenous and exogenous respiration rates were evaluated.

The endogenous respiration rate is defined as the rate of oxygen consumption in the absence of exogenous substrate and includes consumption for bacterial growth-decay cycle, maintenance energy production and protozoa respiration. The maximum respiration rate is defined as the rate of oxygen consumption achieved when all the individual substrates that can be oxidized by a mixed microbial population are present in excess. This condition is unlikely, but a respiration rate in the presence of an excess of a specific substrate or group of substrates can be measured (Spanjers et al., 1998).

The exogenous respiration rate is then defined as the difference between the maximum respiration rate (measured in presence of exogenous substrate) and the endogenous respiration rate (measured in absence of exogenous substrate).

2.4.1. Experimental unit

MBR sludge samples were immediately placed in the "respirometer" represented in Fig. 1a. The "respirometer" consisted of a bioreactor with a working volume of 1.5 L. The reactor was aerated through a perforated tube placed below the Rushton turbine (300 rpm). The air flow was controlled to maintain a dissolved oxygen concentration of between 2.7 and 4.2 mg/L. The temperature was controlled at 26 °C. The oxygen concentration was measured continuously by a probe (YSI 5739, with an actual temporal resolution of 1.5 s) connected to an oxymeter YSI MODEL 57. The oxygen concentration was recorded continuously with a computer. This device allowed the utilization of the dissolved oxygen concentration of the biological suspension to be observed continuously.

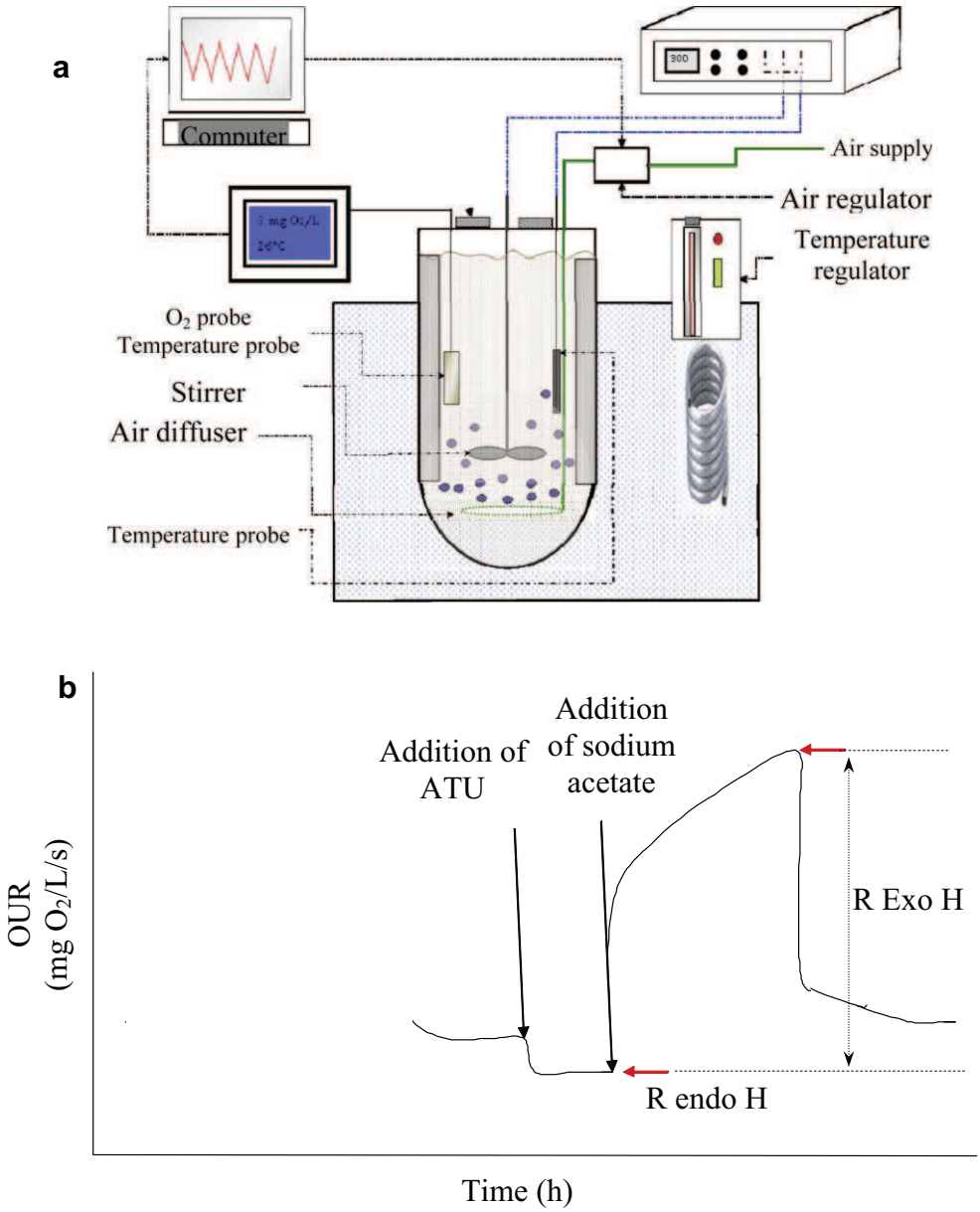

Fig. 1. (a) Experimental set-up for respirometric tests. (b) Course of a respirogram. R: respiration, Endo: endogenous, Exo: exogenous. H: heterotrophic.

The MLSS concentration in the respirometer was adjusted to 2 g/L. This conditions (ratio of concentrations [initial substrate/initial biomass] = 0.1 g COD/g MLSS) was determined in order to get a system response sufficient for interpretation (a reliable "respirogram"). If relatively little biomass is present then the measured respiration rate may be too small and the test would take too long time. If there is more biomass relatively to the amount of substrates, then the respirometric response may be too short for a reliable measurement, or be swamped into the endogenous rate (Vanrolleghem et al., 1999).

2.4.2. Methodology

2.4.2.1. Short-term exposure analysis. The concentrations of pharmaceutical compounds used (CPs cocktail) in the following two respirometer tests (short-term exposure) are presented in Table 2. The concentrations used were almost 35 times those added continuously to the MBR CP during the experimental campaign.

2.4.2.1.1. Test 1. Effect on biological activity after a toxic pulse (CPs). In this experiment we evaluated the influence of a pulse of a relatively concentrated mixture of cyclophosphamide and its principal metabolites on the biological activity of heterotrophic microorganisms by following the evolution of dissolved oxygen in the "respirometer". In this case, the exposure time was very short. This experiment was repeated twice. The respirometric test proceeded as follows.

A sample of sludge from the MBR-control bioreactor was placed in the respirometer (activated sludge sample taken from MBR-con-

Table 2
CPs cocktail. Pharmaceutical compound concentrations in the respirometer. Test assessing the effects of CPs cocktail on microbial activity by short-term exposure.

Compound	Concentration in respirometer (μg/L)
Cyclophosphamide	176
Acrolein	80
Phosphoramide mustard	312
Dechloroethylcyclophosphamide	59
4-Ketocyclophosphamide	37
Nitrogen mustard	18

trol on day 80). The MLSS concentration in the respirometer was adjusted to 2 g/L. Respiration data were calculated from the slope of decrease in dissolved O_2 observed between two thresholds of 4.2 mg/L and 2.7 mg/L. A continuous supply of air for several hours led to the exhaustion of the exogenous substrate present in the supernatant. Following this depletion, the micro-organisms were in the physiological state of endogenous respiration. Once this had been achieved, a known amount of sodium acetate (1 ml of a 230 mg/mL acetate solution) was added into the "respirometer".

The rate of oxygen consumption was recorded versus time. After the exhaustion of the added substrate, the micro-organisms were maintained in endogenous respiration. The CPs cocktail was then added. More than 2 h later, the same amount of sodium acetate was again added to the respirometer. If there was an inhibitory effect, oxygen consumption for total assimilation of the added COD would be lower than after the first addition.

2.4.2.1.2. Test 2. Effect on biological activity of 4 days of exposure to CPs. The biomass remained in contact with the pharmaceutical compounds in relatively high concentrations for 4 days (three times the hydraulic retention time in MBRs, 32 h). An exogenous supply of carbon and nitrogen substrate was provided continuously. This experiment was performed twice. The respirometric test proceeded as follows.

An activated sludge sample from MBR-control (on day 136) was taken. The aim of this experiment was to evaluate the influence of the CPs cocktail on the activity of the biomass during 4 days of exposure. MLSS concentration in the respirometer was 2 g/L in a total volume of 1.5 L. A continuous supply of air for several hours led to the exhaustion of the exogenous substrate present in the supernatant. Respiration data were calculated from the slope of decrease in dissolved O_2 observed between the two thresholds of 4.2–2.7 mg/L. As the residual substrate was exhausted, the sludge reached a stable respiration known as endogenous respiration. A mixture (300 ml) of NH_4Cl and CH_3COONa (sodium acetate) was added continuously through a feed pump at a very low rate (approximately 75 ml/day) to ensure the provision of carbon and nitrogen exogenous substrates for heterotrophic and autotrophic nitrifying micro-organisms (both ammonium and nitrite oxidizers), respectively. The total volume increase in the respirometer due to the addition of exogenous substrate was 20%. The dilution effect on the CPs concentration was negligible with respect to the toxic effect under study.

The CPs cocktail was added once the oxygen consumption rate in the respirometer had reached a roughly constant value. If the cocktail had an inhibiting effect on the biological activity in the conditions studied, the rate of oxygen consumption should decrease.

2.4.2.2. Long-term exposure analysis. Fig. 1b gives an overview of the respirometric experiment performed to measure the endogenous and exogenous respiration of heterotrophic bacteria.

Prior to the experiment, the investigated sludge was aerated for 3 h so that all readily degradable substances were consumed. Respiration was calculated from the slope of the decrease in dissolved O_2 between two thresholds of 4.2–2.7 mg/L. As the residual substrate became exhausted, the sludge reached a stable respiration known as endogenous respiration. A solution of allylthiourea (ATU) at 10 g/L was added to the activated sludge as a selective inhibitor of nitrification. ATU is a selective inhibitor of nitrosobacteria (Nitrosomonas), bacteria that convert ammonium to nitrite. After the stabilization period of endogenous respiration (Fig. 1b, R endo H), a carbon substrate was injected into the reactor for heterotrophic micro-organisms. In this study, sodium acetate was selected as the carbonaceous substrate (CH_3COONa) because it is readily biodegradable by most heterotrophic populations. The addition of substrate caused an increase in the rate of oxygen consumption. The oxygen consumption rate was proportional to the kinetics of aerobic biodegradation of the substrate by the heterotrophic bacteria of the activated sludge. If the concentration of substrate was sufficient, the rate of oxygen consumption reached its maximum value, corresponding to the maximum respiration rate. Once all the substrate had been oxidized, the activated sludge returned to the endogenous physiological state.

The data were then analyzed with the Respiroexpert software developed at the laboratory. Mixed liquor suspended solids (MLSS) were measured in (g/L) before the beginning of each experiment, i.e. after the permanent aeration of 3 h.

3. Results

Two laboratory-scale membrane bioreactors (MBR) were run in parallel, one with the cytostatic drugs (MBR-CPs), and one without (MBR-control). A comparison between the two reactors was made for mixed liquor suspended solids (MLSS), observed sludge yield, COD removal and endogenous and exogenous respiration rates. This comparison was intended to check whether the addition of CPs could affect the microbial behaviour in a cross-flow membrane bioreactor. The MBRs were run for 223 days. Day 107 was the first day of addition of CPs into MBR-CPs. In these conditions, it has been checked that before the pharmaceutical cocktail introduction, the both bioreactor were running in a parallel similitude. 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 and any differences between the two bioreactors (running in parallel, same operating conditions, Table 1) would be due mainly to the presence of the pharmaceutical compounds (Cps).

3.1. Evolution of mixed liquor suspended solids (MLSS) concentration and sludge production

Fig. 2a shows the variation of MLSS concentration inside the two reactors during the operations. Before the CPs were added, the MLSS concentrations for both MBRs were very similar. After addition of the CPs, the MLSS concentrations for the two bioreactors increased over time but MLSS concentrations in the MBR-CPs were slightly lower than those observed in MBR-control. From 126 days to 190 days MLSS concentrations in the bioreactor MBR-CPs were slightly lower than in MBR-control. On day 157, the MLSS concentration in MBR-CPs was 8.45 g/L while in MBR-control it was 10.65 g/L.

As suspended solids were completely rejected by the membrane separation, the observed sludge yield in the bioreactor was calculated by measuring the proportions between the quantity of sludge leaving the bioreactor per time unit (g MLSS/d), and the substrate utilized per time unit (g COD/d) (Henze et al., 2001). Fig. 2b compares the observed sludge yield (Y_{obs}) in the two bioreactors calculated using the following equation:

$$Y_{OBS} = \frac{Q_p \cdot X}{Q \cdot (S_e - S)}$$

where Q_P is the sludge withdrawal rate (L/day), Q is the influent flow rate (L/day), X is the MLSS concentration (g/L), S_e is the inlet COD (g COD/L) and S is the permeate COD (g COD/L).

In our experiments, the observed sludge yield increased during the operation as the biomass concentration increased. In Fig. 2b, we see that, before the CPs were added, yields were similar and

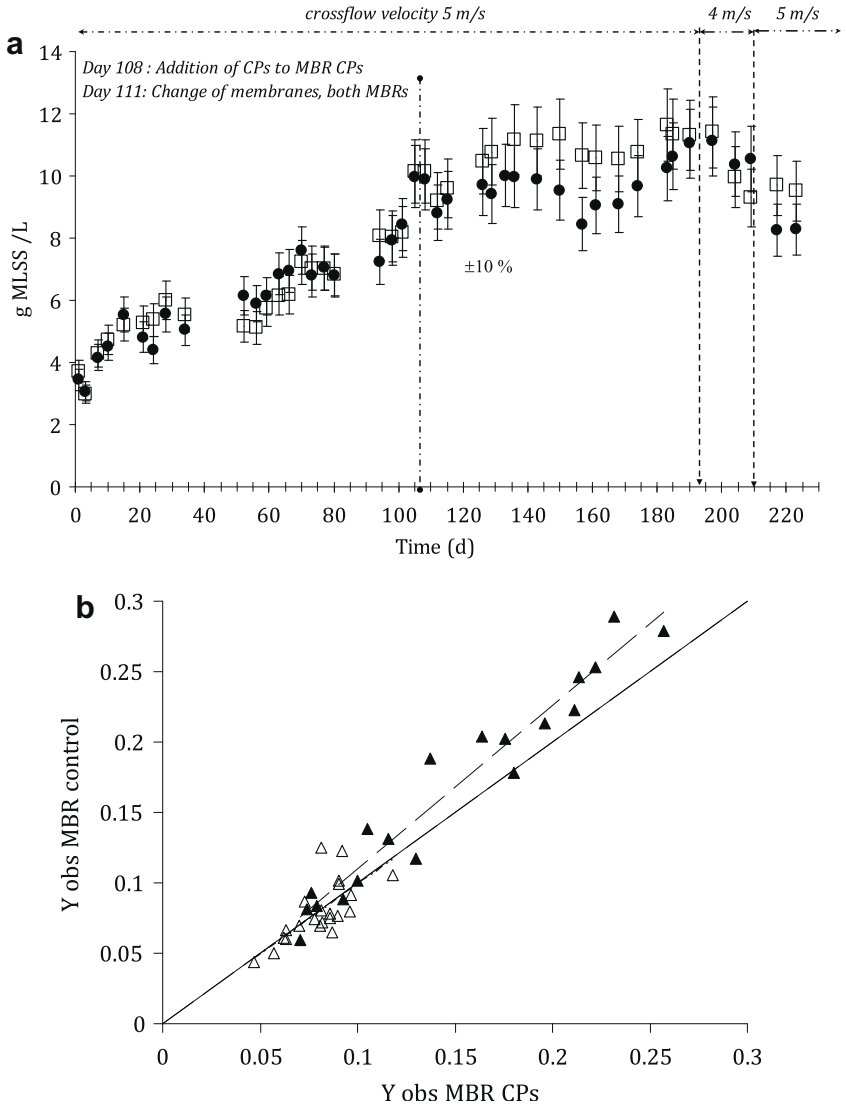

Fig. 2. (a) MLSS concentration. (●) MBR-CPs, (□) MBR-control. (b) Observed sludge yield. (△) Before addition of CPs (▲) after addition of CPs.

always on the bisector line. After the addition of the Cps, the observed sludge yield was higher in the MBR-control than MBR-CPs. This highlights the effect of CPs on the amount of biomass produced: the presence of cytostatics decreased the biomass production rate.

On the basis of these results we also expected alterations in the biological activity. To gain a better understanding of the metabolic mechanisms linked with the presence of cyclophosphamide and its principal metabolites, the microbial activity was measured by respirometric analysis.

3.2. Conventional pollution removal

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 CP reactor. Fig. 3 shows the COD removal variation for both MBRs. In addition, average total nitrogen removal was over 93% for both MBR. The removal efficiencies were almost identical in both MBRs, indicating that the addition of pharmaceuticals had a negligible effect on the efficiency of the treatment under the operating conditions studied.

3.3. Respirometry analyses

The results obtained from respirometric analyses are presented in two parts. The first concerns short-term exposure, the second deals with long-term effects.

3.3.1. Effect of CPs at high concentrations and short-term exposure

3.3.1.1. Effect on biological activity after a pulse of toxics (CPs). Fig. 4a shows the variation of the rate of oxygen consumption. The area under each peak represents the amount of oxygen necessary for the assimilation of the exogenous substrate added. Table 3 shows the calculated values (using the Respiroexpert software developed in the laboratory). We observed that the pharmaceutical compounds, at the concentrations tested, had no immediate influence on the activity of heterotrophic micro-organisms. Furthermore, after adding the cocktail, we observed no change in the endogenous respiration of the micro-organisms.

3.3.1.2. Effect on biological activity during 4 days of exposure to CPs. Fig. 4b illustrates the oxygen uptake rate variation. After an initial phase, the microbial activity stabilized at an oxygen uptake rate of 0.008 ± 0.0006 $mgO_2/L/s$ (14.4 $mgO_2/gMLSS/h$). This value

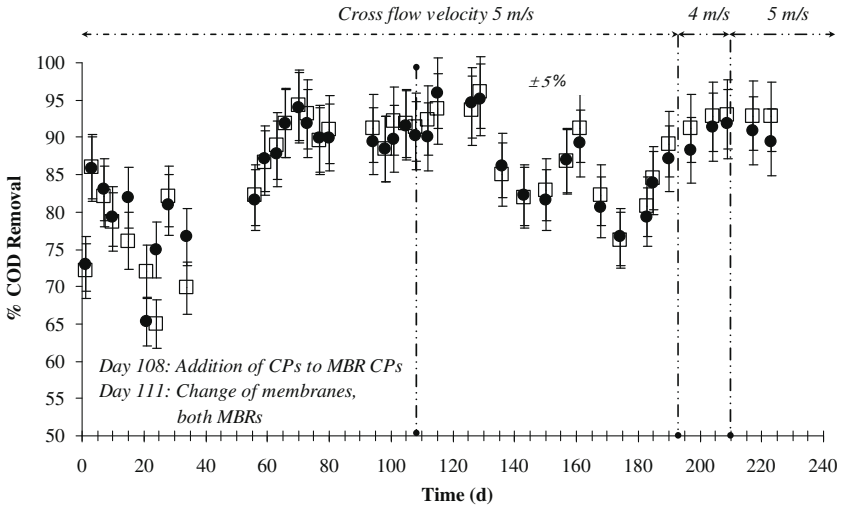

Fig. 3. COD removal. (●) MBR-CPs (□) MBR-control.

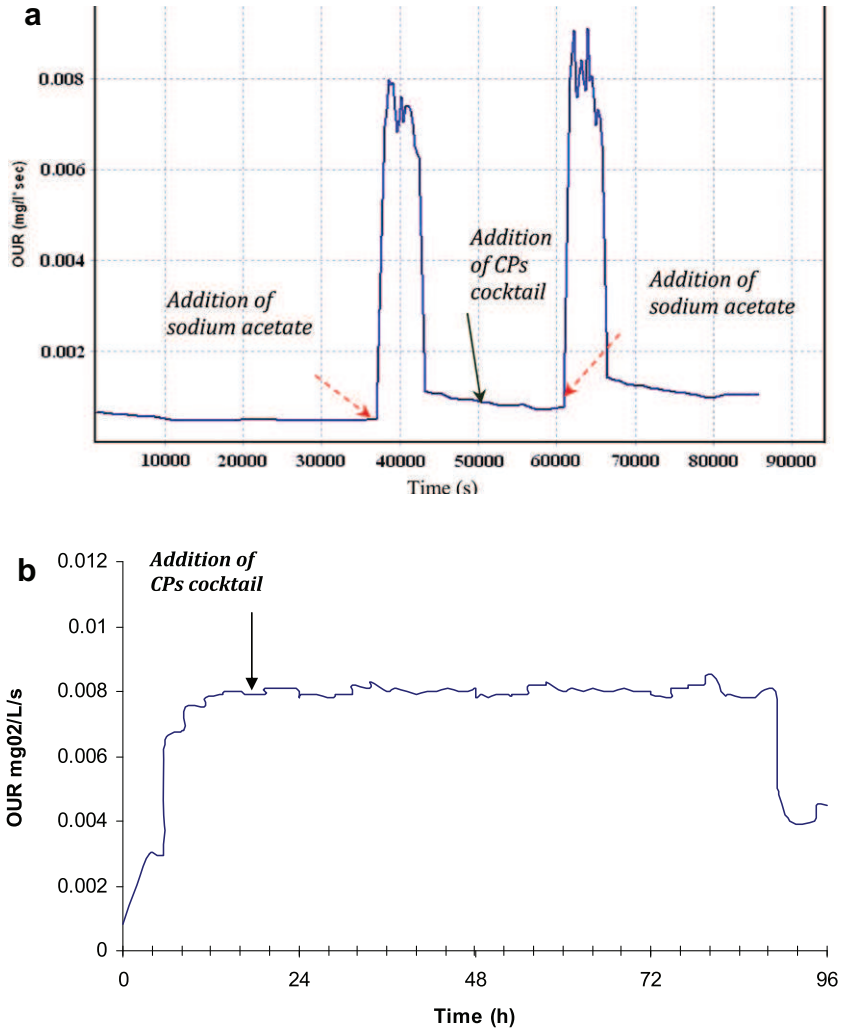

Fig. 4. Oxygen uptake rate variation. (a) Test assessing the effect of CPs on microbial activity after a pulse of cytostatic compounds. (b) Effect of CPs on microbial activity during 4 days exposure time.

remained unchanged throughout the experiment. At the end of the test, the decrease in activity was due simply to the exhaustion of the exogenous substrate added. We did not observe a reduction of microbial activity during the 4 days after addition of the cocktail. However, this experiment cannot exclude the possibility of longer-term effects of the CPs.

Table 3
Oxygen uptake for the assimilation of added exogenous substrate. Test assessing the effect of CPs on microbial activity after a pulse of cytostatic compounds.

	Oxygen uptake (mg O_2/L)
Before the addition of the CPs cocktail	33.2 ± 0.12
After the addition of the CPs cocktail	32.5 ± 0.60

Table 4
MLSS concentration and quantity of substrate added. Long-term exposure respirometric analyses.

Day	MLSS g/L	
	MBR-CPs	MBR-control
29	2.09	2.54
35	1.55	1.89
57	2.1	2.08
112	1.85	1.79
126	1.95	2.14
147	1.7	2.16
220	1.7	1.88
COD added		
Resulting concentration in the respirometer (mg COD/L)		204

3.3.2. Long-term effects of Cps on biological activity

Respirometry analyses were conducted periodically for each of the bioreactors during the experimental campaign. Comparison of the results for the reactor MBR-CPs with those of MBR-control indicated the influence of cyclophosphamide and its principal metabolites on the biological activity.

Table 4 lists the sampling days, MLSS concentration and quantity of substrate added in respirometry analysis. To overcome the differences in MLSS concentrations between respirometry analyses and to facilitate comparisons between bioreactors, the responses corresponding to heterotrophic micro-organisms are expressed as specific values, i.e., normalized by MLSS concentration.

The results were compared for MBR-CPs and MBR-control. Fig. 5a (heterotrophic specific endogenous respiration ratio) and Fig. 5b (heterotrophic specific exogenous respiration ratio) show the variation of the ratio MBR-CPs/MBR-control.

As shown in Fig. 5a, before the addition of CPs, the ratio of specific endogenous respiration was constant. After the addition of CPs, we observed an increase in the endogenous respiration of heterotrophic micro-organisms in the bioreactor MBR-CPs compared to MBR-control. As shown in Fig. 5b, on days 126 and 147, exogenous respiration of heterotrophic micro-organisms in MBR-Cps was lower than in MBR-control. On day 147, maximum inhibition of exogenous respiration was observed in comparison with the

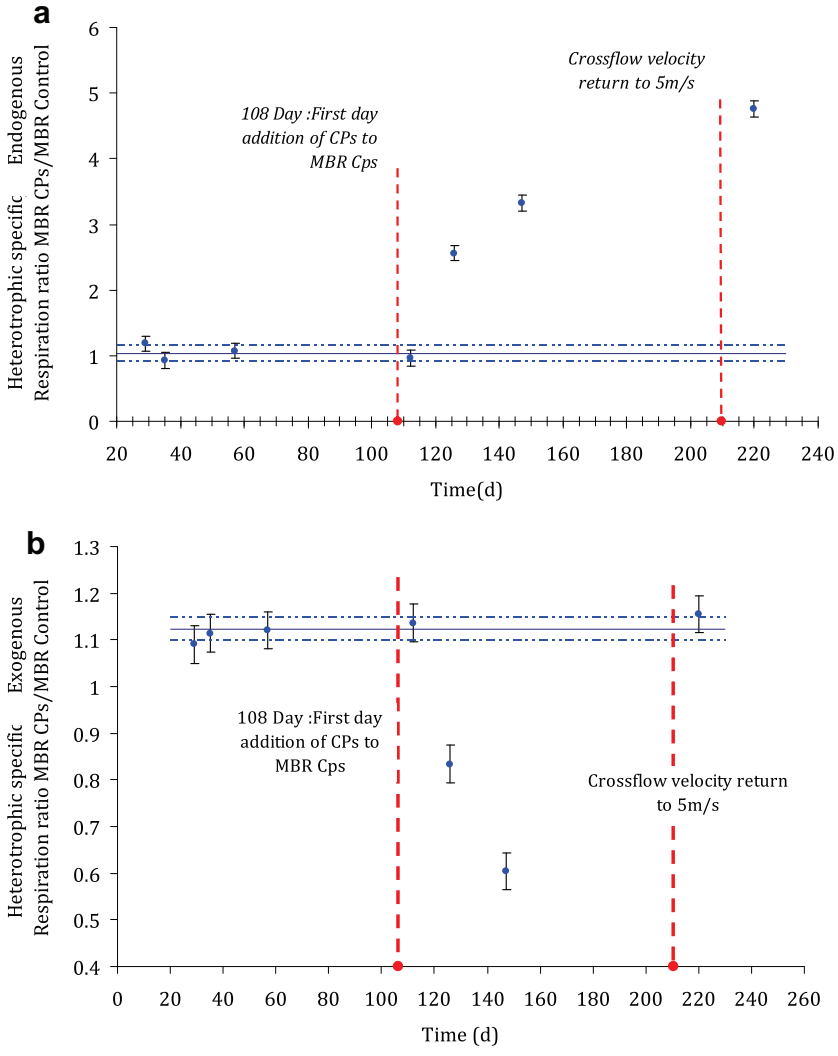

Fig. 5. (a) Heterotrophic specific endogenous respiration ratio: MBR-CPs/MBR-control. (b) Heterotrophic specific exogenous respiration ratio: MBR-CPs/MBR-control.

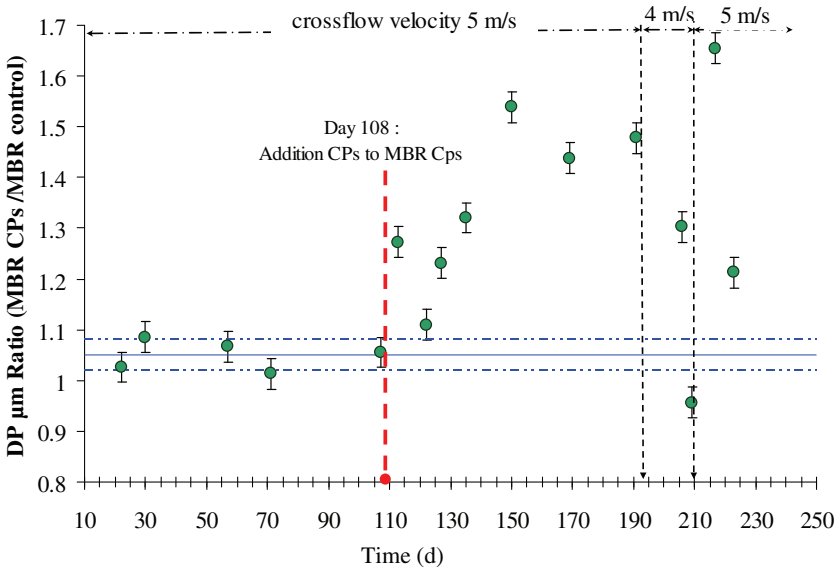

Fig. 6. Mean floc size (µm) ratio: (MBR-CPs/MBR-control).

other days. However, on day 220, exogenous respirations were similar for both bioreactors.

3.4. Effect of CPs on the mean floc size

The microbial floc sizes were monitored during the operation of both MBRs. The mean floc sizes (µm) were compared between the MBR-control and MBR-CPs. Fig. 6 shows the variation of the mean floc size (µm) ratio (MBR-CPs/MBR-control). As shown in Fig. 6, before the addition of CPs, the ratio of mean floc sizes was constant. After the addition of CPs, we observed an increase in the mean floc size in the bioreactor MBR-CPs compared to MBR-control.

4. Discussion

In our study, the feed to micro-organism (F/M) ratio was low (average F/M ratio 0.11 kg CODinlet/KgMLSS/d MBR-CPs and 0.10 Kg CODinlet/KgMLSS/d MBR-control, at steady state). In these conditions, the substrate limits the growth of micro-organisms. This supports the physiological state of endogenous respiration in the micro-organisms. Growth occurs only when the accessible exogenous substrate is greater than the minimum amount necessary to meet energy maintenance needs (Witzig et al., 2002). In limited, low substrate/biomass conditions (low F/M ratios), the literature reports that the substrate is essentially consumed to ensure the cell maintenance requirements rather than growth functions (Low and Chase, 1999a; Pirt, 1975). Lysis (destruction of cell membranes) and cryptic growth (active cell growth on the cell contents provided by the lysis process) mechanisms are also favoured by such limited substrate conditions, which also induces a much greater reduction in sludge production (Low and Chase, 1999b). Nevertheless, despite the same daily average organic loading and the same operating conditions (i.e., same pH and temperature variations), we observed an increase in the endogenous respiration and a decrease in the exogenous respiration of heterotrophic micro-organisms in presence of CPs compared to MBR-control micro-organisms (Fig. 5a and b).

It is often accepted that the cells produce EPS for their survival and in response to environmental stress (Wingender et al., 1999) and the EPS play an important role in sludge flocculation (review of Liu and Fang Herbert, 2003). Moreover, Henriques and Love (2007) found that the EPS matrix inside sludge flocs acted as a protective barrier for bacteria exposed to the chemical toxins octanol and cadmium. Dionisi et al. (2007) found that continuous exposure to micropollutants (a synthetic mixture of 10 organic xenobiotics and two heavy metals) selected for more regular, rounded and compact flocs in an activated sludge process pilot (sequencing batch reactors). It can thus be supposed that micro-organisms produce bound EPS as a protective barrier for better survival in the presence of CPs, promoting the agglomeration of flocs and, in doing so, increasing the average floc sizes as seen in Fig. 6. A detailed study of the effect of cytostatic drug presence on extracellular polymeric substance formation will be the subject of a separate paper (manuscript in preparation).

Consistent with the first two respirometry analysis (short-term effects), the effects associated with the presence of the cytostatic drugs studied on the biomass in the bioreactor MBR-CPs, were observed after several days of exposure. In addition, the presence of CPs increased the maintenance energy requirements of autotrophic (data not shown) and heterotrophic micro-organisms. A decrease in exogenous respiration was observed for both groups of micro-organisms.

The increase in maintenance requirements in the presence of toxic chemical compounds has already been reported. Ray and Peters (2008) studied the impact of chemical stress on the microbial metabolism using chemical models such as 2,4-dinitrophenol (DNP) and pentachlorophenol (PCP). The biological activity of *Pseudomonas aeruginosa* was measured in batch reactors, in the presence and absence of a toxic chemical compound with sub-lethal concentrations. Chemical stress is defined as exposure of a cell to a chemical pollutant at a concentration that is sub-lethal but potent enough to elicit adaptive protective responses. The authors report an increase in the endogenous decay coefficient, b, with increasing concentration of PCP and DNP. Moreover, in this study, Ray and Peters (2008) formulate the following hypothesis regarding the impact of chemical stress on the microbial metabolism: between a lethal and an innocuous concentration, there exists a range of concentrations at which a cell's exposure to a stressor causes a diversion of carbon and/or energy from growth and thereby a decrease in observed biomass yields. Alternatively, there are concentrations at which a stressor has an inhibition effect which would simultaneously slow the rates of growth and substrate utilization,

and there are concentrations at which a stressor has a toxicity effect leading to cell death for some or all of the population.

In this context, the observed increase of endogenous respiration and the reduction of biomass production observed in the presence of cyclophosphamide and its principal metabolites in relation to MBR-control, suggest that part of the of carbon and energy resources are diverted from growth and used in stress management and protection. In addition, the fact that the exogenous respiration also decreased for micro-organisms exposed to CPs in relation to MBR-control (exogenous respiration rate measured in the respirometer, under non-limited substrate conditions), shows that the appropriate CP/metabolites concentration(s) was (were) large enough to decrease the rate of substrate utilization at the same time.

However, due to the complete retention of biomass, MBR can be operated at much higher sludge concentrations and the high sludge retention time (in these conditions, the substrate limits the growth of micro-organisms) allows for adaptation of the micro-organisms in general, and of potentially slow-growing specialist bacteria in particular, and establishes a more diverse microbial community with broader physiological capabilities. Thus this inhibitory effect could be offset by the biomass still active under low ratio (substrate/biomass) conditions in the bioreactor to maintain high overall performance in the removal of COD (Fig. 3) and TN.

The decrease in the rate of substrate utilization by biomass caused by CPs could correspond to an increase in the amount of substrate available for active micro-organism. In limited substrate conditions, an increase of available substrate represents an increase in the oxygen consumption rate of heterotrophic bacteria still active in the bioreactor.

Our results corroborate the previous study on the application of MBR technology in wastewater treatment for removal of cyclophosphamide (Delgado et al., in press). Under steady state conditions (sludge age 50 days), cyclophosphamide removal remained quite stable (75–80%) and COD and total nitrogen removal efficiency were not altered by the anticancer drug toxicity.

5. Conclusion

The analyses performed on microbial behaviour for MBR-CPs and MBR-Control allow us to draw the following conclusions:

- Reduction of sludge production and increase in the endogenous respiration rate suggest that the chemical stress caused by CPs causes a diversion of carbon and/or energy from growth to adaptive responses and protection.
- Reduced exogenous respiration suggests an inhibition of catabolism and anabolism. However, this inhibitory effect can be offset by the biomass still active under low substrate/biomass ratio in the bioreactor, which helped to maintain high overall performance in the removal of conventional pollution.

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References

Buerge, I.J., Buser, H.R., Poiger, T., Müller, M.D., 2006. Occurrence and fate of the cytostatic drugs cyclophosphamide and ifosfamide in wastewater and surface waters. Environmental Science and Technology 40, 7242–7250.

Byrns, G., 2001. The fate of xenobiotic organic compounds in wastewater treatment plants. Water Research 35, 2523.

Delgado, L.F., Dorandeu, C., Marion, B., Gonzales, C., Faucet-Marquis, V., Schetrite, S., Albasi, C., in press. Removal of a cytostatic drug by a membrane bioreactor. Desalination and water treatment.

Dionisi, D., Bertin, L., Bornoroni, L., Capodicasa, S., Petrangeli Papini, M., Fava, F., 2006. Removal of organic xenobiotics in activated sludges under aerobic conditions and anaerobic digestion of the adsorbed species. Journal of Chemical Technology and Biotechnology 81, 1496.

Dionisi, D., Levantesi, C., Majone, M., Bornoroni, L., De Sanctis, M., 2007. Effect of micropollutants (organic xenobiotics and heavy metals) on the activated sludge process. Industrial and Engineering Chemistry Research 46, 6762–6769.

Halling-Sorensen, B., Nors Nielsen, S., Lansky, P.F., Ingerslev, F., Holten Lützhoft, C., Jorgensen, E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. Chemosphere 36, 357–393.

Henriques, I.D.S., Love, N.G., 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. Water Research 41 (18), 4177–4185.

Henze, M., Harremoes, P., Jansen, J.L.C., Arvin, E., 2001. Wastewater Treatment: Biological and Chemical Processes, third ed. Springer Verlag, Berlin Heidelberg, ISBN 3-540-42228-5.

Huitema Alwin, D.R., Cindy, Reinders, Tibben Matthijs, M., Rodenhuis Sjoerd, Beijne Jos, H., 2001. Sensitive gas chromatographic determination of the cyclophosphamide metabolite 2-dechloroethylcyclophosphamide in human plasma. Journal of Chromatography B 757 (2001), 349–357.

Johnson, A.C., Jürgens, M.D., Williams, R.J., Kümmerer, K., Kortenkamp, A., Sumpter, J.P., 2008. Do cytotoxic chemotherapy drugs discharged into rivers pose a risk to the environment and human health? An overview and UK case study. Journal of Hydrology 348, 167–175.

Joqueviel, C., Martino, R., Gilard, V., Malet-Martino, M., Canal, P., Niemeyer, U., 1998. Urinary excretion of cyclophosphamide in humans, determined by phosphorus-31 nuclear magnetic resonance spectroscopy. Drug metabolism and Disposition 26 (5), 418–428.

Kümmerer, K., 2001. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources – a review. Chemosphere 45, 957–969.

Lenz, K., Hann, S., Koellensperger, G., Stefánka, Z., Stingeder, G., Weissenbacher, N., Mahnik, S.N., Fuerhacker, M., 2005. Presence of cancerostatic platinum compounds in hospital wastewater and possible elimination by adsorption to activated sludge. Science of the Total Environment 345, 141–152.

Lenz, K., Koellensperger, G., Hann, S., Weissenbacher, N., Mahnik, S.N., Fuerhacker, M., 2007. Fate of cancerostatic platinum compounds in biological wastewater treatment of hospital effluents. Chemosphere 69, 1765–1774.

Liu, Yan, Fang Herbert, H.P., 2003. Influences of extracellular polymeric substances (EPS) on flocculation, settling and dewatering of activated sludge. Critical Reviews in Environmental Science and Technology 33 (3), 237–273.

Love, N.G., Bott, C.B., 2002. Evaluating the role of microbial stress response mechanisms in causing biological treatment system upset. Water Science and Technology 46 (1–2), 11–18.

Low, E.W, Chase, H.A., 1999a. Reducing production of excess biomass during wastewater treatment. Water Research 33 (5), 1119–1132.

Low, E.W, Chase, H.A., 1999b. The effect of maintenance energy requirements on biomass production during wastewater treatment. Water Research 33 (3), 847–853.

Mahnik, S.N., Lenz, K., Weissenbacher, N, Mader, R.M., Fuerhacker, M., 2007. Fate of 5-fluorouracil, doxorubicin, epirubicin, and daunorubicin in hospital wastewater and their elimination by activated sludge and treatment in a membrane-bio- reactor system. Chemosphere 66, 30–37.

Moore, M.J., 1991. Clinical pharmacokinetics of cyclophosphamide. Clinical Pharmacokinetics 20, 194–208.

Paxeus, N., 1996. Organic pollutants in the effluents of large wastewater treatment plants in Sweden. Water Research 30 (5), 1115–1122.

Pirt, S.J., 1975. Principles of Microbe and Cell Cultivation. Blackwell Scientific Publications, London, UK.

Ray, S., Peters, C.A., 2008. Changes in microbiological metabolism under chemical stress. Chemosphere 71, 474–483.

Rodde-Pellegrin, M.L., Wisniewski, C., Grasmick, A., Tazi-pain, A., Buisson, H., 2002. Respirometric needs of heterotrophic populations developed in an immersed membrane bioreactor working in sequenced aeration. Biochemical Engineering Journal 11, 2–12.

Sarmah, A.K., Meyer, M.T., Boxall, A.B.A., 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65, 725–759.

Sladek, N.E., 1994. Metabolism and pharmacokinetic behavior of cyclophosphamide and related oxazaphosphorines. In: Powis, G. (Ed.), Anticancer Drugs: Reactive Metabolism and Drug Interactions. Pergamon Press, Oxford, UK, pp. 79–156.

Spanjers, H, Vanrolleghem, PA, Olsson, G, Dold, PL., 1998. Respirometry in Control of the Activated Sludge Process: Principles. IAWQ Scientific and Technical Report No. 7. IAWQ Publishing, London, UK.

Steger-Hartmann, T., Kümmerer, K., Hartmann, A., 1997. Biological degradation of cyclophosphamide and its occurrence in sewage water. Ecotoxicology and Environmental Safety 36, 174–179.

Urase, T., Kikuta, T., 2005. Separate estimation of adsorption and degradation of pharmaceutical substances and estrogens in the activated sludge process. Water Research 39, 1289.

Vanrolleghem, P.A., Spanjers, H., Petersen, B., Ginestet, P., Takacs, I., 1999. Estimating (combinations of) activated sludge model No. 1 parameters and components by respirometry. Water Science Technology 39 (1), 195–214.
Wingender, J., Neu, T.R., Flemming, H.C., 1999. What are bacterial extracellular polymer substances? In: Wingender, J., Neu, T.R., Flemming, H.C. (Eds.), Microbial Extracellular Polymeric Substances. Springer, Heidelberg, pp. 1–19.
Witzig, R., Manz, W., Rosenberger, S., Krüger, U., Kraume, M., Szewzyk, U., 2002. Microbiological aspects of a bioreactor with submerged membranes for aerobic treatment of municipal wastewater. Water Research 36 (2), 394–402.