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Biodegradation by activated sludge and toxicity of tetracycline into a semi-industrial membrane bioreactor

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ABSTRACT

Much attention has been devoted recently to the fate of pharmaceutically active compounds such as tetracycline antibiotics in soil and water. Tetracycline (TC) biodegradability by activated sludge derived from membrane bioreactor (MBR) treating swine wastewater via CO_2-evolution was evaluated by means of modified Sturm test, which was also used to evaluate its toxicity on carbon degradation. The impact of tetracycline on a semi-industrial MBR process was also examined and confronted to lab-scale experiments. After tetracycline injection in the pilot, no disturbance was detected on the elimination of organic matters and ammonium (nitrification), reaching after injection 88% and 99% respectively; only denitrification was slightly affected. Confirming the ruggedness and the superiority of membrane bioreactors over conventional bioreactors, no toxicity was observed at the considered level of TC in the pilot (20 mg TOC L^{-1}), while at lab-scale sodium benzoate biodegradation was completely inhibited from 10 mg TOC L^{-1} TC. The origin of the activated sludge showed a significant impact on the performances, since the ultimate biodegradation was in the range −50% to −53% for TC concentrations in the range 10–20 mg TOC L^{-1} with conventional bioreactor sludge and increased to 18% for 40 mg TOC L^{-1} of TC with activated sludge derived from the MBR pilot. This confirmed the higher resistance of activated sludge arising from membrane bioreactor.

1. Introduction

Brittany concentrates 57% of the French pigs' production. The public is becoming increasingly concerned with the livestock industries potential impact on water and air quality, which are damaged by an excess of different chemical compounds. The European project "Zero Nuisance Piggeries' proposes the implantation of a global management of piggeries for the treatment of swine manure in a semi-industrial scale" (Prado et al., 2007).

The system combines a flushing technology of the piggeries; this technology conducts to Dilute Swine WasteWater (DSWW). The flush is followed by a mechanical liquid/solid separation (centrifugation) of the DSWW and a biological treatment by reaction/separation coupling (submerged membrane bioreactor). Regular flushing helps to prevent anaerobic decomposition of the manure, being the main process responsible for the release of odour creating compounds. The bioreactor aims at reducing organic and nitrogen content of the DSWW, while the membrane withholds pathogens and allows the reuse of water for flushing purposes. Membrane bioreactors are used in urban wastewater (Yeom et al., 1999; Shim et al., 2002), industrial wastewater and agricultural wastewater (Cicek, 2003). But only a small part of semi-industrial and industrial processes reuses effectively the treated water.

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). Amongst them, tetracycline (TC) is an important broad-spectrum antibiotic, widely prescribed in pigs' production. Several authors (Halling-Sorensen, 2001; Loke et al., 2002; Sengelov et al., 2003) described non-negligible concentrations of tetracycline found in pig slurry (up to 5 mg L^{-1}). The fate of tetracycline and antibiotics in general in a membrane bioreactor is not widely documented. Several authors studied the interaction between activated sludge processes and pharmaceutically active compounds (Halling-Sorensen, 2001; Göbel et al., 2005; Kim et al., 2005; Joss et al., 2006; Radjenovic et al., 2007). The fate of pharmaceuticals has been demonstrated highly dependant to process parameters (Clara et al., 2005; Kim et al., 2005; Joss et al., 2006).The possibility to control the Sludge Retention Time (SRT) in MBR is better than in Classical Activated Sludge System (CASS). Moreover, due to the higher biomass concentration, MBR are less sensitive to fluctuation than CASS (Radjenovic et al., 2007).

During passage through Activated Sludge (AS) treatment plants, antibiotics removal may occur by hydrolysis, biodegradation and/or sorption to sludge. Sorption to sludge leads to an overestimation

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Nomenclature

AS	activated sludge
Biod%	percentage of biodegradation
$Biod_{res}$	percentage of residual biodegradation
COD	chemical oxygen demand (ML^{-3})
CTP	conventional treatment plant
DSWW	dilute swine wastewater
HPLC	high phase liquid chromatography
HRT	hydraulic retention time (T)
MBR	membrane bioreactor
m_{CO_2P}	mass of CO_2 produced (ML^{-3})
m_{CO_2PE}	endogen CO_2 production (ML^{-3})
m_{CO_2theo}	theorical CO_2 production (ML^{-3})
m_{CiRC}	carbon mass of the referent compound (M)
m_{CiTC}	carbon mass of the target compound (M)
MF	microfiltration
MLSS	mixed liquor suspended solid (ML^{-3})
PVDF	polyvinyl fluoride
SPE	solid phase extraction (T)
SRT	solid retention time (T)
SVU	sealed vessel unit
TC	tetracycline
$ThCO_2$	theorical CO_2 production (ML^{-3})
TN	total nitrogen (ML^{-3})
TOC	total organic carbon (ML^{-3})
TS	total solids (ML^{-3})
TU	trapped unit
VSS	volatile suspended solid (ML^{-3})

of the activated sludge treatment plants efficiency for antibiotics removal and to the release of persistent molecules after biomass death. CO_2-evolution test (OECD 301 B, 1992), formerly known as "Modified Sturm test", allows the evaluation of the biodegradability of antibiotic pollutants by activated sludge via the measure of the produced carbon dioxide. This lab-scale test was carried on 17 antibiotics where the inoculum was derived from municipal sewage treatment (Gartiser et al., 2007) showing that only penicillin had a potential for biodegradation in these conditions. However, the behaviour of tetracycline with another inoculum is not predictable.

In order to understand the impact of MBR acute contamination by tetracycline, a semi-industrial MBR was contaminated in this work. In parallel, the susceptibility of tetracycline to be degraded by AS derived from MBR treating DSWW via CO_2-evolution was evaluated. Modified Sturm tests were also used to evaluate the toxicity of TC on easily biodegradable compound biodegradation. Lab-scale and pilot-scale results were critically confronted to describe the fate of tetracycline into a semi-industrial MBR.

2. Methods

2.1. Pilot scale experiments (Biosep®)

A pilot scale experiment using a setup able to treat up to 0.4 $m^3\,day^{-1}$ of swine wastes (2.4 $m^3\,day^{-1}$ of DSWW) was conducted in Brittany at the Agriculture Applicated Research Centre in Finistere (Prado et al., 2007). A flow-sheet diagram is presented in Fig. 1. The process consists in a centrifugal apparatus, a raw slurry storage tank (1 m^3), an anoxic tank (8 m^3), an aerobic tank (12 m^3), a submerged membrane (microfiltration – MF) tank (2 m^3), a treated water storage tank (10 m^3), two flush tanks (1 m^3 each) and composting equipment. MF tank is provided of 20 polyvinylide fluoride (PVDF) membrane units of 1.4 m^2 each (in reactor 24.4 m^2).

The antibiotics contamination study was carried out under the following experimental conditions:

- the average membrane flux was 9 $L\,m^{-2}\,h^{-1}$,
- the membrane area was 27.4 m^2,
- the SRT was 30 days,
- the HRT was 3 days,
- the average food/microorganisms ratio was 0.088 kg COD kg^{-1} of SS day^{-1},
- the average volumetric load was 0.96 kg COD $m^{-3}\,day^{-1}$ and
- the operational temperature varied from 15.9 °C to 19.7 °C (summer time).

Tetracycline hydrochloride (>96% – HPLC, Fluka–Sigma–Alldrich – St Quentin Fallavier, France) was daily added directly in the anoxic tank. To calculate the quantity, the posology of TC was consulted. In case of respiratory affections, a pig receives 50 mg of TC kg^{-1} and per day during one week. If 72 animals weighing approximately 100 kg each are treated, the addition of TC is around 360 g day^{-1}. Therefore, 100 g of TC day^{-1} were added into the anoxic tank, representing 27% of the total posology, which keeps realistic. It is noticeable that, in France, since January the 1st from the year 2006, it is strictly forbidden to use antibiotics as growth factor. Antibiotics usage is restricted to therapeutic use; however, they are often administrated to a group of pigs. Therefore, the contamination lasted only 5 days and the total quantity introduced in the system was 500 g, corresponding to a TC concentration of approximately 40 mg L^{-1}, namely 22 mg TOC L^{-1} (12 m^3 total water volume in the process).

2.2. Lab-scale experiments (OECD 301 B, norm ISO 9439)

2.2.1. Biodegradation tests

Modified Sturm test (28-days aerobic degradation) was conducted following the method developed by the OECD. To determine heterotrophic activity the CO_2 uptake rate test was applied. Biomass to be characterized was placed into a sealed vessel unit (SVU). The SVU is a 1 L vessel continuously aerated with air CO_2-free (system is composed by six units). Gas from SVU was continuously transferred in CO_2 trapped unit (TU). CO_2 produced by microbial activity was trapped by a solution of barium hydroxide ($Ba(OH)_2$) which precipitate as barium carbonate in presence of CO_2. The remaining barium hydroxide was titrated with 0.05 N standard HCl in presence of phenolphthalein. The CO_2-free air production system consisted of an air compressor, two 200 ml gas wash bottle filled with 4 M NaOH, followed by one 200 ml gas wash bottle filled with 0.0125 M $Ba(OH)_2$. The CO_2-free air was passed on to an air distributor with one input and 6 output channels and through PE-tubes to the SVU. Two $Ba(OH)_2$ traps were connected to each SVU. The 6 reactors were prepared as described on Table 1; the mineral medium described by OECD 302 B was used for dilutions.

Due to the instability of barium hydroxide its concentration was checked with HCl standard solution when traps were changed. Calculations of the mass of CO_2 produced were determined from these parameters. The mass of CO_2 produced (m_{CO_2P}) in SVU reactor was calculated as follows:

$$m_{CO_2P} = (C_{Ba(OH)_2} \cdot V_{Ba(OH)_2} - C_{HCl} \cdot V_{HCl}) \cdot 44 \quad \text{(i)}$$

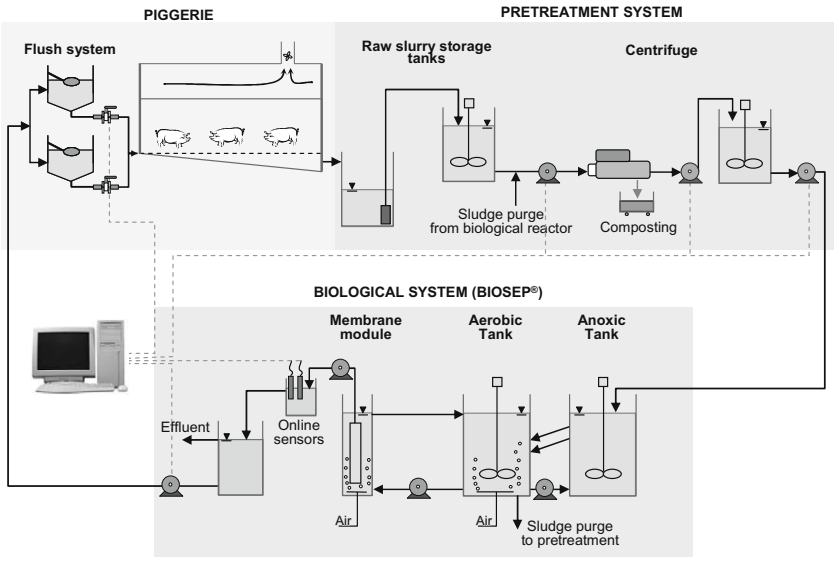

Fig. 1. Flow sheet diagram of the process.

Table 1
Modified Sturm test SVU composition.

SVU no.	Carbon sources	Initial COT (mg L^{-1})	Inoculum (mg L^{-1})	Objective
1	None	0	30	Blank inoculum
2	Sodium benzoate	47	30	Reference
3	Tetracycline	40	0	Abiotic test
4–5	Tetracycline	40	30	Target compound
6	Sodium benzoate	47	30	Inhibition control test
	Tetracycline	40		

The mass of CO_2 produced in the inoculum blank was similarly calculated and written m_{CO_2PE}, with E meaning endogen. The theorical mass of carbon (m_{CO_2theo}) which can be produced if all the available carbon was used (m_{CiTC} meaning carbon mass of the target compound and m_{CiRC} meaning carbon mass of referent compound) was calculated as follows:

$$m_{CO_2theo} = (m_{CiTC} + m_{CiRC}) \cdot \frac{44}{12} \quad \text{(ii)}$$

The ratio 44/12 was the conversion factor of carbon to carbon dioxide. The mass of CO_2 produced (m_{CO_2RC}) from the referent compound corresponded to the following calculation:

$$m_{CO_2RC} = m_{CiRC} \cdot \frac{44}{12} \quad \text{(iii)}$$

Finally the biodegradation percentage was calculated as follows:

$$Biod(\%) = \frac{(m_{CO_2P} - m_{CO_2PE}) \cdot 100}{m_{CO_2theo}} \quad \text{(iv)}$$

It is noticeable that, in some cases, m_{CO_2PE} was higher than m_{CO_2P}, and hence negative results may indicate a toxicity of the tested compound.

2.2.2. Toxicity tests

TC toxicity was evaluated with the same procedure as OECD 301 B evaluation of inhibition. Three inhibition control SVU were filled with increasing concentrations of tetracycline (5, 10 and 20 mg L^{-1}) as described in Table 2.

The toxicity of tetracycline on sodium benzoate biodegradation was evaluated via the calculation of the percentage of CO_2 actually formed in comparison with the total possible CO_2 formation as follows:

$$Biod_{residual}(\%) = \frac{m_{CO_2P} \cdot 100}{Th_{CO_2} + m_{CO_2PE}} \quad \text{(v)}$$

2.3. Analytical method

2.3.1. Tetracycline determination

AS was centrifuged (3600 rpm, 15 min) immediately after collection and filtered to 0.7 μm through Cloup glass microfibre filters (Champigny s/Marne, France) under vacuum. Five millilitre of extraction buffer and 2 ml of methanol were added per 100 ml of sample and were then thoroughly mixed prior to SPE clean-up. SAX cartridge (Sep-Pak Vac 3cc, Waters, France) was preconditioned with methanol (5 ml) and SPE conditioning buffer (5 ml), and then the activated sludge extract was passed through the cartridge at a rate of 100 ml h^{-1}.

Analysis was performed using an HPLC system including a Waters™ 600 controller pump, a Waters 717plus autosampler and a Spectra-Physics UV2000 detector. Separations were performed on a Waters Symmetry C18 5 μm column. A gradient elu-

Table 2
Toxicity test parameters.

SVU no.	Carbon sources	Initial TOC (mg L^{-1})	Inoculum (mg L^{-1})	Objective
1	Sodium benzoate	47	30	Reference
2	None	0	30	Blank inoculum
3	Sodium benzoate	47	30	Toxicity test
	Tetracycline	5		
4	Sodium benzoate	47	30	Toxicity test
	Tetracycline	10		
5	Sodium benzoate	47	30	Toxicity test
	Tetracycline	20		

tion was carried out with tetrahydrofuran (solvent A), acetonitrile (solvent B) and 0.05% trifluroacetic acid in water (solvent C). The flow rate was 1 ml mn^{-1}. The mobile phase was programmed as follows: A–B–C (5:3:92) from 0 to 4 min rising linearly to (5:75:20) from 4 to 18 min and then returning linearly to (5:3:92) from 18 to 20 min. All eluents were degassed with helium prior to and during analysis. The injection volume was 50 μL. Detection of tetracycline was performed at 360 nm.

2.3.2. Chemical analysis

The concentration of total solids (TS), mixed liquor suspended solid (MLSS) and volatile suspended solid (VSS) were determined according to the standard methods (Afnor 78160). The total and soluble chemical organic demand (COD), total nitrogen (TN), ammonium ($N\text{-}NH_4^+$), nitrate ($N\text{-}NO_3^-$) and nitrite ($N\text{-}NO_2^-$) were quantified by colorimetric methods using field tests supplied by HACH/Lange. The colorimetric methods were validated by comparison with standards methods in the laboratory.

3. Results

3.1. Pilot scale experiments

3.1.1. Contamination impact on carbon and nitrogen removal efficiency

The average removal COD yields (Table 3), before and after TC injection, were 92% and 88%. The impact of tetracycline injection on COD removal was weak. Therefore, the process kept sustainable for organic removal efficiency after TC injection. Ammonium removal remained very performing before and after TC injection (Fig. 2). The removal efficiency kept at 99% before and after the injection. Contrarily, the outlet water nitrates and nitrites concentrations tended to increase after the TC injection. Summing up nitrates and nitrites concentrations, the average outlet water before and after injection contained 5 mg L^{-1} of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ and 13 mg L^{-1} of $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$, respectively. One hypothesis is a higher sensitivity of denitrifying biomass than those of nitrifying biomass. However, heterotrophs are usually less sensitive than autotrophs, which is not in accordance with this hypothesis.

It is also noticeable that the COD/N ratio decreased from 18 before the contamination to 8 after. Therefore, before TC injection, nitrogen removal was mainly related to the growth of heterotrophs, and only a part was actually removed by nitrification/denitrification process. After TC injection, the biomass concentration decreased by approximately 10%, and then a higher proportion of ammonia had to be removed by nitrification/denitrification which enhanced the concentration of NO_x in the biological tanks.

However, the whole process may remain at a high level efficiency shortly after the contamination, based on the process behaviour observed during previous disturbances and its ruggedness (Prado et al., 2007).

3.1.2. Tetracycline fate into semi-industrial MBR

Tetracycline was analysed at different points of the process one day after injection (day 96) and at the end (day 99- Table 4). Concerning the repartition of TC, the recirculation of the activated sludge allowed a homogeneous repartition in the different tanks. The tetracycline not adsorbed onto sludge passed the membrane and was find back into the flushing water due to the reuse of the treated water. With no discard of activated sludge, all the TC was kept into the process. The results were summed up to calculate the tetracycline removal efficiency. On day 96, the total tetracycline hydrochloride added was 200 g; leading to a removal efficiency of 79%; on day 99, the total TC added was 500 g and then the removal efficiency was 89%.

Table 3
Pilot scale results before and after TC contamination.

Parameter	Before injection	After injection
Average inlet COD (mg L^{-1})	4795	6072
Average outlet COD (mg L^{-1})	342	685
Average inlet ammonium (mg $N\text{-}NH_4^+$ L^{-1})	266	710
Average outlet ammonium (mg $N\text{-}NH_4^+$ L^{-1})	<1	<1
Average outlet nitrates and nitrites (mg $N\text{-}NO_x$ L^{-1})	5	13

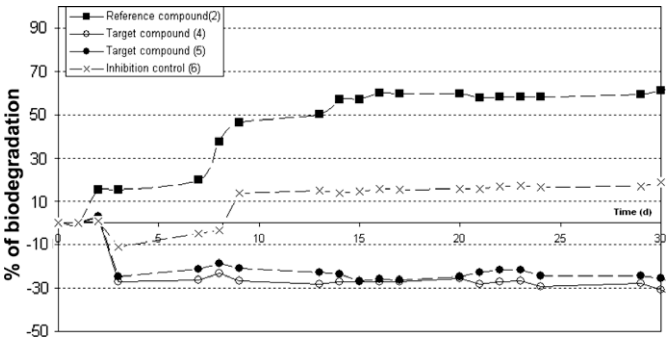

Fig. 2. Modified sturm test results for tetracycline.

Table 4
Tetracycline concentration at different points of the process.

Day	Swine waste water (mg L^{-1})	Anoxic tank (mg L^{-1})	Aerated tank (mg L^{-1})	Membrane tank (mg L^{-1})	Outlet tank (mg L^{-1})	Total (g)
96	3.3	3.3	2.4	2.5	2.6	42.3
99	2	4.6	2.9	3.8	3.9	56.6

3.2. Lab-scale experiments

3.2.1. Biodegradation

A modified Sturm test was carried out with initial carbon concentrations of 47 mg L^{-1} for the reference compound and 40 mg L^{-1} for the target compound (tetracycline) as explained on Table 1. The reference compound, sodium benzoate, was immediately degraded with an average CO_2 production of 38 mg L^{-1} and per day during the first 3 days (Fig. 2). The final biodegradation was approximately 64%, reached in 15 days (Fig. 2). These results confirmed its status of ready biodegradable component. Total biodegradation (100%) cannot be achieved due to the use of a part of the substrate for cellular biosynthesis. The inoculum blank led to the production of 168 mg CO_2 L^{-1}. According to the OECD 301 B guideline, the total CO_2 evolution in the inoculum blank at the end of the test should not normally exceed 40 mg L^{-1}. However, the inoculum source response can be used as far as the high values of CO_2 evolution of the reference compound test and of the inhibitory test are consistent with the inoculum blank value. Moreover, the endogenous CO_2 production was taken into account in the ultimate biodegradation calculation.

The ultimate biodegradations for TC were −35% and −28%, showing that TC can be classified as not biodegradable, in agreement with Gartiser and co-workers (2007). The inhibition control test gave an ultimate biodegradation of 18% after 30 days (Fig. 2).

3.2.2. Toxicity

Modified Sturm test design was used to evaluate the toxicity of TC on sodium benzoate biodegradation. Sodium benzoate was proved being ready biodegradable and inhibition control test led to consider TC as toxic. Increasing concentrations of TC were added

Table 5
Evaluation of tetracycline toxicity on sodium benzoate biodegradation.

Sludge origin	End (days)	TCinitial (mg TOC L^{-1})	$ThCO_2$ (mg TOC L^{-1})	m_{CO_2en} do (mg L^{-1})	m_{CO_2} (mg L^{-1})	Biod (%)	Biodres (%)
Lab-scale pilot	33	5	190	159	287	67	82
Lab-scale pilot	33	10	209	159	48	−53	13
Lab-scale pilot	33	20	245	159	36	−50	9
MBR	30	40	319	168	226	18	46

into different reactors containing the same concentration of inoculum and sodium benzoate (Table 2). The toxicity test lasted 33 days. The results are summarized in Table 5. The total inoculum blank CO_2 evolution exceeded OECD guideline; however, the inoculum source was always the same. No disturbance resulted from 5 mg TOC tetracycline L^{-1}, since the final biodegradation level (68%) was similar to that recorded in absence of TC. TC concentrations of 10 and 20 mg TOC L^{-1} led to a complete inhibition of sodium benzoate biodegradation, showing a high toxic effect (Table 5).

Contrarily, the inhibition control test led to an ultimate biodegradation level of 18% for an initial TC concentration of 40 mg TOC L^{-1} (Fig. 2). This difference might be due to the origin of the inoculum, since for toxicity test the inoculum derived from lab-scale installation, while for biodegradation test the inoculum derived from semi-industrial bioreactor treating swine wastewater. None of them was adapted to tetracycline; however, membrane bioreactors usually show better resistance to operational disorder than conventional bioreactors (Côté et al., 1998; Tazi-Pain et al., 2002).

4. Discussion

Conclusions resulting from lab-scale and semi-industrial-scale experiment are different in term of toxicity. TC injection into the MBR did not affect strongly the biological process behaviour even if lab-scale inhibition threshold has been passed over.

This difference may be due to specific experimental conditions which could not be reproduced in batch tests such as the SRT, temperature variations, the presence of others micropollutants and/or ions (Ca^{2+}) and the salinity. The factor affecting the removal of organic micropollutants including TC antibiotics from wastewater in CTP and MBR have been detailed in an exhaustive review (Cirja et al., 2008). TCs were highly sorbed on sludge and the sorption correlated well with the SRT during adsorption test in batch system (Sithole and Guy, 1987). The adsorption kinetics for TC was determined at various biomass concentrations in sequencing batch reactors at different SRT and HRT (Kim et al., 2005). Between 75 and 95% of applied TC was adsorbed onto the sludge after 1 hour. At long SRT (10 days), the removal of TC was 85%, while the decrease of SRT to 3 days gave a lower removal (78%). According to these results, the long SRT of the MBR (30 days) may enhance the adsorption of TC on sludge surface and reduced its toxic impact.

Cirja et al. (2008) also pointed out general trends of micropollutants removal from wastewater on biological treatment plants: hydrophobic compounds (such as TC) are mostly removed by adsorption and compounds containing toxic groups (such as antibiotics) show higher resistance to biodegradation, a long SRT (at least 8 days) enhances the biodegradation process, the temperature and the pH playing also an important role (low temperature decreases the elimination of micropollutants).

Contrarily to CTP, in MBR not only the biological process plays a role in antibiotics removal; the membrane may also influence the outlet water micropollutants concentration. In this work, the membrane average pore size (0.4 μm) should theoretically allow TC crossed the membrane as far as TC was not adsorbed on activated sludge. However, the organic nature of the membrane material could trap TC on its surface. In their study of TC removal by nanofiltration membrane Koyuncu et al. (2008) showed that TC is strongly adsorbable on membrane surface, and salinity and Ca^{2+} concentration influence its rejection.

From all these, TC was shown to be non-biodegradable and toxic regarding activated sludge on one hand, and on the other hand TC was shown to be removed from the outlet water and did not affect strongly the biological process behaviour. However, the adsorption of TC on sludge surface led to an over estimation of its removal through the process. In fact, composting excess sludge could lead to find back TC in soil and water after the use of the compost for organic amendment.

Therefore, alternative destructive treatment may be considered such as photocatalysis (Reyes et al., 2006) or ozonation (Li et al., 2008). However, both treatments have to be applied at high level or during a long time to achieve total mineralization of the antibiotic molecule. Therefore, they are not cost effective by themselves but may be an initial step of an integrated process, which involve a biological treatment in a second step. The oxidation of TC may produce by-products with a higher potential of biodegradation. The study of coupled processes is an innovative way to manage toxic molecule and to avoid generating antibioresistant strains (Kim et al., 2007).

5. Conclusion

This study demonstrated the ruggedness of a MBR treating dilute swine wastewater facing an acute pollution of tetracycline antibiotics, since the efficiency of the process was not significantly disturbed. Tetracycline removal reached 89%; however, subsequent work at lab-scale is needed to determine the respective parts of biodegradation and biosorption in the removal process. At lab-scale, modified Sturm tests showed the non biodegradability of tetracycline and its toxicity. Contrarily, at pilot-scale, no tetracycline toxicity was observed for this level of TC concentration and this result may be explained by the specific conditions (temperature, pH, matrix content).

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