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Research article

# Removal of pharmaceuticals from municipal wastewater by aerated submerged attached growth reactors

Y. Flores Velázquez [a], P. Mijaylova Nacheva [b, *]

[a] National Autonomous University of Mexico, Morelos Campus, Paseo Cuauhnahuac 8532, Col. Progreso, Jiutepec, Morelos 62550, Mexico
[b] Mexican Institute of Water Technology, Paseo Cuauhnahuac 8532, Col. Progreso, Jiutepec, Morelos 62550, Mexico

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ABSTRACT

The performance of four aerated submerged attached growth bioreactors was studied for the removal of three pharmaceutical micro-pollutants (fluoxetine, mefenamic acid and metoprolol) from municipal wastewater. Two packing materials (polyethylene tapes and polyurethane cubes) were compared and the effects of different organic loads (3.0, 6.0, 9.0 and 12 gCOD $m^{-2}$ $d^{-1}$) and of the effluent recirculation were investigated. The obtained solid retention times were in the range of 4–37 d. The reactors packed with polyurethane cubes allowed 11–26% higher biomass accumulation than the ones with polyethylene tapes and higher solid retention times. The low organic loads, high solid retention times and the implementation of effluent recirculation enhanced the removal of the three pharmaceutical compounds. The highest removals were achieved at organic load of 3 gCOD $m^{-2}$ $d^{-1}$ and 50% of effluent recirculation, with hydraulic residence times of 3.1–4.3 h and the solid retention times of 19–32 d. At this condition, the removals of the fluoxetine, mefenamic acid and metoprolol were up to 95, 82 and 73% respectively. The reactors with polyurethane cubes showed higher removals compared with the ones packed with polyethylene tapes.



## 1. Introduction

Pharmaceuticals are a class of micro-pollutants that may cause acute and chronic effects on aquatic organisms in the concentration range of μg $L^{-1}$ (Escher et al., 2011). Pharmaceuticals have been detected in municipal and hospital wastewater, surface water, groundwater, and even in drinking water (Stuart et al., 2012; Birkholz et al., 2014). Municipal wastewater treatment plants effluents represent one of the main sources of these compounds because most of these plants are not designed to remove them, as they were built with the principal aim of removing biodegradable carbon, nitrogen and phosphorus compounds (Verlicchi et al., 2012; Luo et al., 2014). For these reasons, it is necessary to improve the removal of pharmaceuticals with high environmental risk. Three pharmaceuticals from different classes of action were selected for this study, fluoxetine (psychiatric), mefenamic acid (analgesic/anti-inflammatory) and metoprolol (β-blocker). The model compounds were selected on the basis of their widespread use (Tauxe-Wuersch et al., 2005; Deblonde et al., 2011), their toxicological effects on aquatic organisms (Escher et al., 2011; Roos et al., 2012; Verlicchi et al., 2012; Mansour et al., 2016) and their concentrations in the effluents from wastewater treatment plants, and in the aquatic environment (Ternes, 1998; Miège et al., 2009; Rosal et al., 2010).

Previous studies on micro-pollutant removal in activated sludge wastewater treatment systems have indicated that high removal rates are achieved at solid retention times (SRT) higher than 10 d (Clara et al., 2005; Suarez et al., 2010. The long SRT allow an enrichment of slow growing bacteria such as nitrifying bacteria and the nitrifying activity contributes to the biotransformation of pharmaceuticals (Dawas et al., 2014; Rattier et al., 2014). Cometabolic biodegradation seems to be responsible for the initial biotransformation due to the action of ammonium monooxygenase enzyme, which catalyzes the first step of nitrification by ammonium oxidizing bacteria (Fernandez-Fontaina et al., 2012). Other experiments have indicated that the heterotrophic degradation rather than autotrophic degradation by ammonium oxidizing microorganisms was the main cause for the removal of several compounds including mefenamic acid and metoprolol (Tran et al., 2009; Majewsky et al., 2011; Maeng et al., 2013; Tran et al., 2013; Falås et al., 2016). Therefore, the nitrifying bacteria are capable to

* Corresponding author.
*E-mail address:* petiam@tlaloc.imta.mx (P. Mijaylova Nacheva).

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enhance the biodegradation of pharmaceuticals, but the role of heterotrophic organisms must be considered.

The attached growth processes offer some advantages over activated sludge processes, such as higher biomass concentration and high SRT even operating with low hydraulic residence time (HRT), which allows the development of microorganisms with low specific growth rates, so that high nitrification rate can be achieved (Luo et al., 2014). Falås et al. (2012) showed that moving bed biofilm carriers (Kaldnes K1 and Biofilm chip) have a pharmaceutical reduction potential superior to the activated sludge one. They gave two potential explanations for the observed difference: higher quantity of slow growing pharmaceutical degrading microorganisms (because of the higher SRT in the biofilm carrier's case) and stratification of the microbial community due to the substrate and redox gradients within the biofilm. The microorganisms adapted to easily degradable organic substrates are located in the outer part of the biofilm and microorganisms adapted to the remaining and difficultly degradable organic substrates in the inner part of the biofilm. Later, Falås et al. (2013) observed clear differences between the micro-pollutant removal kinetics obtained with attached and suspended biomass in that higher removal rates were found using attached biomass for most of the studied compounds. For example, mefenamic acid was degraded faster by the attached biomass than using suspended biomass, while the degradation pattern was the opposite for metoprolol. The nitrification capacity per unit biomass was considerably higher for the attached growth biomass than for the suspended growth one. As shown, aerated submerged attached growth reactors are an alternative for the removal of pharmaceuticals, however further research is needed to enhance their performance.

Plastic media are the most frequently used material for biofilm support in aerated attached growth reactors. The selection of packing materials for this study was based on previous work by Mijaylova et al. (2008) which studied the performance of aerobic submerged packed bed reactors for the treatment of domestic wastewater using seven different kinds of packing materials with specific areas in the range of 760–1200 $m^2$ $m^{-3}$. The study concluded that the highest SRT (until 39 d) was obtained in the reactors with polyethylene tapes and polyurethane cubes, and both reactors presented almost 99% $NH_4$-N removal. Mijaylova and Moeller (2010) reported that the biofilm developed in the reactors with polyethylene tapes was thin and this favored the diffusivity and mass transfer in the biofilm, while Guo et al. (2010) indicated that the biomass on polyurethane cubes is retained in two different forms: biofilm developed onto the cube surfaces and biomass deposited or entrapped within the cubes' void spaces. A distinctive dissolved oxygen gradient occurred within the cubes' inward depth, resulting in anaerobic conditions in the space deep inside the cube. The objective of this study was to assess the removal of fluoxetine, mefenamic acid and metoprolol from municipal wastewater by aerated submerged attached growth reactors, comparing the performance of two biomass support materials (polyethylene tapes and polyurethane cubes). The effects of different organic loads and of effluent recirculation were evaluated.

## 2. Material and methods

### 2.1. Experimental set-up and packing materials

The experiments were performed using four aerated submerged attached growth reactors. Each reactor had a cylindrical packed bed zone, a peripheral settling zone and a conical bottom for the extraction of accumulated sludge. Biomass support materials were placed into the cylindrical zone with 0.15 m diameter and a bed height of 0.8 m. Two reactors (PU1 and PU2) were packed with 3250 polyurethane cubes of 1.5 cm edge length and 10 pores per inch; the other two (PE1 and PE2) were packed with 3300 polyethylene tapes of a 5 cm length and 3 cm width. The tapes were supported by a vertical shaft of stainless steel. The specific areas of both packing beds were almost 700 $m^2$ $m^{-3}$. The schematic diagram of the experimental setup is presented in Fig. 1. The reactors were continuously fed with municipal wastewater, the wastewater passed down-flow through the packed bed and up-flow in the peripheral settling zone. The effluent was collected from the upper part of the settling zone and the sludge accumulated in the conic zone was periodically extracted. The aeration was provided by porous stone diffusers installed at the bottom; the dissolved oxygen levels were kept higher than 3 mg $L^{-1}$.

### 2.2. Experimental procedure and analysis

The immobilized biomass was developed by supplying municipal wastewater to all the bioreactors at an organic load (OL) of 3 gCOD $m^{-2}$ $d^{-1}$, without any special inoculation. The addition of the pharmaceutical compounds began after the process stabilization (80% COD and $NH_4$-N removal). The concentrations of the pharmaceuticals in the wastewater were selected according to the reported concentrations in influents to wastewater treatment plants. Tauxe-Wuersch et al. (2005) measured up to 4.54 μg $L^{-1}$ of mefenamic acid in municipal wastewater treatment plants in Switzerland. Deblonde et al. (2011) reported 4.9 μg $L^{-1}$ of metoprolol in influents from wastewater treatment plants. Rosal et al. (2010) reported 1.827 μg $L^{-1}$ of fluoxetine in urban wastewater, while Al Aukidy et al. (2014) reported 2.3 μg $L^{-1}$. Thus, the pharmaceuticals were added to wastewater to obtain almost 2 μg $L^{-1}$ of fluoxetine and 5 μg $L^{-1}$ for mefenamic acid and metoprolol. Pharmaceutical compounds were purchased from Sigma-Aldrich, the CAS numbers were: fluoxetine hydrochloride (56296-78-7), mefenamic acid (61-68-7) and metoprolol tartrate (56392-17-7). The stock solutions were prepared containing one pharmaceutical compound; the correction due to the purity of the compound was taken into account. The mefenamic acid and metoprolol were dissolved in methanol and the fluoxetine in acetone, stirring the solutions during 5 min at 25 °C to create a stock solution of 1000 μg $mL^{-1}$, the solutions were stored in amber vials at 4 °C and used to spike into the municipal wastewater.

The effect of different organic loads on the reactor performance and pharmaceutical compounds removal was evaluated: 3.0, 6.0, 9.0 and 12 gCOD $m^{-2}$ $d^{-1}$. The operational parameters for each experimental phase are presented in Table 1. The effect of 50% effluent recirculation was assessed for all organic loads. Each experimental phase was evaluated for 60 d. The variation of the organic load was performed by increasing the flow rate of the influent to the reactors, thus a decrease of HRT occurred when the organic load was increased.

The changes in the microbial community of the immobilized biomass can show the conditions that benefit the removal of pharmaceuticals in municipal wastewater. The study consisted of five phases, phase 1 (start-up of the reactors) and during the phases 2–5 different organic loads were applied to favor different microbial consortia. The low loaded biofilm processes tend to favor the development of slow growing autotrophic bacteria, such as nitrifying bacteria, which seems promising for the pharmaceutical removal (Falås et al., 2012). The high load condition favor the development of heterotrophs, which grow faster than autotrophs, as a result, the autotrophic nitrifiers can be overgrown by heterotrophs, which cause the nitrification efficiency to decrease (Bassin et al., 2011). According to a previous study, when applying the organic loads of 3 and 6 gCOD $m^{-2}d^{-1}$, high solid retention times (higher than 10 days) are expected and therefore the development

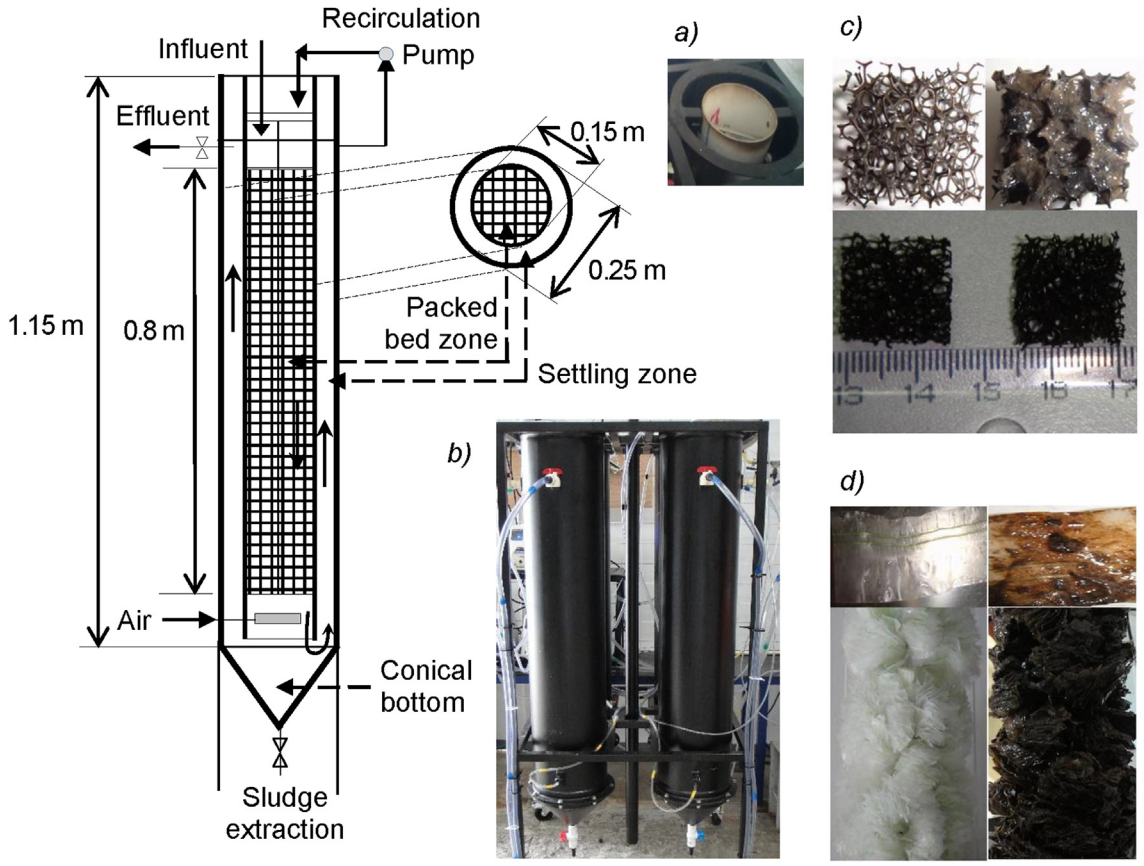


**Fig. 1.** Schematic diagram of the aerated submerged attached growth reactor and general views of the packing materials: *a)* Superior view of the reactor; *b)* Side view of two of the reactors; *c)* Polyurethane cubes; *d)* Polyethylene tapes.

**Table 1**
Operational parameters of the reactors.

| Parameter | Phase 1 Process stabilization | Reactors PE1 and PU1 | | | | Reactors PE2 and PU2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Phase 2 | Phase 3 | Phase 4 | Phase 5 | Phase 2 | Phase 3 | Phase 4 | Phase 5 |
| OL (gCOD $m^{-2}$ $d^{-1}$) | 3.0 | 3.0 | 3.0 | 6.0 | 6.0 | 9.0 | 9.0 | 12.0 | 12.0 |
| Influent flow (L $d^{-1}$) | 96–151 | 83–120 | 79–108 | 200–254 | 199–281 | 248–360 | 236–332 | 401–508 | 398–528 |
| HRT (h) | 2.3–3.5 | 2.8–4.1 | 3.1–4.3 | 1.3–1.7 | 1.2–1.7 | 0.9–1.4 | 1.0–1.4 | 0.7–0.8 | 0.6–0.9 |
| Recirculation (%) | 0 | 0 | 50 | 0 | 50 | 0 | 50 | 0 | 50 |

of slow growing bacteria. When applying the organic loads of 9 and 12 gCOD $m^{-2}d^{-1}$, the solid retention time decrease and the heterotrophic microorganisms have more substrate to grow, benefiting their greater activity. On the other hand, the recirculation reduces the resistance to mass transfer and the decrease of the influent organic matter concentration makes the nitrifiers more competitive. This in turn increases the nitrification efficiency and increases the dissolved oxygen concentration (EPA, 2000).

The traditional one-factor-at-time approach has been used to study the effects of various factors on the treatment process performance and pharmaceutical compounds removal. The independent factors were: OL, biomass support material and recirculation. Statistical analysis of the performance and pharmaceutical removals was performed with Statgraphics software program in order to evaluate the differences between the results obtained with both support materials, applying equal OL and between the conditions with and without recirculation at the same OL for each one of the support materials.

The COD, $NH_4$-N, $NO_2$-N and $NO_3$-N were measured in the influent and effluents three times a week. Total solids (TS) and volatile solids (VS) were determined in the packed beds once every two weeks; the samples were obtained from three different heights of the packed bed (upper, central and lower part), the biomass was detached with methanol and 20 min of sonication. The biomass in each reactor was determined as an average of the dry volatile solids determined at the three heights. In order to determine the SRT, the VS concentrations were measured in the effluents (once a week) and in the extracted sludge (once every two weeks); these parameters were determined according to the standard methods (APHA, 2012). The pharmaceuticals were measured three times a week by Gas Chromatography using Shimadzu TQ8040, fitted with a 30 m DB5-MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness) and connected to triple quadrupole mass spectrometer.

### 2.3. Analysis of the pharmaceutical compounds

Gas chromatography-mass spectrometry method was

developed and validated for the simultaneous detection of the three pharmaceutical compounds (fluoxetine, mefenamic acid and metoprolol) in liquid phase. Solid phase extraction was used to concentrate the pharmaceutical compounds and remove interfering substances, the compounds were extracted on an Oasis HLB cartridge with hydrophilic-lipophilic balance (lipophilic divinylbenzene + hydrophilic N-vinyl pyrrolidone), 200 mg sorbent per cartridge and 30 μm particle size. Cartridges were conditioned with 10 mL of methanol and 10 mL of water (HPLC grade) and the sample was passed through the cartridge by a vacuum manifold. Then the remaining interfering components were washed from the adsorbent with 4 mL of methanol-water solution (5:95, v/v). Later the cartridges were dried under vacuum during 3 h by an air flow to eliminate wetness. The analytes were eluted with 4 mL of methanol. Finally, the eluted extract was concentrated under a gentle nitrogen stream for a subsequent derivatization. The analytes were derivatized by silylation using *N,O*-Bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane, 100 μL of derivatizing agent were used and heating at 80 °C during 60 min. After this drying, the sample was reconstituted with 1 mL of toluene to be analyzed.

Standards and samples concentrates were injected using an automatic sample injector. During the qualitative analysis the characteristics ions were fluoxetine (44), mefenamic acid (223) and metoprolol (72). In terms of operating conditions selected, the injected volume was 1 μL in splitless mode (which means that the whole 1 μL was used for the analysis) at injection temperature of 260 °C and a flow rate of 1 mL $min^{-1}$. The temperature ramping allows to separate the different analytes without the risk of breaking them down. The temperature ramping was initiated at 150 °C for 2 min, then it was increased at a rate of 10 °C $min^{-1}$ up to 250 °C and finally the temperature was elevated at 15 °C $min^{-1}$ up to 290 °C, as a result, a runtime of 14.67 min was obtained. The retention time for mefenamic acid was of 12.291 min, while fluoxetine retention time was 9.270 min and 11.135 min for metoprolol. The following mass spectrometer conditions were chosen: electron impact (EI)-ionization at 70 eV, ion trap temperature of 250 °C, multiplier voltage of 180 V and selective ion monitoring (223 + 44+72).

The analytical methods for fluoxetine, mefenamic acid and metoprolol determination were validated using standard solutions. Calibration curves, response linearity, sensitivity, limit of detection and quantification, recovery and precision of the analytical procedure were calculated. Validation was done using seven replicates at a concentration of 0.01 μg $L^{-1}$. High recoveries of pharmaceuticals was observed after solid phase extraction, mean recoveries of the three compounds were greater than 98%, the acceptance criteria must be 70–130% of the true value for each analyte, thus the acceptance criteria was fulfilled. This means that the method is accurate and OASIS HLB cartridges are suitable for the retention of these compounds. The relative standard deviations of the results of the seven replicates were less than 7%, it must be less than 20%, thus the acceptance criteria was fulfilled and the values indicated that the method is precise. The quantification limits were lower than 0.017 μg $L^{-1}$, while the detection limits were lower than 0.002 μg $L^{-1}$. The results of the validation procedure indicated that the methods allow accurate, precise and reliable determination of the three compounds.

## 3. Results and discussion

### 3.1. Process performance

The biomass development and the process stabilization phase lasted 64 d. The COD and $NH_4$-N removals increased gradually reaching 80% at day 52 from the start up in all the reactors. After 60 d of operation, COD and $NH_4$-N removals were higher than 88% and 91% respectively in all the reactors. After the process stabilization the reactor performance was evaluated applying different OL with and without effluent recirculation. The average COD and $NH_4$-N removals obtained at each experimental phase are presented in Fig. 2. The OL increase resulted in a decrease of the COD removals in all the reactors. The highest removal (of 89%) was achieved with OL of 3 gCOD $m^{-2}$ $d^{-1}$, the lowest removals were obtained with OL of 12 gCOD $m^{-2}$ $d^{-1}$. The organic matter influent concentrations (expressed as COD) were between 200 and 380 mg $L^{-1}$.

The statistical analysis of the COD and $NH_4$-N removals was performed with the Statgraphics program in order to evaluate the significant differences between the removals obtained with different support materials at the same organic load, and the differences between the removals obtained with and without recirculation at the same organic load for each one of the support materials. The values obtained of F-ratio and the P-values of the statistical analysis are shown in Tables 2 and 3 respectively. If the P-value of the F-test is less than 0.05, there is a statistically significant difference between the mean removals from one support material to another, at least at the 95.0% confidence level. Thus, there was a statistically significant difference between the mean COD removals obtained in the reactors with polyethylene (PE) tapes and polyurethane (PU) cubes, except when they were operated with OL of 3 gCOD $m^{-2}$ $d^{-1}$ without recirculation, as well as with OL of 6 and 12 gCOD $m^{-2}$ $d^{-1}$ with recirculation. There was a statistically significant difference between the mean $NH_4$-N removals determined in the reactors with PE tapes and PU cubes during all experimental phases.

The statistical analysis on the differences between removals obtained with and without recirculation at the same organic load, performed for each type of support material, indicated that there were no statistically significant differences between the mean COD removals obtained, with the exception of the case where the reactor with PU cubes was operated with OL of 6 gCOD $m^{-2}$ $d^{-1}$. However, there were statistically significant differences between the mean removals of $NH_4$-N determined with and without recirculation, excepting the case when the reactor with PU was operated with OL of 6 gCOD $m^{-2}$ $d^{-1}$. The low organic loads favored the $NH_4$-N removals. Clear increase of $NH_4$-N removal was observed when the reactors were operated with effluent recirculation. This effect can be attributed to the reduction of the organic matter concentration in the reactors which makes the nitrifiers more competitive, and this in turn increases the nitrification efficiency and the dissolved oxygen concentration (EPA, 2000). Therefore, the highest $NH_4$-N removals (of 98%) were achieved at OL of 3 gCOD $m^{-2}$ $d^{-1}$ and 50% of effluent recirculation in the reactors with both packing materials. The $NH_4$-N removals decreased as the OL was increased; the removals at OL of 12 gCOD $m^{-2}$ $d^{-1}$ and 50% of effluent recirculation were less than 66 and 90% in the reactors PE and PU respectively. The reactors with PU cubes achieved higher removals of $NH_4$-N compared with the ones with PE tapes. The influent concentrations of $NH_4$-N were between 20 and 60 mg $L^{-1}$. These results indicated a good process performance in the reactors. The nitrification rates are presented on Fig. 2c. The reactors with PU cubes showed higher nitrification activity than the PE ones during all the experimental phases. The recirculation improved the nitrification rate in the reactors with both packing materials.

The amount of attached biomass augmented with the increase of the organic loads, thus the reactors PE1 and PU1 (organic loads of 3 and 6 gCOD $m^{-2}d^{-1}$) accumulated lower amounts of biomass compared with the reactors PE2 and PU2 (organic loads of 9 and 12 gCOD $m^{-2}d^{-1}$). The amount of biomass and the calculated SRT are presented in Table 4. The reactors with polyurethane cubes allowed

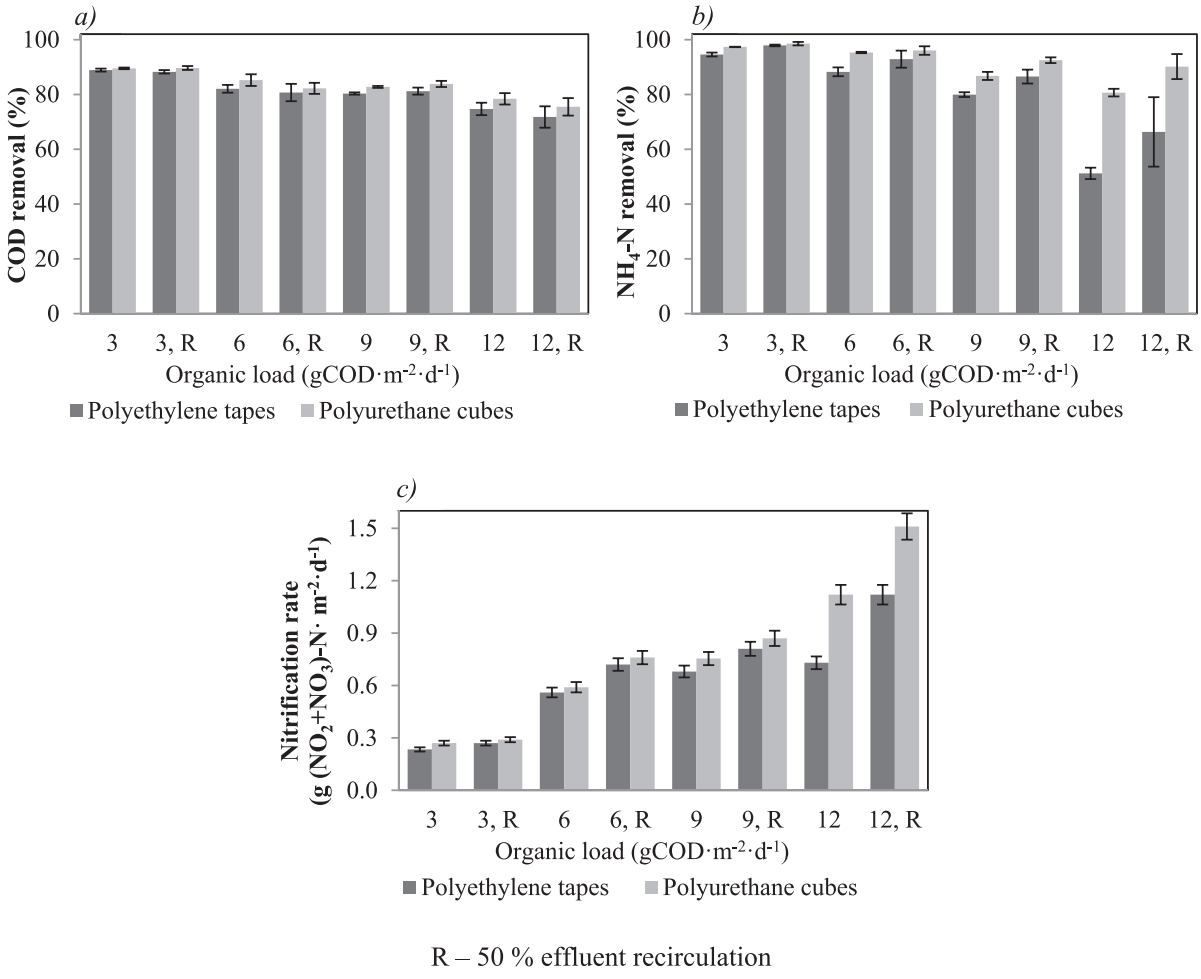


**Fig. 2.** Process performance: *a)* COD removals; *b)* $NH_4$-N removals during the experimental phases; *c)* Nitrification rates.

**Table 2**
Statistical analysis of the differences between the removals obtained with different support materials at the same organic load with and without recirculation.

| OL (gCOD $m^{-2}$ $d^{-1}$) | Recirculation (%) | COD | | $NH_4$-N | |
|---|---|---|---|---|---|
| | | F-ratio | P-value | F-ratio | P-value |
| 3.0 | 0 | 4.72 | 0.0615 | 70.30 | 0.0000 |
| 3.0 | 50 | 12.59 | 0.0040 | 6.10 | 0.0295 |
| 6.0 | 0 | 10.79 | 0.0065 | 133.50 | 0.0000 |
| 6.0 | 50 | 1.20 | 0.2941 | 5.66 | 0.0348 |
| 9.0 | 0 | 92.60 | 0.0000 | 77.98 | 0.0000 |
| 9.0 | 50 | 16.40 | 0.0016 | 33.61 | 0.0001 |
| 12.0 | 0 | 9.82 | 0.0086 | 610.10 | 0.0000 |
| 12.0 | 50 | 3.83 | 0.0741 | 21.90 | 0.0000 |

**Table 3**
Statistical analysis of the differences between the removals obtained with and without recirculation at the same organic load for each one of the support materials.

| Support material | OL (gCOD $m^{-2}$ $d^{-1}$) | COD | | $NH_4$-N | |
|---|---|---|---|---|---|
| | | F-ratio | P-value | F-ratio | P-value |
| PE | 3.0 | 1.28 | 0.2905 | 83.68 | 0.0000 |
| | 6.0 | 1.09 | 0.3163 | 12.28 | 0.0043 |
| | 9.0 | 1.08 | 0.3288 | 28.77 | 0.0007 |
| | 12.0 | 2.95 | 0.1114 | 9.49 | 0.0095 |
| PU | 3.0 | 1.10 | 0.3247 | 19.06 | 0.0024 |
| | 6.0 | 7.29 | 0.0193 | 1.37 | 0.2640 |
| | 9.0 | 3.21 | 0.1107 | 49.53 | 0.0001 |
| | 12.0 | 4.01 | 0.0682 | 27.81 | 0.0002 |

higher biomass accumulation during all the phases. The biomass quantity was 5.8–10.5 gVS $m^{-2}$ in these reactors and 4.9–9.0 gVS $m^{-2}$ in the reactors with polyethylene tapes. The startup of the effluent recirculation was accompanied by a reduction in biomass quantity because the recirculation increases the flow velocities through the reactor, causing greater detachment of excess biofilm.

The SRT were between 12-34 and 15–37 d in the reactors PE1 and PU1 respectively, whereas SRT were between 4-13 and 4–16 d in the reactors PE2 and PU2. The increase of the organic loads was performed by increasing the flow rate of the influent and of the flow velocities in the reactors (from 0.3 to 1.2 m $h^{-1}$). This caused higher biomass detachment and extraction from the reactors, and the SRT decreased in spite of the observed increment of the biomass quantity in the reactors. Under the same organic loads and flow velocities, the biomass quantity and the SRT were always higher in the reactors with polyurethane cubes, which can be attributed to the porous structure of this material and its capability to retain more biomass even at high flow velocities. The highest SRT were found during the phase 2 of the reactors PE1 and PU1 (OL of 3 gCOD $m^{-2}d^{-1}$), and the highest values were determined in the reactors with polyurethane cubes.

**Table 4**
Biomass amount and solid retention times in the reactors.

| OL (gCOD $m^{-2}$ $d^{-1}$) | Phase | Polyethylene tapes | | Polyurethane cubes | |
|---|---|---|---|---|---|
| | | Biomass (gVS $m^{-2}$) | SRT (d) | Biomass (gVS $m^{-2}$) | SRT (d) |
| 3.0 | Stabilization | 5.9–6.2 | 28–31 | 6.8–7.0 | 33–36 |
| 3.0 | Without recirculation | 6.3–6.6 | 26–34 | 7.3–7.9 | 27–37 |
| 3.0 | With recirculation | 5.9–6.0 | 19–28 | 6.3–7.1 | 26–32 |
| 6.0 | Without recirculation | 6.0–6.1 | 14–20 | 6.5–6.8 | 20–25 |
| 6.0 | With recirculation | 4.9–7.0 | 12–18 | 5.8–6.6 | 15–18 |
| 9.0 | Without recirculation | 8.9–9.0 | 11–13 | 9.8–10.3 | 13–14 |
| 9.0 | With recirculation | 7.4–8.1 | 10–13 | 9.0–10.2 | 11–16 |
| 12.0 | Without recirculation | 8.2–8.6 | 8–9 | 10.3–10.5 | 9–10 |
| 12.0 | With recirculation | 6.8–8.3 | 4–6 | 8.8–9.6 | 4–6 |

### 3.2. Pharmaceutical compounds removal

During the first phase, which corresponded to the start-up of the reactors, the immobilized biomass was developed by supplying municipal wastewater at organic load of 3 gCOD $m^{-2}$ $d^{-1}$ (without any special inoculation). This phase lasted until the process stabilization was achieved, as indicated by a constant percentage removal (80% COD and $NH_4$-N removal), then the addition of the pharmaceutical compounds began. The removal of COD reached 80% at day 48 from the start up in the reactors packed with PU cubes and at day 52 in the reactors with PE tapes. The 80% $NH_4$-N removal was reached at day 24 from the start up in the reactors with PU cubes and at day 50 in the reactors with PE tapes. Consequently the biomass development and process stabilization in the reactors took almost two months and the phase 1 lasted 64 days, therefore the addition and determination of the pharmaceutical compounds began at day 65.

The concentrations of the pharmaceutical compounds determined in the influent and effluents from all reactors are presented on Fig. 3. Low pharmaceutical removals were observed in the reactors PE1 and PU1 at the beginning of the evaluation phase 2. The fluoxetine, mefenamic acid and metoprolol concentrations in the effluents progressively decreased until they became almost constant during the last 14 days of this phase. The average removals of fluoxetine, mefenamic acid and metoprolol at OL of 3 gCOD $m^{-2}$ $d^{-1}$, HRT of 2.8–4.1 h and SRT of 26–37 d, were 77.5 ± 1.2, 41.4 ± 3.9 and 59.2 ± 2.9 % respectively in the reactors with PE tapes, meanwhile they were 83.4 ± 1.0%, 60.4 ± 2.7% and 60.6 ± 4.1 % respectively in the reactors with PU cubes. The average removals of the pharmaceutical compounds during the experimental phases are presented on Fig. 4. The highest average removals of the three pharmaceuticals were achieved applying OL of 3.0 gCOD $m^{-2}$ $d^{-1}$ and 50% of recirculation in the reactors with both support materials. The effluent concentrations of fluoxetine, mefenamic acid and metoprolol were 0.14 ± 0.01, 1.43 ± 0.14 and 1.76 ± 0.24 μg $L^{-1}$ respectively for the reactor with PE tapes, obtaining removals of 94.0 ± 0.3, 77.6 ± 2.7 and 67.5 ± 4.3 % respectively during the last 14 days. The highest removals of mefenamic acid were achieved in the reactor with PU cubes, the effluent concentration was 1.17 ± 0.22 μg $L^{-1}$, thus achieving removals of 81.7 ± 3.5%. The fluoxetine removal was 94.9 ± 0.8%, similar to the one obtained in the reactor with PE tapes, and the fluoxetine concentration was of 0.11 ± 0.01 μg $L^{-1}$ in the effluent. The metoprolol removal was 72.7 ± 5.1%, higher than the one obtained in the reactor with PE tapes, with concentrations of 1.47 ± 0.3 μg $L^{-1}$ in the effluent. The performance of the reactors with both packing materials was very good during the experimental phase with OL of 3.0 gCOD $m^{-2}$ $d^{-1}$ and 50% of recirculation, the COD and $NH_4$-N removals were more than 88% and 97% respectively. The average $NO_3$-N concentrations were 11.1 ± 2.7 and 19.0 ± 3.6 mg $L^{-1}$ in the effluents of the reactors with PE tapes and PU cubes respectively. Although the recirculation involved a decrease of the SRT to 19–32 d due to the greater detachment of the excess biomass, it also reduced the resistance to mass transfer (EPA, 2000) and allowed an increase in the reactor removal efficiency.

The OL increase to 6.0 gCOD $m^{-2}d^{-1}$ (without recirculation) caused an increase of the pharmaceutical concentration in the effluents of the reactors with both packing materials. This effect could be attributed to the change of the operational conditions as the influent flow increase caused HRT decrease to 1.3–1.7 h and the SRT was reduced to 14–25 d. Stabilization period of 30–40 d was required to reach relatively constant pharmaceutical concentrations again in the effluents. Removals of fluoxetine, mefenamic acid and metoprolol of 86.5 ± 0.9, 40 ± 1.4 and 58 ± 4.3% respectively were determined in the reactor with PE tapes, however higher removals of 90.4 ± 0.5, 57.3 ± 1 and 64.2 ± 3.5% respectively were obtained in the reactor with PU cubes. The reactors with PU cubes had higher SRT and $NH_4$-N removals compared with those obtained for the reactor with PE tapes. During the next experimental stage, the OL was maintained of 6.0 gCOD $m^{-2}d^{-1}$ but effluent recirculation of 50% was implemented. These operational conditions caused a decrease of the SRT to 12–18 d, which nevertheless increased the pharmaceutical removals in the reactors with both packing materials. The removals of fluoxetine, mefenamic acid and metoprolol were 91.6 ± 0.3, 59.2 ± 2.1 and 68.3 ± 1% respectively in the reactor with PU cubes, higher than those determined in the reactors with PE tapes (88 ± 0.9, 50.3 ± 1.7 and 59.8 ± 1.8 % respectively). This can be attributed to the higher SRT and $NH_4$-N removals obtained in this reactor.

The OL increase from 3.0 to 9.0 gCOD $m^{-2}d^{-1}$ produced instability in the reactors PE2 and PU2 at the beginning of the evaluation phase; 30–40 d were required to obtain relatively constant pharmaceutical concentrations in the effluents. The HRT was of 0.9–1.4 h and SRT of 11–14 d was determined for this experimental phase. The effluent concentrations of fluoxetine, mefenamic acid and metoprolol were 0.69 ± 0.03, 3.95 ± 0.17 and 3.49 ± 0.13 μg $L^{-1}$ respectively for the reactor with PE tapes (removals of 67.1 ± 1.3, 28.8 ± 5 and 40.4 ± 2.7 % respectively). The effluent pharmaceutical concentrations were 0.41 ± 0.04, 2.7 ± 0.07 and 2.99 ± 0.11 μg $L^{-1}$ respectively for the reactor with PU cubes and the removals were calculated of 80.4 ± 1.6, 51.4 ± 2.8 and 49 ± 2.8% respectively. So the PU cubes showed higher removals of fluoxetine and mefenamic acid than the PE tapes. When effluent recirculation was applied while maintaining the same OL of 9.0 gCOD $m^{-2}d^{-1}$, the removals of fluoxetine, mefenamic acid and metoprolol increased in the reactors with both packing materials. Removals of fluoxetine, mefenamic acid and metoprolol of 89.9 ± 0.46, 52.7 ± 3.3 and 55.6 ± 4.0% respectively were determined in the reactor with PE tapes,

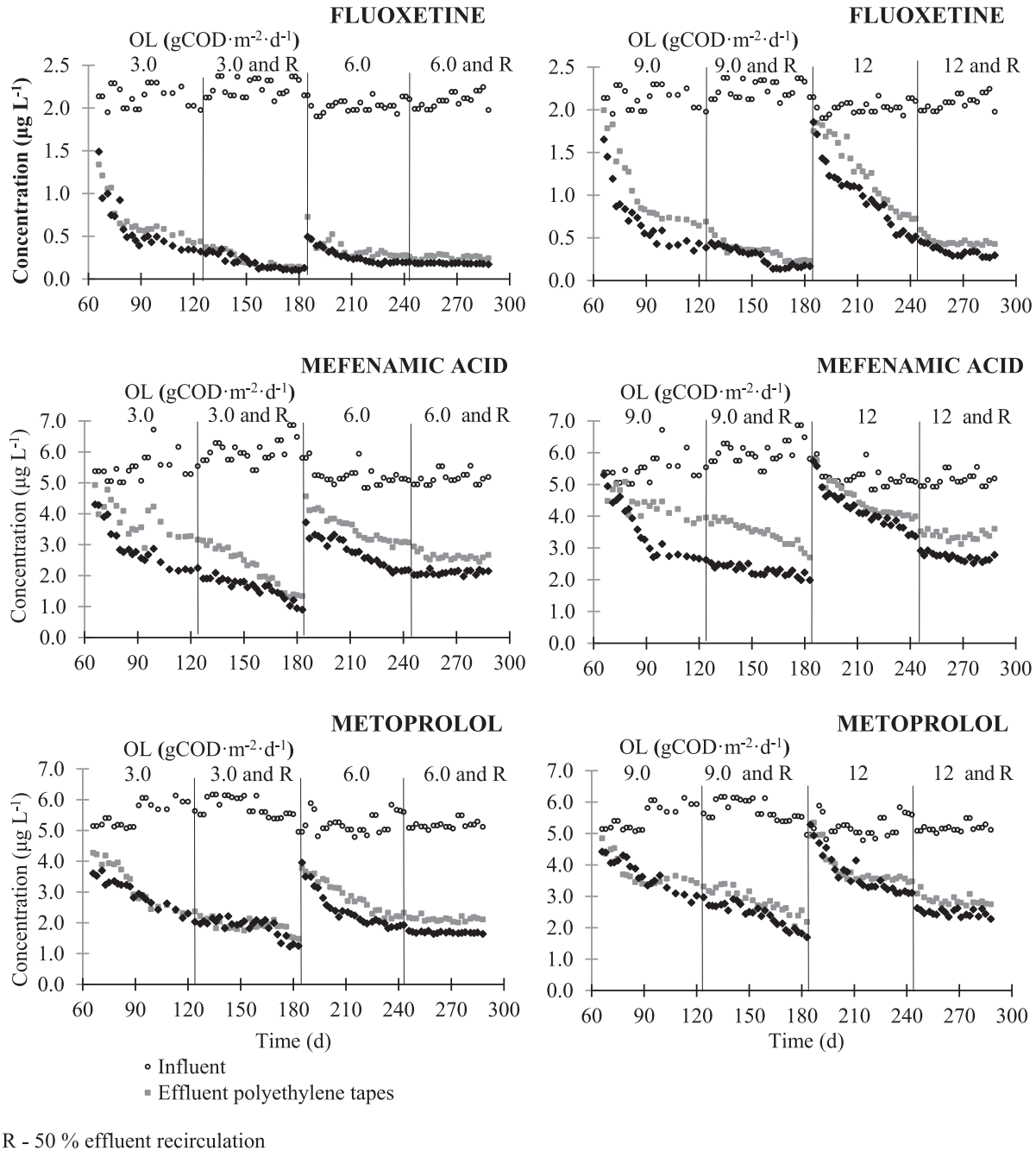


**Fig. 3.** Pharmaceutical compounds concentrations during the experimental phases.

however higher removals of 92.8 ± 0.8, 66.6 ± 3.2 and 65.7 ± 2.3% respectively were obtained in the reactor with PU cubes. The reactors with PU cubes had higher SRT and $NH_4$-N removals compared with the obtained for the reactor with PE tapes.

The organic load increase to 12 gCOD $m^{-2}d^{-1}$ performed on day 184 from the startup of the reactors PE2 and PU2 caused again process destabilization (Fig. 3), which can be attributed to the change of the operational conditions: the HRT was of 0.7–0.8 h and the SRT of 8–10 d. The effluent pharmaceutical concentrations decreased over the time, however almost 50 d were required to reach stability for the pharmaceutical removals in the reactors. The average removals of the fluoxetine, mefenamic acid and metoprolol were 62.5 ± 3.3, 21.4 ± 1.7 and 33.9 ± 4.8% respectively in the reactor PE tapes, and they were 73.3 ± 3.6, 30.2.±3.7 and 41.3 ± 4.6% in the reactor with PU cubes. Better pharmaceuticals removals were obtained in the reactor with the PU cubes compared with the one with PE tapes, however it is important to mention that the lowest $NH_4$-N removals (lower than 80%) were observed in both reactors during this experimental phase. The pharmaceuticals removals increased when the effluent recirculation was applied while maintaining the same OL of 12 gCOD $m^{-2}d^{-1}$. The fluoxetine, mefenamic acid and metoprolol concentrations were 0.43 ± 0.02, 3.42 ± 0.12 and 2.83 ± 0.11 µg $L^{-1}$ respectively and removals of 79.8 ± 1, 33.8 ± 4.5 and 46.3 ± 1.7% respectively were achieved in the reactor with PE tapes. In the case of the reactor with PU cubes, the concentrations were 0.30 ± 0.03, 2.64 ± 0.09 and

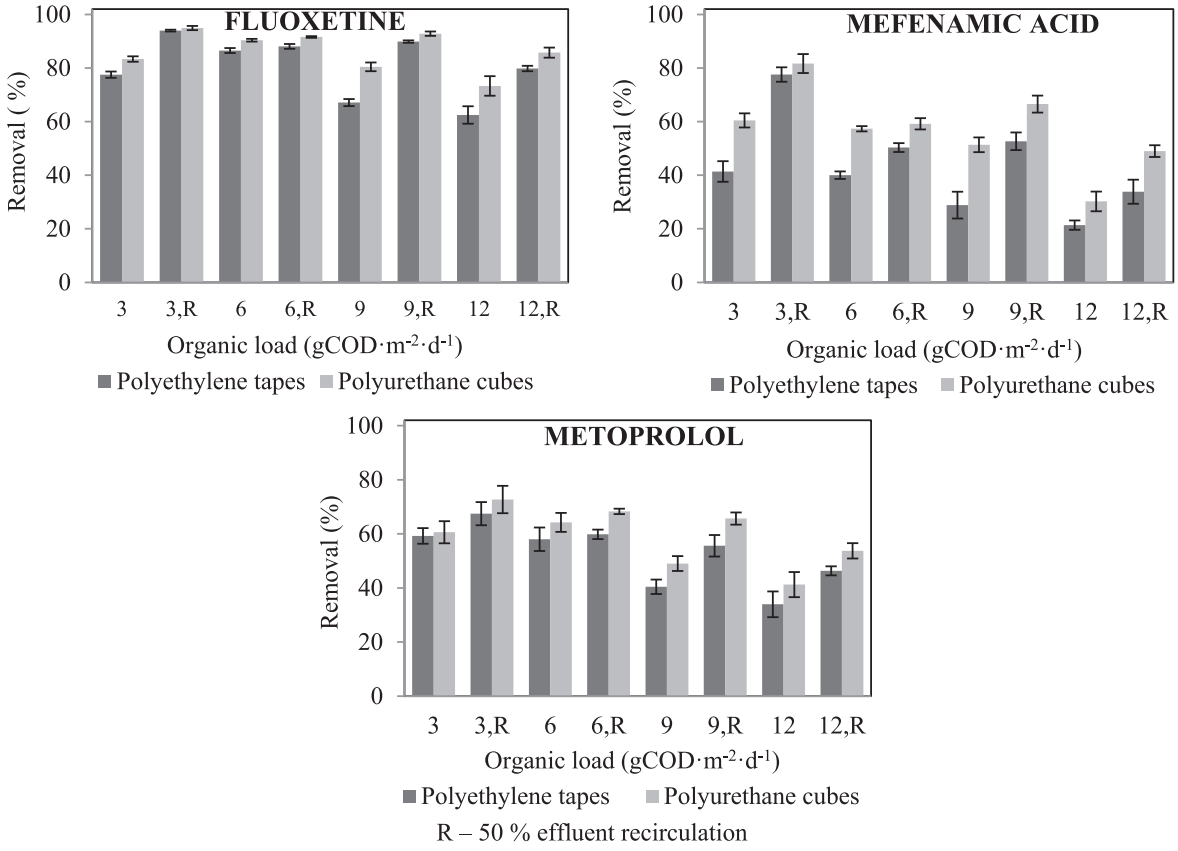


**Fig. 4.** Pharmaceutical compounds removals during the experimental phases.

2.44 ± 0.12 μg $L^{-1}$ respectively and the removals were 85.8 ± 1.9, 49 ± 2.2, and 53.7 ± 2.8 % respectively. The differences in the reactor performances were noticeable and better performance was obtained again in the reactor with the PU cubes, which can be associated with the high SRT. Additionally, it can be observed that the removals obtained with OL of 12 gCOD $m^{-2}$ $d^{-1}$ and 50% recirculation were higher than those determined with OL of 9gCOD $m^{-2}$ $d^{-1}$ without recirculation in the reactors with both packing materials.

The statistical analysis of the pharmaceutical compounds removals in order to evaluate the differences between the removals of each compound using different support materials indicated that there was a statistically significant difference between the mean removals from one support material to another for the three compounds (fluoxetine, mefenamic acid and metoprolol), with the exception of metoprolol removals obtained with OL of 3 gCOD $m^{-2}$ $d^{-1}$ without recirculation. The statistical analysis of the differences between removals obtained with and without recirculation at the same organic load, performed for each type of support material, indicated that there were statistically significant differences between the mean removals obtained for the three compounds, with the exception of metoprolol removals determined with the OL of 6 gCOD $m^{-2}$ $d^{-1}$ in the reactor with PE tapes and the mefenamic acid removals obtained with the OL of 6 gCOD $m^{-2}$ $d^{-1}$ in the reactors with PU cubes.

The most biodegradable compound was the fluoxetine; high removals of this compound were obtained in the reactors with both packing materials (Fig. 4). The highest fluoxetine removal (94–95%) was obtained with SRT of 26–32 d and HRT of 3.1–4.3 h (OL of 3.0 gCOD $m^{-2}d^{-1}$ and 50% recirculation), being the fluoxetine concentrations of 0.136 ± 0.008 and 0.114 ± 0.013 μg $L^{-1}$ in the effluents of the PE and PU reactors respectively. This removal is higher than the one reported by Radjenović et al. (2009), of 33% using SRT of 10 d and HRT of 11.5 h in activated sludge wastewater system, and also higher than the one reported by Suarez et al. (2010), of 90% using SRT>50 d in nitrifying activated sludge. Radjenović et al. (2009) also evaluated two pilot-scale membrane bioreactors and they found fluoxetine removals of 98% which were obtained with HRT>7 h, much greater than the ones used in this study.

The greatest differences between the removals in the reactors packed with different materials were observed for mefenamic acid. Radjenović et al. (2009) found removals of 40 and 35% in two membrane bioreactors (HRT>7 h). The reactors with PE tapes and PU cubes allowed higher removals applying OL of 3 and 6 gCOD $m^{-2}$ $d^{-1}$, using or not effluent recirculation, with SRT of 15–37 d and HRT of 1.2–4.3 h. The reactors with PU cubes reached higher removals of mefenamic acid during all the experimental phases. The highest mefenamic acid removal of 82% was obtained with SRT of 26–32 d and HRT of 3.1–4.3 h (OL of 3.0 gCOD $m^{-2}d^{-1}$ and 50% recirculation) in the reactor with PU cubes. Kovalova et al. (2012) reported removals of 92% for a membrane bioreactor operated with SRT of 30–50 d and HRT of 98 h, much greater than the ones used in this study. Falås et al. (2012) demonstrated that the reactors with Kaldnes K1 biofilm carriers and Biofilm Chip reached higher removal of mefenamic acid compared to nitrifying activated sludge processes, despite the higher nitrification rates of the activated sludge biomass compared with the carrier biomass. It indicates that the difference in mefenamic acid removal was due to a difference in the heterotrophic microbial community, while a clearly positive

trend between the nitrification capacity and the rate constants was observed in the carrier reactors.

Metoprolol presented higher removals than those obtained for mefenamic acid in the reactors with PE tapes during all experimental phases. This relationship was also obtained for PU reactors operated with OL of 6 and 12 gCOD $m^{-2}d^{-1}$ with and without recirculation, almost same removals were obtained at OL of 3 gCOD $m^{-2}$ $d^{-1}$ without recirculation and at OL of 9 gCOD $m^{-2}$ $d^{-1}$ with and without recirculation; however an inverse relationship was obtained at OL of 3 gCOD $m^{-2}$ $d^{-1}$ with recirculation. Radjenović et al. (2009) found metoprolol removals of 24% (SRT of 10 d; HRT of 11.5 h) in activated sludge and removals of 44 and 29% in two membrane bioreactors (HRT>7 h). Higher metoprolol removals were obtained in this study during all the experimental phases. Later, Kovalova et al. (2012) reported metoprolol removals of 55 ± 13 % in a membrane bioreactor at SRT of 30–50 d and HRT of 98 h. The reactors with PE tapes and PU cubes allowed removals higher than 55% applying OL of 3, 6 and 9 gCOD $m^{-2}d^{-1}$, regardless of effluent recirculation, with SRT of 11–37 d and HRT of 1.0–4.3 h. The reactors with PU cubes reached higher removals of metoprolol during all the experimental phases. The highest metoprolol removal of 73% was obtained with SRT of 26–32 d and HRT of 3.1–4.3 h (OL of 3.0 gCOD $m^{-2}d^{-1}$ and 50% recirculation) in the reactor with PU cubes. Vieno et al. (2007) found that there was no clear correlation between the SRT applied in sewage treatment plants and the elimination of the pharmaceuticals like metoprolol. According to another study (Falås et al., 2013), metoprolol was degraded faster by aerobic suspended biomass than attached biomass, where the nitrification was higher for the attached growth than for the suspended growth.

Considering the toxicity studies of pharmaceuticals at environmentally relevant concentrations, negative effects of fluoxetine have been found at low concentrations. Nentwig (2007) verified the effects on the reproduction of snails exposed at 2.25 μg $L^{-1}$ of fluoxetine. The no observed effect concentration and the 10% effect concentration were determined to be 0.47 and 0.81 μg $L^{-1}$, respectively. Mennigen et al. (2010) discovered effects on glucose metabolism in goldfish exposed to 0.54 μg $L^{-1}$ of fluoxetine for 28 days. Fluoxetine concentrations obtained in the effluents of the reactors with both packing materials, operating at OL of 3, 6 and 9 gCOD $m^{-2}$ $d^{-1}$ regardless of effluent recirculation, and also at OL of 12 gCOD $m^{-2}$ $d^{-1}$ with recirculation, were lower than the reported concentrations which cause negative effects on indicator organisms. Hence it has been determined that the submerged attached growth reactors packed with PE tapes and PU cubes are a good wastewater treatment option for the removal of fluoxetine, as they can reach low enough concentrations to avoid negative environmental effects.

Ji et al. (2013) suggest estrogenic potential of mefenamic acid, they showed that mefenamic acid increased the 17β-estradiol and testosterone levels in female zebrafish (*Danio rerio*), while decreased those of testosterone among male fish. After exposure to 10 μg $L^{-1}$ of mefenamic acid, the concentrations of 17β-estradiol in blood plasma increased in male and female zebrafish, also significant up-regulation mRNA was observed in the ovary of female fish. Collard et al. (2013) detected occasionally male sex characteristics in female zebrafish *D. Rerio* exposed to 1 μg $L^{-1}$ of mefenamic acid. The no observed effect concentration in growth was 10 μg $L^{-1}$. The influent concentrations of mefenamic acid in the studied PE and PU reactors were lower than 10 μg $L^{-1}$, but the effluent concentrations were higher than 1 μg $L^{-1}$ of mefenamic acid during all the experimental phases.

*Daphnia magna* exposed to 2.67 μg $L^{-1}$ ($10^{-8}$ M) of metoprolol presented sympathomimetic activity in heart, induced a positive chronotropic effect and a reduced area in diastole (Villegas-Navarro et al., 2003). The PE and PU reactors obtained effluent concentrations lower than this value, when they were operated at OL of 3 and 6 gCOD $m^{-2}d^{-1}$, regardless of effluent recirculation, and at OL of 9 gCOD $m^{-2}d^{-1}$ with recirculation. This relationship was also obtained for the PU reactor operated at OL of 12 gCOD $m^{-2}d^{-1}$ with recirculation. Triebskorn et al. (2007) found effects in the livers of rainbow trout exposed at concentrations of 1 μg $L^{-1}$ of metoprolol. The symptoms included the reduction of glycogen stores, the occurrence of membrane material within the cells, vesiculation, dilation, and irregular orientation of the endoplasmic reticulum. The metoprolol concentrations in the effluents of the PE and PU reactors were between 1.5 and 3.5 μg $L^{-1}$ under the tested operational conditions in this study. Contardo-Jara et al. (2010) examined the changes of gene expression in the freshwater mussel exposed to metoprolol. The concentrations of $2 \times 10^{-8}$ M (5.34 μg $L^{-1}$) caused a significant hsp70 mRNA up-regulation and provoked a P-gp mRNA increase after four days. No effect took place at concentrations of $2 \times 10^{-9}$ M (0.534 μg $L^{-1}$), but it was shown that metoprolol can be accumulated in mussels at this concentration. Gröner et al. (2015) studied the changes on the transcriptional level of enzymes in primary hepatocytes from male Nile tilapia. The metoprolol concentration of $4 \times 10^{-9}$ M (1.069 μg $L^{-1}$) changed Glutathione-S-transferases expression levels significantly, but this concentration did not significantly change multidrug resistance proteins.

The presence of pharmaceuticals in the water poses a human health and environmental risk, as humans are exposed daily via drinking water extracted from groundwaters and surface waters, which are frequently submitted to incomplete treatments using conventional technologies. Another aspect to the presence of pharmaceuticals in drinking water is that the risks have generally been assessed on the base of individual compounds, but additive and/or synergistic/antagonistic effects of the mixture of pharmaceuticals are to be expected (Vulliet and Cren-Olivé, 2011). Therefore, the simultaneous presence of several pharmaceuticals in the environment might result in a greater toxicity than the one predicted for individual active substances (Santos et al., 2010). There are no studies indicating the toxicity of the selected pharmaceuticals to human populations. Pomati et al. (2006) investigated the effects of a mixture of 13 pharmaceuticals with the low concentration profiles detected in the environment (0.01–1 μg $L^{-1}$): atenolol, bezafibrate, carbamazepine, cyclophosphamide, ciprofloxacin, furosemide, hydrochlorothiazide, ibuprofen, lincomycin, ofloxacin, ranitidine, salbutamol, and sulfamethoxazole. They found that the detected environmental levels of pharmaceuticals inhibited human embryonic cells growth by 10–30% compared to controls.

Pharmaceuticals are found in the environment at concentrations ranging from ng $L^{-1}$ to low μg $L^{-1}$, which are significantly lower than most of the Lowest Observed Effect Concentrations (LOECs) for aquatic organisms. However, the chronic toxicity LOECs of a few pharmaceuticals, including fluoxetine, is comparable to concentrations found in wastewater. As an example, studies have determined that maximum concentrations of fluoxetine measured in wastewater effluents were within the range of their toxic LOEC in benthic organisms (Richardson and Kimura, 2016). Verlicchi et al. (2012) analyzed data pertaining to 244 conventional activated sludge systems and 20 membrane biological reactors, and performed an evaluation of the environmental risk posed by pharmaceuticals in the secondary effluent. The comparison was carried out by means of the risk quotient (RQ), which is the ratio between the average pharmaceutical concentrations measured in the secondary effluent and its corresponding predicted no effect concentration. Fourteen compounds pose a high risk: 7 antibiotics (erythromycin, ofloxacin, sulfamethoxazole, clarithromycin, amoxicillin, tetracycline and azithromycin), 2 psychiatric drugs

(fluoxetine and diazepam), 2 analgesics-anti/inflammatories (ibuprofen and mefenamic acid) and 3 lipid regulators (fenofibric acid, fenofibrate and gemfibrozil). Within the group of β-blockers, only metoprolol and propranolol are classified as potentially toxic to aquatic organisms (Cleuvers, 2003; Roos et al., 2012).

## 4. Conclusions

The aerated submerged attached growth reactors with two biomass support materials (polyethylene tapes and polyurethane cubes) were able to remove fluoxetine, mefenamic acid and metoprolol from municipal wastewater up to 95, 82 and 73% respectively. Fluoxetine was the most biodegradable compound. The reactors packed with polyurethane cubes showed a better performance compared with the ones with polyethylene tapes. This difference was considerably noticeable for the mefenamic acid, which can be attributed to the higher solid retention times obtained in the reactors with polyurethane cubes. The low organic loads, high solid retention times and the use of effluent recirculation enhanced the removals of the pharmaceutical compounds. When recirculation was applied, an increase of $NH_4$-N removals and nitrification activity were observed, despite the reduction of the SRT. The highest removals of fluoxetine, mefenamic acid and metoprolol were achieved at organic load of 3.0 gCOD $m^{-2}$ $d^{-1}$ with 50% effluent recirculation (HRT of 3.1–4.3 h; SRT of 19–32 d). The removals were 94.0 ± 0.3, 77.6 ± 2.7 and 67.5 ± 4.3% respectively in the reactor with polyethylene tapes and 94.9 ± 0.8, 81.7 ± 3.5, and 72.7 ± 5.1% respectively in the reactors with polyurethane cubes. The effluent concentrations of fluoxetine were lower than the lowest observed effect concentrations (LOECs) reported for aquatic organisms, which suggests that the aerated submerged attached growth reactors packed with polyethylene tapes and polyurethane cubes are a good wastewater treatment option for the removal of this pharmaceutical compound. The lowest effluent concentrations of mefenamic acid and metoprolol, of 1.17 and 1.47 μg $L^{-1}$ respectively were obtained in the reactor with polyurethane cubes operated with an organic load of 3.0 gCOD $m^{-2}$ $d^{-1}$ and with 50% effluent recirculation. These concentrations are 17% and 47% higher than the LOECs for mefenamic acid and metoprolol respectively. Therefore the aerated submerged attached growth reactors will be able to reach the LOECs for mefenamic acid and metoprolol only if their concentrations in the wastewater are lower than 4.8 and 4.5 μg $L^{-1}$ respectively.

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