# Micropollutants removal in tertiary moving bed biofilm reactors (MBBRs): Contribution of the biofilm and suspended biomass

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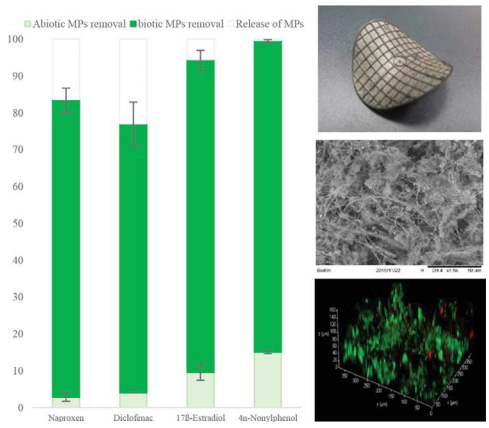
## HIGHLIGHTS

- The potential of tertiary MBBRs was investigated in terms of MPs removal.
- Biofilm's contribution was higher than the suspended biomass for MPs biodegradation.
- The dominant biodegradation mechanism of Estradiol was the competitive inhibition.
- Diclofenac, Naproxen, and Nonylphenol were biodegraded by the cometabolism mechanism.
- MPs sorption onto the suspended biomass was higher than the biofilm.

## GRAPHICAL ABSTRACT




*Keywords:*
Micropollutants
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## ABSTRACT

The performance of tertiary moving bed biofilm reactors (MBBRs) was evaluated in terms of micropollutants (MPs) removal from secondary-treated municipal wastewater. After stepwise establishment of a mature biofilm, monitored by scanning electron and confocal microscopies, abiotic and biotic removals of MPs were deeply studied. Since no MPs reduction was observed by the both photodegradation and volatilization, abiotic removal of MPs was ascribed to the sorption onto the biomass. Target MPs i.e. Naproxen, Diclofenac, 17β-Estradiol and 4n-Nonylphenol, arranged in the ascending order of hydrophobicity, abiotically declined up to 2.8%, 4%, 9.5% and 15%, respectively. MPs sorption onto the suspended biomass was found around two times more than the biofilm, in line with MPs' higher sorption kinetic constants ($k_{sor}$) found for the suspended biomass. When comparing abiotic and biotic aspects, we found that biotic removal outperformed its counterpart for all compounds as Diclofenac, Naproxen, 17β-Estradiol and 4n-Nonylphenol were biodegraded by 72.8, 80.6, 84.7 and 84.4%,

*Abbreviations:* AOB, ammonia oxidizing bacteria; AOP, advanced oxidation process; BAC, biological activated carbon; BS, biofilm solids; CAS, conventional activated sludge; DO, dissolved oxygen; DOC, dissolved organic carbon; EPS, extracellular polymeric substance; GAC, granular activated carbon; HRT, hydraulic retention time; HMDS, hexamethyldisilazane; $k_{biol}$, pseudo-first order degradation rate constant; $K_d$, solid-liquid partitioning coefficient; $k_{de}$, detachment rate constant; $k_H$, henry's law constants; logD, logarithm of the pH-dependent octanol-water partitioning coefficient; LOQ, limit of quantification; MBBR, moving bed biofilm reactors; MLSS, mixed liquor suspended solids; MLVSS, mixed liquor volatile suspended solids; MPs, micropollutants; NF, nanofiltration; NOB, nitrite oxidizing bacteria; OLR, organic loading rate; PAC, powdered activated carbon; PEM, polyelectrolyte multilayer; PSD, particle size distribution; RO, reverse osmosis; $r_{biol}$, MPs transformation rate; $r_d$, detachment rate of the biofilm; SEM, scanning electron microscopy; SF, sand filtration; UF, ultrafiltration; UV, ultraviolet; WWTP, wastewater treatment plant.

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Biodegradation
Co-metabolism
Competitive inhibition
Sorption

respectively. The effect of the changes in organic loading rates (OLRs) was investigated on the pseudo-first order degradation constants ($k_{biol}$), revealing the dominant biodegradation mechanism of co-metabolism for the removal of Diclofenac, Naproxen, and 4n-Nonylphenol, while 17β-Estradiol obeyed the biodegradation mechanism of competitive inhibition. Biotic removals and $k_{biol}$ values of all MPs were also seen higher in the biofilm as compared to the suspended biomass. To draw a conclusion, a quite high removal of recalcitrant MPs is achievable in tertiary MBBRs, making them a promising technology that supports both pathways of co-metabolism and competitive inhibition, next to the abiotic attenuation of MPs.

## 1. Introduction

Nowadays, the high-risk occurrence of micropollutants (MPs), as priority hazardous substances in the aquatic environment, has created a global demand for developing innovative and cost-effective technologies to upgrade current wastewater treatment plants (WWTPs). Since most of the WWTPs are not designed to efficiently eliminate the majority of MPs (Barbosa et al., 2016), secondary-treated effluents have been world-widely recognized as the main source of these hazardous compounds in the water bodies (Margot et al., 2013). To overcome this anxiety, scientists have been trying various types of tertiary treatment technologies such as advanced oxidation processes (AOPs) (Homem and Santos, 2011; Lee et al., 2013), adsorption processes (Bonvin et al., 2016) and membrane filtrations (Taheran et al., 2016) throughout the last decade. As compared to such costly methods in the aspects of investment and operation (Zhang et al., 2015), lower attention has been so far paid to biological treatment of secondary-treated effluents probably due to the not-satisfactory growth of microbial strains at low available carbon sources and nutrients. In spite of this fact, recently, moving bed biofilm reactors (MBBRs) are under the sharp-eyed investigation to see their capability in tertiary treatment of wastewater (Tang et al., 2017a, 2017b). Indeed, the acceptable performance of these versatile reactors have been already proved for carbon oxidation, nitrification, denitrification, and deammonification (Boltz et al., 2017; McQuarrie and Boltz, 2011; Rusten et al., 2006). In addition, Torresi et al. (2016) have lately noticed high potential of tertiary nitrifying MBBRs in MPs removal. They concluded that the thickest nitrifying biofilm (500 μm), attached on *Z*-MBBR carriers, has the highest specific biotransformation rate constants for a broad range of organic MPs due to the high biodiversity found in thick biofilms. Despite this benefit, the time required for development of nitrifying biofilm is long because both types of "ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB)" are autotrophic, grow slowly and have limited abilities to produce extracellular polymeric substance (EPS) (Young et al., 2017) which is known as the main factor of biofilm formation (Boltz and La Motta, 2007). Furthermore, the thick nitrifying biofilm may confine substrate diffusion in the biofilm (Borghei and Hosseini, 2004) and can cause high levels of inorganic precipitates in the biofilm (i.e. scaling). Scaling can lead to the blockage of the biofilm surface by the precipitates, followed by enhancement of the carriers' weight for maintaining in suspension (Piculell, 2016). In view of these points, in this study, the formation of a heterotrophic biofilm was targeted, so the proper conditions for the development of autotrophic biofilm were not provided e.g. by adding the ammonia nitrogen to the influent. We, therefore, initially aimed at developing a heterotrophic biofilm in tertiary MBBRs followed by investigating its potential for MPs removal from secondary-treated effluent. At low substrate availability, however, generation of a thin biofilm was also expected which is logically encountered with lower problematic issues such as scaling. (Regarding the information received from manufacturer of the carriers used in this study (*Z*-MBBR carriers, AnoxKaldnes), 50 to 100-μm biofilms are relatively thin. Whereas, thickness of a thick biofilm can be from 400 to 500 μm). Meanwhile, conversely to autotrophic bacteria, heterotrophic bacteria can have a doubling time of a few hours, making the biofilm establishment faster (Alpkvist et al., 2007; Boltz and Daigger, 2010; Piculell, 2016).

The fate of MPs during the activated sludge processes is controlled by the abiotic and biotic reactions. Photodegradation, air stripping and mostly sorption onto the biomass constitute the abiotic removal of MPs (Jelic et al., 2011), whilst metabolism and co-metabolism are recognized as the biodegradation mechanisms involved in the biotic MPs removal (Margot, 2015). To date, the importance of the biotic MPs removal has been attracted much higher attentions than the role of its counterpart (Andersen et al., 2005), probably due to this fact that MPs biodegradation is a sustainable process and potentially can form end products consisting of inorganic compounds, i.e. mineralization (Maeng et al., 2011). Additionally, MPs biodegradation is often the dominant removal process for the majority of compounds, as compared with abiotic removal drivers (Stevens-Garmon et al., 2011). According to the review paper published by Verlicchi et al. (2012), sorption onto the secondary activated sludge is reported up to maximum 5% for most of the analgesic and anti-inflammatory pharmaceuticals, beta-blockers, and steroid hormones which is too much lower than the role of biodegradation in MPs removal (even up to 100%). On the contrary, the removal percentage of some antibiotics like Ciprofloxacin and Norfloxacin is reported in the range of 70–90% due to the sorption, while below than 10% of these compounds were abated by the biodegradation mechanisms (Golet et al., 2003). Some studies have pointed out the significance of MPs sorption onto the biomass, as this factor is found to have an impact on the MPs bioavailability (Maeng et al., 2011) and causes the occasional negative mass balance of MPs, where MPs desorption from the suspended or attached biomass occurs during the treatment process (Blair et al., 2015). When the waste sludge is going to be used as a fertilizer on an agricultural land, this factor should be also taken into account, knowing that sludge digestion is likely not able to remove the most of persistent MPs (Ternes et al., 2004a, b).

In MBBRs, today's knowledge on the mechanisms of MPs removal is still insufficient in terms of the abiotic and biotic aspects (Casas and Bester, 2015; Grandclément et al., 2017; Torresi et al., 2017). Apart from that, individual contributions of the biofilm and suspended biomass have been rarely studied in MPs removal. To distinguish such contributions, studying the MBBRs would be a good approach as they contain both types of the attached and suspended biomass. Considerable potential of the biofilm for MPs removal is already shown by Falås et al. (2013) who studied the removal of organic MPs in a hybrid biofilm-activated sludge process. They concluded that the attached biomass can contribute significantly to the removal of recalcitrant compounds, such as Diclofenac (Falås et al., 2013).

The main objective of this study was to evaluate the removal of four MPs including two analgesic and anti-inflammatory pharmaceutical compounds (Diclofenac and Naproxen), a steroid hormone (17β-Estradiol) and an endocrine disrupting compound (4n-Nonylphenol) by means of tertiary lab-scale MBBRs, and thereby assess the distinct role of the biofilm and suspended biomass in abiotic and biotic elimination of MPs.

To describe an outline for this research, we firstly tried to develop an efficient biofilm in the reactors that ever worked on the continuous mode. At the same time, the steady-state situation of the reactors fed by the MPs-bearing secondary-treated municipal wastewater was achieved. Subsequently, distributional removal of MPs was comprehensively studied.

## 2. Materials and methods

### 2.1. Chemical compounds

All chemicals used in this study including all salts ($CaCl_2 \cdot 2H_2O$, NaCl, $K_2HPO_4$, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, $KMnO_4$, NaOAc, $NaN_3$), allylthiourea, peptone, meat extract, sucrose, acetone, methanol, hexamethyldisilazane (HMDS), glutaraldehyde, and also all MPs (Table 1S in supplementary data) were analytical grade and obtained from Sigma-Aldrich.

### 2.2. MPs-bearing synthetic wastewater

In order to have a better control on the influent concentrations of chemical oxygen demand (COD), nutrients, and MPs, the reactors were continuously fed by the synthetic wastewater instead of the raw one. Mother stock solution of the chemicals for simulating the secondary-treated municipal wastewater was weekly-prepared according to the "OECD Guideline for the Testing of Chemicals, Part 303B-Biofilms" (Alcantara et al., 2015; OECD, 2001). This solution, fed continuously into the MBBRs, was diluted with the tap water in order to achieve desirable amount of COD, nutrients and MPs. The pH of stock solution was tried to keep at 7 ± 0.5 by using 300 $mg \cdot L^{-1}$ of $CaCO_3$ (in the form of $NaHCO_3$ to provide alkalinity) and NaOH (10 $mg \cdot L^{-1}$) (Torresi et al., 2016). As shown in Table 2S in supplementary data, influent's COD was declined from 500 to 100 $mg \cdot L^{-1}$ in sequential steps. In the final step (COD: 100 $mg \cdot L^{-1}$), total nitrogen (TN) and phosphate ($PO_4^{3-}$) of the influent was 10 ± 1 mg $TN \cdot L^{-1}$ and 1 ± 0.1 mg $P\text{-}PO_4^{3-} \cdot L^{-1}$, respectively.

Several factors were involved in the selection of MPs, including: i) the most commonly detected compounds at the outlet of conventional WWTPs as depicted in many review papers (Boshir et al., 2017; Deblonde et al., 2011; Gorito et al., 2017; Luo et al., 2014b; Sousa et al., 2018), and ii) analytical costs as well as considerations/limitations for measuring the concentration of MPs. Furthermore, diversity of MPs in the aspects of physico-chemical properties (Table 1S in supplementary data) and biodegradability (from the easy-biodegradable e.g. 4n-Nonylphenol (Joss et al., 2006; Luo et al., 2014b; Stasinakis et al., 2010)., to the recalcitrant MP e.g. Diclofenac (Joss et al., 2006; Vieno and Sillanpää, 2014) was taken into account. This point was also the main reason for the selection of 4n-Nonylphenol (the linear isomer) instead of Nonylphenol (mixture of different branched and linear chain isomers). In other words, we aimed at having at least one linear and easy-biodegradable compound in the list of target MPs.

Mother stock solutions of MPs were separately prepared in high-pure methanol with concentration of 1 $g \cdot L^{-1}$, stored in 15-mL amber glass bottles and kept in freezer (−18 °C). An appropriate amount of each MP was added to the mother stock solutions of the wastewater to reach to the target concentration of MPs. Here, the final concentrations of Diclofenac, Naproxen, 17β-Estradiol and 4n-Nonylphenol were considered 0.5, 2.5, 1 and 7 $\mu g \cdot L^{-1}$, respectively, based on available data in literature about concentration of target MPs in effluents of conventional municipal WWTPs, presented in Table 1S in supplementary data along with their physico-chemical characteristics.

### 2.3. Biofilm carriers

Saddle-shaped *Z*-MBBR carriers, produced by AnoxKaldnes company (Lund, Sweden), with a 30 mm diameter, 2190 $mm^2$/carrier protective surface area (PSA), 400 μm grid height and compartment size of 2.3 mm × 2.3 mm were used in this study. Maximum thickness of the biofilm at this so-called *Z*-400 carrier would be 400 μm. Compared to other types of available carriers in the market, I) biofilm expands on the outside of the Z-carriers instead of inside voids, and the exposed biofilm is covered on the entire surface of the carrier (Piculell et al., 2016), and II) these carriers are less prone to the scaling phenomenon, as the formed biofilm is shown to be filled by lower amounts of inorganic precipitates (Piculell, 2016).

### 2.4. MBBR configuration and operation

#### 2.4.1. MBBR set-up

Two identical lab-scale glass MBBR reactors, each with an effective volume of 3.1 L, were operated in parallel under the ambient temperature. Coarse-bubble air distribution was provided from the bottom of each reactor to maintain dissolved oxygen (DO) concentration between 4 and 5 $mg \cdot L^{-1}$ (Honeywell DO Probe), and also provide a proper circulation of the whole carriers inside the reactors. During the continuous running, concentrated wastewater was fed into the reactors by means of an adjustable peristaltic pump (Minipuls 3, GILSON) and a rotameter-based system was used for entering the tap water into the reactors. Applying different ratios between the flowrates of the concentrated wastewater and tap water allowed us to operate MBBRs with favorable values of hydraulic retention times (HRTs) and influent's COD. A glance through the literature indicates that MBBRs have been so far operated in a wide range of HRTs (Boltz et al., 2017; McQuarrie and Boltz, 2011; Rusten et al., 2006) and a definitive value has not stablished yet, in particular, for tertiary MBBRs which are in the beginning steps of the attention. However, in the continuous running of the set-up, HRTs and influent's COD values were stepwisely changed from 20 to 4 h and 500 to 100 $mg \cdot L^{-1}$, respectively.

#### 2.4.2. Start-up procedure & biofilm formation

This part is explained in Section 1S in supplementary data.

#### 2.4.3. Methodology for the assessment of MBBR performance

*2.4.3.1. Overall removal of MPs–contribution of the biofilm and suspended biomass.* After biofilm formation, reactors' feeding with a MPs-bearing secondary-treated wastewater was continued in order to assess the overall removal of MPs at different OLRs. As shown in Table 2Sb in supplementary data, the reactors worked continuously for 5 days at HRT: 4 h, 7.5 days at HRT: 6 h, 10 days at HRT: 8 h and 12.5 days at HRT: 10 h to have the same ratio between the operation time and HRT. Two samples from the influent and four samples from the effluent were collected in the last 2 days of each HRT for COD and MPs analysis. In addition, we also investigated the individual role of the biofilm and suspended biomass in the overall removal of MPs at these applied HRTs (Table 2Sc in supplementary data). For this purpose, colonized carriers from one reactor were transmitted into another identical clean MBBR (filling ratio: 40%), pre-filled with an autoclaved MPs-bearing secondary-treated wastewater. The continuous feeding of the reactor was subsequently started with MPs-bearing secondary-treated wastewater, and parameters of COD and MPs were measured in 2 days in a row. From the difference between the overall removal and MPs removal by the biofilm, we obtained the MPs removal by the suspended biomass.

*2.4.3.2. Abiotic and biotic removal of MPs.* Overall removal of MPs consists of abiotic and biotic aspects of MPs removal. In this research, the biotic removal was obtained from the difference occurred between the overall and abiotic removal. Table 2Sd and Table 3S in supplementary data briefly summarizes our strategy for the assessment of abiotic removal. Taking this into account that sorption onto the suspended and attached biomass, air stripping and photodegradation are involved in abiotic removal of MPs (Jelic et al., 2011), four pre-autoclaved and sealed 1000-mL Erlenmeyer flasks, as described in Table 3S in supplementary data, were incubated in batch mode for 2 h in 120 rpm. Falås et al. (2012) found that sorption of MPs onto the biomass is a fast process in an activated sludge system and can reach equilibrium within just 30 min for acidic pharmaceuticals such as Diclofenac and Naproxen. In the study of Luo et al. (2014a) on in a sponge-based moving bed bioreactor,

some MPs like 4n-Nonylphenol and 17β-Estradiol were eliminated up to 80% during the first 2 h in the batch experiments with colonized sponge that proves sorption has a remarkable role in abiotic removal of these compounds. Moreover, on the basis of a research conducted by Andersen et al. (2005) on the sorption capacity of suspended biomass for steroid estrogens, equilibrium is almost reached after only 30 min and concentrations in the water phase did not change after 2 h. The time used for this batch experiment was therefore set at 2 h to ensure that equilibrium was reached in this test and homogenous samples were collected at regular intervals for MPs analysis. In order to avoid MPs biodegradation throughout the batch experiment, we used 500 $mg \cdot L^{-1}$ sodium azide ($NaN_3$) to suppress aerobic microbial activity, and 5 $mg \cdot L^{-1}$ allylthiourea to inhibit nitrification (Rattier et al., 2014; Torresi et al., 2016).

Experimental design of the batch experiments is given in Section 2S in Supplementary data and Table 3S in supplementary data.

*2.4.3.3. Modeling of biofilm formation.* In the current study, the biofilm growth was experimentally determined using the methodology described in Section 2.7. To go deeper into the biofilm behavior, we used Eq. (1) introduced by Plattes et al. (2008) who developed a zero-dimensional biofilm model for dynamic simulation of MBBRs using Activated Sludge Model 1 (ASM1). They proposed that detachment rate of the biofilm is equal to the biofilm growth rate at steady state condition. Such model was used in continuous mode of operation, at steady-state condition, for evaluating the biofilm growth rate.

$$r_d = k_{de} \cdot (BS)^2 \tag{1}$$

where, BS is concentration of the biofilm solids ($g\ BS \cdot m^{-3}$) (Section 2.7), $r_d$ is detachment rate of the biofilm ($g\ BS \cdot m^{-3} \cdot d^{-1}$), and $k_{de}$ is detachment rate constant ($m^3 \cdot g\ BS^{-1} \cdot d^{-1}$).

*2.4.3.4. Pseudo-first order degradation kinetics.* Biological transformation of MPs in activated sludge-based systems, can be described by pseudo-first order kinetics as expressed as Eq. (2) (Alvarino et al., 2018, 2016). In the MBBRs operating in the continuous mode of operation, this equation was used to calculate $k_{biol}$ of each MP at the steady-state condition (Section 3S in Supplementary data).

$$k_{biol} = \frac{F_{inf} - (F_{eff} + F_{stripped})}{X_S.S.V} \tag{2}$$

where, $F_{inf}$, $F_{eff}$, and $F_{stripped}$ indicate the mass flows of MPs in the influent, effluent, and air-stripped compound, respectively ($\mu g \cdot d^{-1}$). Meanwhile, $k_{biol}$ is pseudo-first order degradation rate constant ($L \cdot g\ VSS^{-1} \cdot d^{-1}$), $V$ is the volume of the reactor (L), and $S$ is soluble compound concentration in the reactor ($\mu g \cdot L^{-1}$). In the present work, in addition to the total $k_{biol}$ (calculated for the both biofilm and suspended biomass), $k_{biol}$ was separately calculated for the biofilm and suspended biomass (methodology is explained in Section 2.4.3.1). For the total $k_{biol}$, $X_S$ is sum of the volatile suspended solids and the biofilm solids ($g \cdot L^{-1}$). Furthermore, $X_S$ is the biofilm solids for the biofilm's $k_{biol}$ ($g\ BS \cdot L^{-1}$), while is the volatile suspended solids for the $k_{biol}$ related to the suspended biomass ($g\ VSS \cdot L^{-1}$).

Parameter of $F_{stripped}$ can be calculated according to Eq. (3).

$$F_{stripped} = Q \cdot H \cdot q \cdot S \tag{3}$$

where, $Q$ is the feed flow rate ($L \cdot d^{-1}$), $H$ is Henry's law constant (dimensionless), and $q$ is the air supply per unit of wastewater ($L_{air} \cdot L^{-1}$ influent).

*2.4.3.5. Solid–liquid partition coefficient.* The solid–liquid partitioning coefficient ($K_d$, $L \cdot kg^{-1}$) of each MP was calculated for the biofilm and suspended biomass by using Eq. (4) and Eq. (5), respectively (Seira et al., 2016).

$$K_d = \frac{S_{sb}}{S_{eb}} \tag{4}$$

$$K_d = \frac{S_{ss}}{S_{es}} \tag{5}$$

where, $S_{sb}$ is the concentration of MP sorbed onto the biofilm ($\mu g\ kg^{-1}{}_{BS}$), $S_{ss}$ is the concentration of MP sorbed onto the suspended biomass ($\mu g\ kg^{-1}{}_{ss}$), $S_{eb}$ is the concentration of MP in the aqueous phase related to the biofilm (μg/ L), and $S_{es}$ is the concentration of MP in the aqueous phase related to the suspended biomass (μg/ L) at equilibrium conditions.

### 2.5. Viability of the biofilm and suspended biomass

During the continuous running of MBBRs, the bacterial viability of the suspended biomass and biofilm was distinguished using "LIVE/DEAD® BacLight™ L7012 Bacterial Viability Kits" (Molecular Probes, Invitrogen Detection Technologies). In order to assess the viability of the suspended biomass, according to the protocol of manufacturer, 3 μL of pre-combined stains (1.5 μL of each stains including SYTO®9 and propidium iodide) was added to 1 mL of the mixed liquor in an amber glass bottle. After mixing, this solution was incubated at room temperature for 15 min. Subsequently, 5 μL of the stained bacterial suspension was trapped between a slide and an 18 mm square coverslip and observed by epifluorescence microscope (LSM 800, ZEISS) equipped with UV light (HXP 200C) (Wan Dagang et al., 2016). On the other hand, for viability assessment of the biofilm, 3 μL of each stain was added to 1 mL of demineralized water. Then 200 μL of staining solution was gently added onto the biofilm sample immediately after picking up the target carrier from MBBRs. Afterwards, the staining dish was covered by the aluminum paper and incubated for 30 min at room temperature. The sample was gently rinsed by demineralized water for removing all excess stain and observed using the confocal microscope (Leica SP2-AOBS) (Hoang et al., 2014).

### 2.6. Biofilm morphology

Throughout the study, the biofilm morphology and its coverage on the surface of carriers were monitored by the Scanning Electron Microscopy (SEM). After gentle cutting of each biofilm-coated carrier into the small pieces, each piece was initially fixed with 2 mL of 4% glutaraldehyde, 1 mL of phosphate buffer (pH: 7.4) and 1 mL of demineralized water for 20 min, and then washed 2 times in 1 mL of phosphate buffer, 2 mL of 0.4 M sucrose and 1 mL of demineralized water for 15 min. In the step of dehydration, sample was immersed in 2-mL acetone-water solution (50%:50%) for 5 min, 2-mL acetone-water solution (70%:30%) for 5 min, and 2-mL acetone-HMDS solution (50%:50%) for 5 min. Finally, the sample was dried overnight under the evaporation of 2 mL HMDS solution. For the following step of metallization, dried sample was coated with 10-nm gold for 60 s via a compact sputter coater (The Scancoat Six, EDWARDS) according to the protocol of manufacture. It was then observed by means of a mini-SEM microscope (TM 3000 tabletop, HITACHI) with different magnifications to assess the biofilm structure.

### 2.7. Quantification of the biomass

To measure the biofilm solids mass, four carriers from each reactor were situated on an aluminum-wrapped cup, dried overnight at 105 °C in a drying oven (Memmert Oven), and weighed. Dried carriers were then washed in 3 M NaOH solution to detach the whole biofilm, and cleaned with demineralized water to rinse excess NaOH solution. Samples were dried again at 105 °C overnight and weighed. Finally,

the biofilm solids were calculated as the weight difference before and after washing of carriers (Escolà Casas et al., 2015). The biomass per area was calculated knowing that each carrier (Z-carriers with maximum biofilm thickness of 400 μm) has a PSA of 2194 $mm^2$ (Piculell et al., 2016). Moreover, mixed liquor suspended solids (MLSS) were measured by filtering through a paper filter (VWR, 516-0348, France) with 0.70 μm pore size succeeded by drying overnight at 105 °C and weight determination. By the way, overnight heating under the temperature of 550 °C in a furnace (Salvis Lab Thermocenter, TC40) was applied in order to measure mixed liquor volatile suspended solids (MLVSS) (Escolà Casas et al., 2015).

### 2.8. Dissolved COD and nutrients measurements

Samples were firstly filtered through 0.70 μm glass fiber filters (VWR, 516-0348, France). Then, the analysis process was done using HACH LANGE kits of LCI 500 or LCK 514 for COD, LCK 341 for total Nitrogen, LCK 304 for $NH_3$-N, and LCK 341 for P-$PO_4^3$, along with DR3900 Benchtop VIS Spectrophotometer equipped with HT200S oven (HACH LANGE, Germany). These parameters were measured in duplicate and the average values are reported.

### 2.9. MPs analysis

For MPs analysis, samples (each with a volume of 250 mL) were firstly filtered using 0.70 μm glass fiber filters (VWR, 516-0348, France), secondly collected in 500-mL amber glass bottles and finally kept in freezer (−18 °C). They were then shipped to the LaDrôme laboratory (France) in a freeze box for analysis within 24 h under the analyzing license of COFRAC ESSAIS. A multi detection procedure including Gas Chromatography (coupled with ECD/NPD mass spectrometry) and Liquid Chromatography (along with DAD, fluorescence, Tandem mass spectrometry) was applied for all MPs with Limit of Quantification (LOQ) of 0.01 μg/L for Diclofenac, Naproxen and 17β-Estradiol, and 0.04 μg/L for 4n-Nonylphenol. Removal values $R$ were calculated according to the Eq. (6), where $C_i$ and $C_e$ are MP concentration in the influent and effluent of the reactors, respectively. Each measurement was performed in duplicate and the average of values with standard deviation are reported.

$$R = \left(1 - \frac{C_e}{C_i}\right) \times 100 \tag{6}$$

## 3. Results and discussion

### 3.1. Biofilm formation

To date, many researchers have found that the process of biofilm formation could be frequently affected by the environmental and operational conditions, such as carbon & nutrients availability, fluid velocity, MLSS, temperature, pH, and surface roughness (Chen et al., 2005). In this research, since we were facing with the challenge of low COD and nutrients availability, the OLR was almost kept constant at different HRTs in order to provide enough food for the biomass generation and maintenance. Fig. 1 indicates that once the COD removal increased >80%, the HRT was reduced to the next step. This procedure was repeated to the final HRT of 4 h, where a stable COD removal and also the food to microorganism ratio (F/M) (Fig. 1S in supplementary data) were observed for 5 weeks in a row.

In addition to this fact that suspended biomass contribute considerably to the overall performance of the MBBR (Piculell, 2016), Plattes et al. (2008) reported that attachment rate of the biomass is a function of the square of the suspended solids ($MLSS^2$) and an attachment rate constant ($k_a$). Hence, both parameters of MLSS and MLVSS/MLSS ratio were monitored during the biofilm formation. As plotted in Fig. 2, at the final HRT of 4 h, the MLSS concentration was remained around 1340 $mg \cdot L^{-1}$ by the conventional recirculation of the gravitational-sedimentated activated sludge into the MBBRs. We also always tried to keep MLVSS/MLSS ratio above 0.7, for instance, about 300 mL of a

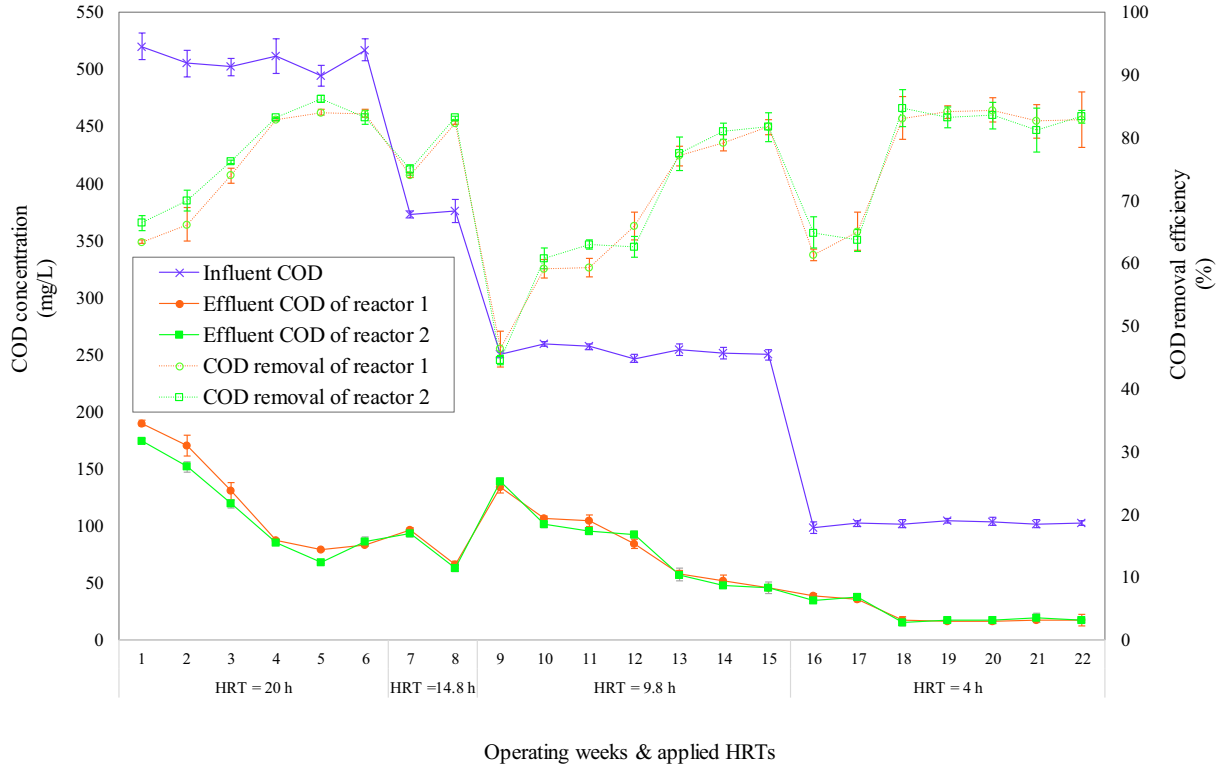



**Fig. 1.** Overall COD conversion in MBBR reactors during the process of biofilm formation.

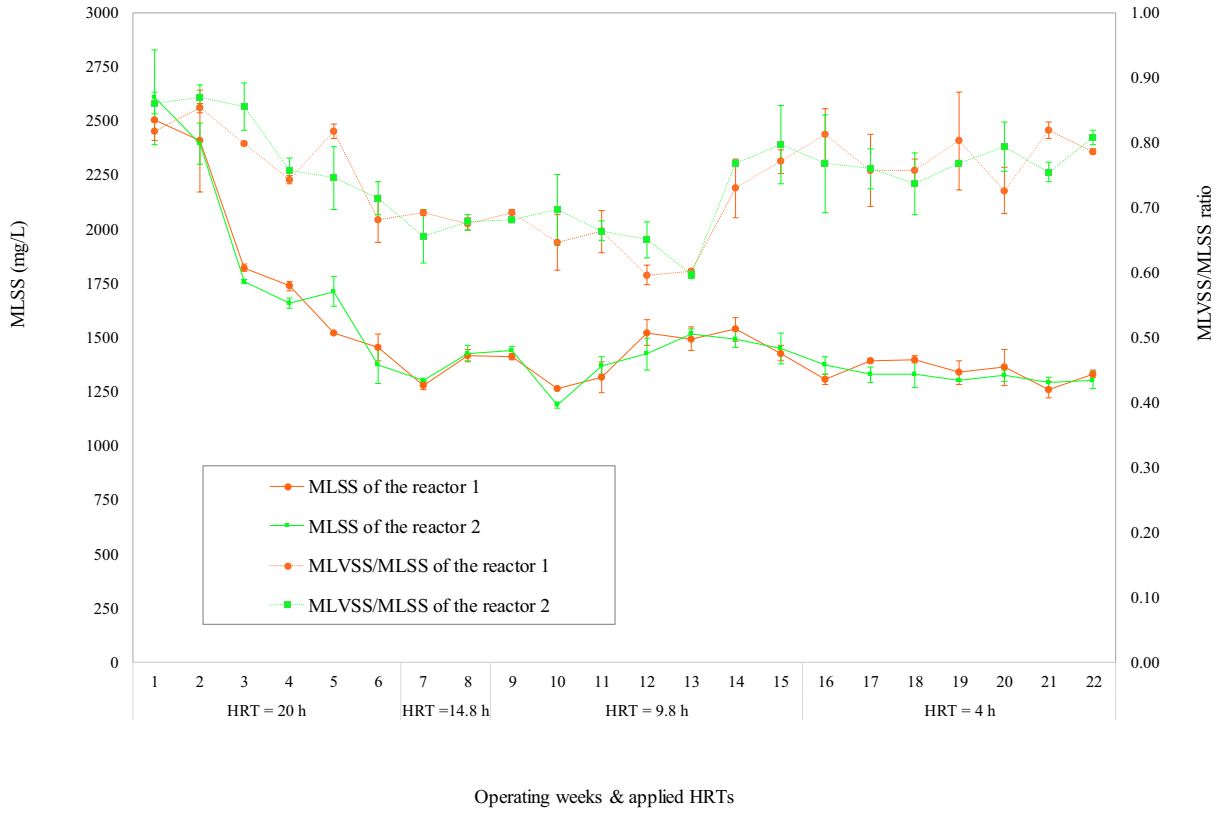
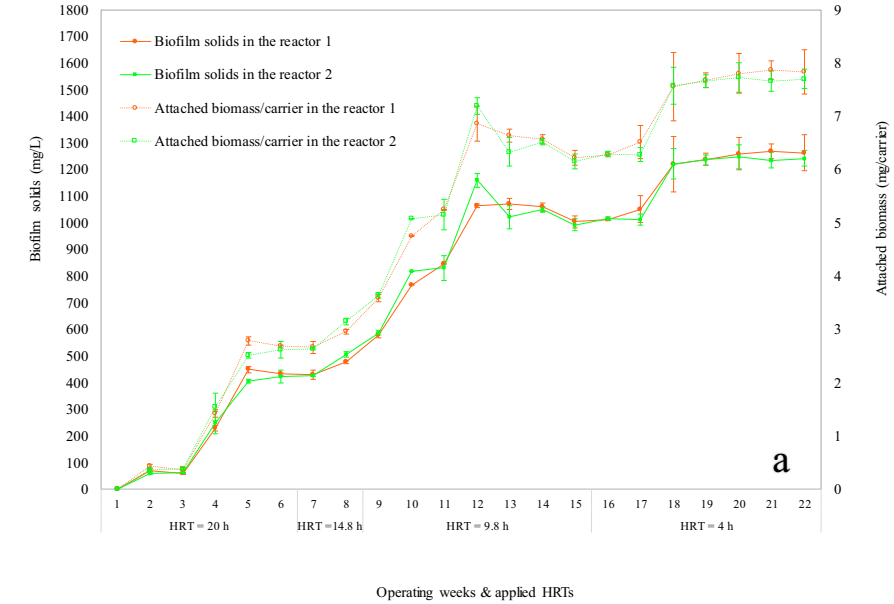


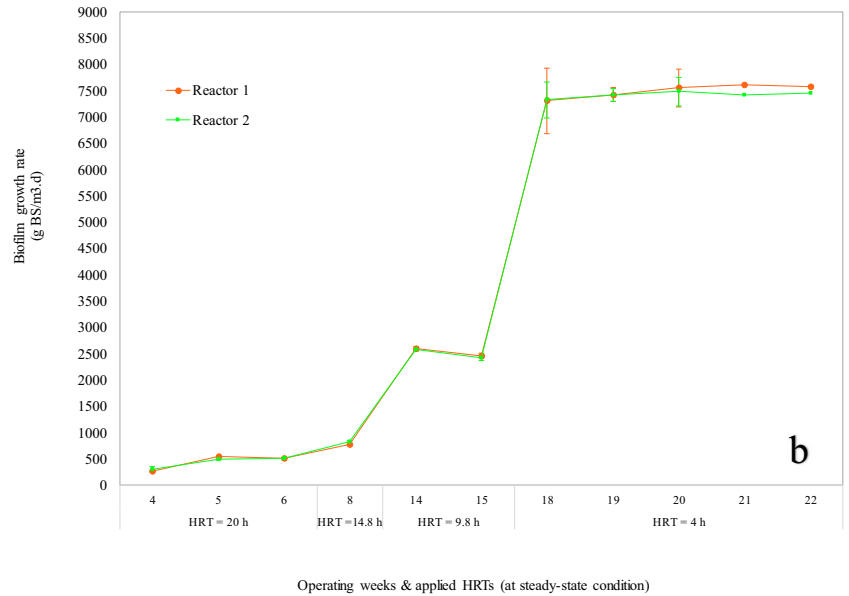

**Fig. 2.** Monitoring of the MLSS and MLVSS/MLSS ratio during the process of biofilm formation.

fresh activated sludge, got from a municipal WWTP, was added into each MBBR in 13th week. Moreover, result of the viability test on the suspended biomass (Fig. 2S in supplementary data) shows that live cells dramatically overcome dead cells at the end of the process of biofilm formation i.e. an HRT of 4 h (pictures are related to 20th week).

In Fig. 3a, we clearly indicate how the biofilm has gradually developed on the surface of carriers up to approximately 7.9 mg/carrier, corresponding to about 1275 mg·$L^{-1}$ biofilm solids inside each MBBR (calculated based on 500 carriers placed in a 3.1-L reactor). In spite of still ongoing studies about the meaning of steady-state condition in biofilm reactors (Lewandowski et al., 2004), assuming that MBBRs are at steady-state condition at the end of HRT: 4 h (COD removal ≈84% for 5 weeks in a row), the detachment rate of biomass can be considered equal to the biofilm growth rate (Piculell, 2016). Here, this hypothesis was used to evaluate the overall and individual biofilm growth rate at each HRT under the steady-state condition. As it can be seen in Fig. 3b, the biofilm growth rate has not fluctuated or changed a little for the last five weeks of the process of biofilm formation. On the other hand, according to Fig. 3c, lower biofilm growth rates were observed in the first applied HRTs compared to the last applied HRT, indicating that initial steps of the biofilm formation are slow and time-consuming. These initial steps are firstly characterized by the loose adhesion of planktonic cells to the surface, secondly the production of EPS, and then the cellular aggregation and the subsequent growth (Kim et al., 2012). The highest proportion of the overall biofilm growth rate belongs to the lowest applied HRT i.e. HRT of 4 h (~67%). Secondary-treated wastewater inherently provides a low mass transfer driving force between the substrate and attached biomass. The use of shorter HRTs in tertiary MBBRs, however, probably promotes substrate diffusion into the biofilm and therefore seems to be more convenient than long HRTs. To better understand the biofilm behavior, Fig. 3d was also plotted using Eq. (1) developed by Plattes et al. (2008). Again, we do see a stable $k_{de}$ for the last 5 weeks of this process (~0.0048 $m^3 \cdot g\ BS^{-1} \cdot d^{-1}$). This value has not been previously reported for tertiary MBBRs in literature, but it is higher than reported values for nitrifying secondary MBBRs (0.001 $m^3 \cdot g\ BS^{-1} \cdot d^{-1}$) (Plattes et al., 2008). In this study, invariable biofilm growth rate and $k_{de}$ in the last weeks probably show a type of balance in the attachment and detachment of the biomass solids from the colonized carriers. After observing this stable situation, next to the steadfast and high COD removal efficiency, we assessed the detailed performance of MBBRs at different HRTs (4, 6, 8 and 10 h) for MPs removal that is discussed in Section 3.2.

Fig. 4 shows different magnifications of SEM images acquired at various HRTs to demonstrate the quantized changes in the biofilm morphology. Under the evolutionary point of view, it is evident that biofilm coverage has increased step by step across the surface of each compartment (magnification of 50×). A filamentous structure with considerable empty spaces was observed in high HRTs by paying a close attention to bigger magnifications in the first steps of the biofilm formation. Then, reduction of HRT appears to reduce the filamentous and openness structure of the biofilm, likely due to the production of EPS that gradually fills the empty spaces (Chen et al., 2005; Huang et al., 2014; Young et al., 2017). Furthermore, the occurrence of large pores is obvious in a fully-covered biofilm at an HRT of 4 h. The porous structure leads to a better substrate penetration into the deeper areas of the biofilm especially in a low substrate availability (Boltz and Daigger, 2010; Van Loosdrecht et al., 1997). Guo et al. (2008) concluded that porous biofilms are convenient for immobilizing of numerous microorganisms and perform well against the biofilm wash-out along with the effluent. To the best of our knowledge, no enough information is still available in the literature on the biofilm's morphology of *Z*-carriers, making comparison with the results of this study difficult. In general, the biofilm morphology, however, is apparently a function of many parameters. For instance, in the case of the biofilm formed by *Pseudomonas aeruginosa*, the biofilm structure can be slab or mushroom-like in shape, depending on the type of carbon source (citrate and glucose, respectively) (Kim et al., 2012). Here, it seems that we have finally prepared a slab-like biofilm.





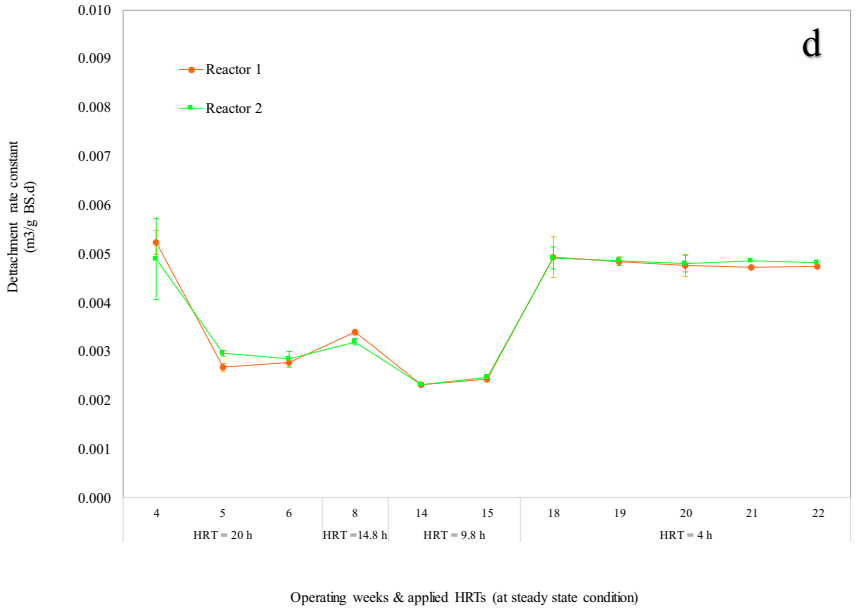



**Fig. 3.** (a): Gradual development of the biofilm on the surface of *Z*-carriers, (b): biofilm growth rate at steady-state condition, and (c): biofilm growth rate at each applied HRT at steady-state situation, (d): $k_{de}$ variations at applied HRTs at steady-state condition.

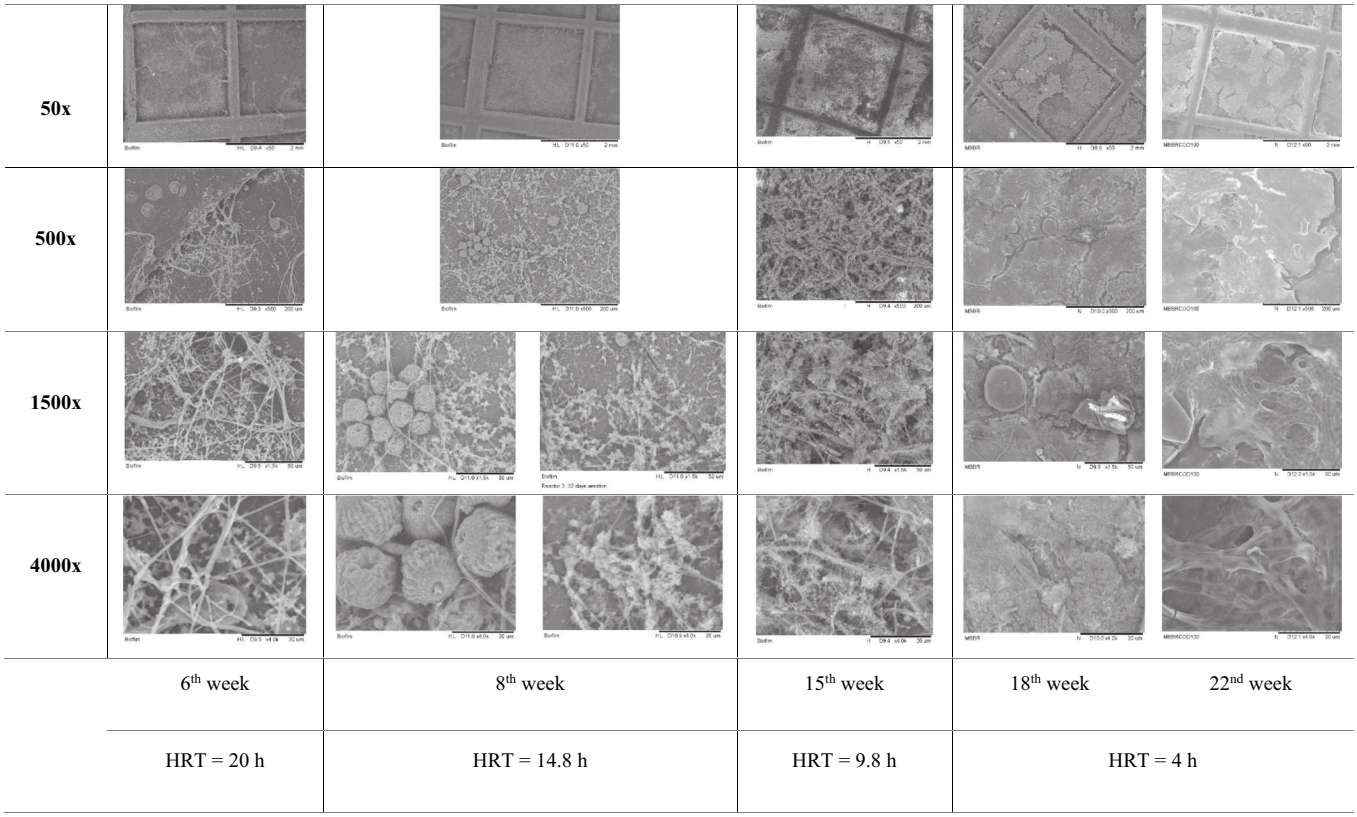



**Fig. 4.** Microscopic observation of the biofilm by the mini-SEM.

Images obtained from the confocal microscopy (Fig. 5), however, proves that we have finally prepared a thin biofilm (average thickness ~ 100 μm) with a high degree of viability even in deepest areas, stating a good penetration of the substrate and oxygen into these areas. In fact, to ensure the high substrate availability throughout the biofilm layers, thin and porous biofilms would be preferable, particularly in the case of low substrate availability (Escolà Casas et al., 2015). Compared to thick biofilms, it has been reported that lower precipitates exist in thin biofilms, and on the another hand the biofilm sloughing and making an odorous biofilm occur rarely in this type of biofilm (Alpkvist et al., 2007; Boltz and Daigger, 2010; Piculell et al., 2016; Van Loosdrecht et al., 1997).

### *3.2. MBBR performance*

#### *3.2.1. Abiotic removal of MPs*

*3.2.1.1. Photodegradation & volatilization.* No MPs removal was occurred in flask 1 during the batch experiments performed in Erlenmeyer flasks (Table 3S in supplementary data), suggesting that neither the photodegradation nor the volatilization were not able to eliminate target MPs in 2 h. More explanations are brought is Section 4S in supplementary data.

*3.2.1.2. Sorption onto the bare carriers.* With non-colonized carriers (flask 3, Table 3S in supplementary data), MPs elimination was not observed due to the absence of biomass. Similarly, no sorption capacity for acidic pharmaceuticals was seen by Falås et al. (2012) on the bare K1 AnoxKaldnes carriers. To our knowledge, except for the paper published by Luo et al. (2014a) who used a sponge-based carriers containing polar and non-polar functional groups in the structure, no research has been reported yet about the considerable sorption capability of bare carriers for MPs.

*3.2.1.3. Sorption onto the biofilm & suspended biomass.* Based on the results obtained from flasks 2 & 4, Fig. 6a is plotted to demonstrate that we can nearly attribute the abiotic removal to the only sorption. In general, two kinds of sorption profoundly occur in activated sludge systems: I) adsorption i.e. electrostatic interactions of the oppositely charged groups (positively charged groups of MPs with the negatively charged surfaces of the microorganisms and sludge), and II) absorption i.e. hydrophobic interactions between the aliphatic and aromatic groups of a compound and the lipophilic cell membrane of microorganisms (Luo et al., 2014b; Siegrist et al., 2005; Ternes et al., 2004a, b). A comprehensive study by Stevens-Garmon et al. (2011) on the sorptive behavior of MPs onto the primary and secondary activated sludge indicates that positively-charged compounds such as Amitriptyline and Clozapine have the highest sorption potential as compared to the neutral and negatively-charged ones. Moreover, sorption onto the biofilm in a nitrifying MBBR was recognized significant only for positively charged MPs in the batch experiments of Torresi et al. (2017). In the current study, regarding the negative charge of Diclofenac and Naproxen, and uncharged situation of 4n-Nonylphenol and 17β-Estradiol at neutral pH (Jermann et al., 2009; Yangali-Quintanilla et al., 2009), no or a little amount of electrostatic interactions is expected due to the phenomenon of charge repulsion. Consequently, in this study, hydrophobic interactions are considered as the main responsible for the abiotic removal. To evaluate the hydrophobicity of MPs at any pH value, the parameter of logD (logarithm of the pH-dependent octanol-water partitioning coefficient) has been proposed (Dang et al., 2014) as compounds with logD >2.6 are referred to as hydrophobic that prefer to accumulate in solid phases instead of being soluble in the aqueous phase, and hydrophilic when logD ≤ 2.6 (Linares et al., 2011). Here, Diclofenac and Naproxen are hydrophilic (logD: 1.77 and 0.34, respectively (Taheran et al., 2016)), while 4n-Nonylphenol and 17β-Estradiol (logD: 6.14 and 4.15, respectively (Dang et al., 2014)) are hydrophobic compounds.

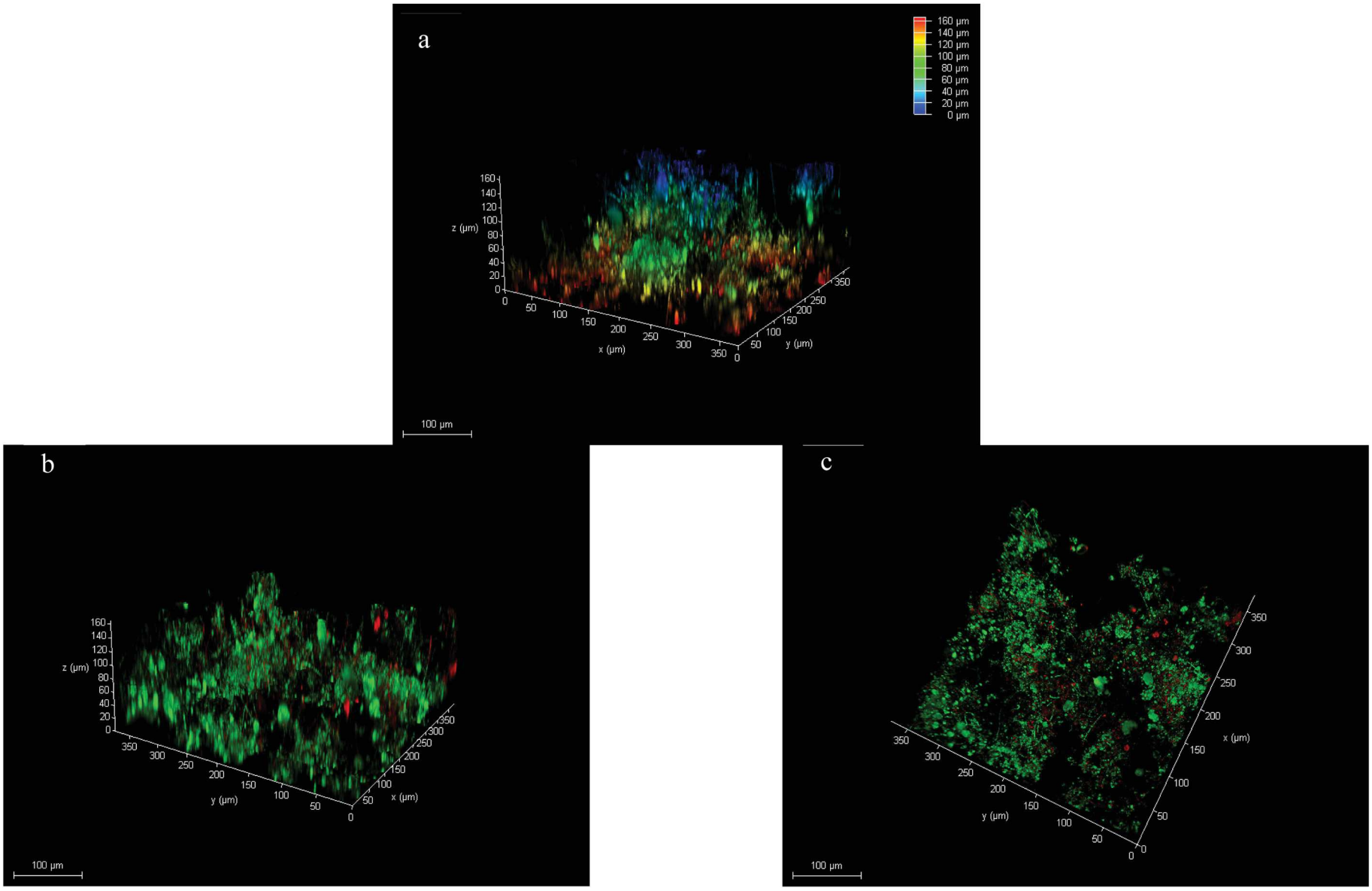



**Fig. 5.** Images of confocal microscopy to assess the thickness (a) and viability of biofilm (b: three dimensional profile, c: top view), at the HRT of 4 h (22nd week).

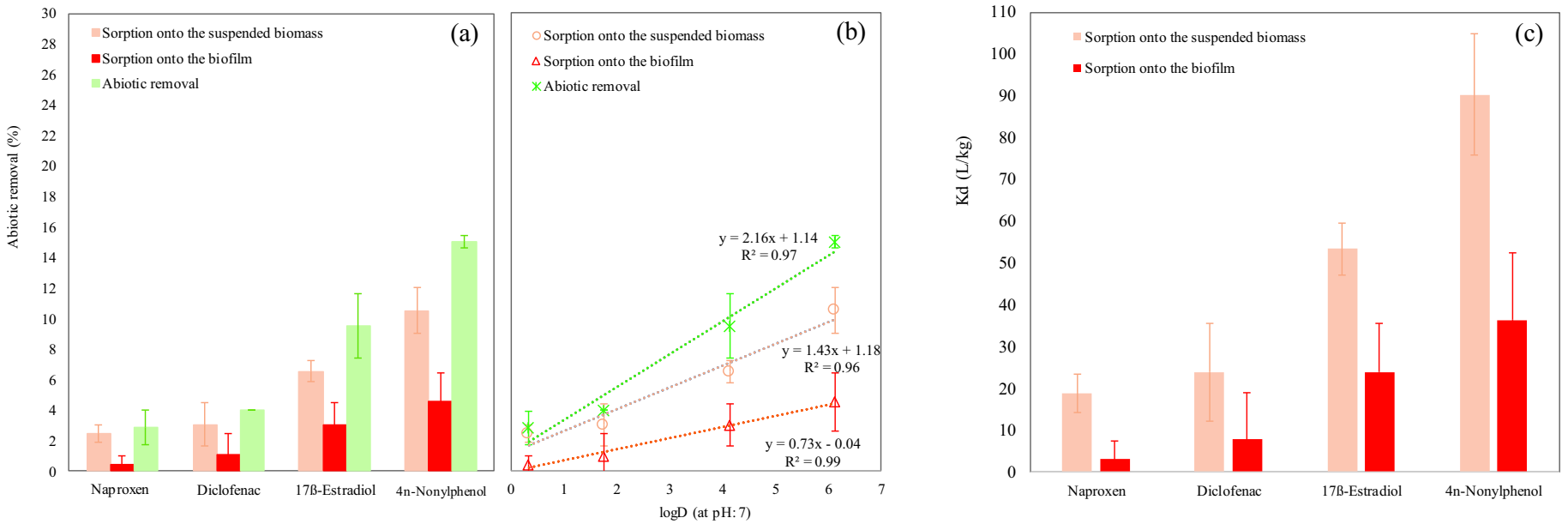



**Fig. 6.** (a) Abiotic removal of MPs, (b) the correlation between the MPs' hydrophobicity and their relevant abiotic removal, and (c) $K_d$ values of MPs for the biofilm and suspended biomass.

As stated above, hydrophobic interactions are recognized to affect the sorption of MPs onto the both suspended and attached biomass in MBBR. To prove this hypothesis, relationship between the abiotic removal of MPs and their relevant logD is plotted in Fig. 6b. From this figure, compounds of higher logD are relatively better absorbed by the both suspended and attached biomass with the R-squared values >0.90, as abiotic removals of 4n-Nonylphenol and then 17β-Estradiol are the highest (15.00 ± 0.4% and 9.50 ± 2.12%, respectively), and for the hydrophilic compounds are the lowest (lower than 4%). These results are in a full agreement with the outcomes of Joss et al. (2005)) who concluded that for pharmaceuticals and fragrances having a logD <2.5, the sorption onto secondary sludge can be deemed negligible.

Apart from the parameter of logD, sorption of MPs onto the biomass depends on the $K_d$ i.e. the ratio of the equilibrium concentration of the chemical on the solids to the corresponding equilibrium aqueous concentration (Stevens-Garmon et al., 2011; Ternes et al., 2004a, b). These values have been obtained according to the calculation in Section 2.4.3.5, and related data is given in Table 4S in supplementary data. When comparing $K_d$ values shown in Fig. 6c, the highest $K_d$ belongs to the 4n-Nonylphenol i.e. 90.4 and 36.4 $L \cdot kg^{-1}$ for the suspended biomass and biofilm, respectively. On the other side, Naproxen had the lowest $K_d$ i.e. 18.9 and 3.1 $L \cdot kg^{-1}$ for the suspended biomass and biofilm, respectively. Stevens-Garmon et al. (2011) noticed that compounds with $K_d$ < 30 $L \cdot kg^{-1}$ are compounds with a poor sorption potential on inactivated sludge (Stevens-Garmon et al., 2011). Meanwhile, a mass balance prepared in a municipal WWTP by Joss et al. (2005) proves that sorption onto the secondary sludge is not relevant for compounds showing $K_d$ value below 300 $L \cdot kg^{-1}$. Regardless of the type of treatment process (i.e. primary, secondary, or tertiary), as shown in Fig. 6c, $K_d$ values of all MPs are lower than 100 $L \cdot kg^{-1}$ that are compatible with low abiotic removal of target MPs (i.e. ~2.8–15%).

In general, variable $K_d$ values are observed in the literature for target MPs. Reported $K_d$ values for Diclofenac (16 $L \cdot kg^{-1}$ (Ternes et al., 2004a, b), <30 $L \cdot kg^{-1}$ (Stevens-Garmon et al., 2011), 32 $L \cdot kg^{-1}$ (Urase and Kikuta, 2005), and 232 $L \cdot kg^{-1}$ (Stasinakis et al., 2013)), and Naproxen (<30 $L \cdot kg^{-1}$ (Stevens-Garmon et al., 2011), 24 $L \cdot kg^{-1}$ (Urase and Kikuta, 2005), and 217 $L \cdot kg^{-1}$ (Stasinakis et al., 2013) can logically justify very low sorption of these compounds onto the biomass. Indeed, low sorption of Diclofenac and Naproxen can be ascribed not only to the low hydrophobicity of such compounds, but also to the electrostatic repulsion with negatively-charged surface of the biomass. $K_d$ value has been reported up to 476 $L \cdot kg^{-1}$ (Andersen et al., 2005) and 533–771 $L \cdot kg^{-1}$ for 17β-Estradiol (Stevens-Garmon et al., 2011), and up to 1149 $L \cdot kg^{-1}$ (Stasinakis et al., 2013) for Nonylphenol, whereby we see their higher sorption than the rest of compounds.

Fig. 6a and Fig. 6c also reveal that sorption capability of the suspended biomass is higher than the biofilm for all MPs. In the case of 17β-Estradiol and 4n-Nonylphenol, a twofold sorption is observed by the suspended biomass compared to the biofilm (e.g. $K_d$ of 17β-Estradiol was 53.5 and 23.9 $L \cdot kg^{-1}$, and its abiotic removal was 6.5 and 3%, for the suspended biomass and biofilm, respectively). Moreover, negligible abiotic removal of Diclofenac and Naproxen by the biofilm (~1%) correlates with their very low biofilm's $K_d$ values (3.1 and 7.9 $L \cdot kg^{-1}$, respectively). Compared to the biofilm, we believe that better performance of the suspended biomass is due to its higher available surface area, providing a great deal of adsorptive sites for the uptake of target MPs. Since the surface of carriers becomes occupied by the ongrowing biofilm, the available sorption sites of the colonized carriers decline by the passing of time, leading to the limited sorption capacity of the biofilm (Luo et al., 2014a). Some studies about particle size distribution (PSD) of the suspended solids (Åhl et al., 2006; Huang et al., 2008; Melin et al., 2005) revealed that MBBR reactors contain smaller solids than activated sludge systems and membrane bioreactors (MBRs). In two parallel-operated MBRs one without carriers and one with carriers (both had the equal MLSS ≈ 5 $g \cdot L^{-1}$), an average diameter of suspended solids without carriers was around 95 μm, whereas with carriers (Filling ratio: 5%) an average diameter of them decreased to 68.3 μm after 72 h of operation (Huang et al., 2008). The reason of this occurrence is that circulating carriers are continuously shattering the suspended biomass and thereby higher accumulation of MPs in MBBRs' suspended biomass is expected than the above-mentioned treatment methods. It is noteworthy that PSD of MBBR reactors is a function of operational conditions, e.g. lowering HRT in MBBR reactors causes a shift in the average particle size of suspended solids towards smaller particles (Åhl et al., 2006; Melin et al., 2005) that can affect the sorption capacity of MPs. Further studies are, however, required to substantiate this phenomenon. MPs desorption from the biomass should be also taken into account when a saturation state is achieved.

*3.2.2. Overall removal of MPs*

After biofilm formation, two MBBRs were continuously fed by synthetic secondary-treated wastewater (COD: 100 $mg \cdot L^{-1}$) and operated with four HRTs (4, 6, 8 and 10 h) to assess the overall removal of COD and MPs. In general, as shown in Fig. 7a, removal of 4n-Nonylphenol is the highest for all HRTs (below than LOQ, i.e. 99.4%), followed by 17β-Estradiol (61.1–94.2%), and then Naproxen (54–84%) and Diclofenac (45.2–76.8%). In order to make the results comparable with other studies in the literature, Fig. 3S and Table 5S in supplementary data were prepared. A glance at these data indicates that removal of Diclofenac and Naproxen is notably higher than other tertiary biological and hybrid reactors such as MBRs, but it is still somehow lower than tertiary membrane filtrations and AOPs. Interestingly, we can realize that removal of 4n-Nonylphenol and 17β-Estradiol is nearly equal with tertiary membrane filtrations and AOPs. The importance of these results is that we have obtained removal rates in the levels of laborious and costly methods of membrane filtrations and AOPs by means of a biological pathway.

Table 1 and Fig. 7a show as HRT declines (or OLR increases), total $k_{biol}$ values and removal percentages of Diclofenac and Naproxen increase, while a converse behavior is observed for 17β-Estradiol. Such trends reflect that MPs removal deeply depends on the mechanism of MPs biodegradation. Hereafter, we will bring some explanations and/or hypotheses to interpret the results.

In the case of Diclofenac and Naproxen, this increment can be explained by an increased specific activity of the suspended and attached bacteria due to higher substrate availability in lower HRTs (Gieseke et al., 2003). In this so-called co-metabolic mechanism, higher concentration of the substrate accelerates the biodegradation rate of MPs. During this mechanism, MPs are not used as a growth substrate but are biologically transformed, by side reactions catalyzed by unspecific enzymes or cofactors produced during the microbial conversion of the growth substrate (Fischer and Majewsky, 2014; Margot, 2015). Casas et al. (2015) evaluated the ability of a staged MBBR (three identical reactors in series) on the removal of different pharmaceuticals (including X-ray contrast media, b-blockers, analgesics and antibiotics) from hospital wastewater. As a whole, the highest removal rate constants were found in the first reactor while the lowest were found in the third one. The authors noticed that the biodegradation of these pharmaceuticals occurred in parallel with the removal of COD and nitrogen that suggest a co-metabolic mechanism. Besides, in the research of Tang et al. (2017a) on a polishing MBBR, the removal rate constant of some pharmaceuticals such as Metoprolol and Iopromide was dramatically enhanced by adding humic acid salt (30 $mg \cdot L^{-1}$ dissolved organic carbon (DOC)), indicating the role of substrate availability in co-metabolic degradation of these MPs.

In contrast to co-metabolism, higher concentration of the substrate decelerates the biodegradation rate of some MPs in the scenario of competitive inhibition i.e., competition between the growth substrate and the pollutant to nonspecific enzyme active sites (Margot, 2015; Plósz et al., 2010). Here, removal of 17β-Estradiol has obeyed this mechanism as though its highest removal was obtained in lowest organic loading rate. This finding is in accordance with the study of Joss et al. (2004)

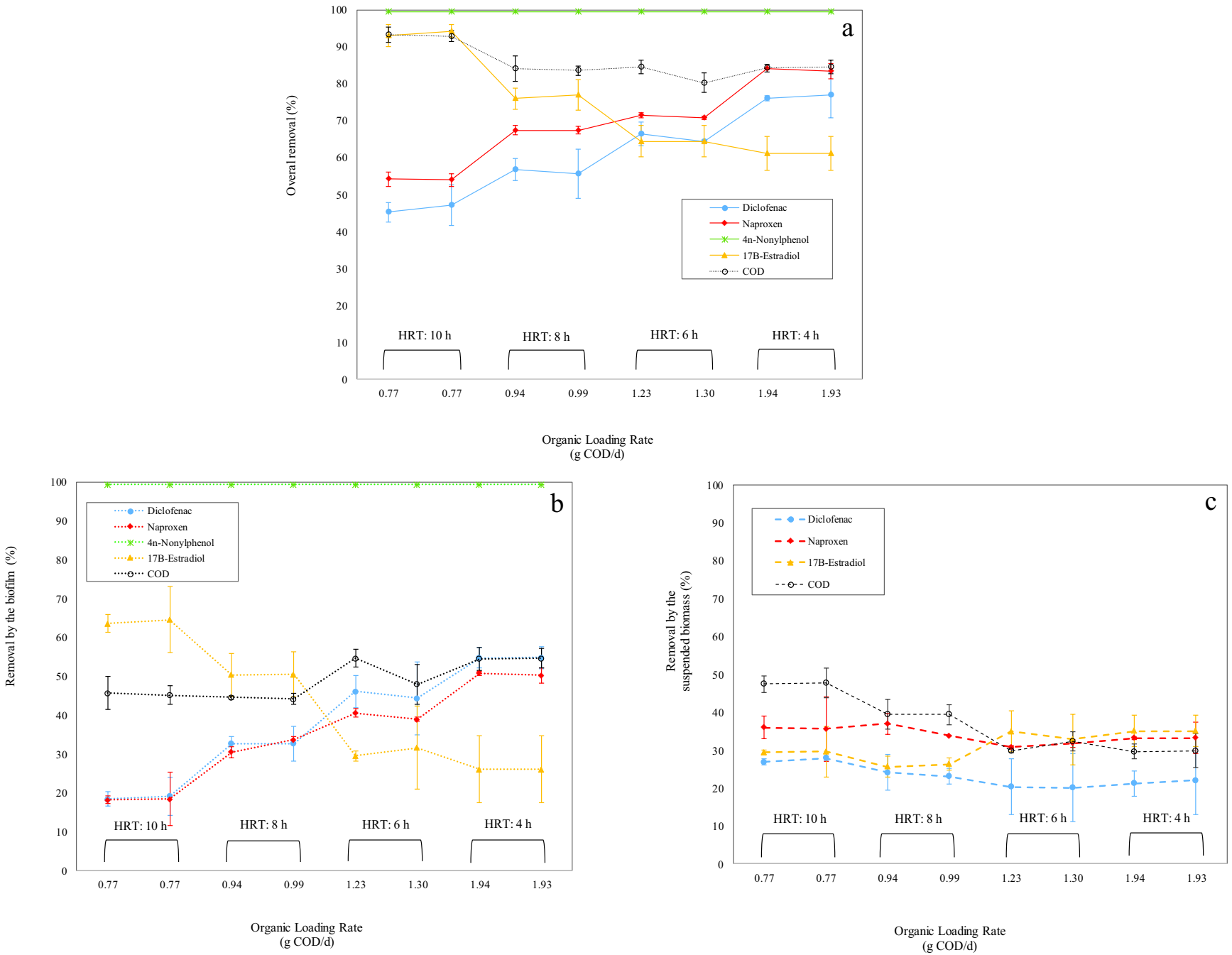



**Fig. 7.** (a): Overall removal of MPs and COD at various OLRs., (b): contribution of the biofilm in terms of MPs and COD removal., and (c): contribution of the suspended biomass in terms of MPs and COD removal.

**Table 1**
$k_{biol}$ values ($L \cdot gVSS^{-1} \cdot d^{-1}$) obtained in this study[a,b,c].

| | Total value (both biofilm & suspended biomass) | | | | Related to the biofilm | | | | Related to the suspended biomass | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HRT = 4 h | HRT = 6 h | HRT = 8 h | HRT = 10 h | HRT = 4 h | HRT = 6 h | HRT = 8 h | HRT = 10 h | HRT = 4 h | HRT = 6 h | HRT = 8 h | HRT = 10 h |
| Naproxen | 10.88 ± 0.68 | 5.35 ± 0.22 | 3.46 ± 0.06 | 1.62 ± 0.09 | 6.79 ± 0.33 | 3.06 ± 0.18 | 1.78 ± 0.13 | 0.76 ± 0.21 | 3.23 ± 0.37 | 1.90 ± 0.04 | 1.69 ± 0.11 | 1.35 ± 0.30 |
| Diclofenac | 10.77 ± 2.15 | 4.11 ± 0.47 | 2.13 ± 0.32 | 1.16 ± 0.18 | 8.09 ± 0.84 | 3.89 ± 0.87 | 1.83 ± 0.25 | 0.77 ± 0.16 | 1.79 ± 0.57 | 1.08 ± 0.49 | 0.94 ± 0.15 | 0.89 ± 0.04 |
| 17β-Estradiol | 4.98 ± 0.82 | 3.78 ± 0.56 | 5.20 ± 0.95 | 6.91 ± 1.74 | 2.36 ± 0.85 | 2.03 ± 0.60 | 3.85 ± 0.83 | 6.10 ± 1.39 | 3.44 ± 0.49 | 2.12 ± 0.57 | 1.02 ± 0.15 | 0.95 ± 0.19 |
| 4n-Nonylphenol | – | – | – | – | 1163.20 ± 23.45 | 809.89 ± 15.17 | 659.27 ± 66.02 | 587.21 ± 5.85 | – | – | – | – |

[a] As discussed in Section 3.2.1, no MPs removal was seen by the air stripping. The mass flow of the air-stripped compound ($F_{stripped}$) was not therefore considered in Eq. (2).
[b] As 4n-Nonylphenol has declined up to LOQ by the biofilm, no $k_{biol}$ values have been reported here for the suspended biomass.
[c] Total $k_{biol}$ has been separately calculated from the biofilm's and suspended biomass' $k_{biol}$. In other words, as $k_{biol}$ values have been measured in separate experiments, there is no a defined relation between the total $k_{biol}$ and individual $k_{biol}$ values. The general methodology is explained in Section 2.4.3.1. Total $k_{biol}$ was obtained from the Table 2Sb in supplementary data, while the biofilm's and suspended biomass' $k_{biol}$ was calculated regarding the Table 2Sc in supplementary data.

who showed the substrate present in the raw wastewater competitively inhibits the degradation of Estrone and 17β-Estradiol in CAS systems. These compounds were then mainly removed in activated sludge compartments with a low substrate loading.

Applying different OLRs did not affect 4n-Nonylphenol removal, leading to make the decision difficult about its removal mechanism only on the basis of a view in Fig. 7. According to the data presented in Table 1, a kind of descending order is observed for 4n-Nonylphenol's $k_{biol}$ values when HRT increases. This manner probably reinforces the hypothesis that the co-metabolic mechanism could govern the removal of 4n-Nonylphenol. Tobajas et al. (2012) found that co-metabolic biodegradation of 4-chlorophenol can be induced by adding carbon sources (phenol and glucose) in a batch test by *Comamonas testosterone*. However, regarding similarities between phenolic compounds of 4n-Nonylphenol and 4-chlorophenol (both contain a single phenol ring and an electron donating group of —OH), we will make sure that 4n-Nonylphenol is biodegraded in a co-metabolic pathway.

A complementary explanation about biodegradation mechanisms of MPs is brought in Section 5S in supplementary data.

*3.2.3. Contribution of the biofilm and suspended biomass in MPs removal*

By the experimental method already explained in Section 2.4.3.1, Fig. 7b,c specifies individual contributions of the biofilm and suspended biomass in overall removal of MPs as a function of OLRs and HRTs.

According to Fig. 7b, when OLR increases we observe that role of the biofilm also increases in the overall removal of Diclofenac and Naproxen (up to around 54% and 51%, respectively). These findings are reinforced when the biofilm's $k_{biol}$ values are under a downward trend in higher applied HRTs (Table 1). To bring an example about Naproxen, the biofilm's $k_{biol}$ values decline from 6.79 to 0.76 $L \cdot gVSS^{-1} \cdot d^{-1}$ by the increase of HRT from 4 to 10 h. Still, 4n-Nonylphenol removal is the highest (~99.4%) and the changes in the biofilm's $k_{biol}$ values likely confirm its co-metabolic biodegradation. In the case of 17β-Estradiol, as HRT is raised from 4 to 10 h, the biofilm's $k_{biol}$ values grow from 2.4 to 6.1 $L \cdot gVSS^{-1} \cdot d^{-1}$, resulting in the increment of the removal from about 26% to 64% under the mechanism of competitive inhibition. Despite of our observations and mathematical calculations, we think there is still some doubts regarding the governance of "competitive inhibition" on the removal of 17β-Estradiol, because of existence of a big difference between initial concentrations of 17β-Estradiol ($1\ \mu g \cdot L^{-1}$) and carbon (COD: $100\ mg \cdot L^{-1}$).

Compared to the efficiency of suspended biomass in MPs removal illustrated in Fig. 7c, it is apparent that the biofilm has wonderfully omitted recalcitrant compounds, as though the biofilm has reduced Diclofenac approximately two times more than the suspended biomass (~54% versus ~23%). In addition, Naproxen elimination by the biofilm is obtained about 20% higher than the suspended biomass. This outcome is in a good agreement with the studies of Mazioti et al. (2015), and Falås et al. (2013) who observed considerably higher MPs removal rates by the biofilm compared to the free biomass. In the study of Falås et al. (2013), the biofilm removed Diclofenac with $k_{biol}$ of 1.3–1.7 L. $gVSS^{-1} \cdot d^{-1}$, while its elimination by the suspended biomass was insignificant ($k_{biol} < 0.1\ L \cdot gVSS^{-1} \cdot d^{-1}$) in a hybrid biofilm-activated sludge process treating municipal wastewater. As it can be seen in Table 1, the biofilm's $k_{biol}$ values are higher than the suspended biomass's ones. The difference between the biofilm's and suspended biomass's $k_{biol}$ values is more salient for the recalcitrant Diclofenac than the rest of compounds. For instance, a nearly fourfold biofilm's $k_{biol}$ value is seen compared to its counterpart for Diclofenac at HRT: 4 h i.e. 8.09 ± 0.84 versus 1.79 ± 0.57 $L \cdot gVSS^{-1} \cdot d^{-1}$.

The main reason behind is that microbial community of the biofilm is too diverse (Biswas and Turner, 2012; Luo et al., 2014a) and this trait would possibly enable the biofilm to outperform the suspended biomass for removal of bio-refractory MPs. Additionally, regarding Fig. 4S in supplementary data, the amount of biofilm solids in the reactor increased from nearly 3 g at OLR = 0.77 $g\ COD \cdot d^{-1}$ to about 4 g at

OLR = 1.94 g COD·$d^{-1}$. Hence, higher amounts of involved attached microbial strains, however, can be another explanation for enhancement of biotic and abiotic removal of persistent MPs in higher OLRs.

While CAS systems is usually dominated by the aerobic or facultative anaerobic heterotrophic bacteria (Schmid et al., 2003), Biswas and Turner (2012) indicated that MBBR reactors treating municipal sewage support the growth of both anaerobic and aerobic organisms inside the biofilm. They also found that the suspended biomass was dominated by aerobic members of the *Gammaproteobacteria* and *Betaproteobacteria*, while anaerobic *Clostridia* and aerobic *Deltaproteobacteria* (sulfate-reducing bacteria) overcame other strains in the biofilm. According to the previous microbiological studies on the biofilm of MBBR reactors, the presence of AOB and NOB bacteria (Torresi et al., 2016), organisms associated with simultaneous nitrification and denitrification (Fu et al., 2010) and Anammox process (Tal et al., 2003), etc. has been proved. Regardless of this fact that richness and evenness of the biofilm's microbial population is found very effectual in MPs removal (Johnson et al., 2015), this widespread biodiversity is able to give a great potential to the biofilm for degradation of MPs. For instance, Torresi et al. (2016), who worked on a nitrifying MBBR, concluded that although thin biofilms favored nitrification activity and the removal of some MPs, MBBR reactors based on thicker biofilms (400–500 μm attached on *Z*-Carriers) that contain more diverse strains should be considered to enhance the elimination of a broad spectrum of MPs. Conversely, a thin biofilm (~100 μm regarding the observation by the confocal microscopy (Fig. 5)) was finally resulted in the present work, whereby substrates diffusion into the biofilm is facilitated (Piculell et al., 2016). High removal of MPs by this thin biofilm probably disaffirms the finding of Torresi et al. (2016) who reported a positive link between the MPs removal and the biofilm's thickness.

Fig. 7c reveals that contribution of the suspended biomass in MPs removal is not influenced by the changes in OLR. We also do not see a notable discrepancy in suspended biomass's $k_{biol}$ values for each MPs at all HRTs, as shown in Table 1. Meanwhile, the amount of suspended biomass in the reactor has been nearly constant in all applied OLRs (~4.2 g, Fig. 4S in supplementary data). In this regard, we observe that Naproxen, 17β-Estradiol and at the last place Diclofenac have been removed up to about 34%, 31% and 23%, respectively by the suspended biomass. As 4n-Nonylphenol is abated until below the LOQ by the biofilm, we are not able to calculate its removal by the suspended biomass. Since the biodegradability of MPs intrinsically relies on the complexity of the structure (Luo et al., 2014b), high removal of 4n-Nonylphenol is expected on the basis of its linear and monocyclic structure possessing electron donating group of —OH. Functional group of —OH embedded in the skeletons of Naproxen and 17β-Estradiol is a good explanation for their removal until about one third of the initial concentration by the suspended biomass (Tadkaew et al., 2011). Persistency of Diclofenac against suspended biomass is mainly related to the existence of an electron withdrawing group named —COOH in the structure (Tadkaew et al., 2011), and its complex pathway for biodegradation/biotransformation leads to see a high variation in elimination rates during activated sludge systems (between 20 and 50%) (Vieno and Sillanpää, 2014).

### *3.2.4. Abiotic and biotic distribution of MPs removal*

Abiotic and biotic distribution of MPs removal is illustrated in Fig. 8. The main message of this figure is that the vast majority of MPs concentration has been mitigated by the biotic reactions, while abiotic mechanisms have no a significant role in MPs removal especially for recalcitrant compounds. Here, the abiotic removal is found to be around 4%, 2.8%, 9.5% and 15% for Diclofenac, Naproxen, 17β-Estradiol and 4n-Nonylphenol, respectively. The low abiotic removal of these MPs were also reported between 0 and 5% in the secondary activated sludge systems (Verlicchi et al., 2012), and from <0.1% to 5.5% in a MBBR reactor treating a medium strength municipal wastewater (Luo et al., 2014a).

When comparing biofilm and suspended biomass, we find that biofilm outperforms suspended biomass in the biotic removal of MPs. due to biodiversity of the biofilm as stated above. While a converse trend exists for the abiotic removal, where suspended biomass overcomes the biofilm because of the accessible surface area for the sorption behavior.

To elucidate the biotic removal further, we are able to refer to a simple classification scheme suggested by Joss et al. (2006) who characterized the biological degradation of MPs using $k_{biol}$ values. In this classification, compounds with $k_{biol} < 0.1$ L·gVSS$^{-1}$·$d^{-1}$ are not removed to a significant extent (<20%), compounds with $k_{biol} > 10$ L·g VSS$^{-1}$·$d^{-1}$ are transformed by >90%, and in-between a moderate removal is expected (Joss et al., 2006). Table 1 indicates none of the target MPs has total $k_{biol} < 0.1$ L·gVSS$^{-1}$·$d^{-1}$. This parameter was obtained in the

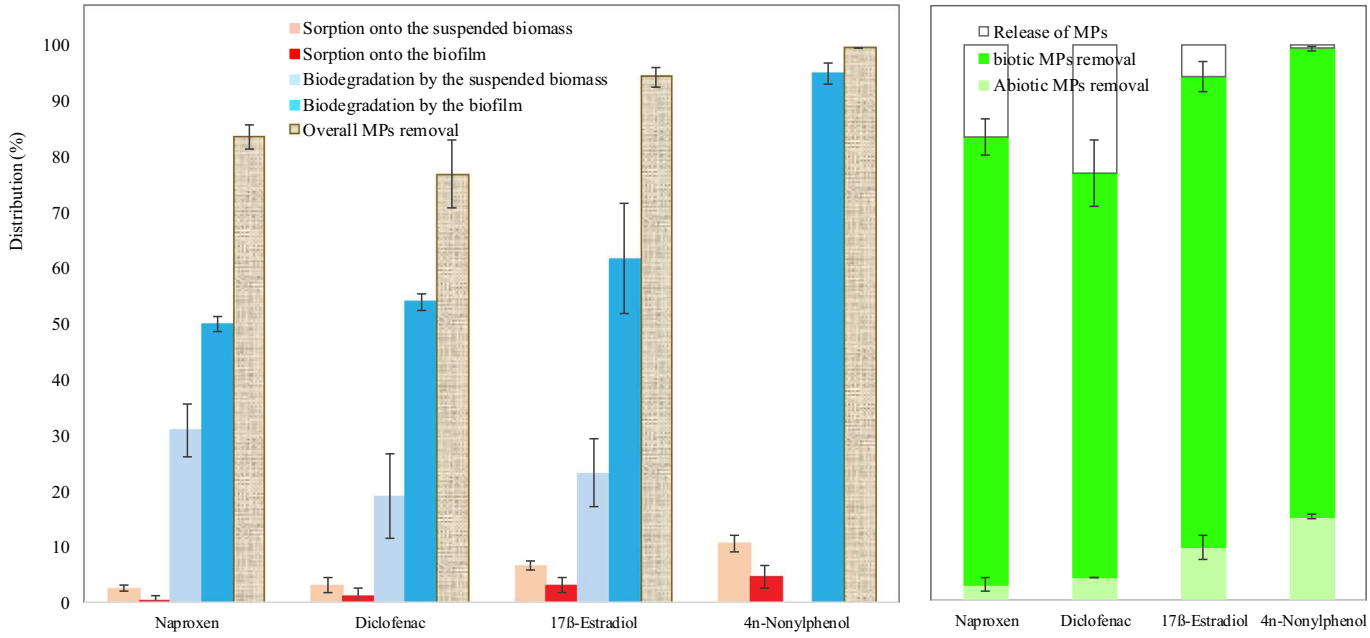



**Fig. 8.** Individual contribution of the biofilm and suspended biomass in abiotic and biotic removal of MPs.

range of 1.6–10.9, 1.6–10.8 and 3.8–6.9 $L \cdot gVSS^{-1} \cdot d^{-1}$ for Naproxen, Diclofenac and 17β-Estradiol, respectively. Meanwhile, very high $k_{biol}$ values for 4n-Nonylphenol are a good explanation for its fantastic biotic elimination. Total $k_{biol}$ values of this study have been compared with what we have found in literature in Fig. 5S in supplementary data.

In the case of Diclofenac, $k_{biol}$ values reported in a staged MBBR reactor treating hospital wastewater (0.62 $L \cdot gVSS^{-1} \cdot d^{-1}$) (Casas et al., 2015), 1.7 $L \cdot gVSS^{-1} \cdot d^{-1}$ in a hybrid biofilm-activated sludge process treating municipal wastewater (Falås et al., 2013), 1.6–2.5 $L \cdot gVSS^{-1} \cdot d^{-1}$ in a nitrifying MBBR (Piculell et al., 2016), and 1.5–5.8 $L \cdot gVSS^{-1} \cdot d^{-1}$ in a nitrifying MBBR treating an ammonium-rich secondary-treated wastewater (Torresi et al., 2016)) are higher than most of the reported values for the CAS systems (˜0.1 $L \cdot g\ VSS^{-1} \cdot d^{-1}$ (Suárez et al., 2012)) and MBR reactors (˜0.1 $L \cdot gVSS^{-1} \cdot d^{-1}$ (Joss et al., 2006)). The above-mentioned values are related to the secondary or tertiary nitrifying reactors and no $k_{biol}$ value has been already reported for MBBR reactors treating a secondary-treated wastewater. A remarkable biotic removal of Diclofenac in this study (~72.8%) is probably linked to an admirable $k_{biol}$ value for tertiary treatment systems where low amounts of carbon and nutrients exist.

Suárez et al. (2012)) calculated $k_{biol}$ values for Naproxen and 17β-Estradiol in a combined preanoxic-CAS up to 3.3 ± 2.8 and 32 ± 6 $L \cdot gVSS^{-1} \cdot d^{-1}$, respectively. Regarding the classification scheme proposed by Joss et al. (2006) and a review paper published by Luo et al. (2014b), we see a moderate removal for Naproxen (40–70%) and a high removal for 17β-Estradiol (>70%) in CAS systems. So far, no work has been carried out to obtain $k_{biol}$ values of these compounds in MBBR reactors. In the present study, about 80.6% and 84.7% of initial concentrations of Naproxen and 17β-Estradiol have been declined, respectively by the biotic reactions, stating again achievement to high $k_{biol}$ values in tertiary MBBRs. As illustrated in Fig. 5S in supplementary data, some researchers have found very high $k_{biol}$ values for 17β-Estradiol in simple CAS and nitrifying CAS systems even up to 350 $L \cdot gVSS^{-1} \cdot d^{-1}$ (Joss et al., 2004). Consequently, it seems that achieving a higher level of $k_{biol}$ values is still doable in tertiary MBBRs by tuning the operational parameters such as HRT. In other words, we believe that applying higher HRTs (even >10 h) can probably elevate $k_{biol}$ values, leading to its supreme biotic removal. Unfortunately, we could not find 4n-Nonylphenol's $k_{biol}$ in the literature but it has been highly removed in CAS even up to 99% (Luo et al., 2014b). Noticeable biodegradation of 4n-Nonylphenol can be probably linked to the high $k_{biol}$ values (587.2–1163.2 $L \cdot gVSS^{-1} \cdot d^{-1}$) obtained in this study. Considerable sorption of 4n-Nonylphenol onto the biomass (Fig. 6) probably accelerates or facilitates its biodegradation, as here such compound declined to below the LOQ. Nevertheless, further studies are still needed to find the key factors behind the scene, such as the quantification of 4n-Nonylphenol or its transformation products in the biofilm network.

Despite the fact that (I) MPs' $k_{biol}$ values are not strongly affected by the SRT (Falås et al., 2016), and (II) the correlation between the SRT and elimination of target MPs is still not clear (Joss et al., 2005; Suárez et al., 2012; Vieno and Sillanpää, 2014), some authors (Casas et al., 2015; Falås et al., 2012; Torresi et al., 2016) have noted that possible high SRTs in MBBRs enable them to remove MPs more efficiently than other tertiary biological methods for the biotic removal of MPs (Fig. 3S and Table 5S in supplementary data). Longer SRTs allow bacterial population to become more diversified and more capable of degrading MPs either by direct metabolism or by co-metabolic degradation via enzymatic reactions (Falås et al., 2016). On the other hand, low F/M ratio emerged by the high suspended and attached biomass and the relative shortage of biodegradable organic matter may force microorganisms to metabolize some MPs with the competitive inhibition mechanism (Sui et al., 2011). We inevitably see that tertiary MBBRs support the main biodegradation mechanisms for the biotic removal of MPs, and the presence of the main substrates for microbial growth is generally neither a main trigger nor a strong inhibitor of MPs degradation.

## 4. Conclusion

In the present work, we provided further insights into the key parameters involved in abiotic and biotic removal of MPs in tertiary MBBRs. No MPs abatement was observed by the both ways of photodegradation and air stripping, revealing that abiotic removal of MPs was completely attributed to the only sorption phenomenon. Compared to the percentages of the abiotic removal (~2.8–15%) that were strongly linked to the compounds' hydrophobicity, biotic removal of MPs was observed to be the principal removal mechanism for all compounds (~72.8–84.7%). Evaluating the effect of the changes in OLRs on the MPs removal and $k_{biol}$ values proved that Diclofenac, Naproxen and 4n-Nonylphenol were mainly degraded by the co-metabolism mechanism, while competitive inhibition was the main mechanism involved in the removal of 17β-Estradiol. Contribution of the biofilm was higher than the suspended biomass in biodegradation of all MPs (specially seen for Diclofenac), while MPs sorption onto the suspended biomass was occurred more than the biofilm.

As a future perspective, regarding the remarkable contribution of the biofilm in biodegradation of recalcitrant Diclofenac and Naproxen (~50%), the establishment of a mature biofilm bio-augmented by appropriate MPs-degrading microorganisms can be suggested for further optimization of MPs biodegradation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.303.

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