Science of the Total Environment 542 (2016) 965–975

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Separate treatment of hospital and urban wastewaters: A real scale comparison of effluents and their effect on microbial communities

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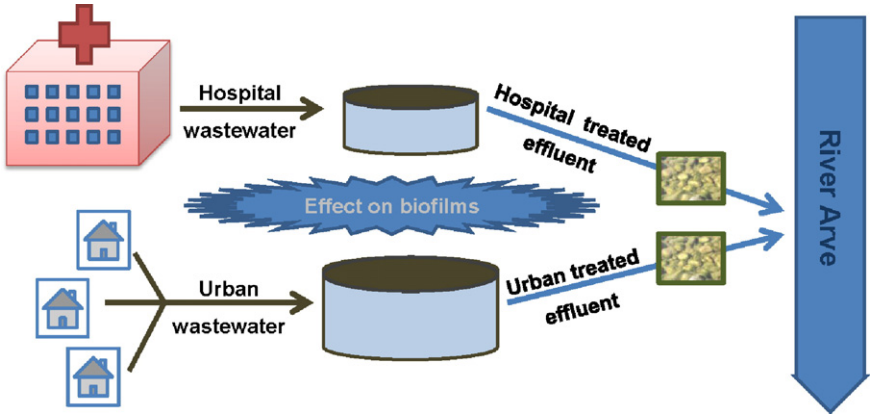
HIGHLIGHTS

- We compared treatment with activated sludge of hospital and urban wastewaters.
- Pharmaceuticals had higher removal efficiency during hospital wastewater treatment.
- Treated hospital effluents still contained higher concentrations of pharmaceuticals.
- Biofilms developed in the two treated effluents had different community structure.
- Biofilms in hospital treated effluents were less developed and had lower diversity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form 30 October 2015
Accepted 30 October 2015
Available online 10 November 2015

Editor: D. Barcelo

Keywords:
Hospital wastewater
Activated sludge

ABSTRACT

Hospital wastewaters (HWW) contain wider spectrum and higher quantity of pharmaceuticals than urban wastewaters (UWW), but they are generally discharged in sewers without pretreatment. Since traditional urban wastewater treatment plants (WWTP) are not designed to treat HWWs, treated effluents may still contain pollutants that could impair receiving aquatic environments. Hence, a better understanding of the effect of pharmaceuticals in the environment is required. Biofilms are effective "biological sensors" for assessing the environmental effects of pharmaceuticals due to their ability to respond rapidly to physical, chemical and biological fluctuations by changes in their structure and composition.

This study evaluated the efficiency of biological treatment with conventional activated sludge system performed parallel on HWW and UWW. Furthermore, six successive monthly colonizations of biofilms were done on autoclaved stones, placed in grid-baskets in the hospital treated effluents (HTE) and urban treated effluents

Abbreviations: AFDM, ash free dry matter; BSA, bovine serum albumin; COD, chemical oxygen demand; COIA, co-inertia analysis; COA, correspondence analysis; DGGE, denaturing gradient gel-electrophoresis; HPLC–MS–MS, high-performance liquid chromatography coupled to a mass spectrometer; HTE, hospital treated effluent; HWW, hospital wastewater; HRT, hydraulic retention time; NSAIDs, nonsteroidal anti-inflammatory drugs; PAO, phosphate accumulating microorganisms; PE, population equivalent; PCA, principal component analysis; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction; TIN, total inorganic nitrogen; TSS, total suspended solids; UTE, urban treated effluent; UWW, urban wastewater; WWTP, wastewater treatment plant; WFD, Water Framework Directive.

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http://dx.doi.org/10.1016/j.scitotenv.2015.10.161
0048-9697/

WWTP effluents
Pharmaceuticals
Antibiotics
Biofilms

(UTE). The biomass of these biofilms as well as the structure and diversity of their bacterial communities were investigated.
Results showed better treatment efficiency for phosphate and nitrite/nitrate during the treatment of UWW. Pharmaceuticals from all investigated therapeutic classes (beta-blockers, nonsteroidal anti-inflammatory drugs, antibiotics, analgesics and anticonvulsants) were efficiently removed, except for carbamazepine. The removal efficiency of the antibiotics, NSAIDs and beta-blockers was higher during the treatment of HWW. HTE and UTE shaped the bacterial communities in different ways. Higher concentrations of pharmaceuticals in the HTE caused adapted development of the microbial community, leading to less developed biomass and lower bacterial diversity. Seasonal changes in solar irradiance and temperature, caused changes in the community composition of biofilms in both effluents.
According to the removal efficiency of pharmaceuticals, the separate treatment was beneficial. However, their high concentrations in the HTE and the following adaptations of biofilm communities identify the importance of adapting wastewater treatment to specific hospital pollutants.

1. Introduction

In recent years, hospital wastewaters (HWW) have become a relevant topic as a contaminating source for freshwater habitats (Perrodin et al., 2013). Compared to urban wastewaters (UWW), HWW contain wider spectrum and higher concentrations of pharmaceuticals, mainly antibiotics and analgesics (Santos et al., 2013; Verlicchi et al., 2012). In a review, Verlicchi et al. (2010) highlighted that concentrations of micropollutants in HWWs are between 4 and 150 times higher than in UWW.

Pharmaceuticals are largely used, but the active substances they contain are metabolized only to a small degree (sometimes only 10%) (Bound and Voulvoulis, 2004). After use they are excreted and released into wastewaters. HWWs are usually discharged into the global sewage systems and then co-treated with domestic wastewaters in the nearest wastewater treatment plant (WWTP) without specific pretreatment (Boillot et al., 2008; Verlicchi et al., 2012) and are then released into the environment. Conventional WWTPs are not specifically designed to eliminate hospital contaminants (Joss et al., 2005) and their effluents may still contain specific active molecules (pharmaceutical compounds as well as their metabolites and conjugates), which are released into aquatic ecosystems and could cause environmental risks. Escher et al. (2011) stated that dedicated treatment of hospital wastewater would reduce the load of pharmaceuticals in the environment. Developing new technologies at WWTPs (e.g. Altmann et al., 2014) or treating hospital wastewater at their source (e.g. Kovalova et al., 2013) are the two main tracks currently envisioned (Verlicchi et al., 2010). To that aim, separate treatment is recommended to avoid dilution of HWW by mixing it with urban sewage (Verlicchi et al., 2010). In European countries, different full scale WWTPs have already been constructed for the dedicated treatment of hospital effluent (Verlicchi et al., 2015). In France, an original approach has been adopted to manage and treat separately the effluent of a new regional hospital (Centre Hospitalier Alpes Léman, Haute Savoie) in order to evaluate the relevance of a separate treatment. With the help of dedicated sewage network, the HWW is conveyed to the near municipal WWTP, where it is treated in a specific line using biological treatment with activated sludge and subjected to continuous monitoring (Perrodin et al., 2013; Sipibel Report, 2014).

Since aquatic microbial communities are the basis of many environmental processes and their structure often reveals environmental pressures, examining the dynamics of these communities exposed to treated effluents with various loads of pharmaceuticals may help to follow the environmental adaptations to these pollutants. In aquatic ecosystems, biofilms that develop on almost every surface respond to physical, chemical and biological fluctuations by changes in their structure, diversity and biomass (Biggs, 1990). This feature makes them good "biosensors" of various environmental pressures. Biofilms are known to reveal both changes in natural environmental factors (such as nutrient concentration, solar irradiance, temperature, chemical oxygen demand (COD), and total suspended solids (TSS)) (Biggs, 1990, 1996) and anthropogenic pollutants (such as pharmaceuticals, heavy metals, and pesticides) (e.g., Corcoll et al., 2015; Villeneuve et al., 2010). Exposure to pharmaceuticals, mainly antibiotics, were shown to lead to negative effects revealed by decrease in biomass and diversity, and changes in community structure as shown by Corcoll et al. (2014) and Proia et al. (2013a). Biofilm microbial communities may though be extremely helpful to compare the response of the environment to treated effluents with different origin and different load of pharmaceuticals.

This work aimed to assess the performance of activated sludge treatment plant on a pure HWW and to compare separately treated effluents from HWW and UWW using the same treatment procedure. UWW, HWW and their treated effluents where characterized. The potential environmental effects of these effluents were compared through the study of periphytic biofilm communities that were in situ exposed to hospital treated effluent (HTE) and urban treated effluent (UTE). We hypothesize that (i) biofilm microbial communities would be shaped differently by the two treated effluents, depending on their origin and the treatment efficiency, (ii) the relative high concentrations of pharmaceuticals that would characterize the HTE would cause less developed biomass and lower diversity in the microbial community of biofilms developed in HTE compared to biofilms developed in UTE and (iii) these biofilm adaptations may be modulated by seasonal changes in environmental factors.

2. Materials and methods

2.1. Study site

The study site SIPIBEL (Site Pilote de Bellecombe, http://www.graie.org/Sipibel/), located at Scientrier, Haute-Savoie, France, includes the pilot WWTP Bellecombe that has been implemented with separate treatments of HWW and UWW, since a new hospital (Centre Hospitalier Alpes Léman, CHAL) was opened in February 2012. The hospital CHAL has 450 beds, 8 operating rooms and diverse services, including emergency, oncology, nuclear medicine diagnosis, internal medical labs, and kitchen (Sipibel Report, 2014). Its effluents flow without special pretreatment, into a collecting system, which routes them directly to the WWTP. The urban network of the WWTP includes around 20,850 inhabitants using the system actively. The system is separated; therefore there should be no contribution of rain events to UWW.

The unique WWTP Bellecombe applies biological treatment with conventional activated sludge system on both HWW and UWW in separate basins (Fig. 1). The parallel treatment using the same process enables the comparison of the two treatments (according to the treatment efficiency and the environmental response to the treated effluents) in the same socio-economical and geographical environment. The WWTP has a capacity for 32,000 population equivalent (PE) and operates with 3 separate basins of 2720 m^3 or 10,600 PE (UWW), 4000 m^3 or 16,000 PE (UWW — not studied here), and 1280 m^3 or 5400 PE (HWW) capacity (Sipibel Report, 2014). Due to the smaller

volumes of the hospital discharge, the smallest basin was dedicated to the separate treatment of HWW. The treatment begins with rough filtration to remove large objects followed by grit and grease separation (Fig. 1). Then the wastewaters reach an activated sludge basin which performs aerobic and anoxic/anaerobic treatment. The supply of oxygen is achieved by an aeration system close to the wastewaters input. Its size is proportional to the capacity of the basin (in m^3), hence it releases the same amount of oxygen per m^3-basin-capacity and per minute in every basin. Its processing time can be adjusted (depending on the flow) to control the release of oxygen and subsequently the presence and duration of anoxic/anaerobic conditions in the basins. After this, the wastewater is pumped into a final clarifier (located in the middle of the tank with activated sludge), where the sludge blanket separates from the clear water and settles down. Finally, the treated effluents are drained off by overflow and directly discharged into the recipient River Arve.

2.2. Sampling procedure

The survey was performed between February and July 2014. Each of the six months (February, March, April, May, June and July) represents one sampling period (corresponding to one colonization period). The study was done on two of the three basins: one basin of 2720 m^3 dedicated to treatment of UWW (urban basin) and one basin of 1280 m^3 dedicated to treatment of HWW (hospital basin). Discharge, hydraulic retention time (HRT) and aeration time of the basins were regularly controlled by the SIPIBEL observatory. Means of all daily measurements were used to calculate means for each of the six sampling periods (Table 1). The total daily discharge of all three basins was 5700 $m^3 \cdot day^{-1}$. The discharge of the studied urban basin (2200 $m^3 \cdot day^{-1}$) was more than 15 times higher than the discharge of the hospital one (140 $m^3 \cdot day^{-1}$), which caused around 7 times shorter HRT in the urban basin (1.3 days) than in the hospital one (9.3 days). Subsequently, the oxidation in the hospital basin had to be adapted to the low flow by applying shorter aeration time of 4.4 h/day instead of 18 h/day. Despite this adaptation, the release of oxygen per m^3 of discharge in the hospital basin is almost twice higher than in the urban basin.

Multiple daily measurements of the meteorological conditions were performed by the observatory of SIPIBEL (for rain) and by the meteorological station of INRA (for solar irradiance and temperature), located 30 km from the WWTP. Means were calculated for each sampling period to characterize the seasonal changes (Table 1).

Global physico-chemical parameters and concentrations for a set of pharmaceuticals in the two wastewaters (HWW and UWW — after filtration and removal of grit and grease separation) and in their treated effluents (HTE and UTE) were quantified by the observatory of SIPIBEL. According to SPIBEL observatory resources, one sampling campaign per month was performed and analyzed. Samplings were performed simultaneously in all four locations (Fig. 1), on days without significant rainfall to avoid dilution effects. Six 24-h campaigns were done between the 18th and 24th from February to July 2014. To insure representative sampling, identical sampling strategies were used for all locations. 24-h composite water samples were collected (a total of 200 subsamples for each location, 100 ml each). The sub-sampling interval was flow–proportional (with basin-specific flow volume) in order to assess the daily variation of the influent concentration. The collected samples were stored under cooled conditions and used for further analysis of physico-chemical parameters and pharmaceutical compounds.

COD, TSS and nutrients (ammonium, nitrite/nitrate and phosphate) were measured following analytical methods, described by the French standard operating procedures (AFNOR, 1997) (Table 2). The same pharmaceutical compounds were measured in all sampling locations and periods. The set of 10 measured pharmaceuticals was characteristic of several therapeutic classes: beta-blockers (atenolol, propranolol), nonsteroidal anti-inflammatory drugs (NSAIDs) (diclofenac, ibuprofen, and ketoprofen), antibiotics (ciprofloxacin, sulfamethoxazole, and vancomycin), analgesics (paracetamol) and anticonvulsants (carbamazepine). Measurements were performed by pressurized liquid extraction, purification by solid-phase extraction (SPE) using HLB columns and analyzed by high-performance liquid chromatography coupled to a mass spectrometer (HPLC–MS–MS) as described in Sipibel Report (2014). Mean removal efficiency for the global physico-chemical parameters and pharmaceuticals (average removal of each therapeutic class) were calculated in order to compare the treatment efficiency in the two basins (Table 2).

To comprehend the effect of released nutrients and pharmaceuticals (measured in the treated effluents) and of seasonal changes on microbial communities, six monthly colonizations of biofilms were done between February and July 2014 (corresponding to the six sampling

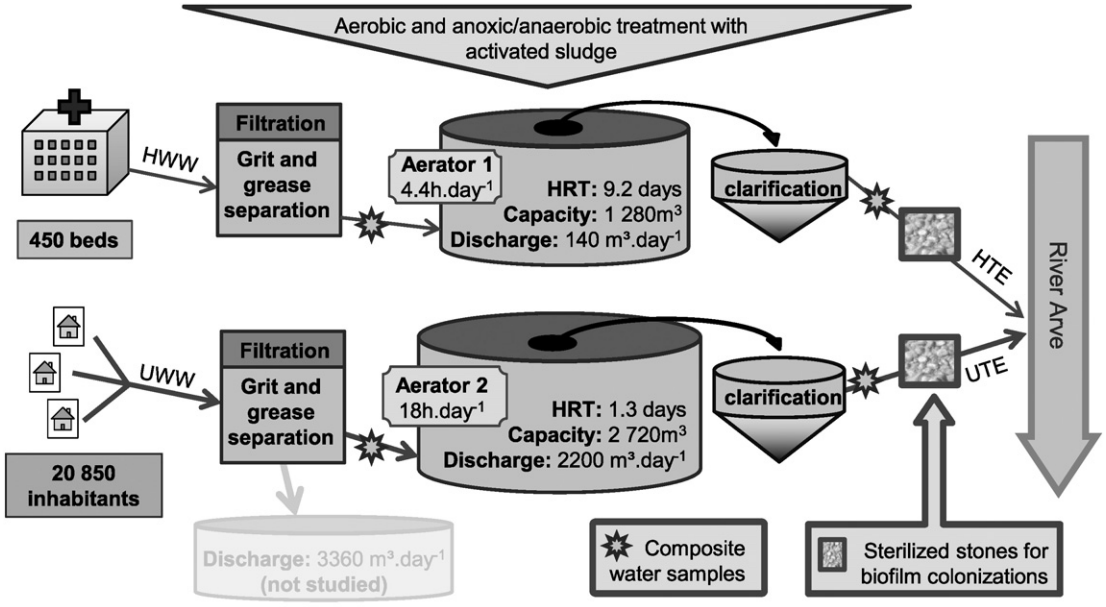

Fig. 1. Schematic of the WWTP Bellecombe (HWW = hospital wastewater; UWW = urban wastewater; HTE = hospital treated effluent; UTE = urban treated effluent; HRT = hydraulic retention time).

Table 1
Mean values from multiple daily measurements of air temperature, solar irradiance, rain, WWTP-discharge and hydraulic retention time (HRT) for each sampling period (February to July 2014) (Data sources: INRA meteorological station* and SIPIBEL observatory).

	Air temp* (°C)	Solar irradiance* ($MJ \cdot m^{-2}$)	Rain ($mm \cdot day^{-1}$)	HRT hospital (days)	HRT urban (days)	Discharge hospital basin ($m^3 \cdot day^{-1}$)	Discharge urban basin ($m^3 \cdot day^{-1}$)
February	4.3	5.2	7.82	8.4	0.8	152	3329
March	6.8	11.4	3.68	8.5	1.1	151	2568
April	10.7	16.9	1.1	10.1	1.6	127	1755
May	12	17.4	4.03	9.9	1.5	129	1871
June	17.2	23.9	4.1	9.4	1.4	136	1927
July	18	20.6	7.34	8.7	1.6	147	1742

periods). Metal grid-baskets with previously sterilized stones (with known surface) were installed in the HTE and UTE, as substrates for natural colonization of biofilms (Fig. 1). These colonization devices were positioned in the HTE and UTE and were exposed to daylight. Three replicates were included. After each colonization period, the biofilms were scraped from the stones and suspended in sterile water. All biofilm samples were brought to the laboratory in cool box for further analysis. Between two colonizations, stones were exposed to a mixture of bleaching water/hydrogen peroxide (50:50) for 2 h, rinsed and autoclaved for 30 min at 121 °C.

2.3. Biomass measurement

Biomass was used as a global measurement of the growth of colonized biofilms, which include autotrophic and heterotrophic communities and EPS matrix. Each replicate was divided into three 15 ml subsamples. For each subsample, the suspension was filtered through previously dried for 24 h at 100 °C glass microfiber filters (Whatman, GFF 25 mm diameter, cat No. 1825–025). Each filter was weighed after drying for 24 h at 100 °C to determine the dry matter content. The dry matter was burned in an oven at 480 °C (Nabertherm P320) for 2 h, and weighed to determine the ash free dry matter (AFDM). For each replicate, results were expressed as 3-subsample means in $mg \cdot cm^{-2}$. Differences in AFDM between sampling locations and dates were tested in a two-way ANOVA followed by Tukey's post-hoc tests.

2.4. Molecular methods

In this experimental approach, we focused on the bacterial community of the biofilm as it may contain taxa that are targeted by pharmaceuticals and may reveal their effect.

Pellets of colonized biofilms were prepared by centrifuging (17,949 g for 3 min, Eppendorf 5430R centrifuge) 6 ml of the initial biofilm suspension. Nucleic acid extraction was performed on the pellets using GenEluteTM-LPA (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, each pellet was mixed with 300 μl of TE buffer and 200 μl of lysis buffer, re-frozen at 80 °C for 15 min, and then thawed into a water bath at 55 °C for 2 min, before being vortexed and placed in a sonication bath for 2 min. 25 μl 20% sodium dodecyl sulfate (SDS) and 10 μl proteinase K ($20\ mg \cdot ml^{-1}$) were added to the pellets which were then incubated at 37 °C for 1 h with gentle stirring and then placed at 55 °C for 20 min. After a quick centrifugation step, the supernatant was collected and DNA was separated from proteins by addition of 50 μl sodium acetate (3 M, pH 5.2) and 1 μl GenEluteTM-LPA (Sigma-Aldrich, $25\ \mu g \cdot \mu l^{-1}$). This was followed by DNA purification through repeated washing out with ethanol. 50 μl TE was added and the DNA was incubated for 1 h at 37 °C. DNA concentration and purity was measured using NanoDrop 1000 spectrophotometer. DNA extracts were diluted with ultrapure water to 25 ng μl^{-1} and stored at −20 °C until analysis.

Table 2
Mean concentrations of six measurements (in HWW, UWW, HTE and UTE) and removal efficiency of global physico-chemical parameters and pharmaceuticals during hospital and urban wastewater treatment measured monthly by SIPIBEL between February and July 2014 (standard deviations in brackets). Requirements for the efficiency of the WWTP functioning set by the Water Framework Directive (European commission, 1991) and/or by a local decree. HWW = hospital wastewater; UWW = urban wastewater; HTE = hospital treated effluent; UTE = urban treated effluent; TSS = total suspended solids; COD = chemical oxygen demand; NSAIDs = nonsteroidal anti-inflammatory drugs.

	Wastewater		Treated effluent		Removal efficiency %		Requirements	
Parameter Analytical method	HWW	UWW	HTE	UTE	Hospital basin	Urban basin	Removal %	Release ($mg \cdot l^{-1}$)
TSS ($mg \cdot l^{-1}$) NF EN 872*	1183 (911)	915 (933)	5.3 (1.7)	4.5 (2.5)	99.6	99.5	90	35
COD ($mg \cdot l^{-1}$) NF T-90-101*	825 (174)	499 (222)	23.9 (2.9)	22.8 (5.1)	97.1	95.4	75	125
Ammonium ($mg \cdot l^{-1}$) NF EN 1484*	53.3 (10.4)	43.5 (22.2)	0.3 (0.4)	4 (3.7)	99.5	90.9	70	15
Nitrite + nitrate ($mg \cdot l^{-1}$) NF EN 26777*/NF EN ISO 10304*	0.3 (0.3)	0.22 (0.21)	65.6 (58.7)	16.5 (9.6)	–	–	–	–
Phosphate ($mg \cdot l^{-1}$) NF EN ISO 6878*	22.9 (14.2)	7.6 (5)	8.9 (1.5)	2.3 (1)	61.1	69.9	–	–
Conductivity (μS/cm) NF EN 27888	2813 (553)	1322 (207)	2450 (57)	1176 (101)	–	–	–	–
NSAIDs ($\mu g \cdot l^{-1}$) HPLC–MS–MS	17 (2.2)	10.6 (1.6)	0.14 (0.04)	1 (0.4)	99.2	90.4	–	–
Beta-blockers ($\mu g \cdot l^{-1}$) HPLC–MS–MS	4.3 (0.5)	2.5 (0.7)	0.2 (0.06)	0.5 (0.19)	95.4	80.9	–	–
Antibiotics ($\mu g \cdot l^{-1}$) HPLC–MS–MS	53.8 (32.9)	0.3 (0.22)	2.6 (1.5)	0.09 (0.06)	95.1	71.8	–	–
Anticonvulsant ($\mu g \cdot l^{-1}$) HPLC–MS–MS	0.3 (0.15)	0.5 (0.1)	0.6 (0.19)	0.5 (0.12)	–	3.5	–	–
Analgesic ($\mu g \cdot l^{-1}$) HPLC–MS–MS	610 (307)	380 (437)	0.9 (1.3)	0.4 (0.31)	99.9	99.9	–	–

* Analytical method described by the French standard operating procedures (AFNOR, 1997).

PCR amplification of 16S rRNA gene fragment was performed using Takara La (Ozyme) reagents, and the primers 358F (CCT ACG GGA GGC AGC AG) (Muyzer et al., 1993) and 907RM (CCG TCA ATT CMT TTG AGT TT; M = A or C) (Schauer et al., 2003), yielding a 549-bp fragment. Amplification reactions were performed with a total volume of 25 μl containing: 1 × buffer, 0.2 mM dNTP, 0.5 μM for each primer, 0.5 $mg \cdot ml^{-1}$ bovine serum albumin (BSA), 0,63 U Takara LA and 25 ng DNA template. For each set of reactions, a negative control was included. After 5 min at 94 °C, samples were subjected to 21 cycles of amplification, as follows: 1 min at 94 °C, 1 min at 65 °C* and 3 min at 72 °C. The PCR cycle used a touch-down profile with a reduction in annealing temperature from 65 °C to 55 °C over the first 10 cycles and at 55 °C for 11 cycles thereafter. The process was completed with a final extension step of 5 min at 72 °C.

The bacterial community was analyzed by denaturing gradient gel-electrophoresis (DGGE) following the manufacturer's protocol Instruction Manual (C.B.S.-Scientific company.inc, DGGE-2001). One-mm thick polyacrylamide gel (6% [wt./vol] acrylamide in 1 × TAE buffer [40 mM Tris, 20 mM sodium acetate, 1 mM EDTA]; pH adjusted to 7.4) was prepared with a linear formamide/urea gradient ranging from 40% to 80% (100% denaturant contained 7 M urea and 40% formamide). It was overlaid with a non-denaturing stacking gel. Each well was loaded with 15 ng PCR product and 4 μl loading buffer. First, one gel including all replicates was made for each sampling date, to assess the diversity of all communities from a single colonization period. Second, replicates corresponding to the same location at the same period were pooled adding equal DNA quantity from each sample, and one single DGGE gel with all 12 pooled samples was run to assess local and seasonal changes by comparing samples from the two locations at all six colonization periods. Electrophoresis was conducted for 16 h at 120 V and 60 °C. Subsequently, the gels were stained in darkness for 40 min in 1 × TAE buffer with 2× SYBR gold solution as specified by the manufacturer. The DGGE profiles were analyzed using GelCompar II software (Applied Math NV) and percentage of DGGE-OTUs, specific for each of the locations, were calculated using qualitative matrix obtained from pooled replicates.

2.5. Statistical analyses

The quantitative matrix of the DGGE profile obtained from pooled replicates was analyzed by correspondence analysis (COA) to compare bacterial communities from different locations and colonization periods in treated effluents. Data were previously log-transformed to normalize their distribution. The richness and the Shannon diversity index of the bacterial communities were estimated using the DGGE bands from the gel with pooled samples as a proxy (fossil package, Vavrek, 2011).

Principal component analysis (PCA) was performed to assess the main tendencies in the distribution of environmental and technical factors in the two treated effluents, where colonizations of biofilms were performed. PCA included technical data about the functioning of the 2 basins (WWTP discharge and HRT), meteorological data (air temperature, solar irradiance, rain) and concentrations of global physico-chemical parameters (TSS, COD, nitrite/nitrate, ammonium, phosphate) and pharmaceuticals (antibiotics, analgesics, anticonvulsants, NSAIDs and beta-blockers) in the HTE and the UTE. Data for PCA were centered and standardized to normalize their distribution.

Furthermore, co-inertia analysis (COIA) was done to interpret the influence of environmental and technical factors on the bacterial biofilm communities. This analysis was favored over redundancy analysis (RDA) or canonical correspondence analysis (CCA) because of its feature to represent species-environment relationships including many factors despite few sampling sites (Dolédec and Chessel, 1994). This multivariate method identifies co-structures between biological and environmental datasets, by analyzing jointly the results of COA and PCA (Dolédec and Chessel, 1994). The resulting graph represents samples with arrows. If the environmental and biological datasets have strong co-structure, short arrows are expected. The occurrence of a significant co-structure was tested by Monte Carlo permutation test (999 permutations).

All statistical analyses were done with R software (3.1.0, R development core team). Principal component analysis (PCA), correspondence analysis (COA) and Co-inertia analysis (COIA) were performed using ade4 package (Dray et al., 2007).

3. Results

3.1. Technical conditions and removal efficiency of the WWTP

Mean values for the meteorological conditions, HRT and discharge, calculated for each colonization period are presented in Table 1. The urban and hospital basins shared the same meteorological conditions. The temperature and solar irradiance exhibited usual seasonal trends and increased in the summer months. The discharge of the two basins was higher in February and March. The discharge of the hospital basin indicates average water consumption of 312 $L^{-1} \cdot bed^{-1} \cdot day^{-1}$ in the hospital.

Average values for physico-chemical parameters and therapeutic classes of pharmaceuticals, measured once a month from February to July 2014 are presented in Table 2. Table S1 presents average values detailed for each measured pharmaceutical compound. The HWW generally exhibited higher concentrations of global physico-chemical parameters and pharmaceuticals and more than twice higher conductivity (2813 μS/cm) than the UWW (1322 μS/cm).

The treatment efficiency varied depending on the basin and compounds (Table 2). Removal efficiency of organic matter (expressed by COD and TSS) and ammonium were above 90% in both basins and slightly higher in the hospital one. The concentrations of nitrite/nitrate increased after treatment, especially in the hospital basin. This resulted in an increase of total inorganic nitrogen (TIN) (ammonia + nitrate + nitrite) from 53.6 $mg \cdot l^{-1}$ in HWW to 65.9 $mg \cdot l^{-1}$ in HTE after treatment. Conversely, in the urban basin, TIN decreased from 43.7 $mg \cdot l^{-1}$ to 20.5 $mg \cdot l^{-1}$ after treatment. Phosphate removal efficiency was 61% and 70% for the hospital and urban basin, respectively. This resulted in its higher concentrations in the HTE than in the UTE.

The analgesic paracetamol was detected in highest concentrations among all pharmaceuticals in both wastewaters (Table 2), but also showed the highest removal efficiency (99.9%). Conversely, the anticonvulsant carbamazepine showed lowest concentrations, but was hardly removed during the treatment (3.5% in the urban basin). Carbamazepine concentrations in the HTE were partially higher than these measured in the HWW (Table S1). All other therapeutic classes showed removal efficiency over 72%.

The HWW exhibited a higher overall load of pharmaceuticals from all therapeutic classes. After paracetamol, the antibiotics showed second highest concentrations in the HWW. Despite their good removal in the hospital basin (23% higher than in the urban basin), their concentrations in the HTE still represented 59% of all measured pharmaceuticals. Beta-blockers and NSAIDs were also better removed in the hospital basin. This resulted in their lower concentrations in the HTE than in the UTE, despite initially higher concentrations (Fig. 3).

Depending on the concentrations present in the two wastewaters and the removal efficiency, we obtained HTE and UTE with very different composition of nutrients and pharmaceuticals (Figs. 2 and 3). The total concentration of nutrients was higher in the HTE (Fig. 2A), because of higher concentrations of phosphate and nitrite/nitrate (Fig. 2C, D). Pharmaceuticals follow the same trends (Fig. 3A), especially for antibiotics, which were about 20 times higher concentrated in the HTE (Fig. 3D). However, concentrations of ammonium, NSAIDs and beta-blockers were higher in the UTE (Figs. 2B and 3B, C).

All available parameters concerned with the development of biofilm in the HTE and UTE (discharge, HRT, air temperature, solar irradiance, rain, TSS, COD, nitrite/nitrate, ammonium, phosphate, antibiotics,

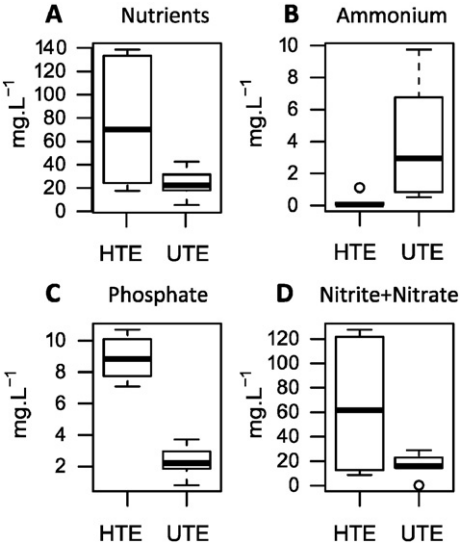

Fig. 2. Boxplots representing the distribution of nutrients (in $mg \cdot l^{-1}$) in the hospital treated effluent (HTE) and urban treated effluent (UTE) between February and July 2014.

analgesics, anticonvulsants, NSAIDs and beta-blockers) were plotted in a PCA (Fig. S1). The most important parameters to axis 1 were HRT, discharge, phosphate, antibiotics, beta-blockers and NSAIDs, which differentiate the two locations (HTE and UTE). Axis 2 represented a seasonal gradient opposing colder to warmer months, mainly linked to air temperature and solar irradiance.

3.2. Biofilm biomass

The biofilm biomass in the UTE was significantly more developed than in the HTE (Fig. 4). UTE-biofilms developed highest biomass in February (0.21 mg AFDM·cm^{-2}) and lowest in April (0.07 mg AFDM·cm^{-2}), and HTE-biofilms developed highest biomass in March (0.08 mg AFDM·cm^{-2}) and lowest in June (0.04 AFDM.cm^{-2}).

A significant effect of the colonization-period (p value = 1.73E-07) and the origin of the treated effluent (p value = 1.34E-08) on periphyton biomass was detected by ANOVA. The Tukey post-hoc test performed for the factor colonization-period showed significant differences for all months except March and July.

Temporal fluctuations in biomass development responded slightly to the evolution of concentrations of antibiotics and analgesics in both treated effluents. The highest biomass developed in the UTE corresponded to the lowest concentrations of antibiotics and analgesics, while the lowest biomass corresponded to the second highest concentration of antibiotics. Similarly, in the HTE, the highest and the lowest biomass corresponded to the second lowest concentration of analgesics and to the highest concentrations of antibiotics and analgesics, respectively.

3.3. Bacterial community composition and diversity

When analyzed individually, DGGE matrices of non-pooled samples revealed that communities from replicate samples exhibited high similarity at all colonization periods (data not shown), which provided evidence for reliable experimental design and sampling procedure. DGGE matrix of pooled samples showed that the bacterial communities differed in their richness, diversity and composition according mainly to the effluent. A total of 71 different DGGE-OTUs were detected in the 12 samples with pooled replicates. The bacterial communities exposed to the two effluents exhibited 34% of common DGGE-OTUs. 41% of the OTUs were present in the UTE only, and 25% only in the HTE. The richness and the Shannon diversity index obtained from DGGE-OTUs were higher in the UTE-communities, except for February (Table 3).

Correspondence analysis (COA) on DGGE matrix of pooled samples, performed to highlight similarities between bacterial communities, confirmed that local similarities were stronger than seasonal ones (Fig. S2). Communities from the two locations (HTE and UTE) were placed oppositely on the first axis. Biofilm communities, developed in the UTE (UTE-communities) were more heterogeneous than the ones in the HTE (HTE-communities) and followed seasonal trends visible on the second axis.

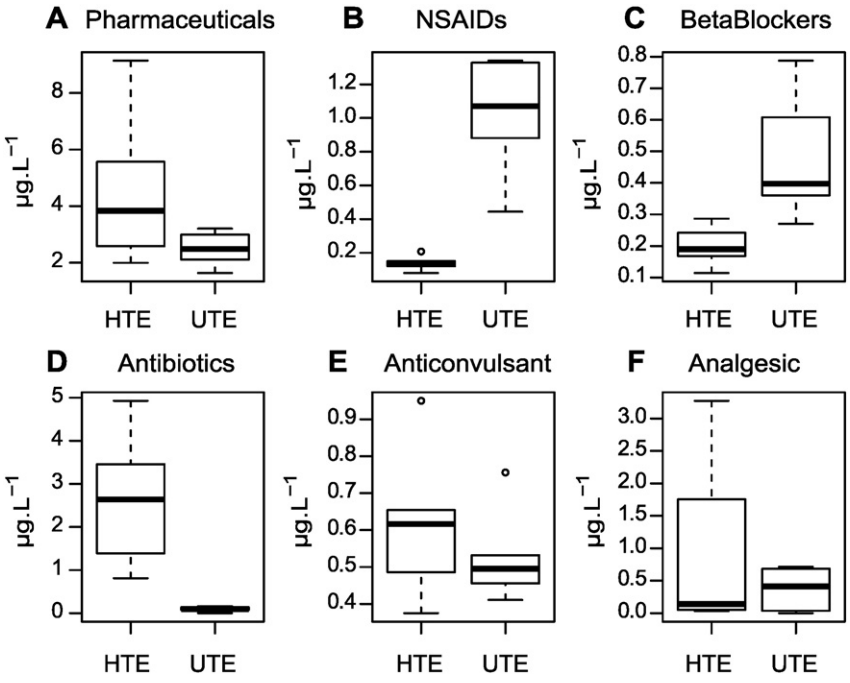

Fig. 3. Boxplots representing the distribution of pharmaceuticals (in $\mu g \cdot l^{-1}$) in the hospital treated effluent (HTE) and urban treated effluent (UTE) between February and July 2014.

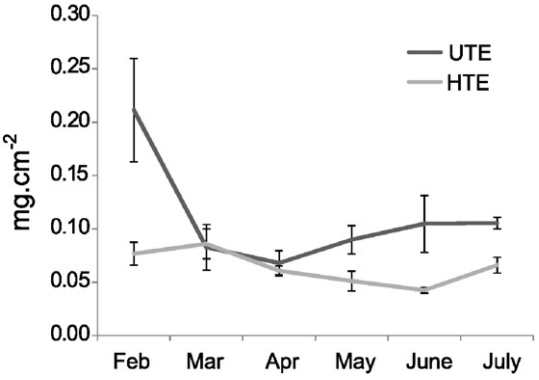

Fig. 4. Biomass measurements (AFDM) for biofilms colonized in hospital treated effluent (HTE) and urban treated effluent (UTE) six times between February and July 2014. Vertical error-bars indicate standard deviations of three replicates.

3.4. Linking environmental and biological data

Co-inertia analysis found significant co-structure between bacterial community (COA) and environmental conditions (PCA), which suggested that communities were influenced both by the origin of the treated effluent and by seasonal changes (Fig. 5). The short or even missing arrows illustrated the strong concordance between the environmental and biological datasets. The significance was tested with Monte Carlo permutations ($p = 0.001$).

Communities developed in the UTE and the HTE were located opposite on the first axis which evidenced stronger local dissimilarities, caused by differences in the treated effluents. UTE-communities were strongly associated with the higher basin discharge, and the higher presence of ammonium, beta-blockers and NSAIDs. HTE-communities were influenced more by the long hydraulic retention time and by the higher presence of phosphate, nitrite/nitrate and the therapeutic classes of antibiotics, analgesics and anticonvulsants. Among all investigated classes, antibiotics seemed to be the most important for the structuring of HTE-communities.

Communities from colder and warmer months were located oppositely on the second axis. This clear seasonal gradient was caused mainly by seasonal fluctuations of air temperature and solar irradiance.

4. Discussion

4.1. Performance of the WWTP

4.1.1. Hospital water management and its influence on the treatment

In the hospital CHAL strategies for economization of water consumption were applied in order to avoid wastage. Such strategies are for example: new water-pipe system nearly free of lacks, technical mechanisms (like dual-flush toilets and showers with economy-button, etc.), and application of hand-sanitizer for hands cleaning instead of water. Furthermore, professionals were asked to avoid unnecessary water consumption. The targeted application of these strategies led to much lower water consumption than average for hospital ($500\ l^{-1}\cdot bed^{-1}\cdot day^{-1}$) (Laber, 1999). Thus, the discharge of HWW was lower and more concentrated, as revealed by its conductivity.

As mentioned in the Materials and methods, the substantial difference between the amount of urban and hospital discharge leads to differences in the treatment process (e.g., HRT and oxygen concentration). These differences, together with the initial different composition of the two wastewaters resulted in specific removal efficiencies in the two basins and specific composition of the two treated effluents.

However, the separate mode of treatment was in accordance with all norms and legal requirements. The WWTP Bellecombe respected the standard guidelines given by the Water Framework Directive (WFD) (European commission, 1991), concerning the removal efficiency of COD and its concentration in the treated effluents. Particular conditions concerning the removal and release of ammonium and TSS set by a local decree specific to the WWTP Bellcombe (Sipibel Report, 2014) were respected too (Table 2).

4.1.2. Nitrogen and phosphorus removal

Opposite patterns in the behavior of ammonium and nitrite/nitrate during the two treatments show their different functioning. The different forms of nitrogen have different stability depending on the presence of oxygen. During the wastewater treatment, ammonium is generally first transformed to nitrite/nitrate under aerobic conditions (nitrification), and then reduced to nitrogen under anaerobic conditions (denitrification) (Pai, 2007). The high concentrations of ammonium in the HWW come from effective ammonification of organic nitrogen (like urea, cell material, proteins and amino acids). The nitrification of ammonium during the wastewater treatment is favored further by the higher presence of oxygen in the hospital basin. This leads to very high nitrate/nitrite concentrations, which cannot be effectively denitrified to nitrogen, because of the less developed anaerobic conditions in the hospital basin. This results in an increase of TIN during the treatment of HWW. Conversely, the lower presence of oxygen in the urban basin provides better nitrification/denitrification conditions. Consequently efficient transformation of ammonium to nitrate/nitrite and then to nitrogen is achieved, which results in a high elimination of TIN during the treatment of UWW.

The high concentrations of phosphate in the HWW could be explained by the release of pharmaceuticals containing phosphatic compounds (like biphosphonates and aminophosphonates) and antineoplastic from the hospital. Four detergents with high contents of phosphorus have been regularly used in the hospital and could therefore also explain the input of phosphate into the HWW (detergent-disinfectant "Eco Bac Super foam", powder for renovation of stainless steel "Assure", rinsing product "Enocare" and oxygen bleaching agent "Oxyflash"). Between 5% to 20% of their composition was represented by phosphates, phosphoric acid or 2-phosphonobutane-1,2,4-tricarboxylic acid. The presence of these products in the treated effluents and their effects on the microbial communities were not investigated, but they might have an influence on the biofilms. These substances have been discharged into the hospital basin where the presence of oxygen is higher compared to the urban basin. Precise oxygenation is essential for the efficient elimination of phosphorus. In an activated sludge, phosphate accumulating microorganisms (PAO) require an anaerobic phase, during which they accumulate P in their cells (so called "luxury uptake"). The less developed anaerobic phase in the hospital basin could disadvantage the uptake of phosphorus (Wagner and Loy, 2002) and consequently its removal. The longer hydraulic retention time in the hospital basin is also unfavorable for the removal of phosphorus (Smolders et al., 1996). This results in lower removal efficiency and higher concentrations of phosphorus in the HTE, compared to the

Table 3
Community richness and Shannon diversity index calculated from DGGE-OTUs deduced from 16S rRNA gene DGGE patterns of pooled replicate samples of periphytic bacteria communities colonized in hospital treated effluent (HTE) and urban treated effluent (UTE) between February and July 2014.

	Richness (number of DGGE-OTUs)		Shannon Diversity Index	
	UTE	HTE	UTE	HTE
February	36	38	3.3	3.5
March	38	33	3.3	3.2
April	44	37	3.5	3.1
May	35	26	3.2	2.7
June	39	30	3.2	2.8
July	43	23	3.4	2.8

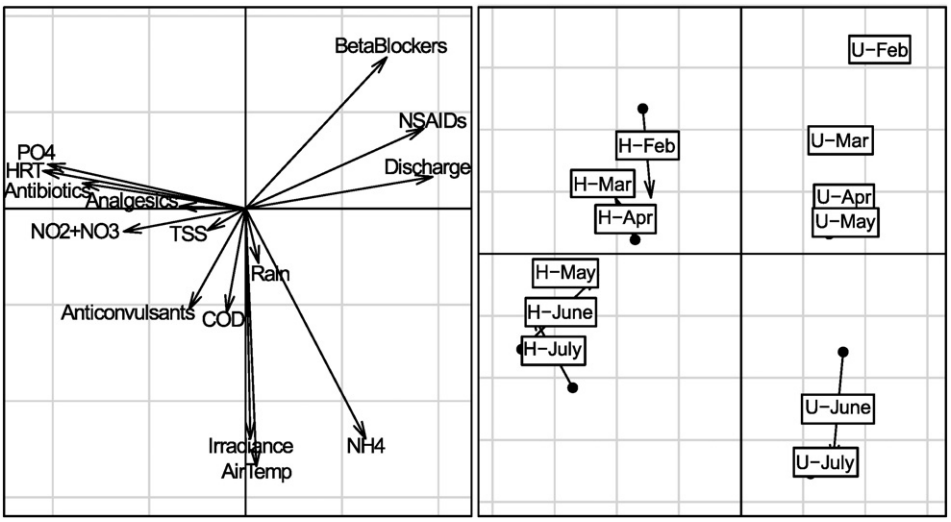

Fig. 5. Two-dimensional plots of COIA based on quantitative DGGE matrices with pooled samples of 16S rRNA gene amplicons for periphytic bacteria communities (developed in hospital (H) and urban (U) treated effluents) and the following variables: basin discharge, hydraulic retention time (HRT), air temperature, irradiance, rain, TSS, COD, nitrite/nitrate, ammonium, phosphate and pharmaceuticals (antibiotics, analgesics, anticonvulsants, NSAIDs, beta-blockers). The first two axes explained 61% and 17% of the variability, respectively.

urban basin. Although no further strategies (like iron chloride or activated carbon) were applied, relatively high phosphorus removal in both basins was achieved due to biological processes only.

4.1.3. Removal efficiency of pharmaceuticals

The regular use of pharmaceuticals in the hospital is mirrored by their higher concentrations in the HWW than in the UWW. Kosma et al. (2010) also reported highest concentrations of paracetamol among all investigated molecules as well as its high removal efficiency in both HTE and UTE.

The removal efficiency of each molecule strongly depends both on its hydrophobicity (logP) and charge (pKw) and its biodegradability. Certain molecules are known to be well removed (e.g. paracetamol), but others remain hardly eliminated after treatment (e.g. carbamazepine). We reported high removal for all studied molecules, except for carbamazepine.

The low removal efficiency of carbamazepine, also reported in other studies (e.g. Radjenović et al., 2009), is mainly explained by the specific characteristics of the molecule like resistance to degradation (Ternes et al., 2007) and low capacity to attach to the sludge (Zhang et al., 2008). Increase in the concentrations of carbamazepine after treatment could be explained by regeneration of its metabolites (e.g. Zhang et al., 2008). During the metabolism, carbamazepine is to 70% transformed to different metabolites, which are excreted via urine (Lertratanangkoon and Horning, 1982). The predominant human metabolites of carbamazepine (10,11-dihydroxy-CBZ and N-glucuronide) cannot be detected during the specific measurement of carbamazepine. However, during wastewater treatment they can regenerate into the parent compound and finally be detected in the treated effluents (Zhang et al., 2008).

Metabolites and conjugates of molecules are common problem when treating wastewaters and evaluating the efficiency of the treatment. Beside carbamazepine, diclofenac and sulfamethoxazole are also likely to form metabolites. In the presence of nitrate (in the case of wastewater treatment for example), they can temporarily form nitro-diclofenac and 4-nitro-sulfamethoxazole, which leads to decrease in their measured concentrations (Barbieri et al., 2012). We did not measure these nitro derivates, but we estimated higher removal of diclofenac and sulfamethoxazole in the hospital basin (Table S1), where higher concentrations of nitrite/nitrate were present. This might have favored the transformation of the parent molecules into their derivates, and therefore caused the decrease of diclofenac and sulfamethoxazole in the HTE. In the recipient aquatic environment where lower concentrations of nitrite/nitrate are present, the derivates could be easily retransformed into the parent compounds (Barbieri et al., 2012). Hence, the investigation of nitro-diclofenac and 4-nitro-sulfamethoxazole is of high importance in order to avoid overestimation of removal efficiency during treatment and report the realistic release of diclofenac and sulfamethoxazole in the environment.

Beta-blockers are known as substances with no or very low removal during the treatment (Luo et al., 2014; Santos et al., 2013). However, we demonstrated high removal, especially in the hospital basin. As beta-blockers are not known for forming conjugates (Gabet-Giraud et al., 2014), we suppose that their high removal is not concerned by this problem.

The removal depends not only on the molecule, but also on the treatment conditions. The high removal of ibuprofen (NSAIDs) in the hospital basin for example might be benefited by the longer hydraulic retention time (Kosma et al., 2010). Differences in physico-chemical processes such as coagulation-flocculation and flotation in the two basins might influence differently the removal of specific pharmaceuticals compounds as well (Carballa et al., 2003).

Several pharmaceutical compounds are used for prescription for hospital use only; therefore they should be detected in HWW only. Among the studied molecules, this is the case of the antibiotic vancomycin only. Vancomycin was detected in the HWW only, but it represented only 0.8% of the total concentrations of measured antibiotics. Hence, the higher concentrations of antibiotics in the HWW are not caused and hardly influenced by the presence of this tracer of hospital contamination.

The different composition of the two treated effluents resulted from the specific concentrations of the HWW and UWW and different removal efficiency during the treatment. We hypothesized that the specificity of these treated effluents will cause specific responses by the biofilms, leading to different bacterial community composition.

4.2. Adaptation of biofilm communities to HTE and UTE

The colonized biofilms developed different adaptations to HTE and UTE, expressed by different biomass development, as well as changes in bacterial diversity and community composition.

4.2.1. Biomass development

The biomass development we observed was generally low, but still in the range reported in other field studies (e.g., Corcoll et al., 2015). This low level is probably linked to a poorly developed autotrophic

compartment as these biofilms grow in an artificial ecosystem (HTE and UTE) with no or low autotrophic cell colonization from the natural environment.

Surprisingly, phosphorus and nitrite/nitrate, higher concentrated in the HTE, did not stimulate the biofilm growth. However, with the higher discharge of the UTE more nutrients passed by the colonized biofilms, which could be an advantage for their development. Furthermore, the higher urban discharge causes faster water flow, which is known to facilitate the nutrients uptake by the microbial communities in biofilms (Battin et al., 2003; Biggs and Hickey, 1994). Despite the effect of nutrients on the biofilm in the treated effluent, the main controlling factors for the biomass development appear to be antibiotics and analgesics. They provoke a response of biofilm development not only according to the location (less developed biomass in the HTE), but also through the successive colonization periods. This influence is explained by the feature of paracetamol to cause negative effect on photosynthesis (Proia et al., 2013b) and antibiotics to increase the mortality rate in bacteria and decrease their enzymatic activity (e.g., Proia et al., 2013a).

4.2.2. Community composition: seasonal changes and immigration of bacteria

Seasonal changes in microbial biofilm communities caused by fluctuations in temperature and solar irradiance have already been described by Lange et al. (2011) and Larras et al. (2013), respectively. However, the HTE-community showed weaker response to seasonal changes. This was probably linked to the higher concentrations of nutrients and pharmaceuticals which took the accent as principal shaping factors.

Differences between bacterial communities in HTE and UTE were higher than seasonal changes. They might be influenced by immigration of bacteria (from the two wastewaters as well as from the activated sludge), and by the direct exposure of biofilms to two treated effluents with specific concentrations of nutrients and pharmaceuticals. According to their origin, HWW and UWW might contain different bacteria species with specific resistance to the present pharmaceuticals and to the treatment processes. Furthermore, the activated sludge in the two basins might also consist of different bacterial communities, as a result of the specific wastewaters and technical conditions in the two basins (Wagner and Loy, 2002). Part of the bacteria, specific for the HWW and UWW, and for the hospital and urban sludge might be released with the treated effluents and embed in the biofilms, leading to different bacterial communities in the HTE-biofilm and the UTE-biofilm. However, these biofilms are directly exposed to the specific composition of nutrients and pharmaceuticals in the two treated effluents and further shaped by them. The resulting different communities have developed specific adaptations to the pollutants load of each effluent. These adaptations may impact their environmental functional abilities. Though, if released to natural aquatic ecosystem they may be vector of the potential effect of the two treated effluents on the environment. However, in this study we focus on the adaptation of the biofilms to the environmental conditions in the treated effluents.

4.2.3. Effect of pharmaceuticals on biofilm diversity and community composition

Antibiotics are designed to target directly bacteria, which makes them very important for the shaping of bacterial communities, especially in the hospital basin (Proia et al., 2013a). The feature of antibiotics to eliminate bacteria makes them controlling factor for their species richness and diversity and might explain their lower presence in the HTE-communities (Table 3). Species found in the UTE only, are probably less resistant to antibiotics and have been eliminated in the HTE (Proia et al., 2013a). Contrary, species specific to the HTE could be naturally occurring bacteria which built up stronger resistance to antibiotics during their stay in the hospital (Rodriguez-Mozaz et al., 2015) or in the hospital basin. Thereby they withstood the wastewater treatment and were released in the treated effluent. These HTE-specific species might also be pathogenic bacteria, which were released by the hospital (Wingender and Flemming, 2011).

Most of the investigated therapeutic classes of pharmaceuticals are not designed to target directly bacteria and might only show indirect effect on biofilm communities. This might explain for example the low influence of paracetamol on the bacterial community composition. However, pharmaceuticals might also affect autotrophic communities that also developed in biofilms, which may cause indirect effects on the bacterial community composition (Bonnineau et al., 2010). The concentrations of paracetamol found in the HTE and UTE are sufficient to discriminate green algae and favor cyanobacteria (Proia et al., 2013b). Beta-blockers have toxic effect on bacteria, but also inhibitory effect on algal photosynthesis (Bonnineau et al., 2010). Corcoll et al. (2014) reported that NSAIDs at the concentrations measured in the UTE, might also influence the community composition.

In our study, a panel of 10 pharmaceutical compounds representing five therapeutic classes was investigated in the HTE and UTE. However, a large diversity of other pollutants can be found in treated effluents and streams (e.g. Orias and Perrodin, 2013; Proia et al., 2013a; Rodriguez-Mozaz et al., 2015). Hence, the presence of other pharmaceutical compounds (and their metabolites and conjugates) as well as compounds like detergents, pesticides and heavy metals, which were not analyzed in this study, might also have played a role in the differential shaping of biofilm communities (e.g., Corcoll et al., 2012; Larras et al., 2014; Tlili et al., 2011).

5. Conclusion

HWW treated with activated sludge in urban WWTP removed more efficiently pharmaceuticals, than equally treated UWW. Despite the better removal during treatment, antibiotics and analgesics were still higher concentrated in the HTE, because of their initial concentrations. Removal of phosphate and nitrite/nitrate in the hospital basin was less efficient than in the urban one, which resulted in their high concentrations in the effluent. Hence, we recommend that the operational condition of the WWTP should be carefully considered with the low discharge of the hospital to avoid oxidation problems during the treatment. Such problems could lead to excessive excretion of nitrite/nitrate and phosphate in the environment, which may favor eutrophication. Considering the low flow of the HWW, operators could either adjust the hydraulic retention time and oxygenation time in the hospital basin according to the ratio between the hospital discharge and volume of the basin, or supplement this hospital basin by mixing some UWW to ensure sufficient flow.

Potential effects of the UTE and HTE on the environment were compared using colonizations of experimental biofilms. Results indicated that the two treated effluents caused different environmental adaptations of bacterial communities due to different occurrence of pharmaceuticals and nutrients in the effluents. Biofilms exposed to HTE exhibited less developed biomass and lower diversity. These results highlight the potential hazard of HTE on the environment and the importance of adapting wastewater treatment to specific hospital pollutants.

Further studies and experimental investigations, including the monitoring of a wider range of pharmaceuticals and other compounds are required. Metabolites and conjugates of molecules known to form byproducts (such as carbamazepine, diclofenac and sulfamethoxazole) should be investigated as well to better evaluate the quality of the wastewater treatment and avoid overestimation of the actual elimination (Barbieri et al., 2012). Furthermore, monitoring of pharmaceuticals in the sewage sludge would enable the evaluation not only of their removal, but also of their degradation (Carballa et al., 2007).

Higher sampling frequency or use of biofilms as biological captures or of passive samplers is recommended to better characterize the presence of pharmaceuticals and comprehend their effects on the microbial communities.

The investigation of the microbial community based on fingerprint methods was powerful to compare communities exposed to effluents from the separate wastewater treatments. However, only preliminary insights could be obtained regarding the adaptations of biofilms to different concentrations of pharmaceuticals. Better characterization of community changes could be obtained using next-generation sequencing in order to identify patterns of bacteria species, and associate them to functional patterns. This would help to trace the origin of species and to describe the adaptation of the communities to the presence of pharmaceuticals and unveil functional impacts that may affect further environmental processes.

Even after better removal of pharmaceuticals in hospital wastewaters owing to the separate treatment, their high concentrations in the treated effluent influenced the shaping of the biofilm communities. The hospital treated effluent is discharged in the recipient river together with the urban one and further diluted with the river water. Hence, it might have only a limited effect on the environment. However, water from the recipient River Arve is used for drinking water production for Geneva (Switzerland) via bank filtration. This sensitive situation requires further environmental investigations in the river, to comprehend how the experimentally observed adaptations of biofilms due to the presence of separated treatments and pharmaceutical loads may be transferred and threat the health of the aquatic ecosystem.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.10.161.

Acknowledgments

This study was done as part of the SIPIBEL field observatory on hospital's effluents and urban WWTPs and was funded by ANSES (French National Agency for Food, Environmental and Occupational Health & Safety), in the framework of the program "PERSIST-ENV" (Effluents hospitaliers et persistance environnementale des médicaments et de bactéries pathogènes). This work was done in collaboration with V. Lecomte (GRAIE). Molecular analyses were carried out at the molecular biology platform of INRA CARRTEL under the supervision of C. Chardon. We thank R. Sommaruga and anonymous reviewers for their constructive comments on the manuscript.

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