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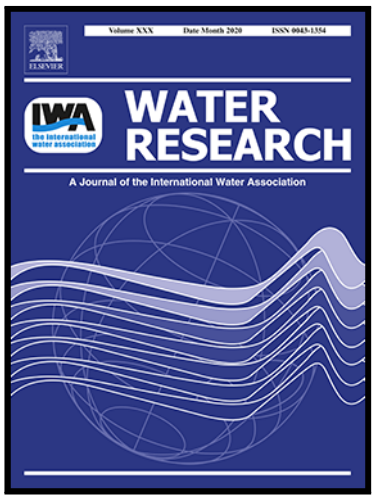
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Reclaimed wastewater reuse in irrigation: role of biofilms in the fate of antibiotics and spread of antimicrobial resistance

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Highlights

- Antibiotics degradation by interactions between planktonic and biofilm
- Antibiotic exposure had no impact on the planktonic bacterial communities.
- Resistance selection and propagation in biofilms are generate from bacterial community composition and diversity
- Antibiotic exposure does not driven forces for resistance selection and propagation in biofilm

Abstract

Reclaimed wastewater associated biofilms are made up from diverse class of microbial communities that are continuously exposed to antibiotic residues. The presence of antibiotic resistance bacteria (ARB) and their associated antibiotic resistance genes (ARGs) ensures also a continuous selection pressure on biofilms that could be seen as hotspots for antibiotic resistance dissemination but can also play a role in antibiotic degradation. In this study, the antibiotic degradation and the abundance of four ARGs (*qnr*S, *sul*1, *bla*TEM, *erm*B), and two mobile genetic elements (MGEs) including *IS*613 and *intl*1, were followed in reclaimed wastewater and biofilm samples collected at the beginning and after 2 weeks of six antibiotics exposure (10 µg L^{-1}). Antibiotics were partially degraded and remained above lowest minimum inhibitory concentration (MIC) for environmental samples described in the literature. The

most abundant genes detected both in biofilms and reclaimed wastewater were *sul*1, *erm*B, and *intl*1. The relative abundance of these genes in biofilms increased during the 2 weeks of exposure but the highest values were found in control samples (without antibiotics pressure), suggesting that bacterial community composition and diversity are the driven forces for resistance selection and propagation in biofilms, rather than exposure to antibiotics. Planktonic and biofilm bacterial communities were characterized. Planktonic cells are classically defined "as free flowing bacteria in suspension" as opposed to the sessile state (the so-called biofilm): "a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living. surface" as stated by Costerton and co-workers (1999).

The abundance of some genera known to harbor ARG such as *Streptococcus*, *Exiguobacterium*, *Acholeplasma*, *Methylophylaceae* and *Porphyromonadaceae* increased in reclaimed wastewater containing antibiotics. The presence of biofilm lowered the level of these genera in wastewater but, at the opposite, could also serve as a reservoir of these bacteria to re-colonize low-diversity wastewater. It seems that maintaining a high diversity is important to limit the dissemination of antimicrobial resistance among planktonic bacteria. Antibiotics had no influence on the biofilm development monitored with optical coherence tomography (OCT). Further research is needed in order to clarify the role of inter-species communication in biofilm on antibiotic degradation and resistance development and spreading.

Keywords: Biofilms, ARGs, antibiotics, reclaimed wastewater reuse, irrigation

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1. Introduction

The use of reclaimed wastewater for irrigation has become an attractive alternative water resource to alleviate water scarcity. However, there are several potential environmental- and health-related risks associated with this practice. One risk is the introduction of antibiotics and their associated antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) into the agro(eco)systems (Kampouris et al., 2021; Marano et al., 2021; Subirats et al., 2019). Bacterial infections are treated with antibiotics, and, after administration, part of these compounds is excreted unchanged in the urine and feces (Junker et al., 2006). This is a problematic issue because antibiotic residues can promote resistance in bacteria during wastewater treatment process (Watkinson et al., 2007). Antibiotic resistance can then be acquired by soil-bacteria (Gatica & Cytryn, 2013) or even reach the food chain (Christou et al., 2017) when using reclaimed wastewater for crop irrigation. The dissemination of this resistance in the environment might end up with inefficiency of antibiotics use (Lucas et al., 2016). For these reasons, the development and dissemination of antibiotic resistance are today issues attracting great attention from the general public and has been classified as one of the major challenges for the global health by the WHO (WHO, 2014).

Conventional wastewater treatment plants (WWTPs) are currently not designed to fully remove antibiotics and ARB&Gs, likely due to the cost and difficulty of treating complex waste mixtures. ARGs have been detected in wastewater treatment plants (WWTPs) (Cacace et al., 2019; Yang et al., 2012) drinking water treatment system (Zhang et al., 2020), surface water (Tang et al., 2016) and groundwater (Zainab et al., 2020) indicating a broad distribution and contamination of different water environments. Even if it has been reported that low-energy anaerobic–aerobic treatment reactors, constructed wetlands, and disinfection processes have shown good removal efficiencies (Barancheshme & Munir, 2019), ARGs are frequently detected in WWTPs effluents.

Traditionally, it has been suggested that urban WWTPs act as hotspots of ARGs and also mobile genetic elements (MGEs) (Guo et al., 2017; Rizzo et al., 2013). In a recent review including data from WWTPs worldwide, a positive correlation between antibiotics and ARGs commonly detected in WWTPs was observed, except for β-lactam antibiotics and *bla* genes (Wang et al., 2020b). The most frequently detected antibiotics are macrolides (clarithromycin, erythromycin, azithromycin and roxithromycin),

quinolones (ofloxacin, ciprofloxacin and norfloxacin), and sulfonamides (sulfamethoxazole), trimethoprim and tetracycline while *bla* (blaCTXM, blaTEM), *sul* (sul1, sul2), *tet* (tetO, tetQ, tetW) and *erm*B genes are the ARGs commonly reported in WWTPs (Wang et al., 2020a).

Yet, other recent studies have shown controversial results, suggesting that in fact the core resistome of biological wastewater treatment units is different from other parts of urban water ecosystems and not necessarily a hotspot for gene transfer (Munck et al., 2015; Quintela-Baluja et al., 2019). It is therefore important to study the composition and behavior of microbial communities involved in wastewater treatment and transport processes, from the WWTP to the downstream receiving water bodies or in the case of agricultural reuse, to the field and crops.

Biofilms are biological structures made up of bacteria, organic matter and some inorganic matter that together constitute three-dimensional polymer niches (Flemming & Wingender, 2010). Biofilms may also be associated with the accumulation of absorbed chemical and pathogenic agents and would thus act as potential reservoirs of materials that would affect the safety of irrigated food produced. However, they lead also to self-depuration due the ability to accumulate organic compounds (Huerta et al., 2016; Ruhí et al., 2016; Wilkinson et al., 2018) at concentrations up to few hundred ng g^{-1}, as well as to degrade them (Desiante et al., 2021;Writer et al., 2011 Sabater et al., 2007).

It has been suggested that ARB can proliferate within irrigation pipe-based biofilms (Blaustein et al., 2016) and that biofilms in water environments are thought to be hotspots for horizontal gene transfer of ARGs (Abe et al., 2021). In fact, biofilms are formed when bacteria are under stressed conditions. In the distribution system, the presence of antibiotics, residual concentration of chlorine will imply a stress and then will favour biofilm development with growth of bacteria and extracellular polymeric substance (EPS) production. As in activated sludge system, this bacterial growth and activity could favour gene transfer. This is a hypothesis that is discussed in the recent paper of Abe et al (2021), but was also proposed by Pruden et al (2014) for the spread of ARG in biofilm grown in drinking water pipe system due to opportunistic pathogens growth. Literature on wastewater transport pipes or drip irrigation systems with reclaimed wastewater is still scarce. A study performed in sewers transporting raw wastewater showed that community composition rather than antibiotic concentration is the main factor

driving the diversity of the sewage resistome (Auguet et al., 2017). Lequette and coworkers (2020) have also shown that community composition shifts between different parts of a treated wastewater irrigation system such as pipes and drippers (Lequette et al., 2020). However, no link has been established between antibiotic concentration, community structure, biofilm development and ARGs occurrence.

In this work we address some of these questions in an attempt to get a comprehensive overview of the effect of antibiotics on the dissemination of antibiotic resistance thanks to a deep characterization of biofilms growing in treated wastewater. Therefore, biofouling, microbial activity, community composition and abundances of four ARGs and 2 MGEs were monitored in biofilms exposed to a cocktail of six antibiotics commonly found in wastewater. The role of planktonic bacteria in the spreading of resistance in interaction with biofilms was also investigated by determining antibiotic concentration and microbial activity and composition in the liquid phases during the 14 days of antibiotics exposure.

2. Material and methods

2.1 Reagents and chemicals.

Six antibiotics were selected in this study: amoxicillin (AMX), ofloxacin (OFX), sulfamethoxazole (SMX), trimethoprim (TMP), clarithromycin (CLA) and cephalexin (CEF)) as they are among the most abundant in WWTP effluents and are frequently quantified in the range of ng/L to µg/L and its presence is addressed in many countries. Some of them such as ciprofloxacin can be detected also at concentration around 8 µg/L (Anjali et al., 2019).” Moreover, amoxicillin, ciprofloxacin, sulfamethoxazole, and trimethoprim are included in the surface water watch list adopted by the European Commission under the Water Framework Directive (2020/1161). Reference standards for all compounds were obtained from Sigma-Aldrich (St Quentin-Fallavier, France) and were of high purity (> 95%). Individual stock solutions of standards (1000 mg L^{-1}) were prepared individually in 100% methanol and stored at -20 °C. Intermediate solutions (100 to 1 mg L^{-1}) were prepared in serial dilution in methanol and stored at −20 °C. Working solutions were prepared in serial dilution in water by mixing all compounds and

immediately used for the treatment. All LC-MS grade organic solvents and HPLC water were purchased from Carlo Erba Reagents (Val de Reuil, France) and were of purity higher than 99.9%.

2.2 Biofilm growth conditions.

The development of 6-weeks old biofilms was performed in three reactors where 8 high density polyethylene (PE) disks (0.5 cm thick and 5 cm in diameter) were fixed on a grid immerged in the reactor. The choice of PE has been selected to be similar to the material conditions used in drip irrigation. Each reactor was filled with 4 L of treated wastewater, and water level was kept constant by means of constant water addition using a peristaltic pump ensuring a hydraulic retention time equal to 24 h and no external nutrients were supplied for biofilm growth. Treated wastewater was collected from the wastewater treatment plant operated on the basis of stabilization ponds with three successive lagoons (13,680, 4784 and 2700 m^3, respectively), without addition of disinfection processes and had a nominal capacity of 1500 Inhabitant Equivalent (Manasfi et al. 2021). The reactors were operated in dark in order to reproduce the irrigation pipe conditions in anaerobic conditions (O_2< 0.1 mg L^{-1}) by continuously feeding with treated wastewater stored in 200 L tanks in the laboratory (T = 20 ± 2 °C). The mix in the reactor was maintained under magnetic stirring. Fresh treated wastewater was changed once per week in the storage tank.

2.3 Experimental set-up, design and operation.

The experimental set-up was divided into two groups: treated wastewater (W) and sterilized wastewater (SW) . W-group gathered set-up made with treated wastewater coming from a secondary biological treatment, applied after a lagoon treatment (Montpellier, France). The SW-group gathered set-up made with the same treated wastewater that was sterilized (120°C, 30 min). After 6 weeks, two PE disks colonized by biofilm were taken out from the reactor and were placed in each set-up made of glass container filled with 650 mL of the treated (W) or sterilized treated (SW) wastewater spiked with selected antibiotics at a final concentration of 10 µg L^{-1} (each). Control containers were made using wastewater (C_BW) and sterilized wastewater (C_BSW) without antibiotics. The experiments were made in triplicate.

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The experiments were conducted using identical glass containers. Each container was operated in the dark at room temperature, under anoxic conditions, and under slight shaking using an orbital shaker at 30 rpm. Anoxic conditions were created by purging the air trapped in closed containers with nitrogen. Anoxic conditions were chosen to mimic conditions found for biofilms inside drip-irrigation pipes. The experimental set-up (Table 1SM) consisted in 14 containers divided as follows: (1) one control with biofilm in wastewater (C_BW); (2) one control with biofilm in sterilized wastewater (C_BSW); (3) 3 containers with biofilm exposed to spiked wastewater (BW); (4) 3 containers with biofilms exposed to spiked sterilized wastewater (BSW), (5) 3 containers with spiked wastewater, and, (6) 3 containers with spiked and sterilized wastewater. All containers were incubated during two weeks and water was changed after 7 days, with fresh antibiotic solution for the spiked containers. Analysis were performed at the start and end of experiment.

2.3 Analytical procedures

Physicochemical characterization of the treated wastewater.

Treated wastewater was collected from WWTP located at Murvièl -Les- Montpellier (Hérault, France). pH, dissolved oxygen, conductivity, nitrate, nitrite, ammonia, total nitrogen, phosphate, suspended solid (TSS) and chemical oxygen demand (COD) concentrations were monitored to follow the wastewater treatment performances. COD, ammonia, nitrate, nitrite, total nitrogen and phosphorus concentrations were measured with a spectrophotometer (DR1900, Hach Company, Loveland, CO, USA) using Hach reagents® after filtration using glass filters according to the Standard Methods (APHA, 2005). Conductivity, dissolved oxygen and pH were measured with probes (TetraCon® 925 and pH-Electrode Sentix® 940, WTW, Wilhelm, Germany). The physicochemical characteristics of the treated wastewater are listed in Table 2SM.

Antibiotic analysis.

Liquid samples (100 mL) were collected from each glass container. Collected samples were filtered through 0.45 µm syringe filters, frozen and stored at - 20°C until analysis. For the analysis of antibiotics, extraction and purification were implemented through HLB OASIS cartridges (200 mg sorbent / 6cc)

from Waters. The conditioning phase was performed with 4 mL MeOH followed by 4 mL UPW, 100 mL of filtered sample was passed through the cartridge followed by 4 mL of UPW for washing. Cartridges were dried under vacuum until total dryness and finally retained analytes were eluted with 4 mL of MeOH. Eluates were totally evaporated under a gentle nitrogen flow at 55°C and residues were reconstituted in 200 µL of LC-MS mobile phase (ACN/water, 5/95%, v/v) and filtered with 0.22 µm PTFE syringe filters before injection.

Antibiotics were quantified using LC-HRMS in positive mode of ionization using an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). Specifically, 10 µL of each sample was injected on a Zorbax Eclipse XDB-C-18 column (3 i.d. × 150 mm length × 5 µm particle size) at a flow rate of 0.150 mL min^{-1}. Samples were eluted using a water (A): acetonitrile (B) mobile phase as follows: start at 5% B, increase to 100% B over 15 min, held for 5 min and back to 5% B in 1 min. Full scan MS mode with a scan range from 90 to 900 m/z, at a resolution of 50,000 FWHM, Automatic Gain Control (AGC) target of 1E6 ions at injection time of 50 ms provided the optimum parameters for high sensitivity together with sufficient data point per peak.

2.4 Biofilm characterization.

Biofilm samples were collected by rubbing a sterile swab over the PE disk with the help of a cylindrical hollow punch (inner surface of 0.8 cm^2). For each container, 2 biofilms samples were collected, one on each PE-disk. Samples were stored at -20°C until DNA extraction.

Determination of biofilm thickness.

At the beginning and end of the spiked studies, the biofilm thickness and quantity was compared in the same measurement area. Three-dimensional Optical Coherence Tomography (OCT), Thorlabs GANYMEDE II OCT (LSM03 lens, lateral resolution: 8 µm; Thorlabs GmbH, Lübeck, Germany), was used to investigate the biofilm evolution as non-invasive imaging. The axial voxel size in water (n = 1.333) of GANYMEDE II was 2.1 µm and center wavelength was 930 nm.

Firstly, 3-D OCT datasets were processed in Fiji (running on ImageJ version 1.50b (Schindelin et al., 2009)). Secondly, an in-house code was used to detect the pixels associated to the biofilm using

MATLAB R2018r (MathWorks, Natick, MA, USA, version 2018b). A threshold (adapted to each dataset) was applied to binarize the dataset and the region above the threshold intensity losses during simple penetration. The intensity contrast between the fouling pixels and the remaining parts (PE plates or water) was significant to allocate these last areas to the back-ground (black). In addition, suspended solids and outliers were removed applying the 'clean and majority functions' of Matlab. For each position (x and y coordinates), the pixels associated with the fixed biofilm (up to the threshold) were summed (on the z coordinate) to obtain the biofilm volume.

In order to compare the mean thickness of biofilm, the biofilm quantity was normalized by surface of the corresponding measurement zones. Taking into account the pixel and mm scale ratios, the mean biofilm thickness (mm) was calculated for each measuring volume after data analysis according to Equation 1:

$$\text{Mean biofilm thickness} = V_{fouling} / S_{measurement} \quad 1)$$

where $V_{fouling}$ is the volume of biofilm measured from OCT acquisitions (mm^3) and $S_{measurement}$ is the surface of OCT acquisitions of the sample determined by the taken image (mm^2).

Quantification of microbial activity.

Microbial activity in biofilm and wastewater samples was determined by adenosine triphosphate (ATP) measurement. The ATP concentration was determined in water and biofilm samples by measuring the amount of light produced in a luciferin-luciferase assay. The generated light signal is measured as Relative Light Units (RLU) by using the luminometric assay DENDRIDIAG kit (GLBiocontrol, France). The ATP data from water were expressed in ng L^{-1} and from biofilm in pg cm^{-2}.

2.5 Microbial community analysis.

DNA isolation.

Water (25 mL) and biofilm (0.8 cm^2) samples were collected. Water samples was vacuum filtered through polycarbonate track-etch membrane filters with a diameter of 50 mm and a pore size of 0.22 µm (Millipore, FRANCE). Filters were then transferred to a bead tube of the FastDNA™ Spin Kit for

soil (MP biomedicals, Solon, USA) following the manufacturer's instructions. DNA purity and concentrations were estimated by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). The extracted DNA was stored at -20 °C.

Quantification of total bacteria and ARGs by qPCR.

The amplification reactions were performed in triplicates, and for two dilutions to check for the absence of PCR reaction inhibition. Reaction mixtures (12.5 µL) contained 6.5 µL of SsoADV Univer Probes or Sybr Super Mix (Biorad) and primers at concentrations indicated in Table 2SM. Total bacterial quantification was performed by qPCR targeting the V9 region from 16S rDNA. 100 nM forward primer BAC338 (5'-ACTCCTACGGGAGGCAG-3'), 250 nM of reverse primer BAC805 (5'-GACTACCAGGGTATCTAAT CC-3') and 50 nM of probe BAC516 (Yakima Yellow – TGCCA GCAGC CGCGG TAATA C – TAMRA) (Yu et al., 2005). The cycling parameters were 2 min at 95 °C for pre-incubation of the DNA template, followed by 40 cycles at 95 °C for 15 s for denaturation and 60 °C for 60 s for annealing and amplification.

Four ARGs and two genes linked to dissemination of antibiotic resistance were quantified by qPCR using Sybr Green chemistry: qnrS (reduced susceptibility to fluoroquinolone); sul1 (resistance to sulphonamides); blaTEM (resistance to β-lactams), ermB (resistance to macrolide), intl1 encoding the integrase of Classe I integrons used as a proxy for the potential capacity of the bacterial community to disseminate resistance (Ma et al, 2017) and IS613 (transposon). The selected genes were quantified in wastewater and biofilm DNA using qPCR systems and PCR programs described in Table 3SM. Specificity of amplification was validated both by melting curve analyses and by checking the size of the amplicons on 2100 Bioanalyzer (Agilent, Waldbronn, Germany). All qPCR assays were performed with high R^2 values (0.9999 in average), high efficiency (90-100%) validating the gene quantification. Non-parametric statistical tests (Kruskal-Wallis and Conover tests) were performed to compare concentrations of ARGs.

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Analysis of bacterial communities by Illumina sequencing.

The PCR amplified the V4-V5 region of 16S rRNA gene with 30 cycles (annealing temperature 65°C) using the primers 515U (5′-GTGYCAGCMGCCGCGGTA-3′) and 928U (5′-CCCCGYCAATTCMTTTRAGT-3′) (Wang and Qian, 2009). Adapters were added for multiplexing samples during the second sequencing amplification step. The resulting products were purified and loaded onto the Illumina MiSeq cartridge for sequencing of paired 300 bp reads according to the manufacturer's instructions (v3 chemistry). Sequencing and library preparation tasks were performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). Mothur (version 1.39.5) (Schloss et al., 2009) was used to associate forward and reverse sequences and clustering at four different nucleotides over the length of the amplicon. UCHIME (Edgar et al., 2011) was used to identify and remove chimera. Sequences that appeared fewer than three times in the entire data set were removed. Remaining sequences were aligned using SILVA SSURef NR99 version 128 (Schloss et al., 2009). Taxonomic assignment was done using Blast (http://www.ncbi.nlm.nih.gov/BLAST/) to locate publicly available sequences closely related to the sequences obtained from the samples. Sequences with 97% similarity were sorted into operational taxonomic units (OTUs) (Nguyen et al., 2016). The chloroplast sequences from 16S rRNA sequences were removed from the raw data and represented less than 1%. Reads were grouped at the 97% similarity level.

The Shannon diversity index, the Simpson index, and the nonparametric richness estimator Chao1 were calculated (Chao, 1984). To compare bacterial community structures, a dissimilarity matrix (Bray-Curtis) was performed and visualized using principal coordinate analysis (PCoA). The sequencing data analysis was processed under R v3.4 (www.r-project.org) through R-Studio (http://www.rstudio.com/) using the phyloseq package (McMurdie and Holmes, 2012). Relative abundancies of putative antibiotic resistant bacteria were determined by looking for taxonomic groups of interest based on scientific literature (Table 3SM). Concentrations were obtained by multiplying relative abundancies by bacterial concentration given by the measurement of total 16S rDNA by qPCR.

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3. Results and discussion

3.1 Degradation of antibiotics by planktonic and biofilm bacteria.

Belonging to the most frequently detected antibiotics in wastewater (Wang et al., 2020a), CLA, OFX, SMX and TMP were included in this study together with two beta-lactam antibiotics, CEF and AMX. Among these six antibiotics, AMX, CEF and OFX have bactericidal activity, whereas CLA, SMX and TMP are mainly bacteriostatic. However, SMX and TMP can be bactericidal when used together and CLA may also be bactericidal depending on the concentration and targeted organism.

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation. By taking advantage of the fact that an antibiotic concentration that kills or inhibits growth of at least some bacteria will, by consequence, be selective at the community level. Bengtsson-Palme and Larsson determined the upper boundaries for minimal selective concentrations and suggested individual safety margins for antibiotics based on the extent of available MIC data (Bengtsson-Palme & Larsson, 2016). Predicted no-effect concentrations (PNECs) were calculated from available MIC values in the public EUCAST database and were used as an indicator for resistance selection. For the six antibiotics used here, the PNECs calculated were (in $\mu g\ L^{-1}$): AMX (0.25), CEF (4), CLA (0.25), OFX (0.5), TMP-SMX (0.5). The concentration used in this study (10 μgL^{-1}) is in any case above the estimated PNECs values, setting favorable conditions for resistance selection in our experimental conditions.

However, the concentrations of antibiotics are dynamic in time (degradation and sorption) and space (microhabitats in biofilms). The mechanisms of resistance selection could be thus interrupted depending of these concentration variations. Antibiotic concentrations were thus monitored in the medium after the 14 d of incubation and removals were calculated as shown in Table 1. The removal was reported as variation of the concentrations during the experiments. It was not possible to separate adsorption process from degradation due to limited quantity of biofilm mass. Whatever the conditions and molecules, the calculated removals were all below 50% underlying low degrading activity of the different ecosystems but in counterpart it enables favorable conditions for resistance selection. Moreover, removals were observed even in the sterilized wastewater set-up underlying a residual bacterial activity of these

systems. This residual activity can be explained by the results on microbial community characterization (3.3) that showed a loss of diversity (due to sterilization) but a highest absolute abundance of 16SrDNA (growth of opportunistic bacteria) in planktonic bacteria under this condition. In addition, among the selected antibiotics, the natural fate of them is well studied. Due their characteristic physic-chemical, the only antibiotics that can readily undergo hydrolysis in aquatic environments due to their unstable beta-lactam ring are the beta-lactams (cephalexin and amoxicillin) while no hydrolysis for sulfamethoxazole was detected at several pH studied by Trovô et al; (2009); Similar was observed for Trimethoprim by Lam and co-workers (2004), where dark hydrolysis and biodegradation in the microcosms over the course of the study from 1.5 to 82 d demonstrated any important in the loss of the compounds. For this reason, we are strongly convince that the biodegradation is mainly driver from specific bacteria communities.

CEF and AMX were totally removed in all reactors and therefore no differential behavior between the applied conditions could be inferred. In fact, beta-lactams are antibiotics that can readily undergo hydrolysis in aquatic environments due to their unstable beta-lactam ring.

TMP removal was very low (<25%) and observed only in reactors without biofilm, suggesting a main role of planktonic bacteria in its degradation. Addition of biofilm may modify the interactions and metabolism of both communities preventing the TMP removal. In the literature, degradation of TMP has been mainly attributed to nitrifying conditions which are far from our conditions (Batt et al., 2006), so it is mean that nitrifying bacteria play an important role on its degradation. So, the low removal of TMP is in agreement with Luo et al. (2012) and Perez et al. (2005) also reported that no biodegradation was observed in conventional activated sludge treatment. In addition, it was demonstrated by Kong et al. (2017) that the presence of TMP, the protein and polysaccharide contents increased to protect the cells from unfavourable conditions. This can justify why the low decrease of its concentration was observed in our experiments.

For SMX, the removal (ranging between 6 and 38.5%) increased in wastewater with biofilm by comparison to wastewater without biofilm. This removal could be assigned in part to biodegradation by biofilm communities and in part to sorption processes. Antibiotic sorption to biofilm is strongly

dependent on physicochemical properties of biofilm and also on antibiotic structure. Wunder and co-workers (Wunder et al., 2011) demonstrated that speciation and size of the antibiotic are important factors affecting the interactions between antibiotics and biofilm. The degradation of SMX is also well documented in the literature (Chen & Xie, 2018; Larcher & Yargeau, 2012). Several studies have focused on biological degradation of SMX under aerobic conditions but few have been investigated under anaerobic and anoxic conditions. Baumgarten and co-workers (Baumgarten et al., 2011) reported anaerobic and anoxic column tests of contaminated water at 0.25 and 4.5 µg $L^{-1}$of SMX and observed 27% removal under anoxic condition (49-day half-life) and 51% under anaerobic conditions (16-day half-life).

OFX removals were observed in all reactors between 30 and 49%, excepted when biofilm was present in wastewater; it supposed a main contribution of the planktonic bacteria and potential competition for co-substrates when biofilm is added in presence of a more diverse community. Fluoroquinolone compounds have a fluorine atom that contributes to their recalcitrance in the environment (Natarajan et al., 2005) and only few studies reported their biodegradation. Few bacteria and fungi species were shown to be able to transform fluoroquinolones (Kim et al., 2011; Manasfi et al., 2020), also under anaerobic conditions (Shu et al., 2021).

Clarithromycin showed opposite trend to OFX. Its removal, ranging between 11 and 47%, increased in presence of biofilm in wastewater, as observed for SMX, and decreased in presence of biofilms in sterilized wastewater.

To summarize, planktonic bacteria were able to degrade the six antibiotics in sterilized wastewater when bacterial community is less diverse but more active, suggesting specific metabolism towards these compounds. Interestingly, SMX was less degraded by planktonic bacteria when wastewater was not sterilized. A plausible explanation could be that low abundant degrading bacteria, generally outcompeted by dominant species in wastewater planktonic communities, might become dominant after sterilization. The presence of biofilm had a positive effect on removal of SMX and CLA in wastewater and only in SMX in sterilized wastewater. Of special interest is the observation that biofilm impacted negatively the removal of OFX and TMP in wastewater and of TMP and CLA in sterilized wastewater

revealing interactions between biofilm and planktonic bacteria. Such inter-species interactions have been shown to contribute to antibiotic tolerance in complex bacterial communities (Aranda-Díaz et al., 2020).

3.2 Impact of antibiotics on biofilm thickness and activity

Biofilm thickness was determined by three-dimensional optical coherence tomography (OCT) imaging. Before exposure to antibiotics, the mean initial thickness averaged 1.76 mm. After 14 days, the OCT measurements show that the mean biofilm thickness (which could give biofilm biomass) was not affected by the presence of antibiotics and the sterilization of wastewater (Figure 1aSM). Therefore, the 6 added antibiotics at a concentration of 10 µg L^{-1} each did not promote the detachment of biofilms from the PE support. However, OCT results are not representative of changes induced at the bacterial community or activity level.

Often, bacterial respiration measured as total ATP produced is used as a parameter for microbial activity measurement (van der Wielen & van der Kooij, 2010). ATP concentration was monitored both in planktonic communities (liquid phase) and in biofilms before and after the antibiotic exposure (Figure 1b,cSM). The average ATP concentrations differed in each experimental group (W and SW). When biofilm was put in presence of treated wastewater, microbial activity was in general lower than in t0. Exposure to antibiotics induced a slight increase of microbial activity. The opposite effect was observed when biofilms were put in presence of sterilized wastewater where the presence of antibiotics induced a reduction in microbial activity in the biofilm (from 7.37 to 6.96 Log eq.bact./ml). Besides, the activity was in any case higher in biofilms put in presence of SW than in W.

Concerning planktonic bacteria, their activities showed different results. Exposure to antibiotics induced an increase on microbial activity in wastewater (5.85 to 6.26 Log eq.bact./ml). This effect disappeared in samples containing biofilms (5.71 to 5.51 Log eq.bact./ml). When using SW, a reduction in microbial activity was observed at t0 due to sterilization (2.97 Log eq.bact./ml) but recovered after 14 days to reach 5.72 Log eq.bact./ml which was similar to the initial ATP value in wastewater. In SW samples containing biofilm, the effect was similar but to a lower extent (5.19 to 6.02 Log eq.bact./ml).

Bacterial cellular respiration measured as production of ATP is today considered a good indicator of microbiological activity and since antibiotics are designed to kill or inhibit the growth of bacteria, it is expected that bacterial cellular respiration is reduced after exposure to antibiotics. However, Lobritz and coworkers identified two different mechanisms depending on antibiotic bacteriostatic or bactericidal effect. Growth inhibition from bacteriostatic antibiotics was associated with suppressed cellular respiration whereas cell death from most bactericidal antibiotics was associated with accelerated respiration. However, in combination, authors observed that suppression of cellular respiration by the bacteriostatic antibiotic was the dominant effect, blocking bactericidal killing (Lobritz et al., 2015). Following this observation, we can conclude that the increase observed on ATP production after 14 days in planktonic bacteria in W and SW or in biofilms in presence of wastewater corresponds to cell proliferation. This bacterial growth in presence of antibiotics could be explained by resistance, degradation or both mechanisms. TMP could have a predominant role since it is not degraded and it is known to inhibit bacterial DNA synthesis.

Anyway, even if ATP assay detects the activity of various types of microbial species, the methodology also has its limitations, as some pathogenic microbes may be present without eliciting a response in ATP; since they are present in relatively low numbers compared to the total number of bacteria (Vang et al., 2014). To overcome this limitation, characterization of bacterial communities was carried out.

3.3 Characterization of bacterial communities

Richness and diversity indexes in planktonic bacterial communities were determined (Table 2). As expected, sterilization had a relevant impact in richness and diversity, being SW the group with lower richness and diversity indexes. The presence of biofilm increased the richness of planktonic communities in both BW and BSW but only the diversity of BSW communities. Overall, exposure to antibiotics had almost no impact on richness. Antibiotics impacted differentially the diversity of planktonic communities. Whereas antibiotic exposure increased the diversity of planktonic communities in BSW, it reduced it in BW.

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Sterilization and presence of biofilms both had an impact on the structure of planktonic bacterial communities (Figure 1). Samples corresponding to the four tested conditions BSW, BW, SW and W formed four independent clusters. SW cluster was very distant from the other three groups, showing that SW community was the most different from the others. Inter-cluster distances between SW and W was bigger than between SW and BSW and also bigger than between W and BW, showing that **sterilization was the most dominant factor in deciphering planktonic community structure**. The biggest inter-cluster distance was found between SW and BW, revealing that in addition to sterilization, the presence of biofilm introduced also differences in the community structure. Interestingly, inter-cluster distance between BSW and W and between BSW and BW were similar indicating that **when biofilms were present, the disruption caused by sterilization was in part restored**. Intra-cluster distances were similar in all groups. Control samples (non-exposed to antibiotics) C_BSW and C_BW, clustered together in their respective group BSW and BW, revealing that antibiotic exposure had no impact on the structure of the planktonic bacterial communities.

In order to go deeper in the bacterial structure, taxonomic classification in biofilm and planktonic communities was performed at genera level (Figure 2 and 2SM) and phyla level (figure 3SM). Fig 2 presents the 30 most abundant genera in all biofilm samples. **The type of wastewater (sterilized or not) had an impact on the bacterial communities of biofilms**. Indeed, BW and C_BW samples were dominated by several genera like *Comamonadaceae*, *Anaerolineaceae* (including some but not all BW and C_BW samples), *Arenimonas*, *Flavobacterium*, *Thiocapsa*, *Run-SP154* and an unculture bacteria as dominant taxons. When wastewater was sterilized, the biofilms were exclusively dominated by the *Comamonadaceae* family. The predominance of *Comamonadaceae* family is common in biofilm evolved in chlorinated drinking water distribution system and the family was the main colonizers of biofilms from chlorinated drippers (Lequette et al., 2021).

Investigation of the most dominant genera in wastewater (Figure 2SM) showed that initial sterilization reduced the richness and shifted communities from dominance of taxon ‘12up’ (Rhodocyclaceae) and *Chitinophacaeae* to dominance of *Flavobacterium* (Flavobacteriaceae), *Fluviicola* (Cryomorphaceae) and *Pedobacter* (Sphingobacteriaceae). Relative abundancy of *Pirellula*, *Algoriphagus*,

Stenotrophomonas and *Rickettsiales* also increased in sterilized wastewater samples. **The presence of biofilms impacted the planktonic community underlying interactions between both media**. It reduced the dominant taxons and improved the diversity in both W and SW samples, with increased abundancy of *Arenimonas, Planctomycetaceae, Cloacibacterium, Romboutsia, TM6-Dependentiae, Methyloversatilis, Legionella, Thermomonas*, and *Zoogloea*, probably due to detachment and/or growth events. The dominant genera in biofilm (*Comamonadaceae*) did not colonized the planktonic community. Four types of bacterial communities were then identified in wastewater: W first with dominance of Rhodocyclaceae, SW with low diversity and dominance of *Flavobacterium* or *Pedobacter*, and finally a group gathering BW and BSW. Interestingly, the planctonic community in C_BSW was more similar to the one of SW than the one of BSW, with a remaining dominance of *Flavobacterium*. To compare, the planktonic community in C_BW was very similar to the one of BW. It seems thus that an impact of the presence of antibiotic is only observable when sterilized wastewater was used inducing loss of diversity and richness.

Taxonomic annotation was employed to also estimate the relative bacteria abundance at the phylum level (Fig 3SM). Whereas Proteobacteria and Bacteroidetes were the predominant phyla in planktonic (average of 55 and 30% respectively) and biofilm (average of 55 and 20% respectively) bacterial communities, the abundance of other minor phyla was different among them. In biofilm samples, the other detected phyla were Chloroflexi, Firmicutes, Planctomycetes and Acidobacteria in similar relative abundances between control and exposed samples and Actinobacteria, Gemmatimonadetes and Latescibacteria at lower abundances (Fig 3SM b). In wastewater, only Actinobacteria and Firmicutes were also present and when biofilm was present other phyla as Chloroflexi, Planctomycetes and TM6_Dependentiae were present (Fig 3SM a). Previous studies reported that these phylogenetic groups were ubiquitous in both laboratory and pilot-scale anaerobic bioreactors (Calderón et al., 2011; Gao et al., 2010; Khuntia & Chanakya, 2020; Lu et al., 2012) and fit with the regular structure pattern of bacterial communities in WWTPs effluents (Do et al., 2019). Moreover, Proteobacteria, followed by Firmicutes, Bacteroidetes, and Actinobacteria were found to be the most mobile phyla associated with the transfer of ARGs and MGEs (Hu et al., 2016). Some phyla like Firmicutes, Bacteroidetes, and Beta-

Proteobacteria were also depicted to be highly correlated with some antibiotic resistant strains such as ciprofloxacin resistant heterotrophs, ciprofloxacin-resistant enterococci, and sulfamethoxazole-resistant enterobacteria (Novo et al., 2013).

Experiments with sterilized wastewater were performed with the aim of reducing the number of planktonic bacteria (only 3 to 4 phyla were present), thus limiting the competition for nutrients in the reactors in favor of biofilm-forming bacteria. In sterilized wastewater conditions, by comparison to non-sterilized one, relative abundances of Actinobacteria, Planctomycetes, and TM6_Dependentiae were drastically reduced almost to extinction whereas Firmicutes increased. In reactors containing biofilms (BW), relative abundances of planktonic Firmicutes, Planctomycetes, Chloroflexi and TM6_Dependentiae phyla increased. TM6_Dependentiae and Planctomycetes phyla increased also in reactors containing biofilms and sterilized wastewater (BSW) but not Firmicutes, and this increase was higher in presence of antibiotic. Following these observations, TM6_Dependentiae and Planctomycetes can be phyla with a potentially relevant role in antibiotic resistance or degradation among planktonic bacteria. Furthermore, biofilm-forming bacteria seem to be involved in this mechanism since TM6_Dependentiae and Planctomycetes were not present in reactors containing only SW but their abundance increased in presence of antibiotics only when biofilms are present in the reactors.

Wastewater shaped the biofilm communities and conversely biofilm communities influenced the planctonic one. Exchange of taxons may occur on both sides. However, less changes are observed when diversity and richness are higher in both media.

3.4 Abundance of ARGs and putative ARBs in biofilms and wastewater

Numerous studies have investigated the occurrence and prevalence of ARGs through wastewater treated by different technologies (Conte et al., 2017; Lee et al., 2017). Biofilm grown under certain levels of antibiotic concentration are expected to develop a bacterial community more tolerant to the direct stress that is made up by antibiotics. Nevertheless, to our knowledge, the present study is the first one investigating the abundance of ARGs and their variation in biofilm and wastewater after long exposure with the most common detected antibiotics in wastewater.

The total and relative abundances of four ARGs (*sul1*, *blaTEM*, *qnrS*, *ermB*), and two MGEs (*intl1*, IS613) were estimated in biofilms and wastewaters collected from all the reactors. The abundance of 16S rDNA gene was also determined to quantify the total bacterial population.

As shown on the figure 4SM, copy numbers of bacterial 16S rDNA genes were $3*10^7$ copies/ml in the treated wastewater and was statistically lower than the one in the sterilized water ($6.8*10^7$ copies/ml); this higher abundance for this less diverse planktonic community is probably due to the emergence and growth of opportunistic bacteria in absence of bacterial competition (Fahrenfeld et al., 2013). The presence of biofilms lowered these 16S abundances in both wastewaters whereas it increased diversity and change the microbial structure. This growth reduction in presence of biofilm may be due to the competition for substrates and/or higher growth into the biofilm by comparison to the planktonic communities as also shown with ATP measurements (fig 1SM b,c). The abundance of bacterial 16S rDNA genes was also determined in biofilm and it was in a range from 1.3 to $3.3*10^9$ gene/cm^2 (figure 4SM). No statistical differences were observed between the conditions, even with the abundance at the beginning of the experiment, but the lowest abundance was observed for the controls (C_BW, C_BSW).

Abundance of ARGs in wastewater

On the 6 targeted ARGs, *qnrS*, *blaTEM* and IS613 were below the quantification limits at the start or at the end of the experiment and were thus not considered in the data analysis (Table 5SM).

In the wastewater, the ARG concentration was globally 1.5log lower that the one measured in biofilm (fig 5SM and fig 3). Moreover, the absolute abundances of *sul1* and i*ntl1* were 2log higher than the one of *ermB*. The absolute abundance of *ermB* was not statistically different from one condition to another but tendencies were similar to the two other ARGs. For *sul1* and i*ntl1*, their absolute abundances were similar in sterilized and non-sterilized wastewater; as the 16S increased in SW, the relative abundance was thus lower in SW which may suggest that the opportunistic bacteria did not carry these ARGs. Their absolute abundances were however significantly reduced (as similarly observed for 16S rDNA) by the presence of biofilm only in presence of non-sterilized wastewater. In the presence of sterilized wastewater, even if biofilm reduced the 16S planktonic abundance, the ARG absolute abundance

remained similar, which ended up with a highest relative abundance. It underlines that when activity, competition and diversity are reduced between planktonic and biofilm communities, the dissemination of ARGs is favored.

Abundance of ARGs in biofilm

Similarly, to the wastewater samples, two ARGs (*sul1* and *ermB*) and one MGE (*intl1*) were detected in all biofilm samples during the experiment, *ermB* being always the less concentrated one (2log lower). The three other genes were not quantified as their values were always below detection limits. Comparison between the different conditions was thus performed and checked statistically based on Kruskall Wallis-Conover tests (p-value<0.05) excepted for the controls because of low number of data (Fig 3).

The absolute abundance of *sul1* was similar whatever the conditions and no increase was observed during the 14 days even if SMX concentration remained at a high level (Table 1). The spread of *sul* genes by horizontal gene transfer usually occurs rapidly in natural communities thanks to their linkage to class 1 integrons (Gillings et al., 2014; Sköld, 2000) that was not observed in our experiment. However, its relative abundance significantly increased during the 14 days and was higher in presence of sterilized WW. For the two other genes, a significant increase of the absolute and relative abundances was observed during the 14 d in presence of sterilized wastewater and for *ermB* these abundances were higher than the one observed in presence of non-sterilized wastewater. It seems that this condition, biofilm in presence of SW, is favorable to the gene dissemination in the biofilm compartment. It reveals that the differences in structure and diversity between planktonic and biofilm communities play a key role in the development and dissemination of resistance.

Considering all ARG results on wastewater and biofilm samples, we can first say that exposure to antibiotics is not the main parameter driving resistance selection and propagation which is in agreement with previously published data (Auguet et al., 2017). Indeed, community structure, diversity and activity are the driven force. The condition that favors dissemination of ARG either in planktonic or biofilm communities is when wastewater is sterilized and put in presence of biofilm, underlying the important

role of the biofilm. In such condition, the presence of biofilm allowed recovering a certain diversity of the planktonic community with a specific structure, dominated by Planctomycetaceae, Flavobacteriaceae and Xanthomonadaceae (Fig 2SM), that may influence the biofilm community dominated by Comamonadaceae (Fig 2). When using non-sterilized WW, the planktonic community also evolved in presence of biofilm but also the biofilm community, meaning that both communities influence each other. But these interactions seem to be different when diversity is previously modified and decreased as if the ecosystem was less efficient or resistant allowing more gene dissemination. It means that it is not only the genes themselves but which bacteria harboring these genes that must be considered to understand the dynamic of antibiotic resistance in wastewater and biofilms in contact with wastewater.

Putative ARB in biofilm

To further investigate the effect of the different conditions on less-dominant bacteria, genera known to harbor antibiotic-resistant-genes ('putative ARBs') based on the literature (Table 3SM) were looked for in sequencing data. Their absolute abundancies in wastewater are gathered in Figure 4.

Among genera known to harbor ARG, some were not detected in control wastewater but were abundant in wastewater spiked with antibiotics. *Streptococcus*, *Exiguobacterium*, *Acholeplasma*. *Methylophylaceae* and *Porphyromonadaceae* were identified in control but their absolute concentrations were higher in wastewater spiked with antibiotics. Similar observation was reported by Shen et al. (2019), where the abundance of *Methylophilaceace* increased after exposure. One possible explanation may be related to biodegradation properties of bacteria in the order of *Methylophilales* (Liu et al., 2015). Additionally, the genus *Exiguobacterium* has been recently characterized as a carrier of some ARGs encoding resistance to beta-lactam and sulfonamides (Carneiro et al., 2012). The presence of antibiotics may then have favored these 5 groups of putative ARBs. Others, such as *Mycobacterium*, were found in controls and wastewater but not in sterilized wastewater. The effect of the presence of biofilms on the concentration of *Mycobacterium*, *Clostridium* sensus stricto 1 and *Porphyromonadaceae* is noticeable: absent in SW, they were found at the same concentration as in W in the presence of biofilms (BSW), showing that the biofilm is a reservoir of putative ARBs that can colonize the planktonic phase. Finally,

the presence of biofilm decreased the concentration of some putative ARBs, as it was also seen at a global level for total 16S rDNA. Concentration of most putative ARBs (*Erysipelotrichaceae*_UCG-004, *Geobacter*, *Porphyromonadaceae*, *Methylophylaceae*, *Streptococcus*, *Exiguobacterium*, *Acholeplasma*) were lower in BW than in W (Fig 4).

Biofilms can then act in two opposite ways depending on the wastewater microbiome composition and diversity: on one side, biofilms can lower the level of ARB in wastewater but on the other side, it can also serve as a reservoir of ARB and re-contaminate low diversity wastewater exposed to these biofilms.

This means also that dissemination is a matter of diversity in between ecosystems and in our case of interaction between planktonic and biofilm microbial communities.

In this sense, a strategy to limit the proliferation of the ARGs in biofilms are needed. Existing methods used to reduce clogging include precipitation, filtration, chlorination, acidification and pressure flushing. The chlorination procedure has been recognised as the least expensive method to treat biological-growth related clogging but it has several disadvantages such as the formation of by-products. Recently, biological method based on the role of quorum sensing molecules such as N-acyl-homoserine lactones on biofilm formation in wastewater has been r studied by several groups (Maddela et al, 2019). Finding a way to interrupt quorum sensing (a process called quorum quenching) would be a tool to counteract proliferation of ARGs in biofilms growing in wastewater pipes. Lately, several approaches have been studied to regulate biofilm formation, including the use of exogenous molecules such as quorum sensing inhibitors or enzymes degrading quorum sensing molecules (Sun et al, 2022) or strategies using or inducing specific bacterial species with this ability (Noori et al, 2022). All these methods are well studied in membranes but application in irrigation pipes is still poorly studied, and the latest method can be an interesting future perspective.

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4. Conclusions

- Antibiotics were partially degraded and remained above lowest MIC for environmental samples maintaining conditions for resistance selection. Degradation driven by interactions between planktonic and biofilm bacteria is hypothesized.
- Biofilm thickness was not affected by the 6 added antibiotics at a concentration of 10 µg L^{-1} each.
- Based on ATP measurements, the presence of antibiotics by comparison to the C_BSW and C_BW did not affect the microbial growth of planktonic bacteria after 14 days in W, SW and BSW but reduced microbial activity in biofilms exposed to SW.
- Antibiotic exposure had no impact in the structure of planktonic bacterial communities. Sterilization was the most dominant factor explaining the planktonic community structure.
- Bacterial community composition and diversity are the driven forces for resistance selection and propagation in biofilms, rather than exposure to antibiotics.
- The abundance of some genera known to harbor ARG like *Streptococcus*, *Exiguobacterium*, *Acholeplasma*, *Methylophylaceae* and *Porphyromonadaceae* increased in reclaimed wastewater containing antibiotics.
- Interactions between planktonic, biofilm microbial communities and antibiotic resistance dissemination should be further investigated.

  Strategies to combat the proliferation of ARGs in biofilms are needed. These could be developed to module biofilm bacterial diversity, based on quorum quenching, or using quorum sensing inhibitors

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1: Antibiotic removal (in %) in containers with and without biofilm in treated and sterilized wastewater. Values are means of the three experimental replicates (n=3). Cephalexin and Amoxicillin were completely removed whatever the conditions. None of the antibiotics were quantified in the controlled container, BCWW and BCSWW.

	OFX	SMX	TRI	CLA
	% removal			
W	48.9 ± 0.6	6.2 ± 0.1	18.7 ± 0.4	31.6 ± 1.1
BW	0.0	38.5 ± 0.3	0.0	46.8 ± 0.4
SW	30.5 ± 0.9	15.3 ± 0.4	24.2 ± 0.8	35.6 ± 0.6
BSW	30.9 ± 0.12	20.5± 0.34	0±0.01	11.3 ± 0.11

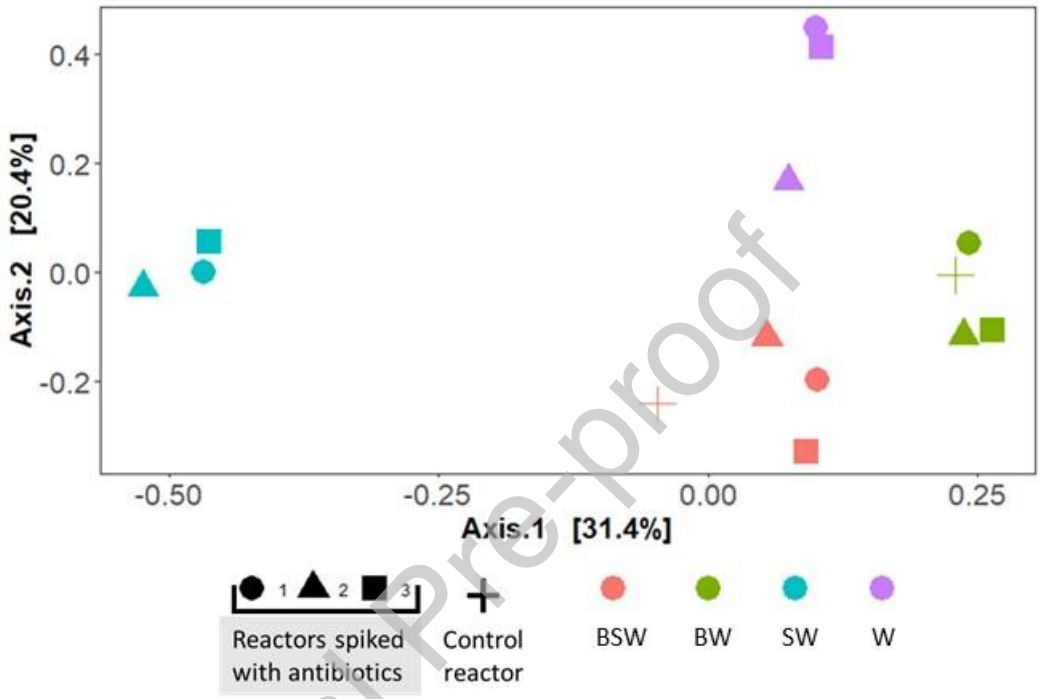
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Table 2: Richness and diversity indices in wastewater samples after two weeks of experiment (average and standard deviation for the three containers per condition, one value per control container – in bold)

	Richness indices		Diversity indices	
	Observed	Chao1	Simpson	Shannon
C_BW	552	577	0.98	4.89
BW	550±33	593±50	0.95±0.04	4.36±0.41
W	462±51	496±44	0.95±0.02	4.22±0.41
C_BSW	345	374	0.88	3.18
BSW	349±46	371±48	0.95±0.01	3.83±0.23
SW	134±7	142±6	0.88±0.02	2.93±0.21

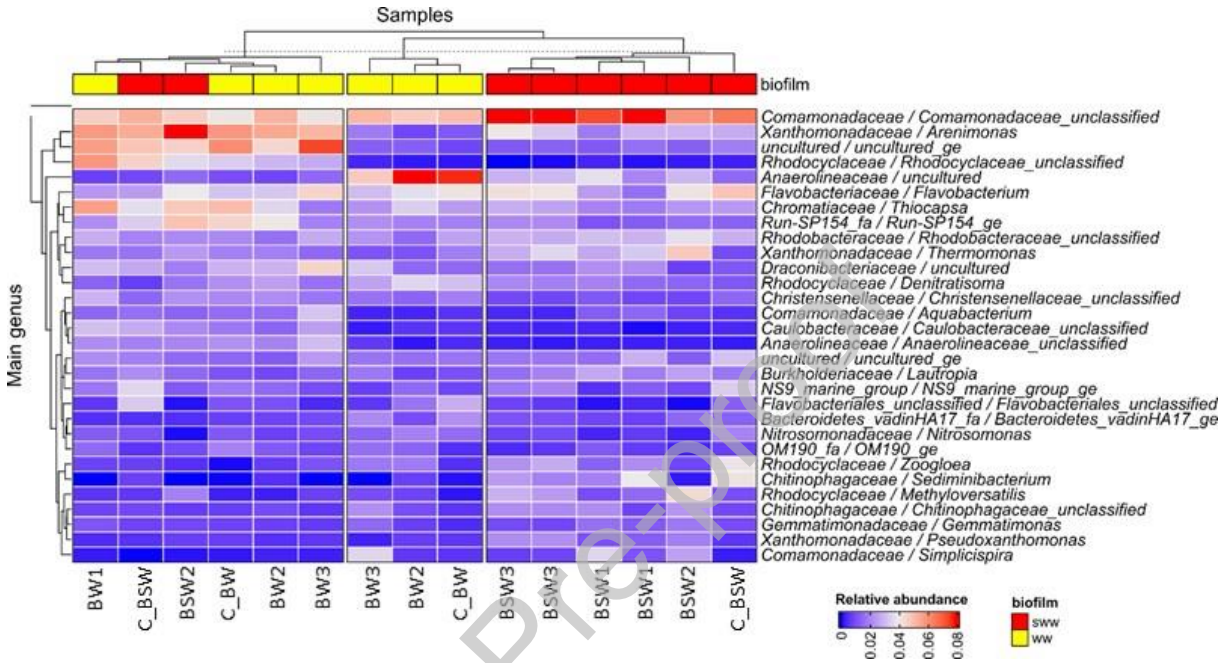
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Figure 1. Principal coordinate analysis (PCoA) of the bacterial communities structure in wastewater samples after 2 weeks of antibiotics exposure (excepted control).



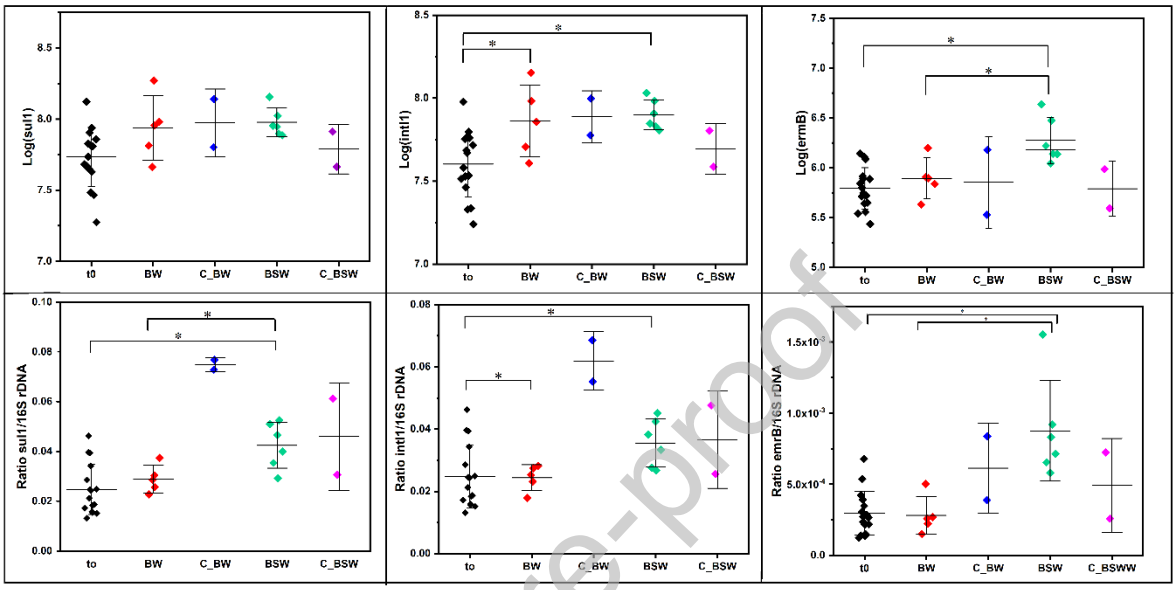
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Figure 2. Heatmap showing the relative abundance of the 30 most dominant bacterial genera in biofilms after 2 weeks of antibiotics exposure (excepted control).



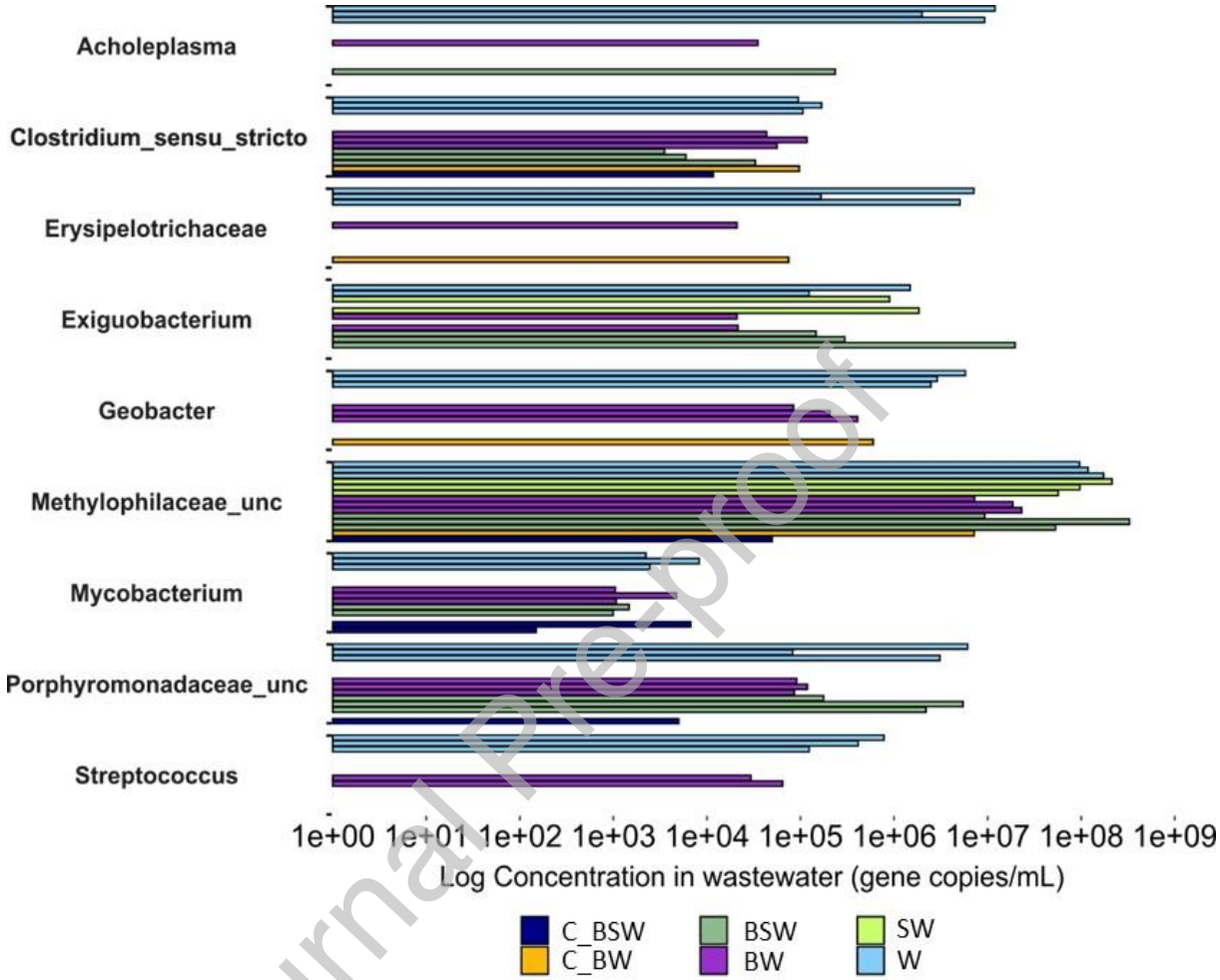
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Figure 3: Absolute (cm^2) and relative ([16S rDNA]) abundance of *sul1*, *intl1* and *ermB* in biofilms before and after 2 weeks of antibiotics exposure (excepted control). Average and standard deviation values were obtained from 6 values (2 duplicated biofilms in 3 containers), apart for control biofilms (average of 2 duplicated biofilms). *: significance differences between conditions (Kruskal-test, $p<0.05$).



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Figure 4. Concentration in wastewater of genera known to harbor ARB ('putative ARB') after two weeks of antibiotics exposure (excepted control).



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