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Effects of the chlorination and pressure flushing of drippers fed by reclaimed wastewater on biofouling

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Abstract

Milli-channel baffle labyrinths are widely used in drip irrigation systems. They induce a pressure drop enabling drip irrigation. However, with a section thickness that is measured in mm², they are sensitive to clogging, which reduces the performance and service life of a drip irrigation system. The impact of chlorination (1.5 ppm of free chlorine during 1 h application) and pressure flushing (0.18 MPa) on the biofouling of non-pressure-compensating drippers, fed by real reclaimed wastewater, was studied at lab scale using Optical Coherence Tomography. The effect of these treatments on microbial composition (bacteria and eukaryotes) was also investigated by High-throughput DNA sequencing. Biofouling was mainly observed in the inlet, outlet and return areas of the milli-labyrinth channel from drippers. Chlorination reduced biofilm development, particularly in the mainstream of the milli-labyrinth channel, and it was more efficient when combined with pressure flushing. Moreover, chlorination was more efficient in maintaining water distribution uniformity (CU <95% compared to less than 85% for unchlorinated lines). It reduced more efficiently the bacterial concentration (≈ 1 log) and the diversity of the bacterial community in the dripper biofilms compared to the pressure flushing method. This latter significantly modified the microbial communities, promoting chlorine-resistant bacteria such as *Comamonadaceae* or *Azospira*. Inversely, several bacterial groups were identified as sensitive to chlorination such as Chloroflexi and Planctomycetes. Nevertheless, one month after stopping the treatments bacterial diversity recovered and the chlorine-sensitive bacteria such as Chloroflexi phylum and the Saprospiraceae, Spirochaetaceae, Christensenellaceae and Hydrogenophilaceae families re-emerged in conjunction with the growth of biofouling, highlighting the resilience of the bacteria originating from drippers. Based on PCoA analyses, the structure of the bacterial communities still clustered separately from non-chlorinated drippers, showing that the effect of chlorination was still detectable one month after stopping the treatment.

Keywords: Drip irrigation; biofilm; cleaning methods, Optical Coherence Tomography;
High-throughput DNA sequencing, water reuse

Journal Pre-proof

1. Introduction

Due to the scarcity of water resources, the use of reclaimed wastewater for crop irrigation is significantly increasing worldwide, particularly in arid and semi-arid countries (Ait-mouheb et al., 2020). Drip irrigation, combined with the use of reclaimed wastewater, has several advantages, as it provides water to the plant (e.g. roots) in optimal quantities and frequencies for plant growth (Goyal, 2018; Wang et al., 2013). The drippers are generally characterized by a narrow labyrinth channel (internal flow section around 1 mm², which enables the development of a turbulent regime composed of a main high velocity flow and vortex zones in channel corners (Al-Muhammad et al., 2019, 2016; Wei et al., 2012). This milli-labyrinth flow path can become clogged by suspended particles, chemical precipitation and biofilms (Green et al., 2018; Y. Li et al., 2019; Oliveira et al., 2017, 2020; Rizk et al., 2019; Zhou et al., 2018). Dripper clogging not only greatly reduces irrigation uniformity, but also affects crops, by severely limiting the large-scale development of drip irrigation. Biofouling caused by biofilm growth, increases physical and chemical clogging (Li et al., 2013; Tarchitzky et al., 2013), and is considered a key factor in the clogging of drip irrigation systems using reclaimed wastewater (Song et al., 2017; Wang et al., 2017). Therefore, the evaluation of methods to control and limit the development of biofilms in these systems is necessary (Lamm et al., 2007).

Existing methods used to reduce clogging include precipitation, filtration, chlorination, acidification and pressure flushing (Duran-Ros et al., 2009; Katz et al., 2014; Puig-Bargués et al., 2010; Song et al., 2017). Although chlorination has been recognised as the least expensive method to treat biological-growth related clogging, the chlorination schemes recommended in the scientific literature differ according to the type of reagent used (liquid sodium hypochlorite (NaOCl), calcium hypochlorite (Ca(OCl)₂), gaseous chlorine (Cl₂)), the

concentration and injection interval application (Goyal, 2018; Rav-Acha et al., 1995). Indeed, chlorination can be applied in concentrations ranging from 0.4 ppm (Batista et al., 2009) to more than 100 ppm (Chauhdary et al., 2015). Both chlorine concentrations and injection frequency have an impact on clogging reduction efficiency. However, the application of high chlorine concentrations can intensify clogging by releasing clogging constituents that were previously stuck to the pipe wall (Rav-Acha et al., 1995), and can negatively impact crop growth, since a high chlorine concentrations in the soil may lead to toxicity (Li and Li, 2009). Therefore, the use of low free-chlorine concentrations (1–2 ppm) at repeated frequencies (weekly, twice a week) has proven its effectiveness in limiting dripper fouling (J. Li et al., 2010; Li et al., 2012; Song et al., 2017). Other studies have shown that the over frequent use of chlorine (once or twice a week) can also promote the development of a biofilm resistant to chlorination (J. Li et al., 2010). However, little is known about the mechanisms and microorganisms involved in this process. An in-depth analysis of the chlorination effects on the microbiome of drippers would be required to better understand the mechanisms of biofilm development and optimise the control of biofilm development (Zhou et al., 2017). Pressure flushing consists in washing water pipelines by increasing the hydraulic shear force within the pipe system. To be effective, flushing must be carried out often enough, and at an appropriate velocity rate, to dislodge and transport accumulated sediment. The minimum flow velocity for flushing the drip irrigation system is 0.3-0.6 m s⁻¹, in order to remove clogging and particulate matter (Han et al., 2018; Lamm et al., 2007; Li et al., 2018). As for chlorination, the frequency of flushing (weekly to monthly) directly influences the efficiency of the method and the service life of the drip irrigation system (Lamm, 2013; Li et al., 2015, 2018; Puig-Bargués et al., 2010). Flushing the system as often as possible is recommended (Lamm et al., 2007), but this may favour the development of a flushing-resistant biofilm caused by specific bacteria (Li et al., 2015). Thus, despite studies aimed at optimizing application parameters,

the problem of clogging has yet to be fully resolved, and the mechanisms involved are poorly understood.

The effectiveness of chlorination and pressure flushing methods on the biofouling of drip irrigation systems is often studied, but generally separately. However, several studies on drinking water systems have shown that when two methods are combined, they were more effective in reducing the level of colonisation than when they were used separately (Mathieu et al., 2014; Tsvetanova, 2020). Thus, studying the effects of these methods, when used separately and in combination, will lead to a better understanding of the mechanisms involved.

The objectives of the present study were to determine the effects of chlorination and pressure flushing, when combined or used alone, on (1) biofilm kinetic development and (2) on the microbial communities in biofilms formed in irrigation systems fed by reclaimed wastewater (RWW). To evaluate if the effects of the treatments were transient or permanent, biofilms were also analysed 1 month after stopping the cleaning operations. A non-destructive optical time-monitoring observation system was developed to study biofilm growth in commercial drippers (1 l.h^{-1}) by optical coherence tomography (OCT) (Lequette et al., 2020). For the first time, the combined use of the OCT method and high-throughput sequencing made it possible to monitor biofilm development according to the cleaning methods used, while also evaluating the impact of these methods on the bacterial compositions. Although eukaryotes can influence the formation and development of biofilms (Parry, 2004; Parry et al., 2007), data on eukaryotes in drippers supplied by reclaimed wastewater remain scarce (Dehghanisani et al., 2005). Therefore, the impact of these cleaning methods on eukaryotes was also investigated.

2. Materials and Methods


Experimental setup

Experimental setup and irrigation procedure

Non-pressure-compensating (NPC) drippers (model D2000, Rivulis Irrigation SAS, Lespinasse, France) with a flow rate of 1 l.h⁻¹ (Table 1) were used. The NPC drippers were placed in a transparent tube (internal diameter 15 mm, TubClair, Vitry-le-François, France), so that the analysis of the biofilm development along the channel could be performed over time without disturbance. More details on these experiments are presented in Lequette et al. (2020). Four irrigation lines with nine dripper systems per line were placed on the test bench: control (C-without cleaning events), a pressure flushing (PF), chlorination line (Cl) and pressure flushing/chlorination (PFCI) (see Cleaning procedures for details). Each of the four lines was connected to a separate tank (total volume 60l) and a pump (JetInox 82M, DAB, Saint-Quentin-Fallavier, France) (Figure 1.B). A disk filter (mesh size 0.13mm) was installed to reduce the physical clogging of emitters according to the technical recommendations for this type of dripper. The inlet pressure was set at 0.08 MPa (nominal working dripper pressure) using a pressure gauge. A gutter system, connected to each respective tank, was installed below each lateral line to collect the water discharged from the drippers during the experiments. The lines were supplied twice a day, five days per week for 1 h, with an interval of 6h off. Discharge measurements were performed each week to evaluate emitter performance and clogging. The relative average discharge of drippers (Li et al., 2015) was used to assess the drip irrigation performance and was calculated in Equation 1:

$$Dra = \frac{\sum_i^n q_i^t}{n q_i^0} \times 100 \quad (1)$$

where q_i^0 indicated the nominal flow of drippers (l.h⁻¹), q_i^t the measured flow rate (l.h⁻¹) and n the total number of experimental emitters.

					(mm ³)				
1	103.4	1.01	0.8	1.02	127	305	0.4	1.4	

Physicochemical and microbiological quality of TWW

The irrigation lines were supplied with reclaimed wastewater from the Murviel-Les-Montpellier treatment plant located in the South of France (43.595034° N, 3.757292° E). The wastewater treatment plant design is based on stabilisation ponds with three successive lagoons (13 680 m³, 4784 m³ and 2700 m³) and a nominal capacity of 1,500 Inhabitant Equivalent. RWW was placed in a 60l tank and changed twice a week to maintain the quality close to that of the wastewater from the treatment plant. Each week (n=16), several physico-chemical and microbiological analyses were performed to evaluate RWW quality. Chemical oxygen demand (COD), ammonia, nitrate, and phosphorus concentrations (mg l⁻¹) were measured using a spectrophotometer (DR1900, Hach Company, Loveland, CO, USA) using LCK Hach reagents[®]. Conductivity and pH were measured with probes (TetraCon[®] 925 and pH-Electrode Sentix[®] 940, WTW, Wilhelm, Germany). Faecal coliforms, *E. coli*, and *Enterococci* were quantified using the IDEXX method (Colilert18 and Enterolert, IDEXX Laboratories, Westbrook, ME) according to the supplier's recommendations. The main effluent properties are listed in Table 2.

Table 2 Physico-chemical and microbiological characteristics of the RWW

Characteristics	Units	Mean (n=9)	SD
COD (LCK 614)	mg l ⁻¹	159.7	25.1
Total suspended solids	mg l ⁻¹	65.9	13.8
Ammonia (LCK 303)	mg l ⁻¹	28.1	11.8
Nitrate (LCK 339)	mg l ⁻¹	3.1	1.1
Phosphorus (LCK 350)	mg l ⁻¹	7.0	0.8

Conductivity	$\mu\text{S cm}^{-1}$	1319.3	82.1
Dissolved oxygen	mg l^{-1}	8.8	0.4
pH		7.9	0.1
Total coliforms	MPN/100 mL	3.10E+05	1.60E+05
<i>Escherichia coli</i>	MPN/100 mL	1.08E+05	0.8E+05
<i>Enterococci</i>	MPN/100 mL	2.73E+04	1.72E+04

Cleaning procedures

Cleaning event tests were divided into two periods: a cleaning period (71 days) followed by a period without cleaning events (33 days) in order to study the interruption of these treatments on biofilm regrowth. During the cleaning period, chlorination and pressure flushing were applied once a week for 1h.

Concerning the disinfection method, liquid sodium hypochlorite (NaOCl) with a residual chlorine concentration >9% was used for chlorination. NaOCl was added directly to the tank in order to reach 1.5 ppm of free chlorine at the outlet of the drip irrigation line. After running the system for 1h, the residual free chlorine concentration was tested every 10 min at the end of the drip irrigation lines using LCX 510 Hach reagents[®]. Depending on the measurements, NaOCl was added in the tanks to maintain the 1.5 ppm of free chlorine in the system.

The maximum working pressure used for the drippers (1 l.h^{-1}) was 0.25MPa according to the manufacturer's recommendations. However, the use of the transparent tubes around the dripper systems meant that this pressure rate could not be reached without damaging the system. Therefore, for lateral flushing procedures, the valves at the end of the drip-line were closed and the pressure was increased to 0.18MPa. The pressure was controlled using a manometer placed at the inlet of the dripline. After each cleaning event, RWW from the test bench was removed and replaced by new RWW.

Image acquisition and processing

Image acquisition

Optical coherence tomography (OCT) was used to study f biofilm kinetics inside the drippers, and along the milli-channel throughout the experimental period (Table 3) after 3 weeks of running. Measurements were made in situ and non-invasively through the transparent tube. For the measurements, dripper system valves were closed to keep the drippers in the water. The dripper system was then disconnected from the irrigation line. Measurements were taken at least once every two weeks. Due to the number of drippers, the monitoring of the 36 drippers (9 per lines) by OCT was carried out over one week. After the OCT measurement, each dripper was returned to its original location on the test bench. The three-dimensional OCT measurements were acquired using a Thorlabs GANYMEDE II OCT (LSM03 lens, axial resolution= 2.1 μm , lateral resolution= 8 μm ; Thorlabs GmbH, Lübeck, Germany). The size of the axial voxel in water ($n = 1.333$) of the GANYMEDE II is 2.1 μm . OCTs have a center wavelength of 930 nm.

Table 3 Analysis and sampling schedule. *: After one month, one dripper from the control line was totally clogged. After 29 days this dripper was sampled along with 3 randomly chosen ones. This explains why the number of drippers monitored and sampled at 104 days is lower than in the other conditions.

Date	Week				Sampling dates		
	Cleaning			No-cleaning	Cleaning		No-cleaning
	3	6	10	15	29	71	104
Number of drippers per condition (n=)	9	5*-6	5*-6	2*-3	4*-3	3	2*-3
Analyses	OCT (inlet, outlet & return areas)				OCT (inlet, outlet & return areas) &		

Image analysis

OCT acquisition was performed in order to monitor biofouling development over time (volume and thickness) depending on the treatment used. First, 3-D OCT datasets were processed in Fiji (running on ImageJ version 1.50b, Schindelin et al. (2009)) and converted into 8-bit grayscale. The datasets were resliced from top to bottom into image stacks, and regions of interest (inlet, outlet and return areas) were selected (Figure S1 in Supplemental material). The remaining parts were allocated to the background (black). Secondly, an in-house code was used to detect the pixels associated with the plastic tube and removed using MATLAB R2018r (MathWorks®, version 2018b). A threshold (adapted to each dataset) was applied to binarize the dataset and the region above the interface was quantified as biofilm. For each position (x, y), the pixels associated with the biofilm (up to the threshold) were summed (on z) to obtain biofilm thickness. The biofilm volume (mm³) was calculated for each area according to Equation 4:

$$\text{Volume of biofilm} = \frac{V_{biofilm}}{V_{areas}} \quad (4)$$

where $V_{biofilm}$ is the biofilm volume and V_{areas} is the volume of the area of interest (inlet, outlet, return).

After the first visualisation of the data, an additional data-driven approach was used to enable biofilm quantification along the labyrinth, which was divided into two zones (around the edges/around the middle). Zones were created using a ‘median split’ procedure. Labyrinth width was determined for each x-step and divided by four in order to split the labyrinth into two equivalent areas: i) the middle zone corresponds to the two central quarters, ii) the edge zone corresponds to the two side quarters summed (Figure S2 in Supplemental material).

Analysis of microbial communities

DNA extraction

DNA was extracted using the PowerWater® DNA Isolation Kit (Qiagen, Hilden, Germany). The dripper and filter samples were placed in 5 ml tubes containing beads. The manufacturer's instructions were then followed. DNA concentrations were measured and purity levels were estimated by spectrophotometry (Infinite NanoQuant M200, Tecan, Austria). The extracted DNA was stored at -20 °C.

Bacterial quantification by qPCR

Total bacterial quantification was performed by qPCR on biofilms taken from drippers targeting the V9 region from 16S rDNA. The amplification reactions were performed in triplicate, and for two dilutions to check for the absence of PCR reaction inhibition. Reaction mixes (12µl) contained 2.5µl of water, 6.5µl of Super Mix qPCR (Invitrogen), 100nM forward primer BAC338 (5'-ACTCCTACGGGAGGCAG-3'), 250nM of reverse primer BAC805 (5'-GACTACCACGGTATCTAATCC-3') and 50nM of probe BAC516 (Yakima Yellow – TGCCA GCACC 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 sec for denaturation and 60 °C for 60 sec for annealing and amplification.

Illumina sequencing

The PCR amplified the V4-V5 region of 16S rRNA genes with 30 cycles (annealing temperature 65 °C) using the primers 515U (5'-GTGYCAGCMGCCGCGGTA-3') and 928U (5'-CCCGYCAATTCMTTTRAGT-3') (Wang and Qian, 2009). A PCR amplified the 18S rRNA genes (30 cycles, annealing temperature 56 °C) was also performed using the primers 5'-GCGGTAATTCCAGCTCCAA-3' and 5'-TTGGCAAATGCTTTCGC-3'

(Hadziavdic et al., 2014) on dripper biofilms at the end of the cleaning period. 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 chimeras. Sequences that appeared fewer than three times in the entire data set were removed. In all, 16S rRNA sequences were aligned using SILVA SSURef NR99 version 128 (Schloss et al., 2009). Finally, 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 respectively 2.8% and 16.5% of the sequences in biofilms and RWW samples. Finally, BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used to locate publicly available sequences closely related to the sequences obtained from the samples. A total of 2,281,644 reads were grouped in 3213 OTUs at the 97% similarity level. The rarefaction curves indicated that the sequencing depths of all samples were satisfactory (Figure S3 in Supplementary material).

Statistical analyses

Non-parametric statistical tests (Kruskal and Wilcoxon tests) were performed to compare the biofilm volume and the biofilm distribution along the labyrinth depending on the treatment used. The Shannon diversity index, the reciprocal Simpson index, and the nonparametric richness estimator Chao1 were also calculated for each RWW and biofilm sample. Chao1 richness estimates were based on singletons and doubletons as described by

Chao1 (Chao, 1984). Kruskal-Wallis tests were performed to compare indices of diversity and richness according to sample origin, and the biofouling rate between the drippers. To compare bacterial community structures, a dissimilarity matrix (Bray-Curtis) was performed and visualised using principal coordinate analysis (PCoA). A one-way analysis of similarities (ANOSIM) was used to identify significant differences in community assemblage structure between samples based on the origin of the sample (Clarke, 1993). The genera that contribute most to the divergence between two habitats were identified using Similarity Percentage (SIMPER) analysis (Clarke, 1993). 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).

3. Results

Biofilm development kinetics under chlorination and pressure flushing treatments, either combined or used separately, were monitored for 71 days using OCT measurements and compared to each other as well as to non-treated controls. The drippers were collected after 29 and 71 days in order to characterise the effect of these treatments on the bacterial composition of the biofilms. After 71 days, the treatments were stopped, and drippers were subsequently analysed a day 104 to evaluate if the effects of the treatments were permanent or transitory.

Biofouling kinetic analysis

Dynamic changes in dripper outflow ratio variation

Changes in discharge ratios (Dra) along the lateral are shown in Figure 2. Dra decreased mainly for control (C) and pressure flushing (PF) lines over time, reaching about 80% after 2 months. The Dra of the chlorinated lines (Cl and PFCl) was close to 100% after 2 months of treatment, meaning that chlorination offered better control of flow rates than purging alone.

For the same treatment, the Dra sometimes increased between two measurements, which indicates that the dripper clogging can be temporary and that detachment can occur. In the control line, most drippers (7/9 after 71 days) had discharge velocity rates below 75% and one dripper was totally clogged after one month (no outflow). In the pressure line, 4/9 drippers were considered clogged after 71 days while none were clogged for chlorinated lines (chlorination alone or combined with pressure flushing). After 1 month, without any cleaning steps, the Dra from the C and PF lines increased but were still lower than the Dra from the chlorinated lines.

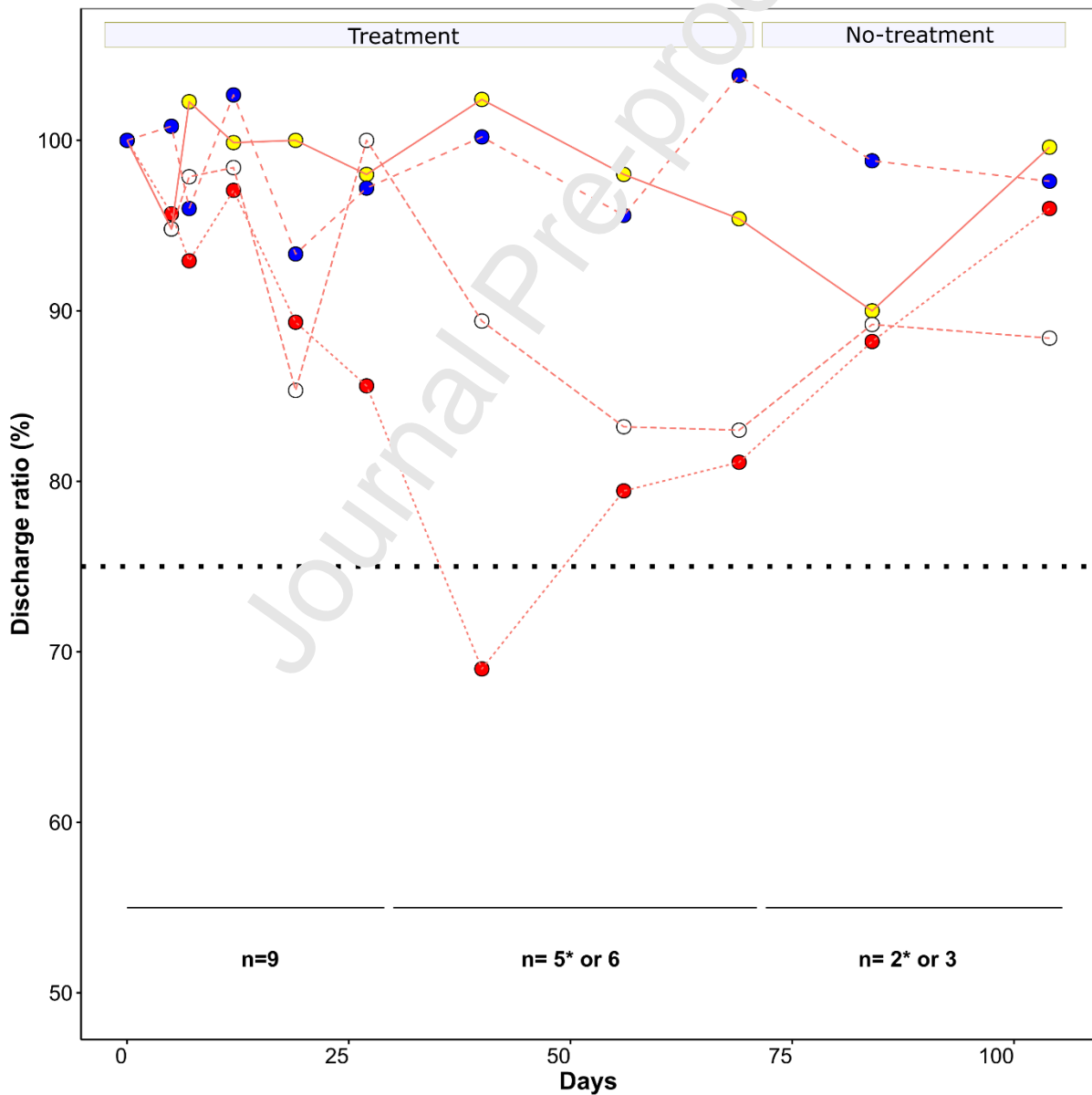


Figure 2 Discharge ratio variation according to the cleaning procedure used. Control (●), Pressure Flushing (○), Chlorination (●) and Pressure Flushing combined with Chlorination (●). The drippers were considered as clogged when the discharge was less than 75% (dotted line). n corresponds to the number of drippers for each line (*: data control line).

Chlorination combined with pressure flushing decreased biofouling

The volume and thickness of the biofilm were determined using OCT in channel areas most sensitive to fouling: the inlet, outlet and return zones.

Biofilm volumes increased during the cleaning phase (p-value <0.05, Figure 3). Although the average volume was lower in the PFCI condition after 10 weeks, there were no statistically significant differences between conditions, and this indicates that the treatments had no significant effect on the overall biofouling level. This may be due to significant variability between the triplicates. After one month, and without cleaning steps (at week 15), the mean biofouling volume increased in PF, CI and PFCI conditions and volume was higher in the PF condition than in PFCI (p-value = 0.06) (Figure 3).

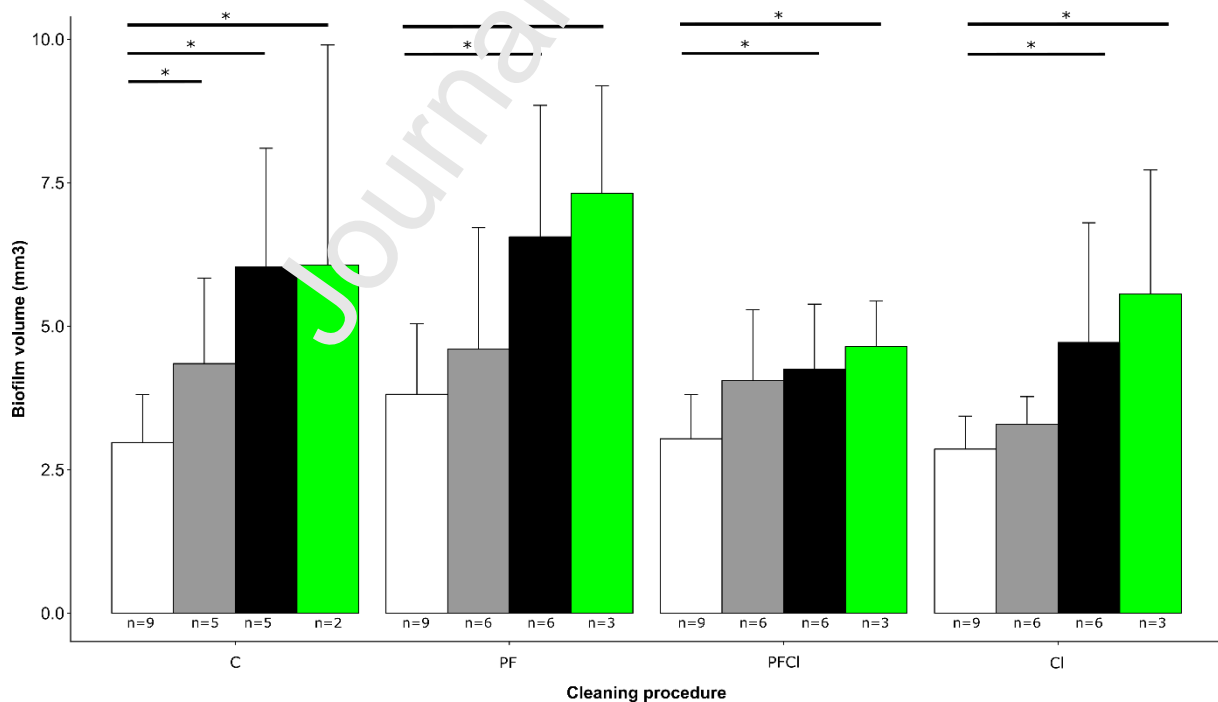


Figure 3 Changes in biofilm volumes over time according to the cleaning method used (at weeks 3, 6, 10 (cleaning phase) and 15 (no-cleaning phase); white, grey, dark grey and green respectively). C= control, PF= Pressure flushing, PFCI= Pressure flushing combined

with chlorination, Cl= Chlorination. n refers to the number of drippers. The biofouling volume was quantified as the sum of the fouling volumes developed for the inlet, return and outlet areas. * shows significant differences (p-value <0.05) on the conover test.

The study of clogging volumes in the areas of interest shown that biofouling tended to be higher in the inlet areas, whatever the treatment concerned (Figure S4). Moreover, dripper outflow was negatively correlated to the increase in inlet biofouling volumes after 10 weeks (Pearson's test, $r=-0.44$, $p<0.05$), which may explain the decrease in Dra observed for lines C and PF (Figure 2).

Although overall biofilm volumes measured in drippers and sensitive areas were statistically similar over time between the cleaning methods, the biofilm thickness was significantly influenced by the type of treatment used. Figure 3 shows an increase in biofouling thickness in the inlet areas during the cleaning period at weeks 3, 10 and 15. The channel inlet was the most sensitive area, in particular in the first baffle (p-value <0.05) where the average thickness increased by more than 0.5 mm (depth of the channel: 0.8 mm). Biofouling of the C drippers increased along the labyrinth, but did not exceed 0.3-0.4 mm (Figure 4). OCT images showed that biofouling thickness decreased mainly: in the middle of the channel when chlorination was applied (Cl and PFCI lines), at the inlet (Figure 4), outlet (Figure S5) and close to the return area (Figure S6) when compared to C and PF lines.

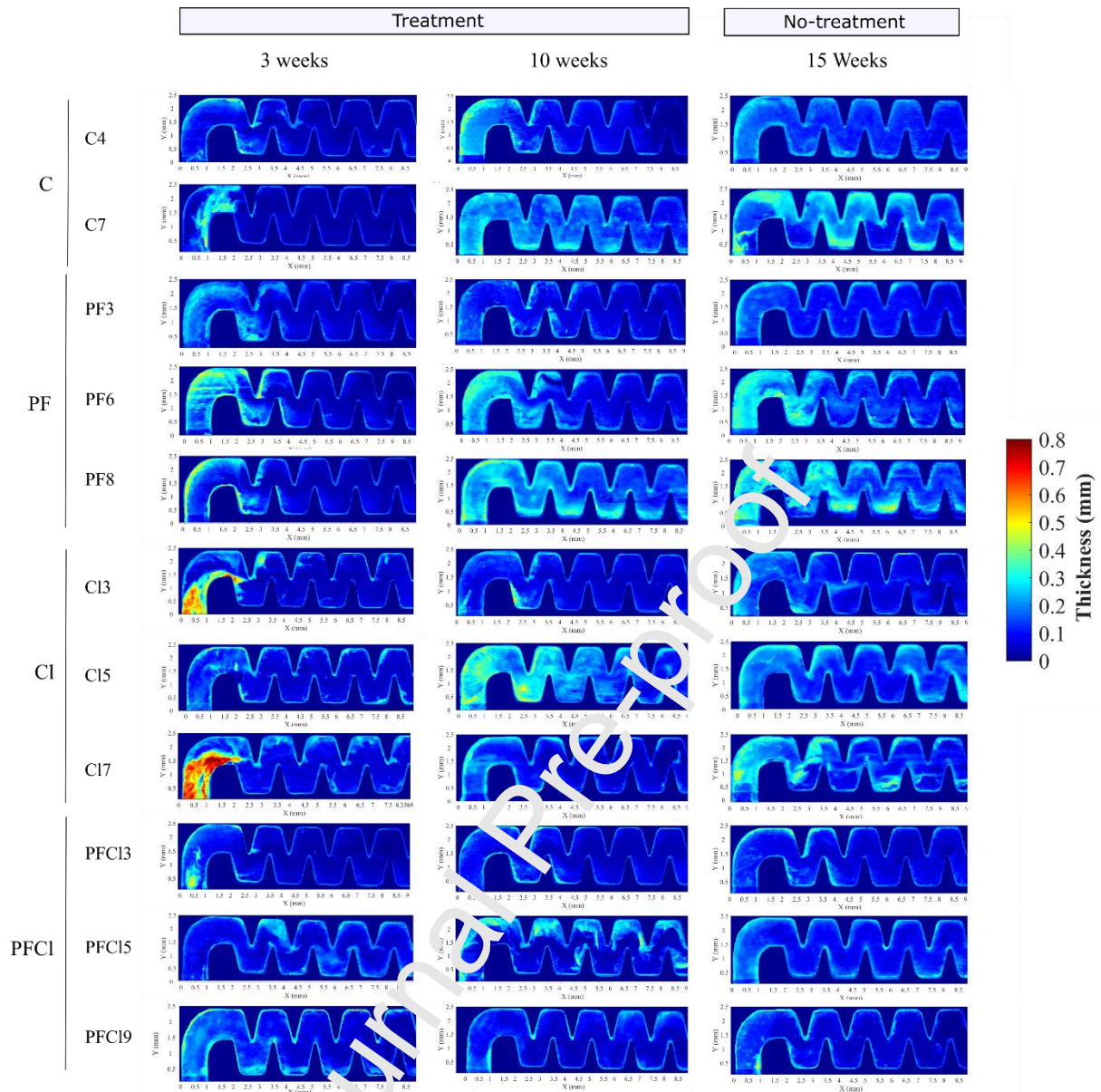


Figure 4 Biofilm thickness at the inlet of drippers under C (Control), PF (Pressure flushing), CI (Chlorination) and PFCI (Pressure flushing/Chlorination) conditions measured after 3, 10 and 15 weeks. The drippers presented are those also analysed by high-throughput sequencing at 104 days.

To further explore differences in biofilm locations according to conditions, the inlet area was divided along the labyrinth into 2 parts: the middle and the edges. The number of pixels associated with biofouling was then determined for each of these areas. Figure 5 shows the mean pixel quantity for biofouling along the inlet channel in the middle, and along the edges at the end of the cleaning step (10 weeks). When chlorination was combined with

pressure flushing (PFCI), the number of pixels associated with biofouling along the inlet channel was statistically lower in the middle and close to the edges compared to the C, PF and CI lines. Biofouling rates tended to be lower in the middle part than in the edge part. After 1 month without cleaning events, the number of pixels associated with biofouling along the inlet channel was similar in the middle and on the edges for all tested conditions (Figure S7). This means that the use of chlorination combined with increasing pressure, affected the location of the biofilm (edges or middle zone) rather than the overall amount of biofouling in the inlet zone. Moreover, this means that the combination of chlorination and pressure flushing was more efficient than chlorination alone at limiting biofilm growth.

For some chlorinated drippers (CI3, CI7), biofilm thickness between the 19th and 71st days decreased around the first inlet baffle, suggesting that some detachment of biofilm occurred (Figure 4).

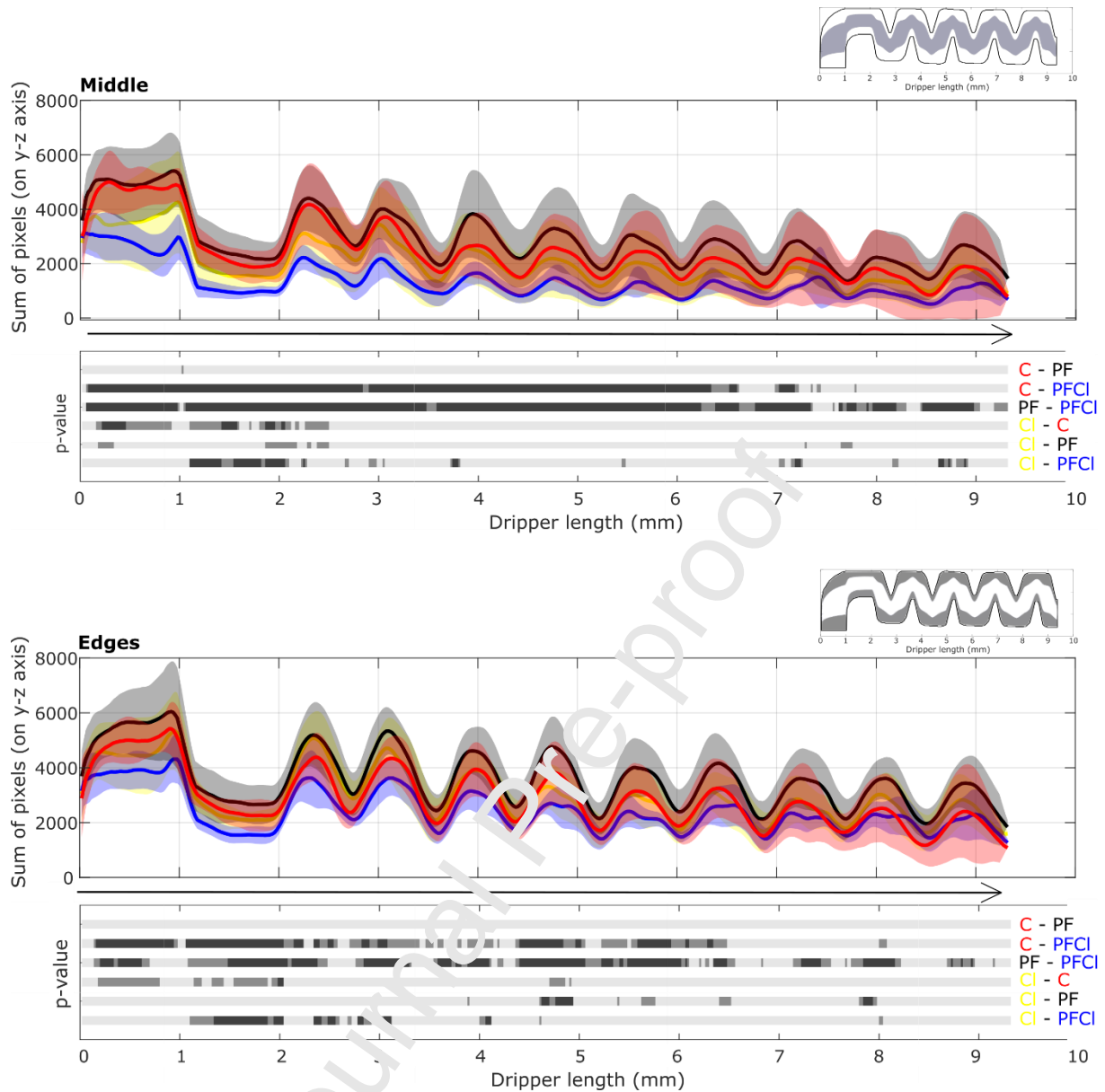


Figure 5 Means and standard deviation of pixels associated with biofouling in the middle and on the edges of the inlet dripper channel after 10 weeks of treatment. For each position of x, pixels in y-z are summed. Grey areas on inlet schemes at right-top indicate the zone of interest. Control (C-●), Pressure flushing (PF-●), Chlorination (Cl-●) and Pressure flushing combined with Chlorination (PFCI-●); the arrow indicates the direction of the flow along the channel; n=6 per condition. P-value graphs show the results of the Wilcoxon tests with ■: non-significant, ■: p<0.1, ■: p<0.05.

Biofouling level and Bacterial quantification

The quantity of bacterial cells in the drippers during the cleaning period was between 10^9 and 10^{10} copies of 16S rDNA at 29 days and between 10^{10} and 10^{11} copies at 71 days (Figure 6A) (background level of 10^3 copies of 16S rDNA at t₀). The number of copies of

16S rDNA increased significantly between days 29 and 71 for the C, PF and PFCl conditions (p -value <0.05), but not for the Cl condition (p -value >0.05). At 29 days, the number of copies of 16S rDNA in the control (C) and Cl conditions were higher than in the PFCl condition (p -value <0.05). At 71 days, the number of copies of 16S rDNA remained higher in the C condition than in the PFCl condition, which means that the combination of chlorination and pressure flushing reduced the number of bacteria more efficiently than pressure flushing (PF) or chlorination alone (Cl). Regardless of the protocol studied, the fouling level given by the volume of biofilm was positively correlated to the number of bacteria recorded during the cleaning period (Pearson's test, $r=0.66$) at 71 days, meaning that the number of bacteria in the dripper increased along with biofouling (Figure 6B).

After one month without cleaning events, the number of copies of 16S rDNA was similar to that recorded at the end of the cleaning period for each condition and concentrations remained higher in the C and PF conditions when compared with the PFCl condition.

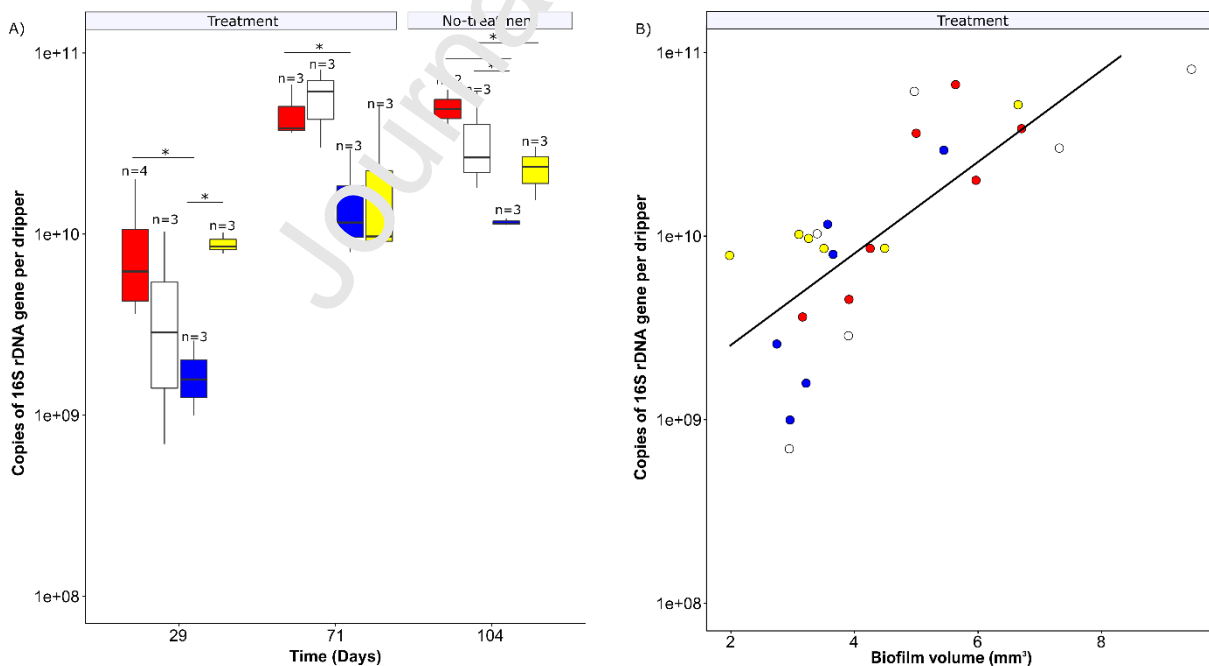


Figure 6 Evolution of bacterial quantities recorded for each dripper according to the cleaning method used (left) and biofilm volume (right). Control (●), Pressure flushing (○), Chlorination (●) and Pressure flushing combined with Chlorination (●). The biofouling

volume was quantified as the sum of the fouling at the inlet, return and outlet areas. * indicates significant differences (conover-test, p-value <0.05).

Bacterial Community Structure Analysis

The bacterial communities in dripper biofilms collected after 29 and 71 days were compared using 16S rDNA Illumina sequencing to evaluate the treatment effects. They were also compared with the communities present in reclaimed wastewater used to supply the dripper systems and renewed every week. After 71 days, treatments were stopped and biofilms were sampled again at 104 days to investigate the resilience of the communities, i.e. to test if the biofilms would then become closer to those estimated for the control condition.

Structure of bacterial communities

During the cleaning period, community richness increased significantly between 29 days and 71 days in conjunction with the biofouling level for all conditions (Table 4, conover test, p-value <0.05). The diversity indices remained similar (conover test, p-value >0.05) except for the CI condition where the diversity indices increased after 71 days.

The richness and diversity indices for the C and PF conditions were higher than in both chlorinated conditions (CI and PFCl) at 29 days and 71 days (Table 4) indicating that the structure of the bacterial community was impacted by the treatment used. After the cleaning period (104 days), the richness and diversity indices were similar between the drippers of the different lines. However, community richness had increased in the CI and PFCl lines while it remained stable in the C and PF lines. The Shannon index increased in the PF, CI and PFCl conditions and was statistically higher than in the control (C) condition. In addition, the 1/Simpson's index for the C line decreased from 71 days to 104 days.

Table 4 Richness and diversity indices according to the cleaning method used

Period	Day	Methods	Richness indices		Diversity indices	
			Observed	Chao1	Shannon	1/Simpson
Treatment period	29	C (n=4)	478 ± 59 ^a	642 ± 53 ^a	4.6 ± 0.1 ^a	47.8 ± 1.9 ^a
		PF (n=3)	469 ± 17 ^a	677 ± 85 ^a	4.6 ± 0.1 ^a	50.9 ± 4 ^a
		PFCI (n=3)	443 ± 34 ^a	549 ± 30 ^{bc}	4.1 ± 0.2 ^{bc}	18 ± 6.1 ^b
		Cl (n=3)	309 ± 49 ^a	441 ± 53 ^b	3.4 ± 0.1 ^c	11.4 ± 1.2 ^b
	71	C (n=3)	597 ± 46 ^a	851 ± 40 ^a	4.9 ± 0.1 ^a	59.9 ± 7.2 ^a
		PF (n=3)	572 ± 41 ^a	786 ± 9 ^a	4.7 ± 0.1 ^a	45.8 ± 8.3 ^a
		PFCI (n=3)	520 ± 37 ^{ab}	698 ± 49 ^{bc}	4.2 ± 0.1 ^b	18.9 ± 4.6 ^b
		Cl (n=3)	408 ± 85 ^b	578 ± 147 ^b	4.2 ± 0.1 ^b	26.0 ± 2.5 ^b
No treatment period	104	C (n=2)	512 ± 53 ^a	751 ± 133 ^a	4.4 ± 0.1 ^a	26.9 ± 4.5 ^a
		PF (n=3)	665 ± 103 ^a	924 ± 159 ^a	5.1 ± 0.1 ^b	63.3 ± 4.3 ^b
		PFCI (n=3)	741 ± 102 ^a	946 ± 73 ^a	5.0 ± 0.1 ^{bc}	48.7 ± 10 ^{ab}
		Cl (n=3)	578 ± 49 ^a	938 ± 91 ^a	4.8 ± 0.1 ^c	32.9 ± 6.3 ^a
		RWW (n=9)	501 ± 67	671 ± 81	4.5 ± 0.3	36.3 ± 15.2

PCoA confirmed that the bacterial community changed depending on the cleaning method used, and over time (Figure 7). Both the cleaning method and time were significant factors that help explain the differences in community structure ($p < 0.05$, Adonis). Pressure flushing, when compared to the control condition, had no effect on the bacterial community structure. This was also true for pressure flushing combined with chlorination (when compared to chlorination alone). Thus, during the cleaning period, bacterial population clustering was correlated to the use of chlorine (Adonis, $p < 0.05$, $R^2_{29 \text{ days}} = 0.58$ and $R^2_{71 \text{ days}} = 0.68$). At 29 days, the bacterial populations from the C dripper biofilms were

significantly different from the Cl and PFCI dripper biofilms (Adonis, $p < 0.05$, $R^2_{C-Cl} = 0.73$ and $R^2_{C-PFCI} = 0.72$ respectively). After 1 month without cleaning events, clustering, linked to the use of chlorine, was maintained (Adonis, $p < 0.05$, $R^2_{104 \text{ days}} = 0.46$) but there was no statistical difference between the chlorinated and non-chlorinated conditions (Adonis, $p > 0.05$).

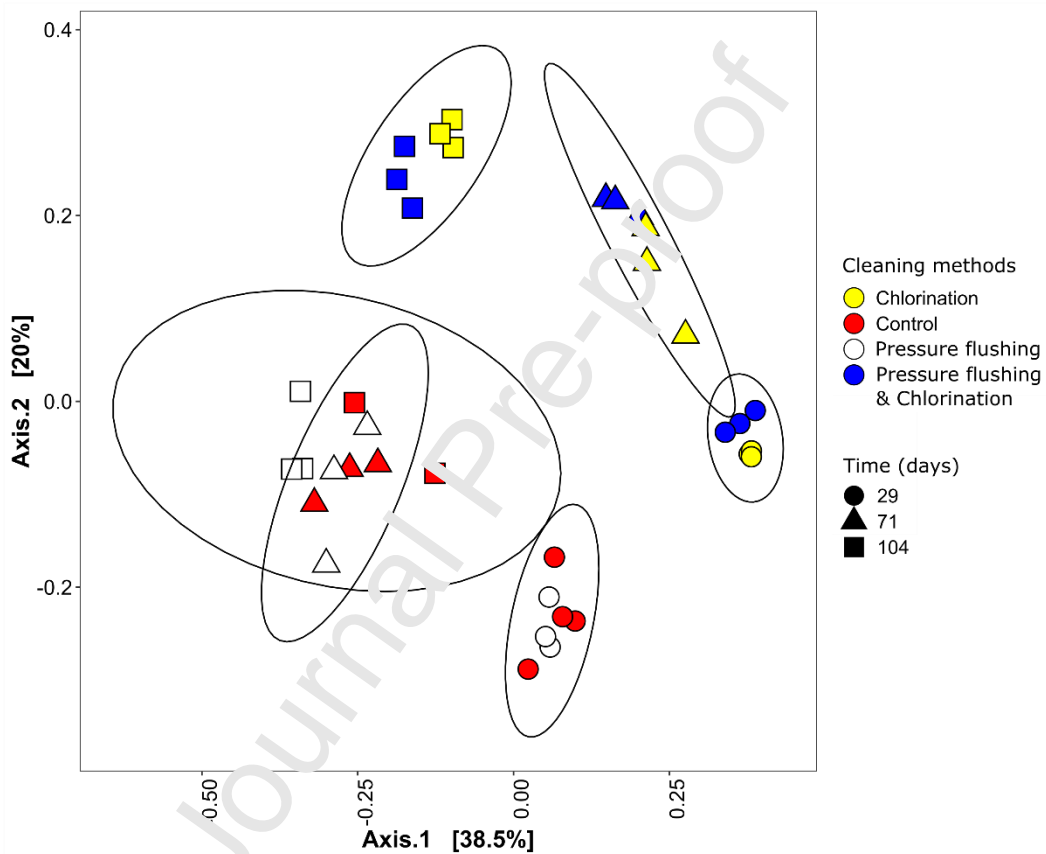


Figure 7 Principal coordinate analysis (PCoA) of microbial communities from dripper biofilms at genus level. The ellipsoids represent the 95% cut off similarity levels among samples.

Composition of bacterial communities in RWW and dripper biofilms

Biofilms and RWW microbiomes were both dominated by Proteobacteria (mainly β - and γ -Proteobacteria), Bacteroidetes, Actinobacteria and Firmicutes (Figure 8A and 8B). However, some phyla were more abundant in RWW, and other, more specific to biofilms. Acidobacteria were mainly detected in samples of dripper biofilms whereas Actinobacteria

and Verrucomicrobia phyla were more abundant in RWW samples. In addition, the relative abundance of the Spirochaetes phylum increased over time in the dripper biofilms, but represented less 1% of the overall RWW bacterial community. This means that the dripper environment induced bacterial selection. From 70 days onwards the Synergistetes phylum appeared in RWW, explaining its increase in two drippers sampled at 104 days. This means that RWW bacterial composition dynamics have an effect on the bacterial community found in dripper biofilms.

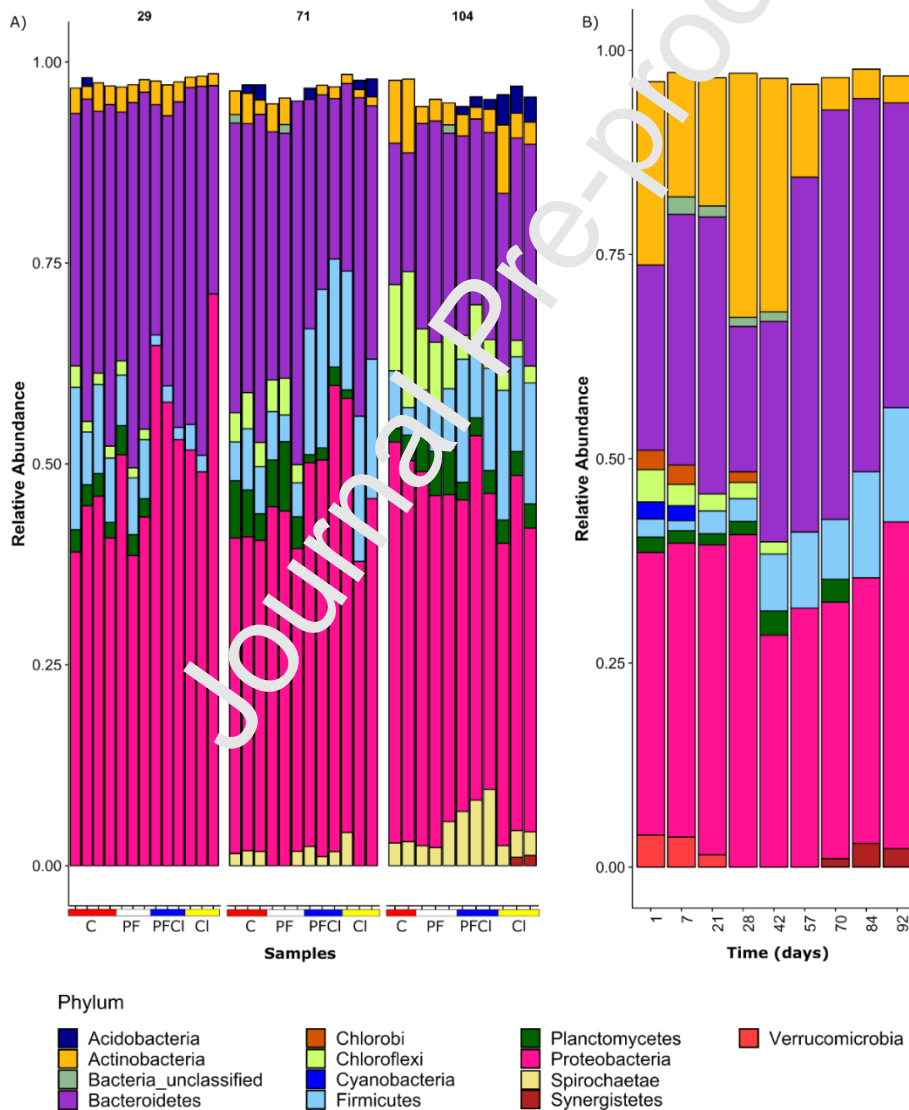


Figure 8 Relative abundance of bacterial phyla (>1%) in dripper biofilms over time (A) and in reclaimed wastewater (B).

Both the chlorination and pressure flushing methods modified bacterial community structure and composition. The relative abundance of Proteobacteria phylum was higher in chlorinated biofilms over time (Kruskal-test, $p < 0.05$), while the relative abundance of Chloroflexi and Planctomycetes was lower (Kruskal-test, $p < 0.05$). Indeed, Chloroflexi and Planctomycetes phyla were under-represented ($< 1\%$ of the global community) in the biofilms sampled on the chlorinated lines when compared to those recorded in non-chlorinated biofilms during the cleaning period (Figure 8A). These phyla were once more detectable in the 104 days samples once the treatment was stopped, but were still less dominant than in the non-chlorinated lines (mean relative abundances: 14% and 7% in C and PF respectively against 2% and 4% in Cl and PFCl respectively). This means that chlorination induced a bacterial selection and, although sensitive bacteria were able to grow again in the biofilm, chlorination effects on the communities persisted 1 month after the treatments were stopped. Thus, the composition of the bacterial community found in the biofilms was principally governed by two factors: the dynamics of the RWW bacterial composition and the selection of some adapted taxa able to cope with chlorination.

As suggested with Figure 7, the bacterial composition profiles of C and PF dripper biofilms were similar and dominated by the same genera as members of Comamonadaceae family, *Dechloromonas*, env.OPS_17 group at 29 days and *Terrimonas*, *Dechloromonas* and *Denitratisoma* at 71 days (Table S1). In addition, the SIMPER analysis showed that there were very few genera that revealed any significant differences between the structure of C and PF communities (Figure S8), which confirmed that the bacterial structure and composition were similar between C and PF dripper biofilms. There were also few differences between the Cl and PFCl conditions (Table S1), indicating that chlorination was mainly responsible for the bacterial composition, as suggested in Figure 7.

The phyla whose abundance was most affected by the treatments were studied at family and genus levels in the dripper biofilms. Proteobacteria were mainly composed of β -Proteobacteria, followed by γ -Proteobacteria, δ -Proteobacteria and α -Proteobacteria. Relative abundances of β -Proteobacteria and α -Proteobacteria were statistically higher in chlorinated biofilms (Cl and PFCl, Kruskal test, $p < 0.05$) while γ -Proteobacteria were not impacted by the cleaning method used (Kruskal test, $p > 0.05$). Inversely, δ -Proteobacteria were statistically higher in non-chlorinated drippers (C and PF) with 2% against $< 1\%$ recorded for chlorinated drippers (Cl and PFCl). Among the β -Proteobacteria and α -Proteobacteria, the Comamonadaceae family members, *Azospira*, Sphingomonadaceae, *Caulobacter* and *Hyphomicrobium* (α -Proteobacteria) were more abundant in chlorinated biofilms (Kruskal test, $p < 0.05$) whereas others were more abundant in the C and PF such as *Denitratisoma* and *Aquabacterium* (Kruskal test, $p < 0.05$) (Figure 9, Table S1). SIMPER analysis indicated that *Azospira*, and an unclassified genus from Comamonadaceae, were the main genera responsible for bacterial community clustering depending on the use of chlorination (Figure S8) during the cleaning period. The genus *Pseudoxanthomonas* (γ -Proteobacteria) was more abundant in Cl and PFCl (3–6%) conditions compared to C and PF conditions ($< 1\%$) whereas the Run-SP154_ge was more abundant in non-chlorinated drippers (C and PF).

The relative abundance of Firmicutes (dominated by Clostridia class) were significantly higher in chlorinated biofilm compared to non-chlorinated biofilm (Kruskal-test, $p < 0.05$) at 71 days (4–6% in C-PF against 16–17% in Cl and PFCl respectively, Figure 8A). Moreover, *Fusibacter* genus (Clostridia class) drove the separation of the bacterial community structure according to the use of chlorine (Figure S8).

Chloroflexi (mainly composed of one Anaerolineaceae member's family, *Leptolinea*, Chloroflexi SBR2076 and SJA-15 group) and Planctomycetes phylum (mainly comprised of

the OM190 group, *Planctomyces* and *Schlesneria* genera) were significantly less abundant in the chlorinated biofilms (C1 and PFC1) during the treatment period (Figure 8A).

Composition of eucaryotic communities in dripper biofilms

At the end of the cleaning period, the 18S rRNA sequence analysis also showed a treatment-related effect on the structure and composition of the eukaryotic communities (Figure S9). The Ciliophora and Fungi classes were the most heavily represented. Among the Ciliophora class, the family Tokophryidae was mainly present in the biofilms of non-chlorinated drippers, while the families Vorticellidae and Peritrichia were mainly present in chlorinated dripper biofilms. The fungi, which was dominated by the Dothideomycetes or Pezizomycotina families, were less present in chlorinated drippers whereas the Sordariomycetes family was mainly present in chlorinated biofilms.

Impact of cleaning stoppage on the bacterial community composition

SIMPER analysis at 104 days showed that there was no difference between C and the other conditions (Table S8), which confirmed that bacterial communities converged (Figure 7) when the treatments were stopped.

Chloroflexi and Planctomycetes phyla increased in chlorinated dripper biofilms from C1 and PFC1 lines once chlorination was stopped (Figure 8A). The genera and taxonomic groups belonging to Chloroflexi such as the Anaerolineaceae family members: *Leptolinea*, SBR2076 and SJA-15 groups settled in chlorinated dripper biofilms. Other minor bacterial families in chlorinated drippers sampled during the cleaning period re-emerged such as Saprospiraceae (Bacteroidetes), Spirochaetaceae (Spirochaetae), Christensenellaceae (Firmicutes) and Hydrogenophilaceae (β -Proteobacteria) at similar levels found in biofilms from non-chlorinated drippers (Figure 9). Inversely, the relative abundance of the main genera

from chlorinated biofilm during the cleaning period decreased such as *Azospira*, *Flavobacterium* and *Cloacibacterium* (Figure 9).

A shift was observed in the bacterial compositions from the C drippers at 104 days compared to 71 days, mainly due to an increase in the relative abundances of Chloroflexi (from 3% to 14%) and Actinobacteria (from 3 to 8.5%), which were higher than those recorded in the other conditions (p-value <0.05). On the contrary, the relative abundance of Bacteroidetes phylum (represented by *Terrimonas* genus) decreased from 37% to 16%. The dominant genera previously found during the cleaning period increased such as *Dechloromonas* and *Aquabacterium* (β -Proteobacteria) (p value > 0.05), which could explain the decrease in 1/Simpson's index observed (Table 4). The relative abundances of Chloroflexi, mainly composed of Anaerolineaceae family members, and Actinobacteria, were higher in the C condition than in the others.

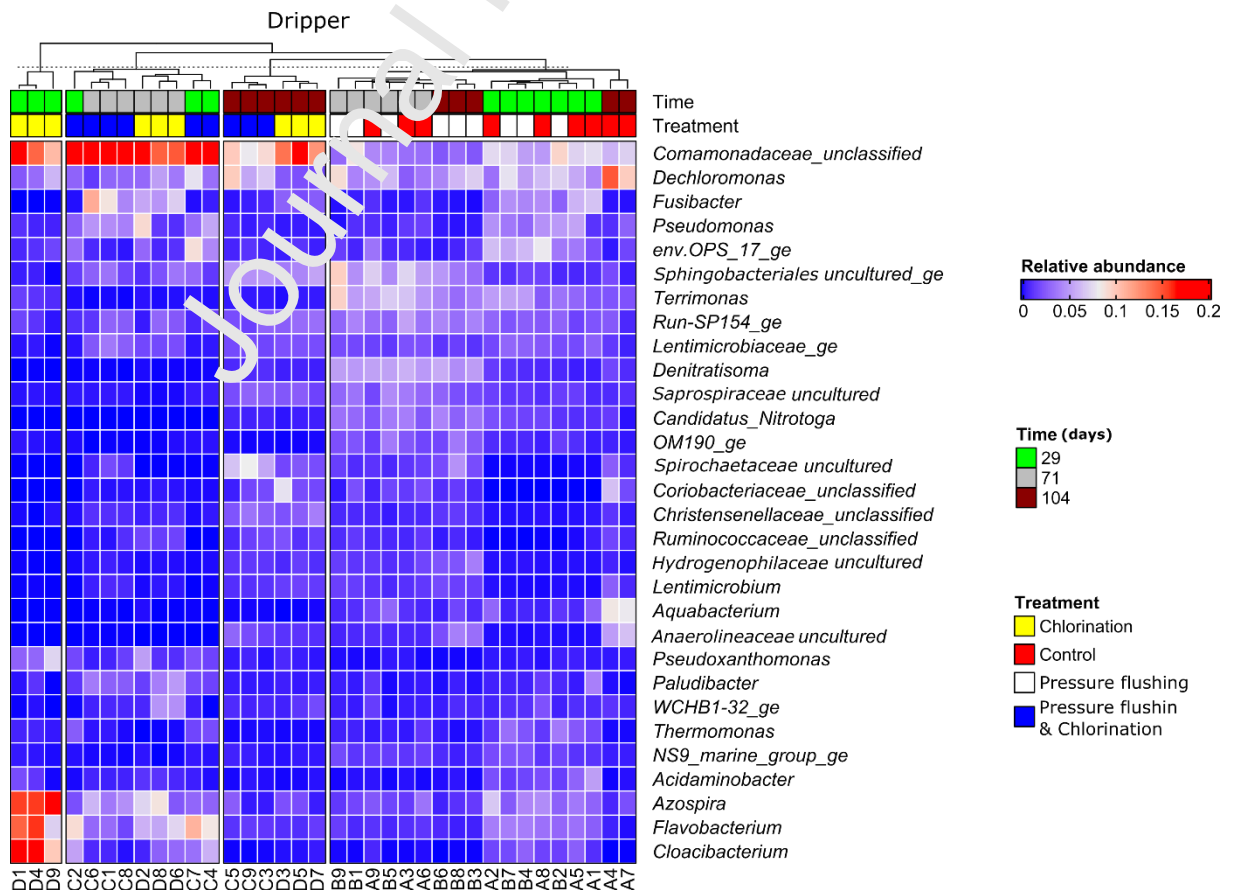


Figure 9 Heat map of bacterial genera from dripper biofilms. Genera with the top 30 relative abundance are shown.

4. Discussion

In this study, the effects on biological clogging and microbial communities, resulting from different cleaning methods (chlorination (Cl), pressure flushing (PF), and a combination of both methods (PFCI)), were investigated. Commercial flat drippers (1 l.h^{-1}) used in agriculture were installed on a test bench and fed with reclaimed wastewater. After a 2-month period of treatments, cleaning events were interrupted in order to study the impact on biofouling regrowth and bacterial communities.

Chlorination, unlike purge-only treatment, helps maintain flow rates

The effectiveness of cleaning methods is often looked at from the perspective of flow maintenance (Feng et al., 2018; Han et al., 2018; Song et al., 2017). Chlorination (1.5 ppm), alone or in combination with pressure flushing (Cl and PFCI), maintained flow rates and reduced distribution heterogeneity throughout the cleaning period. Conversely, the uniformity of water distribution decreased over time under non-chlorinated conditions (C and PF), which means that chlorination is preferable in order to maintain expected flow rates. Chlorination results were consistent with those of J. Li et al. (2010) who showed that chlorinating (2.5 ppm of free chlorine) once a week, maintained the expected dripper flow rates supplied by reclaimed wastewater for a longer period of time. However, the impact of pressure flushing is not consistent with Yu et al. (2018) who showed that increasing the pressure from 0.1MPa to 0.3MPa for 2 minutes helped maintain the expected flow rates for longer periods by removing large particles ($<6.6 \mu\text{m}$ in diameter). The pressure increases used in our experimental work may not have been sufficient to promote a detachment of biofilm

and maintain flow rates. However, biofilm is considered to be an elastically deformable, but quasi-static structure that enables it to deal with an increase in pressure and flow velocity (Picioreanu et al., 2018). For instance, Li et al. (2015) showed that EPS concentrations found in dripper biofilms were higher when purging was performed once rather than twice a week. This suggests that purging too often does not optimally reduce biofilm development. Moreover, Zhang et al. (2020) showed that lateral flushing changes deposit biomass and the microbial environment rather than the attached biomass, which may explain the nonsignificant effect of purging on biofilms.

Biofilms can also increase pressure loss due to friction along the labyrinth (Li et al., 2016): the biofilm formed in the labyrinth increases roughness, which in turn increases friction, resulting in increased flow resistance, and thus increased the pressure loss with a consequent decrease in distribution uniformity.

Cleaning procedure impacts on biofouling

Purging had no impact on bacterial concentrations and biofouling (volume, thickness). A recent study showed that flushing (1h per day) with reclaimed wastewater is not effective at limiting dripper clogging when compared to freshwater flushing due to the presence of suspended solids and other organic compounds (N. Li et al., 2019). Thus, the use of freshwater could offer a solution to improve the efficiency of the pressure flushing method. However, in the case of field irrigation with treated wastewater, it is not always possible to have access to a second water resource.

Conversely, the level of biofouling and bacterial concentrations in the chlorinated lines (Cl and PFCI), mainly in the PFCI line, tended to decrease when compared to those observed for non-chlorinated lines. The combination of pressure flushing and chlorination seemed to offer a better control of biofouling than chlorination alone. This decrease was mainly due to a

significant decrease in biofouling in the middle of the channel, where flow velocity was higher (Ait-Mouheb et al., 2018; Al-Muhammad et al., 2016; Yu et al., 2018a). Increasing the flow velocity by increasing the pressure favours mass transfer (Beyenal and Lewandowski, 2002; Moreira et al., 2013) and influences mixing (Khaydarov et al., 2018; Naher et al., 2011). Mathieu et al. (2014) showed using a rotating disc reactor that the shear stress and cohesive force necessary to detach biofilm decreased after chlorine application (10 ppm during 1h). This is probably due to the breaking of the hydrogen bond, as well as polymer and hydrophobic interactions, caused by chlorine (Xue and Seo, 2013), thus weakening the biofilm and makes it easier to detach. Therefore, chlorine mass transfer caused by hydrodynamic conditions in the main flow zones (middle of the milli-channel) may explain why the biofouling level was lower under PFCI conditions than in C and PF conditions in the middle and corners of the channel, and why bacterial concentrations were lower under this condition.

However, the combination of the two methods did not completely stifle the development of biofouling. One explanation is that the increase in the PFCI condition favours the biofilm compaction, and the resistance of the basal layer in the central and corner areas (Blauert et al., 2015; Rochex et al., 2008; Valladares Linares et al., 2016; Wagner and Horn, 2017), which reduced the chlorine effect (Lee et al., 2018). Lee et al. (2018) showed that the free chlorine penetration at 10 ppm of Cl_2 was low in a biofilm of 2000 μm (7 days to reach the substratum) and tended to promote the sloughing off of the superficial layers of the biofilm. Thus, the concentration of free chlorine used (1.5 ppm) may be insufficient to reduce the level of clogging, especially in corner areas where biofilm thickness was higher. However, studies have shown that the long-term use of a high-concentration of chlorine can have more significant adverse effects on soil health than for the lower ones (Song et al., 2019). Further studies on chlorine transfer, whether combined or not with a purge, while taking into account

agricultural practices and soil health, are needed to find cleaning strategies, which are efficient and with limited environmental impacts.

Chlorination strongly modified microbial communities in biofilms

In this study, total bacteria, diversity and richness indices in conjunction with the level of clogging.

Bacterial diversity was lower in chlorinated conditions compared to those of the control and pressure flushing lines. The bacterial diversity of biofilms found in drinking water distribution pipes was also previously shown to decrease with the increasing concentration of chlorine (0.05 to 1.76 ppm) (Fish and Boxall, 2018; Mi et al., 2015).

Pressure flushing had no impact on the bacterial community when compared with the non-treated control line, which is consistent with Li et al. (2015) who studied the microbial composition of biofilms using T-LFA analysis. On the other hand, the use of chlorine, whether combined or not with pressure flushing, has significantly altered the structure of the bacterial community during the cleaning step. Proteobacteria and Firmicutes phyla appeared to contain bacterial members resistant to chlorine treatment whereas Chloroflexi phylum appeared to be sensitive to chlorine, which is consistent with Song et al. (2019). Chloroflexi phylum includes many filamentous bacteria such as Anaerolineaceae member's family and *Leptolinea* genus detected in sequencing data and responsible for clogging membrane bioreactors in wastewater treatment plants (Li et al., 2008) and drippers supplied by RWW (Lequette et al., 2019). Chloroflexi may have a key role in the dripper clogging.

Chlorinated dripper biofilms were largely dominated by OTUs belonging to the Comamonadaceae family (β -Proteobacteria), followed by *Flavobacterium* (Bacteroidetes), *Pseudomonas* and *Pseudoxanthomonas* genera (γ -Proteobacteria). β -Proteobacteria are

frequently found in chlorinated drinking distribution systems and wastewater biofilms (Douterelo et al., 2016; Navarro et al., 2016; Shaw et al., 2014), and their abundance can increase in conjunction with the chlorine concentration (Wang et al., 2019b). The Comamonadaceae family was commonly found in water and chlorinated biofilms (Wang et al., 2019a; Zhang et al., 2019). *Pseudomonas* and *Hydrogenophaga* (Comamonadaceae) are known to exhibit resistance to disinfection (Jia et al., 2015; Wang et al., 2019a). Increases in relative abundance of *Pseudomonas* have been observed in water samples after chlorination (Jia et al., 2015) and *Hydrogenophaga* are known to colonise chlorinated distribution pipes fed with reclaimed wastewater (Wang et al., 2019b).

Firmicutes predominated in the chlorinated drippers after 2 months of treatment. At the class level, the bacterial community was mainly composed of Clostridia (abundance between 10 and 14%), which includes many members capable of producing endospores. These endospores are highly resistant to a variety of environmental challenges, such as heat, solvents, oxidising agents, UV irradiation and desiccation (Abecasis et al., 2013). The resilience of endospores allows them to remain viable in a hostile environment for long periods of time, and contributed to their survival and proliferation in chlorinated environments (Douterelo et al., 2016).

The eukaryotic communities, which are mainly composed of Ciliophora and Fungi, were also influenced by chlorine use. Sordariomycetes, Tremellomycetes and Chytridiomycetes were still present in chlorinated biofilms, while Dothideomycetes or Pezizomycotina were only recovered in control and purge drippers. Sordariomycetes, Tremellomycetes and Chytridiomycetes are commonly found in the effluent of wastewater treatment plants and are associated with water treatment (Assres et al., 2019). Sordariomycetes has also been detected, and deemed to be dominant, in biofilms found in drinking water distribution systems (0.05 ppm to 0.8 ppm of free chlorine) (Fish and Boxall, 2018). These results suggest that

among fungi, some are more tolerant to chemical disinfection. The resistance to chemical disinfection of some fungi can be explained by their thick melanized cell walls, which increases resistance to mechanical damage and restricts intrusion of biocides into the cell (Hageskal et al., 2012), or may be due to their ability to form spores (Sonigo et al., 2011). Ciliophora were affected by chlorine use, with chlorine-sensitive families (e.g. Tokophryidae), whereas others were still present in chlorinated drippers (e.g. Vorticellidae). Ciliophora contains bacteria predators that can significantly reduce bacteria concentrations (Parry et al., 2007) and influences biofilm morphology (Böhme et al., 2009; Derlon et al., 2012). Although eukaryotic communities found in dripper biofilms are poorly studied, their role in biofilm development and resistance to cleaning processes may be important, and must be integrated in biofilm control studies. In addition, the microbiome of reclaimed water evolves over time, and depends on the treatment used. For this reason, additional studies to investigate the effect of the wastewater treatment (i.e. activated sludge, membrane bioreactor) as well as the role of bacterial predators on the microbial composition of a biofilm should be performed.

Resilience of microbial communities once treatments are stopped

1 month after the stoppage of cleaning events, several bacterial families, deemed to be chlorine-sensitive, re-emerged such as Saprospiraceae, Spirochaetaceae, Christensenellaceae and Hydrogenophilaceae at levels similar to those measured in biofilms from non-chlorinated drippers. The relative abundance of Chloroflexi phylum also increased significantly after this period. Bacteria concentration, diversity and richness indexes also increased. This suggests that treatment stoppage reduces selection pressure and makes it easier to recruit new species. Fish and Boxall (2018) obtained a similar result for biofilm development in drinking water distribution systems, and this would explain the increase in dripper clogging after the

interruption of chlorination observed by Katz et al. (2014) (10 ppm of free chlorine). This also illustrates the resilience of the bacterial community in its ability to deal with chlorine used (Allison and Martiny, 2009) in a drip irrigation system. However, PCoA analyses showed that the drippers in the chlorinated lines still form a different bacterial cluster than non-chlorinated lines 1 month after treatment stoppage, which shows that the treatment, although finished, has residual effects on the structure of the communities, and that resilience was only partial.

5. Conclusion

The combined use of OCT and high throughput sequencing shed light on the impact of the two cleaning procedures (pressure flushing, chlorination) on the biofouling of RWW-fed drip-irrigation systems:

- The pressure flushing method alone did not reduce the biofouling of the drippers and did not influence the biofilm microbiome.
- Conversely, chlorination application alone or combined with pressure flushing steps reduced dripper biofouling, mainly in the centre of the labyrinth.
- Some bacteria commonly known to clog systems, such as *Chloroflexi* and *Planctomycetes*, appear sensitive to chlorine and susceptible to regrowth after chlorination is discontinued.
- Others, such as the members of the *Comamonadaceae* and *Clostridia* (Firmicutes), are less sensitive. Further research on the mechanisms governing sensitivity is needed to improve biofilm control.
- Treatment suspension leads to an increase in the bacterial diversity of the initially chlorinated biofilms, and to a convergence of the communities towards non-chlorinated biofilms, although differences in the structure remain. This indicates that the bacterial community is resilient to chlorine, but that the community at a global scale keeps the ‘memory’ of the chlorination.

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Disclosure statement

No potential conflict of interest was reported by the authors. All authors have approved the final article.

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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.

The authors declare the following financial interests/personal relationships which may be

considered as potential competing interests:

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Credit Author

Kévin Lequette : Conceptualization, Investigation, Methodology, Formal analysis,

Visualization, Writing - Review & Editing

Nassim Ait-Mouheb : Supervision, Methodology , Writing - Review & Editing

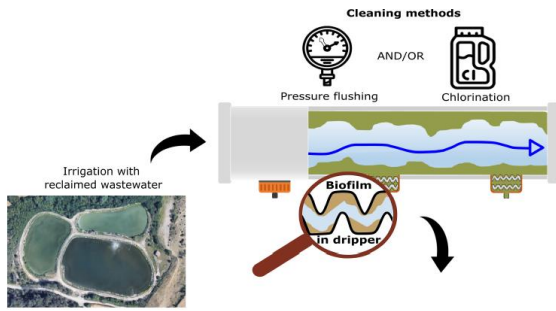
Nicolas Adam : Formal analysis, Visualization

Marine Muffat-Jeandet : Methodology, Ressources

Valérie Bru-Adan : Ressources

Nathalie Wéry : Supervision, Methodology, Writing - Review & Editing

Graphical abstract



BIOFILM	Cleaning methods			
	No treatment	Pressure flushing	Chlorination	Pressure flushing AND/OR Chlorination
Volume	=	=	=	=
Thickness	↑	↑	↓	↓
Shift the microbial composition	×	×	✓	✓

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Highlights

- Chlorination and lateral flushing are commonly used to prevent dripper biofouling
- Biofilm thickness was lowest when chlorination was combined with pressure flushing
- The β -Proteobacteria and Firmicutes contain chlorine resistant bacteria
- The decrease of Chloroflexi (filamentous bacteria) by chlorination was transitory
- The bacterial community was resilient after the intervention of cleaning events