Microbially healthy water in greenhouse horticulture

Prevention and removal of biofilm formation in zero discharge cultivation systems

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Report WPR-905

WAGENINGEN UNIVERSITY & RESEARCH

Referaat
Behandeling van biofilms in irrigatieleidingen is een belangrijk aspect in het bereiken van een emissieloos teeltsysteem, zoals de doelstelling voor de Nederlandse glastuinbouw in 2027. Een testsysteem (in drievoud) en protocol zijn ontwikkeld en gebouwd voor het testen van de effectiviteit van technologieën en producten in het voorkomen van biofilmvorming en het verwijderen van bestaande biofilms. Onder tuinbouwomstandigheden (temperatuur, watersamenstelling, materiaal irrigatieleiding, systeemlengte, etc.) hebben het systeem en het protocol laten zien in staat te zijn een reproduceerbare biofilm in de drie parallelle systemen te kunnen produceren. Vijf technieken zijn getest met dit systeem:

- (1) Oxyl-PRO S zilver gestabiliseerde H_2O_2 heeft laten zien effectief te zijn in het voorkomen van biofilmvorming, maar heeft geen significant effect in het verwijderen van een bestaande biofilm laten zien.
- (2) SureFlow ClO_2 heeft laten zien effectief te zijn in zowel het verwijderen van een bestaande biofilm als het voorkomen van biofilmvorming.
- (3) Antibacteriële leidingen, (4) AQUA4D® en (5) SonoPure Ultrasoon hebben geen significant effect laten zien in zowel voorkomen van biofilmvorming als verwijderen van een bestaande biofilm.

Geen van de onderzochte technieken gaf een selectief effect op de samenstelling van de microbiële gemeenschap in de biofilm.

Abstract
Management of biofilms in irrigation lines is an important aspect to achieve Zero Liquid Discharge cultivation systems, as is the goal for Dutch greenhouse horticulture by 2027. A test system (in triplicate) and a protocol were developed and build to test the effectiveness of technologies and products for removal and prevention of formation of biofilms. Under greenhouse circumstances (temperature, water composition, irrigation line material, system length, etc.) this system and protocol have shown to be capable to produce a reproducible biofilm in three parallel test systems. Five technologies have been tested with this system:

- (1) Oxyl-PRO S silver stabilised H_2O_2 has shown to be effective to prevent biofilm formation, but did not show a significant effect in biofilm removal.
- (2) SureFlow ClO_2 has shown to be effective in both removal of an existing biofilm and prevention of biofilm formation.
- (3) Antibacterial pipelines, (4) AQUA4D® and (5) SonoPure Ultrasound did not show a significant effect in both prevention of biofilm formation and removal of an existing biofilm.

None of the tested technologies had a selective effect on the composition of the microbial community in the biofilm.

Reportinfo

Report WPR-905
Projectnumber: 3742257900
DOI number: 10.18174/527781
Theme: Water & Emission

This project/research has been made possible, in part, by the contribution of Topsector Tuinbouw & Uitgangsmateriaal, Topsector Water and Stichting Programmafonds Glastuinbouw.

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Summary

Dutch horticulture is working towards zero liquid discharge irrigation systems in 2027 to achieve the goals of the European Water Framework Directive (WFD, 2000). To reach the state of zero liquid discharge, quality of the recirculation nutrient solution, the correct working of irrigation equipment and the prevention of disease spreading via the irrigation system is of major importance. Biofilms can affect the correct working of irrigation equipment by clogging drippers and sprinklers and can be a safe haven for pathogenic micro-organisms to spread through the irrigation system. Therefore it is important for growers to have strategies to remove existing biofilms and to prevent the formation of biofilm in clean irrigation lines.

The goal of this project was to develop strategies for the prevention and removal of biofilm from the inside of irrigation lines and to test their efficacy. Technologies as applied in these strategies should fit the goal of zero liquid discharge and therefore preferably do not add chemicals to the nutrient solution that do not add to crop development, as their concentration can build up during recirculation.

A test system was designed to be representative for a greenhouse irrigation system, containing water buffer tanks, pumps and 50 m of irrigation lines. Part of this system was a biofilm monitor, in which the formation of biofilm could be followed in time. This system was built in triplicate, to be able to compare strategies with a control treatment. UV-disinfected rain and desalinated well water (treated with reverse osmosis) was added to the buffer tanks, together with nutrients according to a nutrient recipe for tomato cultivation, a sodium acetate solution as carbon source and actual greenhouse drain water as a bacterial inoculum. In the biofilm monitor rings of material from the insides of irrigation lines were added, for measurement of biofilm formation. Water circulation was applied during daytime (8.00-17.00) during 12 minute cycles (2 minutes circulation, 10 minutes stagnant), and during the night the system remained stagnant, to imitate irrigation turns. Temperature in the climate room was constant at 24 °C. To evaluate prevention of biofilm formation in irrigation lines, technologies were started immediately at the start of the test run. To evaluate removal of existing biofilm from irrigation lines, test systems ran for six days before technologies were started. In this way, a sufficient amount of biofilm could be formed. The efficacy of technologies was analysed by measurement of ATP in water and biofilm, additional analyses were performed on the microbial community in the biofilm.

Comparison of the control treatments in separate test runs have shown that there is no significant difference between the amount of biomass in biofilms in different systems during a test run. Biofilm formation in consecutive test runs could have significantly different amounts of biomass in the biofilm. By testing the technologies in duplicate for removal and for prevention of biofilm formation in two different test systems, this effect could be filtered out of the results.

Five technologies were tested for their capacity to remove a biofilm or prevent a biofilm from forming:
- Oxyl-PRO S (Cindro): Oxyl-PRO S is a hydrogen peroxide formulation containing 50% H_2O_2, stabilised with a coating of silver nanoparticles. Dosage of 20 mg/L of H_2O_2 (40 mg/L Oxyl-PRO S) resulted in prevention of biofilm formation in clean irrigation lines. Removal of an existing biofilm did not show a significant effect.
- SureFlow ClO_2 (CH2O B.V.): chlorine dioxide (ClO_2) is produced on site by combining hydrochloric acid and sodium chlorite. Addition of 0.5 mg/L ClO_2 prevented biofilm formation in clean irrigation lines and was capable of removing an existing biofilm.
- Antibacterial irrigation lines (MVP-Starmaker): The antibacterial irrigation lines are produced from regular LDPE-material, to which a zinc-additive is added during the production process. No chemicals dissolve in the water during application as irrigation line. This technology can only by applied to prevent biofilm formation, however, no significant effect was found compared to the control treatment.
- AQUA4D® (Planet Horizons Technologies): The AQUA4D® technology generates very low-frequency (a few kHz) electromagnetic resonance fields (<20 mT) with very low intensity to treat the nutrient solution. No significant effect was found on prevention of biofilm formation or removal of an existing biofilm by application of this technology.
- Ultrasound (AWWS): the technology creates low frequency ultrasonic sound waves (25 kHz) that propagate through the irrigation lines to reach the biofilm, creating a mechanic effect to remove the biofilm. However, application of this technology in this test system did not show a significant effect on the prevention of biofilm formation and on the removal of an existing biofilm in irrigation lines.

None of the technologies showed selective removal of certain bacterial classes from biofilm communities.

The project results show that from the tested technologies, the traditional (chemical) methods (SureFlow ClO_2 and Oxyl-PRO S H_2O_2) were most successful in preventing and removing biofilms under the conditions tested here, representative for greenhouse circumstances.

Samenvatting

De Nederlandse glastuinbouw werkt toe naar een emissieloze teelt in 2027 om de doelen van de Europese Kaderrichtlijn Water (WFD, 2000) te halen. De kwaliteit van de recirculerende voedingsoplossing, de correcte werking van het irrigatiesysteem en het voorkomen van de verspreiding van ziektes via het irrigatiesysteem zijn van groot belang om deze status van zero liquid discharge te bereiken. Biofilms kunnen de correcte werking van het irrigatiesysteem verstoren door het verstoppen van druppelaars en sproeiers en kunnen daarnaast bijdragen aan de handhaving en verspreiding van pathogene micro-organismen in het teeltsysteem. Het is daarom van groot belang dat telers technieken en strategieën hebben om de vorming van biofilm tegen te kunnen gaan of een bestaande biofilm te kunnen verwijderen uit het irrigatiesysteem.

Het doel van dit project was het ontwikkelen van strategieën voor het voorkomen en verwijderen van biofilm aan de binnenzijde van irrigatieleidingen en het testen van hun effectiviteit. Technieken die worden toegepast in deze strategieën moeten passen binnen de doelstellingen van een emissieloze teelt en daarom bij voorkeur geen chemicaliën aan de voedingsoplossing toevoegen die niet in het teken van gewasgroei staan, omdat deze in concentratie kunnen toenemen tijdens recirculatie.

Een test systeem was ontworpen, representatief voor een glastuinbouw irrigatiesysteem. In het systeem zitten waterbuffers, pompen en 50 meter irrigatieleiding. Een biofilmmonitor was een onderdeel van dit systeem, waarin de biofilmvorming in de verschillende strategieën gevolgd kon worden in de tijd. Het systeem is in drievoud gebouwd, zodat behandelingen vergeleken konden worden met een controlebehandeling. Een mix van regenwater en met omgekeerde osmose behandeld bronwater is ontsmet met UV en toegevoegd aan de buffertanks, samen met nutriënten volgens een recept voor tomatenteelt, een natriumacetaat oplossing als koolstofbron voor micro-organismen en glastuinbouw drainwater als bacterieel inoculum. In de biofilmmonitor zijn ringen geplaatst van het materiaal van de binnenzijde van irrigatieleidingen, waarmee de vorming van biofilm gemeten kon worden. Het water werd gedurende de dag (8.00 – 17.00) rondgepompt in 12 minuten durende cycli (2 minuten circulatie, 10 min stilstaand), gedurende de nacht stond het systeem stil, waarmee de irrigatie in een teeltsysteem werd nagebootst. Een constante temperatuur van 24 °C is aangehouden in de klimaatruimte. In testruns voor preventie van biofilmvorming werden de technieken direct na de start van de testrun ook opgestart. In testruns voor verwijdering van een bestaande biofilm werden de technieken pas na zes dagen opgestart. Op deze manier kon een voldoende dikke biofilm opgebouwd worden om de effectiviteit van de technieken vast te kunnen stellen. Meting van ATP uit levende cellen is toegepast om de vorming van biomassa in water en biofilm te kunnen volgen, aanvullende analyses zijn uitgevoerd om ook de samenstelling van de microbiële gemeenschap te kunnen vaststellen.

Een vergelijking van de controlebehandeling in de afzonderlijke testruns heeft laten zien dat er geen significant verschil is in de vorming van biofilm in de verschillende systemen tijdens een testrun. In opeenvolgende testruns kan wel een significant verschillende hoeveelheid biofilm opleveren. Door de technieken in verschillende testsystemen toe te passen in de duplo-uitvoeringen van de behandeling, werd dit effect uit de resultaten gefilterd.

Vijf technologieën zijn getest voor hun effectiviteit in het verwijderen van een bestaande biofilm en het voorkomen van biofilmvorming in schone leidingen:
- Oxyl-PRO S (Cindro): Oxyl-PRO S is een formulering bestaande uit 50% H_2O_2, gestabiliseerd met een coating van zilver nanodeeltjes. Dosering van 20 mg/L H_2O_2 (40 mg/L Oxyl-PRO S) zorgde voor het tegengaan van de vorming van biofilm in schone leidingen. Verwijdering van een bestaande biofilm heeft geen significant effect aangetoond.
- SureFlow ClO_2 (CH2O B.V.): chloordioxide (ClO_2) wordt gemaakt uit zoutzuur en natriumchloriet. Toevoegen van 0.5 mg/L ClO_2 voorkwam vorming van biofilm in schone leidingen en was ook in staat om een bestaande biofilm uit leidingen te verwijderen.
- Antibacterieel leidingwerk (MVP-Starmaker): de antibacteriële irrigatieleidingen zijn geproduceerd uit standaard LDPE-materiaal, waaraan tijdens het productieproces een zink additief is toegevoegd. Dit additief zit vast in het materiaal en lost tijdens gebruik niet op in de voedingsoplossing. De technologie kan alleen toegepast worden voor het voorkomen van biofilmvorming, maar hiervoor werd geen significant effect aangetoond.

- AQUA4D® (Planet Horizons Technologies): de AQUA4D® technologie maakt zeer laagfrequente (een paar kHz) elektromagnetische resonantievelden (<20 mT) met een zeer lage intensiteit om de voedingsoplossing te behandelen. Er is geen significant effect aangetoond van deze technologie in het verwijderen van een bestaande biofilm en het voorkomen van de vorming van een biofilm in schone leidingen.
- Ultrasoon geluid (AWWS): de technologie creëert laag frequente ultrasone geluidsgolven (25 kHz) die zich voortplanten door het water in de irrigatieleidingen, om via mechanische stress door cavitatie de biofilm los te trillen van de binnenzijde van leidingen. Toepassing van deze techniek in het testsysteem heeft geen significant effect aangetoond voor het verwijderen van een bestaande biofilm en het tegengaan van de vorming van biofilm in schone leidingen.

Geen van de technieken heeft een significant selectief effect laten zien in de verwijdering van bepaalde groepen micro-organismen uit de gemeenschappen in de biofilm.

Het project heeft aangetoond dat van de geteste technieken de traditionele (chemische) methoden (SureFlow ClO_2 en Oxyl-PRO S H_2O_2) het meest succesvol waren in het voorkomen van biofilmvorming in nieuwe leidingen en het verwijderen van een bestaande biofilm, in de omstandigheden zoals hier getest, representatief voor glastuinbouw omstandigheden.

1 Introduction

1.1 Background

The European Water Framework Directive (WFD, 2000) urges member states to reach a good (chemical and ecological) surface water quality by ultimately 2027. Greenhouse irrigation systems offer opportunities to close the water cycle at the company level by cultivation in substrates out of the soil, thereby decreasing emissions to the environment. Around 90% of Dutch commercial greenhouse companies grow their crops in such substrates (e.g. stonewool, cocopeat or peat moss) and reuse drainage water. Nevertheless, growers still decide to discharge their nutrient solution (Beerling *et al.,* 2014). With this so called discharge water, horticulture has an effect on surface water quality by discharging drain water, possibly also containing plant protection products, to surface water. The Dutch greenhouse sector made a deal with the government to decrease the impact on surface water quality by application of obligatory discharge water purification (Hoofdlijnenakkoord waterzuivering in de glastuinbouw, 2015) for the removal of plant protection products, with January 1st 2018 as starting date. This increases the cost of discharging drain water and increases the incentive for recirculation. Also emission standards for nitrogen (Rijksoverheid, via infomil.nl) were agreed upon by the sector, to decrease the emission of nitrogen and phosphorus to the environment. According to this regulation, all crops need to be produced within zero liquid discharge cultivation systems by 2027. Both of these regulations force growers to try to understand internal water quality better, to be able to decrease discharge of drain water and increase recirculation.

Although current cultivation systems are generally successful in recirculating drain water, sometimes cultivation problems make discharge necessary. For most of these problems, solutions are developed or are under investigation, but issues related to microbiology in the irrigation system make zero-emission cultivation a too big of a risk. Recent results show that the composition of organic matter (root exudates, secreted by plant roots) plays an important role in the microbiological composition of recirculated irrigation water and thus can contribute to the microbiological water quality (see results of WP1; https://www.glastuinbouwwaterproof.nl/onderzoeken/kwr2018007-microbieel-gezond-water-in-de-glastuinbouw/p/4/). This can also affect the physical composition of the water, e.g. by consumption of oxygen or changing the pH, thereby having a direct effect on crop production.

A more indirect effect of microbiology in the irrigation system on crop production is the formation of a biofilm. Conditions in the irrigation system are often ideal for growth of micro-organisms and thereby for the formation of biofilms: water temperatures of 20-40 °C, easily consumable organic matter, nutrients, a high ratio between surface area and volume and long periods of stagnant water. Bacteria and fungi (not all are pathogenic) will stick to the insides of a pipeline, but also for example to connection points, corners or on rough surfaces (e.g. caused by nutrient depositions). Once stuck to the surface, the micro-organisms will start to grow and produce an extracellular matrix, a slimy layer, to protect against the (harsh) environment. Also other bacteria or fungi (possibly pathogenic) in the water can colonize this existing biofilm, thereby becoming a source of infection for the entire irrigation system (Pachepsky *et al.,* 2012). It is hypothesised that *Rhizobium rhizogenes*, initiator of 'crazy roots' in vegetable cultivation, spreads through the irrigation system via biofilms. It is also reported that viruses can colonize a biofilm (Skraber *et al.,* 2005). Mechanically a biofilm can clog drippers or sprayers, either by growing into it or by transport of flocks of biofilm with the water flow. This causes uneven supply of nutrient solution to the crop within the greenhouse: some plants may not have enough water whereas others will be overirrigated.

The problem of mechanical clogging of irrigation lines and drippers/sprayers due to the presence of biofilm is recognised by growers. A variety of strategies is applied to clean the irrigation lines or keep them free of biofilm, either during the cultivation season or at crop interchange. This can be done by using antimicrobial products such as hydrogen peroxide, chlorine dioxide and electrochemically activated water (ECA-water) (Van Os en Kromwijk, 2014; Hofland-Zijlstra *et al.,* 2011), but also by physical water treatment such as very low-frequency electromagnetic resonance fields (Mercier *et al.,* 2016) or ultrasound treatment (Lambert *et al.,* 2010). Not all of these products are suitable for use in zero-discharge cultivation systems, as they may be directly harmful to the crop or by-products can increase in concentration due to infinite recirculation. Also effectivity in the removal of biofilm in the entire length of the irrigation system is currently unknown.

KWR has developed a biofilm monitor to measure the effect of different treatments on biofilm formation in water supply systems for drinking water, cooling water or surface water (Van der Kooij *et al.,* 1995; 2009). With the biofilm monitor and a test protocol it is possible to create a controlled biofilm and thereby test strategies to prevent biofilm formation or remove an existing biofilm (Van der Kooij *et al.,* 2003).

1.2 Goals

Goals of this project include:

- Designing and building an installation for controlled formation of biofilm, representative for greenhouse cultivation circumstances.
- Development of a test protocol for controlled biofilm formation, with reproducible results on biofilm formation for consecutive test runs.
- Develop and test technologies/strategies for their effectivity in the removal of an existing biofilm or prevention of biofilm formation in a clean irrigation system. Tested technologies/strategies should fit within a zero emission cultivation system.

1.3 Project partners and financers

This report describes one out of two work packages of the project "Microbieel gezond water in de glastuinbouw" (microbially healthy water in greenhouse horticulture). This project is financed as a public private partnership (PPS) within the Topsector Tuinbouw & Uitgangsmaterialen (TKI T&U) and the Topsector Water (TKI Water Technology), within the programme Greenhouse Horticulture Waterproof (Glastuinbouw Waterproof). This specific work package (WP2) is on prevention and removal of biofilm formation in zero discharge cultivation systems. This work package is financially supported by Dutch greenhouse entrepreneurs via Stichting Programmafonds Glastuinbouw. Project partners AWWS, Planet Horizons Technologies, Cindro, MVP-Starmaker and CH2O also participated financially in this work package.

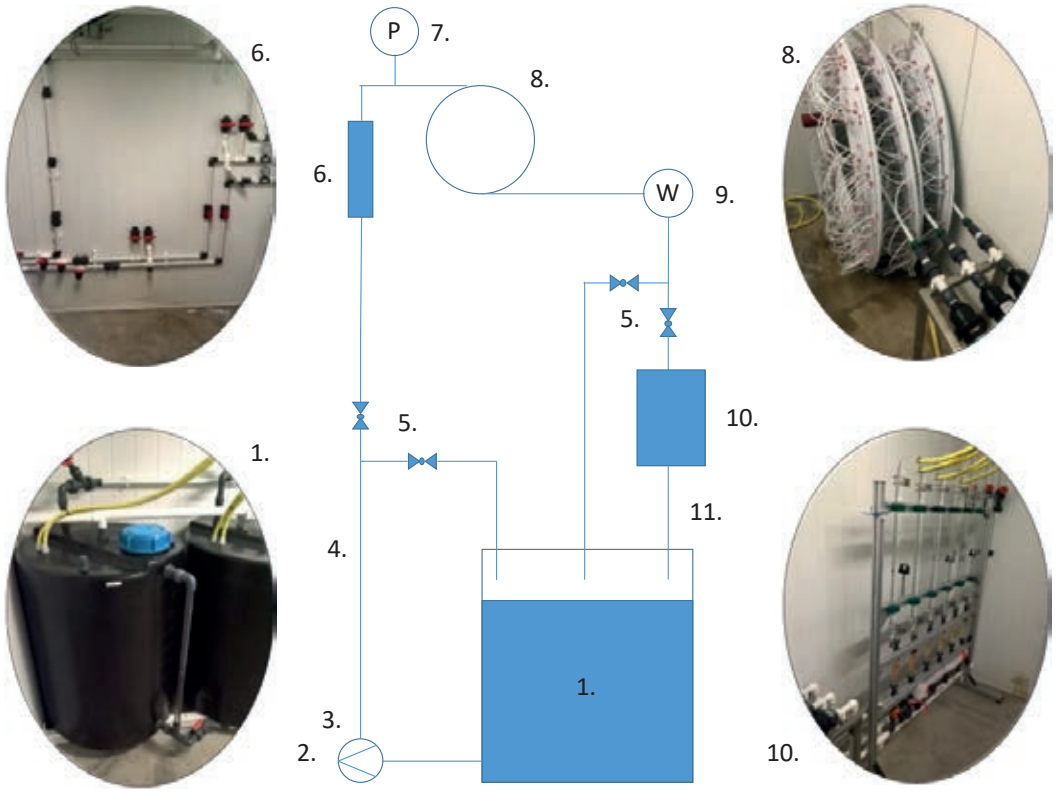
2 Materials & methods

2.1 Experimental set-up

In Annex 1 the complete protocol for the experimental setup can be found (in Dutch). In this chapter, all elements are briefly explained.

2.1.1 Test system

The test system consisted of three parallel systems for the production and treatment of biofilm formation. Each system contained a buffer tank for storage of nutrient solution. Water from this buffer tank was pumped towards the technology to be evaluated, and after that this water was transported through an LDPE-irrigation line (50 meters) and an analogue water meter. Manual valves allowed for control of the water flow through the pipelines. The water then flowed through a biofilm monitor, in which the water of each system was split over two parallel glass columns (replicates A and B) with valves and a rotameter in each column to control the flow. In these glass columns, alternately rings of the inside material of irrigation lines (LDPE) and glass rings were placed (Figure 2). The LDPE-rings (surface area of around 17 cm^2) were used to sample the developing biofilm, whereas the glass rings were used as weights to hold the LDPE-rings in place. After the biofilm monitor the water was returned to the buffer tank to start the (re)circulation cycle again (Figure 1).



1. Buffer tank 500 L
2. Self-priming pump 230 V/1.6 kW
3. Time/pulsrelais 24/240 volt ac/dc 2VA
4. Pipe work EPDM PVC 32 mm
5. Y-valve hand controlled, 4/system D32 NNT EPDM PVC-U
6. Insertion point for technologies
7. Manometer glycerin filled: 0-4 bar
8. Irrigation pipeline: 50 m 16 mm LDPE white/black: 4 bar, Dripper Kameleon High 2 L
9. Water meter analogue: capacity 1.5 m^3/h, dP 100
10. Biofilm monitor, 220 l/h
11. Tricoflex soft & flex 12.5 mm inner diameter, PVC

Figure 1 *Schematic overview of the installation for controlled biofilm formation, with pictures of parts of the installation from the lower left picture clockwise: buffer tank, insertion point for technologies, irrigation pipeline with analogue water meter and biofilm monitor.*

2.1.2 Cleaning protocol

Before the start of each test run, the installation was cleaned and disinfected. As start of the cleaning and disinfection cycle, leftover rings were removed from the biofilm monitor and remaining water was pumped out of the system. Then, each system was filled with 300 L clean water and 3 L of hydrogen peroxide (50% concentrate, no additives, Cindro). Then the water was circulated through the system from 8:00 until 17:00 (9 hours) in 12-minute cycles, consisting of two minutes recirculation and ten minutes stagnance. During the remaining 13 hours (17:00-8:00 [next day]) the water remained stagnant. After this initial cleaning, the water with 0.5% hydrogen peroxide was pumped out of the system and the system was flushed with clean water to remove remaining hydrogen peroxide, until no more residual H_2O_2 could be measured.

Used glass rings were placed in 3 L water with 30 mL hydrogen peroxide (50% concentrate, no additives, Cindro) for 4-6 hours. After that, these rings were rinsed with tap water for 1 hour, left to stand in tap water for 24 hours and rinsed again with tap water for 1 hour. New LDPE rings had the same cleaning treatment: 1 hour rinsing with tap water, leaving in tap water for 24 hours and another hour of tap water rinsing. A stainless steel wire was disinfected with a 70% ethanol solution and placed in each of the biofilm monitor glass pipes. This wire prevented the movement of the rings during operation of the system. Cleaned rings were placed in the biofilm monitor, using sterile gloves and a sterile stainless steel wire with a hook for easy placement. LDPE and glass rings were placed alternately, starting with an LDPE-ring to stop the LDPE-rings from floating. In each pipe of the biofilm monitor 12-14 LDPE-rings were placed for measuring biofilm formation. After this cleaning protocol, the system was ready for the start of a test.

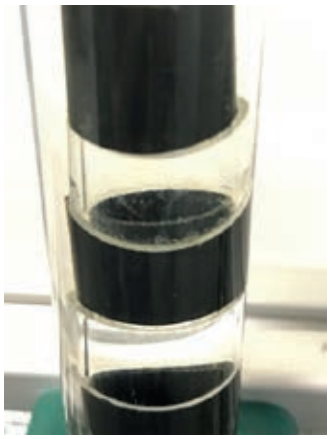


Figure 2 *LDPE and glass rings in a glass pipe of the biofilm monitor.*

2.1.3 Test water composition

At the start of each test run, 300 L of tomato nutrient solution was prepared, according to the recipe in Table 1. The medium was based on a mixture of reverse-osmosis-treated well water and rain water (after storage in a closed water basin and treatment with UV). Depending on the season and the availability of rain water, a higher or lower percentage of well water was used. For nutrients, two 100x concentrated stock solutions were prepared (A and B). The buffer tank was filled with 150 L of water, then three litres of each nutrient stock (A and B) was added, after which the buffer tank was further filled to a level of 300 L. The EC of the nutrient solution was 1.9 mS/cm, and pH was corrected to pH 5.5 after filling the buffer tank. This was done by either adding Baskal (YaraTera Substrafeed Baskal, mixture of 20-25% potassium carbonate and 15-20% potassium hydroxide) to achieve a pH raise, or by adding nitric acid for lowering the pH.

Table 1

Composition of the nutrient solution, standard nutrient solution for tomato (De Kreij et al., 1999).

NH_4	K	Ca	Mg	NO_3	SO_4	H_2PO_4	Fe	Mn	Zn	B	Cu	Mo
mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L
1.0	6.5	2.75	1.0	10.75	1.5	1.25	15	10	4	20	0.75	0.5

When the water had the desired composition, a bacterial inoculum of 20 mL was added. For this purpose, 2 L of water from the bottom of the drain pit of the research station in Bleiswijk was collected on March 25, 2019. This sample was homogenised and split in subsamples of 20 mL, which were stored at 4 °C in the dark. Sodium acetate (10 µg C/L) was added to the water to act as carbon source for the bacteria to grow.

Sodium acetate

Sodium acetate solution (10 µg C/L) is added to the nutrient solution as carbon source for the micro-organisms. It is prepared with:

- Sodium acetate (CH_3COONa) (CAS nr. 127-09-3) 1 g.
- MilliQ water 5000 mL.

Dissolve the sodium acetate in the water. Sterilise during 15 minutes at 121 ± 3 °C. This solution is stored at 5 ± 3 °C and it keeps for 6 months at most.

Sterile tap water

Sterile tap water is used to collect the biofilm from the LDPE rings.

Protocol for production of sterile tap water:

- Use a tap for drinking water and flush the tap for at least 30 minutes.
- Fill the 1 litre bottle with the water.
- Add to the bottle 1 mL NTA-solution (50 g/mL) (this is to neutralise the toxic effect of eventually present copper).
- Sterilise during 15 minutes at 121 ± 3 °C.
- Closed bottles can be stored up to 6 months at room temperature. Open bottles cannot be used for longer than one day. There could be some sediments at the bottom of the bottle, this is calcium and can do no harm.

Trisodium nitrilotriacetate (NTA)

NTA is used to neutralise eventually present copper in tap water. This solution is prepared using the following protocol:

- Trisodium NTA ($Na_3C_6H_6NO_6$) (CAS nr. 5064-31-3) 50 g.
- water 1000 mL.

Dissolve the trisodium-NTA in the water. Sterilise during 15 minutes at 121 ± 3 °C. This solution is stored at 5 ± 3 °C and it keeps for 9 months at most.

2.1.4 Protocol

The effectivity of technologies was evaluated in two ways: prevention of biofilm formation and removal of an existing biofilm. Protocols for testing of both principles are described below (see Annex 1 for Dutch version). Samples were taken from both nutrient solution (water) and biofilm (biofilm monitors).

2.1.4.1 Generic settings

The test system described in 2.1.1 was placed in a climate chamber at Wageningen University & Research Business Unit Greenhouse Horticulture in Bleiswijk. Temperature in the climate chamber was kept constant at 24 °C, equal to the temperature of a spring day in a tomato greenhouse. The climate room was completely dark, except for during biofilm measurements. During the test runs, an irrigation schedule for tomato culture during spring/summer was applied. Water circulation was applied during daytime (8.00-17.00) during 12 minute cycles (2 minutes circulation, 10 minutes stagnant), and during the night the system remained stagnant. This process was automated with a timer. Flow rate through the pipes of the biofilm monitor was 200-220 L/h during circulation.

2.1.4.2 Prevention of biofilm formation

To evaluate prevention of biofilm formation in irrigation lines, technologies were started immediately at the start of the test run. At day 0, the system was filled with nutrient solution. Before the first measurements, nutrient solution was circulated for five times two minutes with a manual start. After these measurements, technologies were started. Table 2 shows the general planning of the test runs for prevention of biofilm formation. In some cases, test runs were extended, or sometimes additional measurements were done, in order to answer additional questions from project partners.

Table 2

Planning of tests for prevention of biofilm formation.

		pH	EC	T	ATP		DNA		
		Water	Water	Water	Water	Biofilm	Water	Biofilm	
day	-1	Cleaning protocol							
day	0	x	x	x	x	x	x	x	Before startup of technologies
day	1								
day	2	x	x	x	x	x			
day	3								
day	4								
day	5								
day	6	x	x	x	x	x			
day	7								
day	8	x	x	x	x	x	x	x	

2.1.4.3 Removal of existing biofilm

To evaluate removal of existing biofilm from irrigation lines, test systems ran for six days before technologies were started. In this way, a sufficient amount of biofilm could be formed. At day 6, before technologies started, the amount of biofilm formed was measured. In Table 3 the general planning for these tests is shown.

Table 3

Schedule of tests for removal of an existing biofilm.

		pH	EC	T	ATP		DNA		
		Water	Water	Water	Water	Biofilm	Water	Biofilm	
day	-1	Cleaning protocol							
day	0	x	x	x	x	x			
day	1								
day	2								
day	3								
day	4								
day	5								
day	6	x	x	x	x	x	x	x	Before startup of technologies
day	7	x	x	x	x	x			
day	8								
day	9	x	x	x	x	x			
day	10	x	x	x	x	x	x	x	

2.1.5 Test schedule

All the technologies were evaluated at least twice on prevention of biofilm formation and on removal of an existing biofilm (if relevant for the respective technology). In each test run the technology was installed in a different test system, to minimise the effect of differences in biofilm formation in the consecutive systems.

Table 4 gives an overview of the performed test runs. Test -2 and test -1 were used to check the protocol, the reproducibility of results in the separate test systems (system variance) and successive test runs (testrun variance) and the effectiveness of the cleaning protocol.

Table 4

Test schedule.

		Start date	System 1	System 2	System 3
-2	Pre-test	2-4-2019	Control 1	Control 2	Control 3
-1	Pre-test	17-4-2019	Control 1	Control 2	Control 3
1	curative	28-5-2019	Electromagnetic waves	Silver stabilised H_2O_2	Control
2	curative	25-6-2019	Control	Ultrasonic waves	Electromagnetic waves
3	curative	30-7-2019	Silver stabilised H_2O_2	Control	Ultrasonic waves
4	preventive	27-8-2019	Control	Silver stabilised H_2O_2	Antibacterial irrigation lines*
5	preventive	24-9-2019	Ultrasonic waves	Control	Silver stabilised H_2O_2
6	preventive	7-10-2019	Control	Electromagnetic waves	Antibacterial irrigation lines
7	preventive	21-10-2019	Electromagnetic waves	ClO_2	Control
8	preventive	4-11-2019	Control	Ultrasonic waves	ClO_2
9	curative	18-11-2019	ClO_2	Control	Control extra sodium acetate
10	curative	2-12-2019	Antibacterial irrigation lines	ClO_2	Control

* After the testrun it became clear that material from the wrong batch was used, so that no anti-bacterial properties were implemented. Therefore, the results of this testrun are not taken into account for this technology.

Test runs 1, 2, 3, 9 and 10 were to evaluate removal of an existing biofilm (curative), whereas tests 4-8 were to evaluate prevention of biofilm formation. For the anti-bacterial pipelines test run 10 was also used to check the effectiveness of pipe material in the prevention of biofilm formation. This test run ran for 30 days, in order to have a longer time span to evaluate the technology.

In test run 9 an additional control treatment was added, to evaluate the effect of an increased concentration of sodium acetate (C-source, 80 µg C/L, eight times higher concentration than standard) on microbial growth.

2.2 Analyses

2.2.1 Sampling

2.2.1.1 Water

The following protocol for sampling of water was used:

- Switch off the system pumps.
- Write down time and meter readings from the water meters.
- Use a Bunsen burner to sterilise the sampling tap until droplets appear.
- Let water run from the tap to cool down the sampling tap.
- Take a water sample of 250 mL for each system in a sterile bottle.

2.2.1.2 Biofilm

The following protocol for sampling biofilm was used:

- Add 10 mL of sterile tap water to a test tube.
- Lower the water level in the biofilm monitor columns to underneath the cylinders to be harvested.
- Remove the cap of the column.
- Sterilise a stainless steel rod with a Bunsen burner and let it cool down in the air.
- Use this rod to take an LDPE ring from the column and to transfer the ring into the test tube. Be careful not to let the ring touch the wall of the column.
- Close the column by replacing the cap.
- Restart the pumps.

2.2.2 ATP

ATP (adenosine triphosphate) is a measure for the total biomass present in water or in biofilm and was chosen as a parameter to check the effectivity of technologies in the removal of an existing biofilm or prevention of biofilm formation. ATP is present in cell structures and can dissolve in water as cell walls are broken down. Luminultra Quench-Gone Aqueous technology was selected for measuring ATP (detection limit 0.1 pg/mL), as this method is capable of measuring ATP from living cells only. The LuminUltra method consisted of filtrating sample water through a filter and the filtrate was discarded. In this way, dissolved ATP from broken and dead cells was filtered from the samples before measurement. Subsequently, 1 mL LuminUltra UltraLyse was filtrated through the same filter and this filtrate was mixed with 9 mL LuminUltra UltraLute buffer. Then 100 µL of this solution was added to 100 µL LuminUltra Luminase enzyme and measured on the LuminUltra PhotonMaster.

The graphs in the results section are shown both on a logarithmic scale and a normal scale. KWR and WUR prefer to use the logarithmic scale, as it shows trends more than exact results, as uncertainties in microbial analyses can be relatively large and this is the normal way to present microbial data. The project partners prefer a normal scale, as these graphs are easier to read and understand.

2.2.2.1 Biofilm

The test tubes with the LDPE-rings in 10 mL of sterilised tap water were placed in an ultrasound water bath (Eumax, 40 kHz, 50 W) for 2 minutes. The water from the test tube was poured in a 50 mL sterile greiner CELLSTAR Centrifuge tube, the PE-ring remained in the test tube. The greiner tube was placed on ice for storage. Again 10 mL of water was pipetted in the test tube, placed for two minutes in an ultrasonic water bath and poured in the same greiner collection tube. These steps were repeated another two times, until 40 mL of water was collected in the same greiner tube. From the collected water, 20 mL was used for measuring ATP with the Luminultra QGA test kit. The PE-ring was stored for surface area measurement. The remaining 20 mL of replicates A and B were pooled in one of the sterile greiner tubes and stored on ice for transport to KWR for DNA/ NGS analysis.

2.2.2.2 Water

From the collected water sample of 250 mL, 50 mL was used for measurement of ATP from living cells with the Luminultra QGA test kit, according to the method described above. In the remaining water sample, EC and pH were determined. For DNA-analyses, an additional sample of 250 mL was taken. For determination of the concentration of H_2O_2 and ClO_2 additional water was taken from the system.

2.2.3 EC & pH

In the water remaining after ATP-analysis, EC and pH were measured with calibrated equipment to control the process.

2.2.4 H_2O_2

A separate sample (100 mL) was taken from the tap at the bottom of the water columns in the biofilm monitor during running time of the pump to measure the concentration of H_2O_2. Concentration of H_2O_2 was measured using Quantofix peroxid 0-25 mg/L.

2.2.5 ClO_2 and total oxidants

Concentration of ClO_2 and total oxidants were manually measured. For ClO_2 a Palintest ClO_2^+ meter was used (see Annex 5 for method, in Dutch). Total oxidants is the sum of ClO_2, chlorite and chlorate, the last one being a disinfection by-product of ClO_2. Method for measurement of total oxidants was supplied by CH2O B.V. and was a titration method (see Annex 4 for method, in Dutch).

2.2.6 Microbial community analyses

For the biofilms studied in our research, we were not only interested in the biomass (the amount of living matter present in the biofilm) but also in the identity of the bacteria present, and how this was affected by the different treatments. This information can be derived from the genetic material present in the bacteria (DNA). In our research, this was done by analysis of the variability of the 16S rRNA gene, a piece of genetic information (gene) which is present in all bacteria and can be used to assess the identity of bacteria. When knowing the identity of many species in a sample, the so-called microbial profile of this sample can be described. The microbial profile is as a fingerprint of a microbial community in a sample: each profile is unique, and yet some communities are more similar to each other than other ones. All water samples (250 mL) and biofilm samples (40 mL) were send from Bleiswijk to KWR in Nieuwegein on ice. The samples were filtered through a 0.22 µm membrane filter at KWR. After having added the appropriate buffers (MBL and FB), samples were stored at -20 °C. At a later time point, DNA from the filters was isolated using the DNeasy PowerBiofilm kit (Qiagen). The DNA isolation was carried out according to the manufacturer's protocol.

16S rRNA gene amplicons were generated using previously described primers 515F and 806R (containing Illumina adapter overhangs as described by Illumina) targeting the V4 hypervariable region of the 16S rRNA gene (Caporaso *et al.,* 2011). Amplicons were generated in duplicate by performing PCR reactions with 5 µl DNA in a reaction volume of 25 µL. Duplicate amplicon reactions were pooled, and 25 µL of this mixture was cleaned, indexed and sequenced as described in the Illumina MiSeq 16S metagenomic sequencing library preparation protocol (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf, June 2016). Amplicons were loaded on the Illumina MiSeq system at a final concentration of 4 pM and supplemented with 10% bacteriophage PhiX DNA (Illumina; San Diego USA) for control and to add diversity. Negative controls (DN/RNase free water) were included in every experiment to monitor the presence of contaminating DNA.

The DNA sequences from the Illumina MiSeq system are processed using the specialist computer program Qiime2 (Boleyn *et al.,* 2019). To describe the variety and variability of microorganisms (=biodiversity) within and between samples, different methods can be used. Alpha diversity describes the total number (absolute number) and/or relative abundance (percentage number) of different species present within samples. Alpha diversities can be the same in different samples, yet the identity of the species can be different. Therefore, we used the beta diversity to compare diversity between different samples. Beta diversity shows the difference between microbial communities from different environments, which in our study means the different treatments. Main focus in beta diversity is on the difference in taxonomic abundance profiles from different samples. To compare beta diversity, weighted UniFrac distances between the communities were compared, which were based on both identity and abundance information. These distances are first represented in a principal coordinate analysis (PCoA). PCoA is an ordination technique which places different samples as dots in a plane in different dimensions, and reflects the similarity of the biological communities. The distances were turned into points in space and closer samples are considered more similar and vice-versa. The alpha diversity and beta diversity analyses were performed with Qiime2. The PCoA plots were made for the beta-diversity, using the program R with the package phyloseq and is based on the Bray-Curtis distance.

To get an impression of the different bacterial classes present in the samples, the results were analysed through class taxonomical level barplots (Annex 7). Barplots are a representation of the relative abundances for each treatment (based on the averages of their replicates). Only classes >2% are shown in the graphs; classes with lower abundances were grouped together as 'others'.

2.2.7 Statistical analyses

Because the data on biomass over time may not always give a clear picture on the effectivity of different methods and strategies, we performed separate analyses on start and end points of the different experiments. Results of start and end of the same strategy (prevention or removal) of each method were combined across different experimental runs. Because of large differences in biofilm densities (ATP) and in order to homogenize variance, results were analysed by performing a t test on log-transformed values.

The closer the dots in PCoA analyses, the more similar the communities are, and vice versa. Additional analyses were done on these data in order to test the differences or similarities in beta diversity between certain treatment groups, using permutation analysis of variance (PERMANOVA). The permutational multivariate analysis of variance (PERMANOVA) is a nonparametric method to conduct multivariate ANOVA and test for differences between samples. PERMANOVA first tests whether there is a significant overall difference (is any treatment group significantly different from the others), followed by pairwise comparisons of the different treatments to each other. The same analysis tests which factors or their interactions contribute more for the differences in samples.

2.2.8 Greenhouse measurement

To see if the amount of biomass in biofilm in the test system is comparable to values in an actual greenhouse situation, measurements were done from irrigation lines in a greenhouse compartment of Wageningen University & Research in Bleiswijk growing gerbera's. The irrigation lines were in use for at least a year in the production of gerbera, it was not clear if this cultivation started with new irrigation lines or if cleaning was applied on beforehand. During the cultivation year, no measures were taken to remove a biofilm or prevent biofilm formation. Drain water was recirculated without disinfection.

Three rings in total were cut from the end of three separate irrigation lines after disinfection of the outside of the irrigation line with ethanol (70%). The rings were collected in separate erlenmeyers in 40 mL of sterile tap water. The rings in the erlenmeyers were treated in an ultrasonic bath to get the biomass in the sterile tap water. The water was collected from the erlenmeyer and stored on ice. Again 40 mL of water was added to the ring in the erlenmeyer and the ultrasonic treatment was repeated. This was done four times to make sure all the biomass was collected from the irrigation line, with in total 160 mL of sterile tap water. Afterwards, 50 mL of the sterile tap water was measured for ATP, according to the same method as in the test protocol.

2.3 Technologies that have been evaluated

2.3.1 Oxyl-PRO S (Cindro)

Oxyl-PRO S is a hydrogen peroxide formulation containing 50% H_2O_2, stabilised with a coating of silver nanoparticles. Oxyl-PRO S (https://toelatingen.ctgb.nl/nl/authorisations/13251) was added to the nutrient solution to remove biofilm from irrigation lines with 40 mg/L (20 mg/L H_2O_2). For this test, Oxyl-PRO S was added continuously to the recirculating nutrient solution. Dosing was based on pulse measurement of water passing a water meter. Every 0.5 L of water passing a water meter created an electronic pulse, after 8 electronic pulses the dosage pump injected a pulse of Oxyl-PRO S. After start-up of the system, the concentration of hydrogen peroxide was frequently determined manually, aiming to reach a value of 20 mg/L. Following these measurements, the dosing pump was switched off at too high concentrations and switched on when H_2O_2 concentrations dropped below this value.

To reach the end of the dripper line with this concentration in a commercial greenhouse, a higher concentration needs to be applied. The last dripper in line will only receive this concentration of hydrogen peroxide if nutrient solution is applied to the crop, as dripper lines normally have a dead end. Therefore, the root zone at the first drippers in line is also subject to these concentrations of hydrogen peroxide, which is a potential risk for damage to the plant roots. It was not part of this research to investigate the effect on the crop. This could be overcome by application of a circular irrigation line, in which the applied solution can be circulated through the irrigation lines without opening of the drippers to the plants. In this way, the irrigation lines could be cleaned and the solution in the irrigation lines replaced by a nutrient solution before application to the crop. For example, application of this principle before the first irrigation of the day. In commercial greenhouses, the product is applied in a low concentration to slowly remove an existing biofilm layer by layer. The concentration is monitored continuously to make sure the biofilm is not removed in large parts, thereby preventing clogging of drippers or sprinklers.

H_2O_2 reacts with organic matter to water molecules, which could be taken up by the crop. For zero emission irrigation systems, this would be perfect. However, the silver nanoparticles in the Oxyl-PRO S commercial product could potentially accumulate in the irrigation water or in the crop in closed irrigation systems (not a subject in this research). The faith and the effect of silver nanoparticles on the plant are yet unknown.

2.3.2 SureFlow ClO_2 (CH2O B.V.)

Chlorine dioxide (ClO_2) is produced on site by combining hydrochloric acid and sodium chlorite by the SureFlow technology of CH2O and is added to nutrient solution to remove biofilm from irrigation lines (https://toelatingen.ctgb.nl/nl/authorisations/15238). In this test, a dosing pump was connected to the biofilm system to inject ClO_2 during runtime of the system pump, based on pulses from a pulse generating water meter. After start-up of the technology, the concentration of ClO_2 and total oxidants (remaining chlorite and reaction product chlorate) were measured, as well as pH, as acid was added during the process. If necessary, Baskal (YaraTera Substrafeed Baskal, mixture of 20-25% potassium carbonate and 15-20% potassium hydroxide) was added during regular measurements to stabilise pH of the solution. During the testruns it was aimed to keep the concentration of ClO_2 at 0.75 mg/L. When this concentration was reached, dosing was temporarily stopped. The same as for stabilised hydrogen peroxide, the applied concentration of chlorine dioxide could be harmful to the rootzone of the crops.

Chlorine dioxide is often confused with sodium hypochlorite, although it has completely different reaction pathways and disinfection effectiveness (not part of this research). Reaction by-products from sodium hypochlorite include perchlorate and trihalomethanes, which are not formed from chlorine dioxide reactions. Chlorinated rest-products are reported to be few for chlorine dioxide, potentially the unreacted chlorite and reaction product chlorate can still be formed by the reaction with organic matter in the nutrient solution, of which the effects on the system are insufficiently known. Recently, a new MRL (maximum residue limit) was reported for chlorate in food products (https://ec.europa.eu/food/plant/pesticides/chlorate_en). This could become a problem in completely closed irrigation systems, but was not part of this research. Also small amounts of sodium are added to the irrigation system by this technology.

2.3.3 Antibacterial irrigation lines (MVP-Starmaker)

The antibacterial irrigation lines from MVP-Starmaker can be a replacement of regular irrigation lines. The antibacterial irrigation lines are produced from regular LDPE-material, to which a zinc-additive is added during the production process. This makes sure the antibacterial effect is present throughout the entire material, so that if the material is damaged due to piercing of the drippers the antibacterial effect will still be present. The material is supposed to kill bacteria when they make contact with the material. As this technology is not meant to remove an existing biofilm, it was only tested during preventive tests. In the preparation of a test run, the conventional irrigation line of 50 m was replaced with the antibacterial pipeline and was cleaned together with the rest of the system. We did not receive single layer material to create rings to put in the biofilm monitor. As only the inner layer of these irrigation lines have antibacterial properties (just like normal irrigation lines, these lines consist of two layers of material), it was not possible to put antibacterial rings in the biofilm monitor. To measure the amount of biofilm on the material, a different protocol was followed:

- The outside of a part of the 50 m irrigation line was disinfected with ethanol (70%) and allowed to evaporate.
- Around 2 cm of irrigation line was cut off in twofold with a sterilised knife.
- These rings were treated in the same way as the PE-rings for the other technologies. In calculating the amount of ATP/cm^2 only the inner surface area of the ring was used, as the outside of the material was sterilised with ethanol (70%).

2.3.4 AQUA4D® (Planet Horizons Technologies)

The AQUA4D® technology generates very low-frequency electromagnetic resonance fields to treat the nutrient solution, to change the structure of water and the behaviour of nutrients and organic matter. AQUA4D® is a physical water treatment technology based on quantum physics and electrodynamics. The Aqua-4D® F-A OO system used in this study is a set composed of two modules:

- Command 30F: The Command 30F is the "brain" of the system. It generates electrical signals of Very Low Frequency (a few kHz) and very low intensity
- TU 60 G-A: The treatment unit TU 60G-A converts the electrical signals generated by the Command 30F to electromagnetic resonance fields (< 20 mT) which are spread within the water.

This should lead to an improved dissolution and hydration of minerals and organic matter, which should prevent biofilm formation and remove existing biofilms in irrigation lines. The AQUA4D® Treatment Unit is implemented right behind the system pump as a flow-through pipe. The Treatment Unit is in hydraulic continuity with the biofilm monitor that means that the water vein is not interrupted between both. Next to that, the biofilm production system is grounded and equipment is installed to get rid of stray currents. From the starting moment of the test of the technology, the AQUA4D® system is switched on until the end of the test run. The technology is not meant to disinfect the water, so no effect on the amount of biomass in water is to be expected.

2.3.5 Ultrasound (AWWS)

The low frequency (25 kHz) Ultrasound waves originating from the SonoPure will affect the water and biofilm in two ways. Ultrasound can have a direct impact on the removal of biofilm due to small shockwaves that propagate through the pipelines. Small cavitation bubbles will collapse in the vicinity of the biofilm and will create small jets that may shear off the biofilm from the pipelines. Ultrasound may also have an indirect effect on biofilm formation: due to electromagnetic activity of the ultrasonic transducer, the formation of strong calcium-rich deposits may be suppressed. Biofilm is using strongly bound calcium deposits to structure themselves and to stick at the pipelines surface. Inhibiting the formation of these strong calcium structures may make the biofilm more susceptible for removal using the ultrasound. These two effects may strengthen the effect of Ultrasound to effectively remove biofilm inside pipelines.

3 Results

3.1 Protocol checks

3.1.1 System x testrun variance

To investigate the possible effects of different starting conditions across the different test systems, and across the different experimental runs, we compared the initial biofilm densities for all these conditions (Figure 3). As can be seen in this Figure, large differences could occur in initial biofilm densities between runs, but less between systems (within runs).

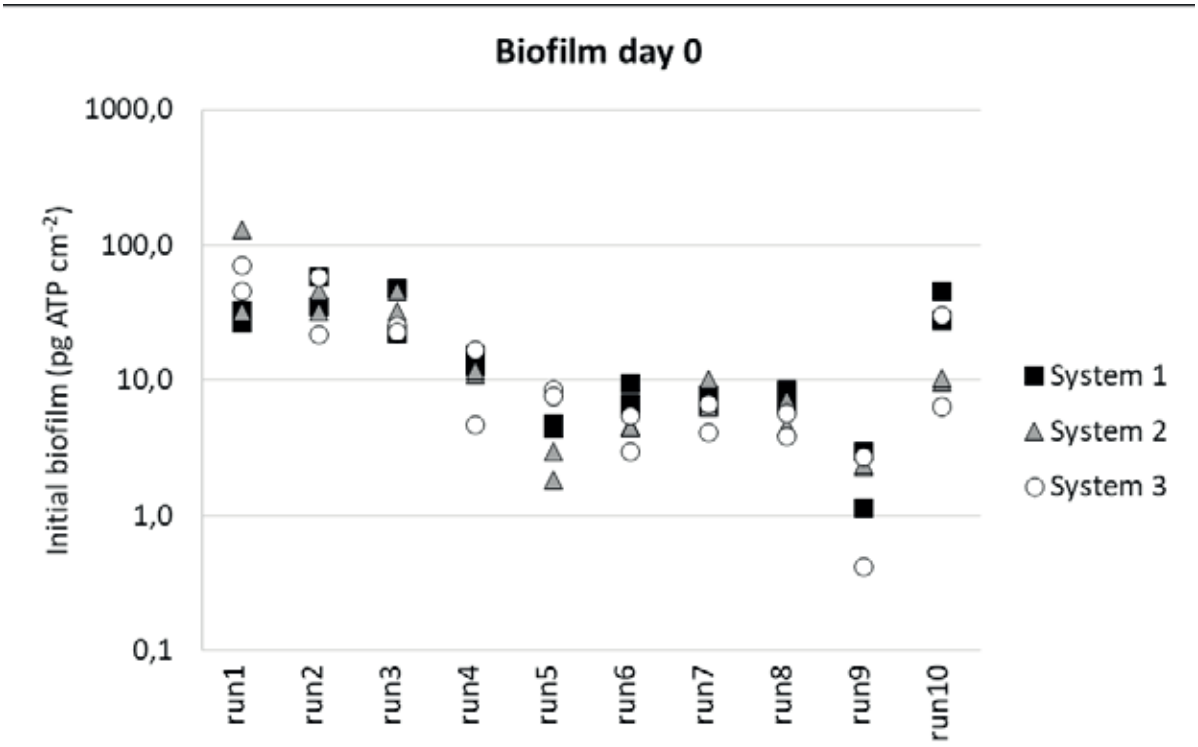


Figure 3 *Initial biofilm densities for the different test runs investigated, and for the different test systems.*

To further analyse these results we ran a two-way analysis of variance (ANOVA) on the initial biofilm densities (Table 5). The results confirm that there is a significant test run effect ($p<0.001$) but no significant system effect, nor a significant interaction between test run and system.

Table 5

Results of the two-way ANOVA on the effects of test run and test system on the initial biofilm density. Given are the sums of squares (SS), the degrees freedom (df), the mean square (MSS), the value of the F-test (F) and the corresponding probability (p-value).

Source	SS	df	MSS	F	p value
Testrun	18652.86	9	2072.54	8.700	<0.001
System	84.31	2	42.15	0.177	0.839
Testrun*System	3694.47	18	205.25	0.861	0.622
Error	7146.98	30	238.23		
Total	29578.61	59			

In order to test the effects of different methods on biofilm removal, a biofilm was allowed to develop during the first 6 days. Therefore, for the analysis of the removal strategies, the actual tests started on the 6th day of the test run. We also investigated the starting conditions of these tests (Figure 4).

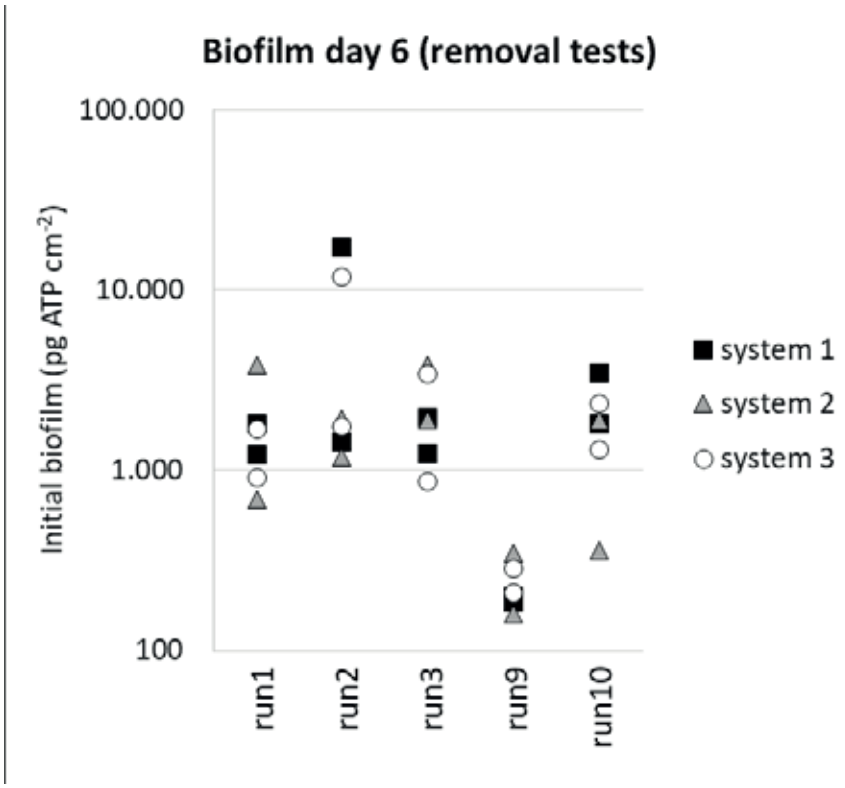


Figure 4 *Initial biofilm densities for the different test runs that investigated biofilm removal, for the different test systems.*

As biofilms have been growing for 6 days, there is a higher probability to obtain differences between the replicate tubes within a system, or between the different systems within one run. As can be seen in Figure 4, sometimes large differences can occur within runs, and (again) also between runs.
To further analyse these results we ran a two-way analysis of variance (ANOVA) on the initial biofilm densities on day 6 of the removal tests (Table 6).

Table 6

Results of the two-way ANOVA on the effects of test run and test system on the initial biofilm density in the removal tests. Given are the sums of squares (SS), the degrees freedom (df), the mean square (MSS), the value of the F-test (F) and the corresponding probability (p-value).

Source	SS	df	MSS	F	p value
Testrun	105293502	4	26323376	2.106	0.131
System	10496791	2	5248395	0.420	0.665
Testrun*System	56466374	8	7058297	0.565	0.791
Error	187528732	15	12501915		
Total	359785398	29			

Despite the large differences between individual runs, no significant test run effect was found for the initial biofilm density in the removal tests; neither was there a significant test system effect or significant interaction ($p < 0.05$).

In all types of tests applied, the initial differences between systems were always smaller than the differences between individual runs. This makes sense because the different systems have been designed to be similar, whereas different runs over time are very unlikely to have the same initial values over and over again, due to factors such as temperature, tap/rain water composition, etc.

3.1.2 Effect of carbon source

Sodium acetate was added to the water as additional carbon source for micro-organisms to grow. To see the effect of the chosen concentration, during test run 9 one treatment received an eightfold concentration (80 µg C/L) of sodium acetate, whereas the control had normal sodium acetate levels (10 µg C/L). The results of this experiment are shown in Figure 5. Additional sodium acetate as carbon source did not seem to result in a higher level of biofilm formation, so biofilm growth inhibition after day 6 is not caused by a lack of carbon. Because biofilm growth did not increase very much after day 6, this day was chosen as starting day for the removal tests.

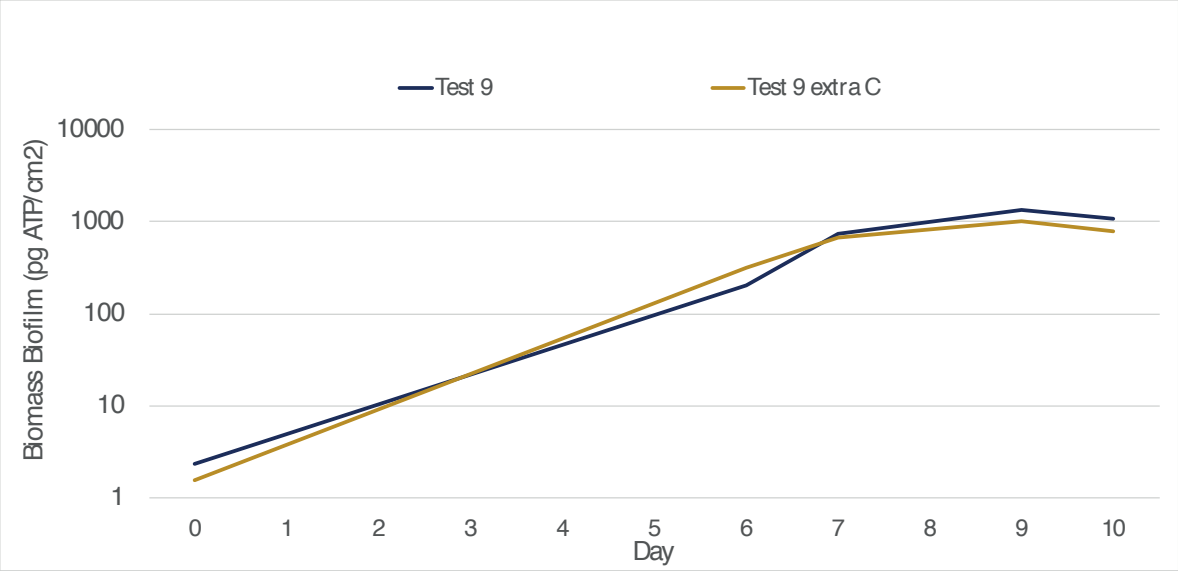


Figure 5 *Biomass of biofilm (pg ATP/cm²) in control treatments of test run 9: comparison of biofilm formation with normal and eight fold dosage of sodium acetate as carbon source.*

3.1.3 Level of biofilm in greenhouse

To compare the final levels of biofilm in the test system with an irrigation line in an actual greenhouse, the biofilm from an irrigation line in a gerbera greenhouse was measured. The average of the three measurements of biomass from biofilm from the greenhouse was 71 pg ATP/cm² of irrigation line, compared to an initial biomass of around 1000 pg ATP/cm² in the test system at day 6 (Figure 4).

3.2 Chemical treatment

3.2.1 Oxyl-PRO S

3.2.1.1 Prevention of biofilm formation

In Figure 6 the results of the testruns for prevention of biofilm formation with the use of Oxyl-PRO S (silver-stabilized hydrogen peroxide) are shown. In both test runs, during the first three days the concentration of biomass in the biofilm (200 pg ATP/cm²), as well as in the water (750 pg ATP/cm²) were comparable to the control treatment. This can be explained by the low concentration of H_2O_2 (Figure 8) during the first couple of days. After day 3, the concentration of H_2O_2 increased to the expected value (20 mg/L) and immediately the biomass in the system approached 0 pg ATP/cm². This shows that a certain minimum concentration of H_2O_2 is required to have an effect on the biomass in the water and in the biofilm. At a concentration of 20 mg/L, H_2O_2 is capable of reducing biomass in water and prevention of biofilm formation.

A low concentration of H_2O_2 during the first two days can possibly be explained by the way of dosing. At the start of the test run, the concentration of H_2O_2 in the 300 L of nutrient solution was 0 mg/L. Dosing took place in a short pulse after each 4 L passing the dosing point, so with long pauses in between, without proper mixing in the nutrient solution. By using it in this way it took quite some time to dose H_2O_2 to the entire volume of water in the system. In the meantime, H_2O_2 reacted with the organic matter in the water, so that the concentration could not increase. Probably it took a while before the concentration of H_2O_2 increased to a level of 20 mg/L.

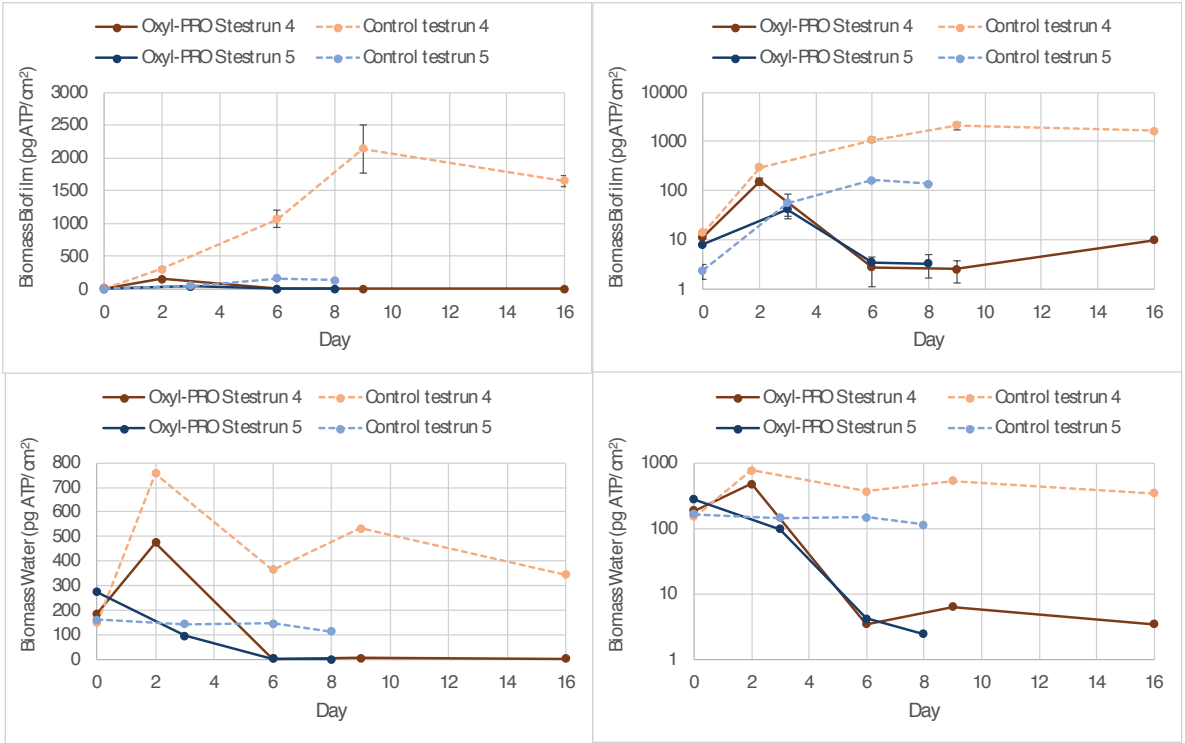


Figure 6 *Microbial biomass in hydrogen peroxide treatments (Oxyl-PRO S) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 4 and 5 to prevent biofilm formation in clean irrigation lines.*

Initial biomass densities in biofilm were not significantly different between the control and H_2O_2 condition (left side of Figure 7) (control: 8.18 ± 6.76 pg ATP cm^{-2}; treatment: 9.75 ± 2.02 pg ATP cm^{-2}; $t(6) = 0.97$, $p = 0.36$), but final densities differed significantly (control: 1139 ± 1172 pg ATP cm^{-2}; treatment: 2.98 ± 1.30 pg ATP cm^{-2}; $t(6) = 6.46$, $p=<0.001$). This showed Oxyl-PRO S was capable to prevent the formation of biofilm in irrigation lines with a concentration of 20 mg/L H_2O_2 (dosing 40 mg/L Oxyl-PRO S).

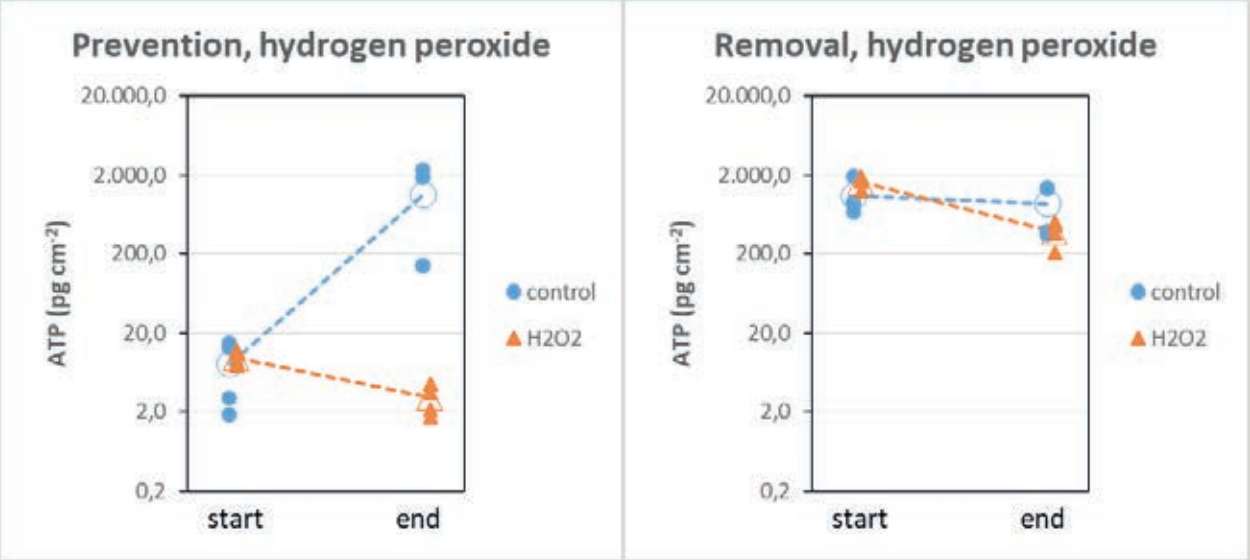


Figure 7 *Results on initial (start) and final (end) biomass densities in biofilm for hydrogen peroxide (Oxyl-PRO S) and the controls for biofilm prevention (left) and removal of existing biofilm (right). Open symbols connected by dashed lines are the average values.*

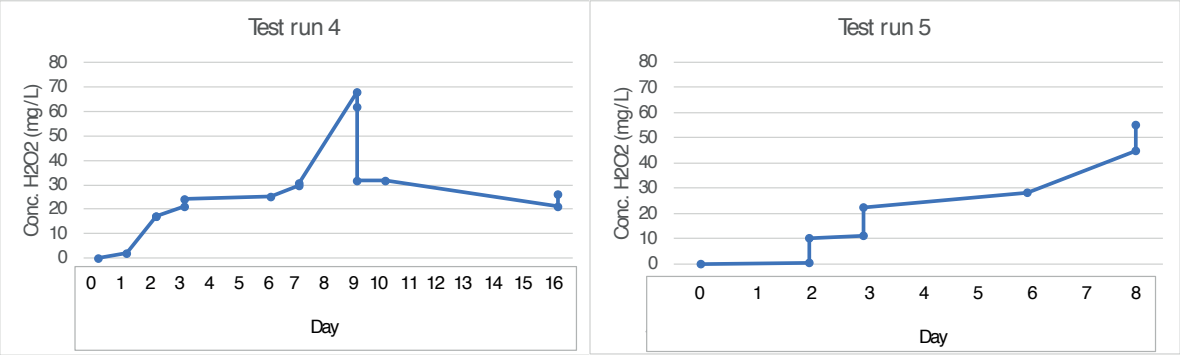


Figure 8 *Concentration of H_2O_2 during test runs 4 and 5. Multiple data points in one day represent measurements at different times.*

With Oxyl-PRO S, the initial microbial profiles for control and treatment were similar at the start, but different between runs (Figure 9). The final Oxyl-PRO S profiles were quite similar, whereas one control was also quite similar to these treatments in the end, but the control from the other run was very different. The PERMANOVA did not reveal any significant results (Annex 6).

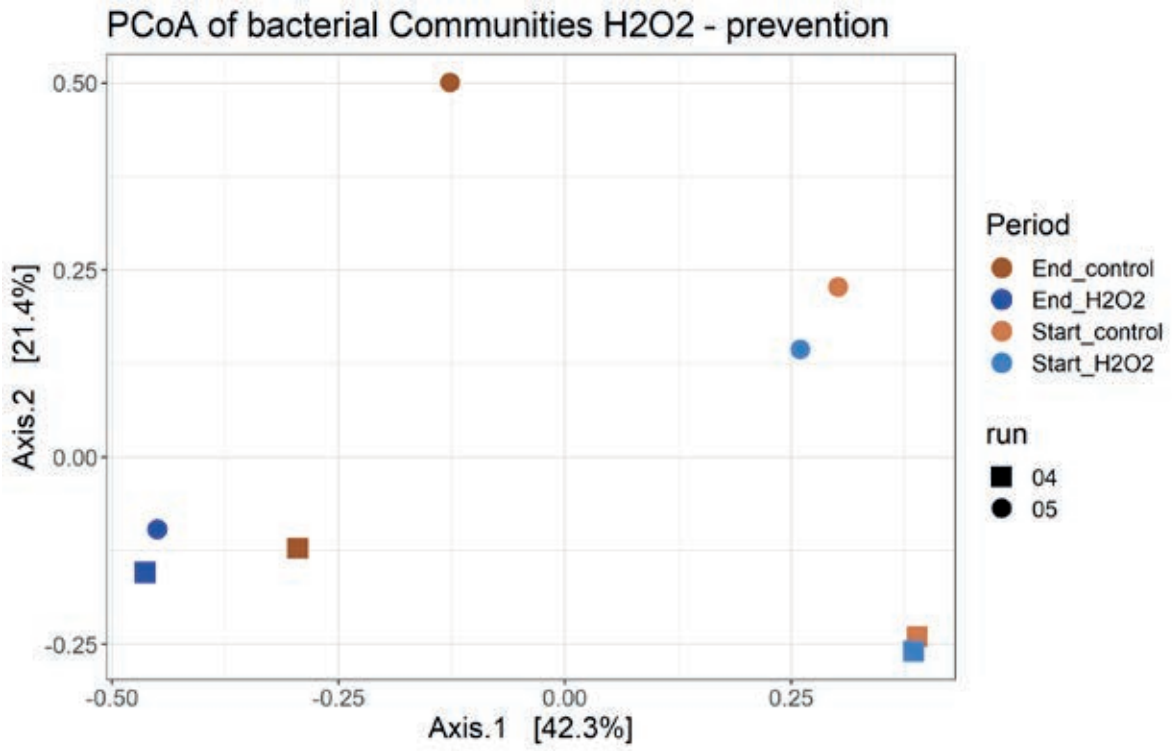


Figure 9 *Results of the principal coordinate analysis (PCoA) for Oxyl-PRO S, prevention.*

3.2.1.2 Removal of existing biofilm

Figure 10 shows the effect of Oxyl-PRO S in removal of an existing biofilm. After start-up of the dosing of Oxyl-PRO S (concentration of H_2O_2 in Figure 11), biomass in water decreased immediately after start of the method. The Figure also shows that it takes a few days before the oxidizing effect of H_2O_2 on the biofilms becomes visible after the method starts. In testrun 1 the absolute difference in biomass in biofilm between the control and the treated system is small; treatment shows a decrease from day 7 – day 10, a sharp decrease in the control at day 9 equalises treatment and control, causing no significance in difference. In test run 1 and in test run 3, after start-up of the technology the concentration of biomass in biofilm clearly decreases. The different bacterial strains that were found in the biofilm can be seen in Annex 7. Treatment had a clear effect on biomass in water.

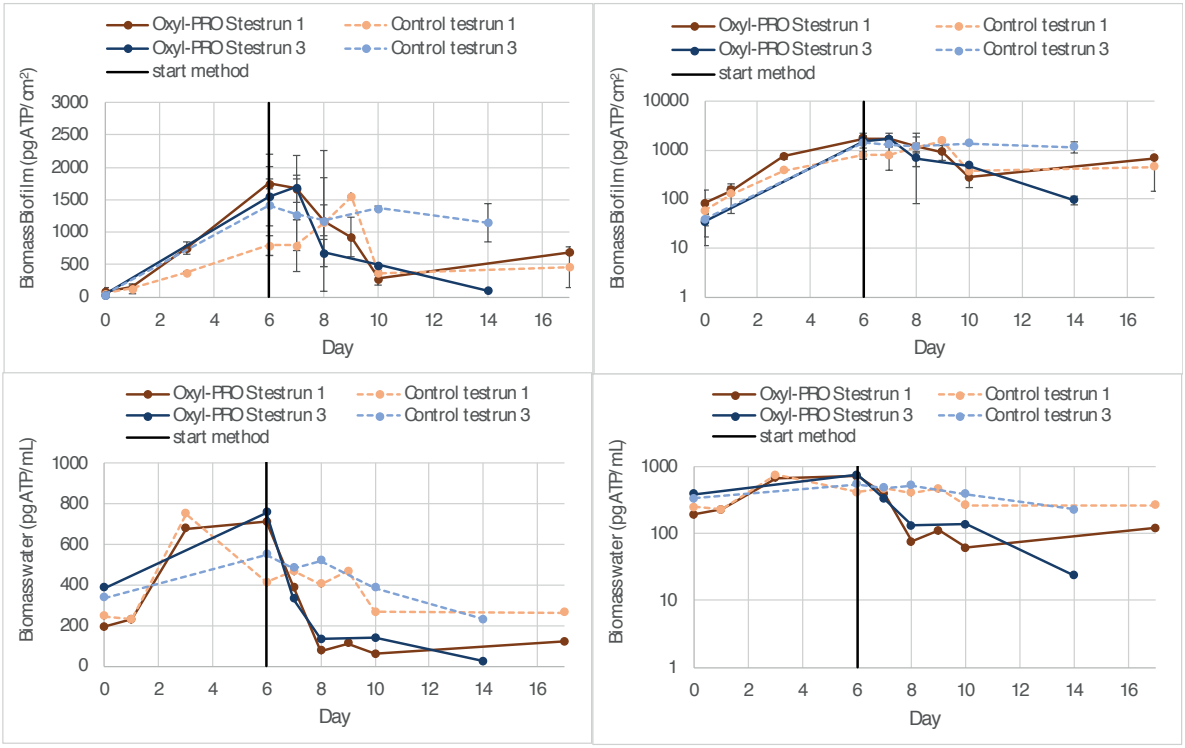


Figure 10 *Microbial biomass in hydrogen peroxide treatments (Oxyl-PRO S) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 1 and 3 to remove an existing biofilm from irrigation lines.*

Initial biomass densities were not significantly different, as is shown on the right side of Figure 7 (control: 1106 ± 582 pg ATP cm^{-2}; treatment: 1649 ± 293 pg ATP cm^{-2}; $t(6) = 1.90$, $p = 0.106$), and neither were final densities (control: 870 ± 576 pg ATP cm^{-2}; treatment: 384 ± 134 pg ATP cm^{-2}; $t(6) = 1.57$, $p = 0.167$).

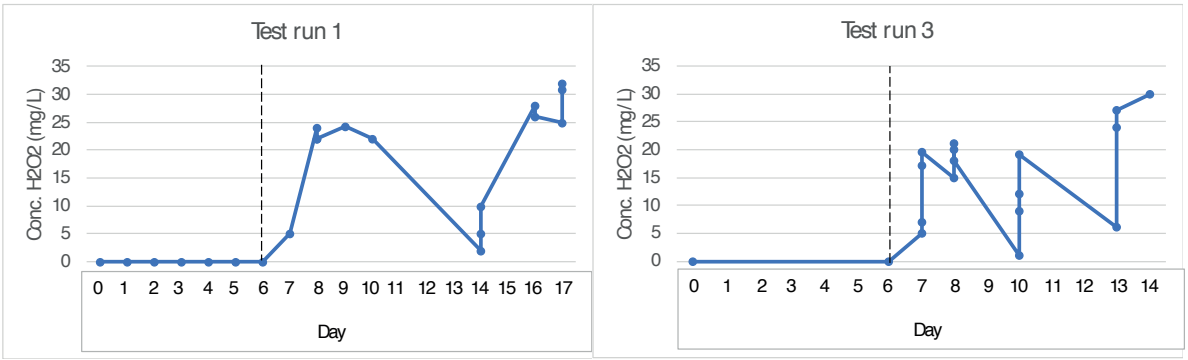


Figure 11 *Concentration of H_2O_2 during test runs 1 and 3. The dotted lines present the starting date of dosing Oxyl-PRO S. Multiple data points in one day represent measurements at different times.*

The microbial composition of treatments and controls for both runs hardly change in the course of time (Figure 12). The initial communities were already similar in run 1, whereas control and treatment were different during run 3, and remained so during the experiment. Not surprisingly, the PERMANOVA did not reveal any significant differences (Annex 6). The biomass composition can be found in Annex 7.

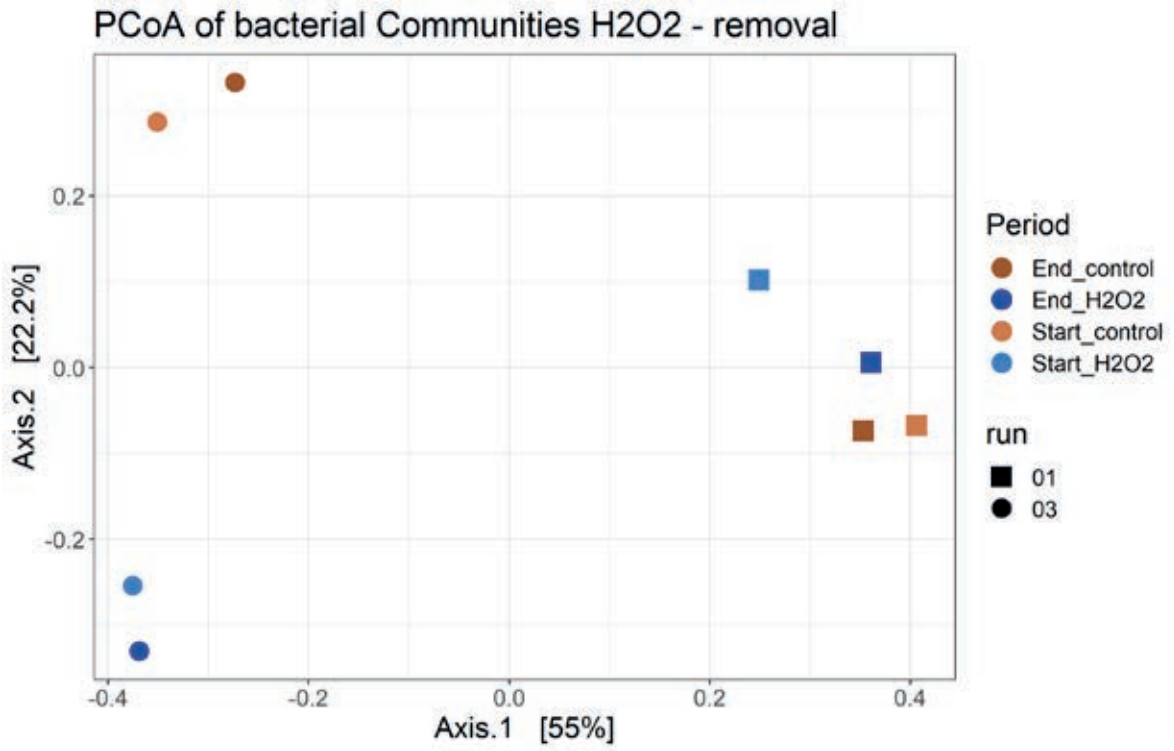


Figure 12 *Results of the principal coordinate analysis (PCoA) for Oxyl-PRO S, removal.*

3.2.2 SureFlow ClO_2

3.2.2.1 Prevention of biofilm formation

In Figure 13 the results are shown for the application of SureFlow chlorine dioxide for prevention of biofilm formation in test runs 7 and 8, compared to the controls. The controls showed a normal biofilm development, whereas treatment with ClO_2 almost completely prevented biomass formation, even at very low concentrations (Figure 15). In test run 7, after day 3 the concentration of ClO_2 in the water increased rapidly during the weekend (3 mg/L). However, even in the first three days, a low concentration of ClO_2 (<0.5 mg/L) showed a low biomass concentration. In testrun 8, chlorine dioxide concentrations also remained <0.5 mg/L and therefore lower than concentrations as advised in commercial greenhouses (0.7 - 0.9 mg/L). Also biomass in water was rapidly removed after dosage of ClO_2.

The concentration of total oxidants (ClO_2, chlorite and other reactive elements formed during the production and reaction of ClO_2) increases rapidly. In practice these reactive elements react with the organic matter in the substrate and rootzone or within the water disinfection installation and are thus removed from the recirculating nutrient solution. In the test system, there is neither a substrate and rootzone, nor a disinfection unit, so these reactive species remain in the system. In this way they will have an effect on biomass in biofilm and water, but there was no way to remove them from the test system without affection biomass formation. The concentration of total oxidants in practice is not known, but is expected to be much lower than in the test system.

Apart from that, an unforeseen effect of the pH of the nutrient solution occurred: low amounts of acid were dosed during dosing of chlorine dioxide, which significantly lowered pH (pH = 3). In this low volume closed loop system without automatic pH control, this led to a decrease in pH of the nutrient solution. This was observed during test run 7, and during test run 8 the pH was repetitively measured and corrected by adding Baskal (mixture of potassium carbonate and potassium hydroxide).

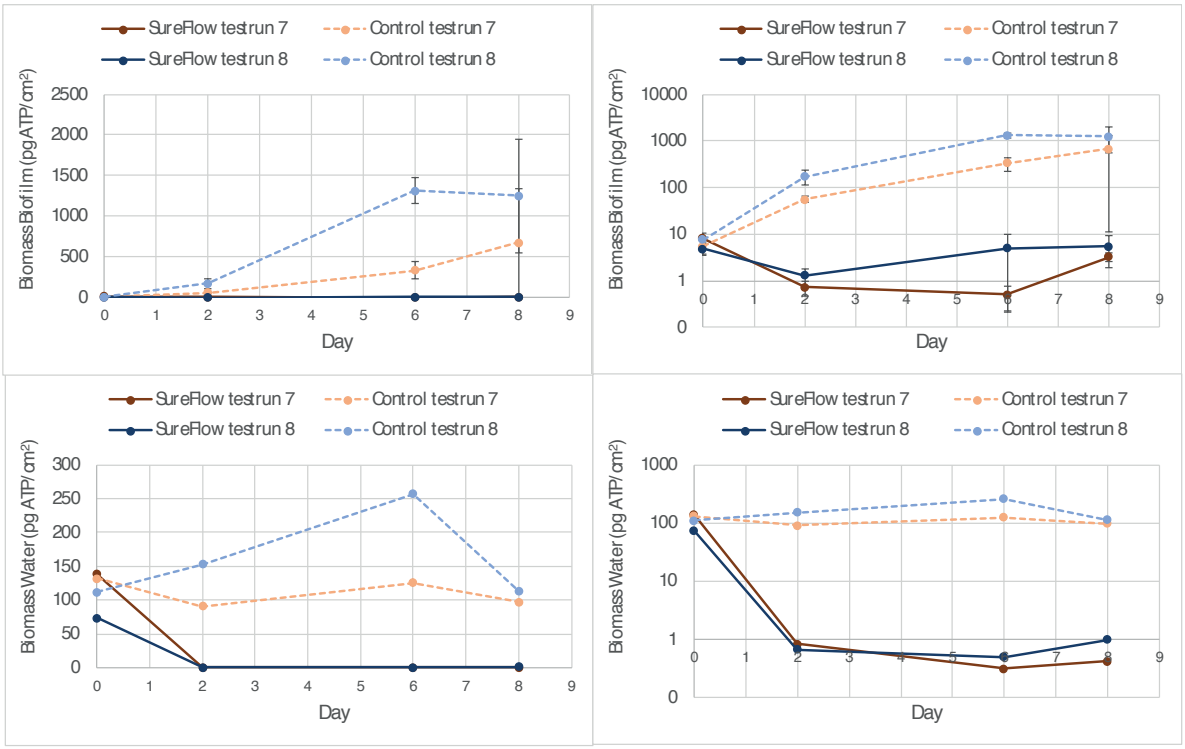


Figure 13 *Microbial biomass in chlorine dioxide treatments (SureFlow) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 7 and 8 to prevent biofilm formation in clean irrigation lines.*

For SureFlow chlorine dioxide, the summarising statistical results are presented in Figure 14. Initial biomass densities were not significantly different (control: 6.54 ± 1.81 pg ATP cm^{-2}; treatment: 6.53 ± 2.56 pg ATP cm^{-2}; $t(6) = 0.10$, $p = 0.92$), but final densities differed significantly (control: 961 ± 646 pg ATP cm^{-2} ; treatment: 4.38 ± 2.46 pg ATP cm^{-2}; $t(6) = 10.0$, $p<0.001$).

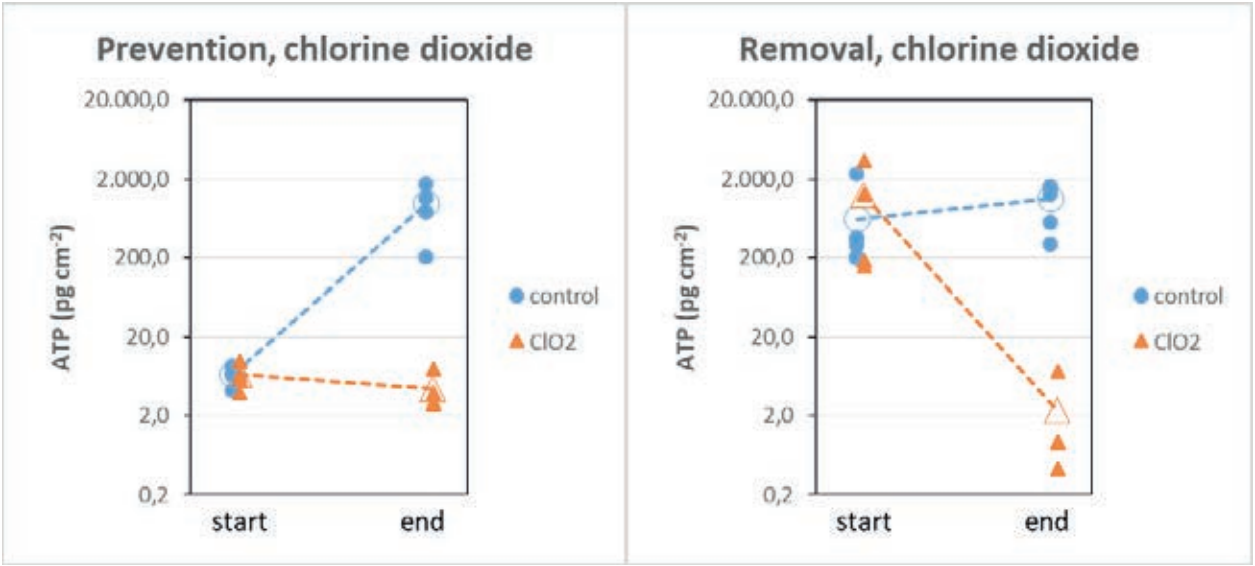


Figure 14 *Results on initial (start) and final (end) biomass densities in biofilm for chlorine dioxide (ClO_2) and the controls for biofilm prevention (left) and removal of existing biofilm (right). Open symbols connected by dashed lines are the average values.*

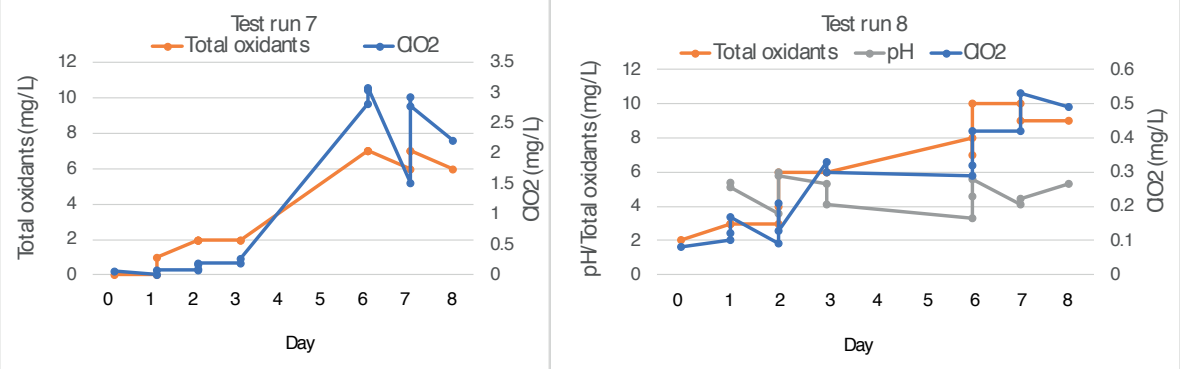


Figure 15 *Concentration of chlorine dioxide (mg/L), concentration of total oxidants (mg/L) and pH during test runs 7 and 8.*

The results of the PCoA for chlorine dioxide, prevention, are shown in Figure 16. The initial differences between different runs are larger than between controls and treatments (lighter coloured symbols). At the end of these runs, both controls (dark blue) cluster in one area of the plot, as well as both treatments (dark brown). This suggests a clear chlorine dioxide treatment effect on the microbial profiles. The PERMANOVA shows a trend (close to but not significant; Annex 6: p=0.066). Pairwise comparisons after that did not show any significant effects (Annex 6). The composition of the biomass can be found in Annex 7.

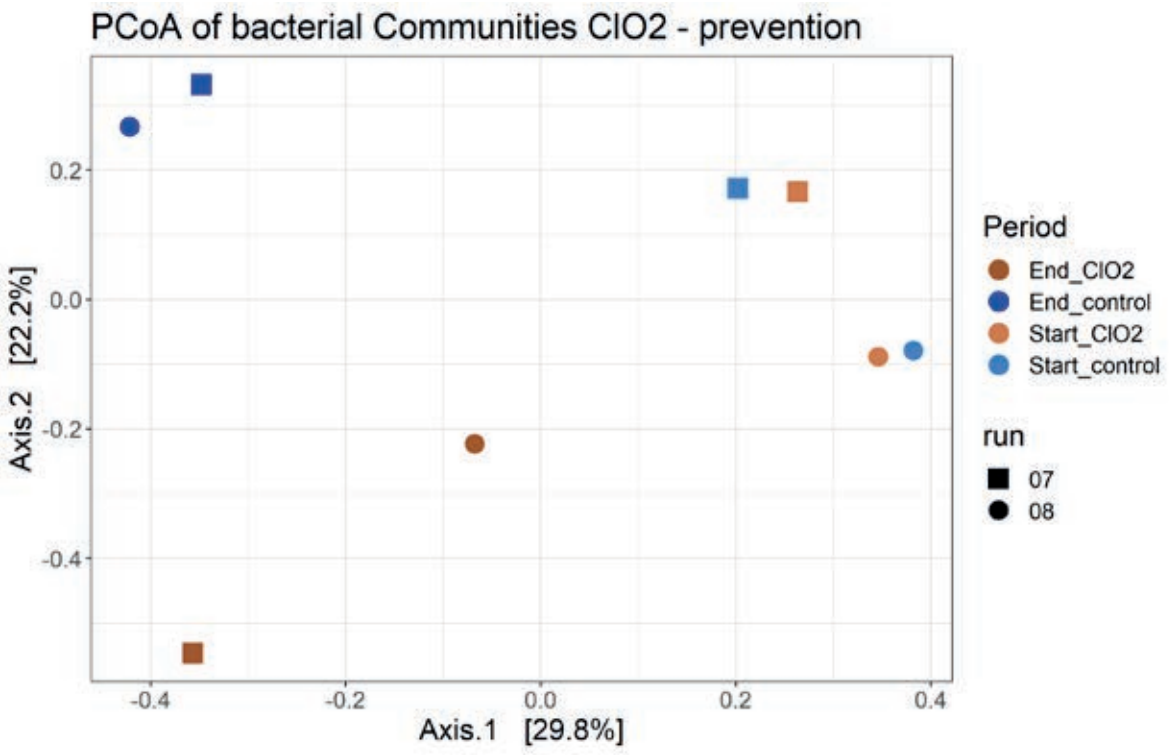


Figure 16 *Results of the principal coordinate analysis (PCoA) for chlorine dioxide, prevention.*

3.2.2.2 Removal of existing biofilm

Figure 17 shows the results of the removal of an existing biofilm by application of chlorine dioxide (SureFlow). Biomass formation during the first six days of both testruns showed a normal growth pattern. Biomass both in biofilm and in water decreased to almost zero directly after start of the method, while concentration of chlorine dioxide remained <0.75 mg/L (no graph, measured data was lost by technical failure).

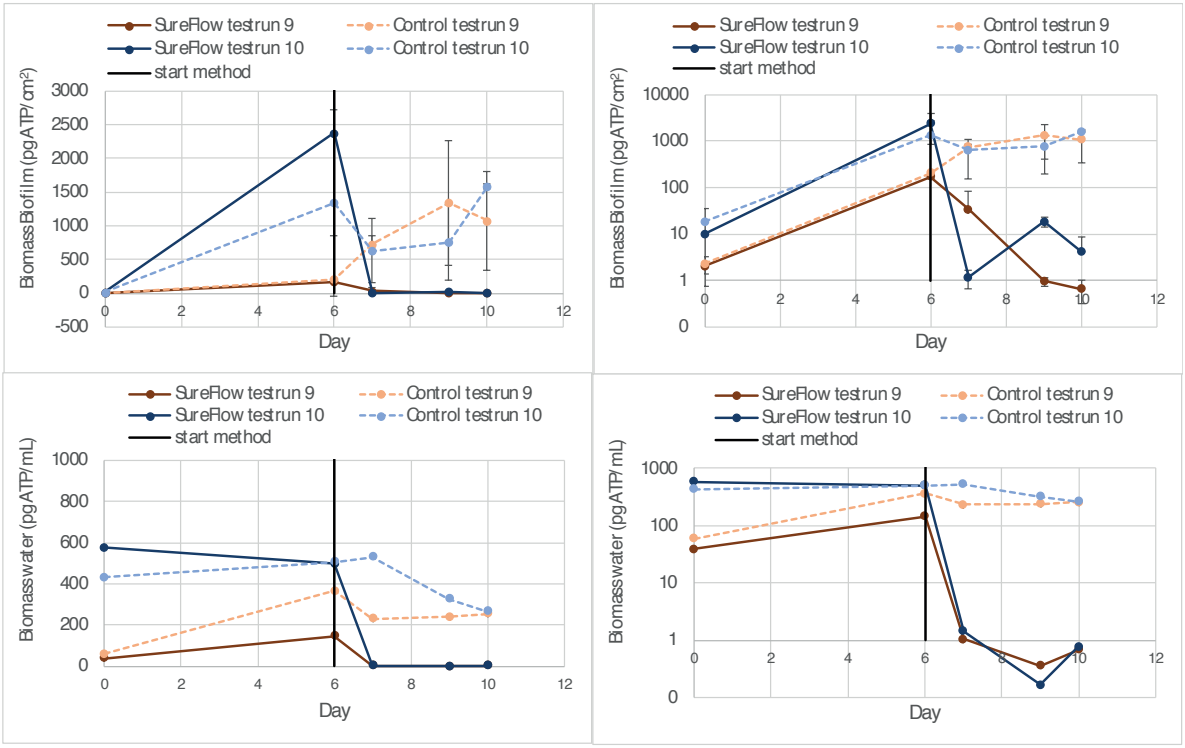


Figure 17 *Microbial biomass in chlorine dioxide treatments (SureFlow) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 9 and 10 to remove an existing biofilm from irrigation lines.*

Initial biomass densities were not significantly different (control: 619 ± 836 pg ATP cm^{-2}; treatment: 1257 ± 1546 pg ATP cm^{-2}; $t(6) = 0.10$, $p = 0.92$), but final densities differed significantly (control: 1149 ± 577 pg ATP cm^{-2} ; treatment: 2.37 ± 3.24 pg ATP cm^{-2}; $t(6) = 11.1$, $p<0.001$). Chlorine dioxide has shown to be effective in the removal of an existing biofilm.

Figure 18 shows the PCoA results for chlorine dioxide, removal. In run 9, treatment and controls initially cluster, but the ClO_2 treatment appeared to have an effect. In run 10 there are initial differences between control and treatment which remain throughout the experimental run. This suggests a variable treatment effect on the microbial profiles. The PERMANOVA did not reveal any significant results (Annex 6). The biomass composition can be found in Annex 7.

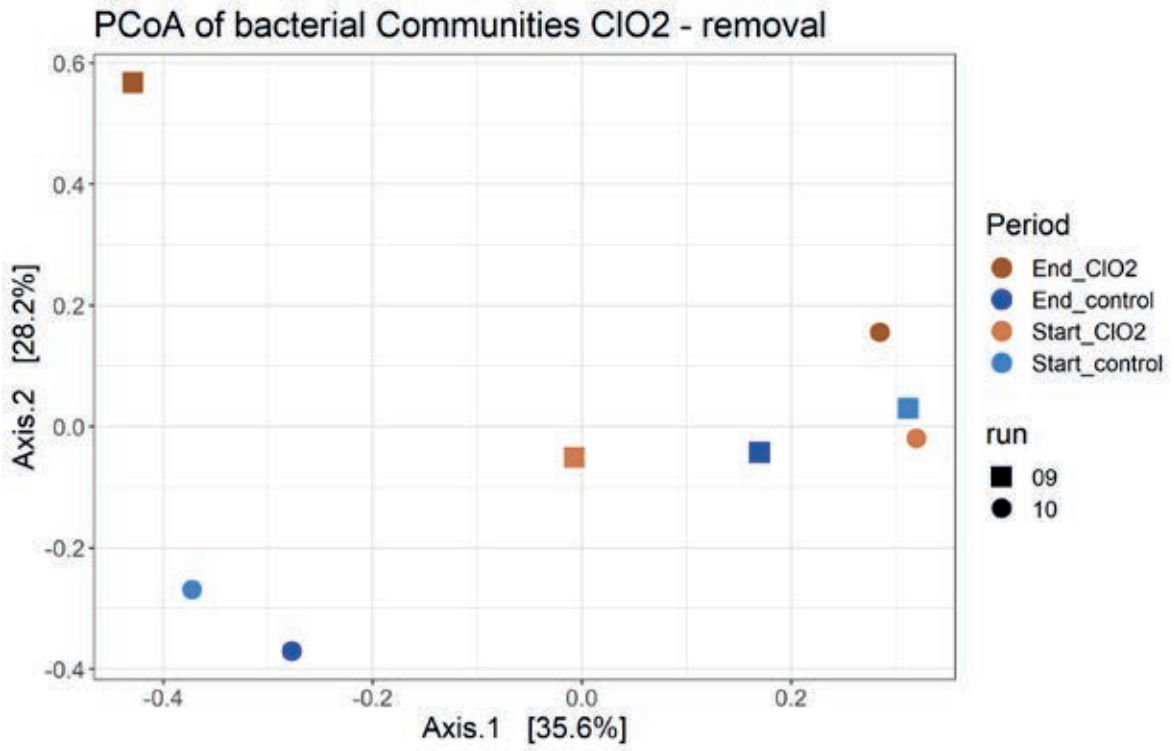


Figure 18 *Results of the principal coordinate analysis (PCoA) for chlorine dioxide, removal.*

3.2.3 Antibacterial irrigation lines

3.2.3.1 Prevention of biofilm formation

The effect of the antibacterial irrigation line on the formation of biofilm and micro-organisms in the water are shown in Figure 19. In both testruns, the trend for the amount of biomass in biofilm is equal for the treatment and the control. It seems from these results that a biofilm is forming in the antibacterial irrigation line as well. After consultation it was hypothesized that effects would appear after a longer time period, therefore testrun 10 has been elongated to 30 days. However, there does not seem to be an effect after a longer treatment period.

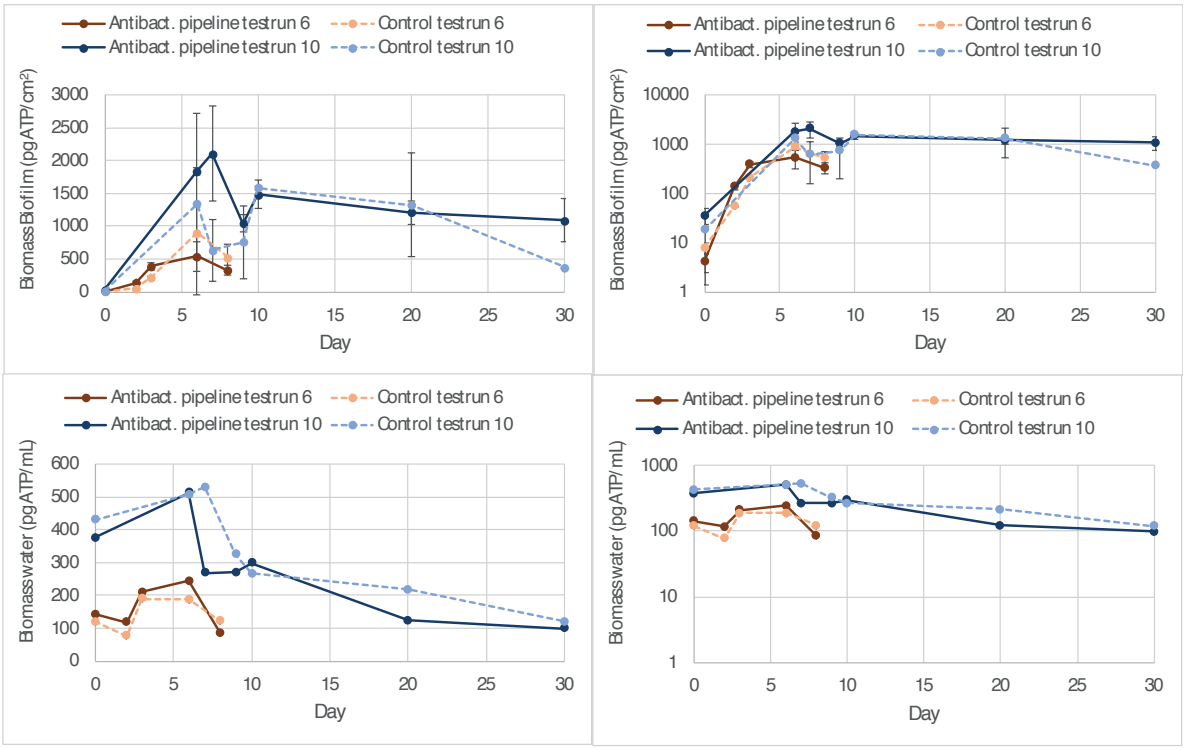


Figure 19 *Microbial biomass in antibacterial irrigation line treatments and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 6 and 10 to prevent biofilm formation in clean irrigation lines.*

For the antibacterial pipelines, the summarising statistical results are presented in Figure 20. Initial biomass densities were not significantly different (control: 13.51 ± 11.54 pg ATP cm^{-2}; treatment: 20.25 ± 19.90 pg ATP cm^{-2}; $t(6) = 0.17$, $p = 0.86$), and neither were final densities (control: 640 ± 368 pg ATP cm^{-2}; treatment: 685 ± 421 pg ATP cm^{-2}; $t(6) = 0.040$, $p = 0.97$).

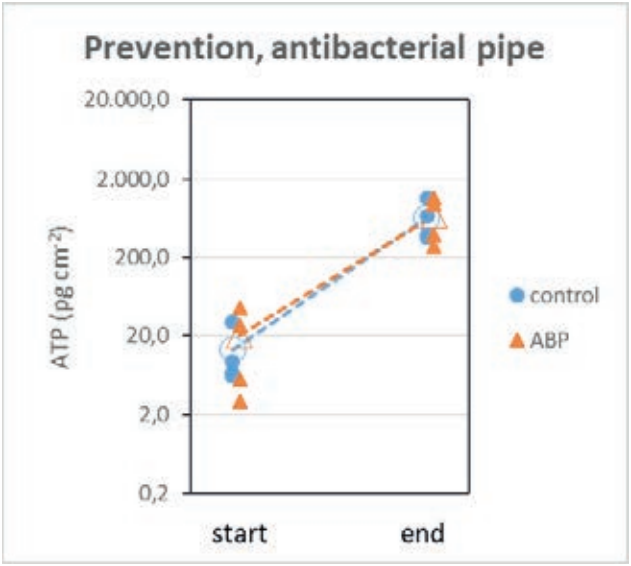


Figure 20 *Results on initial (start) and final (end) biofilm densities for the antibacterial pipe material (ABP) and the controls. Open symbols connected by dashed lines are the average values.*

The PERMANOVA showed a trend (close to but not significant; p=0.090, Annex 6) which seems to be caused by (non-significant) differences between control and treatments (Figure 21), as there were trends (p=0.1) in the pairwise comparisons both at the start and at the end. The biomass composition can be found in Annex 7.

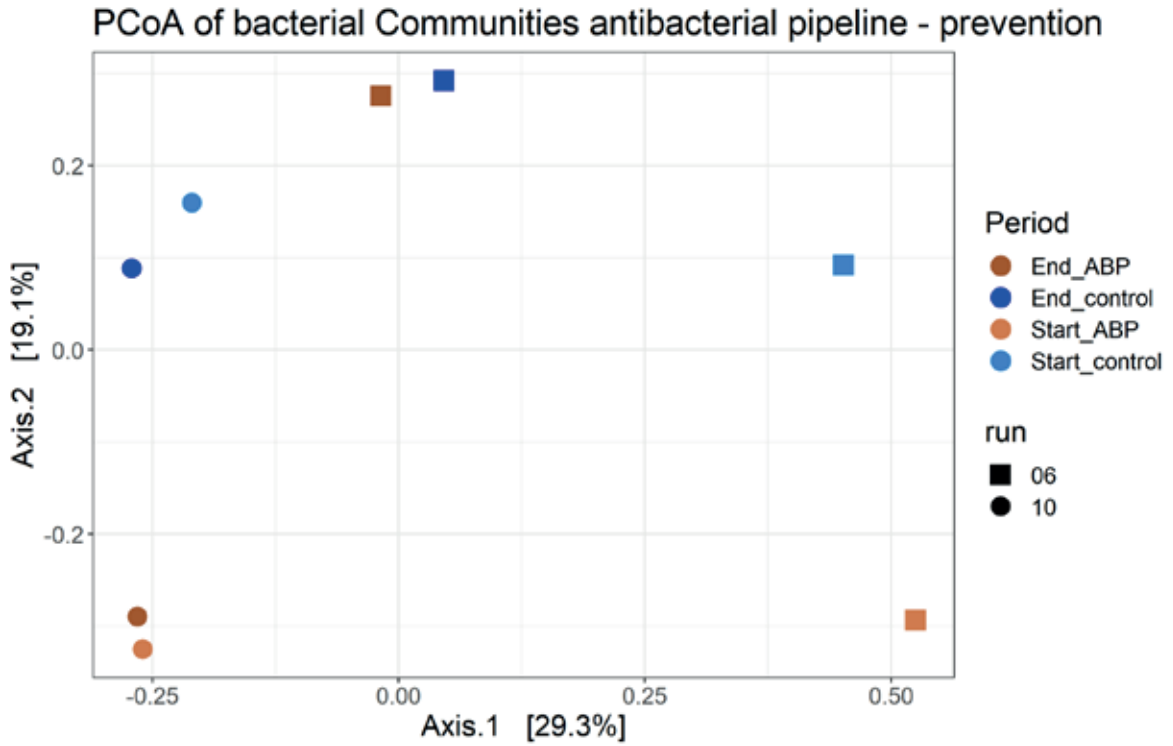


Figure 21 *Results of the principal coordinate analysis (PCoA) for antibacterial pipe material, prevention.*

3.3 Physical treatment

3.3.1 AQUA4D®

3.3.1.1 Prevention of biofilm formation

In Figure 22 the effect of electromagnetic treatment (AQUA4D®) on biomass development is shown. The biomass development in both treatments was quite similar, the technology does not seem to have had an effect on the biomass in the biofilm. As was expected, no effect was observed on the biomass in water, as the technology was not intended for disinfection of water.

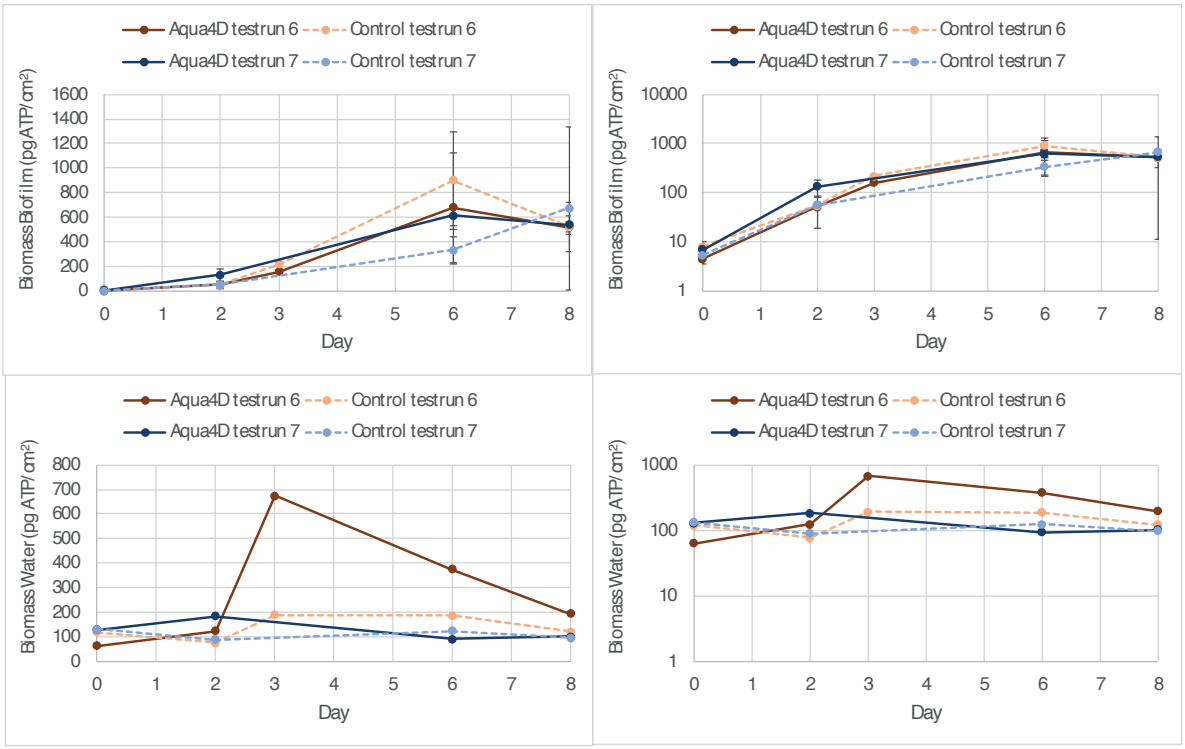


Figure 22 *Microbial biomass in electromagnetic treatments (AQUA4D®) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 6 and 7 to prevent biofilm formation in clean irrigation lines.*

For the AQUA4D®electromagnetic treatment, the summarising statistical results are presented in Figure 23. Initial biomass densities were not significantly different (left side of Figure 23) (control: 6.72 ± 2.18 pg ATP cm^{-2}; treatment: 5.80 ± 1.57 pg ATP cm^{-2}; $t(6) = 0.61$, $p = 0.56$), and neither were final densities (control: 600 ± 410 pg ATP cm^{-2}; treatment: 530 ± 49 pg ATP cm^{-2}; $t(6) = 0.18$, $p = 0.86$). This shows that the electromagnetic waves were not capable to prevent a biofilm from forming in the test system.

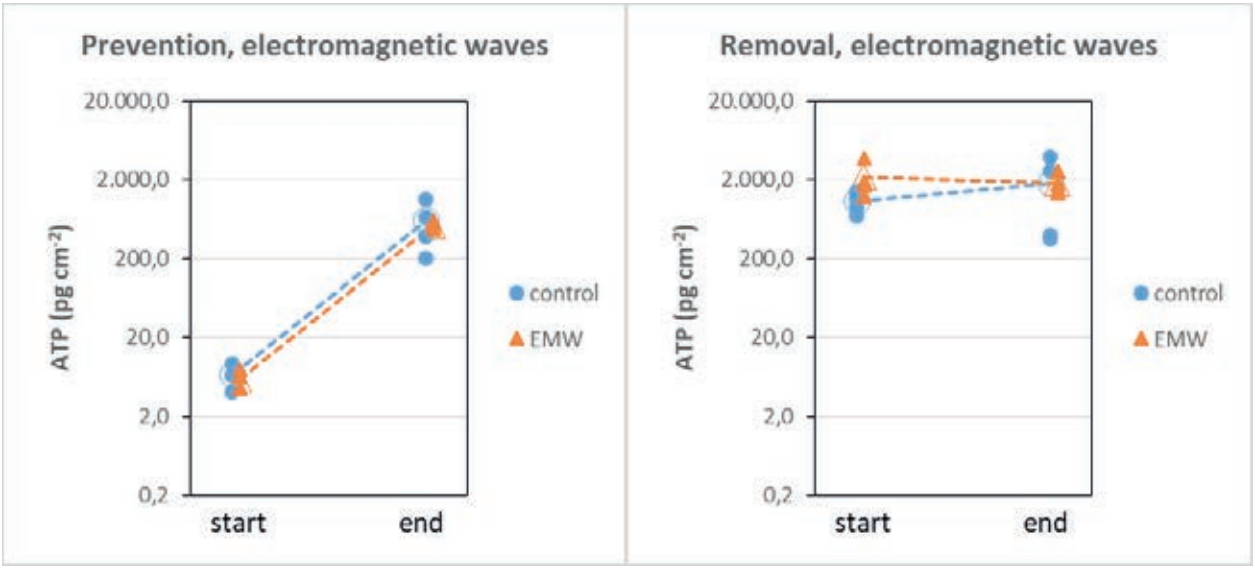


Figure 23 *Results on initial (start) and final (end) biomass densities in biofilm for AQUA4D® and the controls for biofilm prevention (left) and removal of existing biofilm (right). Open symbols connected by dashed lines are the average values.*

With AQUA4D®, prevention, the initial microbial profiles for controls and treatment were quite similar at the start of both runs (Figure 24). At the end of the runs, the communities had shifted: the control of run 7 was most similar to the treatment of run 6, and vice versa. The PERMANOVA did not reveal any significant results (Annex 6). The biomass composition can be found in Annex 7.

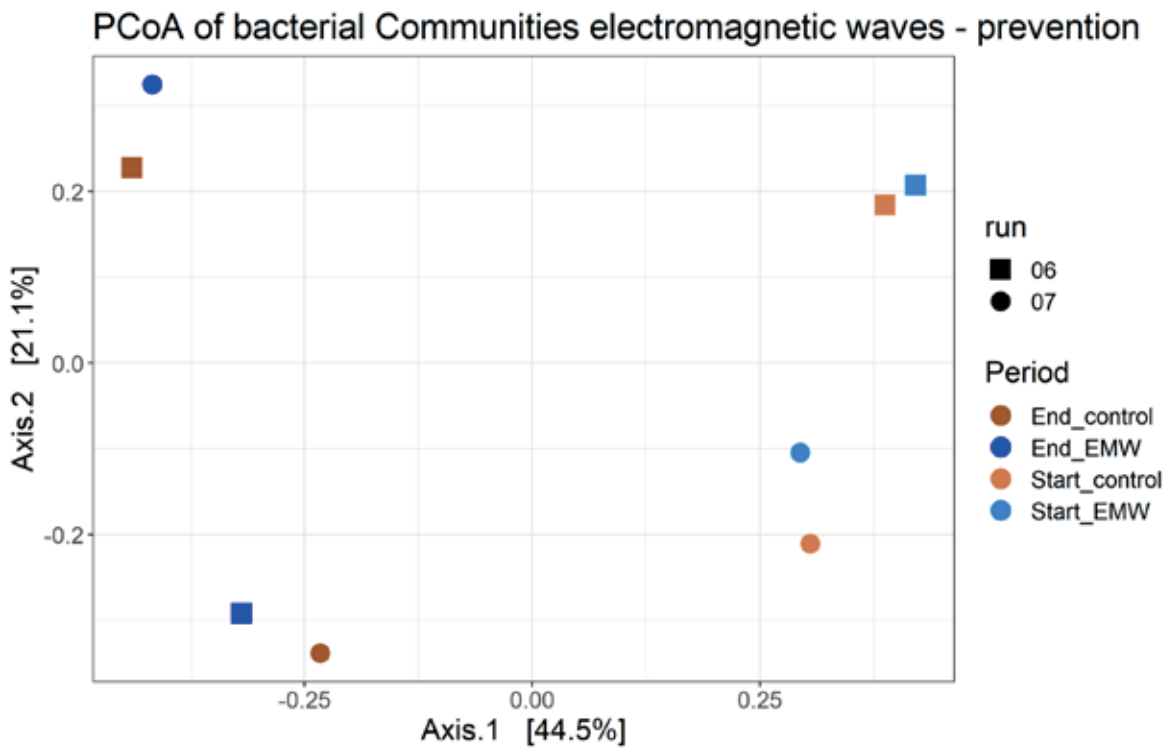


Figure 24 *Results of the principal coordinate analysis (PCoA) for AQUA4D® (electromagnetic treatment), prevention.*

3.3.1.2 Removal of existing biofilm

In Figure 25 the results are shown from testruns 1 and 2 on the removal of an existing biofilm with electromagnetic treatment. The lines for the electromagnetic and control treatment follow exactly the same trend. As expected, there was no effect on the biomass in the water.

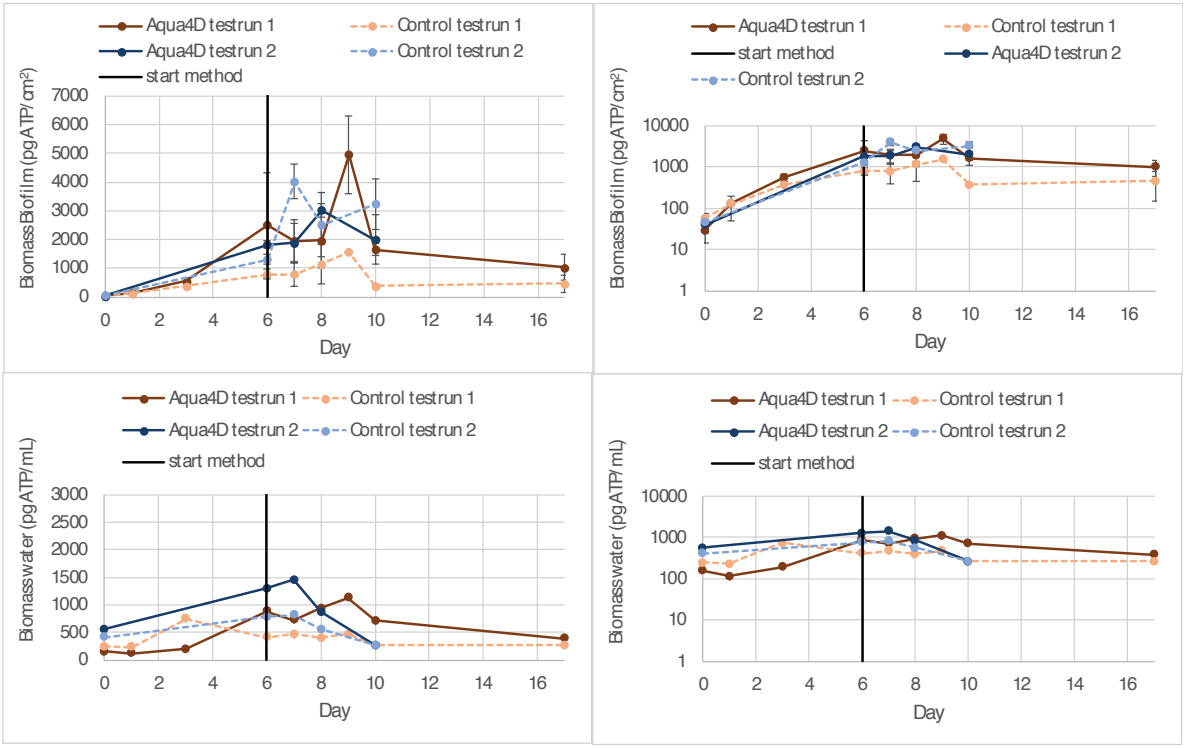


Figure 25 *Microbial biomass in electromagnetic treatments (AQUA4D®) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 1 and 2 to remove an existing biofilm from irrigation lines.*

There was a trend (p-value close to but not significant) to differences in initial biomass densities (right side of Figure 23) (control: 1053 ± 328 pg ATP cm^{-2}; treatment: 2169 ± 1127 pg ATP cm^{-2}; $t(6) = 2.34$, $p = 0.058$), but final densities were not significantly different (control: 1804 ± 1731 pg ATP cm^{-2}; treatment: 1826 ± 546 pg ATP cm^{-2}; $t(6) = 0.76$, $p = 0.47$).

With AQUA4D®, removal, microbial profiles of controls and treatments appeared to be different at the start, and all treatments shifted in the same direction along Axis 2 (Figure 26). The PERMANOVA did not reveal any significant results (Annex 6). The biomass composition can be found in Annex 7.

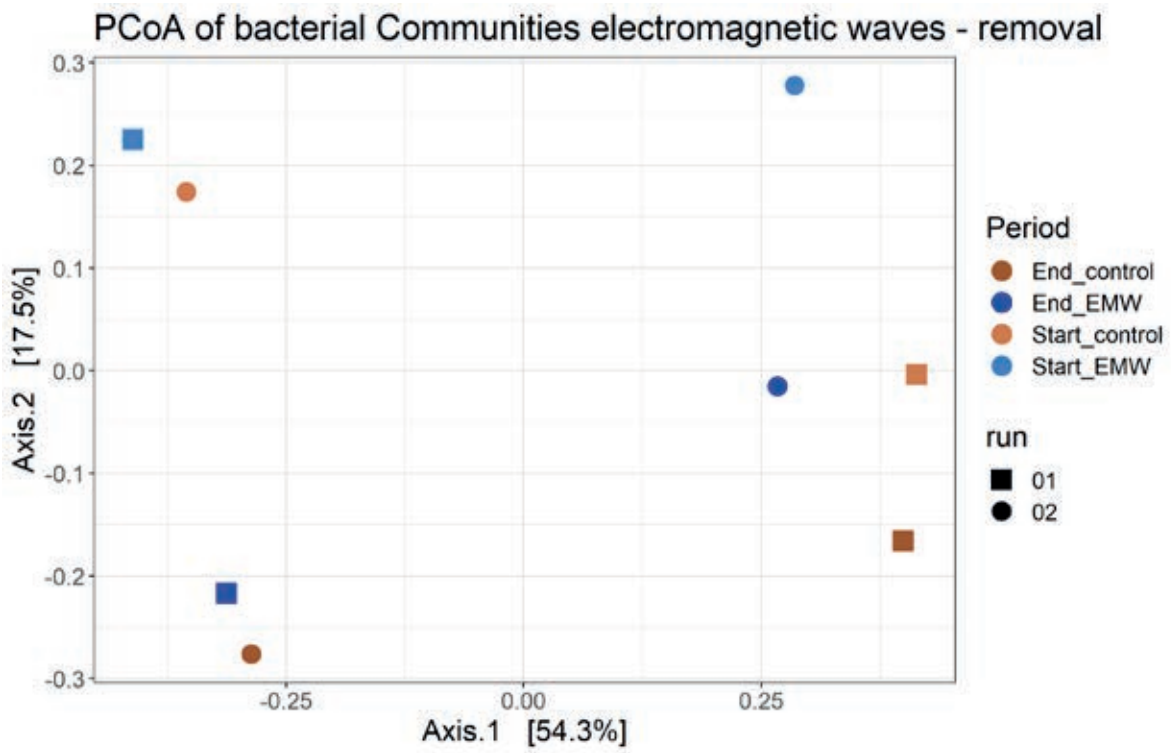


Figure 26 *Results of the principal coordinate analysis (PCoA) for electromagnetic waves, removal.*

In commercial greenhouses applying electromagnetic treatment, the irrigation lines are sometimes flushed with increased velocity water. After consultation it was hypothesised that electromagnetic treatment could loosen the biofilm for easier mechanical removal. To see whether this would create a difference between electromagnetic treatment and the control treatment, we applied a flushing test after the last regular measurement of testrun 2. Therefore, we doubled the water velocity (450 L/h) in the biofilm monitor by closing one of the replicate columns for six minutes. We applied this treatment for the control and ultrasound treatment as well, for both replicate columns. The amount of biomass in the biofilm clearly decreased by flushing the system, but the remaining amount of biomass (as a percentage of amount before flushing) was similar for the three treatments (Figure 27).

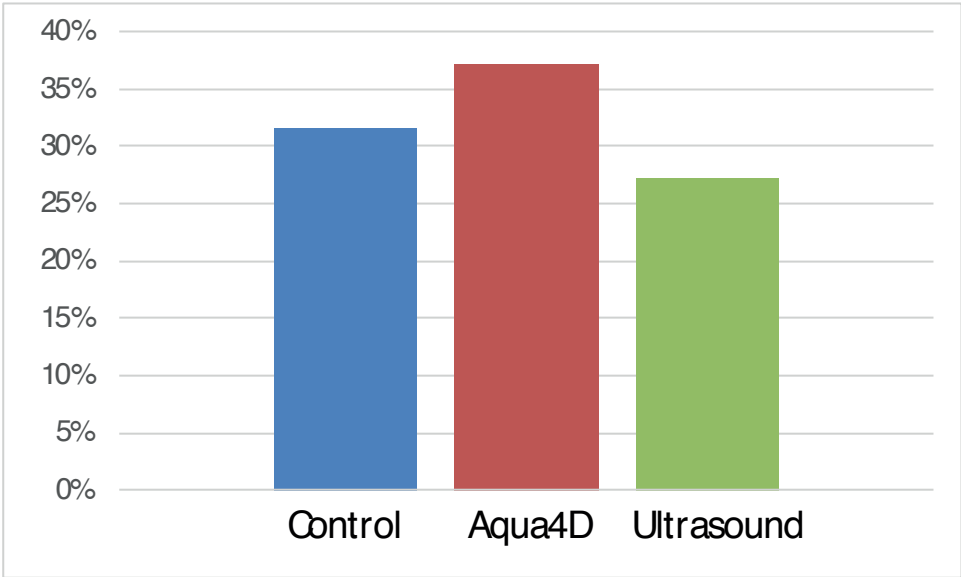


Figure 27 *Remaining amount of biomass in biofilm after flushing the system as a percentage of start value.*

3.3.2 Ultrasonic treatment

3.3.2.1 Prevention of biofilm formation

The effect of ultrasound on formation of biofilm in clean irrigation lines is shown in Figure 28. As can be seen in the Figure, the lines for the control and the ultrasonic treatment follow each other perfectly in testrun 8, while there was quite a difference in total level of biomass in biofilm in testrun 5. However, the trend in testrun 5 is the same for treatment and control. The concentration of biomass in the water in control and treatment were comparable.

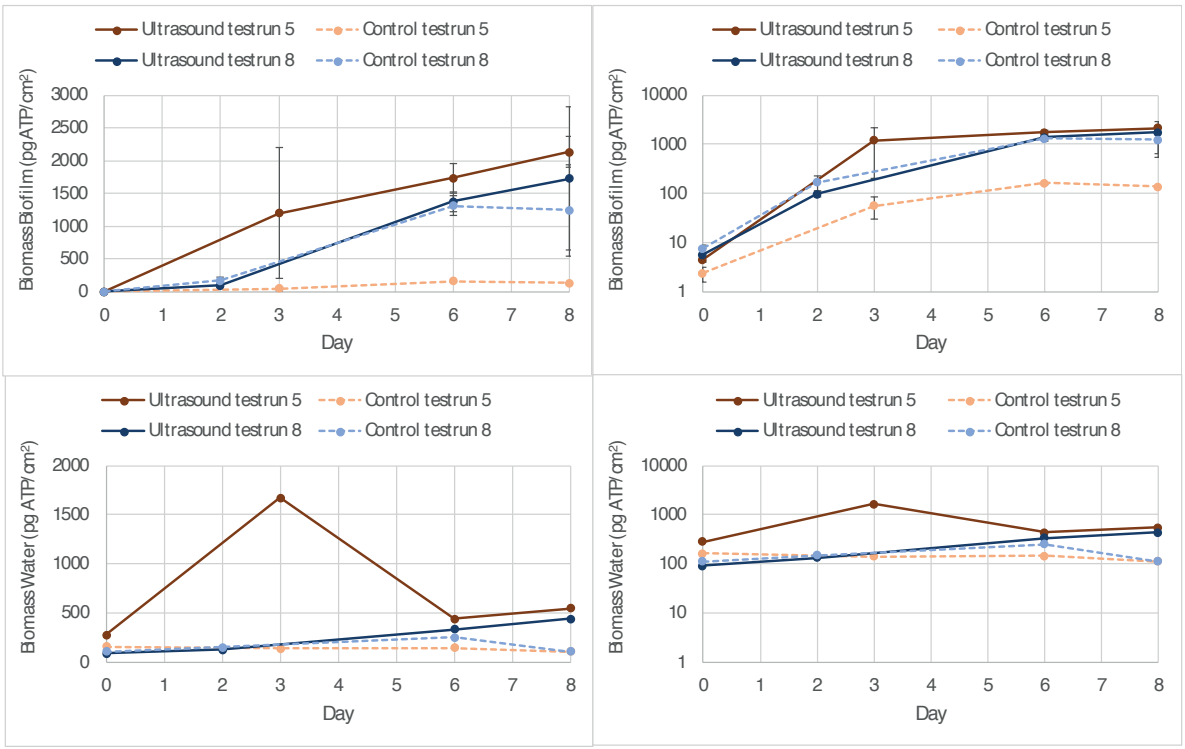


Figure 28 *Microbial biomass in ultrasonic treatments (AWWS) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 5 and 8 to prevent biofilm formation in clean irrigation lines.*

For the SonoPure ultrasonic treatment, the summarising statistical results are presented in Figure 29. Initial biomass densities were not significantly different (left side of Figure 29) (control: 5.05 ± 3.17 pg ATP cm^{-2}; treatment: 5.23 ± 1.27 pg ATP cm^{-2}; $t(6) = 0.51$, $p = 0.63$), and neither were final densities (control: 1693 ± 685 pg ATP cm^{-2}; treatment: 1933 ± 685 pg ATP cm^{-2}; $t(6) = 0.46$, $p = 0.66$). The technology does not seem to have an effect on prevention of the formation of biofilm in irrigation lines. This can be due to sonic extinction caused by particles in the nutrient solution or obstacles like bends and valves in the pipelines.

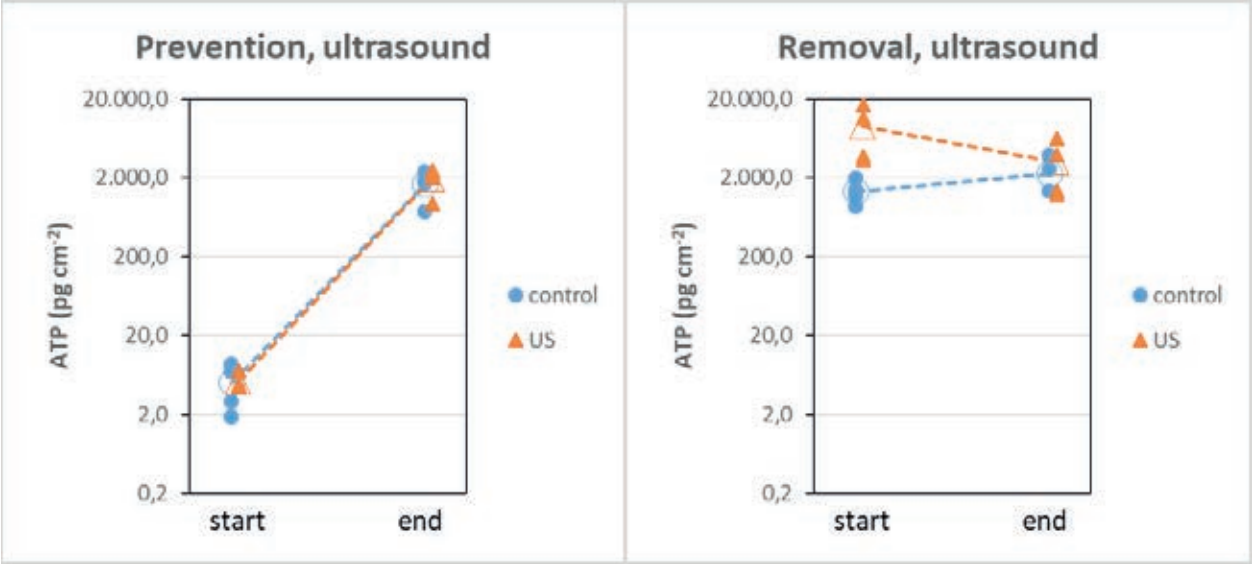


Figure 29 *Results on initial (start) and final (end) biomass densities in biofilm for ultrasound (US) and the controls for biofilm prevention (left) and removal of existing biofilm (right). Open symbols connected by dashed lines are the average values.*

Although the runs were initially different, controls and treatments did not differ much at the start (Figure 30). Over the course of the experimental run, all communities shifted to the right side, i.e. along Axis 1. The PERMANOVA did not reveal any significant results (Annex 6). The biomass composition can be found in Annex 7.

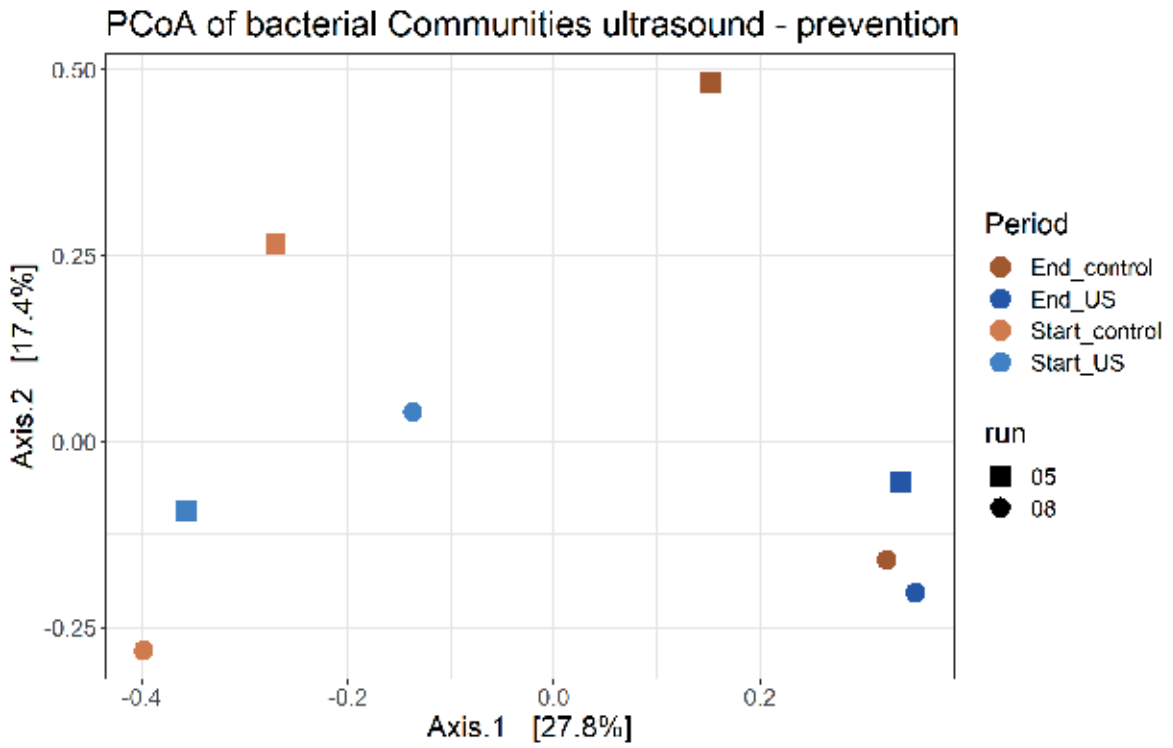


Figure 30 *Results of the principal coordinate analysis (PCoA) for ultrasound, prevention.*

3.3.2.2 Removal of existing biofilm

Figure 31 shows the effect of ultrasound on the removal of an existing biofilm compared to an untreated control. In testrun 3, the trend in the formation of biomass in the biofilm in the ultrasonic treatment exactly follows the trend of the control treatment. In testrun 2, the first measurement after start-up of the technology indicates an effect of the treatment, but in the following measurements the amount of biomass in the biofilm remains more or less the same.

For removal (Figure 29, right side), initial biomass densities were significantly different (control: 1363 ± 465 pg ATP cm^{-2} ; treatment: 9028 ± 6634 pg ATP cm^{-2}; $t(6) = 3.87$, $p = 0.008$), but final densities did not differ significantly anymore (control: 2302 ± 1193 pg ATP cm^{-2}; treatment: 3235 ± 2462 pg ATP cm^{-2}; $t(6) = 0.43$, $p = 0.68$). Ultrasound decreased the amount of biomass in biofilm during the test run, but the decrease was not significantly different to the control treatment at the end of the test run.

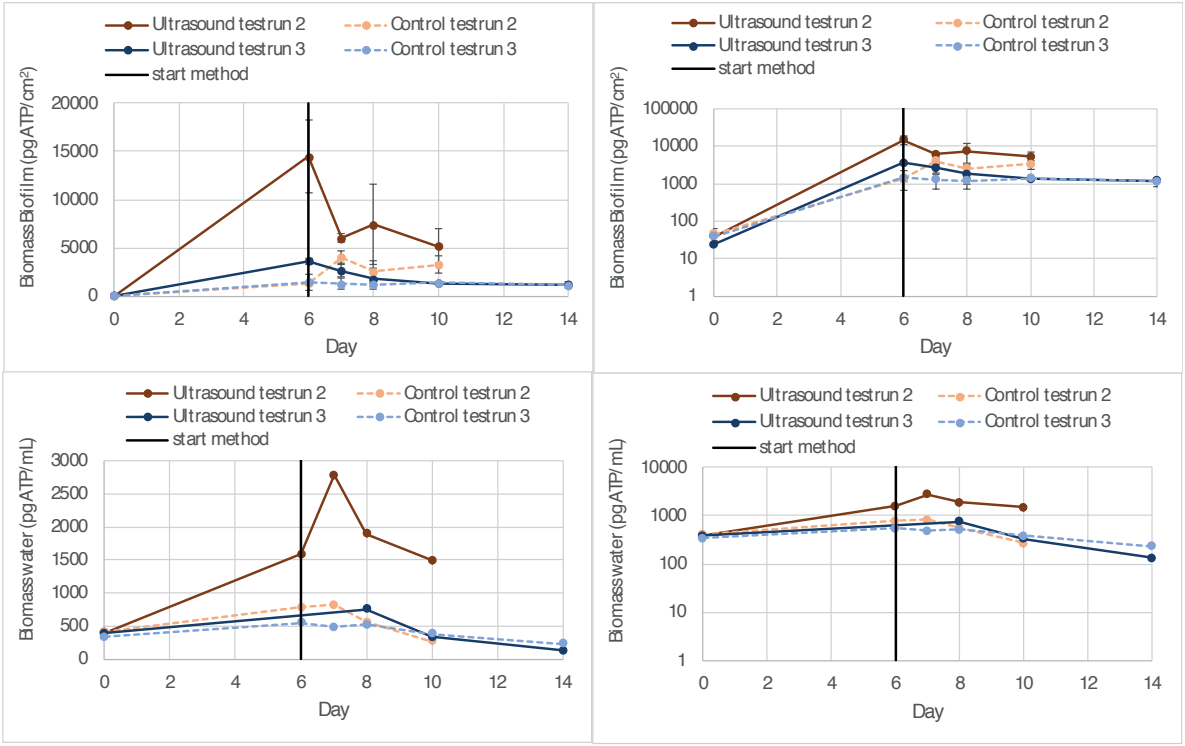


Figure 31 *Microbial biomass in ultrasonic treatments (AWWS) and controls in biofilm (top) and water (bottom) samples on a linear scale (left) and a logarithmic (right) scale, during testruns 2 and 3 to remove an existing biofilm from irrigation lines.*

For both runs, there were initial differences between controls and treatments but all communities more or less remained the same over the experimental runs (Figure 32). The PERMANOVA did not reveal any significant results (Annex 6). The biomass composition can be found in Annex 7.

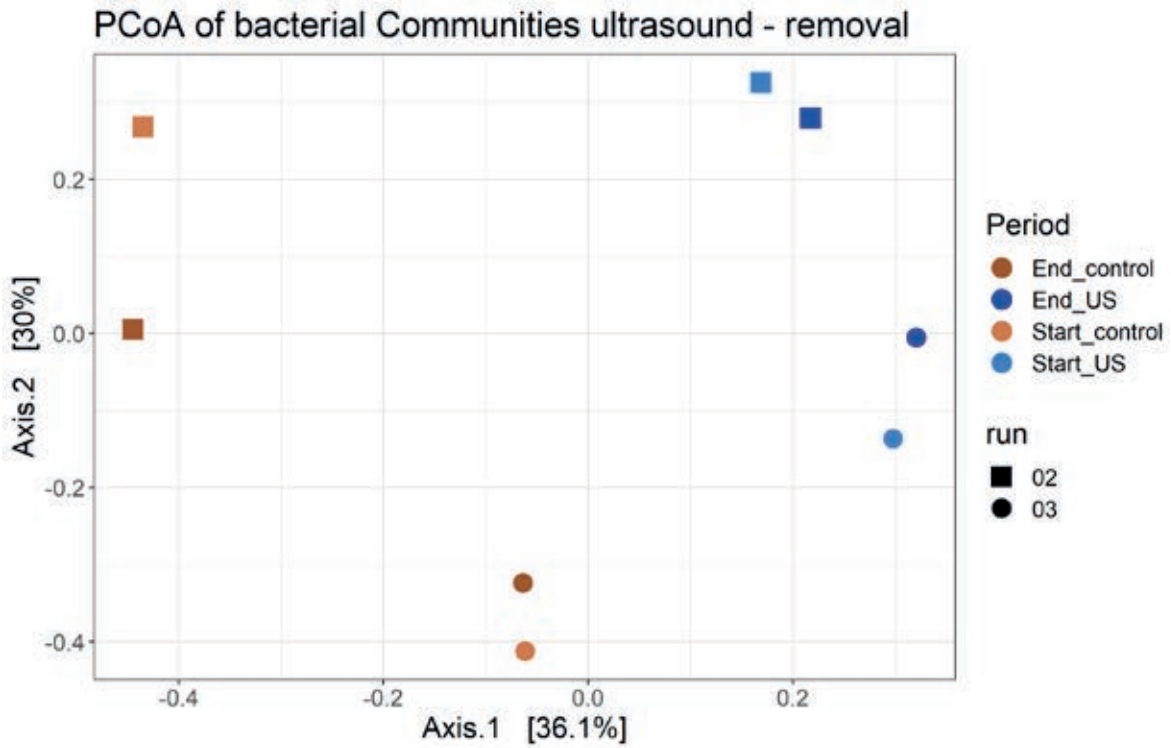


Figure 32 *Results of the principal coordinate analysis (PCoA) for ultrasound, removal.*

4 Discussion

Protocol and installation
The problem of mechanical clogging of irrigation lines and drippers/sprayers due to biofilm formation is recognised by growers. The most important goal for this research was to find effective ways to remove biofilms from the irrigation lines in greenhouse horticulture. Therefore an installation, protocol for controlled production of biofilm and a test protocol have been developed, matching real-life greenhouse conditions such as temperature, nutrient solution, concentration of organic matter, irrigation strategy, water velocity, pipe material and length as closely as possible.

The control treatments have shown quite a good consistency during the different testruns. Maximum biofilm concentrations were reached after 6-7 days, after which a stabilisation or a small decrease was observed. It was hypothesised that a lack of carbon source or decreasing concentrations of oxygen in the nutrient solution would be the reason for this. However addition of extra sodium acetate as carbon source at the start of the testrun did not show any effect (as was shown in Chapter 3.1.1). Occasional measurements of dissolved oxygen in the test systems did not show inhibitory low oxygen levels for the biofilm after day 6 of the testruns. Other (micro) nutrients could have become a limiting factor, but this has not been tested.

The biomass growth in the different systems shows great consistency within the same testruns, meaning that treatments can be compared to controls and outcomes are not dependent on specific test systems. Nevertheless, we found differences between testruns, as conditions such as water source/composition, temperature or other factors may vary over time. The main difference is probably the composition of the water source, as the ratio of rainwater and well water was not consistent throughout the year. The stock solution for nutrients was replenished every 2-3 testruns, so also here small differences might have occurred. Also differences in the effectiveness of the cleaning protocol between testruns could have occurred over time.

The amount of biomass in the installation has shown to be comparable to a greenhouse situation. However, this was only investigated with a very limited amount of samples. A biofilm in commercial greenhouses could have been build up for multiple years and can also contain a lot of (dead) organic matter and nutrient precipitation. We were not capable to recreate this situation in the test system and protocol. For commercial greenhouses it is recommended to replace the irrigation lines if very much (organic) matter is present in the irrigation lines, as treatment could break flocs from the fouling to clog drippers and sprinklers even more. After replacing the irrigation lines, water treatment should aim to keep the new irrigation lines clean.

Oxyl-PRO S H_2O_2
In the tests, 20 mg/L of H_2O_2 was applied for the prevention and removal of biofilm. The applied concentration is an important parameter for the effectiveness of the technology. In testruns 4 and 5, the concentration of H_2O_2 only reached 20 mg/L at the biofilm monitor after two to three days, which was also the point in time when the biomass concentration in the biofilm started to drop in the preventive tests. In the testruns on the removal of an existing biofilm, the concentration of H_2O_2 dropped to below 5 mg/L around day 10. In testrun 3 a clear stagnation in the removal of biofilm and biomass in water was shown during this drop in concentration of H_2O_2. This could be the reason that no significant difference was found between the start and the final concentration of the curative testruns. However, more repetitions should be done to prove the significance in the removal of an existing biofilm.

The effect of Oxyl-PRO S seems to be more direct on the biomass in water than on the biomass of the biofilm. Apparently hydrogen peroxide cannot penetrate deeply into the biofilm and needs to slowly remove the outer side of the biomass in the biofilm.

SureFlow ClO_2

The effect of a low concentration of ClO_2 seems to work very direct on the biofilm. In all testruns the biomass in biofilm and water decreased immediately after start-up of the technology, at a concentration <0.5 mg/L. At that moment the concentration of total oxidants had not increased yet; this only happened during the course of each testrun, so the removal of biomass was most probably not caused by remaining chlorite and reaction product chlorate. Also pH did probably not cause this effect, as it was regularly measured and corrected if needed. The pH drop only happened after at least two days, before which the effect on biomass in the biofilm and in water could already be seen.

AQUA4D® electromagnetic treatment

It is known that electromagnetic treatment can prevent calcium carbonate precipitation from forming on the inside of pipelines (Kobe *et al.,* 2002). As precipitated salts can be a starting point for biofilm formation on the inside of irrigation lines, this could be the working principle for the effect of electromagnetic treatment on biofilm formation in commercial greenhouses, as was reported by the supplier of the technology (Planet Horizons Technologies). In the testruns of this project, we did not observe an effect on biofilm removal with this technology, which could implicate that there was no calcium precipitation (scaling) on the insides of the irrigation lines during the test runs. Calcium precipitation was not checked during the testruns. This suggests that the electromagnetic waves from AQUA4D® treatment do not have a direct effect on biofilm, but a more indirect effect by prevention of calcium precipitates.

SonoPure ultrasonic treatment

Ultrasonic waves (high intensity) are known to be capable of removing both sessile and planktonic bacteria in water by cavitation (Broekman *et al.,* 2010). This effect was not seen during the testruns of this project. Possibly the long distance and obstacles (valves, water counters, bends, etc.) between point of application and point of measurement can be the reason for this effect (by sonic extinction), although it was not measured during the project. Possibly the applied intensity/power of the technology was too low to create an effect on the biofilm in the long irrigation lines.

Antibacterial irrigation lines

The protocol for measurement of biofilm was a bit different than for the other methods, as the supplied product consisted of an inner tube of antibacterial material and an outer tube of normal PE. These could not be separated, so that the material could not be applied as rings in the biofilm monitor. Potentially the method for biofilm measurement of this material (as described in paragraph 2.3.3) could create a difference with the normal measurements. However, as could be seen in the results, the measured amount of biofilm was exactly the same as in the control measurement.

Microbial profiling: overall effects

The PCoAs and PERMANOVAs revealed some shifts in microbial profiles, but no significant changes have been found, only two trends. One trend, for antibacterial pipe material, was caused by initial differences between treatments and controls which persisted throughout the experiment, suggesting no treatment effect. The other trend for prevention using chlorine dioxide indicates a real treatment effect. Perhaps the level of replication (n=2) was too low to bring about any significant effects.

To get an impression of the overall effects of the different treatments, we also made a PCoA of all results together, divided over the different sampling days during the different test runs (Figure 33). This gives a very clear picture of the starting conditions: at day 0 (square symbols), all communities cluster together on the right side of the PCoA, at intermediate height. After that, all communities shift towards the left side of the figure over time (circles, triangles, diamonds and stars). This indicates that the initial community composition at day 0 (start of the prevention runs) is very comparable across different test runs and treatments, but that the initial communities at the start of the removal runs (day 6, circles) already diverged over time. The possible implications of this are that shifts in community composition may be more clear for the prevention runs as compared to the removal runs. Indeed, the clearest effects were found for the prevention runs. Although one could expect a stronger effect of any method on newly developing biofilms (prevention) as opposed to more mature biofilms (removal), it is good to realise that the time elapsed during biofilm formation introduces extra variability in starting conditions for the study of removal, making it more difficult to demonstrate significant results on community composition. This aspect should – in addition to the level of replication - require careful consideration in future experiments.

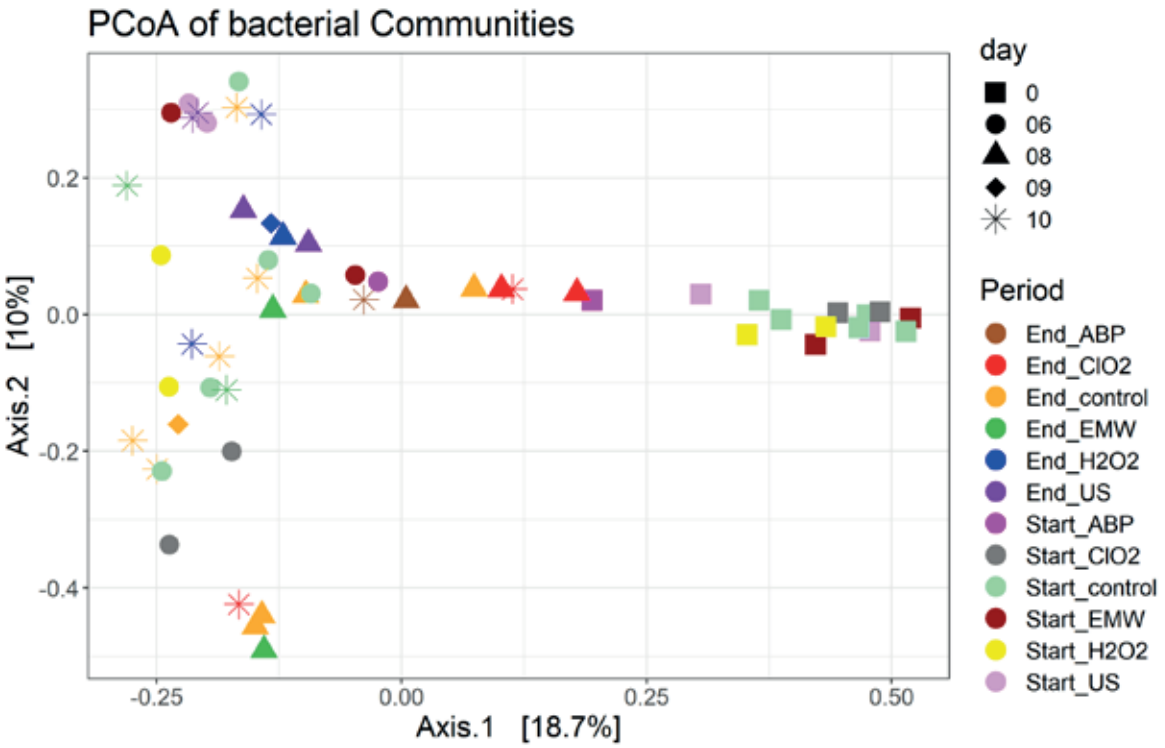


Figure 33 *Results of the principal coordinate analysis (PCoA) for all treatments and al test runs, biofilms only.*

5 Conclusions

Based on the measurements performed in this study we can conclude the following:

- The installation is representative for greenhouse cultivation conditions.
- With the test protocol, controlled biofilm formation could be obtained in consecutive test runs.
- Biomass development in water and in biofilms was comparable in the three parallel systems during a testrun.
- We observed differences in biofilm development between consecutive testruns, but not within testruns, allowing to compare technologies within and across testruns.
- Oxyl-PRO S (silver nanoparticles stabilised H_2O_2, Cindro BV): with a concentration of 20 mg/L H_2O_2 in the nutrient solution, by dosing 40 mg/L of formulated product, this product has shown to be capable of preventing biofilm formation in clean irrigation lines.
- Sure Flow ClO_2 (chlorine dioxide, CH2O BV): with a concentration of maximum 0.5 mg/L ClO_2, Sure Flow ClO_2 was capable of preventing biofilm formation in clean irrigation lines and of removal of an existing biofilm.
- AQUA4D® (water treatment with very low-frequency electromagnetic resonance fields, Planet Horizons Technologies SA): electromagnetic treatment of the nutrient solution did not show an effect on prevention of biofilm formation in clean irrigation lines and did not show an effect on removal of an existing biofilm.
- SonoPure (ultrasound treatment, AWWS): ultrasound treatment of the nutrient solution did not show an effect on prevention of biofilm formation in clean irrigation lines and did not show an effect on removal of an existing biofilm.
- Antibacterial irrigation lines (MVP Waterworks): the antibacterial properties of the irrigation lines did not show an effect in the prevention of biofilm formation.
- We could not demonstrate selective removal of certain bacterial classes from biofilm communities with any of the technologies investigated. Because of the complexity of this question, the experimental design could be reconsidered.

The project results show that from the tested technologies, the traditional (chemical) methods (SureFlow ClO_2 and Oxyl-PRO S H_2O_2) were most successful in preventing and removing biofilms under the conditions tested here, representative for greenhouse circumstances.

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Annex 1 Protocol production biofilm in biofilmmonitor

Klimaatinstellingen
Een hogere temperatuur zorgt voor een snellere vorming van biofilm. In een tomatenkas wordt een gemiddelde etmaaltemperatuur rond de 20 graden aangehouden, maar overdag komen hogere temperaturen voor. In de klimaatruimte zal de temperatuur ingesteld worden op 24 graden.

Schoonmaken ringen
Voor het vullen van de kolommen van de hexamonitor dienen de ringen eerst schoongemaakt te worden. Gesteriliseerde glazen ringen en ongebruikte PVC ringen moeten gespoeld worden met kraanwater voor gebruik. Plaats de ringen de dag voordat de test wordt gestart in een bekerglas en spoel gedurende 60 ± 5 min met kraanwater. Laat de ringen vervolgens gedurende 24 ± 1 uur in het water bij kamertemperatuur staan. Spoel de stukjes op de dag van de test nogmaals 60 ± 5 min met kraanwater. De kraan hoeft niet vol open, maar moet wel goede verversing van het water in het bekerglas hebben. PE drijft, dus leg een rvs grid (of iets dergelijks) op het bekerglas zodat je materialen er niet uit kunnen, maar het water wel. Gebruikte glazen ringen moeten de dag voor uitvoering van een test 4-6 uur in een bekerglas met waterstofperoxide gezet worden (dosering zie schoonmaakprotocol leidingsysteem). Vervolgens op diezelfde dag nog 60 ± 5 min spoelen met kraanwater, 24 ± 1 uur in water op kamertemperatuur laten staan en op de dag van de test nog 60 ± 5 min spoelen met kraanwater.

Vullen met ringen (handschoenen aan)
Als de ringen schoon zijn, vullen we de hexamonitor. Maak de RVS-draad schoon met 70% ethanol en laat uitwasemen. Plaats de RVS draad vervolgens in de kolom tot helemaal onderin (dit zorgt er voor dat de cilinders minder gaan bewegen in de kolom). Met behulp van het steriele (uitgloeien met brander en aan de lucht afkoelen) RVS 'hengeltje' kunnen de ringen nu voorzichtig in de kolom gebracht worden. De onderste ring is van PE, vervolgens om en om glas en PE ringen plaatsen, zodat de PE ringen niet gaan drijven. Netjes boven elkaar stapelen. Iedere kolom wordt volledig gevuld met ringen (lengte ca. 1,5 cm en inwendige diameter ca. 1,5 cm; dus het oppervlakte is ca. 17 cm^2).

Samenstelling water
Een voedingsoplossing van 300 L voor tomaat wordt klaargemaakt volgens het recept in Tabel 1, in ieder van de drie bakken van 500 L in de klimaatruimte. De bak wordt gevuld met 150 L water, hiervoor wordt gebruik gemaakt van water uit de gietwatervoorraad van Wageningen University & Research BU Glastuinbouw, bestaande uit een mengsel van regenwater en bronwater na behandeling met omgekeerde osmose en ontsmet met UV. Nu worden de meststoffen toegevoegd, zoals genoemd in de laatste kolom van Tabel 2. Deze meststoffen zullen we eenmaal afmeten voor 5000 L voedingsoplossing. Hiervoor worden een A- en een B-bak van 50 L gevuld (100x geconcentreerd) met de hoeveelheden uit Tabel 2. Vanuit deze geconcentreerde stockoplossingen moet 3 L uit de A-bak en 3 L uit de B-bak worden gedoseerd in de tank van 500 L. Vervolgens wordt de bak aangevuld met water tot 300 L.

Tabel 1
Samenstelling van de voedingsoplossing, standaardvoedingsoplossing voor tomaat (Bemestings Adviesbasis Substraten, 1999).

NH_4	K	Ca	Mg	NO_3	SO_4	H_2PO_4	Fe	Mn	Zn	B	Cu	Mo
mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L	µmol/L
1.0	6.5	2.75	1.0	10.75	1.5	1.25	15	10	4	20	0.75	0.5

Tabel 2

Hoeveelheden meststoffen toe te voegen per tank van 300 L.

	Per 300 L		per 50 L 100x geconcentreerd (5000 L voedingsoplossing)	
A-bak				
kalksalpeter (l)	203	mL	3.4	L
ammoniumnitraat (l)	17	mL	0.28	L
kalisalpeter	25	g	420	g
ijzerchelaat DTPA 6%	4	g	70	g
B-bak				
kalisalpeter	104		1.7	kg
monokalifosfaat	51	g	850	g
kalisulfaat	26	g	435	g
bitterzout	74	g	1.2	kg
mangaansulfaat	510	mg	8.5	g
zinksulfaat	345	mg	5.8	g
borax	570	mg	9.5	g
kopersulfaat	57	mg	0.95	g
natriummolybdaat	36	mg	0.6	g

Inoculum bacteriën

Slib vanaf de bodem van een vuil draintank is verzameld voor het verkrijgen van een bacterieel inoculum. Hiervan zijn flesjes van 20 mL aangemaakt en opgeslagen bij 4 °C. Aan ieder systeem wordt bij de opstart een flesje toegevoegd, om de groei van biofilm te versnellen en om een samenstelling aan bacteriën te krijgen die ook in een tuinbouwsysteem aanwezig is. Vervolgens wordt 10 µg natriumacetaat toegevoegd aan deze 300 L water, dit dient als koolstofbron voor de bacteriën.

De pH van de voedingsoplossing wordt op 5.5 gebracht door toevoegen van salpeterzuur of baskal.

Instellingen 'watergift'

De watergift bootst een situatie in een tomatenkas na. Tussen 8.00 uur en 17.00 uur worden de pompen steeds twee minuten aangezet en 10 minuten uit. De pompen worden door een tijdschakelaar aangestuurd.

Doorstroomsnelheid op verschillende punten in het systeem

Door hexamonitor moet per buis een waterstroom van 200-220 L/uur gehaald worden, twee buizen per systeem. De monitor kan maximaal 250 L/uur hebben.

Hexamonitor bemonsteringsprotocol

De glazen kolommen dienen voorzichtig behandeld te worden i.v.m. mogelijke breuk.

Benodigdheden
- Ultrasoon bad.
- ATP meetset.
- RVS 'hengeltje' (bijgeleverd bij hexamonitor).
- Brander.
- Reageerbuizen met een diameter van 20 mm en een lengte van +/- 19 cm, voorzien van een plastic kapje en gesteriliseerd (geleverd door KWR).
- Steriel leidingwater (geleverd door KWR).
- 50 mL steriele greiner buizen (geleverd door KWR).
- Steriele monsterflessen (250 mL inhoud) (geleverd door KWR).
- Trapje voor tijdens bemonstering.

Monster nemen
- Zet de pompen uit.
- Schrijf het tijdstip op en de meterstanden van de watermeters.
- Brand de kraan waaruit je gaat bemonsteren af totdat je waterdruppels uit de kraan ziet komen.
- Laat het eerste water weglopen, zodat de kraan kan afkoelen.
- Neem een watermonster van 250 mL per systeem in een steriele plastic fles met rode dop.
- Gebruik uit dit sample 50 mL voor het bepalen van de ATP-concentratie met de ATP-testkit.
- Meet in het overgebleven water de EC en de pH.

Cilinders/ringen
- Breng vlak voor de monsterneming 10 mL steriel leidingwater in 6 reageerbuizen.
- Koppel de kop van kolom los.
- Neem voorzichtig met het steriele rvs 'hengeltje' (uitgloeien met brander en aan de lucht afkoelen) een PE cilinder uit de kolom en plaats deze in de grote glazen reageerbuizen met daarin steriel leidingwater. Zorg ervoor dat het cilindertje de wand van de buis niet (zo min mogelijk) raakt. De glazen ring die er tegelijkertijd uitgehaald wordt kan worden opgeslagen voor later gebruik. Doe dit voor alle testbuizen, twee buizen per systeem.
  - Let op: cilinders nooit met je handen aanraken.
- Koppel de kop van de kolom weer vast.
  - Let op dat er geen lekkages zijn.
  - Eventueel; als de slijpstukken veel biofilm laten zien dan kunnen deze worden schoongemaakt met een schoon doekje.
- Zet de pompen weer aan.
- Zet de tijdschakelaars weer op de juiste tijd.
- Nu staat de monitor weer in bedrijf.

Monster bewerken
- Tril de cilindertjes gedurende 2 minuten in het ultrasone waterbad, niet in een plastic rekje (want dit geleid de trillingen niet goed).
- Schenk vervolgens de vloeistof uit de buis in een steriele greiner buis (de "verzamelbuis") en plaats deze in ijs.
- Voeg opnieuw 10 mL steriel leidingwater toe aan de buis met het cilindertje tril nogmaals gedurende 2 minuten en schenk de vloeistof in de "verzamelbuis".
- Voer genoemde handelingen vervolgens nog tweemaal uit.
- Neem met een pipet van 10 mL in totaal 20 mL van het water uit de verzamelbuis en doe dit in de spuit van de ATP-testkit (nadat hier een filter op is gezet).
- Bepaal in de verzamelde vloeistof (totaal 20 mL) direct de ATP.

DNA-analyses
- Neem voor het bepalen van ATP, pH, EC en T een kleiner volume uit de monsterfles of greinerbuis en pipetteer hieruit zodat de monsterfles zo schoon mogelijk blijft (dit is vooral van belang als we gaan filteren voor DNA).

DNA
- Biofilm (afhankelijk van de andere analyses) ±40 mL (dit is dus gepoold van de twee kolommen).
- Water (afhankelijk van inhoud buis en andere analyses 250 mL.

Monster opslag
Op ijs, of in koelkast (4 °C).

Schoonmaakprocedure leidingsysteem
1. Systeem vullen met 300 L water. Voeg 3 L H_2O_2 (50% concentratie) toe per systeem, en zet het systeem aan. Laat het systeem tenminste 6 uur draaien.
2. Maak systeem 1 & 2 leeg. Zet daarvoor de kranen naar de pomp dicht. Draai de koppeling naar de pomp los (let op, hier komt een klein beetje vloeistof uit, maar bevat flinke hoeveelheid H_2O_2! Trek daarom handschoenen aan en zet een veiligheidsbril op. Vang dit water op in een bakje). Koppel vervolgens het verbindingsstuk en een slang aan het systeem en zet de kraan weer open. Laat het systeem zo ver mogelijk leeg lopen. Als deze systemen leeg zijn, pompen we hier weer water in, terwijl de afvoerkraan open staat. Op deze manier wordt steeds een zo hoog mogelijke concentratie H_2O_2 afgevoerd. Blijf dit doen, totdat de concentratie H_2O_2 ongeveer 2 ppm is. Vul het vat met ongeveer 100 L schoon water, sluit de systeempomp weer aan en koppel de afvoerslang onder de biofilmmonitor aan het systeem. Zet de systeempomp aan en laat het systeem leegpompen.

ATP-test
- Maak de luminase vloeistof klaar door de vaste luminase op te lossen in de buffervloeistof. Zorg dat de luminase vloeistof op kamertemperatuur is als je deze gaat gebruiken.
- Nulmeting voor controle activiteit luminase (eenmaal per 4 uur).
  - Doseer 2 druppels ultracheck in een cuvetje.
  - Pipeteer hier 100 µL luminase bij.
  - Meng met drie korte draaibewegingen.
  - Zet het mengsel in de luminometer en voer een calibratiemeting uit (waarde moet >5000 zijn).
- Zuig 50 mL water op met de monsterspuit.
- Plaats een filter op de monsterspuit en duw het water door het filtertje heen.
- Haal het filter van de spuit en verwijder vervolgens de plunjer uit de spuit.
- Pipeteer 1 mL ultralyse uit de voorraad en sluit de voorraad. Plaats het filter weer op de spuit en pipeteer de ultralyse in de spuit.
- Pers nu de ultralyse door het filter in een buisje met 9 mL ultralute.
- Plaats het dopje op het buisje en kantel het buisje 5x om goede menging te krijgen.
- Pipeteer 100 µL uit het buisje in een cuvetje.
- Pipeteer met een nieuwe pipettip 100 µL luminase aan het cuvetje.
- Meng met drie korte draaibewegingen en plaats het direct in de luminometer en voer de meting uit.

Overige metingen
Gebruik het restant vloeistof om de overige metingen uit te voeren:
1. EC.
2. pH.
3. T.

Annex 2 Memo ‘Methodiek voor het monitoren van biofilmvorming in recirculatiewater in glastuinbouw’

Bestemd voor: TKI projectgroep "Microbieel gezond water in de glastuinbouw”
Betreft: Methodiek voor het monitoren van biofilmvorming in recirculatiewater in glastuinbouw
Van: Ir. Bart Wullings en Dr. Paul van der Wielen
Datum: 22 december 2017

Achtergrond

De microbiologische waterkwaliteit en stabiliteit heeft een grote rol in de microbiële gezondheid van het recirculatiewater in de glastuinbouw. Organische stof uitgescheiden door plantenwortels spelen waarschijnlijk een belangrijke rol in de microbiologische waterkwaliteit van het recirculerende irrigatiewater en kunnen microbiële groei stimuleren. Dit kan leiden tot biofilmvorming in het irrigatieleidingsysteem, wat tot problemen zoals verstopping van druppelaars kan leiden. De mate van biofilmvorming door micro-organismen in het irrigatieleidingsysteem in de glastuinbouw is afhankelijk van de beschikbaarheid van voedsel dat aanwezig kan zijn in het irrigatiewater of het leidingmateriaal. Wanneer biofilm tot problemen gaat leiden, worden curatieve maatregelen genomen om de biofilm te verwijderen met desinfectiemiddelen of biociden. Het is echter onder praktijksituaties moeilijk vast te stellen hoe succesvol dergelijke middelen zijn om de biofilm in het leidingsysteem te verwijderen. Daarom wordt binnen WP2 onderzocht of een standaardopstelling met behulp van een biofilmmonitor kan worden opgezet waarin de effectiviteit van biofilmverwijdering kan worden onderzocht. Het doel van dit document is om een kort overzicht te geven van biofilmsystemen die in het verleden door KWR zijn ontwikkeld voor de drinkwaterindustrie en die ook als een dergelijke standaardopstelling voor de tuinbouw kunnen dienen.

Gestandaardiseerde methodiek voor biofilmanalyse

Bij KWR is een biofilmmonitor ontwikkeld die als doorstroom-testeenheid kan worden gebruikt, maar ook als by-pass kan worden geïntegreerd in het irrigatieleidingsysteem (Figuur 1).

Figuur 1 *Biofilmmonitor.*

Deze biofilmmonitor bestaat uit een glazen kolom met daarin opeengestapelde glazen ringen, een flowmeter, een reduceerventiel, een monstername kraan en een watermeter (figuur 2). De aanwezige slangen zijn gemaakt van teflon, de kranen zijn van roestvrijstaal en zoals eerder is genoemd is de kolom van glas. De keuze voor deze materialen is gedaan om de eventuele bijdrage van de gebruikte materialen aan de groei van bacteriën in de biofilmmonitor te voorkómen. De flowmeter en het reduceerventiel hebben de functie om de stroomsnelheid van het water te regelen en te controleren. Om de condities in de monitor overeen te laten komen met de condities in het buizensysteem wordt de stroomsnelheid in de monitor zo ingeregeld dat het overeenkomt met de stroomsnelheid in het irrigatiebuizensysteem.

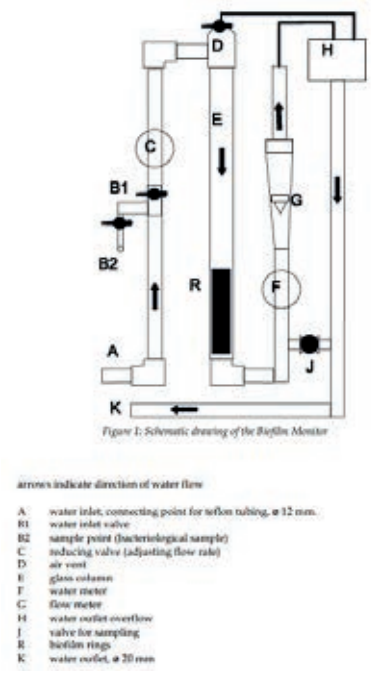

Figure 1: Schematic drawing of the Biofilm Monitor

Figuur 2 *Schematische weergave van de biofilmmonitor.*

In de glazen kolom worden 40 ringen geplaatst. Deze ringen kunnen van verschillende materialen zijn en welk type materiaal wordt gebruikt ligt aan de vraagstelling van het onderzoek waar de biofilmmonitor voor wordt toegepast. Voor het onderzoeken van biofilmverwijdering op leidingen van irrigatiewater binnen de tuinbouw, is het raadzaam om de ringen van het materiaal te maken dat ook wordt gebruikt als leidingmateriaal in het leidingnetwerk. Op deze ringen zal bij doorstroming van de monitor met het te onderzoeken water de biofilmvorming plaatsvinden. De snelheid van biofilmvorming en hoeveelheid biofilm wordt normaliter bepaald door tweewekelijks twee glazen ringen uit de monitor te halen, maar het interval van monstername van ringen kan ook korter of langer zijn. In het laboratorium wordt de biofilm van de ringen verwijderd door de ringen met een kleine hoeveelheid water ultrasoon te trillen. Op de op deze manier verkregen biofilmsuspensie kunnen zowel biologische als chemische analyses worden uitgevoerd. De hoeveelheid gevormde biomassa op de ringen wordt bepaald door de biologische activiteit in het getrilde suspensie te kwantificeren. Hiervoor wordt de concentratie adenosine trifosfaat (ATP) bepaald. Het ATP-molecuul fungeert in de cellen voor energietransport en hoe meer (bacterie)cellen in de suspensie aanwezig zijn en hoe actiever de cellen zijn, hoe meer ATP er wordt gemeten. De ATP-bepaling is relatief eenvoudig en snel met tevens een zeer lage detectiegrens. De biofilmhoeveelheid die op deze manier wordt verkregen wordt uitgedrukt in ATP/cm^2. Naast ATP kunnen ook additionele analyses op de gesuspendeerde biofilm worden uitgevoerd, voorbeelden hiervan zijn: bacteriesamenstelling of microbiële profilering door DNA-sequentieanalyse, directe microscopische celtelling, detectie van pathogene micro-organismen met specifieke detectiemethoden (bv selectieve kweek of qPCR) en chemische analyses (ijzer, mangaan, koolhydraten).

Effectmeting van bestrijding en/of verwijdering van de biofilm

Het principe van de hierboven beschreven biofilmmonitor is in het verleden ook toegepast om effecten van behandelingen tegen biofilmvorming te onderzoeken. Met behulp van de ATP-analyse eventueel aangevuld met microbiële profilering kunnen zowel (1) de afname van de biofilm worden gekwantificeerd maar ook (2) eventuele veranderingen van de microbiële samenstelling van de biofilm. De biofilmmonitor is hiervoor aangepast tot een pentamonitor, waarin vijf parallelle biofilmmonitoren naast elkaar zijn geplaatst (Figuur 3). Iedere biofilmmonitor in deze opstelling kan apart worden bedreven en het is ook mogelijk om per biofilmmonitor een desinfectiemiddel te doseren. Deze testopstelling maakt het daarom mogelijk om in een vergelijkbare conditie als in het irrigatieleidingnet (vergelijkbare waterkwaliteit en leidingmateriaal)verschillende behandelingsstappen naast elkaar te analyseren. Op deze wijze kan relatief snel verschillende condities/ concentraties/middelen worden getest en onderling worden vergeleken.

Figuur 3 *De Pentamonitor met doseermogelijkheid voor desinfectiemiddelen.*

Standaardopstelling binnen wp2

Voorgesteld wordt om de bij KWR aanwezige pentamonitor als standaardopstelling te gebruiken voor onderzoek naar het effect van desinfectie- en biocidemiddelen op biofilmverwijdering. De biofilmmonitoren zullen hiertoe worden gevuld met ringen van materiaal dat normaal wordt toegepast in het irrigatieleidingwerk en zullen doorstroomd worden met water dat vergelijkbaar is met irrigatiewater. In de eerste stap van het onderzoek wordt onderzocht of dit systeem leidt tot de ontwikkeling van reproduceerbare biofilm en binnen welk tijdsbestek zich een stabiele biofilmconcentratie heeft ontwikkeld. In het vervolg kunnen dan de effecten van desinfectiemiddelen en biociden op de biofilm worden onderzocht met deze opstelling.

Annex 3 Biomass in water

For biomass in water the results are shown in Figure 34 and Figure 35. It can be seen that the curve for the increase of biomass in water flattens already after day 2 and remains fairly constant with a slight decrease after day 10. Only a small difference can be seen between systems, so it can be concluded that the results from different systems can be compared. It also shows that differences between a treatment and the control treatment during one testrun are not caused by differences in the test system.

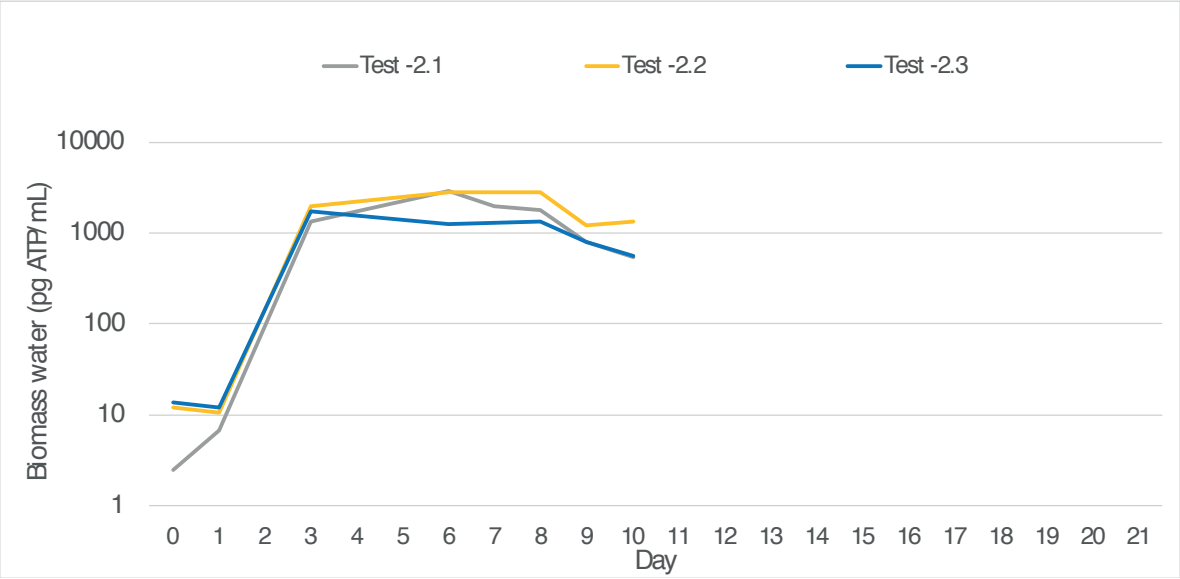

Figure 34 *Biomass in water (pg ATP/mL) in three parallel test systems during test run -2; no anti-biofilm technologies were applied during this test run.*

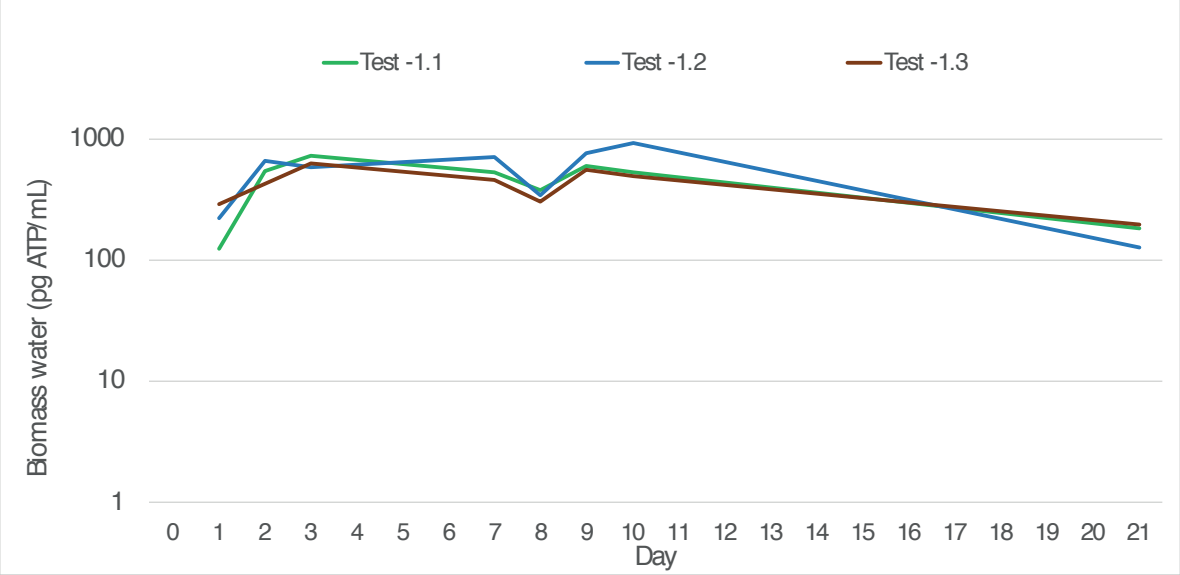

Figure 35 *Biomass in water (pg ATP/mL) in three parallel test systems during test run -1; no anti-biofilm technologies were applied during this test run.*

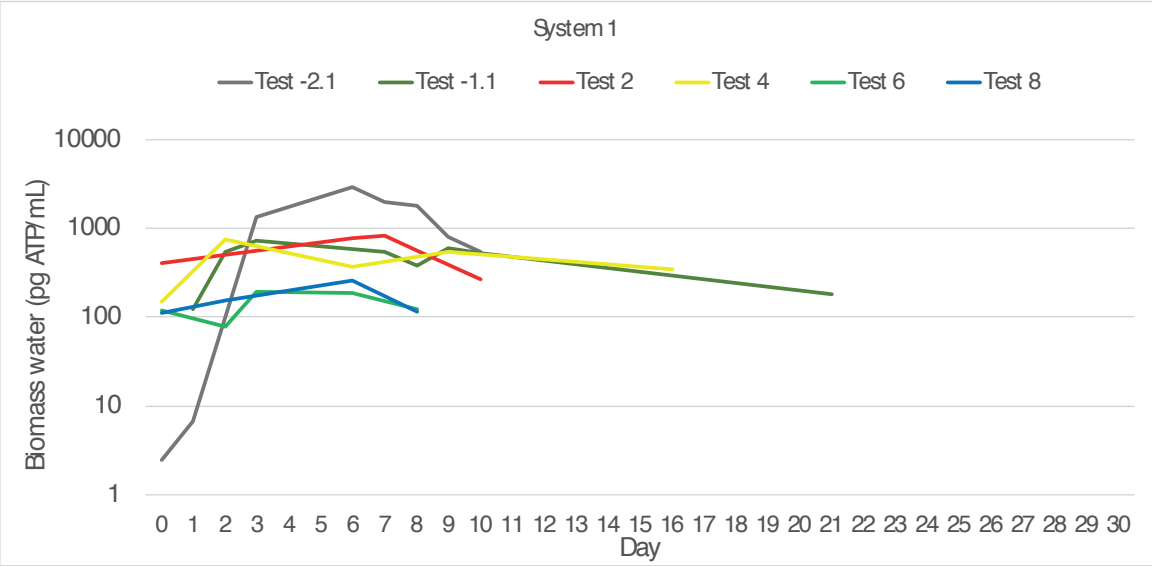

Figure 36 *Biomass in water (pg ATP/mL) in system 1: comparison of biomass development in six test runs without treatment.*

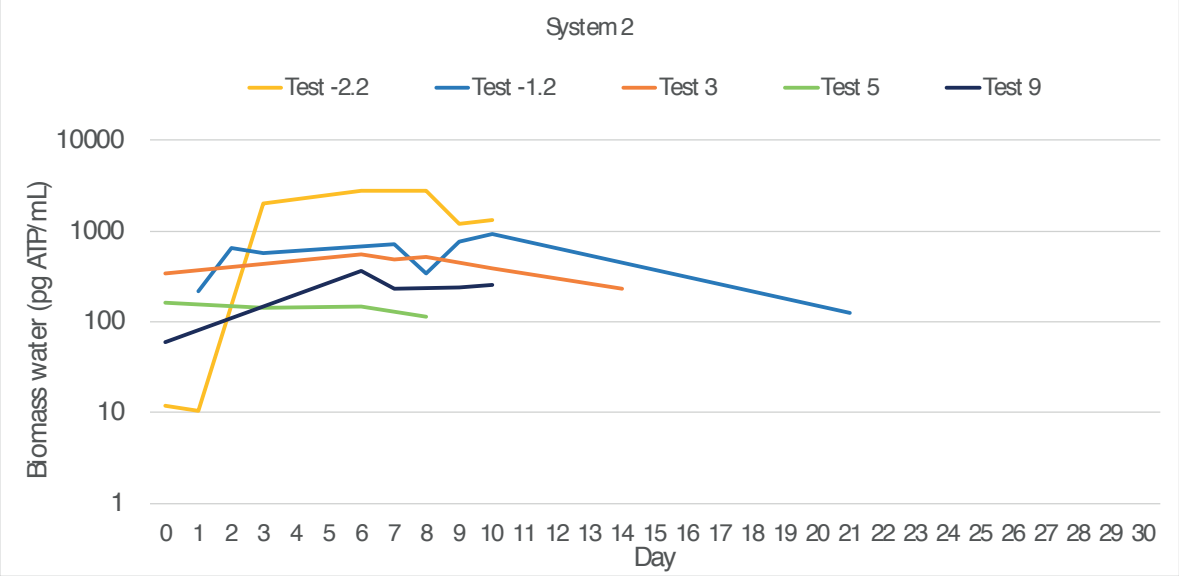

Figure 37 *Biomass in water (pg ATP/mL) in system 2: comparison of biomass development in six test runs without treatment.*

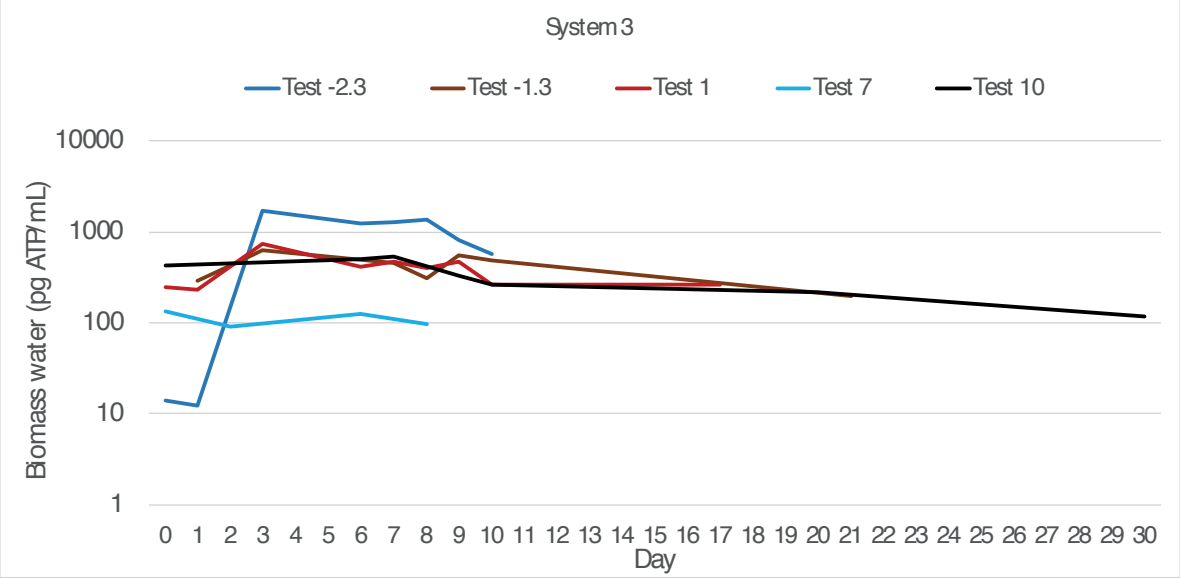

Figure 38 *Biomass in water (pg ATP/mL) in system 3: comparison of biomass development in six test runs without treatment.*

For biomass in water we see a maximum of 500 – 900 pg ATP/mL, reached at day 2-3. The level stabilises after day 2-3 and only slightly decreases later on. In most test runs the increase in the first days is not as fast as in the formation of biomass in biofilms. The maximum levels in test runs -2, 5 and 7 differ from the control treatments in test runs.

Annex 4 Total oxidants measurement

CH2O B.V.

MAKING WATER WORK FOR YOU

Totale Oxidanten meting

Benodigdheden voor de test:

- Wit flesje "Total Oxidant #1"
- Wit potje "Total Oxidant #2"
- Transparant flesje "Total Oxidant #3"
- Plastic maatbekertje
- Maatlepeltje 0,2 gram

De test werkt als volgt:

1. Spoel het maatbekertje twee maal en vul de maatbeker met 10 ml water, waarbij de onderste lijn op de 10 ml lijn moet staan.
2. Voeg bij het maatbekertje 5 druppels toe uit het witte flesje Total Oxidant #1 en schud de maatbeker even kort.
3. Voeg 1 maatlepel poeder uit het witte potje Total Oxidant #2 toe aan de maatbeker en zorg er voor dat je de poeder zo goed als mogelijk oplost in het water.
4. Als het Emitter Clean product/chloordioxide in het water aanwezig is dan zal het water paars gaan kleuren. Dit vertelt twee dingen. Het eerste is dat het product daadwerkelijk meegegeven wordt en de het tweede is dat hoe donkerder de kleur, hoe hoger de gedoseerde concentratie.
   Kleurt het water niet dan kan dit komen doordat er geen product geïnjecteerd wordt (actie: check of slangetje geel is) of dat de test niet goed is uitgevoerd.
5. Voeg druppels toe uit het transparante flesje Total Oxidant #3. Iedere druppel is 1 ppm aan Totale Oxidanten. Het totaal aantal druppels wat nodig is om het water helder te maken geeft de totale hoeveelheid ppm's aan Totale Oxidanten van het geteste water weer.

- **Bij twijfel of wanneer er een afwijkende meting gedaan is herhalen**
- **Aub de meting of het gemiddelde van twee metingen op het registratie formulier invullen.**

Bij vragen of mogelijke problemen aub contact opnemen met CH2O BV op 0174-504785 of een mail sturen naar info@ch2o.nl

Linnewever 32 - 2292 JH Wateringen - T +31 (0) 174 - 50 47 85 - E info@ch2o.nl - W www.ch2o.nl
IBAN NL73 RABO 0368 7860 48 - BTW NL 851 709 965 B01 - KvK 554 346 22

Annex 5 Chlorine dioxide measurement

CH2O B.V.

MAKING WATER WORK FOR YOU

Palinmeter Chloordioxide meting

1. Spoel beide flesjes twee maal en vul 1 flesjes met 10 ml water, waarbij de onderste lijn op de 10 ml lijn moet staan.
2. Druppel 4 druppels van het **Glycine** flesje in het flesje gevuld met water en roer deze even door met het roerstaafje.
3. Veeg de vingerafdrukken van het flesje af en zet deze met de het vierkantje/ruitje naar voren in de Palinmeter en doe de zwarte dop er op.
4. Nu kun je de Palinmeter aanzetten met de knop onderaan de meter.
5. Dan kies je middels de menu knop **ClO2 10** (middelste knop boven in)
6. Druk daarna de linker knop in van het "lege" flesje. Dit is om de nulmeting te kunnen doen. Als het goed is staat er nu 0.00 in scherm.
7. Dit flesje mag nu uit de meter en je mag dit water vervolgens gebruiken om 2 tot 3 mm water in het lege flesje over te gieten.
8. Daarna een **DPD 1** pil pakken en deze in het flesje met de 2 tot 3 mm water fijn maken en oplossen.
9. Als de DPD 1 pil redelijk opgelost is, mag de rest van al het water bij de oplossing met de DPD1 pil. Als er chloordioxide in zit kleurt de vloeistof roze. Hoe donkerder hoe hoger de concentratie.
10. Nogmaals goed roeren en daarna de vingerafdrukken van het flesje afhalen.
11. Het flesje met de roze oplossing met het vierkantje naar voren weer terug in de Palinmeter zetten.
12. **Vervolgens 1 minuut wachten**
13. Daarna de rechter knop met het "volle" flesje op de Palinmeter indrukken.
14. Nu staat de meting in het scherm van de hoeveelheid chloordioxide wat in het water zit. Dit is aangegeven in ppm.

- **Bij twijfel of wanneer er een afwijkende meting gedaan is herhalen**
- **A.u.b. de meting of het gemiddelde van twee metingen op het registratie formulier invullen.**

Bij vragen of mogelijke problemen a.u.b. contact opnemen met CH2O BV op 0174-504785 of een mail sturen naar info@ch2o.nl

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Annex 6 PERMANOVA results

Chlorine dioxide, prevention

Table 1

Overall PERMANOVA results for chlorine dioxide, prevention.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	1.49697
p-value	0.0664
number of permutations	9999

Table 2

Results of the pairwise PERMANOVA results (comparisons) for chlorine dioxide, prevention.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2	Samples	Permutations	Pseudo-F	P value	Q value
End_ClO2	End_control	4	9999	1.496858	0.3305	0.4032
	Start_ClO2	4	9999	1.472125	0.3360	0.4032
	Start_control	4	9999	1.361221	0.3298	0.4032
End_control	Start_ClO2	4	9999	2.164032	0.3328	0.4032
	Start_control	4	9999	2.103757	0.3345	0.4032
Start_ClO2	Start_control	4	9999	0.374148	0.6722	0.6722

Chlorine dioxide, removal

Table 3

Overall PERMANOVA results for chlorine dioxide, removal.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	0.779371
p-value	0.7084
number of permutations	9999

Table 4

Results of the pairwise PERMANOVA results (comparisons) for chlorine dioxide, removal.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_ClO2	End_control	4	9999	1.266013	0.3362	0.80172
	Start_ClO2	4	9999	0.876915	0.6612	0.80172
	Start_control	4	9999	0.759838	0.6681	0.80172
End_control	Start_ClO2	4	9999	0.935109	0.6605	0.80172
	Start_control	4	9999	0.269221	0.6606	0.80172
Start_ClO2	Start_control	4	9999	0.517739	1.0000	1.00000

Table 5

Overall PERMANOVA results for electromagnetic waves, prevention.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	1.36342
p-value	0.2507
number of permutations	9999

Table 6

Results of the pairwise PERMANOVA results (comparisons) for electromagnetic waves, prevention.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_EMW	End_control	4	9999	0.313201	1.0000	1.00000
	Start_EMW	4	9999	1.869551	0.3299	0.49695
	Start_control	4	9999	1.772629	0.3285	0.49695
End_control	Start_EMW	4	9999	2.080789	0.3313	0.49695
	Start_control	4	9999	1.909311	0.3257	0.49695
Start_EMW	Start_control	4	9999	0.260381	1.0000	1.00000

Table 7

Overall PERMANOVA results for electromagnetic waves, removal.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	0.441069
p-value	0.8251
number of permutations	9999

Table 8

Results of the pairwise PERMANOVA results (comparisons) for electromagnetic waves, removal.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_EMW	End_control	4	9999	0.318943	1.0000	1.0
	Start_EMW	4	9999	0.529352	0.6622	1.0
	Start_control	4	9999	0.524632	0.6675	1.0
End_control	Start_EMW	4	9999	0.755157	0.6756	1.0
	Start_control	4	9999	0.327073	0.6630	1.0
Start_EMW	Start_control	4	9999	0.198067	1.0000	1.0

Hydrogen peroxide, prevention

Table 9

Overall PERMANOVA results for hydrogen peroxide, prevention.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	0.402876
p-value	0.9143
number of permutations	9999

Table 10

Results of the pairwise PERMANOVA results (comparisons) for hydrogen peroxide, prevention.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_H2O2	End_control	4	9999	0.561606	1.0000	1.0
	Start_H2O2	4	9999	0.223176	0.6688	1.0
	Start_control	4	9999	0.441789	1.0000	1.0
End_control	Start_H2O2	4	9999	0.581575	1.0000	1.0
	Start_control	4	9999	0.226515	0.6633	1.0
Start_H2O2	Start_control	4	9999	0.392278	1.0000	1.0

Hydrogen peroxide, removal

Table 11

Overall PERMANOVA results for hydrogen peroxide, removal.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	0.402876
p-value	0.9143
number of permutations	9999

Table 12

Results of the pairwise PERMANOVA results (comparisons) for hydrogen peroxide, removal.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_H2O2	End_control	4	9999	0.561606	1.0000	1.0
	Start_H2O2	4	9999	0.223176	0.6688	1.0
	Start_control	4	9999	0.441789	1.0000	1.0
End_control	Start_H2O2	4	9999	0.581575	1.0000	1.0
	Start_control	4	9999	0.226515	0.6633	1.0
Start_H2O2	Start_control	4	9999	0.392278	1.0000	1.0

Table 13

Overall PERMANOVA results for ultrasound, prevention.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	1.20261
p-value	0.2109
number of permutations	9999

Table 14

Results of the pairwise PERMANOVA results (comparisons) for ultrasound, prevention.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_US	End_control	4	9999	0.935118	1.0000	1.00000
	Start_US	4	9999	1.448427	0.3433	0.51495
	Start_control	4	9999	1.552266	0.3350	0.51495
End_control	Start_US	4	9999	1.290880	0.3279	0.51495
	Start_control	4	9999	1.287175	0.3271	0.51495
Start_US	Start_control	4	9999	0.663307	1.0000	1.00000

Ultrasound, removal

Table 15

Overall PERMANOVA results for ultrasound, removal.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	8
number of groups	4
test statistic	1.16747
p-value	0.3552
number of permutations	9999

Table 16

Results of the pairwise PERMANOVA results (comparisons) for ultrasound, removal.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_US	End_control	4	9999	2.223895	0.3265	0.6548
	Start_US	4	9999	0.461315	0.6659	0.6680
	Start_control	4	9999	1.426113	0.3274	0.6548
End_control	Start_US	4	9999	1.783980	0.3270	0.6548
	Start_control	4	9999	0.524769	0.6680	0.6680
Start_US	Start_control	4	9999	0.961324	0.6669	0.6680

Antibacterial pipe material, prevention

Table 17

Overall PERMANOVA results for antibacterial pipe material, prevention.

PERMANOVA results	
method name	PERMANOVA
test statistic name	pseudo-F
sample size	10
number of groups	4
test statistic	1.34214
p-value	0.0898
number of permutations	9999

Table 18

Results of the pairwise PERMANOVA results (comparisons) for antibacterial pipe material, prevention.

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	Group 2					
End_ABP	End_control	6	9999	1.752795	0.0955	0.2066
	Start_ABP	5	9999	0.756309	0.7015	0.7015
	Start_control	5	9999	0.900121	0.5985	0.7015
End_control	Start_ABP	5	9999	2.371845	0.1033	0.2066
	Start_control	5	9999	2.085041	0.0997	0.2066
Start_ABP	Start_control	4	9999	0.827601	0.6667	0.7015

Annex 7 Microbial profiling

The five different disinfection strategies were analysed with microbial profiling. In Figures 38-42 the relative abundance of bacterial strains were shown for the different treatments in water and biofilm for the removal and the prevention tests. In general, taxonomic analysis revealed that most of the sequences in all the samples were associated with the prevalence of the phyla Proteobacteria (58–43%), Actinobacteria (23-6.8%) and TM7 (13.1-6.4%).

Oxyl-PRO S (H_2O_2)
The barplots show that removal strategy with Oxyl-PRO S lowered the proportion of Betaproteobacteria, Sphingobacteria and TM7-3 in biofilm and increased the abundance of Cytophagia in water (Figure 38, top). The prevention strategy with Oxyl-PRO S showed that there is a reduction of Betaproteobacteria in biofilm and an increase in water (Figure 38, bottom). There is a significant increase in both matrices of Alphaproteobacteria and a decrease Saprospirae and Cytophagia, and of Gammaproteobacteria in water.

SureFlow Chlorine dioxide (ClO_2)
Based on the class abundances of the barplot (Figure 39), water samples comprised comparable dominant taxa as the respective biofilms and variances were related to the higher abundance of Betaproteobacteria in biofilm and Saprospirae in water. Comparing the different strategies, removal had similar abundance profiles at the start for both water and biofilm, with a decrease in abundance of Alphaproteobacteria and Gammaproteobacteria for both matrices and an increase of Actinobacteria over time (Figure 39, top). A similar pattern was seen for prevention: the proportion of Actinobacteria was highest in the end in both water and biofilm when compared to day 0. In water, Betaproteobacteria, Gammaproteobacteria, SJA-4 and Saprospirae were reduced while TM7-3 increased (Figure 39, bottom).

Antibacterial pipe material (ABP; prevention only)
The bacterial composition the barplot (Figure 40) reveals that differences between water and biofilm were due the dominance of SJA-4 in water samples than biofilm and due to the higher abundance of Planctomycetia and Saprospirae in biofilm when compared to water. Large differences in community composition over time were observed between runs. The main alterations were found with the increase of TM7-3, Betaproteobacteria and decrease of Gammaproteobacteria in both matrices. Interestingly, Planctomycetia decreased until 8 days and then increased over time in the biofilm.

AQUA4D® (EMW)
In Figure 41 the bacterial classes are shown for removal (top) and prevention (bottom) strategies. In general, when comparing the same sampled days, biofilm and water samples were characterized by similar bacterial profiles. On average, Betaproteobacteria were more abundant in biofilms and Saprospirae and SJA-4 had higher relative abundance in water. The removal strategy showed an On day 10, the abundance decreased over time of Alphaproteobacteria for both matrices (biofilm and water) and Flavobacteria mainly in biofilm when compared to day 6. The prevention strategy Day 8 showed an increase of Betaproteobacteria and Sphingobacteria when compared to day 0 over time. The bacterial community composition with the prevention strategy of day 0 and 8 remained relatively stable over time despite the variations in proportions of TM7-3, Betaproteobacteria in water and biofilm and SJA-4 in water and the reduction of Gammaproteobacteria and Saprospirae in water and biofilm. The bar graphs confirm the results of the PCoA: shifts in bacterial community composition occur over time, but no clear EMW treatment effects are visible.

Ultrasound (US)
The bacterial composition in water and biofilm was similar (Figure 42). For the removal strategy (top), a higher abundance of Betaproteobacteria and a decline of Alphaproteobacteria in water was the main difference between water with and biofilm. After removal there was an increase of Sphingobacteria and TM7-1 for in both matrices. With prevention (bottom), there is an increase of TM7-1, TM7-3, Betaproteobacteria and SJA-4 in water and a decrease of Gammaproteobacteria and Saprospirae.

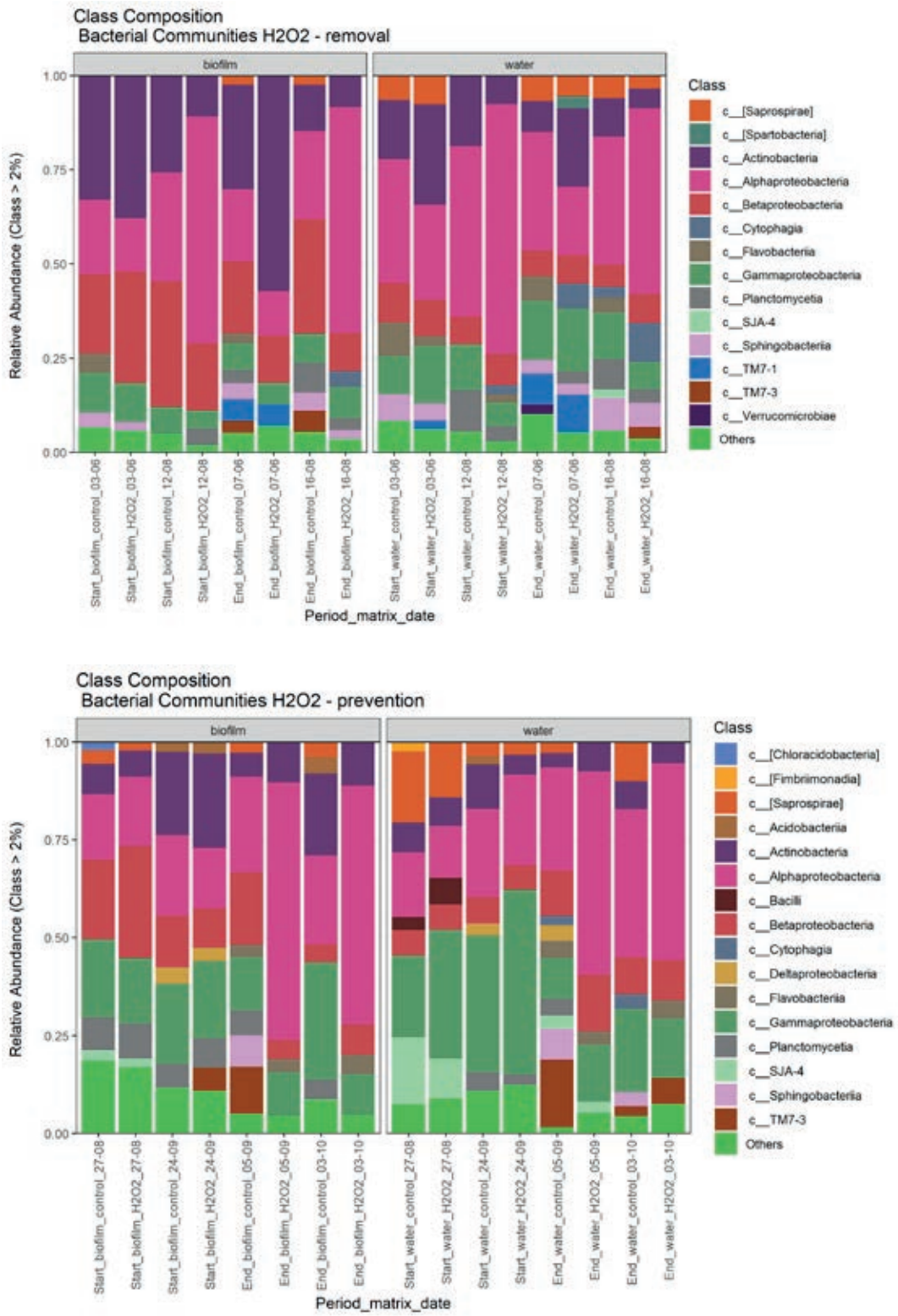


Figure 39 *Relative abundance (> 2%) of water and biofilm of bacterial class for silver stabilized H_2O_2. Top: Removal strategy (start and end), bottom: prevention strategy (start and end). Start and end results are grouped by run date. Classes lower than 2% are grouped in 'others'.*

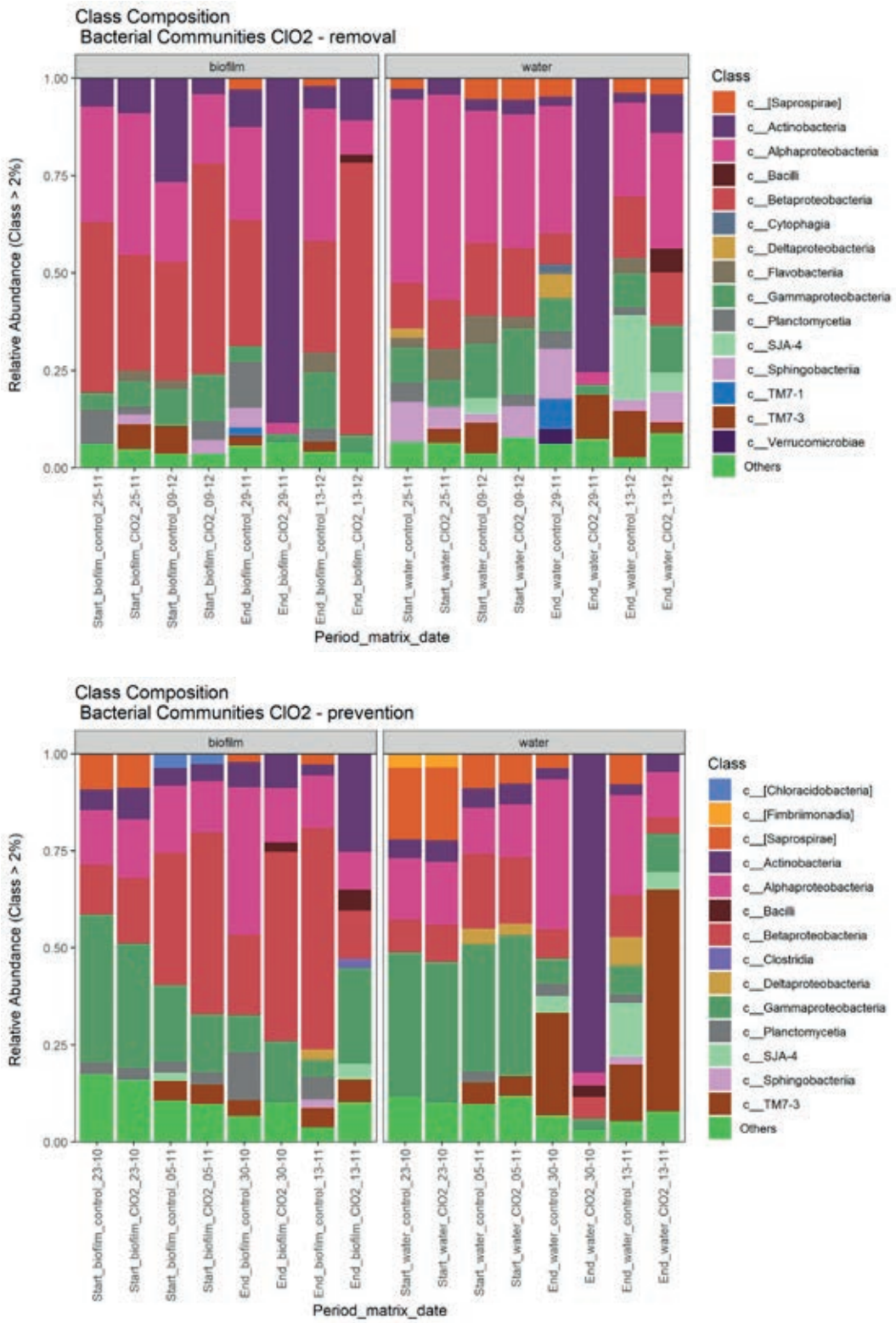


Figure 40 *Relative abundance (> 2%) of water and biofilm of bacterial class for ClO_2. Top: Removal strategy (start and end), bottom: prevention strategy (start and end). Start and end results are grouped by run date. Classes lower than 2% are grouped in 'others'.*

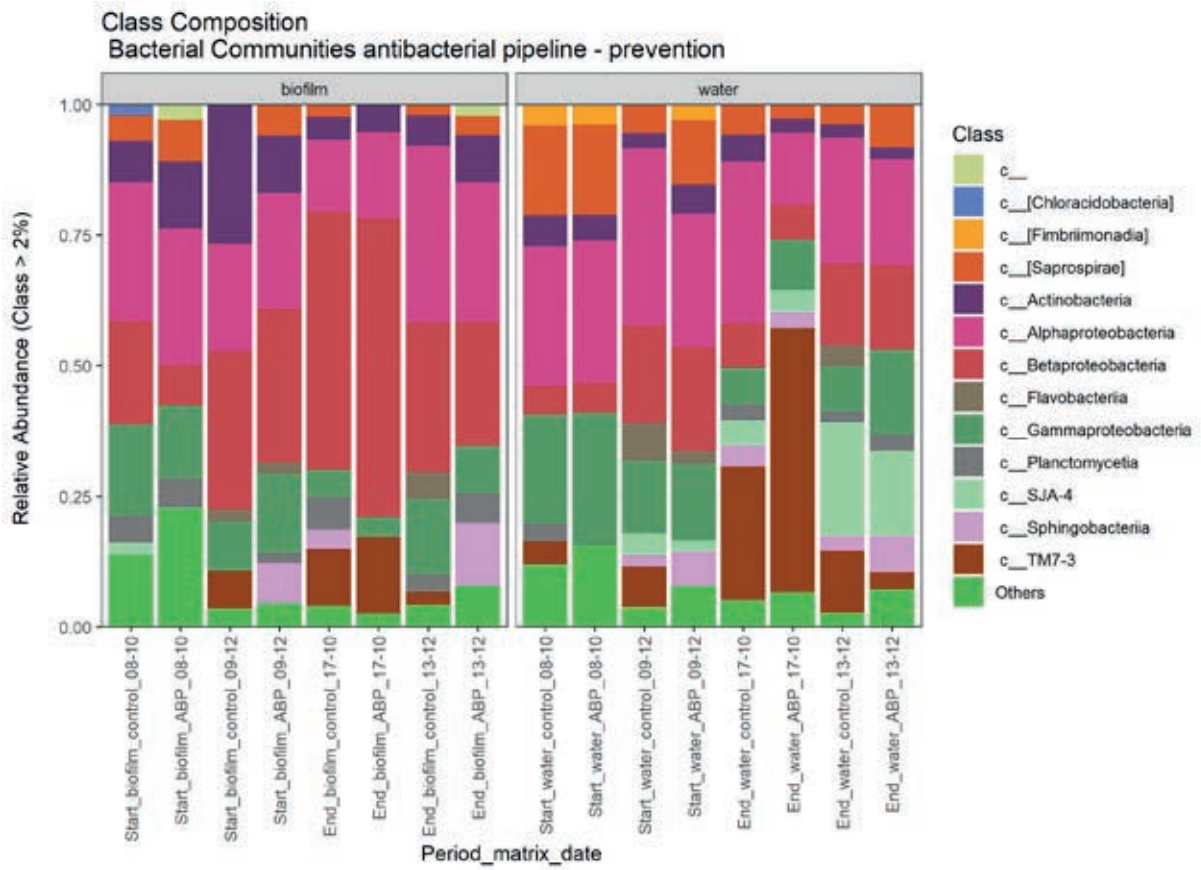


Figure 41 *Relative abundance (> 2%) of water and biofilm of bacterial class for antibacterial irrigation pipes, prevention strategy. Start and end results are grouped by run date. Classes lower than 2% are grouped in 'others'.*

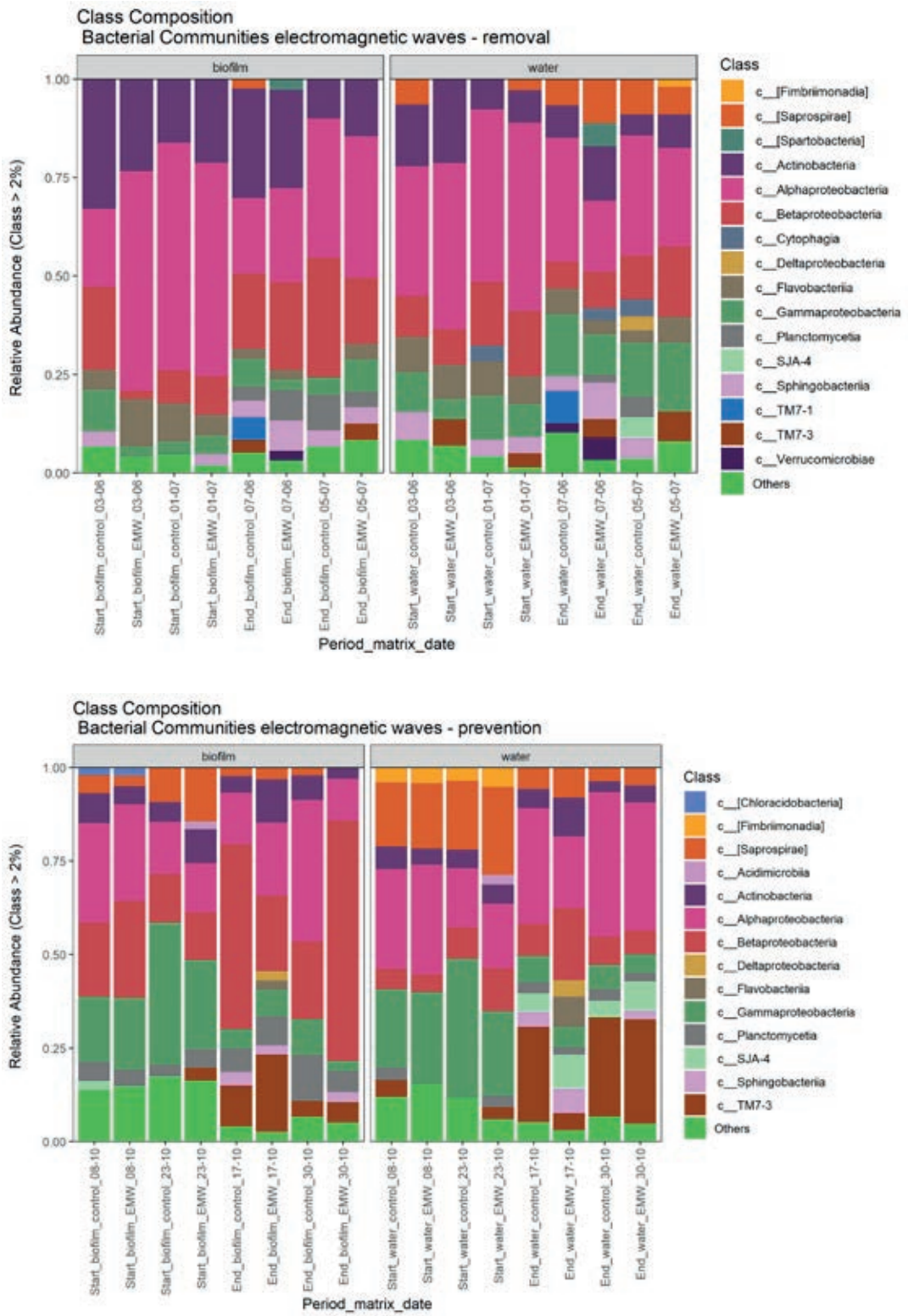


Figure 42 *Relative abundance (> 2%) of water and biofilm of bacterial class for electromagnetic waves. Top: Removal strategy (start and end), bottom: prevention strategy (start and end). Start and end results are grouped by run date. Classes lower than 2% are grouped in 'others'.*

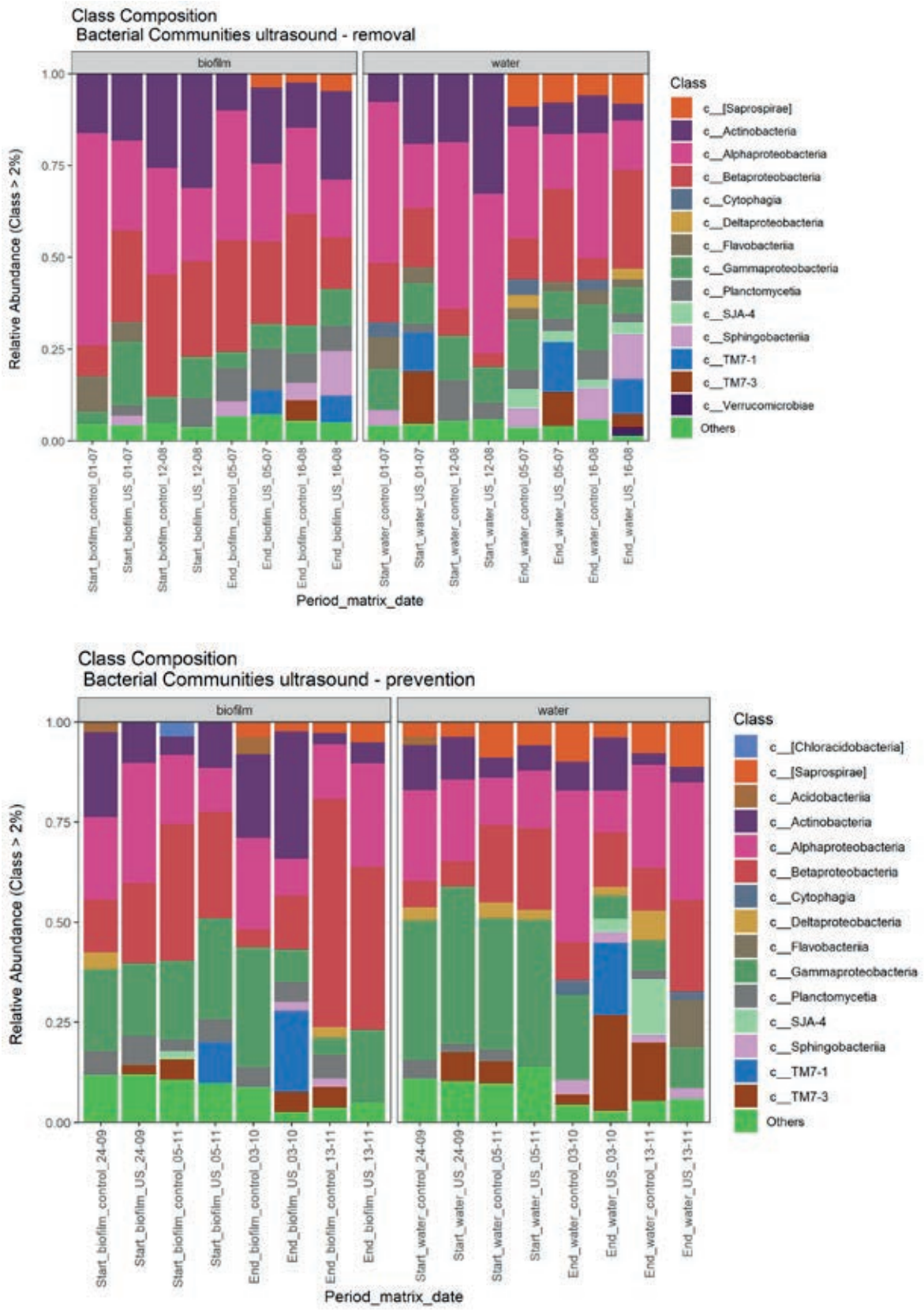
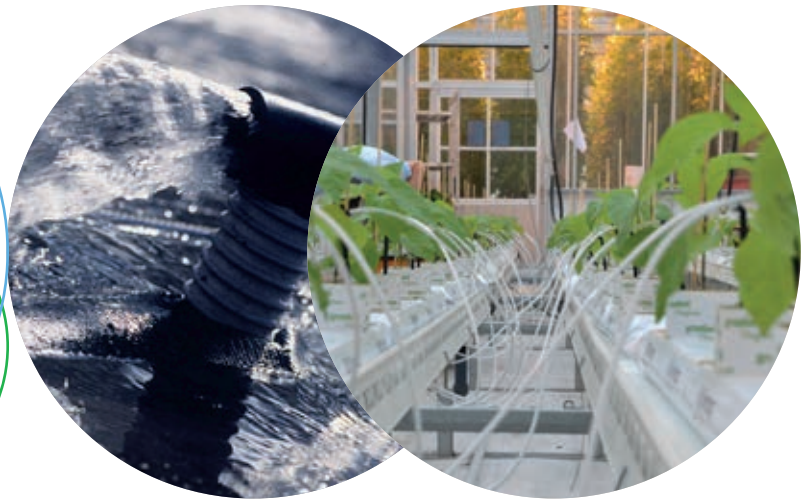


Figure 43 *Relative abundance (> 2%) of water and biofilm of bacterial class for ultrasound strategy. Top: Removal strategy (start and end), bottom: prevention strategy (start and end). Start and end results are grouped by run date. Classes lower than 2% are grouped in 'others'.*

To explore the potential of nature to improve the quality of life



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Report WPR-905

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