Biofilm growth in strong electric fields

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2011-02-10
'No book is ever finished. It is abandoned.'
- Joyce Carol Oates
Abstract

Bacterial surface associated communities, so called biofilms, located on the inside of drinking water distribution pipes, are believed to be responsible for lowering the drinking water quality by releasing bacteria into the water stream. As there were some indications that a strong electric field along the pipes inner walls could deter and possibly kill bacteria trying to adhere to the surface, it was the aim of this thesis to investigate that effect and its possible application in tap water.

A closed system was set up with three cylindrical containers, made from short pieces of the pipe intended to be used in water distribution systems, with isolated electrodes on the inside. The voltage (0 – 10 kV) as well as pulse length (0 – 110 ms) and pulse repetition rate (1 Hz or 5 Hz) was varied in order to find the optimal settings. Through the system, liquid acetate enriched minimal media inoculated with *Comamonas dentitrificans* 110 as model organism was being pumped. Each run lasted for a week, after which the biofilm in the containers were stained with 1 % crystal violet, and the biofilm formation analyzed.

The above system was not ideal for studying any effects of the electric field; the results were inconclusive though a trend showing biofilm deterrence could be seen, with long pulses at a high pulse repetition rate being the optimal setting.

Bacterial concentrations and ion strengths in the three container system could not be kept comparable to that in distribution pipes, so a lone container, was set up in an open system, with only tap water (adjusted to 30°C) running through it for 4 months, in order to assess the biofilm thickness and the speed at which it forms. A thin but evenly distributed biofilm was seen on the inside of the tap water container indicating that further testing could be done in tap water with a very low risk of biofilm not forming inside the control container.

To clarify how the electric field affected biofilm, small volumes of cultures containing detached pieces of biofilm were put into a 1 ml cuvette over which an electric field similar to that in the big system was applied. Electrolysis was made using similar solutions so that comparisons could be made.

Little disturbance could be observed in the structures when applying an electric field, but the effects seen in these test was very subtle compared to electrolysis were almost all biofilm fragment had lost their initial structure.

Whether strong electric fields will prevent biofilm formation is unclear, it seems unlikely, but not impossible. The field will have a wider range in tap water and maybe boost the effect of chlorine, but will also go up against much tougher bacteria.
Abbreviations

PE Polyethylene
MDPE Medium-density polyethylene
HDPE High-density Polyethylene
EVOH Ethylene vinyl alcohol
NA Nutrient Agar
NB Nutrient Broth
OD Optical Density
CFU Colony Forming Units
UV Ultra violet
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1. Introduction

Biofilm growth in drinking water distribution systems is a problem. The water leaving the distribution plants have to be very clean (less than 10 bacteria/ml) [1]. Despite hypochlorite or other chloride containing disinfectants being added to the water in order to inhibit bacterial growth, the bacterial concentration has multiplied when the water reaches the consumer. This is probably due to resistant biofilms on the inner walls of the distribution pipes [2][3]. In Sweden the highest tolerated number of bacteria in drinking water reaching the consumer is 100/ml, there is no limit as to how much biofilm that is allowed within the distribution pipes [1].

Cleaning the insides of biofilm infested pipes with strong oxidizing chemical agents have, even if they claim to be effective against biofilm, a very limited effect [4]. To the extent that these disinfectants are effective, it applies to certain laboratory grown biofilms, typically formed in a short period of time by specific model organisms. A tougher approach, involving physically cleaning the pipes, is usually necessary when dealing with the mature biofilms found in water distribution systems.

KPS Petrol systems specialize in the manufacture of electrically conducting pipelines, which are used in oil distribution to prevent the buildup of static electricity within the distribution pipes as the oil rubs against the inner surfaces. Indications exist from previous trials that a pulsating electric field in a water distribution pipeline could be used to fight biofilm buildup, especially if it is used continuously, as a preventive measure. By modifying the KPS pipes and connect theses to a high voltage generator, a strong electric field can be upheld along the walls on the inside of the pipes and possibly prevent the buildup of biofilm.

To investigate how biofilm growth is affected inside KPS pipes, three containers of pipe material were connected in series trough tubing in a closed circulation system. One container serves as control the two others are connected to a high voltage generator with the purpose of creating strong electric fields within the containers.

The aim of this thesis was to evaluate the effect of electric fields on bacteria, primarily by using the above setup, to ultimately be able to decide whether such a system could be used to reduce the occurrence of biofilm inside water distribution pipes.
2. Background

2.1. Biofilm

Biofilms are according to J. W. Costerton: “... defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces” [5]. The often very mature biofilms encountered in nature are usually multilayered communities within a highly hydrated, porous, extracellular matrix. Biofilms were recently characterized as the most successful forms of life on earth [6]. Regardless of whether biofilms are *forms* of life – and not formed, most bacteria on our planet exist in biofilms. But forming biofilm is a means of survival, a stress response; it is the safest, not the fastest, mode of growth. The absence of selection mechanisms promoting biofilm, is the reason why bacteria gradually lose their ability to form biofilm, when cultured in laboratory environments for successive generations [7].

The development of a biofilm starts with coordination to and subsequent attachment of a few cells to a surface or interface. This taxis is often motivated by the occurrence of organic and/or inorganic nutrients inherent to, or loosely adhered to, the surface [8][9]. The initial attachment of the cells is usually a very weak, but highly coordinated effort, often mediated by flagella, where the cells are evenly distributed across the surface forming a thin monolayer [10]. After attachment microorganism looses its flagella and the cellular machinery is instead focused on making components of an extracellular matrix that is used to provide structure to the biofilm and facilitate the introduction of even more layers upon the initial one. The production of this matrix, in conjunctin with new layers of cells, gives rise to what is called a mature biofilm with an intricate porous network spurring different niches and bacterial heterogeneity. The niches are facilitated by a wide range of different gradients (pH, oxygen, nutrient) [11] that the extracellular matrix, through consisting of up to 97% water and complex irrigation systems, give rise to [12]. After this stage, detachments of biofilm will begin to occur, eventually reaching a quasi-stationary equilibrium with biofilm growth.

The extracellular matrix consists of a wide range of substances, mostly polysaccharides, proteins and nucleic acids. Of these macromolecules, the polysaccharides are the most studied group, and they are widely considered to be the main structural component of the extracellular matrix providing it with rigidity [13]. The matrix polysaccharides are usually called *exopolysaccharides* and are abbreviated EPS. That abbreviation could cause, and has given rise to, much confusion as all matrix associated macromolecules are included in the much more broadly defined *extracellular polymeric substance* with the same abbreviation, therefore the term EPS will not be used in this paper. The role of the matrix proteins and nucleic acids are largely unknown, though it has been shown that bacteria living in biofilms retain their excreted enzymes in close proximity due to the lower diffusion rates inside the extracellular matrix and the closeness to the surface. With such a scheme they can increase the efficiency of their nutrient uptake [6].

The reason microorganisms aggregate to form these complex societies are several, but all have to do with survival. Biofilms have proven highly resistant to several types of antibiotic and chemical
disinfectants. Mature biofilm can be extremely rigid and hard to get rid of even by rough physical forces. In the diffusion limited environment the matrix provides, there is a much more efficient exchange of information through quorum sensing and sharing of genetic information through conjugation, than for planktonic bacteria in solution [10]. The matrix also works as sieve in nutrient poor environments by working as an ion exchanger capturing charged molecules.

2.2. *Comamonas denitrificans* 110

*Comamonas denitrificans* 110 (figures 1 and 2), has shown great potential as an organism to be utilized in wastewater treatment studies, as a biomarker, due to its superior denitrifying ability [14] and willingness to form biofilm [15]. It is a gram negative, rod shaped, facultative anaerobic bacteria with flagellum in both ends [14]. When grown in a minimal media utilizing acetate as its carbon source, *Comamonas denitrificans* 110 has been shown to produce thick biofilm rapidly, in contrast to when glucoses is used, which cuts biofilm production by more than half [16]. Thus acetate seems to be a key ingredient for inducing biofilm development in *Comamonas denitrificans*.

![Figure 1. Monolayer of biofilm made by Comamonas denitrificans when grown in a minimal media with acetate as carbon source at 1000 times magnification.](image)
2.3. Drinking water

The concentration of bacteria in water distribution pipes is by law required to be very low [1]. Different ways of disinfecting the waters are used, all with varying success, the most common being chlorine in the form of hypochlorite.

The mechanism behind the bactericidal effect of chlorine is unclear, though it is particularly effective against gram negative bacteria, implying an interaction with the cell wall barrier [17]. Even though the introduction of chlorine into water distribution systems in the developed countries has contributed to the eradication many waterborne diseases, possible side-effects caused by long term exposure to toxic disinfection by-products have led to a shift towards other types of chemical disinfectants - like ozone or chloramines, and other ways of disinfecting the system – like ultraviolet (UV) light [18]. Because of their quick ability to adapt to harsh conditions, just changing the disinfectant could drastically improve the
drinking water quality, at least temporarily. Bacterial resistance to chlorine is widely spread within water distribution pipelines [19].

All methods for disinfection have their drawbacks and none are particularly effective when it comes to deterrence of biofilm formation inside the water distribution pipes [18][2]. Especially bad is UV radiation, which is dependent on a high surface exposure of the bacteria to have a bactericidal effect as the radiation does not penetrate far enough into the biofilm [20].

The problem is bigger in pipes where the diameter gets smaller and flow rate is slow. It is a particular problem in dental units that, even though regular washing with disinfectants is done, host high amounts of biofilm [21]. Despite of the problem, because of the absence of an effective solution, there are no laws regulating the amount of biofilm that is allowed within the water distribution pipes.

2.4. Electric fields

Even though using electric fields in biotechnology research is quite common, with electrophoresis probably being the most common, few studies concentrate on electric fields alone. In most experiments medium, to strong, currents are used.

As to the nature of an external electric field, originating from isolated electrodes, it should make the influence of the inherent electric potential of the isolating material negligible. In contact with a solution, the surface electric potential influences the chemical environment directly adjacent to it, but its range is normally very short unless solutions with very low ionic strengths are used [11].

It has been reported that strong pulsed electric fields, though leaving cell morphology intact, have a significant bactericidal effect, but is unclear as to why that is [22]. It might be possible that the field, if bacteria come sufficiently close to it, induces electroporation, causing molecular transport of charged molecules across the membrane. The prevailing theory is that electroporation is caused by a favouring of dipole interactions in the direction of a strong electric field and that the phospholipids’ heads situated along the cell wall perpendicular to the field, by having large dipole moments, being forced to make a hole in the membrane structure in order to align themselves with the field [23]. For electroporation to be successful a low ion strength of the liquid is of importance, not only because ions shield the bacteria from the field but because of the presence of charged molecules in between the phospholipids’ heads making it harder for them to align with the field, as revealed by computer modeling [24].

Another hypothesis is that the field through affecting ion gradients, and thus flagella, disrupts the bacteria’s ability to navigate and thus weakens the biofilm structure during attachment and subsequent buildup of multilayered biofilm.

Setting up a system connected to a high voltage generator with isolated electrodes allows for very low currents but creates a strong electric field while leaving a negligible magnetic field. In the absence of literature dealing with effects of this kind, good data becomes imperative.
3. Materials & Methods

3.1. The three-container system

![Diagram of the three-container system](image)

*Figure 1. Model of a closed system consisting of a Duran flask with nutrient media and three containers connected in series via a peristaltic pump.*

To evaluate the impact of electric fields on biofilm formation, a closed system was set up (figure 1): A 10 liter Duran flask containing liquid culture, a peristaltic pump and three standing cylindrical containers (about 1 liter each) with a rubber plug in each end was connected in series by rapid fittings and polyurethane tubing. The flask was placed on a magnetic stirrer, at a position lower than the rest of the setup. Between the pump and the first container (always the control) an air inlet was located, releasing small bubbles of filtrated air into the system. Each container was filled up through the bottom to minimize the air space inside. Taps where located close to the bottom of each container, enabling them to be emptied separately. One of the containers served as a control while the other two were hooked up to a high-voltage generator. The voltage adjustment (0-10kV) was common to both containers, while
pulse length (0-110 ms) and pulse repetition rate (1 or 5 Hz) could be set individually. From the top where liquid media was flowing out of the containers, samples could be taken from the system through rubber septa. A sensor for measuring temperature was situated at the top of the last container. The system was built on a laboratory bench, all electronic settings were done on controls separately connected to the high voltage source, and the peristaltic pump was covered in Styrofoam for noise reduction (figure 2). To reduce power consumption which also increased safety, a very low current (less than 1 µA) was used. A very high voltage (10 kV) was applied along the inside of the pipes.

Figure 2. Picture of assembled three-container system. On the bench, from the left: High, voltage generator, pulse controller, voltage controller, temperature meter and Styrofoam enclosed peristaltic pump.

All containers were short plastic pipes (length: 300 mm, inner diameter: 76 mm), similar to those that KPS plan to use in water distribution systems. Thick rubber plugs with rapid fittings covered both ends of each container making an inner volume of about 0.8 dm³ and an inner wall surface area of 4.3 dm². The inside of the containers were covered by a thin removable membrane of HDPE (High-density Polyethylene) attached to a thin layer made of a copolymer of EVOH (Ethylene vinyl alcohol) and PE. By being removable, the inner layer facilitates an easy way to analyze biofilm growth patterns after staining. The two plastic layers isolate the underlying electrodes (six stripes of HDPE filled with carbon black, 2 cm wide, and 2 cm apart from each other), preventing oxidation-reduction reactions from occurring. The containers were covered by an outer casing made of MDPE (Medium-density polyethylene).
Before building the system before each run, all fittings were autoclaved, the containers and rubber plugs were thoroughly washed and new polyurethane tubing was used. Deionized water was pumped through the system at first to provide an initial cleaning and to make sure that no water was leaking out and no air was leaking in. The air inlet (a rubber septa with a needle connected to a 0,22 μm filter) was then set up and the diameter of the needle modified with pair of pliers so as to give a continuous flow of bubbles. This was followed by double distilled autoclaved water being pumped through the system for about two hours before bacteria and growth media were added.

*Comamonas denitrificans* 110 were cultured in acetate minimal media for 20 - 24 hours before being added to 6 liters of media that was subsequently connected to the three-container system. The initial bacterial concentrations were all well below 1000 colony forming units per milliliter (CFU/ml) to avoid the results from an earlier run where the inner membranes were completely clean after one week (Appendix, previous runs, run three, page ). It was assumed, based on earlier plate counts that the bacterial concentration of a bacterial solution with and optical density at 620 nm of 0.1 was about 7 000 000/ml (appendix, correlating optical density to colony forming units, page 67) and the initial concentration in each run was accordingly adjusted to 200 CFU/ml. Most bacterial concentrations were double checked with plate counting, as it is very hard to correlate bacterial concentration to optical density for a biofilm forming organism as pieces of biofilm tend to affect the absorbance values as well as the number of colonies present to a great extent.

The media used in all runs was, except where it says otherwise, was an acetate minimal media [26] consisting of 1% (w/v) sodium acetate trihydrate, (w/v) 0.24 % HEPES, (w/v) 0.21 % NaHCO3, (w/v) 0.0033% Bushnell-Hass Broth, (w/v) 0.05% yeast extract and (w/v) and 0.05 % Casamino acids. The media was made in a 10 liter Duran flask that later would be connected to the three-container system. A magnet was added to enable an even distribution of nutrients within the flask.

After each run, which lasted for about a week, the system was emptied through taps under each container. The liquid culture was then replaced with deionized water, the taps were closed and the water was pumped through the system for 2 hours. The system was subsequently emptied once again, the tubing was thrown away, the plugs were removed from the containers and the fittings were washed and left to dry. The containers were dipped in deionized water three to five times in order to remove planktonic bacteria and loosely attached biofilm. If any biofilm could be seen, a sample was taken to be analyzed under the microscope. The containers were then lowered into a crystal violet solution 0.1 % (w/v water) for 45 min, which stains both bacteria and extracellular matrix [25], subsequently the containers were again washed with deionized water. The containers were allowed to dry in room temperature overnight and then the inner membranes were removed, revealing the extent of biofilm formation on the inside.
Runs in three container system

Run X1
The system was built as described above. A flow meter was used in this run, consisting of a graduated one liter flask with an outlet in the bottom connected in series with the system just before the media returned to the 10 liter Duran flask. The flow was measured by stopping the flow between the flow meter and the Duran flask with a ball valve, making the flow meter fill up and with a stop watch time the interval between each increment of 250 ml.

The voltage was set to 9 kV; one container was connected to a continuous electric field, the other was connected to 110 ms long electric pulses with a pulse repetition rate of 5 Hz. During the run, optical density was measured from samples taken from liquid leaving all containers. The voltage as well as flow rate and temperature were also monitored.

Run X2
The voltage was adjusted to 10 kV. Due to a high similarity between optical densities, no liquid samples were taken from the control but only from the two containers affected by an electric field. A dilution was made on the culture after the run was finished. Otherwise the procedures were the same as in the previous run.

Run X3
The voltage was lowered to 7 kV. To reduce the impact on contaminants to the system, only temperature and voltage was monitored during this run. To lower the initial number of contaminants, 5 liters of 1% Virkon (peroxygenic acid) was circulated through the system for 30 min after deionized water had been used to check for air leaks. It was followed by 3 times 5 liters of autoclaved deionized water preceding the nutrient media. No attempt to do a bacterial count was made before the run was started but the media was diluted the same way as before run X1 and run X2, as described on page 12.

Run X4
This run was started about a month after the previous run had been finished and by this time all efforts to sterilize the system had been abandoned. The main focus was now to test whether it is the pulse itself and not the length of the pulse that is responsible for any observed effects. To also investigate how early contaminations begin to affect the system, microscopic analysis were done on one of the samples taken each day. A portion of each samples were also diluted and plated on NB as a way to further check for impurities by looking at the shapes and relative occurrence of distinct colonies.

The voltage was set to 10 kV, the pulse repetition rate to 5 Hz and the pulse lengths to 110 ms and 6 ms. The flow rate was adjusted to 3 ml/s before the run started. The flow could not be measured during the run as parts necessary for the flow meter were missing.
Run X5
The media was changed to one containing: 0.1% (v/w) yeast with 0.5% (v/w) sodium acetate trihydrate and 0.5% (v/w) sodium nitrate, which had shown promise during earlier testing to see if we could get a more reliable biofilm by favouring denitrifying bacteria. The pulse length of the shortest pulse was increased to 11 ms to see if that would make any difference in the result. New media was connected to the system after 5 days and was pumped in while simultaneously pushing out the old media.

Sterilization of system
An antibacterial liquid had to be developed in order to sterilize the system after it had been assembled. The requirements put on the bactericidal liquid being developed was that the resulting disinfectant would be only mildly corrosive, would be able to kill all bacteria and their spores within 12 hours and that it would not give off any dangerous gases. Because of the large volumes to be used (>5 liters), product must be reusable and preferably not require any special handling when discarded as waste.

Two earlier isolated bacterial contaminants from the three container system, one endospore-forming, the other psychrophilic, were inoculated along with Bacillus mycoides at 30 degrees for at least 48h in 5 ml nutrient broth. The shape of their colonies where studied before any testing started.

Of the contaminant solution, 100 µl was added to 10 ml of different solutions, incubated at room temperature for up to 24 hours. During that time samples were taken out and were analyzed on agar plates incubated at 30 degrees for 24 hours.

The disinfectants tested were: brine; ddH2O, ethanol at different concentrations; dichloromethane in H2O; 1% Virkon (Peroxygenic acid); Yes (Surfactant based dishwashing liquid, also known as Fairy) and other detergents at different concentrations; lemon juice; different pH achieved by adding NaOH and various kinds of acids. Only key discoveries will be discussed in results.

The most effective mixture (10% YES in 50% ethanol, at pH 3) was inoculated with contaminants at 30°C for 24 hours; the 10 ml solution was then diluted 50 times in sterile NB and incubated for another week at 30°C. The solution was then checked for turbidity and inoculated with a fresh set of spore forming contaminants. This constitutes a simplified version of standard dilution suspension tests used when testing the potency of different disinfectants [27].

Nutrient media
Because sterilization attempts were proving unfruitful some attempts were made to alter the nutrient media used in order to consistently get a thick biofilm so as to confidently determine the relative effectiveness of the fields in each run even if the effectiveness of the fields between different runs could not be reproduced.
First a crude method was utilized by simply inoculating from plate to media trying to find key ingredients promoting biofilm. In the end a biofilm formation assay earlier developed at the department of environmental biotechnology [16], were cultures are grown in triplets on a 96 well plate with biofilm cultures diluted to the same initial OD\textsubscript{620 nm} are grown overnight, then stained by crystal violet and destained by ethanol, was used to in an attempt to quantify the results.

The different media used were: Tenfold diluted nutrient broth with 1% (w/v) sodium acetate trihydrate added; the same acetate minimal media used in the three-container system [26]; 0.1% (v/w) yeast with 0.5% (v/w) sodium acetate trihydrate; 0.1% (v/w) yeast with 0.5% (v/w) sodium nitrate; 0.1% (v/w) yeast with 0.5% (v/w) sodium acetate trihydrate and 0.5% (v/w) sodium nitrate.

### 3.2. Tap water container

![Model of the container through which tap water was continuously running](image)

To check the extent of biofilm formation in tap water, a standing container was set up, connected to a water tap, the temperature was adjusted to 30 degrees giving a flow rate of about 7 ml/s and was left to run for 115 days (figure 3). The temperature was checked weekly. Samples of 100 µl were taken sporadically in doublets from water leaving the container, put on Petri dishes and inoculated at 30°C for a week.
After the run was finished the container was dipped five times in deionized water to wash away loose bacteria and biofilm. It was subsequently stained in crystal violet for 45 min, washed in deionized water again and then the inner membrane was taken out.

3.3. Electric field across cuvette

A cuvette (inner bottom dimension of 4 mm x 10 mm) was modified by putting aluminum electrodes on each side (6 mm distance between electrodes). The cuvette was cleaned with ethanol, dried with compressed air and sterilized under UV-light for 30 min. A sample (700 µl, about 17 mm in height) of the liquid culture from a mature (over a week old) C. denitrificans biofilm sample, with much detachment, was added to the cuvette. The volume to surface quotient when the sample was added was 1.4 mm.

The modified cuvette was connected to the same high-voltage generator used for the three-container system and the electronics was adjusted to produce identical field strengths over the cuvette as those present on the inner containers inner membranes (2.5 kV, 5 Hz, 110 ms). Tests were done for 30 min with an old mature biofilm and the results were investigated with phase-contrast microscopy. A separate test were performed where an overnight culture was diluted to an OD$_{620}$nm of 0.1 and placed in the electric field for 30 min. That culture was diluted further 10000 times and three 100 µl samples were taken out and plated before and after testing, to see if any bactericidal effect could be observed.

3.4. Electrolysis

Electrolysis was performed at 15 V for 15 minutes with two platinum coils lowered into a solution of falcon tubes filled with detached biofilm in solution (over a week old) C. denitrificans in acetate minimal media. The experiment was repeated three times and microscopic analysis was preformed of the biofilm before and after electroporation.
3. 5. The test container

A system consisting of only a lone container, but otherwise similar to the three-container system was set up to be able to reliably test hypotheses for maximizing biofilm development in the three-container system. A disinfecting liquid as well as two types of biofilm inducing media were tried.

![Diagram of closed system](image)

*Figure 4. Model of closed system, containing a lone container connected to Duran flask with bacterial culture, being pushed through the system by a peristaltic pump.*

A single container was connected to a two liter Duran flask by polyurethane tubing in a closed system (figure 4). A short string of a silicon rubber tube was used to connect two ends of the polyurethane tubing over a peristaltic pump that was used to force media through the system. Before the tubing was connected to the fittings situated on rubber plugs sealing the container it made a loop at the top of which an air inlet was situated.
Runs in test container

Sterilization run
To test whether the sterilization protocol developed was working the equipment was assembled, washed with deionized water followed by five liters of disinfectant for 24 hours. Crystals were forming in the disinfectant, to get rid of them the system was then washed for three hours with 95% ethanol.

A culture of C. denitrificans was grown in 10 times diluted NB supplemented with 1% (v/w) acetate for 24 hours at 30°C. The culture was then diluted in the same media giving a volume of 2 liters and a bacterial concentration of about 100 CFU/ml. The experimental run was ended after one week, then the inner membrane was stained with crystal violet, removed, and the biofilm pattern was analyzed.

Test container - run 2
The culturing procedures were the same as the previous run except media containing 0.05% (v/w) yeast, 0.05% (v/w) casamino acids, 0.5 % (v/w) sodium acetate trihydrate, and 0.5 % (v/w) sodium nitrate were used. The initial bacterial concentration was below 100 CFU/ml.

Test container - run 3
Because the casamino acid buffered media did not produce sufficient biofilm the yeast media containing 0.1% (v/w) yeast, 0.5 % (v/w) sodium acetate trihydrate, 0.5 % (v/w) sodium nitrate was tried instead. The run was for six days with the culture media being replaced after three days. The materials and protocols were the same as those in the previous run.
4. Results & Observations

4. 1. The three-container system

4. 1. 1. Runs in the three-container system

Run X1
The optical density was identical regardless of where it was measured. It rose sharply the first 42 hours, to then plummet to a local minimum after 72 hours, after which a slow but steady increase in optical density could be observed (figure 5).

![Optical density as a function of runtime for samples taken from all three containers](image)

*Figure 5. Optical density as a function of runtime for samples taken from all three containers*

The voltage varied between 8.9 and 9.1 kV. The flow rate stayed between 2.5 and 2.6 ml/s. Temperature followed room temperature and stayed between 21.5 and 22 °C (Appendix, raw data from three-container systems, page 63). Large fragments began to appear in the flow meter after 42 hours and stayed there until 114 hours when there were only trace amounts left (Figure 6). They reappeared at 167 hours, just before the run was ended.
The initial bacterial concentration in the system was about 400 CFU/ml. The pH was 7.4 when the run was started and 8.0 when the run was finished.

The inner membranes showed a clear difference between the control container and those under the influence of an electric field (Figures 7-9). Both electric field containers were very clean making it hard to see any difference between them. The membrane from the pulsed field container does seem to house a little bit less biofilm but it is unclear whether this difference is the result of chance or actual biofilm deterrence.
Figure 7. Inner membrane taken out of control container from run X1, stained with 0.1 % crystal violet.
Figure 8. Inner membrane taken out of pulsed field (9 kV, 5 Hz, 110 ms) container from run X1, stained with 0.1 % crystal violet.
Figure 9. *Inner membrane taken out of continuous field (9 kV) container from run X1, stained with 0.1 % crystal violet.*
Run X2

The optical density of both containers mirrored each other, except for minor deviations occurring at 20 and 60 hours (figure 10).

![Graph showing optical density against runtime](image)

*Figure 10. Runtime plotted against Optical density. Samples from pulsed field container are represented by light gray squares, samples from continuous field by dark gray circles.*

The voltage varied 9.7 to $10 \text{kV}$. Flow rate went down from 2.5 to 2.3 ml/s while temperature followed room temperatures, staying between 22 and $21.5 \degree \text{C}$ (Appendix, raw data from three-container systems, page 63). Different bacterial colonies were observed when culture media was plated at the end of the run. The initial bacterial concentration were found to be about 200 CFU/ml. Fragments were observed in the flow meter after 37 hours, reached a low after 58 hours and then stayed fairly constant until the end of the run.

Less biofilm was observed during this run. The control still shows more biofilm than the other membranes but it is even harder to see any difference between pulsed field and continuous field though pulsed still seem a little cleaner (figures 11-13)
Figure 11. Inner membrane taken out of control container from run X2, stained with 0.1 % crystal violet.
Figure 12. Inner membrane taken out of pulsed field (10 kV, 5 Hz, 110 ms) container from run X2, stained with 0.1 % crystal violet.
Figure 13. Inner membrane taken out of continuous field (10 kV) container from run X2, stained with 0.1 % crystal violet.
Run X3
Voltage varied between 6.8 and 7.0 kV, the temperature varied between 22.5 and 22 °C (Appendix, raw data from three-container systems, page 64)

No biofilm could be observed on the membranes (figure 14-16).

Figure 14. Inner membrane taken out of control container from run X3, stained with 0.1 % crystal violet.
Figure 15. Inner membrane taken out of pulsed field (7 kV, 5 Hz, 110 ms) container from run X3, stained with 0.1 % crystal violet.
Figure 16. Inner membrane taken out of continuous field (7 kV) container from run X3, stained with 0.1 % crystal violet.
Run X4
The initial bacterial concentration was about 900 CFU/ml. A sharp increase in optical density (620 nm) was observed during the first 48 hours, followed by a slow decline for 80 hours and then a second surge the last 40 hours (figure 17).

![Figure 17. Optical density variation during run X4. Light gray squares represent samples from 110 ms pulsed container, while the dark gray circles represent samples from 6 ms pulsed container.](image)

The pH showed an initial increase the first 24 hours followed by a small decrease over the next 24 hours. The pH then steadily increased until reaching its peak after a runtime of 128 hours (figure 18).

![Figure 18. The variation of pH during run X4.](image)
The voltage varied between 9.8 and 10.0 kV. Temperature varied between 22 and 23 °C (Appendix, raw data from three-container systems, page 64).

Strong biofilm growth could be observed on all membranes, the control clearly having the thickest, followed by the short pulse container (6 ms) preceding the longer pulsed container (110 ms) (figures 19-21)

Figure 19. Inner membrane taken out of control container from run X4, stained with 0.1 % crystal violet.
Figure 20. Inner membrane taken out of pulsed field (10 kV, 5 Hz, 6 ms) container from run X4, stained with 0.1 % crystal violet.
Figure 21. Inner membrane taken out of pulsed field (10 kV, 5 Hz, 110 ms) container from run X4, stained with 0.1 % crystal violet.

A high but unmeasured optical density was observed in the 10 liter Duran flask before the run was ended (figure 22).

Figure 22. Bacterial culture pumped through the system just before Run X4 was finished
Washing the system after the run revealed large flakes of biofilm evenly distributed inside the Duran flask that had detached themselves from the container walls (figure 23).

Figure 23. Detached biofilm showing up in the deionized water after washing.

Microscopic analysis showed contaminants being present after 24 hours and it was evident that a rod shaped contaminant had taken over after 48 hours when pieces of biofilm began to appear containing primarily that bacteria (figure 24) with show a distinct morphology from C. denitrificans (figures 1 and 2).
From samples take after the run it also seem that *Comamonas denitrificans* form distinct patches in the biofilm despite being underrepresented in the sample taken from the container during the experimental runs (figure 25).
Most of the biofilm taken from the containers show a thick and complex structure where distinct morphologies very hard to make out (figure 26).
The agar plates, on which diluted samples from the system had been smeared out, showed an early prevalence of a fast growing bacterium. After reaching peak population the population of this contaminant stayed fairly constant, while slower growing bacteria, like *C. dentificans*, gradually increased in numbers (figure 27).

![Figure 27. Diluted plated samples, taken after 82 hour (to the left) and 168 hours (to the right)](image)

At least three different contaminants could be found on the agar plates (figure 28).

![Figure 28. Microscopic analysis of two different colonies found on agar plates on which diluted samples of liquid culture from the three-container system had been spread out.](image)
Run X5

The optical density (620 nm) show a very rapid increase over the first 36 hour and then gradually decline until new media is put into the system at 110 hours. It then shows a small surge for about 20 hours and then falls into gradual decline for the remainder of the run (Appendix, raw data from three-container systems, page 64) (figure 29).

![Figure 29. Variation in optical density during run X5. Fields with pulse lengths of 11 ms is in light gray while pulses of 110 ms hides in the background, coloured dark gray. New media were added after 110 hours.](image)

The pH show a steady increase from 6,7 to 7,15 until new media was added resulting drop in pH followed by another steady increase (figure 30).

![Figure 30. Variation in pH during run X5. New media is added at 110 hours.](image)

The voltage varied between 9.8 and 10.0 kV. Temperature varied between 22 and 22.5 °C (Appendix, raw data from three-container systems, page 64).
The biofilm formation was not great but good enough to discern a pattern. The control showed least biofilm formation of all followed by the membrane exposed to shortest electrical pulses (11 ms) while the most biofilm was found on the membrane most exposed to the electric field (110 ms) (figures 31-33).

Figure 31. Inner membrane taken out of control container from run X5, stained with 0.1 % crystal violet.
Figure 32. Inner membrane taken out of pulsed field (10 kV, 5 Hz, 10 ms) container from run X5, stained with 0.1 % crystal violet.
Figure 33. Inner membrane taken out of pulsed field (10 kV, 5 Hz, 110 ms) container from run X5, stained with 0.1 % crystal violet.
Contaminants were found on the agar plates when colony forming units were to be determined (figure 34). They were traced to the yeast media used when making and subsequently diluting the overnight cultures.

*Figure 34. One of three $10^5$ dilution plates made from the bacterial solution that was used as inoculums for run X5.*

Microscopic analysis showed it to be chains of cell (figure 35) forming a biofilm structure quite distinct from that made by *Comamonas denitrificans* (figure 2).
Aggregates of biofilm showed very similar structure when they first appeared in the samples after 60 hours, as it did after 130 hours when the amount observed biofilm went down (figure 36).

![Figure 35. Biofilm structure formed chains of bacteria found in yeast media.](image1)

![Figure 36. Microscopic analysis of samples taken after 60 hours (to the left) and 130 hours (to the right) at 400 times magnification.](image2)
Plated samples showed a similar pattern as that of the previous run with only fast growing colonies being present on the first plate and slower growing colonies becoming more numerous for every day that passes (figure 37).

![Figure 37. Samples taken after 36 hours (to the left) and after 180 hours (to the right) smeared out on NA plates.]

Microscopic analysis on biofilm from all three containers at the end of the run showed similar structures (figure 38).

![Figure 38. Biofilms found at the bottom of all three containers. From the left: Control, pulsed field with a pulse length of 110 ms, and pulsed field with a pulse length of 10 ms.]

A summary of all runs show pulsed electric field as the one with the least biofilm, the last run being the exception (table 1.)

Table 1. Summary of runs 1 to 2 and X1 to X5 (appendix, page 63 - 65).

<table>
<thead>
<tr>
<th>Run (nr)</th>
<th>Initial (CFU/ml)</th>
<th>Nutrient Media</th>
<th>Voltage (kV)</th>
<th>Pulses second* container</th>
<th>Biofilm relative control**</th>
<th>Pulses third* container</th>
<th>Biofilm relative control**</th>
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<tr>
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<td>-</td>
</tr>
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<td>Acetate minimal</td>
<td>4.3</td>
<td>Continuous</td>
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<td>98/2 s on/off</td>
<td>-</td>
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<td>-</td>
</tr>
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<td>5 Hz, 6 ms</td>
<td>-</td>
</tr>
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<td>Yeast-acetate-nitrate</td>
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<td>5 Hz, 110 ms</td>
<td>++</td>
<td>5 Hz, 11 ms</td>
<td>+</td>
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</tbody>
</table>

*Denotes the container position relative to the pump, the control being in the first position.

**Denotes biofilm formation relative to that of the control. The notation is the following: 0 = biofilm formation equal to control, - = less than control, -- = least biofilm of all containers in that particular run, + = more biofilm formation than control, ++ = most biofilm of all containers in that particular run.

4. 1. 2. Sterilization

The shapes of *B. mycoides* colonies are very characteristic showing radial filaments curved counterclockwise. The other sporulating bacteria make compact big colonies that appear very fast. The psychrophilic bacteria have a slower growth rate than the other two bacteria at 30 degrees and makes small yellow colonies.

Double distilled water and brine doesn’t seem to have any bactericidal effect, all three bacteria survived such treatment. Low pH (around 3) and ethanol killed the psychrophilic bacteria leaving only the two spore forming bacteria (figure 39).
1 % Virkon was the most effective disinfectant killing all bacteria within two hours. In Yes all bacteria survived, but the endospore forming bacteria located themselves on the fringes of the smear made on the agar plate (figure 40).

Crystallization of an unknown compound was observed in YES solutions were the pH had been lowered. Washing liquid from old Yes bottles did not have that problem, and were just as, if not more, effective.

Acidic conditions turned out to be generally more effective than alkaline conditions. The most effective solution turned out to be the following combination: 10% YES in 50% ethanol, at pH 3. Only a few (between 0 and 4 colonies) bacteria managed to survive that combination for 4 hours. After being diluted in NB 50 times, it showed no growth after a week of inoculation at 30°C. After a fresh inoculum was added there was a change in turbidity within 48 hours.
4.1.3. Optimizing nutrient media

The diluted nutrient broth media did not reach a high enough OD$_{620}$ (> 0.1) in 20 hours even though the biofilm was comparable to that of the other cultures except for the yeast-nitrate media that did not show any biofilm at all. After the rest had been plated and subsequently stained. Unfortunately the plate reader had been reset and right plate dimensions with a precision to the tenth millimeter could not be found anywhere. It was evident from just looking at the plate that the media promoting most biofilm was the media containing 0.1 % yeast, 0.5 % acetate and 0.5% nitrate, followed by the 0.1 % yeast, 0.5 % acetate media and then the minimal media regularly used in the three-container systems.

4.2. Tap water container

Temperature varied between 24 and 30 degrees during the test period. NA plates done 14 days after the tap water system was started and incubated for a week showed 12 and 5 colonies. After 21 days the number of colonies had risen to 24 and 13. After one month only 10 and 1 colony grew and after two months the number of bacteria found in the tap water had declined to 7 and 3 colonies.

After staining and taking out the inner membrane, an evenly distributed, and thin but clearly visible biofilm could be seen (figure 41).
Figure 41. Stained inner membrane from tap water experiment run for 115 days.
4.3. Electric field across cuvette

Large pieces of biofilm containing some filamentous bacteria, looking like *C. denitrificans*, could be seen in the liquid cultures before being put into the pulsed electric field (figure 42).

![Image of biofilm before pulsed electric field](image1)

*Figure 42. Detached pieces of biofilm. The two pictures at the bottom are different magnifications of the same biofilm structure.*

After being in the pulsed electric field for 30 min none of the very big clusters of biofilm could be seen but a lot of medium sized rounded biofilm were still present (figure 43). Some indications that the larger filaments were less centered inside the biofilms and instead located in fringe regions could be seen.
Plate counts from before applying the electric field gave a 95% confidence interval of 151±12 CFU, while the interval after electric field treatment was 147±24 CFU. Therefore no significant difference between the cultures could be established. Further statistical analysis showed the difference to be 4 CFU ± 42 with a confidence level of 95%.
4. 4. Electrolysis

The mature detached biofilm showed very round or roundish structures, presumably because they all arise from a single bacterial cell dividing evenly in all directions (Figure 44).

*Figure 44. Some examples of mature biofilm structures from solutions before electrolysis have taken place in the liquid. Each row is from before a different trial. The two pictures on top and the two pictures in the middle are different magnifications of different biofilm structures.*
Biofilm after electrolysis showed lots of disrupted biofilm even though pieces of round biofilm still existed in solution (figure 45).

Figure 45. Biofilm after electrolysis. Each row show different magnifications of biofilms from different electrolysis trials.
A braking of the biofilm was observed in all cases, it seems extracellular matrix were ripped and bent apart. Bacteria found adjacent to these braking points seem to be a lot more active (observed shaking behavior) than planktonic bacteria in the vicinity.

The difference between undisturbed and interrupted biofilm was a lot more subtle in the electric field case than in the electrolysis case. Preserved cell morphology was observed in all bacteria, even after electrolysis.

4. 5. The test container

Microscopic analysis at the end of sterilization trial in test container revealed that endospore-forming contaminants had taken over the system (figure 46). The inner membrane, taken out after the run showed a little biofilm located at the top of the container (figure 47).

![Figure 46. Contaminations found in sterilization test after one week](image)
Membrane from the casamino acid buffered yeast media (figure 48) showed more biofilm than the previous sterilization test but still a lot less compared to the one used in the three-container system.

Figure 47. Biofilm after sterilization test stained with crystal violet.

Figure 48. Membrane stained with crystal violet taken out of test container after test run with casamino acid buffered yeast media.
A thin but clearly visible layer of biofilm was observed on the upper half of the container membrane after run 3 was finished (figure 49).

![Figure 49. Membrane taken out of test container after test run 3.](image)

The biofilm growth observed after run 3 was not extensive but if regularly produced would be enough to make a better analysis than with the more ambiguous media so far used in the three-container system.
5. Discussion

5.1 The three-container system

From runs X1 and X2, it seems the pulsed field is more effective in preventing biofilm formation, but it is unclear whether this difference is the result of chance or actual biofilm deterrence since all membranes were very clean. It is congruent with results from previous runs though (Results, table 1, page 46), the only difference being the increased effectiveness of the continuous field at higher voltages.

It is very likely that the samples taken from the liquid coming out of the top of the electric field containers don’t represent what goes on inside the container. LIVE/DEAD tests for example always showed all bacteria as being alive. This can be attributed to live bacteria being more buoyant than dead bacteria and thus overrepresented in any samples taken. In addition to that, detached biofilm are known to be released from mature biofilms but due to them being composed by non-motile bacteria it is likely that they will be underrepresented in the samples as well. Given the tremendous growth observed, especially after run X4, it seems unlikely that any extensive bactericidal effect is at play, though it cannot be ruled out.

Still, while not being representative, the general variations in optical density need to be explained. All graphs start with a high initial growth followed by a decline and ends with optical density increasing again. What is seen is probably the superposition of two growth curves, one with a rapid initial growth followed by a stationary phase and a death phase, the other one observing a long lag phase followed by sudden exponential growth. From agar plates it could be seen that at least one other bacterium was slowly increasing in numbers, such an increase could account for the second growth peak. The decline could be bacteria dying from local lack of nutrients which probably is a part of the story, it is also possible that these starvation conditions causes the bacteria to aggregate - which was observed in the microscope - making them heavier, so they can’t reach the top of the container quite as easy as before. It would also change the relationship between optical density and bacterial concentration making us think there are fewer bacteria than there actually is. The same is true of the plated bacteria where one aggregate instead of one bacterium would give rise to one colony.

The position of the containers should be taken as possible source for discrepancies. The control is first in line from the pump and are always (with the exception of the last run) showing most growth. In the runs done before this thesis got started (appendix, previous runs, page 65) the constant field container was positioned in the middle while the pulsed container was positioned last. With the new setup the pulsed and continuous container changed places. This could explain the sudden efficiency of the continuous field (Results, table 1, page 46), though still not as effective as the pulsed field. To really get to the bottom of this more data is needed, a randomization of the container placement seems likely place to start.

Given that the effect seen was not just dependent on the position of the containers, it seems reasonable to assume that the effect of a constant field on biofilm growth is very sudden, passing a certain voltage
level. That was also the reason why the voltage was lowered for run X3, as well as seeing if lowering the voltage would mean an overall higher rate of biofilm formation in all containers.

The high influences of what seems to be biofilm antagonistic contaminants were made apparent after run X3. A contaminant-free system would increase the reproducibility of the results so that much more qualified conclusions could be drawn. A clean system was also a prerequisite for being able to do controlled experiments, where other bacteria would be introduced into the system, in order to study synergistic effects and the likely much stronger biofilm formation it would result in.

Having the test container certainly made it easier to test promising media and without it would not have been possible to do any testing at all as the polypropylene surface of clean falcon tubes were too different from the non-sterile conditions inside the polyethylene containers.

It was hypothesized that nitrate could help biofilm growth of *C. denitrificans* even though it would raise the availability of nitrogen, possibly reducing the stress felt by the bacteria. The reason behind this thinking was that as it is able to reduce nitrate instead of oxygen, it would select for denitrifying bacteria, giving them an advantage over contaminants not being able to do that. It would also help spurring heterogeneity within the *C. denitrificans* species as it would give it two options for metabolism. Another quality of the yeast-acetate-nitrate media was that it could be autoclaved in a single step.

It is unclear why such deviating results came from run X5. It could be that the yeast media selects for other bacteria and that in this particular case they happened to be drawn to electric field environments. It is also possible that the new media that was put into the system disrupted the biofilm formation; even though the opposite should be true, new media usually means that biofilm can have a chance to grow more. Maybe the new media induced biofilm detachment; in that case the control would be affected the most by being closer to the media flask, but this explanation is still unsatisfactory as the longer pulsed container that showed most growth, was next in line.

A contamination of the system was expected to occur owning to the use of non-sterile parts in the system such as the containers, plugs and tubing. Therefore some effort needed to be put into disinfecting the system after assembly and efficiency of different cleaning schemes needed to be assessed.

There were concerns that 1% Virkon, even though being the most effective, was too fast acting, not being able to penetrate possible biofilms and that a slower acting, surfactant based detergent would over time be able to loosen up the biofilm structures. Virkon was also very acidic (pH around 2) and would only be active for a week. Disposing of 5 liters of 1% Virkon on a weekly basis did not seem a viable alternative. The reason 1% Virkon was not effective when used in run X3 in the three container system could have to do with the shorter time period (30 min) it was given to effect the three-container system compared to when tested in tubes (2 hours) and the probable existence of biofilms on the end plugs. There is also the possibility that residues of Virkon stayed inside the containers due to insufficient washing and affected the end results.
The fact that spore forming bacteria only grow in the fringe regions of a yes agar plate was taken as a sign that they could only grow were the concentration of Yes were not so high.

It turned out to be impossible to create sterile initial conditions for the system, resulting in very early contamination. While that is not a problem when looking at efficiency of a system that ultimately is to be used in a non sterile environment, the non-reproducibility of conditions makes studying the effects hard. The effect might come very sudden as one increased voltage and/or number of pulses. To be able to see the difference between biofilm structures under different voltage settings is therefore important but as the biofilm structures are likely to differ each time the system is run, because of different contaminations, it becomes very hard to try to draw any conclusions from the biofilm structures taken from the system. Other factors contributing to the non-reproducibility of the system was the long duration of each test run and the fact that the amount of air let into the system could not be controlled by other than crude methods.

That the strongest disinfectant mixture did not show any bacterial growth, even when it was diluted, was interpreted as a result of the mixture not merely deterring growth but also killing the bacteria. The problem with the final solution was that it, in addition to not effectively sterilizing the system, a slow crystallization process was beginning as a result of the pH being lowered. The cause of the crystallization is unknown but it was later shown that it could be helped by adding NaCl to the solution, making it a more effective sterilizer but then you instead get a problem with precipitation of NaCl.

5.2 Electric field

Since it is the biofilm on the container surface, as well as the media directly adjacent to that surface that are of interest, and these areas are blocked while running the three-container experiments, separate tests had to be performed. It also has to do with the samples taken from the top of the three-container system not being representative of what is happening near these target surfaces. This last aspect of the sampling problem can be expressed as a problem due to a high inner volume to wall surface quotient which in the three-container system is equal to half the inner radius (about 19 mm.) A sterile system with a much smaller quotient was set up in order to study the effects of the electric field in more detail.

To deal with the surface to volume problem, an electric field was instead applied over a cuvette with an inner volume to wall surface quotient of 1.4 mm. No indication of high electric fields actually killing bacteria have been demonstrated in these experiments, though such effects cannot be ruled out. The effect also seems to be very subtle, so the type of interactions affected are not likely to be very strong and from the amount of biofilm that detached itself after run X4 it is evident that young biofilm is held together by primarily weak forces.

It would be interesting to do a LIVE/DEAD test on the ruptured biofilm to study the bacteria situated close to where the biofilm pieces have been broken apart. Such an examination was planned but
technical difficulties with the microscope prevented further analysis as these experiments were done at the very end of the experimental period.

As the mark of a dead bacteria in the LIVE/DEAD test is the diffusion of fluorescent molecules through the ruptured membrane, such a test should shed light on the question as to whether we are dealing with an electroporation mechanism.

5.3 Tap water container

Since the three-container system cannot mimic the water distribution system in terms of nutrient availability, ionic strength and the variety of bacterial species present, a similar test system with tap water should be set up after the electronic equipment and settings have been optimized. The field in tap water will, due to the lower ion strength, though still stronger along the inner walls of the container, extend further into the pipe (Appendix, electric field inside container, page 67). Since such a system is likely run for a very long time (over three months) it is imperative to know that sufficiently thick biofilm is formed within that timeframe in the control container to guarantee good results.

It was postulated that the biofilm growth inside the container could be monitored by checking the number of bacteria coming out of the system. This hypothesis was abandoned as the tap water plates differed too much in bacterial concentration. It could be because biofilm fragments only give rise to one colony on the agar plates and sonication might be used to amend that problem in the future. The declining number of bacteria could also be because of the coming of winter and the fact that samples were taken at different times of the day.

6. Conclusions

It seems a pulsed field is more effective at preventing biofilm than a continuous field though the difference between them is smaller at higher voltages. From run X4 one can deduce that it is not the transient, the pulse itself in contrast to the length of the pulse, which is responsible for the reported effects. It seems likely that the optimal effect can be reached by increasing the number of pulses while simultaneously trying to keep the pulses as long as possible.

The three-container system was a good system in terms of adjusting electronic parameters, as the end result was very easy to analyze by looking at the inner membranes. However, in terms of studying how the nature of the electric fields affects formation and maturation of biofilm, the system turned out to be inadequate. Optimizing the electric field parameters for maximal efficiency must be done independently from trying to mimic the natural environment in the distribution systems as well as trying to study the effect itself and its cause. Mimicking the natural environment must be done with tap water over a period of three months or more. Studying the cause of biofilm formation disruption requires systems
with a low volume to surface quotient, preferably shorter runtimes and in an ideal scenario the ability to film bacteria inside a strong electric field while under the microscope.

The results from electric fields applied over the small cuvette were unclear but at any rate it seems that a distinction must be made between optimizing electronic equipment and studying its effects.

Regarding electric fields, the following question comes to mind: Do we know of any electric field effect that does not vary with distance, that wouldn’t cause a striped pattern on the inside of the containers alongside the electrodes?

The only resemblance to an answer I can think of is by considering an effect I have only observed and not read about: The tendency for a mature biofilm, when breaking, to break rather like a cobweb, as if a strain was held at bay by mechanical locking points within the biofilm. When enough of these safeguards give away, big chunks of biofilm are first rolled up and the releases from the surface. That would be an example of an effect that by acting locally affects the entire system evenly.

As for the success of the present system in water distribution systems it seems too early to tell. However, the microorganisms inhabiting these relatively unfriendly environments are very efficient survivors and it seems to me more likely that they will find a way to survive rather than perish. It should also be remembered that nutrients adhered to the walls in water distribution systems are likely become more concentrated if no bacteria are there to break it down so that a selection for possible “electric field-loving” bacteria are likely to occur in water distribution systems. Studies have shown that using a low intensity electric field increases the antimicrobial efficacy of antibiotics, the assumption being that the field increases the permeability of the biofilm matrix[28]. Even though a similar effect could not be observed when applying a low intensity electric field to chlorine and biofilm in water distribution systems, if any effect is seen, the possibility of an increased efficacy of chlorine must be taken into account[29].

From trying hard to sterilize the system, two conclusions can be drawn:

It seems possible to make an environmentally friendly sterilization mixture with a long sustained potency. But even if such a disinfectant would be developed, it would not be enough to sterilize the system, due to the high resistance of the contaminants involved, presumable as a result of thin but strong biofilms being present.

If a sterile system is wanted you need autoclavable or already sterile parts and you need to be able to assemble the system in a sterile environment. To render a system sterile, if desired, is not a feature that you can add on afterwards without changing your system and thereby making your new results incomparable to your earlier results. Further pursuits in sterilizing the system are therefore not recommended.
7. Further studies

The tap water system seemed to work well for its intended purposes and a similar setup to the one used should serve sufficient to get a clear idea as to whether this method of preventing biofilm growth has any merit. Considering the long runtime of such experiments, and the likelihood of disappointing results to discourage further testing, great care should be taken in designing and planning such a trial. The most important aspect is making sure that the field strength is the highest possible after taking into account the risk for discharges.

Since the field is strongest close to the electrodes, the effects of the electric field could be further optimized by reducing the volume to surface quotient by using a thinner cuvette and do a LIVE/DEAD test. A variant where the biofilm is allowed to grow inside the cuvette is also possible.

It would be interesting to find out if electric fields affect bacterial flagella. To find that out conclusively would involve being able to study bacteria under the influence of an electric field while being studied under the microscope. A more suitable model organism than *C. denitrificans* would have to be found, preferable one where the flagella is visible without any previous staining techniques being used.
6. Appendix

Raw data from the three-container systems

Table 1. Raw data from run X1

<table>
<thead>
<tr>
<th>Runtime (hours)</th>
<th>Optical density (620 nm)</th>
<th>Voltage (kV)</th>
<th>Flow rate (ml/s)</th>
<th>Temperature (Celsius)</th>
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Table 2. Raw data from run X2

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<td>22.0</td>
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<td>9.7</td>
<td>2.4</td>
<td>22.0</td>
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<td>2.4</td>
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<td>2.4</td>
<td>21.5</td>
</tr>
<tr>
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<td>9.7</td>
<td>2.3</td>
<td>21.5</td>
</tr>
<tr>
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<td>9.9</td>
<td>2.4</td>
<td>21.5</td>
</tr>
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<td>9.7</td>
<td>2.3</td>
<td>21.5</td>
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</tbody>
</table>
**Table 3. Raw data from X3.**

<table>
<thead>
<tr>
<th>Runtime (hours)</th>
<th>Voltage (kV)</th>
<th>Temperature (Celsius)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.0</td>
<td>22.5</td>
</tr>
<tr>
<td>24</td>
<td>6.9</td>
<td>22.5</td>
</tr>
<tr>
<td>44</td>
<td>6.9</td>
<td>22.0</td>
</tr>
<tr>
<td>73</td>
<td>6.9</td>
<td>22.5</td>
</tr>
<tr>
<td>90</td>
<td>6.9</td>
<td>22.5</td>
</tr>
<tr>
<td>123</td>
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</tr>
<tr>
<td>140</td>
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<td>22.0</td>
</tr>
<tr>
<td>168</td>
<td>6.9</td>
<td>22.0</td>
</tr>
</tbody>
</table>

**Table 4. Raw data from X4.**

<table>
<thead>
<tr>
<th>Runtime (hours)</th>
<th>Optical density at 620 nm</th>
<th>pH</th>
<th>Voltage (kV)</th>
<th>Temperature (Celsius)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00 0.00</td>
<td>7.4</td>
<td>9.9</td>
<td>22.5</td>
</tr>
<tr>
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<td>9.8</td>
<td>22.5</td>
</tr>
<tr>
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<td>9.8</td>
<td>22.0</td>
</tr>
<tr>
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</tr>
<tr>
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<td>10.0</td>
<td>22.5</td>
</tr>
<tr>
<td>128</td>
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<td>7.9</td>
<td>10.0</td>
<td>22.5</td>
</tr>
<tr>
<td>157</td>
<td>0.60 0.63</td>
<td>7.9</td>
<td>10.0</td>
<td>23.0</td>
</tr>
<tr>
<td>168</td>
<td>0.70 0.86</td>
<td>7.8</td>
<td>10.0</td>
<td>23.0</td>
</tr>
</tbody>
</table>

**Table 5. Raw data from X5.**

<table>
<thead>
<tr>
<th>Runtime (hours)</th>
<th>Optical density at 620 nm</th>
<th>pH</th>
<th>Voltage (kV)</th>
<th>Temperature (Celsius)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10.1</td>
<td>22.5</td>
</tr>
<tr>
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<td>10.0</td>
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<tr>
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<td>22.0</td>
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<tr>
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<td>9.9</td>
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<tr>
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</tr>
<tr>
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<td>9.8</td>
<td>22.0</td>
</tr>
<tr>
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<td>9.9</td>
<td>22.5</td>
</tr>
<tr>
<td>167</td>
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<td>10.0</td>
<td>22.5</td>
</tr>
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<td>0.44 0.44</td>
<td>7.1</td>
<td>10.0</td>
<td>22.0</td>
</tr>
</tbody>
</table>

*The media was changed after 110 hours. New conditions: OD: 0.07; pH 7.2; 23 degrees Celsius.
Data from previous runs

First run

Voltage: 4.2 - 4.3 kV.
Pulsed field: 98 seconds on, 2 seconds off
Initial concentration: ~400 CFU

Figure 1. Inner membranes from first run. From the left: Continuous field, control, pulsed field.

Second run

Voltage: 4.2 - 4.3 kV
Pulsed field: 98 seconds on, 2 seconds off
Initial concentration: ~200 CFU

Figure 2. Inner membranes from second run. From the left: Continuous field, control, pulsed field.

Third run

Voltage: 4.2 - 4.3 kV
Pulsed field: 98 seconds on, 2 seconds off
Initial concentration: ~1000 CFU
No visible biofilm formation at all after staining with crystal violet (figure 3).

![Image](image.png)

*Figure 3. Control from third run. No visible biofilm.*

**Correlate optical density to colony forming units**

Four overnight cultures diluted to an optical density of 0.1, was further diluted 10 000 times and three samples of 100 µl were taken out from each dilution and subsequently plated overnight on nutrient agar. The average number of colonies on plates coming from each of the four cultures was 150, 70, 65 and 72; the total average being 89. Given that the number of colonies from the first culture’s values differed so much from the rest the average of culture 2 to 4 was calculated as being 69. Taking all dilutions into account that gives concentration of 6 900 000 bacteria/ml, roughly 7 000 000 bacteria/ml.

**Table 6. Number of colonies on each plate for each culture and the calculated average for each culture**

<table>
<thead>
<tr>
<th>Culture</th>
<th>Number of bacteria on each plate</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>134</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>79</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>72</td>
</tr>
</tbody>
</table>
The electric field inside the containers when tap water is used

Electric fields present inside each container when connected to the high voltage generator.
7. References

[1] "Livsmedelsverkets föreskrifter om dricksvatten", SLVFS 2001:30