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Growth and control of *Pseudomonas aeruginosa* in a multi-species biofilm

Růst a kontrola *Pseudomonas aeruginosa* ve vícedruhovém biofilmu

Master thesis

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Acknowledgements

To my parents, thank you for supporting me through my studies.

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Abstrakt

UNIVERZITA KARLOVA, Farmaceutická fakulta v Hradci Králové
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Biofilmy sú základný spôsob rastu väčšiny baktérií. Mikróby sa obalia v matrici, zloženej najmä z extracelulárnych polymérnych zlúčenín. Biofilm sa môže skladať z viacerých druhov baktérií. Prostredie biofilmovej matrice indukuje rozličné fyziologické zmeny, ako zmena do latentného stavu či indukcia biofilm-špecifických génov. Vyzretý biofilm je heterogénny, kvôli rozdielom v spotrebe živín a distribúcii mikróbov naprieč biofilmom. Matrica poskytuje baktériám veľa výhod, napríklad zachytávanie a transport živín alebo ochranu pred extrémnymi podmienkami.

Skúmané boli viacdruhové biofilmy z umyvadlového sifonu so zameraním na *P. aeruginosa*. Tento oportunistický patogén spôsobuje nozokomiálne infekcie, hlavne u imunokompromitovaných pacientov, so značnými zdravotnými a socio-ekonomickými dopadmi. Bola stanovená spojitosť medzi umývadlami a prostredím kontaminovaním *P. aeruginosa.* Dekontaminácia prostredia môže byť prístup ako znížiť spomínané dopady.

Bola vyvinutá nová metodológia na popis vertikálneho pohybu baktérií prostredníctvom šírenia biofilmu.

Abstract

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Biofilms are a default mode of growth for most bacteria. Microbes encapsulate themselves within a matrix, composed mainly of extracellular polymeric substances. A biofilm can be composed by multiple species. Matrix environment induces various physiological shifts, such as switch to dormant state or expression of biofilm-specific genes. Mature biofilm's heterogeneous, due to differences in spatial microbe distribution and spatial nutrient utilization across the matrix. Matrix provides many advantages to bacteria, like nutrient capture and transfer or protection against extreme conditions.

Washbasin U-bend multi-species biofilms were investigated with a special focus on *Pseudomonas aeruginosa*. This opportunistic pathogen causes nosocomial infections, mainly in immunocompromised patients, with considerable health and socio-economic impacts. A link between sinks and environmental *P. aeruginosa* contamination has been established. Environmental decontamination may be an approach to lessen aforementioned impacts.

A new methodology to describe the ability of bacteria to perform vertical movement *via* biofilm spreading was developed.

1. Abbreviations

CA – Cetrimide Agar

CFU – Colony Forming Unit

DEB – Dey-Engley Broth

EPS – Extracellular Polymeric Substance(s)

FAC – Free Available Chlorine

HCAI – HealthCare Associated Infection

HOCl – Hypochlorous acid

ICU – Intensive Care Unit

MSDS – Material Safety Data Sheet

NaDCC – Sodium DiChloroisoCyanurate

NaOCl – Sodium Hypochlorite

PAA – Peracetic Acid

Pa – *Pseudomonas aeruginosa*

PSB – Phosphate Buffer Saline

rpm– Rotations Per Minute

TSA – Tryptone Soya Agar

TSB – Tryptone Soya Broth

VCC –Viable Cell Count

2. Aims and objectives

This thesis had two main aims with associated objectives:

1. Investigation of the fate of *P. aeruginosa* embedded in multi-species drain biofilm following various disinfectant treatments

- Embedment of *P. aeruginosa* in established drain biofilm model
- Disinfectant efficacy testing
- Investigation of *P. aeruginosa* and drain biofilm recovery post treatment

2. Assessment of bacterial ability to perform vertical movement

- Development of methodology for testing bacterial motility
- Determination of upward growth speed of *P. aeruginosa* and drain culture biofilms

3. Introduction

3.1. Bacterial biofilm properties and functions

3.1.1 What is a biofilm?

Biofilms are classified as clusters of microorganisms living in self-secreted microenvironment, usually adhered to a surface. Physiology of biofilm embedded bacteria is different from planktonic cells. [1]

It is default mode of growth for most bacteria, providing many benefits to cells within the biofilm, such as enhanced survivability. [2] Vast majority of bacteria exist in this setting. [3,4]

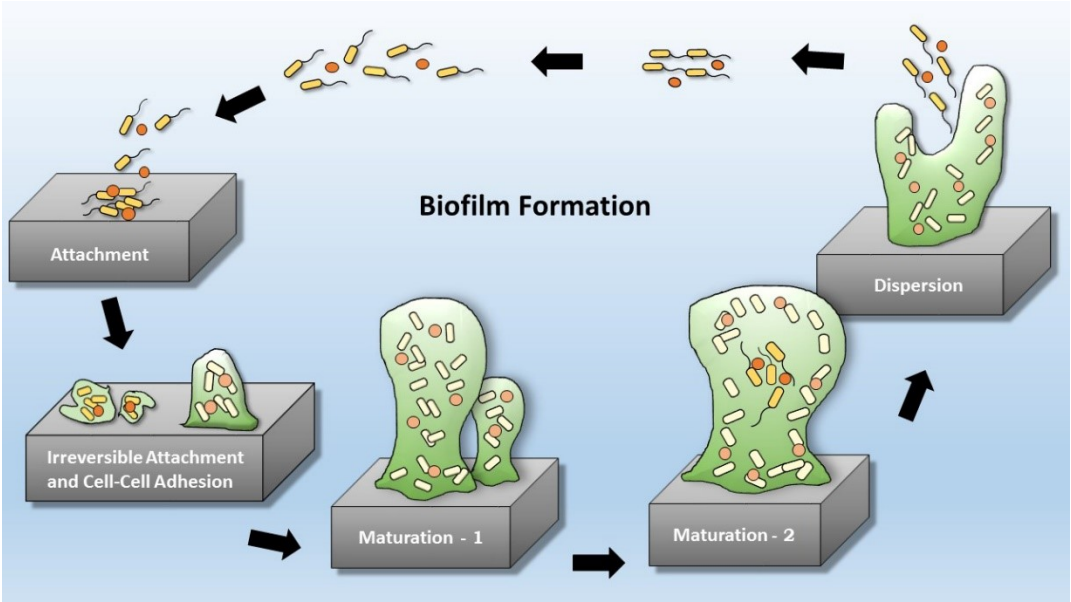
There are two theories why bacteria form biofilm: developmental and individualist. Developmental model says bacteria cooperate together, preferring group's interest before their own. [5] On the other hand, individualist model claims biofilms form as local ecological adaptation of individual cells.[5] Regardless which theory is true, biofilm mode of growth enhance microorganism's survivability, posing a challenge for those who want to overcome pathogenic biofilms.

3.1.2 Biofilm formation

Sauer *et al.* identified five life cycle stages, based on changes in bacterial phenotype (Figure 1) of *P. aeruginosa* biofilm: a) reversible attachment (of planktonic cells), b) irreversible attachment, c) maturation-1, d) maturation-2 and e) dispersion. Attachment happens as soon as possible (>0 min), irreversible attachment was observed after two hours, maturation-1 in three days, maturation-2 stage was observed after six days. At the end of life cycle, approximately in 9–12 days for *P. aeruginosa,* planktonic cells are dispersed into the environment. [6]

Earliest times of onset for each of the biofilm life cycles stages were found to be reproducible. At these breakpoints, majority of biofilm cells display the same phenotype. At different stages, bacteria are physiologically distinct to cells in other stages. Changes are apparent due to different protein expression over time. In a mature biofilm, all stages of development might be present at the same time. [6]

Figure 1: Biofilm formation, adapted from Andrew Thelwell [7]



3.1.3 Biofilm structure

Biofilm structure is influenced by environment, respiration, genetics and species composition. Biofilm matrix consists of extracellular substances (EPS), lipids, nucleic acids and bacteria, which create a hydrated matrix. EPS creates a two-phase system from physiochemical point of view. [8]

Ratio of viable cells to total biomass decreases from upper to deeper layers of biofilm matrix. Microbes are suspended to various degrees, organized in microscale spatial organisation. [9] This organisation creates multiple distinct layers. [10]

Natural pores can be found within a biofilm. Pores can be either horizontally or vertically oriented and have consequences for transport of substrates and products. [11]

3.1.4 Biofilm permeability

Channels and pores in a biofilm certainly help substrate transport. While voids facilitate flow, to reach a cell cluster, a molecule must be able to diffuse through it. [12–14]

Permeability is an essential property for mass transport in a matrix. The ease of mass transport depends on barriers it must pass. Density of a biofilm matrix increases from top to bottom. [11] Higher density of matrix should deteriorate diffusion rate. However, it has been proven, that molecular weight of antimicrobial substance has no effect on tolerance to antimicrobials of biofilm embedded microbes. [15,16]

3.1.5 Biofilm heterogeneity

One way to define biofilm is by its heterogeneity, which on its own has many implications for applied sciences dealing with microbial biofilms. Many properties and features, such as biofilm tolerance (Figure 2), are derived from biofilm's heterogeneity. Main driving force behind heterogeneity is everchanging microenvironment around each individual cell. Bacteria adapt to their microenvironment by inducing genetic or physiological shifts.

Imagine a biofilm as a unit, existing in an environment. There is certain availability of substrates outside the matrix, such as oxygen or nutrients. When they diffuse into the biofilm, they will be most likely used up by bacteria in the upper biofilm layer. [10] A probable scenario is that metabolic demand of upper layer cells uses up most nutrients, leaving lower layers to adapt to the situation [9,10]. At the same time, upper layer cells excrete their own substrates and waste products. This has two implications: a) Introducing even more heterogeneity to all respective microenvironments. b) keeping the diffusion gradient of nutrients favourable for the matrix.

Individual cells use what they are given, in their microenvironment, creating and enhancing even more diffusion gradients. Thus, we can find many physiological differences among microbes even in a single species biofilm. Differences are apparent both spatially and over-time. [17]

3.1.6 Biofilm protective functions

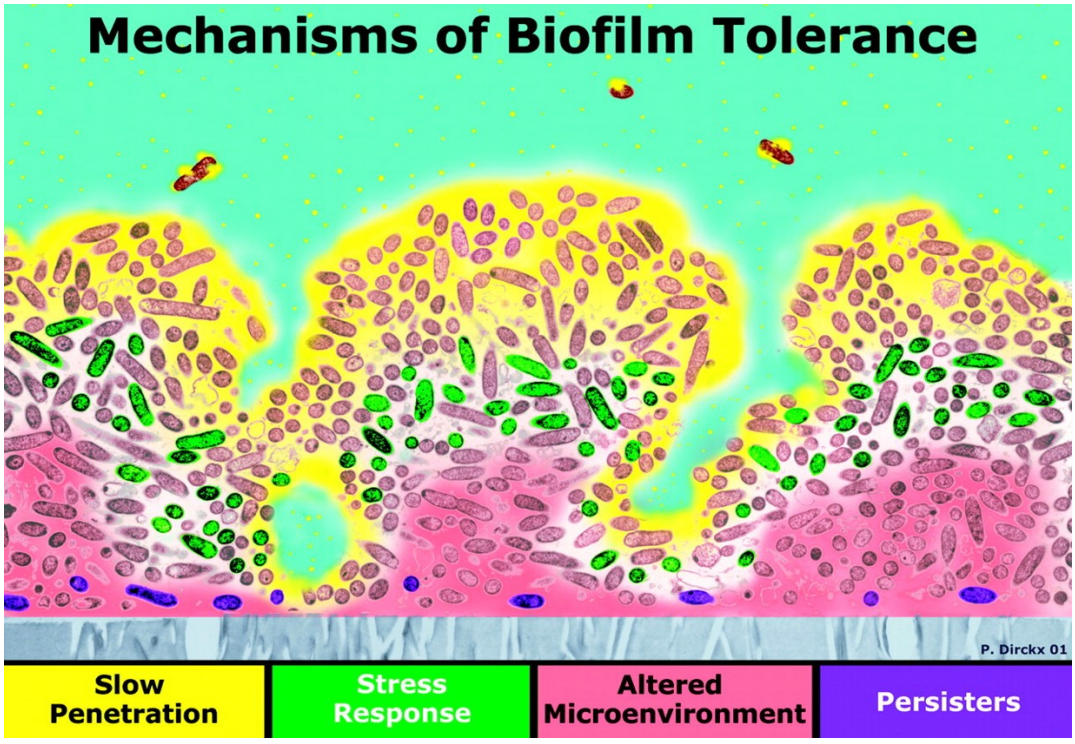
Microbes gain many benefits by creating and living within a biofilm. Matrix serves as a general protection against any extreme conditions. [18,19]

In the scope of this thesis, protective function of biofilm is the most important. One of the indirect evidences on biofilm matrix providing protective function to microorganisms is *Acinetobacter baumannii* higher susceptibility to antibiotics when grown in planktonic state, as opposed to its growth in biofilm. [20]

Bacteria within a biofilm both compete and cooperate. Globally, competition might be more prevalent. [21] On the other hand, bacteria are able to aid each other in ways relevant for decontamination efficacy. [2]. On certain occasion, multiple species can participate in augmented stress resistance of the bulk. [2] Study on tobramycin penetration suggests protective intra-species cooperation. [18]

Another significant process is, that a fraction of matrix population is able to be induced into dormant state, a process happening stochastically, by expressing certain genes. [23,24] In dormant state, cell's metabolic requirements decrease and resulting implication is, that treatments, such as ribosome-inhibiting antibiotics will not affect the dormant cell, since the ribosome is practically inactive. Also called persister cells (Figure 2). Persister cells display a multidrug tolerant phenotype due to reduced growth rate. [24] Dormancy mechanism is different in comparison to antimicrobial resistance. Compared to resistant bacteria, persisters are not mutants. Neither mobile genetic elements play a role in mechanism of persister cell dormancy. [25] They emerge as a result of phenotypic switch induced by presence of antimicrobial molecules through multiple pathways. [24,26]

Figure 2: Mechanisms of biofilm tolerance by Jason D. Chambless *et al.* [27]



Biofilm-specific resistance genes were identified in *P. aeruginosa.* [28] Extracellular DNA (eDNA), a component of biofilm matrix, can contribute to inducible antibiotic resistance, moreover it has antimicrobial properties in higher concentrations. [29]

3.2 Epidemiology and environmental sources of *P. aeruginosa* in hospitals

3.2.1 Environment as a reservoir

There are multiple ways of *P. aeruginosa* transmission. Patients, staff and environment are all potential source of *P. aeruginosa*.[30]

It has been known for over four decades, that one of the important environmental reservoirs of *P. aeruginosa* are water drainage systems. [31]

Multiple studies have proven colonization of hospital environment by *P. aeruginosa.* [32,34,35,36] An environmental study at Burns care ward and critical care ward in the UK shows how *P. aeruginosa* colonizes various surfaces. Three different environments, screened for *P. aeruginosa*, were defined in the study. Half of water samples were positive for *P. aeruginosa*. Wet environment was positive in a quarter of cases. Only one in twenty dry surfaces screened positive for *P. aeruginosa.* [32] This comes as no surprise as *P. aeruginosa* fares better in water environment.

Before the emergence of genotypic methods, association between environmental and infectious isolates was poorly understood.[33] As genotypic methods are more sensitive, several studies were able to identify similarities in clones colonizing patients and water taps, suggesting potential risk of tap-water to human transmission. [32,34,35]

Carriage of *P. aeruginosa* is both source and consequence of tap water contamination. A prospective intensive care unit (ICU) screening showed, more than 50% of *P. aeruginosa* carriage in patients was acquired *via* tap water or cross-transmission. [30]

A multicentric French study evaluated contaminated water environment exposure at the entry room as a risk factor for *P. aeruginosa* infection with a hazard ratio of 1.66, meaning that patients in a room with contaminated hydric environment are 1.66 more likely to get infected compared to patients staying in rooms where uncontaminated water environment is present. [37]

3.2.2 Environmental cleaning as a part of infection prevention and control

Prevention is better than treatment, when justifiable. Minimizing of life-threatening infections caused by pathogenic bacteria in a hospital environment is a long-term goal. Preventive measures need to be taken to minimize damage caused by hospital-acquired infections.

According to World Health Organisation (WHO), “Infection prevention and control (IPC) is a scientific approach and practical solution designed to prevent harm caused by infection to patients and health workers”.[38] Among other interventions, such as hand hygiene or injection safety, environmental cleaning is strongly recommended in WHO IPC guidelines.[39]

Effective IPC reduces Healthcare-associated infections (HCAIs) by at least 30%. [40] This can be further supported by outbreaks, where implementing environmental cleaning decreased the number of *P. aeruginosa* infection cases. [41,42]

3.2.3 Epidemiology of *P. aeruginosa* in hospital setting

Drainage systems are part of everyday life. While using them, many might not realise how much they actually interact with microbes colonizing plumbing. Flush down residues, such as coffee leftovers or dead skin cells can serve as nutrients for bacteria living there [44].

Users introduce new species into the drain system. Drain community can move vertically [43], colonizing everything outside the drain, including patients in hospital wards. [30,34,35,37]

Observational study focused on characterization of handwashing sink activities in an ICU revealed multitude of various actions taking place, discovering that majority of activities were not hand-hygiene related, moreover some actions could potentially enhance biofilm growth or patient item contamination. [44]

P. aeruginosa outbreaks are gaining an increasing importance in hospitals. [41,42,46] It is one of the major causes of NIs (nosocomial infections). Based on anatomic location, *P. aeruginosa* is contributing to approximately 13% of NIs, where culture was proven. [47]

Risk is elevated in ICU wards. According to surveillance reports, *P. aeruginosa* causes 10—20% infections, depending on type of infection, of all hospital-acquired ICU infections in Europe. [48]

In the years 2016/2017 there were approximately 834 000 HCAIs recorded in England alone with 28 500 patient deaths. [49]

High mortality scores are alarming, considering most of patients received prompt therapy. Overall mortality from *P. aeruginosa* blood stream infections (PABSI) was 37%. [50] High mortality suggests antimicrobial therapy is not very effective against *P. aeruginosa* infections [50].

At the same time there is an emergence of multi-drug resistant (MDR) *P. aeruginosa* strains worldwide. [41,42,45,46] A meta-analysis compared clinical impacts and showed in-hospital all-cause mortality. Results indicate that infections caused by MDR *P. aeruginosa*, closely followed by resistant isolates, yield significantly higher mortality compared to susceptible strains of *P. aeruginosa*. [51]

Naturally, we should look for alternative treatments and prevention methods. One of the options worth exploring is limitation of patient contamination from the environment. [30,36,37] This can be partially achieved by better water drainage system sanitation.

3.3 Socio-economic impacts

There are several points of view from which you could look at *P. aeruginosa* infections: social, economic, etc. Any health issue in general, will have a negative impact. Reducing incidence of diseases improves quality of life across whole society.

Burden of infection involves not only population at risk but overall population, as worrying about your loved ones certainly does not benefit you in any way. [52,53]

According to WHO antibiotic resistance is one of ten threats to global health in 2019. [54] Prevention and better understanding of infections promotes more rational use of antibiotics.

Apart from social impacts, illness in general also affects the economy. There are certain costs connected to treatment of every disease. Infected patients usually have prolonged hospitalisation, need additional treatment or procedures, which is increased in cases of both MDR pathogens and HCAIs. [51,55,56] Among others, these factors compose increased costs connected to *P. aeruginosa* infections. [55]

To put a perspective to these costs, *P. aeruginosa* infections increase associated costs in thousands to ten-thousands euros in Western Europe. [55,56] In 2016/2017 National Health Service (NHS) recorded approximately 834 000 HCAIs with total expense of 2.7 billion pounds. [49] A very rough estimate, calculating with 10% of HCAIs being caused by *P. aeruginosa* and disregarding different costs of various anatomic location infections, around 270 million pounds might be spent on tackling consequences of *P. aeruginosa* infections annually. United States have higher costs with medians somewhere along 50 000 dollars. [83]

3.4 Biocides

3.4.1 Sodium hypochlorite (NaOCl)

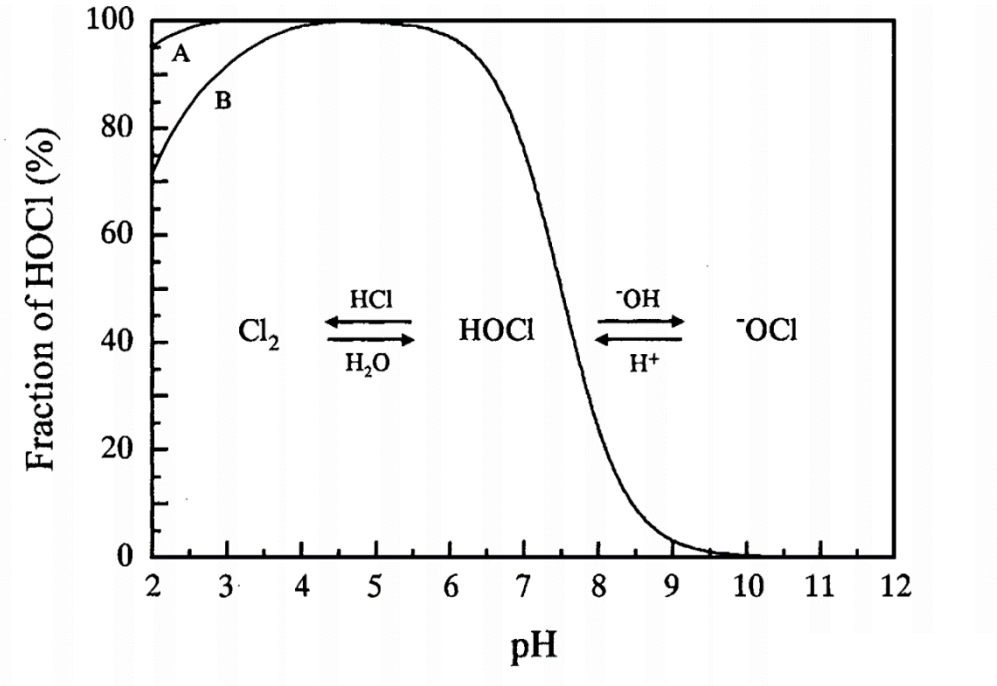
Bleach has been known and used in multiple ways for several centuries. NaOCl is one of the disinfectants most commonly used in hospitals.

NaOCl reacts with water, forming hypochlorous acid (HOCl). HOCl molecule is similar to water (H-O-H/H-O-Cl), thus it penetrates bacterial cell wall and membrane

effortlessly. [57] HOCl is a weak acid with pK_a value 7.497. [58] It exists in three different forms in aqueous solution, based on pH. Sum of all three forms is called free available chlorine (FAC). This equilibrium is important (Figure 3), since only undissociated HOCl is able to pass cell membranes and is thought to be the active molecule. When HOCl is used up, equilibrium shifts and FAC replenishes HOCl. Therefore, antibacterial effect is achieved by combined concentration of protons (H^+) and FAC. [59]

Hypochlorous acid derives its germicidal activity from being a strong oxidizing agent. It reacts with wide spectrum of biomolecules, denaturing proteins, rendering their function and thus, killing the cell. [60-63]

Figure 3: Distribution of HOCl in aqueous solution based on pH by US Coast Guard [65]



3.4.2 Sodium dichloroisocyanurate (NaDCC)

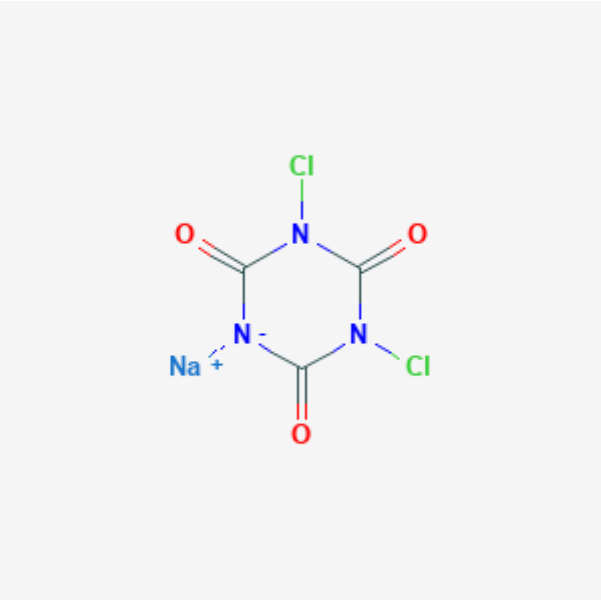
NaDCC is a white crystalline solid with bleach-like odour. [66] It is water soluble organic salt. Like sodium hypochlorite, NaDCC (Figure 4) is a carrier for hypochlorous acid, making it an alternative FAC source.

Difference between these two compounds lays in their breakdown. While NaOCl releases all its chlorine as FAC, in the case of NaDCC approximately 50% is released immediately

and rest is ‘stored’ in form of chlorinated isocyanurates. With FAC depleting, equilibrium shifts and more FAC is released. [59]

Another advantage of NaDCC is easier storage and application of solid compound compared to highly reactive and unstable aqueous solution of NaOCl.

Figure 4: Sodium dichloroisocyanurate molecule by PubChem [66]



3.4.3 Peracetic acid (PAA)

Being a peroxide of acetic acid, peracetic acid (PAA) is a strong oxidizing agent. PAA is thermodynamically unstable, releasing a radical oxygen when it decomposes, forming acetic acid and hydrogen peroxide in aqueous solution.

Biocidal activity of PAA is pH dependent. It is more potent in acidic environment, as it shifts equilibrium towards undissociated PAA, which is able to pass cell membranes.

Main advantage of PAA over other chlorine-based biocides is low environmental burden, since PAA forms little amount of disinfection by-products. [67,68] On the other hand, PAA decomposition produces acetic acid, serving as a nutrient source for some microbes after the treatment.[69]

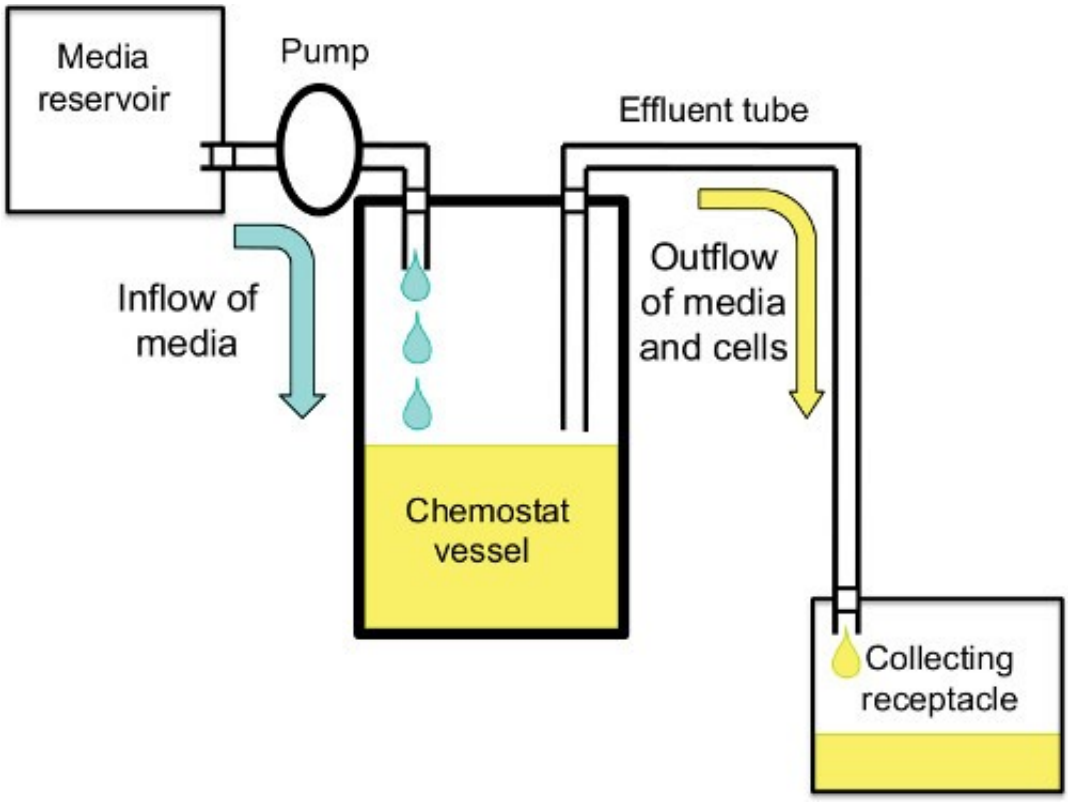
3.5 Chemostat

Chemostat was invented in 1950s and its creators describe it as ‘a device for keeping a bacterial population growing at a reduced rate over an indefinite period of time’. [70]

A diagram of a chemostat system is presented in Figure 5. Growth happens in chemostat vessel. Inflow of fresh media is ensured by a pump connected to a media reservoir. Media reservoir can be sealed with a 0.22 µm syringe filter to prevent environmental contamination. Pump timer and speed can be adjusted based on experiment performed, combined with media concentration, nutrient availability can be manipulated. Excess solution exits vessel by effluent tube and is collected in waste bottle.

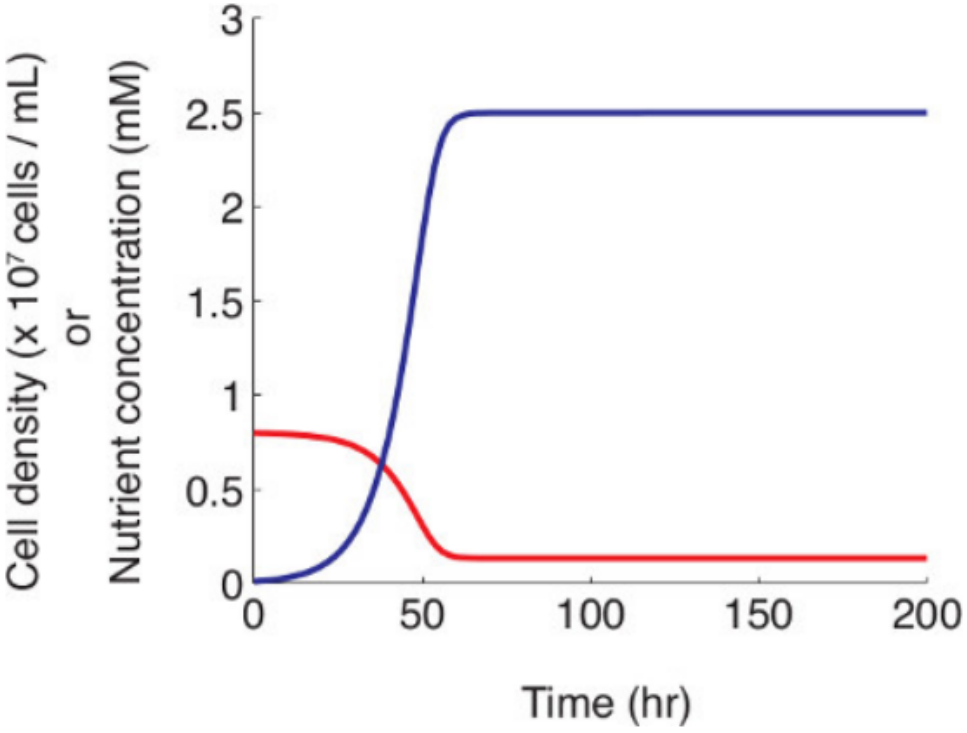
Big advantage of this system is, that apart from effluent tube, it is sealed from environment, minimizing potential contamination.

Figure 5: Chemostat diagram by Naomi Ziv *et al.* [70]



Its main function lies in the ability to control microbial growth rate in defined and controlled environment. This is achieved by the principle of growth-limiting nutrient(s). Cells growing within a chemostat, achieve a steady state, defined by rate of dilution. Thus, by altering growth-limiting nutrient(s) supply, various steady-states can be established after a certain period of time (Figure 6). [70]

Figure 6: Mathematical model of a steady state. Nutrient concentration (red) and cell density (blue) by Naomi Ziv *et al.* [70]



4. Materials and methods

4.1 Media and materials preparation

A summary of chemicals and media used is listed in Table 1. All media and materials were sterilised by autoclaving at 121 °C for 15 min in Astell AMB230D (Astell, UK). Dey-Engley broth (DEB) with 100 mg/ml proteinase K solution, was filter-sterilised through a 0.22 µm syringe filter (Minisart®, Germany).

Table 1: List of chemicals and media used

Media	Brand and manufacturer	Preparation
Tryptone soya broth (TSB)	Oxoid, Thermo Fisher Scientific, Newport, UK	30 g of TSB dissolved in 1 liter of distilled water
Dey-Engley broth (DEB)	Neogen® Corporation, Ayr, UK	39 g of DEB dissolved in 1 liter of distilled water
Phosphate buffer saline (PBS) 0.01 M	Fisher Bioreagents™, Fisher Scientific, Loughborough, UK)	One PBS tablet dissolved in 200 ml of distilled water
Cetrimide agar (CA)	Sigma-Aldrich, St Louis, USA	46.7 g of cetrimide dissolved in 990 ml of distilled water and 10 ml glycerol (Sigma-Aldrich, St Louis, USA)
DEB with 100 mg/ml proteinase K	New England, BioLabs®Inc., Ipswich, USA	Mixed sterilised DEB with proteinase K using a vortex
Tryptone soya agar (TSA)	Oxoid, Thermo Fisher Scientific, Newport, UK	40 g of TSA dissolved in 1 liter of distilled water

4.2 Sample preparation and storage

4.2.1 Drain biofilm culture

Drain biofilm culture was obtained from a trap below a communal room sink at the School of Pharmacy and Pharmaceutical Sciences, Cardiff University. Culture was diluted in PBS to a 1:10 ratio and glycerol was added to obtain a 30% solution of glycerol. Afterwards it was stored at -20 °C.

Prior to the experiment, overnight cultures were prepared by diluting 5 ml of defrosted drain biofilm with 45 ml of 1:10 TSB, close to a Bunsen Burner. The suspension was mixed using a Fisherbrand™ vortex shaker (Fisher Scientific, Loughborough, UK) and incubated overnight in an IOX402 Orbital shaking incubator (Sanyo, Osaka, Japan).

4.2.2 *Pseudomonas aeruginosa* ATCC® 10145 GFP™

The culture was received from American Type Culture Collection (ATCC, Middlesex, UK). Overnight cultures were produced near a Bunsen Burner, by transferring a single colony forming unit (CFU) to 20 ml of TSB using a 10 µl disposable culture loop (Microspec Ltd, Bromborough, UK). The suspension was incubated overnight at 37 °C in a shaking incubator. The culture was cultivated weekly by streaking one CFU on TSA plate using a 10µl disposable culture loop. The plate was inverted and incubated at 37 °C overnight in Universal Oven 100-800 (Memmert, Schwabach, Germany) then wrapped in Parafilm® M (Sigma-Aldrich, St Louis, USA) and stored at 4 °C.

4.3 Establishment of sample VCC

Initial overnight samples serial dilutions in PBS were spread-plated on TSA in 2 technical replicates. Plates were inverted and incubated at 37 °C overnight. VCCs were obtained by counting CFUs the following morning.

Two more serial dilutions were performed in 1:10 TSB (4.2.1) and TSB (4.2.2), in order to measure absorbance. Absorbance of both serial dilutions was measured at 600 nm with Amersham 2100 Pro UV Vis Spectrophotometer (Biochrom Ltd, Cambridge, UK). A calibration curve was made, plotting absorbance and consequent variable, VCC. [data not shown] Subsequent overnight cultures had their absorbance measured and their VCC was calculated from the calibration curve. Inoculum ratios (4.4.1) were derived from absorbance measurements.

4.4 Complex drain biofilm formation and growth

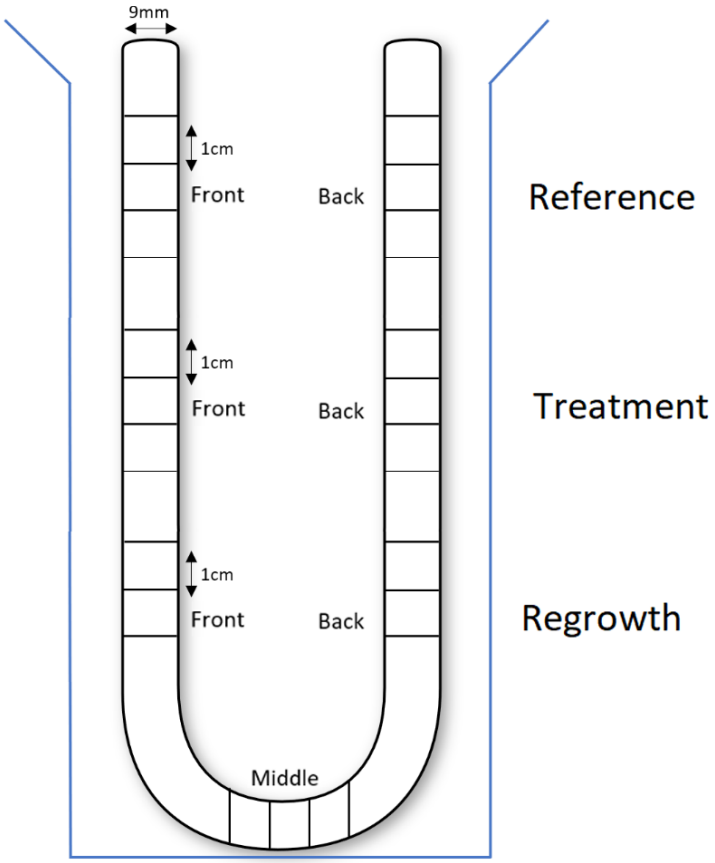
4.4.1 Inoculation phase

Sterile Silicon Rubber Platinum-cured Tubing of 9 mm diameter (ø) (Fisherbrand™, Fisher Scientific, Loughborough, UK) and approx. 41 cm length was cut, and 1 cm sections were marked (Figure 7). The tubes were autoclaved at 121 °C for 15 min.

Overnight cultures of drain biofilm culture (4.2.1) and *P. aeruginosa* (4.2.2) were mixed to a 1:10 Pa:drain cell ratio using a vortex shaker. Inoculation was done near a Bunsen Burner, to minimize environmental contamination. Tubing was placed in a beaker, in order to copy the shape of a U bend. To inoculate tubes, 43 ml of the culture was used, so that marked sections were under inoculum level. Fisherbrand™ Solid Rubber Stoppers ø 7-9 mm (Fisher Scientific, Loughborough, UK) were rinsed with 70% ethanol (Honeywell, Fisher Scientific Ltd., Loughborough, UK) to minimize contamination risk,

before sealing the tubes. Tubes were left standing for two days at room temperature in order to let bacteria attach and form a biofilm.

Figure 7: ø 9 mm silicone tubing set in a glass beaker to copy the shape of a U bend located below domestic sinks.



4.4.2 Sink trap model set-up and media supply phase

After 2 days of attachment time (4.4.1), a multi-species drain biofilm was grown using the FH100M Multichannel Peristaltic Pump (Fisher Scientific, Loughborough, UK), which could simultaneously hold six sink trap models described below. Whole set-up was conducted near a flame to minimize environmental contamination.

Saint-Gobain Sterile Silicone Tubing ø 3 and 5 mm, (Fisher Scientific, Loughborough, UK) and Silicon Rubber Platinum-cured Tubing ø 7.2 mm (Fisherbrand™, Fisher Scientific, Loughborough, UK) were used to connect the sink trap model. The tubing was autoclaved at 121 °C for 15 min prior to the set-up day.

The ø 3-5-7.2 mm front tubing connected 500 ml of 1:10 TSB media in Duran™ Clear Glass Laboratory Bottles (Fisher Scientific, Loughborough, UK) to sink trap models. Kinesis™ Omnifit™ "T" Series Bottle Caps (Fisher Scientific, Loughborough, UK) were

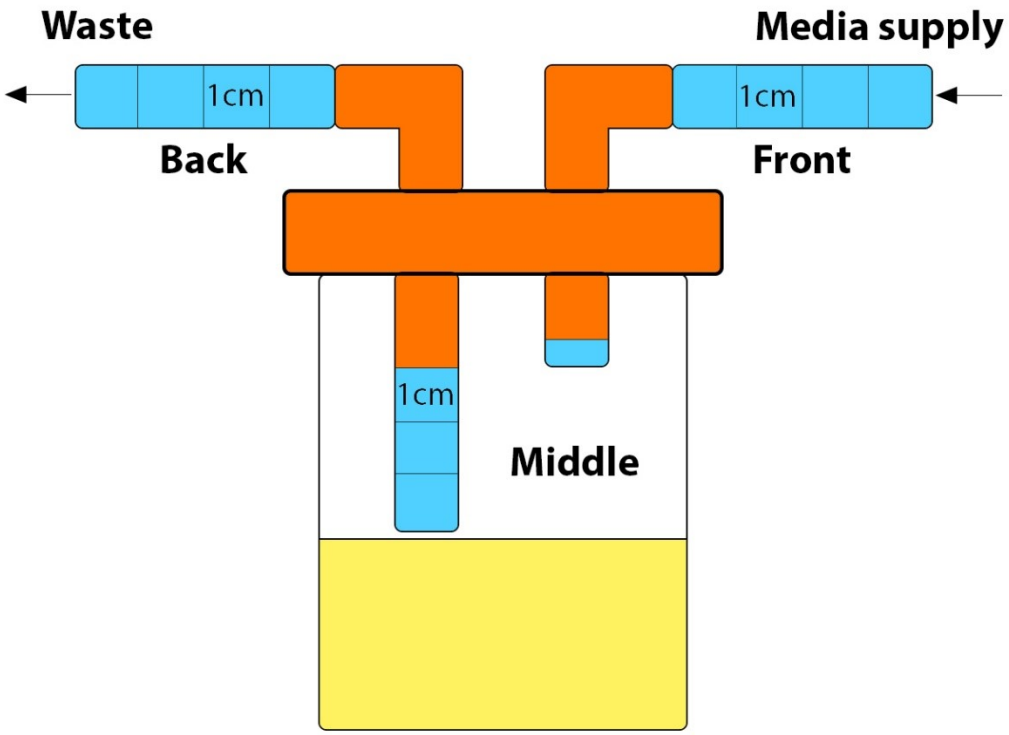
used to allow the ø 3 mm tubing to be pulled through one hole using sterile tweezers until it sank to the bottom of the bottle. Second hole was sealed with a 0.22 µm filter to prevent contamination from the environment. The ø 7.2 mm section of the front tubing was placed on the pump rails and was secured with holders.

The back ø 5-7.2 mm tubing connected the system to a 1 l Duran waste bottle. An insulator tape was used to make the whole system airtight.

The sink trap model is a 100 ml Duran bottle with a GL45 Screw cap Twin Hose Connector for Pyrex GL 45 media-lab bottle (Fisher Scientific, Loughborough, UK). All junctions were sealed with an insulator tape.

ø 9 mm silicone tubing containing inoculum (4.4.1), was unsealed after two days of attachment period and connected to the front and back tubing. Afterwards, the pump was started to drain the ø 9 mm tubing. Drained ø 9 mm tubing was cut to 4 parts with scissors, flame-sterilised after every cut, to prevent cross-contamination. Cuts were connected to the trap model (Figure 8). Pump was started until media dripped to the waste bottle, indicating that the whole system is rehydrated (Figure 8). The pump was then set to run for 10 seconds at 30 rotations per minute (rpm) every 2 hours for 6 days.

Figure 8: Sink trap model. ■ ø 9 mm silicone tubing with 1 cm sections marked ■ screw cap ■ liquid level



4.5. Disinfectant and regrowth test

4.5.1. Reference samples

On day 8, the tubing system was drained by pulling the ø 3 mm tube above medium level and running the pump at 30 rpm until tubing drains. Subsequently, 1 cm reference sample was cut from front, middle and back sections each and transferred into separate, sterile McCartney's bottle containing 1 g of sterile glass beads and 2 ml of Proteinase K/DEB solution (1 mg/10 ml). Samples were incubated for 1 hour at 37 °C. After 1-hour incubation, McCartney's bottles, containing samples, were mixed using a vortex shaker for 2 min. Afterwards, serial, 10-fold dilution in PBS, was performed. Multiple dilutions were plated. For TSA plates, spread-plating method was used. 100 µl of each serial dilution was transferred to a pair of plates and spread with L-shaped spreaders (Microspec Ltd, Bromborough, UK). Drop-plating method was used for CA plates. Three 10 µl drops of a single dilution were dropped on a plate. Plates were inverted and incubated overnight at 37 °C. VCCs were obtained the following day. Every step was conducted by a Bunsen burner, in order to minimize environmental contamination.

4.5.2 *P. aeruginosa*:drain day 8 ratio

A hypothesis was established, that biofilm composition may influence biocide efficacy. Based on the hypothesis, selection criterium chosen was Pa:drain cell ratio on day 8, in order to compare biofilms with similar microbe composition. Reference sample VCCs were used to calculate cell ratio of *P. aeruginosa*:drain on day 8.

4.5.3 Disinfectant test

New disinfectant agents (Table 2) were prepared according to manufacturer recommendations on the day. Both, NaOCl and NaDCC were prepared as 1000 ppm solutions. PAA's formulation (composition of formulation not disclosed for proprietary issue), with 4000 ppm concentration, was weighed (6.3 g). 20 ml of each disinfectant solution, sterile water in case of PAA, were poured to Fisherbrand™ 50 ml Sterile Disposable Centrifuge Tubes (Fisher Scientific, Loughborough, UK).

Table 2: Disinfectants used for experiments

Abbreviation	**Main Active Ingredient[1]**	**Concentration of the Main Active [2]**	**Excipients (from MSDS)[1]**	**Type**
NaDCC	Sodium dichloroisocyanurate	1000 ppm	Adipic acid, Na, toluene	Commercial
NaOCl-Ref[3]	Sodium hypochlorite	1000 ppm	-	Reference[3]
PAA	Peracetic acid	4000 ppm	(composition of formulation not disclosed for proprietary issue)	Commercial

1. Main active ingredient and excipients mentioned in the Material Safety Data Sheet (MSDS) information of the commercial products used in this study
2. The concentration of available chlorine was measured with Pocket Colorimeter™ (HACH®, Manchester, UK) (regardless of the product claim on the label)
3. Unformulated sodium hypochlorite solution (1000 ppm) used as reference

The ø 3 mm tube was pulled above the media liquid level. The pump was started until front and back sections drained dry. Pump was stopped immediately after they drained.

All ø 3 mm tubes were removed from media and put to Fisherbrand™ 50 ml Sterile Disposable Centrifuge Tubes containing respective disinfectant solutions. PAA's formulation was administered in a different way. A portion was put directly into the ø 9 mm front section, the rest, that didn't fit, was introduced directly into trap bottle. Pump was started, until all disinfectant solution got sucked up. 15 min of contact time was tracked afterwards.

In the meantime, 20 ml of sterile DEB was poured to Fisherbrand™ 50 ml Sterile Disposable Centrifuge Tubes. Disinfectant tubes were changed for DEB tubes. After 15 min of disinfectant contact time, the pump was started to neutralize the system with DEB. Pump was stopped after all DEB has been sucked in and 5 min contact time was tracked. System was drained after 5 min of neutralization contact time.

Scissors were sterilised after every cut by exposing them to a flame for a moment. One cm sample was cut from each ø 9 mm section of the sink trap model and transferred into separate, sterile McCartney's bottle, containing 1 g of sterile glass beads and 2 ml of Proteinase K/DEB solution (1 mg/10 ml).

Samples were incubated for 1 hour at 37 °C. After 1-hour incubation, Samples were mixed using a vortex shaker for 2 min. Multiple 10-fold dilutions were spread-plated on TSA (2 technical replicates) and drop-plated on CA (3 technical replicates). Plates were inverted and incubated overnight at 37 °C. VCCs were obtained the following day. Every step was conducted next to a Bunsen burner, in order to minimize environmental contamination.

4.5.4 Regrowth test

After conducting the disinfectant test, the sink trap model was reconnected. Duran bottles with 1:10 TSB medium were changed for 500 ml Duran bottles with sterile water. Bottle caps and the 0.22 µm filters were changed for sterile ones. Ø 3 mm tubes were moved to Duran bottles with sterile water. Pump was set to run for 10 seconds at 30 rpm every 2 hours for 24 hours. The system was drained 24 hours after the disinfection by pulling the ø 3 mm tube above media level and running the pump at 30 rpm.

Scissors were sterilised after every cut by exposing them to a flame for a moment. One cm sample was cut from each ø 9 mm section of the sink trap model and transferred into separate, sterile McCartney's bottle, containing 1 g of sterile glass beads and 2 ml of Proteinase K/DEB solution (1 mg/10 ml).

Samples were incubated for 1 hour at 37 °C. After the 1-hour incubation, Samples were mixed for 2 min using a vortex shaker. Serial dilution of samples in PBS was conducted following the mixing. Each sample was 10-fold diluted multiple times. Dilutions of each sample were spread-plated on TSA (2 technical replicates) and drop-plated on CA (3 technical replicates). Plates were inverted and incubated overnight at 37 °C. VCCs were obtained the following day.

4.5.5 Result presentation

4.5.5.1 Disinfectant test

Disinfectant efficacy is presented as $\log_{10}$ reduction. Log_{10} reduction is the difference between VCCs obtained from reference samples and corresponding VCCs recovered from disinfection samples.

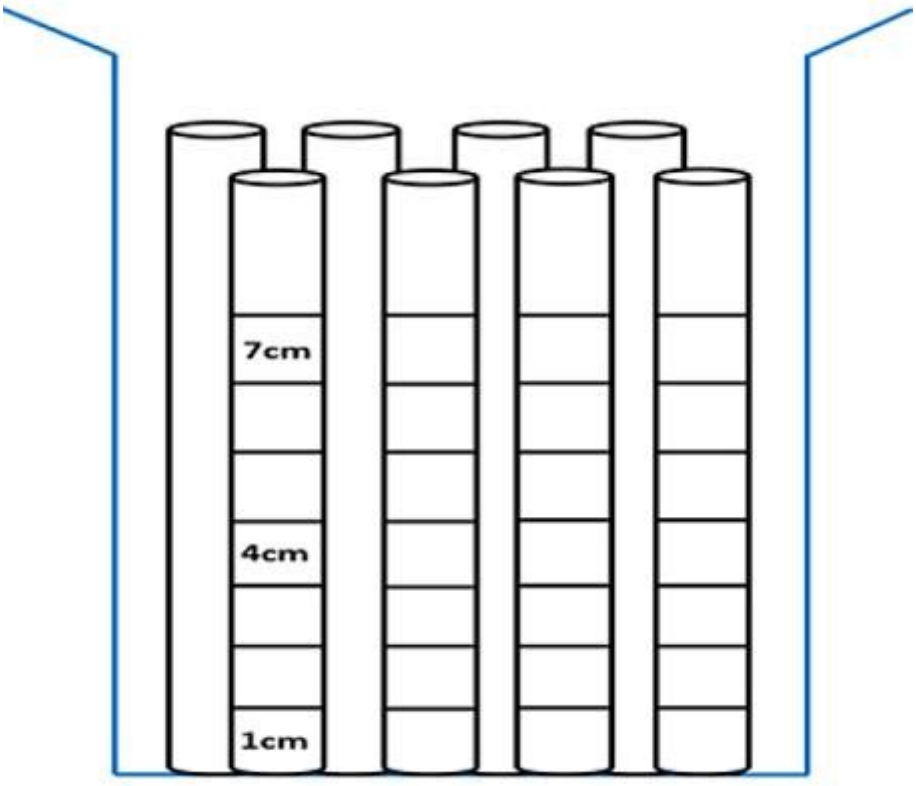
4.5.5.2 Regrowth test

Results of the regrowth experiment are expressed as $\log_{10}$ of VCC recovered directly after a treatment and corresponding VCC recovered 24 hours after the treatment. The results show effect of respective disinfectants on multi-species biofilm's ability to reinstate in a day's time. No selection criteria were applied to the results.

4.6 Motility test

Ø 9 mm tubing of approx. 10 cm length was cut and 1 cm sections were marked. Eight 10 cm tubes were inserted into a 250ml glass beaker (Figure 9). The beaker was covered with aluminium foil and autoclaved at 121 °C for 15 min. Foil was removed only near a flame after sterilisation, to prevent environmental contamination.

Figure 9: Diagram of silicone tubing in a beaker. Aluminium foil was used to cover the beaker.



Overnight cultures of drain (4.2.1) and *P. aeruginosa* (4.2.2) were diluted to 3 $\log_{10}$ CFU/ml in 1:10 TSB (4.2.1) or TSB (4.2.2). Pre-determined volume (2 ml per tube; 16 ml total) of 3 $\log_{10}$ CFU/ml dilution of either Pa or drain, was used to inoculate separate beakers with a Sterile Polystyrene Disposable Serological Pipet (Fisherbrand™, Fisher Scientific, Loughborough, UK). Special care was taken while inoculating, in order to prevent contamination of tubes *via* splash.

Every 24 hours (up to day 3), two tubes (2 biological replicates) were removed from the beaker with sterile tweezers. Two tubes were removed immediately after inoculation (day 0). Sterile scissors were used to cut 1 cm, 4 cm and 7 cm section from each tube. Samples were transferred into separate, sterile McCartney's bottles containing 1 g of sterile glass beads and 2 ml of PBS. Samples were incubated at 37° C for 1 hour. Afterwards, all samples were mixed for 2 min and serially diluted in PBS. Dilutions were spread-plated on TSA in 2 technical replicates, inverted and incubated overnight at 37 /°C. VCCs were obtained the following morning.

4.6.1 Motility test data presentation

VCCs, displayed as $\log_{10}$, obtained from 1, 4 and 7 cm sections every 24 hours were plotted, resulting in three linear functions (one for each distance), showing the change in VCCs recovered, over time. [data not shown] Functions were used to calculate the time, when significant VCC breakpoints occurred, at every analysed section (1, 4 and 7 cm).

In order to get a function showing a change in distance over time, $\log_{10}$ had to become a constant. Two breakpoints were chosen for both Pa and drain motility tests. The breakpoints show VCC deemed significant, 4 $\log_{10}$ and 6 $\log_{10}$.

The significant breakpoint times (calculated from linear functions), were used to plot new linear functions, showing a change in distance over time for each of the two breakpoints. Gradients of these functions represent speed of upward motility in cm/hour.

4.7 Statistical analysis

MS Excel 2020 was used for statistical analysis and graphic figures. Bar and line charts represent the mean standard deviation. Data were evaluated in MS Excel 2020 by t-Test: Two Sample Assuming Equal Variances and t-Test: Two Sample Assuming Unequal Variances. F-test Two-Sample for Variances was used to check whether variance is equal or unequal. Statistical significance in VCCs retrieved at CA and TSA agars was evaluated

using t-Test Paired Two Sample for Means. Both one-tail and two-tail α values below 0.05 were deemed statistically significant.

5. Results

5.1. Day 8 *P. aeruginosa:*drain reference sample ratio

Pa:drain day 8 ratios calculated from reference sample VCCs for 5 biological replicates conducted, are presented in Table 3. Each tenfold is regarded as a separate group and disinfectant test data are compared within these groups.

Cases where the result is negative were disregarded. In these cases, VCC on CA was greater than VCC on TSA. Suggesting, *P. aeruginosa* may have completely overtaken the biofilm.

Table 3: Reference Pa:drain day 8 ratio ■ 0.01-fold $\log_{10}$ ratio ■ 0.1-fold $\log_{10}$ ratio ■ 1-fold $\log_{10}$ ratio ■ 10-fold $\log_{10}$ ratio ■ 100-fold $\log_{10}$ ratio negative $\log_{10}$ ratio

	NaOCl			NaDCC			PAA		
Replicate	Front	Middle	Back	Front	Middle	Back	Front	Middle	Back
1	0.05	11	-2	-17	13	1.2	-2	4.6	1.4
2	0.9	-21	-7	1.3	4.8	3.8	-4	45	140
3	0.5	0.3	0.1	1.9	0.2	1.5	0.2	1.7	0.8
4	2.9	6.9	-6	24	-11	3.2	3.1	2.1	8.3
5	3.8	1.2	-5	-6	34	2.9	1.0	2.9	1.9

5.2 Disinfectant test

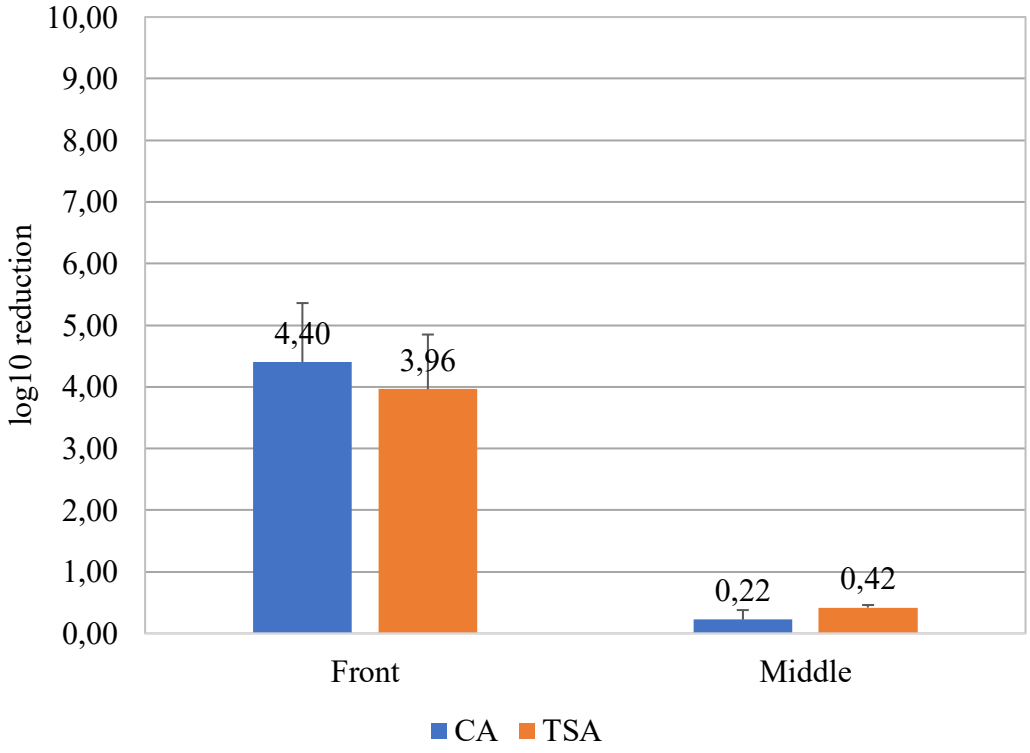
Five biological replicates were conducted. Results show means and positive standard deviations of the means.

5.2.1 Disinfectant efficacy on 1-fold Pa:drain ratio biofilm

5.2.1.1 NaOCl

Figure 10 shows log_{10} reduction observed following 15 min treatment with NaOCl 1000 ppm.

Figure 10: log_{10} reduction observed following 15 min treatment with NaOCl 1000 ppm: ■ Cetrimide Agar (Pa), ■ Tryptone Soy Agar (Pa+drain)



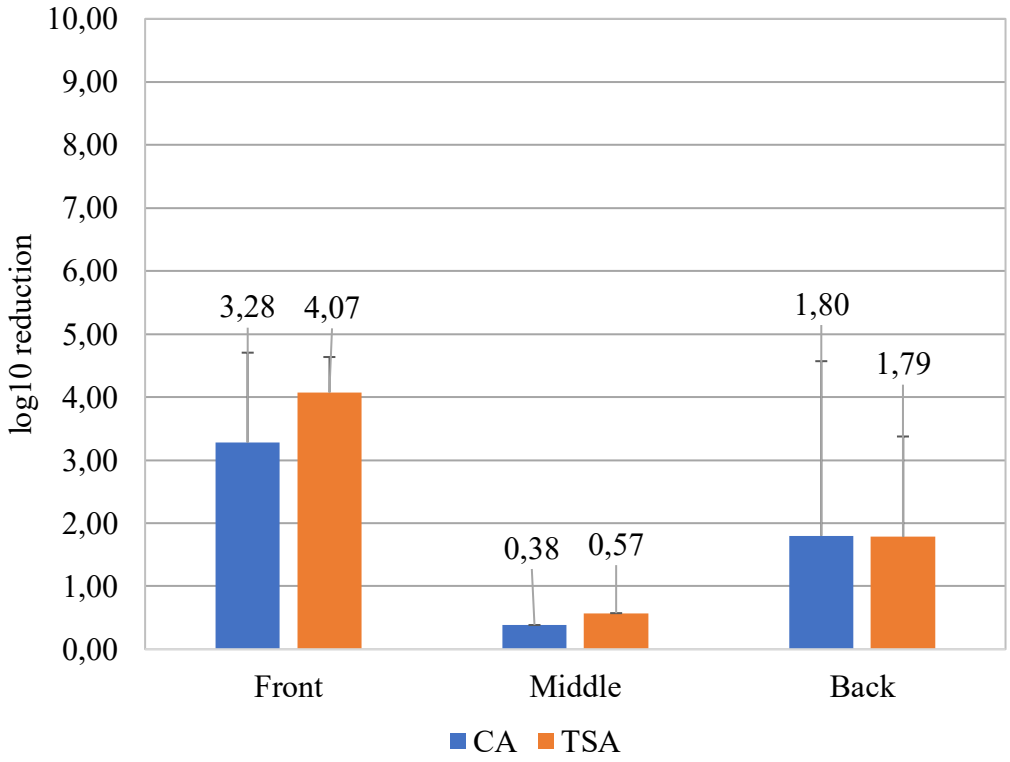
Log_{10} reductions observed following 15 min treatment with NaOCl 1000 ppm: Front section (4.40 ± 0.96 CA; 3.96 ± 0.89 TSA); Middle section (0.22 ± 0.16 CA; 0.42 ± 0.04 TSA)

Statistically significant difference was observed between sections for both (CA:CA) ($p=0.026$, t-Test: Two-Sample Assuming Equal Variances) and (TSA:TSA) ($p=0.03$, t- Test: Two-Sample Assuming Equal Variances). No statistical significance was observed within sections (CA:TSA)

5.2.1.2 NaDCC

Figure 11 shows log_{10} reduction observed following 15 min treatment with NaDCC 1000 ppm.

Figure 11: log_{10} reduction observed following 15 min treatment with NaDCC 1000 ppm: ■ Cetrimide Agar (Pa), ■ Tryptone Soy Agar (Pa+drain)



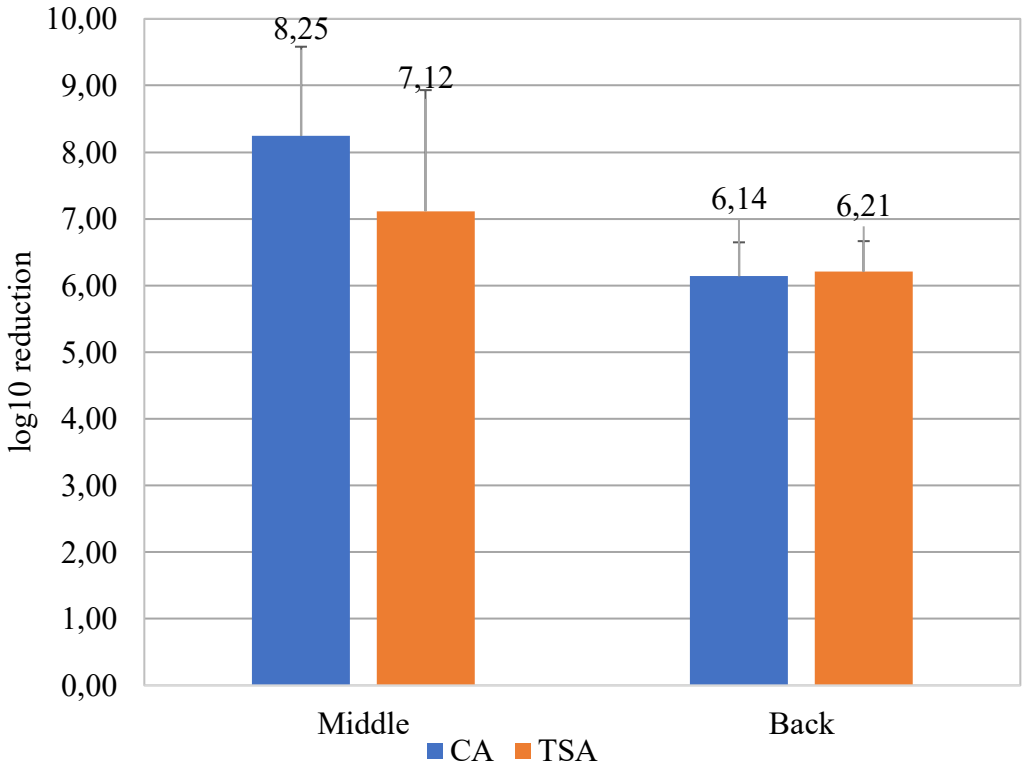
Log_{10} reductions observed following 15 min treatment with NaDCC 1000 ppm: Front section (3.28 ± 1.42 CA; 4.07 ± 0.56 TSA); Middle section (0.38 CA; 0.57 TSA), Back section (1.80 ± 2.77 CA; 1.79 ± 1.59 TSA).

No statistically significant difference was observed between sections (CA:CA) ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances) nor within sections (CA:TSA) ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances).

5.2.1.3 PAA

Figure 12 shows log_{10} reduction observed following 15 min treatment with PAA 4000 ppm.

Figure 12: log_{10} reduction observed following 15 min treatment with PAA 4000 ppm: ■ Cetrimide Agar (Pa), ■ Tryptone Soy Agar (Pa+drain)



Log_{10} reductions observed following 15 min treatment with PAA 4000 ppm: Middle section (8.25 ± 1.34 CA; 7.12 ± 1.82 TSA), Back section (6.14 ± 0.51 CA; 6.21 ± 0.46 TSA).

No statistically significant difference was observed between sections (CA:CA) ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances) nor within sections (CA:TSA) ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances).

5.2.1.4 Comparison of disinfectants

No significant difference in log_{10} reduction at front section was observed between NaDCC and NaOCl ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances).

PAA shows significantly better log_{10} reduction at middle section compared to NaOCl for both CA (p=0.004, t-Test: Two-Sample Assuming Equal Variances) and TSA (p=0.004, t- Test: Two-Sample Assuming Equal Variances).

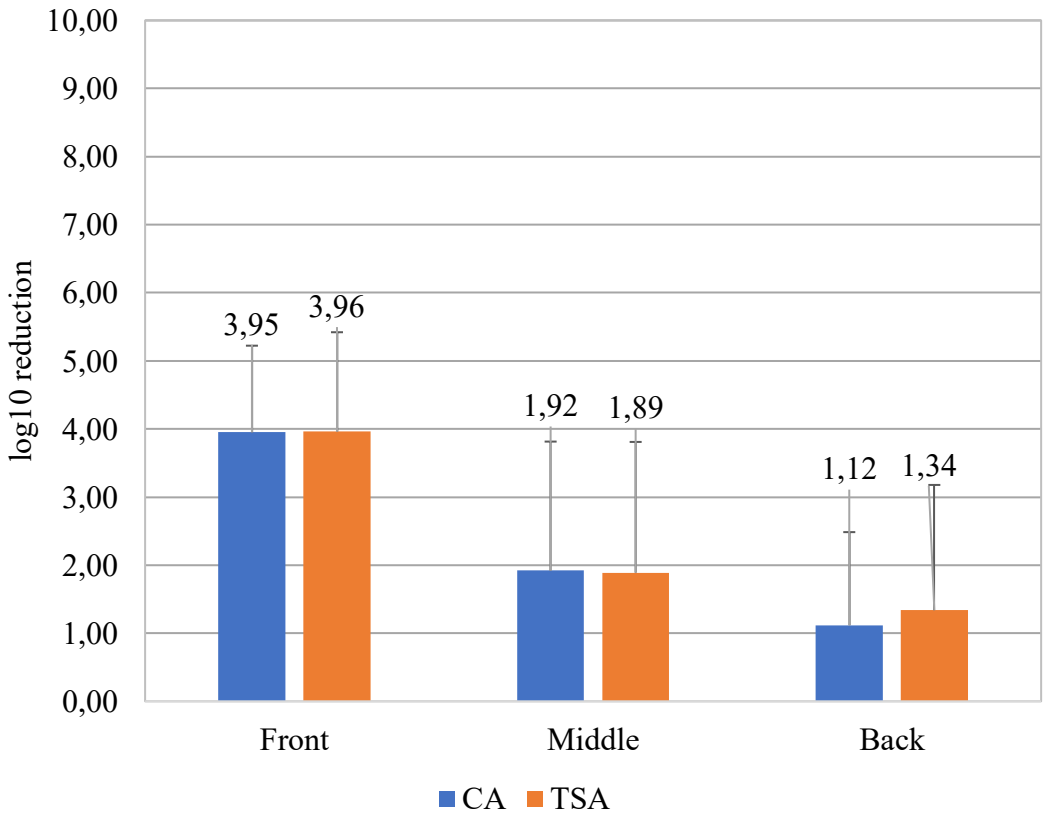
PAA shows significantly better log_{10} reduction at back section compared to NaDCC for for TSA (p=0.013, t-Test: Two-Sample Assuming Equal Variances) but not for CA (p>0.05, t-Test: Two-Sample Assuming Equal Variances).

5.2.2 Disinfectant efficacy on 0.1-fold Pa:drain ratio biofilm

5.2.2.1 NaOCl

Figure 13 shows log_{10} reduction observed following 15 min treatment with NaOCl 1000 ppm.

Figure 13: log_{10} reduction observed following 15 min treatment with NaOCl 1000 ppm: ■ Cetrimide Agar (Pa), ■ Tryptone Soy Agar (Pa+drain)



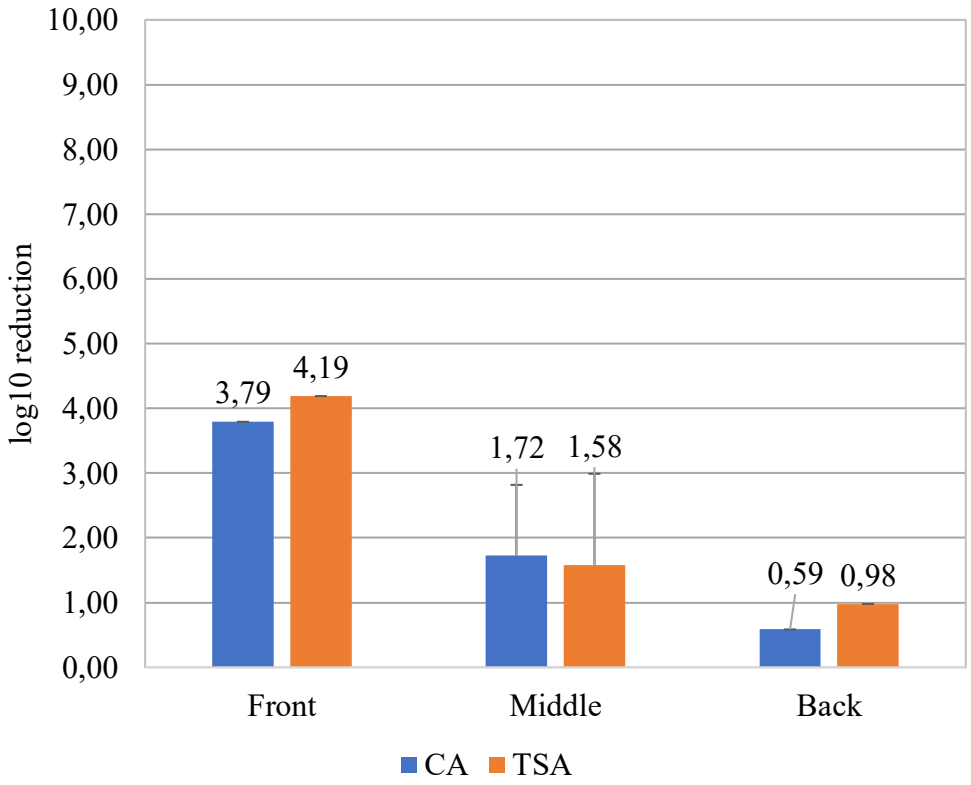
Log_{10} reductions observed following 15 min treatment with NaOCl 1000 ppm: Front section (3.95 ± 1.28 CA; 3.96 ± 1.46 TSA) Middle section (1.92 ± 1.90 CA; 1.89 ± 1.92TSA), Back section (1.12 ± 1.37 CA; 1.34 ± 1.84 TSA).

No statistically significant difference was observed between sections (CA:CA) ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances) nor within sections (CA:TSA) ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances).

5.2.2.2 NaDCC

Figure 14 shows shows log_{10} reduction observed following 15 min treatment with NaDCC 1000 ppm.

Figure 14: log_{10} reduction observed following 15 min treatment with NaDCC 1000 ppm: ■ Cetrimide Agar (Pa), ■ Tryptone Soy Agar (Pa+drain)



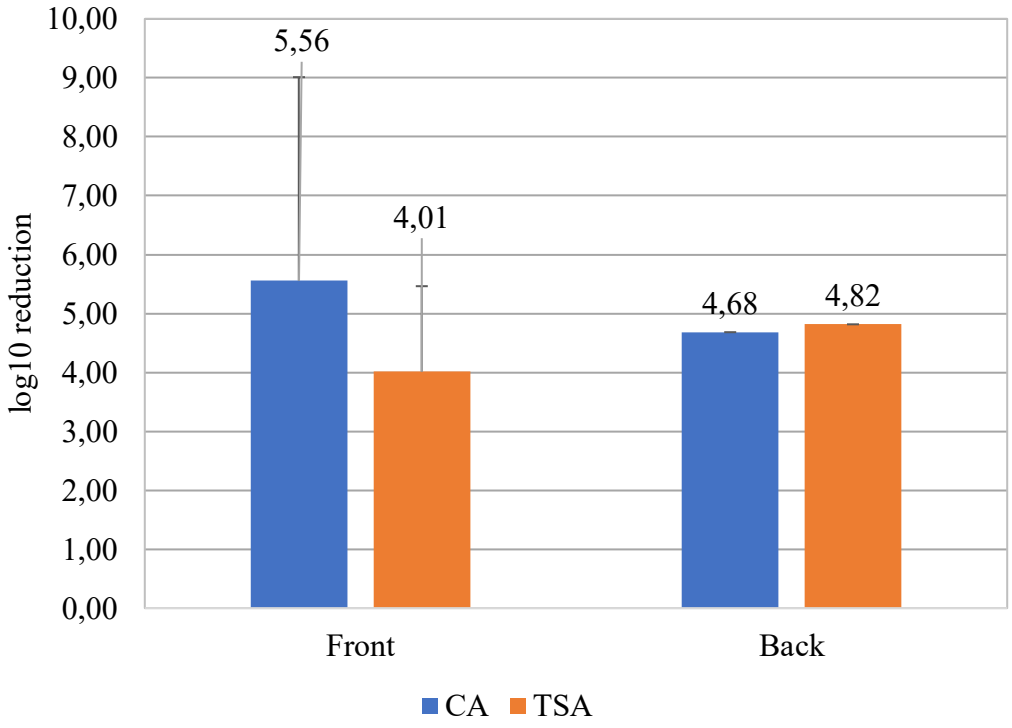
Log_{10} reductions observed following 15 min treatment with NaDCC 1000 ppm: Front section (3.79 CA; 4.19 TSA) Middle section (1.72 ± 1.09-CA; 1.58 ± 1.41-TSA), Back section (0.59 CA; 0.98 TSA).

No statistical significance was observed within middle section ($p>0.05$; t-Test: Two Sample Assuming Equal Variances).

5.2.2.3 PAA

Figure 15 shows log_{10} reduction observed following 15 min treatment with PAA 4000 ppm.

Figure 15: log_{10} reduction observed following 15 min treatment with PAA 4000 ppm: ■ Cetrimide Agar (Pa), ■ Tryptone Soy Agar (Pa+drain)



Log_{10} reductions observed following 15 min treatment with PAA 4000 ppm: Front section (5.56 ± 3.45 CA; 4.01 ± 1.45 TSA) Back section (4.68 CA; 4.68 TSA).

No statistical significance was observed within front section (CA:TSA) ($p>0.05$; t-Test: Two-Sample Assuming Equal Variances).

5.2.2.4 Comparing disinfectants

No significant difference in log_{10} reduction at front section was observed between PAA and NaOCl ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances).

No significant difference in log_{10} reduction at middle section was observed between NaDCC and NaOCl ($p>0.05$, t-Test: Two-Sample Assuming Equal Variances).

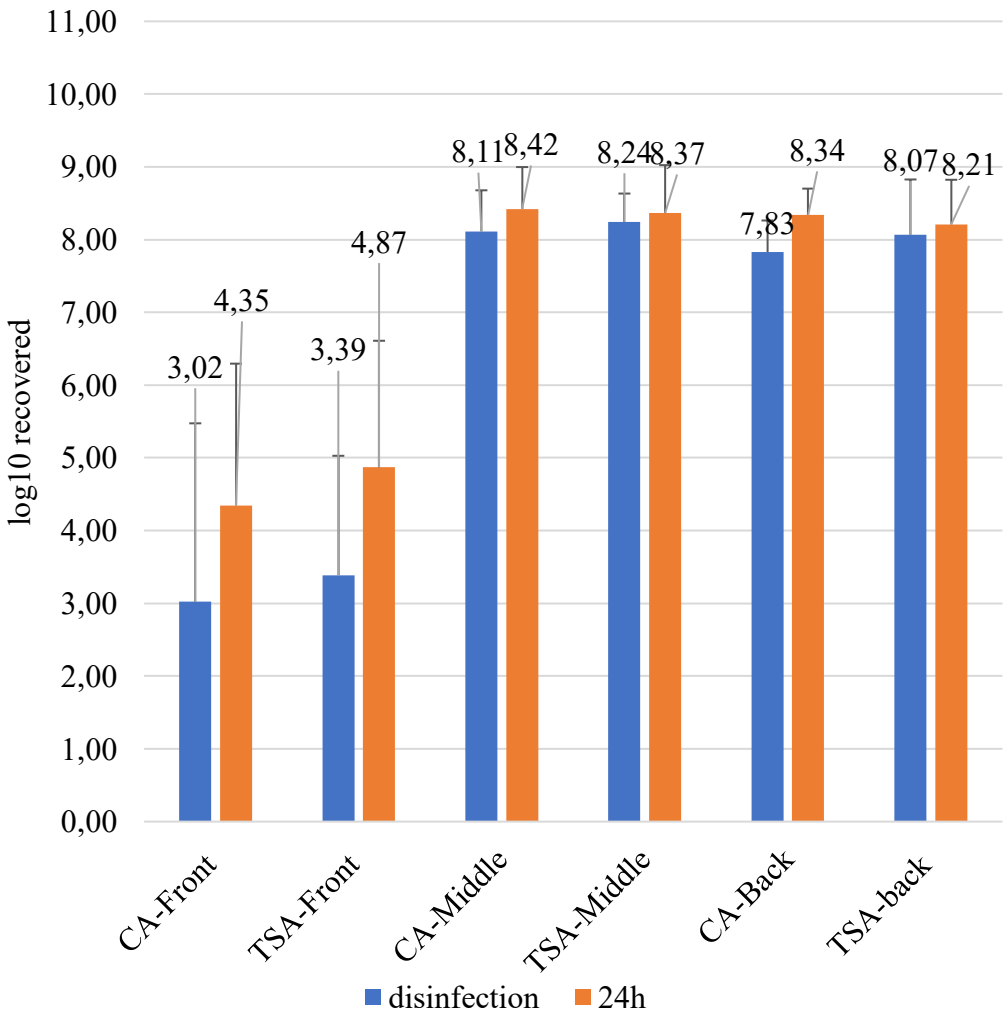
5.3 24 hours regrowth

Five biological replicates were conducted. Each biological replicate was carried out in two technical replicates. Results show their mean in log_{10} and standard deviation of the mean.

5.3.1 NaOCl

Figure 16 shows log_{10} recovered directly and 24 hours following 15 min treatment with NaOCl 1000 ppm.

Figure 16: log_{10} recovered directly and 24 hours after the NaOCl treatment: ■ log_{10} recovered directly after treatment ■ log_{10} recovered 24 hours after treatment CA (Pa) TSA (Pa+ drain)



Front section log_{10} recovered directly after a treatment (3.02 ± 2.45 CA; 3.39 ± 1.64 TSA) and 24 h after the treatment (4.35 ± 1.95 CA; 4.87 ± 1.74 TSA).

Middle section $\log_{10}$ recovered directly after a treatment (8.11 ± 0.56 CA; 8.24 ± 0.39 TSA) and 24 h after the treatment (8.42 ± 0.58 CA; 8.37 ± 0.66 TSA).

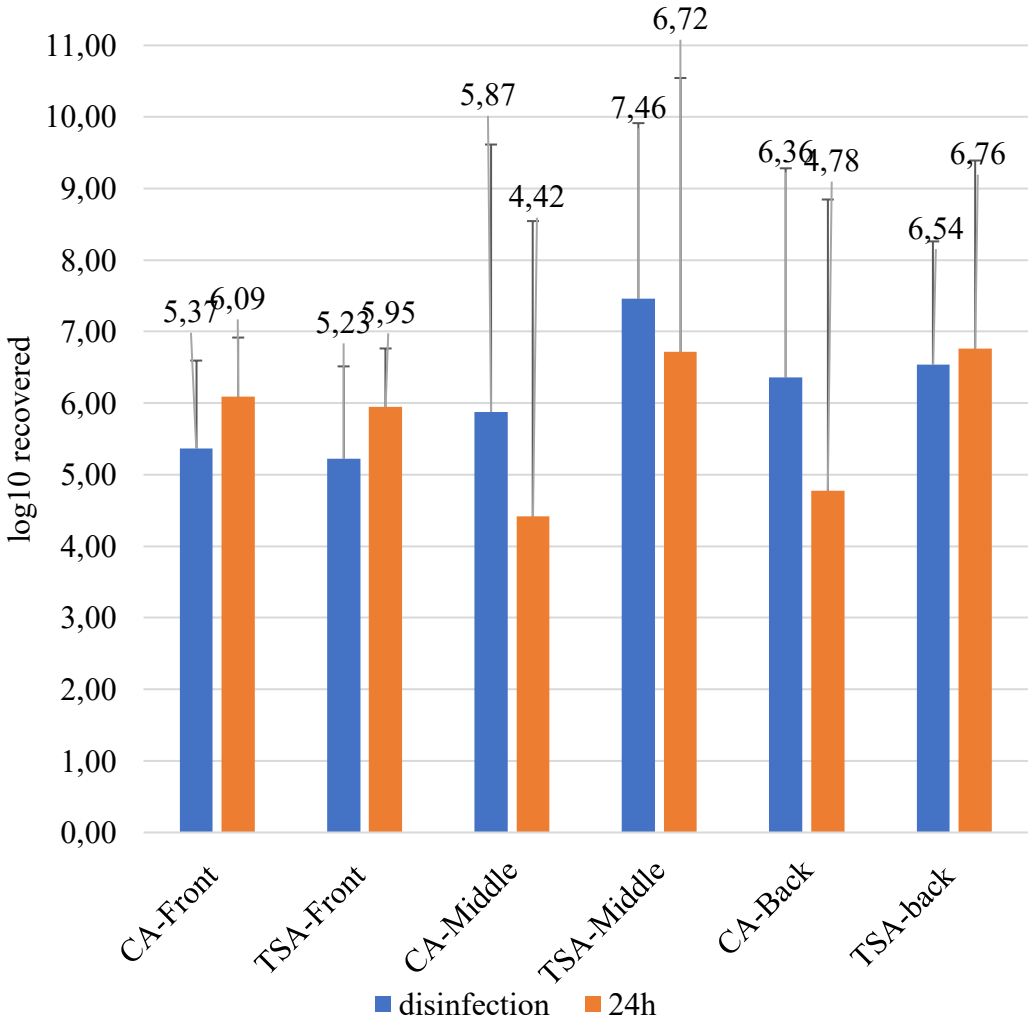
Back section $\log_{10}$ recovered directly after a treatment (7.83 ± 0.43 CA; 8.07 ± 0.76 TSA) and 24 h after the treatment (8.34 ± 0.36 CA; 8.21 ± 0.61 TSA).

No statistically significant difference was observed between $\log_{10}$ recovered directly and 24 hours following 15 min treatment with NaOCl 1000 ppm. ($p>0.05$ two-tailed t-Test: Paired Two Sample for Means).

5.3.2 NaDCC

Figure 17 shows log_{10} recovered directly and 24 hours following 15 min treatment with NaDCC 1000 ppm.

Figure 17: log_{10} recovered directly and 24 hours after NaDCC treatment: ■ log_{10} recovered directly after a treatment ■ log_{10} recovered 24 hours after the treatment CA (Pa) TSA (Pa+ drain)



Front section log_{10} recovered directly after a treatment (5.37 ± 1.23 CA; 5.23 ± 1.29 TSA) and 24 h after the treatment (6.09 ± 0.83 CA; 5.95 ± 0.82 TSA).

Middle section log_{10} recovered directly after a treatment (5.87 ± 3.74 CA; 7.46 ± 2.46 TSA) and 24 h after the treatment (4.42 ± 4.16 CA; 6.72 ± 3.64 TSA).

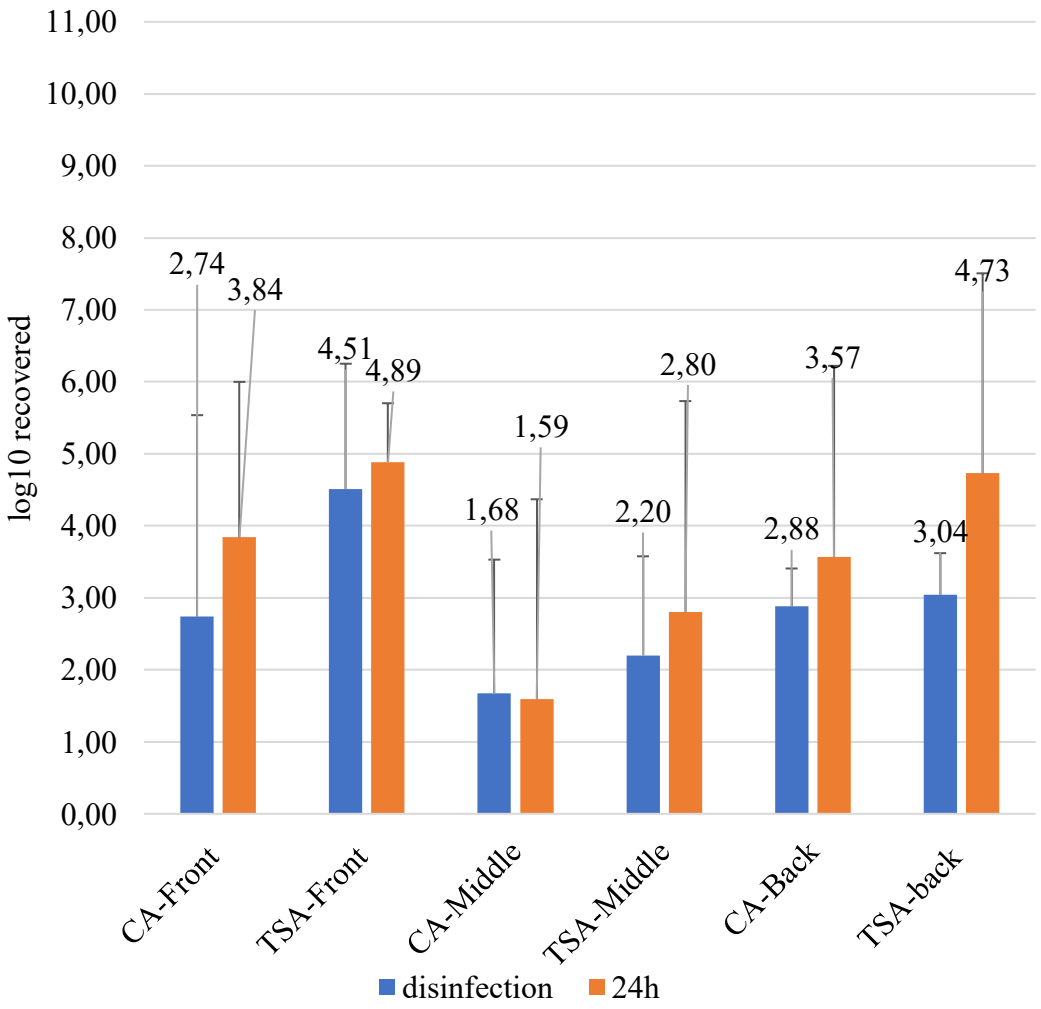
Back section log_{10} recovered directly after a treatment (6.36 ± 2.92 CA; 6.54 ± 1.72 TSA) and 24 h after the treatment (4.78 ± 4.07 CA; 6.76 ± 2.63 TSA).

No statistically significant difference was observed between log_{10} recovered directly and 24 hours following 15 min treatment with NaDCC 1000 ppm. ($p>0.05$ two-tailed t-Test: Paired Two Sample for Means).

5.3.3 PAA

Figure 18 shows log_{10} recovered directly and 24 hours following 15 min treatment with PAA 4000 ppm.

Figure 18: log_{10} recovered directly and 24 hours after PAA treatment ■ log_{10} recovered directly after a treatment ■ log_{10} recovered 24 hours after the treatment CA (Pa) TSA (Pa+ drain)



Front section log_{10} recovered directly after a treatment (2.74 ± 2.80 CA; 4.51 ± 1.74 TSA) and 24 h after the treatment (3.84 ± 2.16 CA; 4.89 ± 0.82 TSA).

Middle section $\log_{10}$ recovered directly after a treatment (1.68 ± 1.85 CA; 2.20 ± 1.37 TSA) and 24 h after the treatment (1.59 ± 2.78 CA; 2.80 ± 2.93 TSA).

Back section $\log_{10}$ recovered directly after a treatment (2.88 ± 0.53 CA; 3.04 ± 0.58 TSA) and 24 h after the treatment (3.57 ± 2.65 CA; ± 2.78 TSA).

No statistically significant difference was observed between $\log_{10}$ recovered directly and 24 hours following 15 min treatment with PAA 4000 ppm. ($p>0.05$ two-tailed t-Test: Paired Two Sample for Means).

5.4 Motility test

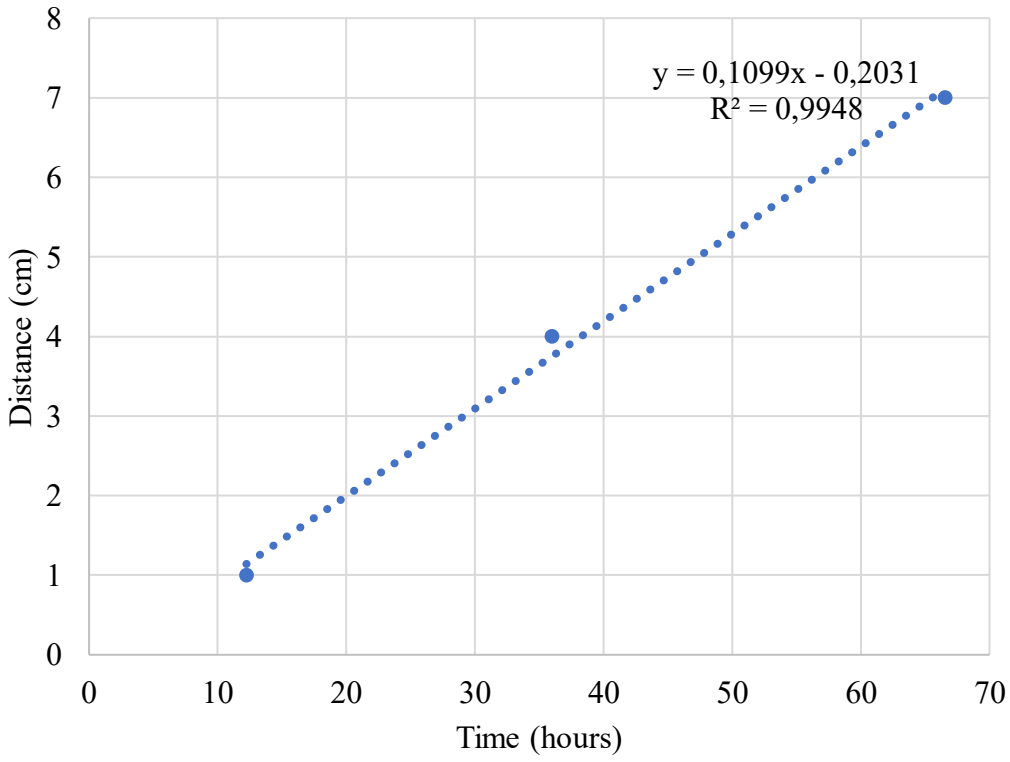
Four biological replicates were conducted with both *P. aeruginosa* culture and drain culture.

5.4.1 *P. aeruginosa* motility test

5.4.1.1 *P. aeruginosa* 4 $\log_{10}$ breakpoint motility

Figure 19 shows *P. aeruginosa* 4 $\log_{10}$ breakpoint motility speed.

Figure 19: *P. aeruginosa* 4 $\log_{10}$ breakpoint motility speed

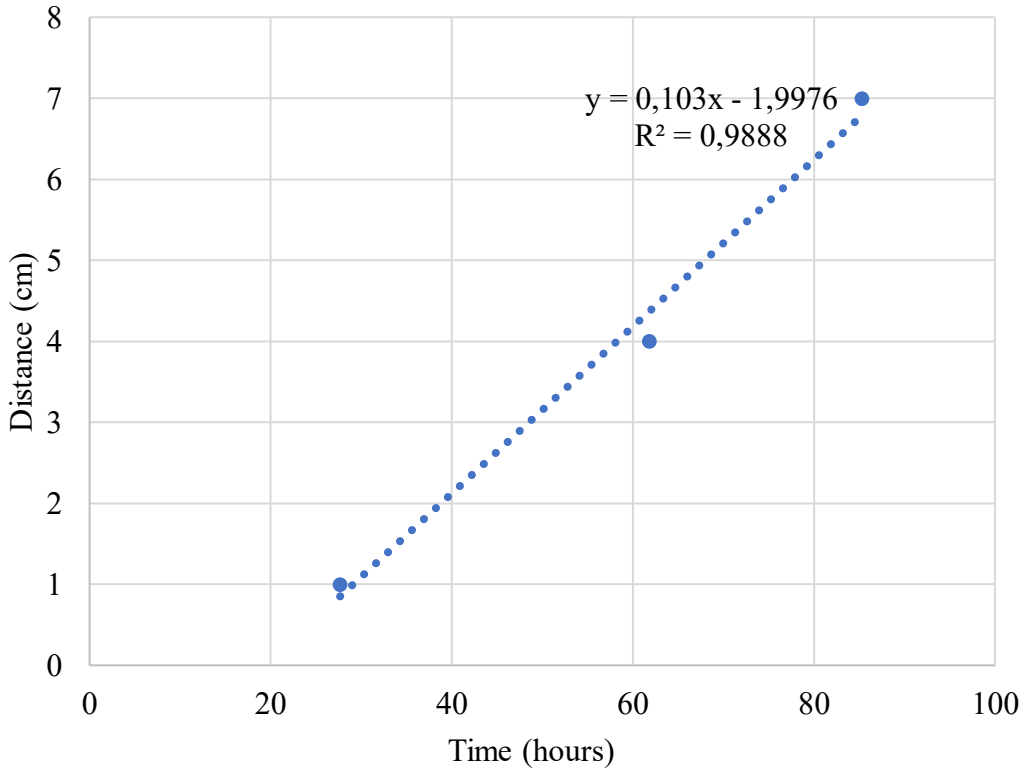


Derived from gradient (Figure 19), speed of upward motility for *P. aeruginosa* 4 $\log_{10}$ breakpoint is 0.11 cm/hour.

5.4.1.2 *P. aeruginosa* 6 $\log_{10}$ breakpoint motility

Figure 20 shows *P. aeruginosa* 6 $\log_{10}$ breakpoint motility speed.

Figure 20 *P. aeruginosa* 6 $\log_{10}$ breakpoint motility speed



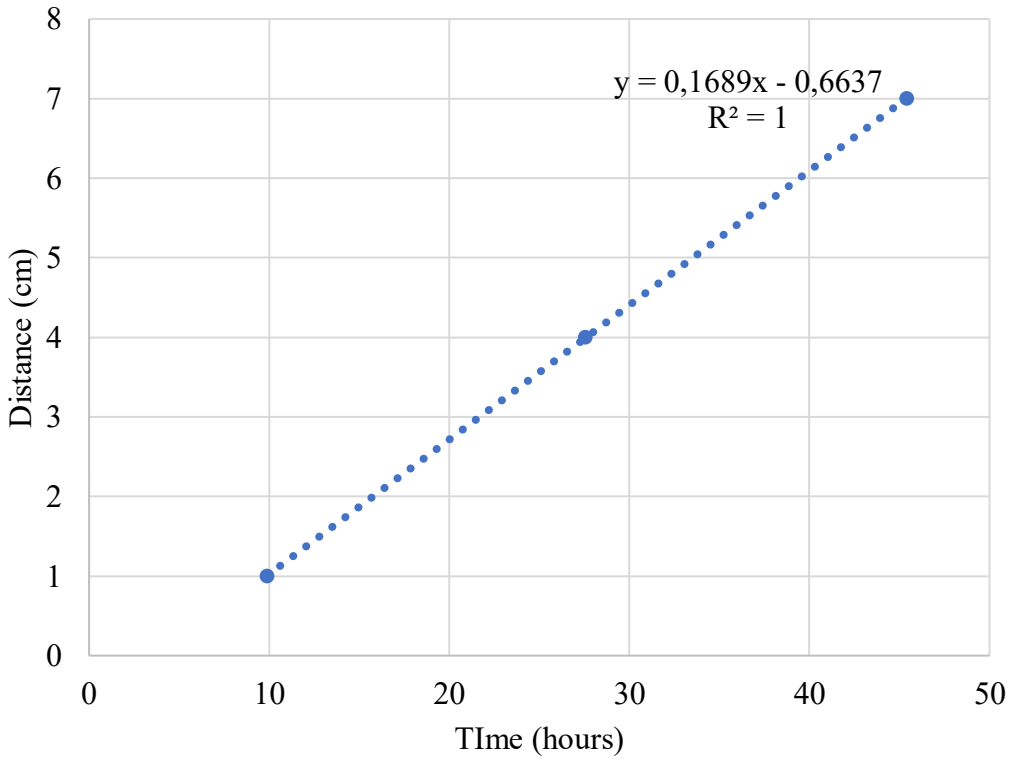
Derived from gradient (Figure 20), speed of upward motility for *P. aeruginosa* 6 $\log_{10}$ breakpoint is 0.10 cm/hour.

5.4.2 Drain culture motility test

5.4.2.1 Drain culture 4 log_{10} breakpoint motility

Figure 21 shows drain culture 4 log_{10} breakpoint motility speed.

Figure 21: Drain culture 4 log_{10} breakpoint motility speed

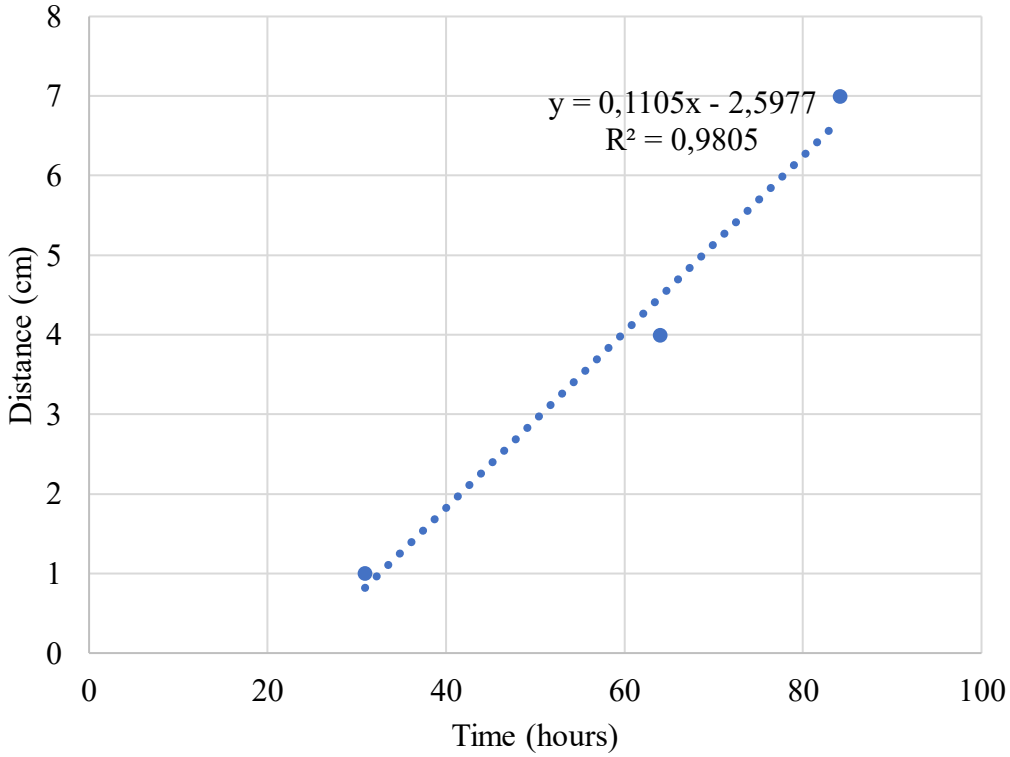


Derived from gradient (Figure 21), speed of upward motility for drain culture 4 log_{10} breakpoint is 0.17 cm/hour.

5.4.2.2 Drain culture 6 log_{10} breakpoint motility

Figure 22 shows drain culture 6 log_{10} breakpoint motility speed.

Figure 22: Drain culture 6 log_{10} breakpoint motility speed



Derived from gradient (Figure 22), speed of upward motility for drain culture 6 log_{10} breakpoint is 0.11 cm/hour.

6. Discussion

Aims of this thesis were assessment of multi-species bacterial biofilm's susceptibility to decontamination, description of its ability to regrow after use of various disinfectants and report on vertical motility of cultures used.

6.1 Biofilm composition

In the protocol used for the disinfectant experiment, inoculum is let to attach for two days and biofilm grows for another six days. This correlates with findings about maturation-2 stage of biofilm ending in approximately 6 days. [6]

P. aeruginosa is able to dominate mixed-species biofilms. [71,72] At the end of drain biofilm formation, *P. aeruginosa* dominates over drain culture, regardless the initial inoculation ratio. Overnight inoculum ratio, which produced most constant day eight

complex biofilm ratio was established in prior experiment. [data not shown] 1:10 Pa to drain culture cell ratio was chosen as inoculation ratio. Consistent *P. aeruginosa* to drain culture day eight complex biofilm ratios were not achieved. One of the reasons might be that drain culture used, was a highly complex community of microorganisms, consisting of eighty-four different bacterial species. Described by Ledwoch *et al.* [73] Heterogeneity of the culture might have contributed to variation in biofilm composition, as well as error introduced by the conductor. Some sections even display negative Pa to drain culture ratio. In these cases, reference cell count on *P. aeruginosa*-selective agar was higher than on non-selective agar, suggesting that *P. aeruginosa* has totally overtaken those biofilms (Table 3).

6.2 Disinfection

Results obtained, seldom show statistically significant difference. This is mainly due to insufficient number of comparable biological replicates relative to variance which was observed.

6.2.1 NaOCl

NaOCl showed significantly better ability to reduce $\log_{10}$ of a 1-fold ratio multi-species *P. aeruginosa* and drain culture biofilm at front section compared to middle section when applied to the sink trap model used in the thesis. This can be contributed to the nature of the experiment and disinfectant formulation (solution). Front section was exposed to original concentration of NaOCl solution. Middle and back sections were exposed to solution that has been diluted in trap bottle, lowering NaOCl concentration, therefore biocidal activity at these sections. Also, NaOCl decomposes faster than NaDCC or PAA which may have resulted in its worse performance. [59, 74] No significant difference in $\log_{10}$ reduction was observed between CA (Pa) and TSA (Pa + drain) at either section.

In the case of 0.1-fold *P. aeruginosa*:drain cell ratio samples, no significant difference in $\log_{10}$ reduction was observed neither between sections, nor within sections. 0.1-fold ratio biofilm results show relatively high variance and it is possible, that number of biological replicates obtained are not robust enough to see a significant difference.

No studies using an *in vitro* flow model, similar to sink trap model used in the thesis, observing biocide efficacy on removing multi-species biofilm have been conducted to the best of my knowledge. There is a study by Yap *et al.* studying NaOCl's biocidal efficacy on multi-species biofilm grown on dentine. Testing was performed in 24 well

plate where 0.9% NaOCl was introduced. They show 10-fold reduction of CFU/µg protein. That would be 1 $\log_{10}$ reduction. Dentine and species inhabiting it are different to sink drain and its micro-environment, thus it is difficult to draw any comparisons. [75]

However, there are studies describing NaOCl's effect on *P. aeruginosa* single-species and dual-species biofilms. Lineback *et al.* have achieved better $\log_{10}$ reduction (8.75) compared to this study, testing a single-species *P. aeruginosa* biofilm, that was grown on borosilicate coupons. There are three factors contributing to difference in achieved results. Single-species biofilm does not gain advantage in the form of inter-species cooperation and its composition is limited to molecules produced by the species composing it. Disinfectant was also introduced in a different way compared to this thesis. Last factor is concentration. Lineback *et al*. mentions 1.3 % NaOCl product concentration, 52-fold bigger than NaOCl concentration used in this study. It comes as no surprise; their results are superior. [76]

Another study is testing disinfectant efficacy in a very similar chemostat model. Behnke *et al.* achieved 3.90 $\log_{10}$ reduction with 2 ppm NaOCl solution, and 6 $\log_{10}$ reduction with 30 ppm. In this case concentration of NaOCl used is lower by several folds, but their contact time is twice as long (30 min), compared to this study. Their methodology adjusts biofilm CFU/ml to 7 $\log_{10}$ while biofilms grown for this study were unadjusted and counted approximately 8 $\log_{10}$ CFU/ml. All these differences could add up to different performance of NaOCl observed. Behnke *et al.* also tested planktonic cells and show that biofilm requires approximately ten times higher dose of chlorine compared to planktonic cells. [77]

6.2.2 NaDCC

NaDCC seems to achieve better $\log_{10}$ reduction at front section of the sink trap model. However, not enough biological replicates have been obtained to show statistically significant difference between sections.

NaDCC's performance on *P. aeruginosa* single-species biofilm and planktonic cells has been described previously using a flow cell, apparatus similar to set-up used in this thesis. Several different concentrations were used with different magnitude compared to experiments presented here. Planktonic cell CFU/ml in the Morgenthau *et al*. [78] study is 8 $\log_{10}$, similar to viable counts retrieved from biofilm reference samples presented here. On the other hand, they did not assess viable cell counts of biofilm samples, making

it difficult to compare results for biofilm control. They show approximately 5 $\log_{10}$ reduction for planktonic *P. aeruginosa* cells after 30 min exposure to 5% NaDCC. This somewhat correlates with results presented in this thesis. Higher concentrations of NaDCC, above 60% completely inhibited growth of both biofilm and planktonic cells, suggesting that higher concentration of NaDCC might yield better results than results obtained in this thesis.

6.2.3 PAA

PAA was used in 4-fold greater concentration compared to chlorine-based disinfectants. Therefore, PAA-product's better $\log_{10}$ reduction was expected. Statistically significant difference was found between NaOCl's $\log_{10}$ reduction and PAA's $\log_{10}$ reduction at middle section, in the case of 1-fold Pa:drain ratio biofilm. There is also significant difference between NaDCC and PAA for back section, but only for TSA samples, probably because there was higher variance for NaDCC's efficacy at back section and data obtained are not robust enough to show statistically significant difference. Log_{10} reduction at front section was lower, probably because not all PAA product fit the front section tube. Product that didn't fit, had to be introduced directly to the trap bottle.

Zhang *et al.* present that PAA performed worse at removing single-species biofilm compared to chlorine-based products. They furthermore prove that PAA reacts less with organic content compared to bleach, because it performed better in presence and worse in absence of organic matter in comparison to bleach. [74]

Zhang *et al.* findings, that PAA reacts less with organic matter and/or does not decompose as quickly as free chlorine are align with data presented in this thesis, where PAA is dominant in the ability to remove biofilm. [74]

6.3 24 hours regrowth

6.3.1 NaOCl

No statistically significant difference was observed between $\log_{10}$ recovered directly and 24 hours following 15 min treatment with NaOCl 1000 ppm. This could suggest inadequate effect of NaOCl on biofilm's regeneration after a single treatment.

To the best of my knowledge there are no published studies displaying $\log_{10}$ recovered any time after NaOCl treatment, to compare the thesis with.

A study conducted by Buchan *et al.* followed sink drains in ICU unit one day and 7 days after disinfection with bleach and hydrogen peroxide foaming product. They don't show $\log_{10}$ reduction, rather CFU/ml before disinfection, one day after disinfection and 7 days after disinfection. Furthermore, they have used MacConkey's agar, selective for gram-negative bacteria. Similarly, to this study there is rather high variance in results obtained, since they worked with multi-species biofilm as well. [79]

Zhang *et al.* have conducted a study on regrowth of single-species biofilm after disinfection. However, they present how much NaOCl and PAA is needed to prevent biofilm regrowth after a treatment. [74]

6.3.2 NaDCC

No statistically significant difference was observed between $\log_{10}$ recovered directly and 24 hours following 15 min treatment with NaDCC 1000 ppm. This potentially suggests low effect of NaDCC on multi-species biofilm's regrowth after a single treatment.

Ledwoch *et al.* have published the only study, to the best of my knowledge, where biofilm's ability to regrow after a NaDCC treatment is assessed. However, there are major differences in ways how their study was conducted compared to this thesis. Ledwoch *et al.* work with dry-surface *Candida auris* biofilms. *C. auris* is a yeast, with huge physiological differences compared to bacteria. Their results track delay in biofilm post-treatment re-emergence whereas $\log_{10}$ recovered has been followed and presented here. [80]

6.3.3 PAA

No statistically significant difference was observed between $\log_{10}$ recovered directly and 24 hours following 15 min treatment with PAA 4000 ppm. This could suggest inadequate effect of PAA on biofilm's regeneration after a single treatment.

Limited data are available on PAA's ability to reduce biofilm regrowth. To the best of my knowledge there is only one study by Zhang *et al.* Single-species biofilm was used and they didn't measure log reduction, rather concentration of PAA to inhibit biofilm regrowth. [74]

6.4 Motility test

This experiment shows and quantifies speed of vertical motility *via* biofilm spreading, that was observed over 3 days.

P. aeruginosa culture 4 $\log_{10}$ breakpoint's vertical growth was 0.11 cm/h while 6 $\log_{10}$ breakpoint grew upwards at a rate of 0.10 cm/h.

Drain culture culture 4 $\log_{10}$ breakpoint grew upwards at a rate of 0.11 cm/h while 6 $\log_{10}$ breakpoint's vertical growth was 0.10 cm/h.

Kotay *et al.* present that *E. coli,* inoculated in a P-trap, reaching a strainer in 7 days when nutrients were introduced. Same starting inoculum (10^3 CFU/ml) was used as in this thesis. Their findings show 2.54 cm/day speed of vertical motility, with. It's the same speed (0.11cm/h) as was obtained with motility test methodology developed in Cardiff.

Motility test methodology developed in Cardiff has its main advantage in simple conduction. At the same time, it takes up neither many resources nor much time of a scientist. This methodology could be used to describe movement *via* biofilm spreading of different species and/or multi-species biofilms.

7. Conclusion

It has been established, that gram-negative bacteria inhibit water drainage systems and are able to colonize environment outside the sink, for example patients.

Issue of sink-drain to patient contagion is better observable in hospital setting due to several factors. Multi-resistant species might emerge from antibiotic-residue flushes. Immuno-compromised patients are more prone to get infected. Combined with the fact that the size of hospital scales with the amount of people and traffic, the probability of contagion and its spreading increases.

This thesis evaluates multi-species biofilm's resistance and ability to reinstate after a single disinfectant treatment.

Three disinfectants were compared on their ability to remove bacteria from multi-species biofilms and impact multi-species biofilm's regrowth 24 hours after the treatment. Product containing PAA had better efficacy at removing biofilm and preventing its regrowth. More studies focused on repeated treatments and tracking regrowth at later stages should be conducted to better describe multi-species biofilm's properties and behaviour.

A simple, cheap, yet effective methodology to investigate upward bacterial movement *via* spreading of bacterial biofilm has been developed. Results obtained on upward

motility further support environmental and genetic studies focusing on whether bacteria in hospital wards come from water drainage systems.

8. References

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