



Degree Project in Biotechnology

Second cycle, 30 credits

Understanding the Dynamics of Microbial Growth and Disinfection Strategies in Granular Activated Carbon Filters for Drinking Water Treatment

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Abstract

Norrvatten is a Swedish drinking water producer and is currently in the process of building a complementary waterwork, which partly involves a combination of ultrafiltration (UF) followed by granular activated carbon (GAC) filtration. The consecutive order of these entails a risk of unwanted and harmful microbial growth in the GAC, due to low competition. Therefore, this study aimed to understand the potential growth of microbes in GAC filters and how different contamination sources could accelerate this growth. Sodium hydroxide (NaOH) was also evaluated for the disinfection and regeneration of GAC beds, as a potential point of action upon unwanted microbial growth. New GAC was spiked with different water sources from the current waterwork streams and reference material from the Swedish Food Agency (SFA) and then submerged in permeate from an UF pilot plant. Bacterial growth was followed in both spiked and non-spiked carbon by flow cytometric measurements of total and intact cells (TCC, ICC) in the GAC and liquid phases. The resulting carbons were then treated with NaOH at varying concentrations and times. Effects on different groups of microorganisms were measured by analyzing GAC for total and intact cells, microfungi, heterotrophic plate counts, coliforms and *Escherichia coli*, before and after treatment. It appeared that UF-permeate and GAC made an excellent growth matrix for bacteria where the strongest contributors to growth in non-spiked carbon beds were the carbon itself, followed by keeping the system open to air. A non-spiked and open GAC bed also surpassed all other spiked beds, in terms of liquid phase growth. Results showed that NaOH could effectively disinfect bacteria attached to GAC, with concentrations down to 0.1 w/v% being greatly effective against at least 2.8 million ICC/g carbon. Conclusions about microfungi, coliforms and *E. coli* could not be drawn due to the absence of these in GAC samples. NaOH also showed promising regeneration capabilities in terms of the removal of TCC from GAC. However, the relationship between concentration, time and TCC reduction was irregular, suggesting that there were one or more additional factors affecting regeneration efficiency.

Keywords

Desorption, disinfection, DW, DWTP, GAC, microbial growth, regeneration, ultrafiltration

Sammanfattning

Norrvatten är en svensk dricksvattenproducent och är nu i en process av att bygga ett komplimenterande vattenverk som delvis består av en kombination av ultrafiltrering (UF) följt av filtrering genom granulerat aktivt kol (GAC). Den på varandra följande ordningen av dessa innebär en risk för oönskad tillväxt av skadliga mikroorganismer i kolfiltren, på grund av låg konkurrens. Denna studie syftar därför till att förstå den potentiella tillväxten av mikroorganismer i kolfilter och hur olika kontaminationskällor skulle kunna påskynda tillväxten. Natriumhydroxid (NaOH) utvärderades också för desinfektion och regenerering av kolfilter, som en potentiell punktåtgärd vid oönskad mikrobiell tillväxt. Nytt kol spetsades med olika vattenkällor från det nuvarande vattenverkets strömmar och med referensmaterial från livsmedelsverket för att sedan läggas i filtrat från en UF pilot. Bakteriell tillväxt följdes sedan i spetsat och icke-spetsat kol genom att med flödescytometri mäta totala och intakta celler (TCC, ICC) i GAC och vattenfaserna. De resulterande kolen behandlades sedan med NaOH i olika koncentrationer och behandlingstider. Effekten på olika grupper av mikroorganismer mättes genom att analysera totala och intakta celler, mikrosvamp, heterotrofer, koliformer och *Escherichia coli* i kolprover, innan och efter behandling. Det kunde visas att UF-filtrat tillsammans med GAC utgjorde en utmärkt tillväxtmiljö för bakterier, där de största bidragande faktorerna till tillväxt i icke-spetsat kol var kolet självt, följt av att hålla systemet öppet mot luft. En icke-spetsad och öppen kolbädd visade även en tillväxt starkare än alla spetsade bäddar, med avseende på tillväxt i vattenfaserna. Resultaten visade att NaOH är ett effektivt desinfektionsmedel för bakterier bundna till GAC, där koncentrationer ner till 0.1 v/v% var mycket effektiva mot som minst 2.8 miljoner ICC/g kol. Slutsatser kunde inte dras om mikrosvamp, koliformer och *E. coli* eftersom dessa inte var närvarande i kolproverna. NaOH visade dessutom lovande regenereringsförmågor med avseende på borttagning av totala celler i GAC. Däremot var sambandet mellan koncentration, tid och reduktion av totala celler oregelbundet, vilket tyder på att det fanns en eller flera ytterligare faktorer som påverkade effektiviteten av regenerering.

Nyckelord

Desorption, desinfektion, dricksvatten, dricksvattenverk, GAC, mikrobiell tillväxt, regenerering, ultrafiltrering

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Abbreviations

Biologically active carbon	BAC
Cellulose acetate	CA
Design of experiments	DoE
Drinking water	DW
Drinking water treatment plant	DWTP
Flow cytometry	FCM
Granular activated carbon	GAC
Heterotrophic plate count	HPC
Intact cell count	ICC
Most probable number	MPN
Norrvatten's future water production	NFVP
Polytetrafluoroethylene	PTFE
Propidium iodine	PI
Swedish Food Agency	SFA
Swedish Veterinary Agency	SVA
SYBR green	SG
Total cell count	TCC
Total organic carbon	TOC
Ultrafiltration	UF
Ultrafiltration pilot plant	UFPP
Yeast extract agar	YEA

1 Introduction

Norrvatten is a Swedish municipal association producing and distributing drinking water to around 700,000 people in northern Stockholm, several large hospitals and Arlanda airport. Water is taken from Lake Mälaren in Görvälnverket waterworks, where it is purified in several steps to reach high quality. It is then distributed to the 14 member municipalities (Fig 1) which in turn are responsible for delivering the water to the residents (1). Norrvatten must supply the region with clean drinking water to ensure the functionality of the healthcare system, Arlanda airport and the health of people in the region. Therefore, there is a constant investment in research and development within purification technology, climate effects on raw water quality and environmental work (2). Norrvatten is now in the early phase of building a complementary waterwork with state-of-the-art purification technologies to improve capacity, operational reliability and barriers against microbes, viruses and parasites. The project is called Norrvatten's future water production (NFVP) which this study is a sub-project within (3).



Figure 1: Norrvatten's member municipalities (4).

1.1 Current drinking water treatment process

In the current purification process at Norrvatten, raw water is taken at two different depths depending on the season and water quality. It then passes a strainer basket with micro sieves to remove fish and other organic debris before it is pumped to a mixing chute where aluminum sulphate is added. The water then flows into agitated flocculation chambers where the aluminum sulphate joins humic substances, particles, microorganisms and more into flocks. The addition of small amounts of sodium silicate also makes the flocks larger. Thereafter, flocks are removed by sedimentation and flotation in elongated sedimentation basins and processed in a separate sludge facility. The remaining water is then filtered through 1.5 m deep sand beds to remove excess flocks. During sand filtration, the water becomes clear and colorless but might still have taste, odor and contain trace amounts of harmful substances. Therefore, the water is further filtered through 2.5 m deep granular activated carbon (GAC) beds where unwanted substances are reduced. To minimize microbial growth in the distribution system, carbon filtrate is disinfected with ultraviolet light followed by the addition of small amounts of monochloramine. Lime is also added to make the water slightly basic to prevent corrosion in the distribution system. The final water is stored in a reservoir from where it is pumped to the municipalities (Fig 2) (5).

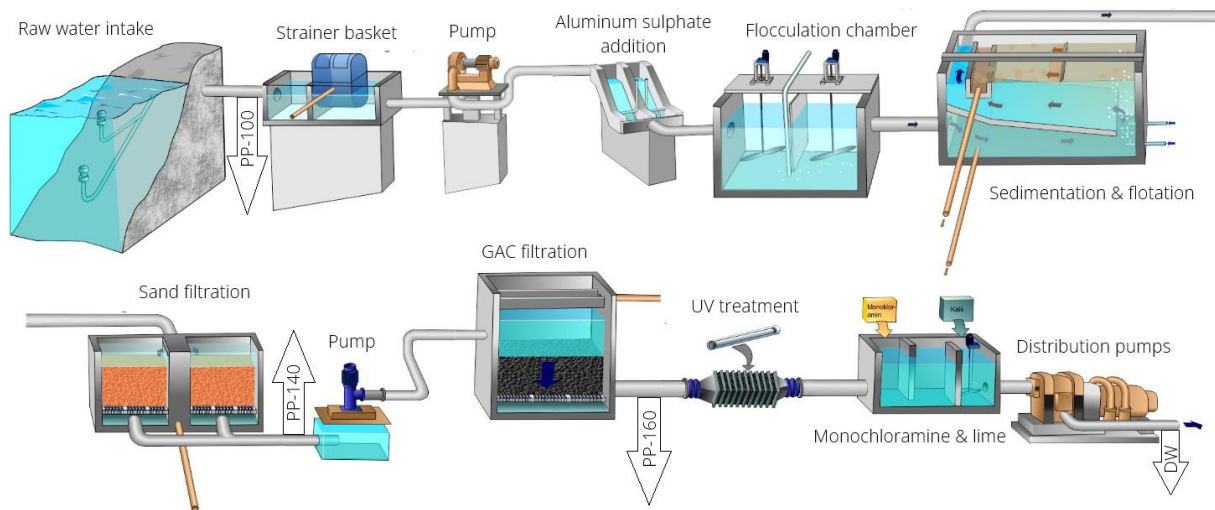


Figure 2: The current drinking water treatment process steps. Arrows indicate sampling points (5).

1.2 Drinking water challenges

Some of the growing concerns for drinking water are the prevalences of per- and polyfluoroalkyl substances (PFAS) and pharmaceuticals in raw water. According to the Swedish Food Administration, guideline values for PFAS 4 and PFAS 21 are 4 ng/l and 100 ng/l respectively, which becomes the legal limits by January 1, 2026 (6). Coagulation, flocculation, sand filtration and several other conventional drinking water treatment techniques are generally ineffective in reducing these types of chemicals. However, methods such as granular activated carbon (GAC) filtration are currently being used and developed worldwide for the adsorption of these contaminants, showing great potential. Even though GAC and other techniques have shown to be effective in removing PFAS from drinking water, the chemicals are adsorbed rather than destroyed. Hence, the captured chemicals create a waste that must be managed and disposed of without releasing the content back into the environment. This is often a complicated and costly process (7)(8). Swedish waterworks are standing in front of a huge challenge considering the stricter limits on PFAS that applies from 2026. The major issues will be how to operate and reactivate GAC filters in a way that ensures these limits are always met. Another challenge is the occurrence of pathogenic and toxinogenic bacteria, viruses and parasitic protozoa in raw water, which bring the risk of outbreaks of waterborne diseases. Waterworks must have sufficient microbial barriers that reduce these pathogens to harmless concentrations, even during high abundance in the raw water. Slow filtration (sand), membrane filtration, primary disinfection (inactivation) and chemical precipitation together with filtration are examples of approved barriers by the Swedish Food Agency. Preferably, separative techniques should be combined with a primary disinfection for a safe and reliable overall barrier (9). Even though microbial barrier effects are highly regulated and controlled, outbreaks still occur every year.

1.3 Future drinking water treatment process

Because of the challenges described above, GAC filtration will have an important role in the future drinking water production at Norrvatten, where it shall reduce odor and taste substances, genotoxic effects and organic chemicals, such as PFAS. To meet and keep up with increasing government demands regarding microbial barriers, NFVP is also planning an implementation of ultrafilters (UF) prior to GAC filtration in the future waterwork (10). Although the process design is not completely determined, the latest proposal is to directly precipitate raw water on the UF membranes. Permeate would then pass through GAC filters and finally be disinfected by UV treatment, followed by the addition of monochloramine and lime (11). Since ultrafilters removes up to 99.7% bacterial cells but only around 46% total organic carbon (TOC), GAC filters in the future waterwork will be supplied with a low bioburden, high TOC feed stream (12)(13). Compared to the current GAC beds, future carbon filters will not have the same competition in terms of microbial load and diversity, illustrated in figure 3.

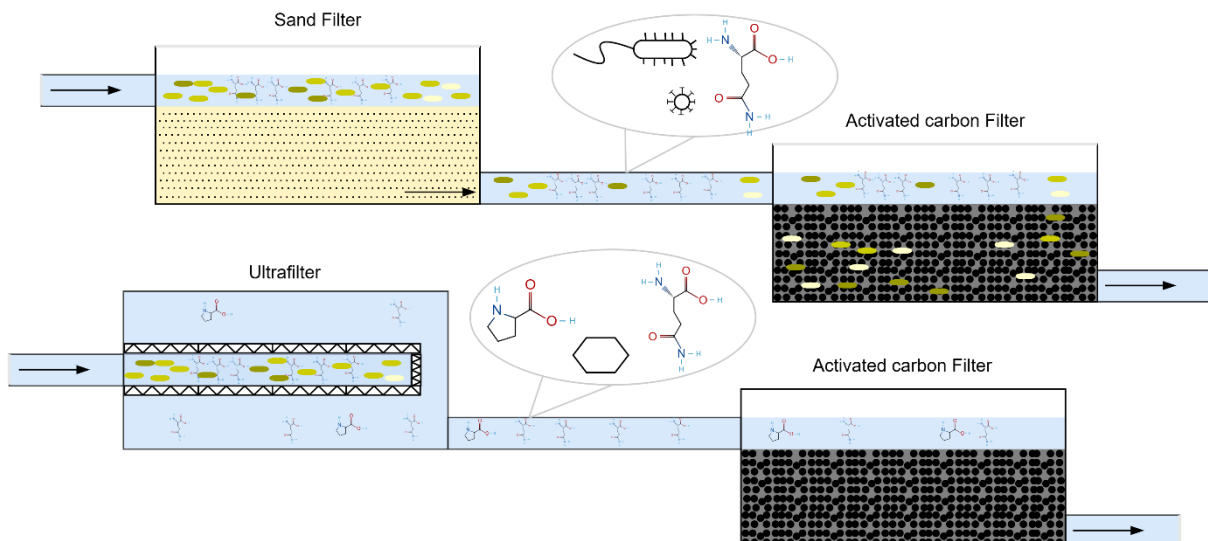


Figure 3: Current GAC filters (top) and future GAC filters (bottom) with prior process steps.

1.4 Scientific aims

The combination of UF and GAC in the future waterwork poses a risk of unwanted microbial growth, such as pathogenic or toxinogenic, so called microbial instability in the GAC beds. Contaminations originating from the carbon or ambient air would have an ideal growth matrix with low competition and high nutrition. Therefore, Norrvatten wants to investigate the microbial growth and disinfection of carbon filters using new GAC as a “sterile” starting point, which will be carried out in the form of this degree project. Along with microbial growth in UF-permeate submerged GAC, it will be investigated whether sodium hydroxide could be used for treating possible microbial instability. If disinfection does not work, a more frequent reactivation of the carbon will be necessary. Hence, the objectives of this study are to:

- 1) Understand the potential microbial growth in GAC filters of the future waterwork.
- 2) Understand what contamination sources could be expected and the risk of establishment in future GAC beds.
- 3) Evaluate sodium hydroxide as a disinfectant and potential regeneration agent for microorganisms attached to GAC.

1.5 Background

1.5.1 Granular activated carbon (GAC) and filtration

Granular activated carbon is made of small and extremely porous particles ranging from 0.4 to 2.5 mm in diameter. It is produced by heating and steaming organic materials, such as wood or coconut shells, to over 800°C in the absence of air (14). Due to the porous structure, GAC has a surface area of around 1000 m²/g and the pores range from visible to molecular size (15). These properties make GAC a great absorber of natural contaminants in drinking water, where the adsorption of specific substances or microbes is dependent on carbon surface chemistry and the volume of pores that fit the contaminant. In new or recently reactivated GAC, the reduction of contaminants occurs through adsorption to the cavities of carbon particles and after a certain time the carbon becomes saturated and must be reactivated to regain its capacity. This is commonly done by exposing carbon to very high temperatures so that adsorbates are volatilized, which is costly and highly energy consuming (14). Between reactivations GAC is exposed to various microbes through inlet water, ambient air and unpredictable sources like the refilling process, leading to microbial attachment in the particle cavities and surfaces. Attached microorganisms proliferate on and within particles and eventually give rise to active biofilms, turning GAC into so-called biological activated carbon (BAC). Although biofilms can improve the reduction of certain unwanted compounds, it may also yield higher concentrations of bacteria in the effluent water due to detachment (16). The adsorption capacity decreases over time while microbial activity, such as biodegradation, increases and eventually constitutes the main removal of dissolved organic carbon (DOC) (17). Since PFAS are resistant to microbial degradation, its removal from drinking water decreases in relation to microbial saturation (18). Activation level, or adsorptive function of activated carbon can be measured in iodine number, which measures the adsorption of iodine from an aqueous solution (19).

1.5.2 Biofilm quantification

The most common method for direct quantification of biofilm is cell enumeration, which involves plate counts, most probable number (MPN) methods, flow cytometry and similar. These methods are also well-established within the quality control of drinking water, for the measurement of cell concentrations. But biofilm could also be quantified indirectly by methods that use markers, for instance proteins, polysaccharides or staining properties to indicate cell amounts. A widely used method, also used in this study, is crystal violet staining where the dye penetrates cell membranes and leaves a color that could be quantified by spectrophotometry (20).

1.5.3 Desorption of cells from GAC particles

To quantify cell numbers in GAC, cells must first be extracted from the particle pores and suspended in a liquid. In a study by Camper et al. (1985) evaluating different procedures to desorb bacteria, it was found that homogenization at 16 000 RPM (4°C) for 3 min using an Omni-mixer homogenizer is the best physical technique for removing cells and protecting viability. The highest desorption efficiency was achieved when combining homogenization with a solution of Zwittergent 3-12 (10^{-6} M), Tris buffer (0.01 M, pH 7), peptone (0.01%) and EGTA (10^{-3} M) for the suspension of released cells. These reagents are used to protect viability and to prevent readsorption of cells. Furthermore, this procedure was shown to desorb 90% of attached bacteria, determined by heterotrophic plate counts. Blending and sonication were also tested for desorption along with a wide range of reagents for the desorption solution. Although blending and sonication were shown to desorb cells, it also killed or injured portions of the desorbed cell population (21). The suggested homogenization method has also been applied in another other study by Pernitsky et al. (1995) with promising results (22).

1.5.4 Reactivation and disinfection strategies for GAC

As described before, conventional reactivation involves the heating of carbon to high temperatures to volatilize adsorbed organisms and substances (14). Reactivation is usually distinguished from regeneration which refers to techniques that partially regain the adsorption capacity of GAC, for instance, removal of biofilm. Reactivation is the total restoration of saturated GAC, like the original production. While heat treatment is the most established method for reactivation, there are other methods suggested for the regeneration of GAC. An example is chemical regeneration which could be implemented on-site (23). One study showed that ethanol together with 0.5% ammonium hydroxide on average could remove 53% of PFOS and 93% of PFOA (included in PFAS 4) from GAC, with possibility to concentrate the waste by distillation for easier management (24). Another review study summarized a wide range of chemicals with potential in PFAS removal from GAC. However, it was also suggested that chemical regeneration at full scale might be problematic from an economical and environmental point of view (25). While chlorine compounds are extensively used for disinfecting bacteria in suspension, it has been shown to have no significant effects on bacteria attached to GAC. Which is suggested to be a result of the protective environment of carbon crevices and the extracellular polymeric substances (EPS) (26). However, an elevated pH by sodium hydroxide (NaOH) has been shown to be

bactericidal to a broad range of strains with only 1 out of 148 surviving the strongest treatment at pH 12 for 72 h, in tryptic soy broth (27). Steam treatments similar to autoclavation could also be used for disinfection since it effectively kills microorganism, although it would require a steam- and pressure resistant filter construction (28).

2 Materials and methods

2.1 Experimental setup and location

Lab scale batchwise pilots were set up using autoclaved 900 ml reagent bottles with plastic caps. For the attachment phase, bottles were filled with fresh GAC (Fig 4) together with different water sources of microbial contamination and let to stand for two weeks, to allow attachment of microorganisms to the GAC pores and surfaces. Thereafter, each liquid phase was exchanged to ultrafilter (UF) permeate, collected from the ultrafiltration pilot plant (UFPP) at Görvålverket, which filters raw water. The permeate was autoclaved prior to use due to microfungus contamination in the UFPP, with the purpose of simulating the future GAC filters that would be fed with non-contaminated ultrafiltered raw water. Following the liquid phase exchange, bottles were left, with regular shaking, for several weeks to allow the development of biofilm inside carbon particles by the growth of attached cells. During the incubation period, microbial growth was monitored by regular flow cytometry (FCM) analysis of the liquid phases (batch 1) or by regular quantification of GAC-attached cells (batch 2). The final carbon beds in bottles from batch 1 were later used for desorption, disinfection, regeneration and regrowth experiments as illustrated in figure 5.

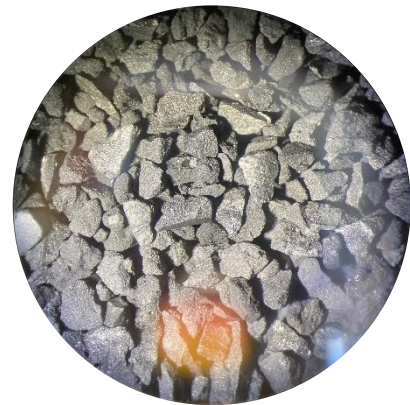


Figure 4: Picture of GAC.

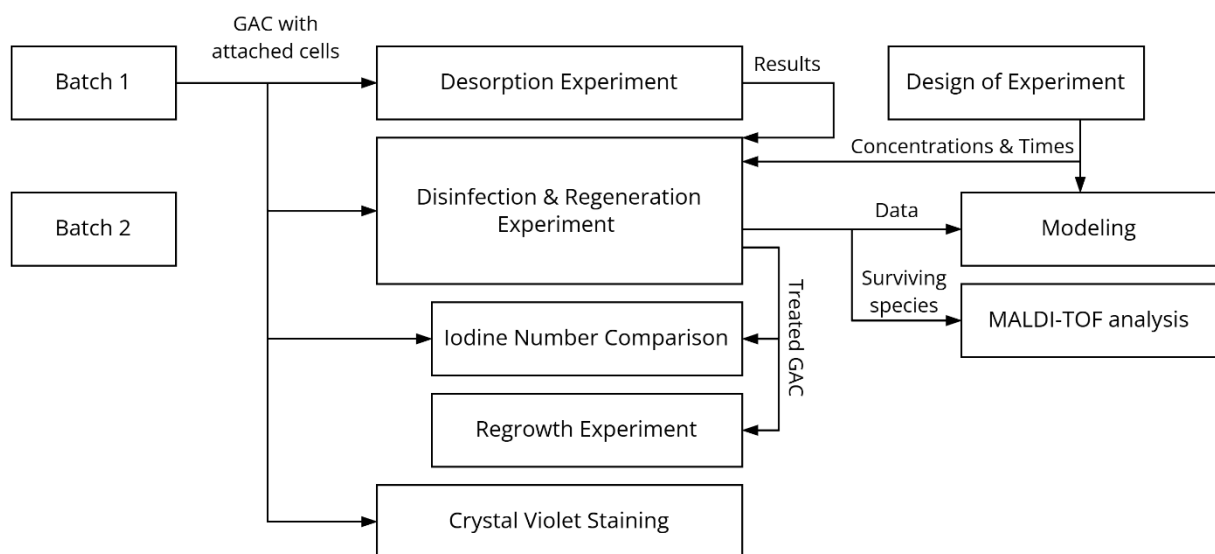


Figure 5: Experimental setup and the connections between different experiments.

2.2 Growth in GAC and liquid phases during batches

Batch 1 consisted of eleven bottles according to figure 6 below. The bottles were filled with 400 ml GAC (FILTRASORB® TL830, Chemviron) and up to the 670 ml mark with water sources (Fig 6) to resemble the volumetric ratio between carbon and inlet water in the current process. After the addition of water sources, bottles were placed inside a sludge processing facility (room temperature) to eliminate the risk of contaminating the DW process line. The bottles were left for 2 weeks (except for 5.1, 6, 7) to allow attachment of microorganisms to the GAC. With regular intervals during these weeks, bottles were inverted ten times and the caps of closed bottles were slightly opened for 2 minutes to allow oxygen and carbon dioxide transfer (Table B2, Appendix B). Samples of the liquid phases were regularly analyzed by FCM to get indications of the level of attachment (Table B2, Appendix B). Bottle 5.1 was only left in raw water for 2 days according to the initial plan, without analyzing attachment by FCM. Bottles 6 and 7 were never exchanged for UF permeate since these were used as a backup in case of poor attachment in the other bottles. Following the attachment phase, the liquid content of each bottle was interchanged with UF permeate, which had been autoclaved for 20 ± 1 min at 120°C , 1 bar using a Sjukhuservice AB Uniclave. The exchange was carried out by carefully pouring out the water until only a few milliliters were left, without losing any GAC particles. 500 ml UF permeate was then poured into each bottle using a clean 500 ml plastic measuring cylinder. Bottle 5.1 was exchanged for UF-permeate by strapping a sieve cloth (Menuett® strainer cloth) over the flask opening, followed by drainage. The sieve cloth had been sterilized in sodium hypochlorite (2 mg/ml) and washed twice in Milli-Q water prior to use. The bottle was then filled with autoclaved UF-permeate until the liquid surface reached 670 ml. The use of a sieve cloth was ignored for the remaining bottles since the cloth had a strong smell of hypochlorite even after washing in Milli-Q. Which in turn meant a risk of introducing sodium hypochlorite inside the bottles and possibly affecting microbial growth. After the interchange, growth in the bottles was followed for 15 days by analyzing the liquid phases for FCM total and intact cell count (TCC, ICC) twice a week. Autoclaved UF permeate was also refilled twice a week during this period by emptying 250 ml and filling 250 ml, except for bottle 5.1, 6 and 7. Concentrations were later adjusted for the dilution factors, including the refills and sample volumes (Eq 1).

$$X_n = C_n \cdot DF_n = C_n \cdot \begin{cases} \frac{498}{248}, & \text{if } n=1 \\ \frac{498}{248} \times \frac{494}{244}, & \text{if } n=2 \\ \frac{498}{248} \times \frac{494}{244} \times \prod_{k=1}^{n-2} \frac{492.5 - 1.5(k-1)}{242.5 - 1.5(k-1)}, & \text{if } n \geq 3 \end{cases}$$

Equation 1: n represents the number of dilutions, X_n is the adjusted and comparable concentration, DF_n is the dilution factor and C_n is the measured concentration after n dilutions.

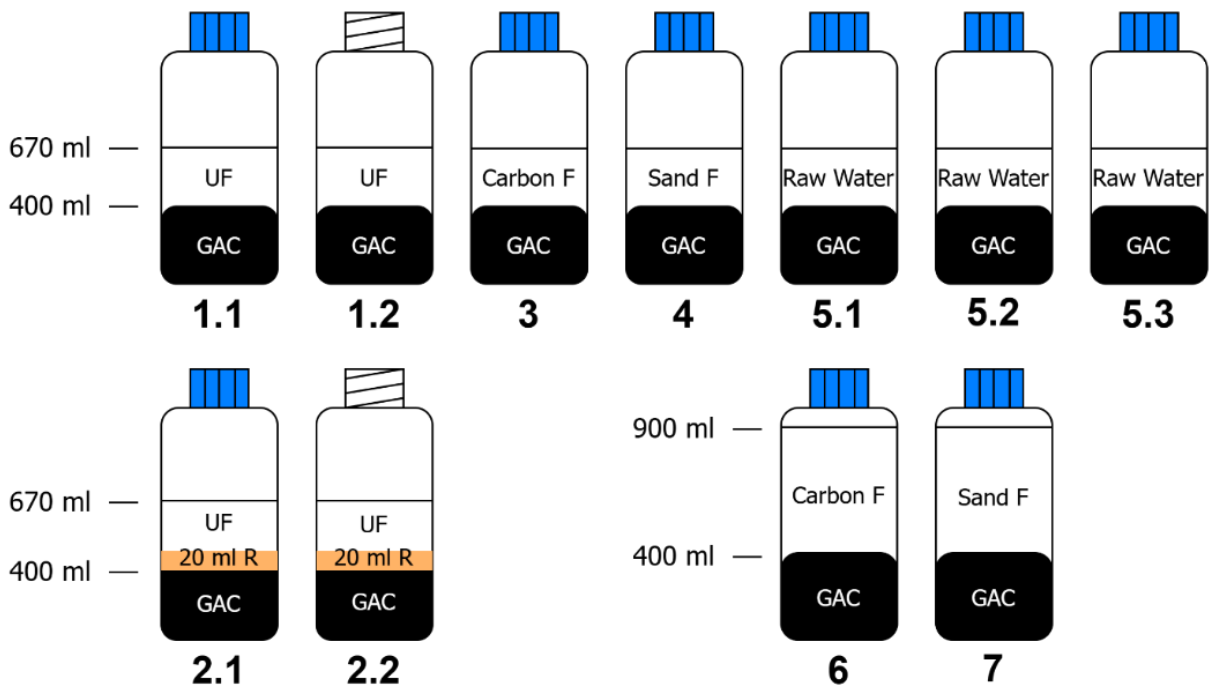


Figure 6: Batch 1 bottles with contents and volumes. Blue caps represent closed bottles. UF refers to ultrafilter permeate, F to filtrate and R refers to Swedish Food Agency reference material.

Batch 2 consisted of three bottles and in contrast to batch 1, the GAC was never contaminated with any water sources. Instead, the three bottles were directly filled to the 700 ml mark with autoclaved UF-permeate following the addition of 400 ml GAC (FILTRASORB® TL830, Chemviron). Furthermore, bottle A was filled with GAC and fresh UF-permeate and autoclaved as a whole, to rule out the growth originating from the GAC itself. The remaining two bottles were prepared with autoclaved UF-permeate as described previously, where one was kept open and the other one closed (bottles B and C respectively). All bottles were then placed in the sludge processing facility as before and the growth inside particles of the respective GAC beds were monitored for 16 days by quantification of GAC-attached cells (see 2.3.2) with FCM twice a week. In addition to inversion during sampling, the caps on closed bottles were also slightly opened for 2 min to allow oxygen and carbon dioxide transfer.

2.3 Sampling

Samples of raw water (tap PP-100), carbon filtrate (tap PP-160), sand filtrate (tap PP-140) and autoclaved UF-permeate (UF pilot) were taken prior to the batches and analyzed by FCM (Fig 2). These measurements were later used to calculate the level of attachment during the attachment phase in batch 1. During the batch experiments, water phase samples were taken by inverting the bottles ten times, followed by pipetting near the water surface in the center of the flask. Furthermore, carbon samples were taken similarly by inverting the bottles ten times and then extracting a mass of carbon using a metal spoon, sterilized with 70% ethanol. All samples were stored in sterile VWR® PP centrifuge tubes (15 or 50 ml) and kept refrigerated until analysis, which was done within two hours after sampling. (Table B2, Appendix B).

2.4 GAC-attached biofilm analysis

2.4.1 *Development of a method for cell desorption*

In order to analyze the cell density inside GAC particles there is a need for a technique that will release attached cells into a surrounding liquid medium. For this study, a sonication bath was chosen for physical desorption due to availability of instruments in the lab. Furthermore, a mixture of 1.25 ml phosphate buffer (68 g/l KH_2PO_4 in Milli-Q), 5.0 ml magnesium sulfate solution (99 g/l MgSO_4 in Milli-Q) and 1 l Milli-Q water was used as the liquid desorption medium to maintain cell viability during desorption. To optimize the time of sonication with regards to maximal released number of cells and minimal cell death, iterative experiments were conducted. In the first attempt, 12 time points ranging from 0-180 min were tested to get a wide screening of the effect of time on total and intact cell count concentrations in the desorption medium. A 25 ml carbon sample was taken from one of the bottles at the end of batch 1 and the carbon was washed five times with 1:1 volume of Milli-Q water, to wash away loosely attached cells. The washed carbon sample was then dried by vacuum filtration for 4 min and aliquoted into twelve 1 g portions in separate glass test tubes. 10 ml of desorption medium was further added into each tube and a 5 ml liquid sample was taken from the 0 min tube. The remaining eleven tubes were then sonicated in a BRANSON 3510 Ultrasonic Cleaner (40 kHz) at KTH and the respective tube was removed at each time point, followed by taking a 5 ml liquid sample. All samples were kept refrigerated and analyzed by FCM within 8 hours. The second attempt was conducted in a similar manner with six time points in duplicates ranging from 0-3 minutes. This time the carbon was vacuum dried for 2 minutes since it was considered enough for the removal of excess liquid. The carbon was also sonicated in 30 ml of desorption medium in 250 ml glass reagent bottles since these were to be used during the disinfection and regeneration experiment. Furthermore, the second and third attempt were conducted at Norrvatten, thus an Elmasonic S 120 ultrasonic bath (37 kHz) was used instead of the previous BRANSON 3510. Finally, the third attempt was carried out with the same procedure as in attempt two, but with four time points in duplicates ranging from 0-45 seconds. All carbon weights were recorded during these experiments, for the adjustment of carbon mass on released cell concentration. The time which gave the highest concentration of TCC and ICC in the desorption medium was chosen for the quantification of GAC-attached cells in experimental samples, described below.

2.4.2 *Quantification of GAC-attached cells*

During batch 2, GAC-attached cells were quantified by vacuum filtering each carbon sample for 40 seconds, followed by measuring 1 g into a glass test tube. Tubes were then filled with 15 ml desorption medium and liquid samples of 1.5 ml were extracted into separate Eppendorf-tubes, by pipetting, prior to sonication. The glass tubes were placed in a rack and sonicated for 30 s in the Elmasonic S 120 ultrasonic bath. Furthermore, a second 1.5 ml liquid sample was extracted from each tube after being sonicated and all samples (before and after) were then analyzed for TCC and ICC by FCM. The carbon cell densities could then be calculated by the difference between the number of cells before and after desorption divided by the mass of carbon, according to equation 2.

$$\text{Carbon cell density} = \frac{(C_{\text{After}} - C_{\text{Before}}) \cdot (V_{\text{DM}} - V_{\text{sample one}})}{m_{\text{Carbon}}}$$

Equation 2: C_{Before} and C_{After} are cell concentrations before and after sonication respectively, V_{DM} and $V_{\text{sample one}}$ are the volumes of desorption medium and the first sample respectively and m_{Carbon} is the carbon mass.

During the disinfection and regeneration experiment, carbon samples were first treated with sodium hydroxide according to 2.8 below. Treated and untreated samples were then analyzed for attached cells by vacuum filtration for 40 s and measuring 2 g of carbon into 250 ml reagent bottles. Each 250 ml bottle was then filled with 250 ml desorption medium and liquid samples of 120 ml were extracted into sterile VWR® PP centrifuge tubes (3 x 50 ml). Bottles were thereafter sonicated for 30 s using the same ultrasonic bath as above, followed by extracting another 120 ml liquid sample. All volumetric transfers were done using measuring cylinders which were sterilized with 70% ethanol and washed with Milli-Q water between samples. This procedure was done for six carbon samples at a time and the respective liquid samples were stored in the fridge until analysis. After sonicating all carbon samples, the liquid samples from before and after were analyzed for coliforms and *Escherichia coli*, heterotrophic plate count (HPC), microfungi and FCM. Carbon cell densities of the respective groups of microorganisms were then calculated according to equation 2.

2.4.3 Crystal violet staining

Before proceeding with the disinfection and regeneration experiment, carbon samples from all bottles in batch 1 were quantitatively analyzed for biofilm by crystal violet staining. New GAC and carbon samples from the current carbon filter 10 (KF10) were also analyzed for comparison. Staining was done by a slightly modified version of a procedure earlier described by D. Djordjevic *et al* (29). All carbon samples were washed three times with 1:1 volume of Milli-Q water to remove non- or loosely attached cells before staining. Thereafter, samples were dried for 1 min by vacuum filtration and portions of 0.5 g were weight into 10 ml glass vials. 500 µl of 0.1% crystal violet solution was then added to each vial by pipetting, so that all carbon was covered. After staining for 45 min in room temperature carbon samples were washed with Milli-Q water by filling the vials, emptying and repeating ten times using a glass pipette. Finally, 1 ml of 96% ethanol was added to each vial and after 5 min, 50 µl of each solution was mixed with 1900 µl 96% ethanol for dilution purposes. 1 ml of the final solutions were then transferred to cuvettes and measured for absorbance at 595 nm in a spectrophotometer. The absorbance of a blank containing 96% ethanol was also subtracted from all measurements.

2.5 Flow cytometry (FCM) for bacterial analysis

2.5.1 Development of a method for carbon fines removal

GAC fines in raw samples had to be removed prior to FCM to prevent interference with readings and clogging of the fluidic system. At first, sample filtration was tested by filtering a DW sample (Fig 2) through 1.2 µm Whatman™ CA, 3 µm BRAND® PTFE and 5 µm Minisart® CA sterile syringe filters respectively, followed by FCM measurements. Results were compared to a control of unfiltered DW with regards to TCC and ICC. Thereafter, centrifugation was evaluated by centrifuging 1 ml DW containing fines in 1.5 ml Eppendorf tubes for 10, 20 and 30 seconds using a MiniStar silverline (VWR) at 2000 rpm. After centrifugation, 0.5 ml of supernatants were removed by pipetting and analyzed by eye for any remaining carbon fines. To validate that centrifugation does not alter the TCC and ICC readings, a DW sample was divided into four 1 ml aliquots in 1.5 ml Eppendorf tubes (SG & PI duplicates). The tubes were centrifuged for 10 s and FCM samples were taken directly from each supernatant by pipetting near the tube wall, opposite to where the carbon fines would sediment. Readings of the centrifuged duplicates were compared to duplicates of untreated DW from the same original sample.

2.5.2 Flow cytometry measurements

Two samples of 1 ml (for SYBR green & propidium iodine) from each flask were vortexed and centrifuged in 1.5 ml Eppendorf-tubes for 10 seconds using a MiniStar silverline (VWR). However, one sample of 1.5 ml was later taken from each flask instead of two separate ones, to reduce the usage of Eppendorf tubes and the number of centrifugations. FCM samples were extracted directly from the supernatants by pipetting near the tube wall, opposite the carbon sediment, into new Eppendorf tubes. 495 µl was taken for SYBR green (SG) staining and 494 µl for the Propidium Iodide (PI) staining. Thereafter, 5 µl of SG solution (1 vol% 10,000x SYBR green in DMSO) and 6 µl of PI solution (16.67 vol% PI stock in SB solution) were added to the respective Eppendorf tube to make a final volume of 500 µl. Tubes were then vortexed and incubated at 37°C for 15 min to allow for proper binding of the dyes, prior to flow cytometry readings. After incubation, tubes were vortexed once again and analyzed at a laser wavelength of 488 nm by detector FL1-A (SYBR green) and FL3-A (PI) using a BD Accuri™ C6 Plus flow cytometer. All samples were read consecutively by Auto Collect in the BD CSampler plus software with SIP rinse in between (Figure B1, Appendix B).

2.6 Coliforms and *Escherichia coli* analysis

Coliform bacteria and *E. coli* in liquid samples were enumerated following the Swedish standard SS-EN ISO 9308-2:2014, Water quality – Enumeration of *Escherichia coli* and coliform bacteria – Part 2: Most probable number method (ISO 9308-2:2012). Colilert-18 medium was added from ampoules to 100 ml sample, sealed in count trays, incubated in 37°C and read after 18-22 hours.

2.7 Heterotrophic plate count (HPC)

Culturable microorganisms in liquid samples were enumerated by 3-day heterotrophic plate count following the Swedish standard SS-EN ISO 6222, Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium (ISO 6222:1999). 1 ml of samples were infused in yeast extract agar in petri dishes, incubated at 22°C and colony counted after 3 days.

2.8 Microfungal analysis

Microfungi in liquid samples were enumerated by membrane filtration methodology following the Swedish standard SS 02 81 92, Water quality – Microfungi in water – Quantitative determination with the membrane filter method. 20 ml of dilution water (the same as desorption medium in 2.4.1) was added to 15 ml of sample and vacuum filtered through a sterile membrane filter. The filter was then put on a petri dish containing rose Bengal agar, incubated at 25°C and colony counted after 7 days.

2.9 Disinfection and regeneration of GAC

During this set of experiments, sodium hydroxide (NaOH) and its ability to disinfect and regenerate GAC with attached cells was investigated. Different concentrations and times of exposure were tested on carbon from batch 1 and effectiveness was measured by quantifying GAC-attached cells (see 2.3.2) in treated and untreated carbon samples. A regrowth experiment was also done to examine whether spores could survive the treatment or not. Iodine number (IN) was determined for treated, untreated and fresh carbon to see if NaOH (5% and 0.1%) would reduce the binding capacity of GAC and how IN is affected by attached microorganisms. The initial plan was to determine iodine number for all treated carbon samples to include potential reductions in the models described below, however there were not enough resources for that. Finally, surviving species from HPC plates were identified by MALDI-TOF. The procedure of each of these experiments are described in detail below.

2.9.1 Design of experiment (DoE) and modeling for optimization

To optimize NaOH concentration and time of exposure, a design of experiments based experimental setup was retrieved using Sartorius MODDE® (in terms of combinations of concentration and time to be tested). In the design wizard in MODDE®, factors and responses were defined according to Table 1, objective was set as optimization and the design used was CCF with 2 center points. Two additional combinations were added manually based on which concentration and time the treatment would, from what was thought, start being effective (to make the model stronger in that area). See the experimental setup in figure 7. The different strengths of NaOH solutions were then prepared in 50 ml PP centrifuge tubes by diluting 25 w/v% NaOH (Brenntag) in Milli-Q water to total volumes of 15 ml. Wet samples of GAC (15ml) from selected bottles in batch 1 were then submerged in NaOH and left in room temperature for 30 to 360 min, see Table B1, Appendix B for chosen bottles. The treated carbon was then washed ten times in 1:1 volume of Milli-Q to remove the sodium hydroxide. The respective untreated GAC samples were also washed five times to remove loosely attached cells. Thereafter, all 24 carbon samples were analyzed by quantification of GAC-attached cells described in 2.4.2. The efficiency of each combination of concentration and time was then calculated by the difference in GAC-attached cells between treated and untreated samples, divided by the untreated number. Disinfection efficiencies for TCC, ICC, HPC, microfungi, coliform and *E. coli* could then be entered into the response section in MODDE® to create an individual model for each group of cells. Regeneration was considered as the removal of TCC while disinfection was measured by the change in the other analysis.

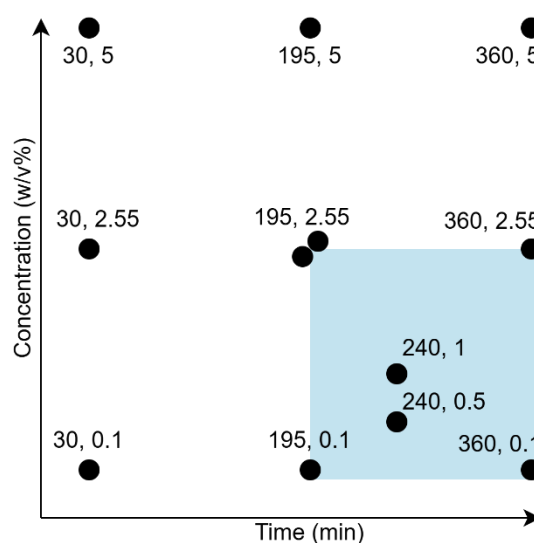


Figure 7: Factor combinations of the experimental design. Outer dots encapsulate the region which is reasonable to stay within, in terms of concentration and time. The blue square represents the area of factor combinations that are most probable to be feasible and effective in large scale application. Time and conc. is given as coordinates (x, y).

Table 1: Definition and settings of factors and responses in MODDE®.

Factors	Units	Type	Settings			
Time	Min	Quantitative	30 to 360			
Concentration	w/v%	Quantitative	0.1 to 5			
Responses	Units	Condition	Objective	Min	Target	Max
Disinfection efficiency	%	Required	Maximize	90	100	
Iodine number reduction	%	Required	Minimize		0	10

2.9.2 Regrowth experiment

To understand the regrowth in NaOH-treated carbon, the extreme concentrations 0.1% and 5% were tested in combination with a contact time of four hours. Solutions were prepared according to 2.9.1 above. A carbon sample from bottle 2.2, batch 1, was then divided into two 15 ml portions and submerged in each solution for four hours. The carbon was thereafter washed ten times in 1:1 volume of autoclaved Milli-Q to remove NaOH, done inside a laminar flow hood to keep sterility. After moving the carbon samples to autoclaved 250 ml reagent bottles, 250 ml autoclaved UF permeate was added. Approximately 50 ml of the Milli-Q was also added to the UF permeate container to make the control. 3 ml samples were taken from the three bottles and analyzed for HPC and FCM. Bottles were then stored closed inside the laminar hood (room temperature) for 1 week, followed by another 3 ml sampling and analysis. The exact same procedure was also repeated for a carbon sample from carbon filter 10 (KF10).

2.9.3 Impact on iodine number

The Iodine number of carbon samples was determined by following a modified version of the standard method D4607 earlier described by ASTM international (19). GAC was not grinded and strained as proposed in the standard. Instead, the contact time with the iodine solution was increased from 30 s to 1.5 hours to compensate for the carbon not being powdered. Calculations were made according to the standard. 20 ml GAC samples were dried for 1 h and 40 min at 100°C and weighed into three portions of 1.125, 0.985 and 0.845 g. These portions were then boiled in HCl, submerged in iodine solution for 1.5 hours, filtered and adsorption was measured by titration of the filtrate, according to the standard. Titration volumes were used to calculate an adsorption isotherm from which the iodine number was determined, described in the standard.

2.9.4 MALDI-TOF analysis of surviving species

Representative colonies were extracted from selected HPC plates from the disinfection experiment and stroked onto new yeast extract agar plates using a sterile inoculation loop. The new plates were then incubated at 22°C until desirable growth had occurred. Thereafter, colonies were refreshed by another isolation streak, incubated until distinct growth and finally sent to the Swedish Veterinary Agency (SVA) in sealed packaging for MALDI-TOF analysis. Results were screened against a database by SVA for species identification. Chosen plates from disinfection experiment can be seen in Table 3.

3 Results

3.1 Bacterial growth during batches

During the attachment period of batch 1, TCC and ICC levels in the liquid phase of studied bottles were decreasing over the two weeks (Fig 8).

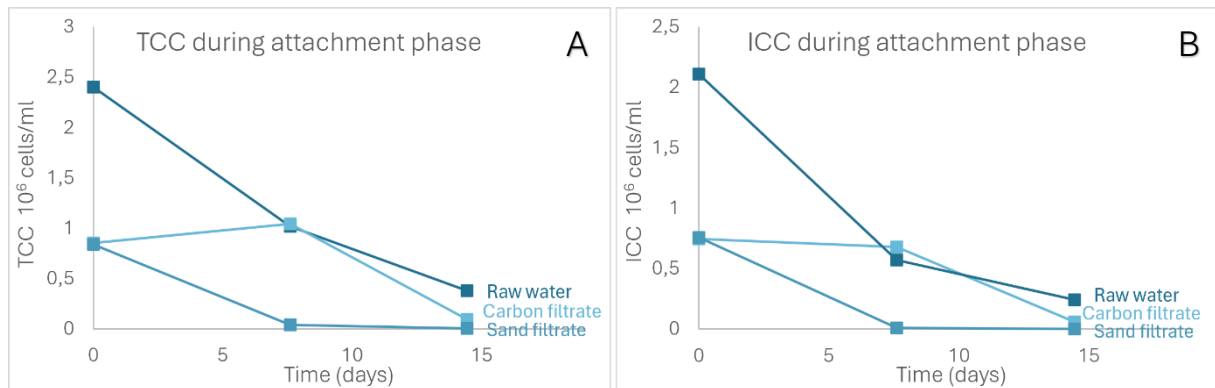


Figure 8: Cell counts during attachment phase. (A) TCC and (B) ICC levels in the liquid phases of carbon that were submerged in raw water, carbon- and sand filtrate. Lines between data points are for illustration.

Upon UF permeate interchange, the levels started rising in all bottles and kept rising exponentially, for the most part, during the two-week incubation period. The top four strongest growth curves were observed for bottles 1.2 (UF open), 2.2 (spike open), 2.1 (spike) and 4 (sand filtrate) where 1.2 clearly showed the fastest increase in TCC and ICC. Incubation growth curves are presented in figure 9 below. In some of the bottles ICC levels were persistently close to TCC while in others the difference was more prominent. However, the difference became larger for all bottles towards the end (Figure C1, Appendix C).

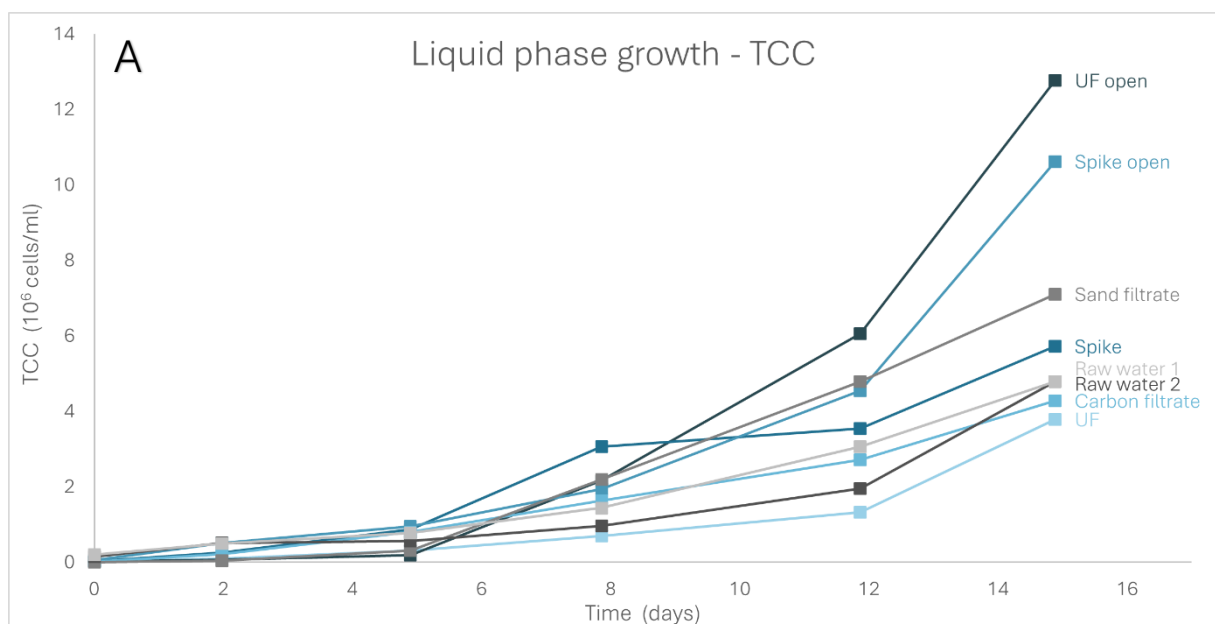


Figure 9: Liquid phase growth. (A) TCC and (B) ICC levels in liquid phases of carbons submerged in UF permeate, with refills, that previously had been submerged in water sources (labels in fig). Lines between data points are for illustration.

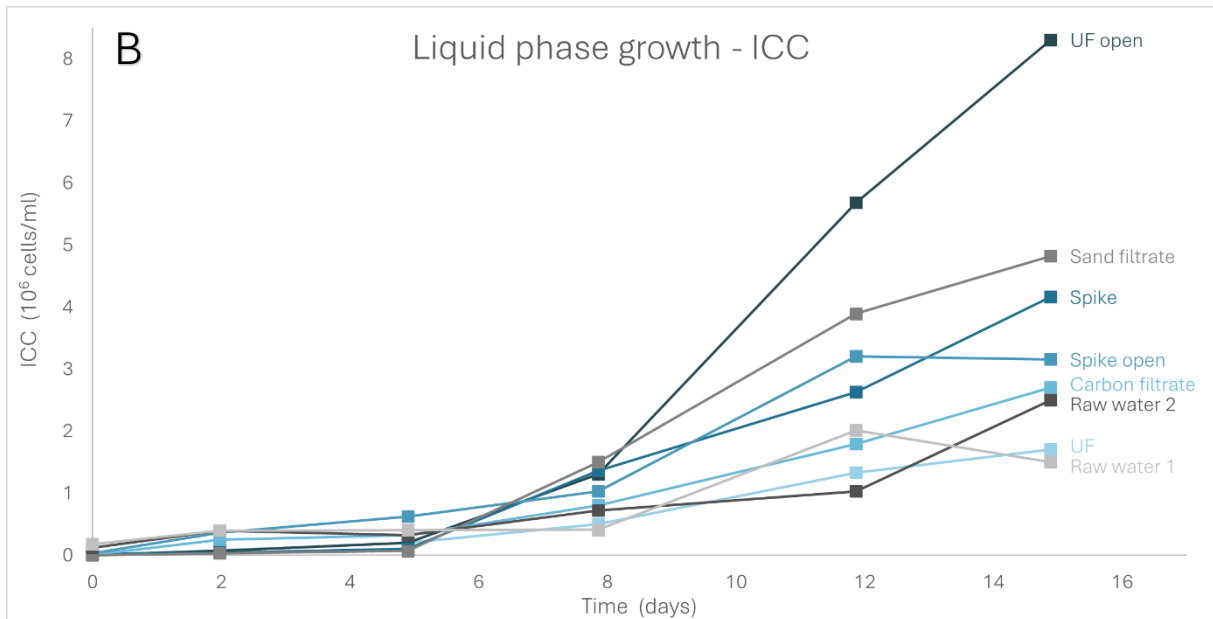


Figure 9: Liquid phase growth. (A) TCC and (B) ICC levels in liquid phases of carbons submerged in UF permeate, with refills, that previously had been submerged in water sources (labels in fig). Lines between data points are for illustration.

In the water phase of bottle 5.1, which was not refilled with UF permeate after the first exchange, TCC and ICC levels increased during the first two weeks but then stopped and levels began to decrease with a pattern of peaks and valleys (Fig 10). Furthermore, an example of the development of FCM fingerprints during attachment and incubation phases is presented for bottle 5.2 in figure C2, appendix C, including cytograms and the gate used.

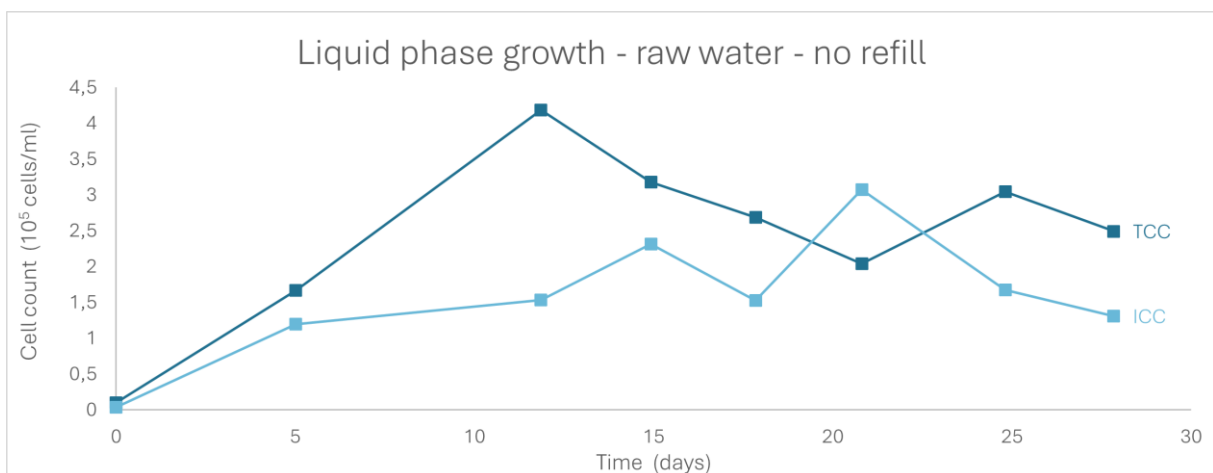


Figure 10: Liquid phase growth – raw water – no refill. The TCC and ICC levels in the liquid phase of carbon submerged in UF permeate, without any refill, that previously had been submerged in raw water. Lines between data points are for illustration.

In batch 2, the growth in all carbon beds was slow during the first ten days regarding both TCC and ICC densities. But then the growth took off dramatically in the open and closed bottles, containing unsterilized GAC, while the totally autoclaved bottle kept showing poor growth. The open bottle had a faster increase in TCC and ICC densities compared to the closed one, however the difference between the autoclaved and closed bottle was prominently larger (Fig 11).

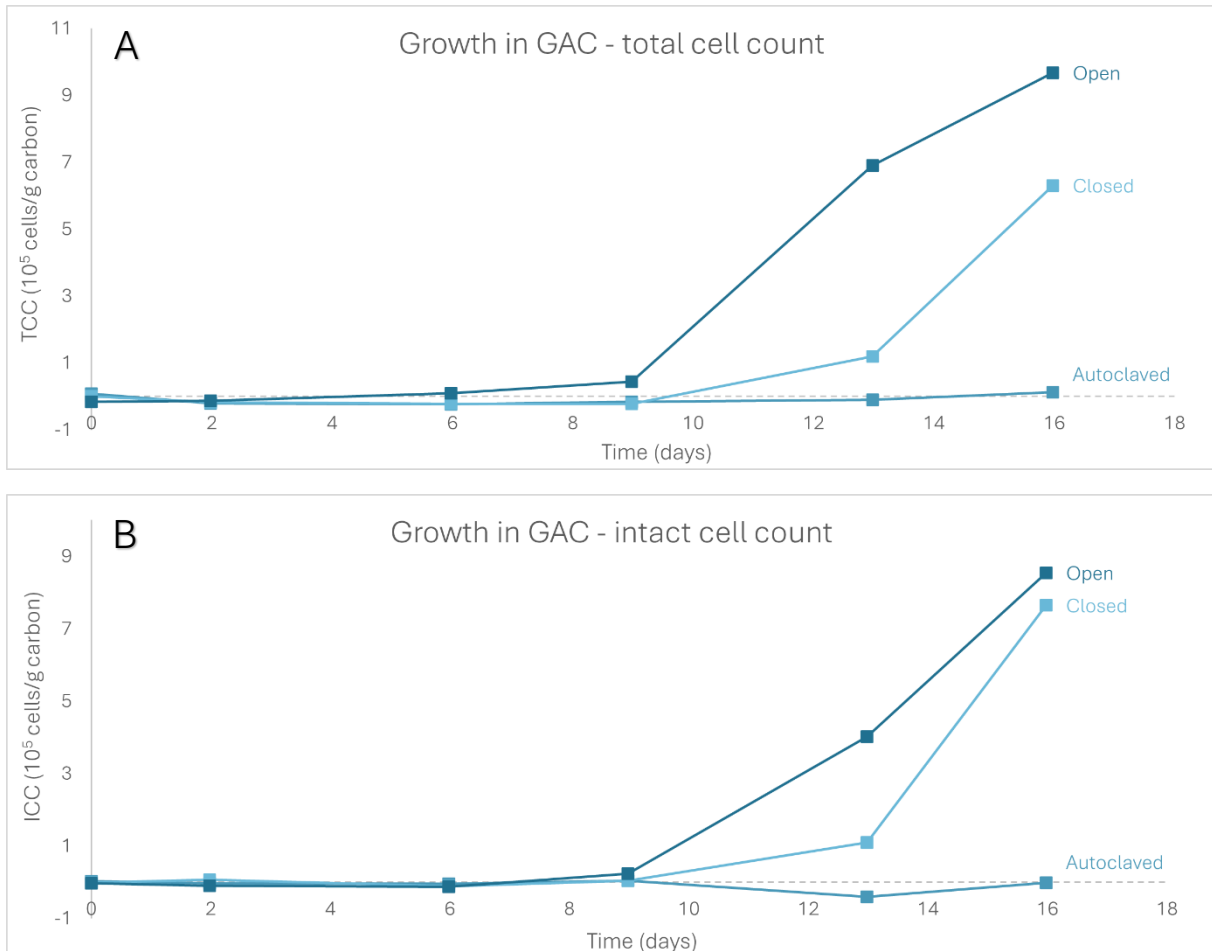


Figure 11: Growth in GAC. (A) TCC and (B) ICC densities in carbons submerged in UF permeate, with refills. The carbons included one that was autoclaved together with UF permeate (Autoclaved), and two that were submerged in autoclaved UF permeate and then kept open (Open) and closed (Closed) respectively. Lines between data points are for illustration.

3.2 Desorption of attached cells

When testing desorption by sonication for 0-3 hours, a distinct peak in TCC and ICC carbon densities were observed at the first measurement, at three minutes. Following the peak, levels decreased over a period of 50 minutes and then stabilized around 2% of the peak levels (Fig 12 A). In the second experiment, focusing on the first four minutes, a new peak was discovered at 45 seconds of sonication, which again was the first measurement. Like before, levels kept decreasing after the initial peak (Fig 12 B). When investigating whether there could be an earlier peak in the third experiment, a new peak was in fact found at 30 seconds. This peak was found at the second measurement, indicating that an even earlier peak is unlikely (Fig 12 C). TCC and ICC densities also showed similar desorption patterns during all experiments, with almost parallel changes between data points (Fig 12).

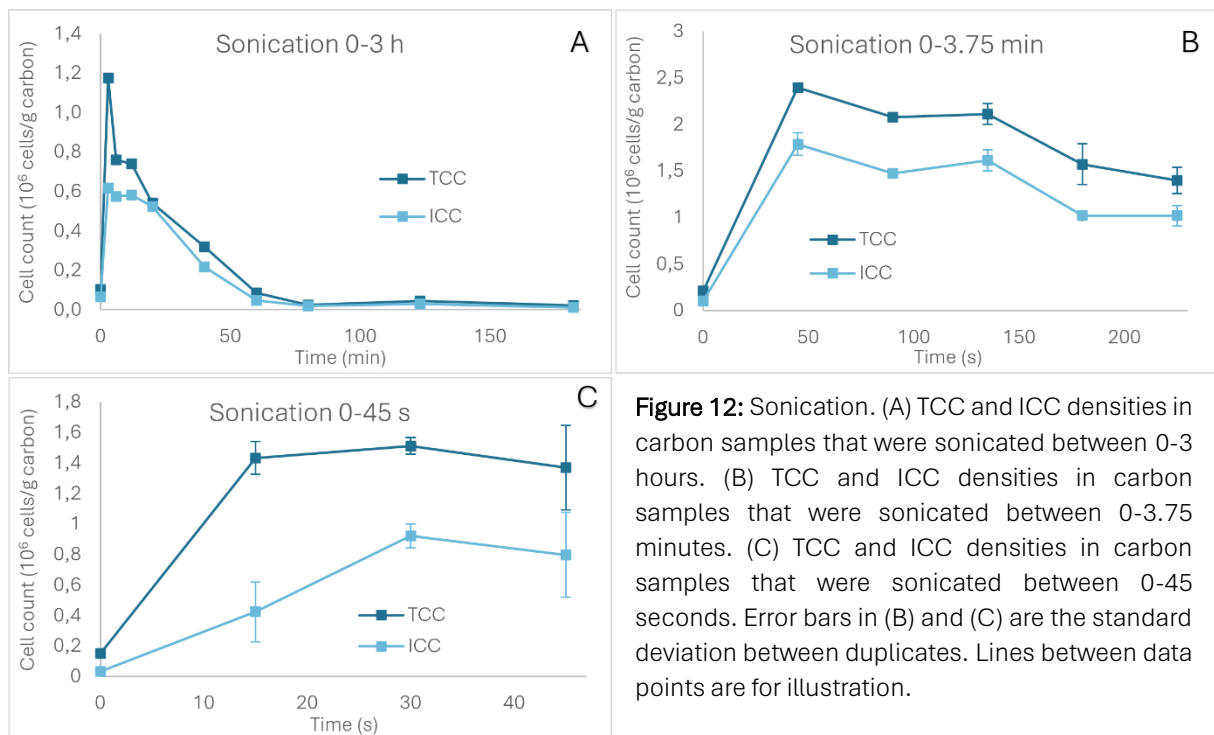


Figure 12: Sonication. (A) TCC and ICC densities in carbon samples that were sonicated between 0-3 hours. (B) TCC and ICC densities in carbon samples that were sonicated between 0-3.75 minutes. (C) TCC and ICC densities in carbon samples that were sonicated between 0-45 seconds. Error bars in (B) and (C) are the standard deviation between duplicates. Lines between data points are for illustration.

3.3 Disinfection and regeneration of GAC

3.3.1 Effect of sodium hydroxide

Sodium hydroxide had a noticeable effect on attached cells in terms of TCC and ICC, where all concentrations and times that were tested resulted in great losses in TCC (up to 95%) and close to 100% losses in ICC. The exception was the weakest treatment of 0.1% and 30 min which apparently had an increase in TCC and ICC between the untreated and treated samples (Fig 13). There was a large variation in TCC and ICC densities between untreated carbon samples originating from different bottles, ranging from 600k to 4700k TCC/g and 50k to 2800k ICC/g. However, the variation between the duplicates was small, in terms of both starting numbers and the reduction of TCC and ICC densities (Fig 13).

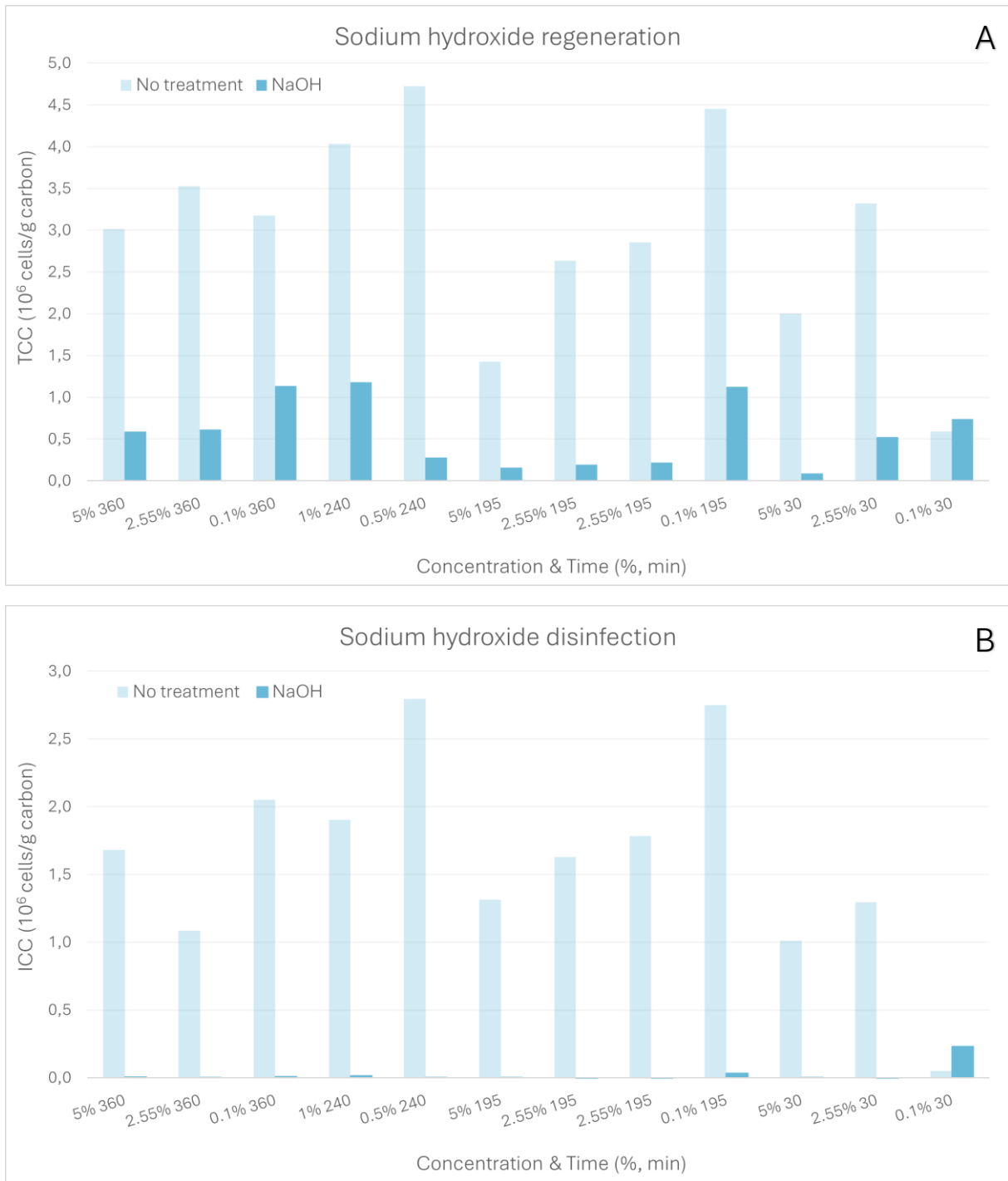


Figure 13: Sodium hydroxide regeneration and disinfection. (A) TCC and (B) ICC densities in carbons before and after being treated with respective concentrations of NaOH and times.

Sodium hydroxide also showed a distinct effect on culturable microorganisms in the three-day heterotrophic plate counts. All combinations of concentration and time resulted in a 100% reduction of CFU, except for the weakest treatment (~ 53% reduction) and the 2.55%, 30 min and 0.1%, 195 min treatments which both showed a minor survival rate (Fig 14). However, the variation of CFU density was large between the untreated carbon samples from different bottles (Fig 14), as for TCC and ICC described earlier.

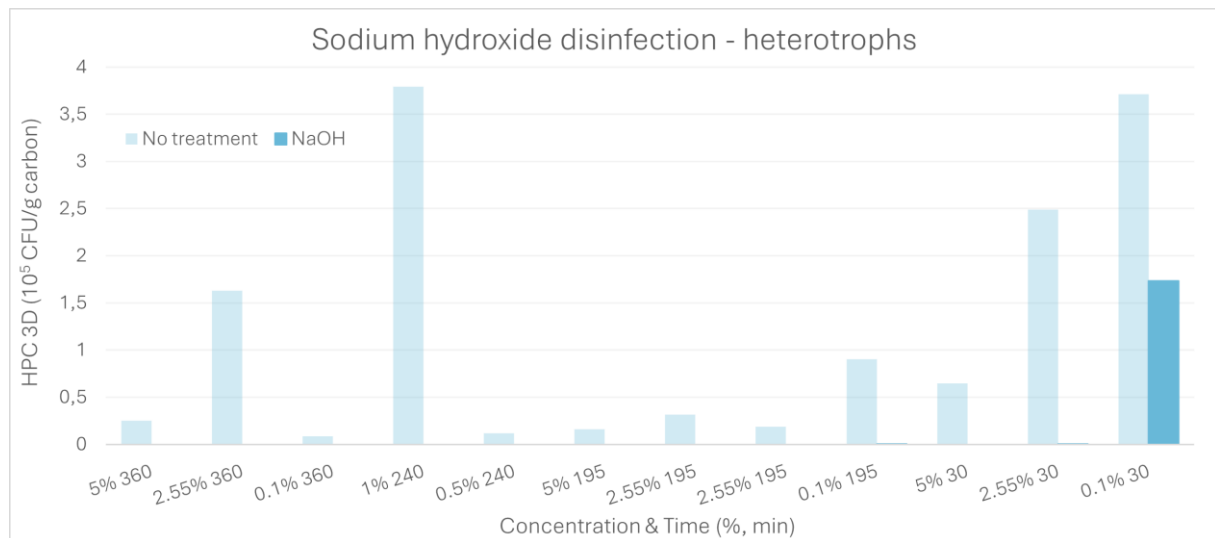


Figure 14: Sodium hydroxide disinfection – heterotrophs. CFU densities in carbons, from heterotrophic plate counts, before and after the carbon was treated with the respective concentrations of NaOH and times.

Microfungal analysis showed zero colonies on all plates for all samples. Coliforms and *E. coli* analysis also gave zero response for both types in all samples, or <1 CFU/ml according to the MPN standard method described earlier.

3.3.2 Modeling of disinfection and regeneration

Unfortunately, TCC data of the regeneration of GAC was too irregular to make a valid model of regeneration. For instance, the 0.5%, 240 min treatment showed a much greater reduction compared to 5%, 360 min and when trying the model this data, any validity could not be achieved. Because of complete disinfection for most factor-combinations in the ICC and HPC data, models would not have any predictability and thus, proper models could not be made. Furthermore, since microfungal, coliform and *E. coli* analysis gave zero responses, these data could of course not be modeled either.

3.3.3 Regrowth

The 0.1% NaOH treatment showed great increases in TCC, ICC and HPC over the regrowth period of one week, for both carbon sources. The starting samples, straight after treatment, also contained a lot of intact and viable cells (Table A2, Appendix A). However, the 5% NaOH treatment showed no regrowth in the carbon from bottle 2.2 (originally spiked with Swedish Food Agency reference material) but one colony appeared on the HPC analysis of the end sample of KF10 carbon, with zero colonies in the starting sample. End samples of KF10 could not be analyzed for TCC and ICC since the liquid had a dark orange color and a pH of

10.9. Furthermore, controls showed a minor increase in TCC and ICC levels during the one-week period, but zero CFU in the start and end samples during HPC.

3.3.4 Iodine number

The iodine number determinations showed that NaOH (5%) had no negative effects on the adsorptive function of GAC. Instead, treated GAC and carbon from bottle 3, batch 1, had higher iodine numbers compared to new GAC (Table 2).

Table 2: Measured iodine number of different carbons

Carbon source	Iodine number (mg/g)
New	649
Treated with 5% NaOH	730
Bottle 3	721

3.3.5 MALDI-TOF

MALDI-TOF analysis showed that *Pseudomonas stutzeri* colonies were growing after sonication after the 0.1%, 30 min treatment, with carbon from bottle 1.1. The respective plate counts for before and after sonication also show that viable *P. stutzeri* must have been present in the carbon after the treatment and that it is not an outer contamination. Similarly, *P. stutzeri* was also present inside the untreated carbon sample from the same source (Table 3). Furthermore, *P. stutzeri* was identified before and after sonication of untreated carbon samples from bottle 1.2 (Table 3). Both these cases concern carbon that has been in contact with autoclaved UF permeate only. Another group of bacteria, *Pseudarthrobacter* species, were identified after sonication after the 2.55%, 30 min treatment and after sonication of the respective untreated carbon sample (Table 3). Again, the plate counts of before and after sonication shows that viable cells must have been present inside each carbon sample. The single colony on the plate representing the 0.1% and 195-min treatment, after sonication, could not be identified by SVA.

Table 3: Species identified by MALDI-TOF

Bottle	Treatment	Sample plate	Identified species
1.1	0.1% 30 min	After sonication	<i>Pseudomonas stutzeri</i>
1.1	-	After sonication	<i>Pseudomonas stutzeri</i>
1.2	-	Before sonication	<i>Pseudomonas stutzeri</i>
1.2	-	After sonication (colony 1)	<i>Pseudomonas stutzeri</i>
1.2	-	After sonication (colony 2)	<i>Pseudomonas stutzeri</i>
2.1	2.55% 30 min	After sonication	<i>Pseudarthrobacter</i> species
2.1	-	After sonication (colony 1)	<i>Pseudarthrobacter</i> species
2.1	-	After sonication (colony 2)	<i>Pseudarthrobacter</i> species
5.2	0.1% 195 min	After sonication	Unidentified

3.4 Crystal violet staining

Crystal violet staining of the different carbon sources showed that GAC itself is a great adsorber of crystal violet, even better than the old, saturated process carbon (Fig 15). The level of adsorption of crystal violet was in between the old and new carbon for most of the carbon samples from bottles in batch 1. However, carbon from bottle 5.3 and 4 showed greater adsorption than the fresh GAC (Fig 15). Furthermore, the differences in adsorption between carbon samples were greatly inconsistent with the differences in TCC densities, measured during the disinfection and regeneration experiments (Fig 15).

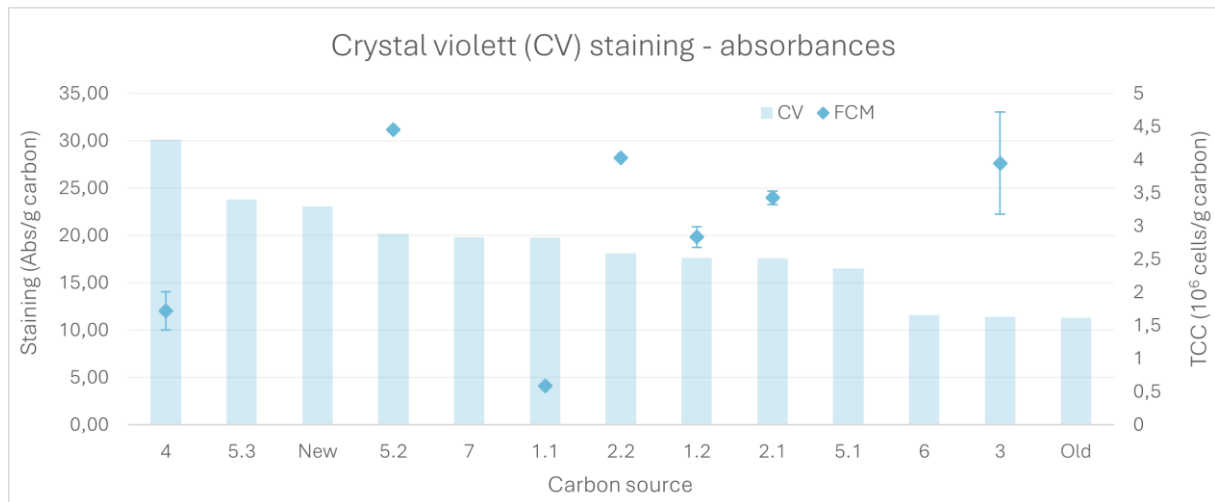


Figure 15: Crystal violet staining. Staining absorbances of different carbon sources in relation to carbon mass. Absorbances are also compared to respective TCC densities of carbons (blue diamonds). Error bars are the standard deviations between duplicates.

3.5 Removal of carbon fines

The DW samples that were filtered through 1.2, 3 and 5 μm filters respectively showed deviations from the unfiltered DW, in terms of TCC and ICC (Table A1, Appendix A). The FCM fingerprints also consisted of straight lines and not the regular clusters of cells. However, the DW sample that was centrifuged for 10 s showed much smaller deviations in TCC and ICC toward the original DW sample (Table A1, Appendix A).

4 Discussion

The decreasing levels of TCC and ICC during the attachment phase in batch 1 indicate that cells were getting attached to the carbon cavities and surfaces, which have been seen earlier (30). However, since the liquid was not exchanged for two weeks a decrease could also be expected from nutrient depletion and it is probably a combination of these explanations (31). The idea of using the volumetric ratio of the current filters was later disregarded since the ratio in the future waterwork is unknown. Furthermore, the lab pilot lacks a continuous flow of water unlike the current process, making the ratio less important since these properties already make the cases incomparable regarding water dynamics. It is also the future waterwork that constitutes the focus of this study. Furthermore, the strong growth curves during the incubation phase of batch 1 show that UF permeate contains accessible nutrients, in accordance with literature (12,13), and that it is an excellent growth medium for a wide range of bacteria. Unfortunately, this means that keeping an UF-permeate fed GAC bed sterile or free from microbial growth will be challenging. The growth in the different bottles also suggests that a wide range of different contaminants will thrive in an environment like this. However, it is evident that keeping the system closed against ambient air will reduce growth dramatically, by looking at the difference between opened and closed bottles in batch 1. Whether these differences are due to higher gas transfers or bacterial contamination from the air, in the open bottles, is hard to tell. But both phenomena are likely contributing to stronger growth. Even though the TCC and ICC levels in the liquid phases are not direct measures of the bacterial content of the carbon particles, it should still give an indication of what growth could be expected inside the carbon. The liquid phase extends inside the particle cavities after all and should therefore support similar growth. Furthermore, the measured growth is probably due to both growth of cells in the liquid phase and the release of attached cells, growing inside the carbon. As described earlier, the differences between TCC and ICC grew over time which means the proportion of damaged cells was increasing. This could only be explained by the rate, of which intact cells became damaged, being larger than the rate of damaged cells becoming non-cellular. Although the damaged rate must have been lower than the growth rate since ICC was increasing. As seen in the beginning of batch 2, negative values were encountered for the TCC and ICC densities in all bottles, which means that the concentrations were decreasing during desorption. This in turn means that more cells were broken down compared to the number of cells that were desorbed, which proves that a proportion of cells are dying during 30 seconds of sonication. However this number must be small since the starting sample of the autoclaved bottle, which should contain close to zero TCC and ICC, resulted in a small negative number. Thus, there should not have been any desorbed cells counteracting the cell death and this negative number should describe the actual cell death by sonication, which from the raw data would be around 1000 cells/ml. Even when measuring the growth inside carbon particles the open bottle showed the greatest growth, in accordance with the findings in batch 1. This further strengthens the idea of stronger growth due to better gas transfer, but also that airborne bacteria end up inside the carbon over time. During the last analysis on day 16, ICC exceeded TCC in the sample from the closed bottle, which is biologically impossible but rather an error in the flow cytometer, a faulty staining or deviation

between the SYBR green and PI samples. Furthermore, the TCC and ICC densities in the autoclaved GAC bed stayed close to zero during the whole experiment. This suggests that the growth in remaining bottles was mainly caused by bacteria that were already present in the GAC beforehand and not due to the handling and sampling. It is also evident that sterilizing the GAC before use has a larger effect on slowing down growth compared to the difference between an open and closed system.

For the desorption of attached cells, it was desired to use a mixer homogenizer together with a specialized desorption buffer since this was found to be the most promising setup according to literature (21), as described in the introduction. However, neither KTH nor Norrvatten had the equipment and purchasing a new one would be too expensive, thus sonication was chosen as it was the second-best alternative. Reagents for the specialized buffer were also unaffordable, and a regular solution of phosphate buffer and magnesium had to be used instead. It should be stated though that ultrasonic treatment is known to break cells, and it is used for lysis purposes, hence it is not the perfect method for cell desorption. As already described, the lysis of cells during sonication was also confirmed in batch 2 and the sonication experiment. However, since this number is small and equal for all samples, the effect on results and conclusion of this study is considered negligible. Furthermore, the time of the peak that was found after the three sonication attempts was considered to give the most representable value regarding the true number of cells inside carbon samples. However, it is not possible to say with confidence that all cells have desorbed after 30 s and strongly attached cells might require more time to desorb. A solution for assessing the level of desorption is presented under future perspectives.

The irregularity in the TCC data from the disinfection and regeneration experiment might have several explanations and there must be at least one unknown factor affecting the removal of TCC. Otherwise, the 0.5%, 240 min or 2.55%, 360 min treatment could never have been more effective than the 5%, 360 min treatment, as an example. One explanation could be that carbon from different bottles contains cells with varying levels of attachment or even variations in cell density within carbon samples, which were seen between duplicates. These types of variations have also been seen in other studies, with a focus on disinfection (32). Since before and after samples are separate, internal variations would affect the calculated removal. Another likely explanation would be that there is an unknown factor related to the method. For instance, released cells and cell fragments could be reabsorbed during the sodium hydroxide treatment since there is no continuous agitation nor extraction of released biomass. Readsorption has been suggested as a possible source of error in literature (21), which is also why a specialized desorption buffer should be used for optimal desorption during analysis. Hence, readsorption during both treatment and desorption is likely to contribute to these variations. Furthermore, an idea of a possible solution for this problem, considering regeneration of GAC filters, is described in future perspectives. However, the small variation between duplicates indicates high reproducibility. There is also a possibility that carbon samples with different cell density have different sonication peaks. For instance, a low-density sample could possibly have released all cells before 30 s while a high-density sample might not have reached its peak yet. Even though the data was not possible to model or optimize, it is apparent that sodium hydroxide

has potential in terms of regeneration of GAC since TCC removal of up to 95% were encountered during the experiment. Furthermore, the ICC and HPC data clearly prove that sodium hydroxide is an excellent disinfectant for GAC beds, even at low concentrations and time of exposure, also seen before (27). Disinfection was also shown to be successful on a broad range of bacteria, with almost 100% disinfection on carbons that were contaminated with raw water, spikes and more. However, it should be noted that these results apply to fairly young GAC which has been feeded with ultrafiltered raw water, in a non-continuous manner. Hence, these concentrations and times will probably not have equally strong effects on well-developed BAC filters but rather directed toward preventing newly installed GAC from developing into BAC. The growth that was seen during batches could also be expected to increase when filters are continuously feeded, because of a higher oxygen and nutrient supply. Since many combinations of concentration and time showed complete disinfection, there is a great chance that these could handle even higher cell densities. As described earlier, complete disinfection is also the reason why predictive models could not be made, because of inadequate response variation. Unfortunately, conclusions on the effect of sodium hydroxide on microfungi, coliforms and *E. coli* cannot be drawn since these analyses gave zero responses in all samples.

The regrowth experiment showed that a 0.1%, 4-hour treatment has poor disinfection properties against carbon from bottle 2.2 and KF10, in terms of ICC and HPC. Because of this, growth was also strong during the following week and probably not due to spores but rather the already present viable cells. However, the 5%, 4-hour treatment showed great disinfection properties and no regrowth except for one HPC colony in the one-week sample from KF10. Since the liquid phase of this bottle was found to have a pH of 10.9, this colony should either represent a surviving spore or an outer contamination in the analysis. The respective liquid phase around the carbon from bottle 2.2 probably had a high pH even though it was not discovered, which could be the reason for no regrowth (27). Therefore, the lack of regrowth might not justify the assumption that spores could not survive the sodium hydroxide treatment. The controls showed minor increases in TCC and ICC but none in HPC, which also confirms that pH in the 5%-bottles affected the TCC and ICC levels. Furthermore, a concern about using sodium hydroxide for GAC disinfection and regeneration is how much water it would require to completely wash out the NaOH. The dark orange color of the liquid phase in the 5% KF10 regrowth bottle might be a result of NaOH reacting with excess aluminum sulphate from the process, originally suggested by Hanna Lundvik, a coworker at Norrvatten (personal communication). Together with the pH, this suggests that NaOH was present in the carbon even after ten washes. Therefore, it might require some time for the washing water to penetrate deeper pores and there should be a liquid-liquid exchange between pores and surrounding water, which requires time to fully interact. Considering the iodine number determinations, it seems that sodium hydroxide does not reduce the binding capacity of carbon, instead the number was higher after treatment. However, it is unlikely that the carbon from bottle 3 (batch 1) would have a higher IN than new GAC, which suggests that these higher numbers might be due to variations in the method and not an actual increase in IN. Since the standard was modified, the expected error is also unknown. Furthermore, the strong presence of *P. stutzeri* after sonication in untreated samples from

bottle 1.1 and 1.2 proves that this species was present in the carbon samples beforehand. Since the species is widely spread in the environment, this is not unexpected (33). However, the HPC results from before and after sonication of treated carbon from bottle 1.1 (0.1% 30 min) and 1.2 (2.55% 195 min) suggest that *P. stutzeri* does not survive sodium hydroxide, but rather that the first treatment is too weak. Similarly, *Pseudarthrobacter* species was present in carbon from bottle 2.2 and the 2.55%, 30 min treatment showed one surviving colony. This group is also found in many environments, such as soil and water so it is not unexpected to find in GAC (34). A stronger treatment of the same carbon gave zero surviving colonies, indicating that this species cannot survive sodium hydroxide either but that the first treatment was weak.

The original thought about crystal violet staining was that carbon samples with higher densities of biomass would show stronger staining. On the contrary, the new GAC showed one of the strongest bindings while the most saturated KF10 carbon had the weakest staining. Since most of the batch 1 samples were in between these two, it was thought that results could be interpreted the other way around. Meaning that a high-density sample would show less staining because of the blockage of carbon binding cavities and vice versa. However, TCC densities of the respective samples were totally inconsistent with the staining properties, suggesting that crystal violet staining might not be a great method for detecting biomass in GAC.

5 Conclusions

- A GAC bed that is supplied with ultrafiltered raw water provides an excellent growth matrix for various bacteria and a contamination could become established within weeks.
- To minimize growth, GAC filters should be kept closed against surrounding air to the greatest extent possible. Newly installed carbon should also be sterilized prior to operation to prolong the time before a first reactivation is required.
- Sonication by an ultrasonic bath will desorb cells that are attached to GAC. However, the time dependency of desorption together with the lysis of cells over time prevents measurements of actual cell numbers.
- Sodium hydroxide is an excellent chemical for bacterial disinfection of GAC filters at the right strengths, where concentrations down to 0.1 v/w% are greatly effective against at least 2.8 million ICC/g carbon. Several hours of exposure is preferable for maximum effect.
- Sodium hydroxide also has great potential in regeneration of GAC with TCC removals of up to 95% in several weeks old GAC. The efficiency of regeneration could potentially be improved by continuous agitation and extraction of released biomass.

6. Future perspectives

With the Swedish Food Administration's stricter limits on PFAS 4 and PFAS 21 starting by 2026, the demand for reactivation will probably increase throughout the country. An in-house regeneration of GAC would exclude or at least lower the dependency on conventional burning, which is highly energy consuming and requires long and heavy transport back and forth to reactivation facilities. Whether sodium hydroxide is applicable in terms of cost, sustainability, water consumption, removal of organic carbon and waste management are hard to tell from this study, but it certainly has technical potential for biological regeneration. Especially in GAC filters like the future waterwork at Norrvatten, where the saturation rate is expected to be lower due to the clean feedwater. A potential way to regenerate GAC would be to apply air for agitation while simultaneously circulating the sodium hydroxide through a microfilter to accumulate released biomass on a membrane (Fig 16). The microfilter could then be backwashed together with the GAC bed by adjusting valves to open a second path through the filter (Fig 16).

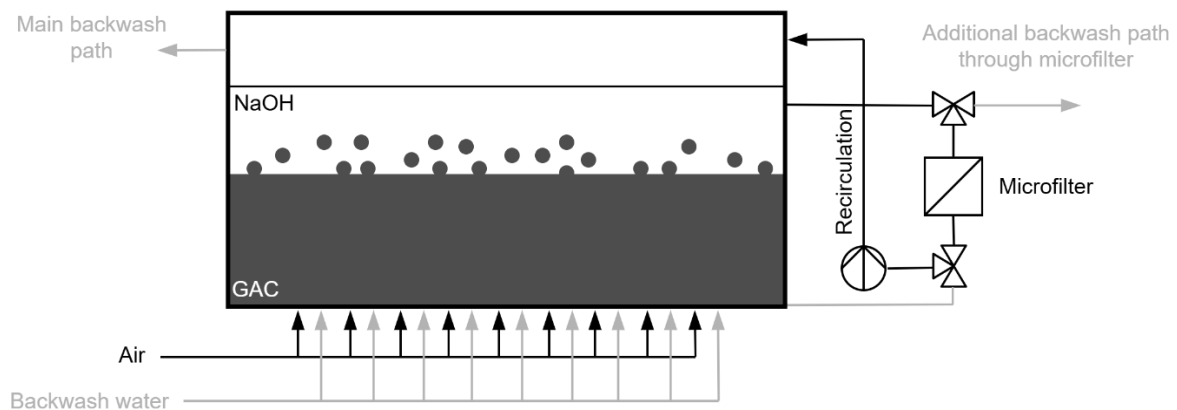


Figure 16: Potential regeneration solution. Black arrows indicate actions that would be taken during the regeneration while grey arrows indicate the backwashing process/removal of excess NaOH.

Regarding the focus of this study, sodium hydroxide disinfection could be used as a point action upon microbial instability in Norrvatten's future carbon filters. But before applying NaOH in disinfection or regeneration, the concentration and time should be optimized toward the specific circumstances. To do this, a future experiment would be to grow biofilm in GAC similarly to the future waterwork and then use the same carbon sample to test various concentrations and times in a screening experiment, using for instance MODDE®. The screening experiment could then be improved iteratively to eventually build a statistically significant and predictable model for optimization. This model could be expanded by spiking GAC with microfungi, coliforms and *E. coli* or other risk microorganisms and conducting a similar disinfection experiment. Predictable models of different risk microorganisms are valuable for preparatory purposes, to know how to treat possible future contaminations.

Furthermore, the ability to quantify different microbes that are attached to GAC would be beneficial in several ways for Norrvatten and other waterworks. It would allow detection and source localization of contaminations, such as microfungi or other problematic microbes. But also make it possible to follow the development and the state of GAC/BAC filters. One way to assess the efficiency of a future desorption method, for instance mixing homogenization, would be to measure TCC and ICC in the desorption medium at several time points as in the sonication experiment. Then create two solutions of cells in fresh desorption medium, which has the same concentrations of TCC and ICC respectively as the peak concentrations from the desorption experiment. These two solutions are then treated by the same desorption method without carbon and analyzed at the same times. If the desorption curves behave like the curves for artificial solutions, the peak constitutes all cells that were in the carbon sample. However, as long the desorption curves show slower decrease in TCC and ICC, compared to the artificial solutions, cells are still desorbing.

Conclusions about the growth in GAC beds are also applicable in decision-making and validation of already taken decisions, regarding the operation and design of Norrvatten's future carbon filters. These results could also be useful for other waterworks that might use similar process designs in the future. Furthermore, the absence of growth in the GAC that was autoclaved suggests that steam treatment might be applicable as a disinfection strategy, even though it does not kill spores.

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Appendix A – Raw data

Table A1: Flow cytometry measurements (IN: attachment phase, UF: incubation phase)

<i>Date & time</i>	<i>Sample</i>	<i>TCC</i> <i>10³</i> <i>cells/ml</i>	<i>TCC</i> <i>Stdv 10³</i> <i>cells/ml</i>	<i>ICC</i> <i>10³</i> <i>cells/ml</i>	<i>ICC</i> <i>Stdv 10³</i> <i>cells/ml</i>	<i>TCC</i> <i>HNA</i> <i>%</i>	<i>ICC</i> <i>HNA</i> <i>%</i>
25/01/21	Carbon filtrate	854,71	21,17	745,85	3,79	34	35,9
25/01/21	Sand filtrate	837,08	2,8	756,61	5,59	33,75	35,65
25/01/21	Raw water	2404,09	7,17	2110,22	0,92	39,75	41,1
25/01/22	Autoclaved UF filtrate	0,02		0		100	0
25/01/23 14:13	5.1 UF T0	10,04		3,94		20,3	43,1
25/01/28 14:25	1.1 IN T1	0,88		0		88,6	0
25/01/28 14:25	1.2 IN T1	7		0,66		99,7	97
25/01/28 14:25	2.1 IN T1	0,54		0,02		40,7	0
25/01/28 14:25	2.2 IN T1	81,08		10,5		99	99,6
25/01/28 14:25	3 IN T1	1042,9		680,34		69,6	36,5
25/01/28 14:25	4 IN T1	41,54		7,76		22,2	12,9
25/01/28 14:25	5.2 IN T1	1021		571,48		57,1	57
25/01/28 14:25	5.1 UF T1	166,82		119,98		78,3	87
25/01/29	1.2 filtered 5µm	55,1		54,8		89,6	91,8
25/01/29	4 filtered 5 µm	81,08		70,88		74,9	81,2
25/01/29	1.2 filtered 5+3 µm	3,46		2,62		57,8	67,9
25/01/29	4 filtered 5+3 µm	17,26		9,16		21,1	21,4
25/01/31	DW unfiltered	788,26		442,86		30,7	36,6
25/01/31	DW filtered 1.2 µm	738,62		438,74		25,5	27,4
25/01/31	DW filtered 3 µm	616,68		390,74		24,2	31,2
25/01/31	DW filtered 5 µm	786,02		479,92		28,3	29,2
25/02/03	DW centrifuged 10s	703,76	0,82	550,31	14,13	25,15	23,4
25/02/03	DW	700,7	0,7	539,5	32,02	24,9	24,25
25/02/04 10:35	1.1 IN T2	14,84		6,88		87,6	92,2
25/02/04 10:35	1.2 IN T2	10,64		7,6		94,4	98,4
25/02/04 10:35	2.1 IN T2	133,9		51,16		92,3	98,4
25/02/04 10:35	2.2 IN T2	185,94		175,74		97,5	98,9
25/02/04 10:35	3 IN T2	97,12		58,06		87,1	51,1
25/02/04 10:35	4 IN T2	4,1		1,06		65,9	54,7
25/02/04 10:35	5.2 IN T2	378,02		242,12		81,5	87,2
25/02/04 10:35	5.1 UF T2	418,34		153,78		93,5	88,9
25/02/05 12:33	1.1 UF T0	11,44		5,92		89,5	95,6
25/02/05 12:33	1.2 UF T0	16,7		11		94,5	96,7
25/02/05 12:33	2.1 UF T0	35,54		15,9		90,5	98,1
25/02/05 12:33	2.2 UF T0	52,06		31,72		93,4	95,3
25/02/05 12:33	3 UF T0	11,22		8,62		83,6	63,6
25/02/05 12:33	4 UF T0	1,68		0,32		67,9	87,5
25/02/05 12:33	5.2 UF T0	150,74		125,8		95,8	96,8
25/02/05 12:55	5.3 UF T0	200,14		183,82		96,6	97,9
25/02/07 12:22	1.1 UF T1	49,82		40,2		93,9	96,7
25/02/07 12:22	1.2 UF T1	36,92		35,82		96,2	97,8
25/02/07 12:22	2.1 UF T1	130,56		22,14		82,6	96,3
25/02/07 12:22	2.2 UF T1	252,6		182,2		95,7	96,8
25/02/07 12:22	3 UF T1	108,14		125,8		94,4	65,2
25/02/07 12:22	4 UF T1	20,98		17,72		95,3	99,5
25/02/07 12:22	5.2 UF T1	252,28		198,82		97,1	95,1
25/02/07 12:22	5.3 UF T1	245,74		197,88		95	95,7
25/02/07 12:22	5.1 UF T3	318,02		231,64		95,7	90

<i>Date & time</i>	<i>Sample</i>	<i>TCC</i> <i>10³</i> <i>cells/ml</i>	<i>TCC</i> <i>Stdv 10³</i> <i>cells/ml</i>	<i>ICC</i> <i>10³</i> <i>cells/ml</i>	<i>ICC</i> <i>Stdv 10³</i> <i>cells/ml</i>	<i>TCC</i> <i>HNA</i> <i>%</i>	<i>ICC</i> <i>HNA</i> <i>%</i>
25/02/10 10:30	1.1 UFT2	154,18		100,56		94,5	98,5
25/02/10 10:30	1.2 UFT2	95,5		100,7		92,8	94,3
25/02/10 10:30	2.1 UFT2	434,88		54,68		93,4	97,1
25/02/10 10:30	2.2 UFT2	473,16		311,14		97,4	98,7
25/02/10 10:30	3 UFT2	398,22		164,42		82,6	82,9
25/02/10 10:30	4 UFT2	152,72		35,56		94,2	93,1
25/02/10 10:30	5.2 UFT2	280,86		164,4		95	90,2
25/02/10 10:30	5.3 UFT2	388,18		201,6		93,5	95,7
25/02/10 10:30	5.1 UFT4	268,86		153,1		96,1	95,5
25/02/13 09:30	1.1 UFT3	171,3		124,48		94,2	97,8
25/02/13 09:30	1.2 UFT3	532,16		321,26		93,2	98,7
25/02/13 09:30	2.1 UFT3	755,32		336,36		96,7	98
25/02/13 09:30	2.2 UFT3	477,08		254,34		92,8	95,8
25/02/13 09:30	3 UFT3	401,92		198,4		83,4	70,3
25/02/13 09:30	4 UFT3	541,08		369,22		92,1	95,7
25/02/13 09:30	5.2 UFT3	239,02		178,92		88	72,1
25/02/13 09:30	5.3 UFT3	350,9		100,3		91,6	87,9
25/02/13 09:30	5.1 UFT5	204,46		307,24		77,6	92,1
25/02/17 09:40	1.1 UF T4	160,38		161,08		95,5	97,1
25/02/17 09:40	1.2 UF T4	733,66		688		96,6	98,3
25/02/17 09:40	2.1 UF T4	430,2		318,8		92,7	94,7
25/02/17 09:40	2.2 UF T4	550,72		388,56		96,1	97,7
25/02/17 09:40	3 UF T4	329,4		217,68		83,3	71
25/02/17 09:40	4 UF T4	580,16		471,7		89	95,6
25/02/17 09:40	5.2 UF T4	237,24		125,48		84,4	81,4
25/02/17 09:40	5.3 UF T4	363,42		238,7		89,7	85
25/02/17 09:40	5.1 UF T6	304,52		167,32		94,1	92,6
25/02/20 10:05	1.1 UF T5	225,32		101,48		95,5	97,9
25/02/20 10:05	1.2 UF T5	759,28		493,98		95,9	98,3
25/02/20 10:05	2.1 UF T5	340,48		247,62		92,8	93,8
25/02/20 10:05	2.2 UF T5	631,6		187,68		94,5	96,5
25/02/20 10:05	3 UF T5	254,66		160,68		77,5	67,2
25/02/20 10:05	4 UF T5	422,56		286,86		87,8	94,8
25/02/20 10:05	5.2 UF T5	284,56		148,86		81,6	63,8
25/02/20 10:05	5.3 UF T5	275,52		86,58		87,5	79
25/02/20 10:05	5.1 UFT7	249,48		131,1		91,8	76,3
25/02/26	Son1: 0	11,12		7,04		82,2	96
25/02/26	Son1: 3	121,18		63,5		69,8	91,5
25/02/26	Son1: 6	82,9		62,66		89,2	92,9
25/02/26	Son1: 6v2	83,4		86,92		89,3	94,2
25/02/26	Son1: 6v	76,86		61,64		88,9	92,6
25/02/26	Son1: 12	82,2		64,52		90	95,8
25/02/26	Son1: 20	58,34		56,64		89,9	94,6
25/02/26	Son1: 40	33,34		22,68		77,3	95,9
25/02/26	Son1: 60	8,84		4,94		87,6	91,9
25/02/26	Son1: 80	2,52		1,98		63,5	80,8
25/02/26	Son1: 120	4,5		2,96		75,6	89,9
25/02/26	Son1: 180	2,2		1,14		54,5	54,4
25/02/27	Son2: 0	7,68		3,72		74,5	89,2
25/02/27	Son2: 0	7,26		3,52		73,6	90,3
25/02/27	Son2: 45	78,58		55,6		87,2	93,8
25/02/27	Son2: 45	81,18		63,68		85,1	93,9

<i>Date & time</i>	<i>Sample</i>	<i>TCC</i> <i>10³</i> <i>cells/ml</i>	<i>TCC</i> <i>Stdv 10³</i> <i>cells/ml</i>	<i>ICC</i> <i>10³</i> <i>cells/ml</i>	<i>ICC</i> <i>Stdv 10³</i> <i>cells/ml</i>	<i>TCC</i> <i>HNA</i> <i>%</i>	<i>ICC</i> <i>HNA</i> <i>%</i>
25/02/27	Son2: 130	69,34		48,1		87,4	95
25/02/27	Son2: 130	69,22		50,2		87,6	93
25/02/27	Son2: 215	66,76		57,54		88,2	92,7
25/02/27	Son2: 215	74,1		49,98		89,4	92,7
25/02/27	Son2: 300	59,76		32,78		88,2	95,2
25/02/27	Son2: 300	49,66		38,62		83,8	89,5
25/02/27	Son2: 345	33,56		24,26		84,3	95,5
25/02/27	Son2: 345	41		30,06		87,4	95
25/02/28	Son3: 0	5,18		1,3		51,4	89,2
25/02/28	Son3: 0	4,98		0,92		57,4	82,6
25/02/28	Son3: 15	51,36		7,64		83	90,6
25/02/28	Son3: 15	44,2		20,7		73,2	89,1
25/02/28	Son3: 30	48,58		28,06		70,2	89,6
25/02/28	Son3: 30	52,22		33,36		77,6	89,9
25/02/28	Son3: 45	36,48		17,32		77,5	92,5
25/02/28	Son3: 45	54,92		35,88		76,4	86,7
25/03/06	Dis: 1.2 B I (I: before)	4,78		0,68		61,1	97,1
25/03/06	Dis: 1.2 B E (E: after)	56,3		28,48		88,5	95,6
25/03/06	Dis: 1.2 B NaOH I	3,3		0,02		52,7	0
25/03/06	Dis: 1.2 B NaOH E	12,2		0,08		19,5	0
25/03/06	Dis: 2.1 B I	2,36		0,16		55,1	62,5
25/03/06	Dis: 2.1 B E	72,5		19,28		74,4	93,8
25/03/06	Dis: 2.1 B NaOH I	4,06		0,12		36,9	0
25/03/06	Dis: 2.1 B NaOH E	13,64		0,18		17	22,2
25/03/06	Dis: 3 B I	3,68		0,66		60,9	90,9
25/03/06	Dis: 3 B E	55,8		34,36		79,6	95,2
25/03/06	Dis: 3 B NaOH I	2,26		0,02		43,4	0
25/03/06	Dis: 3 B NaOH E	19,54		0,16		24,5	0
25/03/06	Dis: 2.2 I	5,6		0,72		50,7	86,1
25/03/06	Dis: 2.2 E	70,4		31,22		86	95,5
25/03/06	Dis: 2.2 NaOH I	2,72		0,06		44,1	66,7
25/03/06	Dis: 2.2 NaOH E	14,94		0,1		15,7	20
25/03/06	Dis: 3 A I	4,2		1,8		68,1	93,3
25/03/06	Dis: 3 A E	81,76		47,68		83,5	95,1
25/03/06	Dis: 3 A NaOH I	1,4		0,02		45,7	0
25/03/06	Dis: 3 A NaOH E	5,7		0,04		6,32	0
25/03/06	Dis: 4 A I	2,48		0,7		65,3	91,4
25/03/06	Dis: 4 A E	24,58		21,04		87,8	95,5
25/03/06	Dis: 4 A NaOH I	1,42		0,02		26,8	0
25/03/06	Dis: 4 A NaOH E	3,86		0,04		15,5	0
25/03/06	Dis: 1.2 A I	4,46		1,12		62,8	94,6
25/03/06	Dis: 1.2 A E	48,28		28,22		89	95,9
25/03/06	Dis: 1.2 A NaOH I	2,06		0,04		55,3	0
25/03/06	Dis: 1.2 A NaOH E	5,38		0,02		16,7	0
25/03/06	Dis: 1.2 C I	4,14		1,08		66,2	85,2
25/03/06	Dis: 1.2 C E	49,36		29,32		91,8	96,7
25/03/06	Dis: 1.2 C NaOH I	2,08		0,06		50	0
25/03/06	Dis: 1.2 C NaOH E	5,56		0,04		23	0
25/03/06	Dis: 5.2 I	3,5		0,66		64,6	81,8
25/03/06	Dis: 5.2 E	72,94		43,56		83,2	90,6

Date & time	Sample	TCC	TCC	ICC	ICC	TCC	ICC
		10 ³ cells/ml	Stdv 10 ³ cells/ml	10 ³ cells/ml	Stdv 10 ³ cells/ml	HNA %	HNA %
25/03/06	Dis: 5.2 NaOH I	1,3		0,04		23,1	0
25/03/06	Dis: 5.2 NaOH E	18,84		0,56		44,2	71,4
25/03/06	Dis: 4 B I	4,14		1,24		65,2	88,7
25/03/06	Dis: 4 B E	35,08		16,82		85,6	92,6
25/03/06	Dis: 4 B NaOH I	2,36		0,04		50	0
25/03/06	Dis: 4 B NaOH E	3,76		0,06		14,4	0
25/03/06	Dis: 2.1 A I	1,76		0,14		39,8	42,9
25/03/06	Dis: 2.1 A E	52,8		20,02		84,1	94,8
25/03/06	Dis: 2.1 A NaOH I	1,72		0,02		45,3	0
25/03/06	Dis: 2.1 A NaOH E	10,16		0		12,2	0
25/03/06	Dis: 1.1 I	2,98		0,12		39,6	100
25/03/06	Dis: 1.1 E	12,16		0,88		65,6	90,9
25/03/06	Dis: 1.1 NaOH I	0,98		0,12		30,6	83,3
25/03/06	Dis: 1.1 NaOH E	12,58		3,76		69,5	93,6
25/04/01 10:00	1 T0 I (I: before)	4,96		0,42		52,8	19
25/04/01 10:00	1 T0 E (E: after)	5,58		0,68		54,5	8,82
25/04/01 10:00	2 T0 I	3,58		0,22		62,6	9,09
25/04/01 10:00	2 T0 E	3,6		0,14		56,1	0
25/04/01 10:00	3 T0 I	4,3		0,58		46,5	0
25/04/01 10:00	3 T0 E	3,16		0,42		54,4	4,76
25/04/03 09:30	1 T1 I	4,98		0,2		55,8	0
25/04/03 09:30	1 T1 E	3,56		0,04		57,9	0
25/04/03 09:30	2 T1 I	4,58		0,48		59,4	0
25/04/03 09:30	2 T1 E	3,24		1,04		53,1	13,5
25/04/03 09:30	3 T1 I	4,82		0,78		56	2,56
25/04/03 09:30	3 T1 E	3,8		0,08		58,4	25
25/04/07 09:30	1 T2 I	6,36		0,86		55,7	4,65
25/04/07 09:30	1 T2 E	4,4		0,52		49,1	0
25/04/07 09:30	2 T2 I	5,3		1,52		49,4	5,26
25/04/07 09:30	2 T2 E	3,5		0,66		57,7	3,03
25/04/07 09:30	3 T2 I	5,64		1,9		44	6,32
25/04/07 09:30	3 T2 E	6,42		0,9		38	17,8
25/04/10 09:30	1 T3 I	4,08		0,2		51	20
25/04/10 09:30	1 T3 E	2,86		0,52		39,9	0
25/04/10 09:30	2 T3 I	4,44		0,46		51,8	56,5
25/04/10 09:30	2 T3 E	2,8		0,8		51,4	62,5
25/04/10 09:30	3 T3 I	4,8		1,8		55,8	57,8
25/04/10 09:30	3 T3 E	8,12		3,6		64,5	82,8
25/04/14 09:30	1 T4 I	5,28		3,76		51,9	20,2
25/04/14 09:30	1 T4 E	4,46		0,34		54,3	29,4
25/04/14 09:30	2 T4 I	7,04		2,04		63,1	71,6
25/04/14 09:30	2 T4 E	16,18		10,46		82,1	88
25/04/14 09:30	3 T4 I	12,72		6,12		80	88,6
25/04/14 09:30	3 T4 E	64,94		36,56		92,4	95,8
25/04/17 09:30	1 T5 I	5,62		0,72		55,5	2,78
25/04/17 09:30	1 T5 E	6,54		0,62		52	0
25/04/17 09:30	2 T5 I	21,76		14,06		85,8	89,2
25/04/17 09:30	2 T5 E	70,96		73,72		89,1	95,3
25/04/17 09:30	3 T5 I	34,6		23,04		89,9	96
25/04/17 09:30	3 T5 E	108,28		88,12		93,8	97,2
25/04/15	Re: 0.1% T0	3,94		2,1		65,5	61,9
25/04/15	Re: 5% T0	3,36		0,42		37,5	0

Date & time	Sample	TCC	TCC	ICC	ICC	TCC	ICC
		10 ³ cells/ml	Stdv 10 ³ cells/ml	10 ³ cells/ml	Stdv 10 ³ cells/ml	HNA %	HNA %
25/04/15	Re: UF+MQ T0	0,22		0,06		9,09	0
25/04/16	Re: KF10 0.1% T0	1815,94		671,14		46,9	79,3
25/04/16	Re: KF10 5% T0	3106,52		1,62		24,7	0
25/04/16	Re: KF10 UF+MQ T0	0,04		0,22		0	9,09
25/04/22	Re: 0.1% T1	2612,62		1834,12		97,1	96,4
25/04/22	Re: 5% T1	1,36		0,3		27,9	13,3
25/04/22	Re: UF+MQ T1	0,92		0,14		8,7	0
25/04/23	Re: KF10 0.1% T1	3597,78		2953,38		81	90,3
25/04/23	Re: KF10 UF+MQ T1	0,2		0,04		70	0

Table A2: Regrowth in NaOH treated GAC samples

Sample	Bottle 2.2 carbon			KF10 carbon		
	TCC 10 ³ cells/ml	ICC 10 ³ cells/ml	3D CFU/ml	TCC 10 ³ cells/ml	ICC 10 ³ cells/ml	3D CFU/ml
0.1% start	3,94	2,1	502	1815,94	671,14	534
0.1% end	2612,62	1834,12	210 365	3597,78	2953,38	110 979
5% start	3,36	0,42	0	3106,52	1,62	0
5% end	1,36	0,3	0	N/A	N/A	1
UF+MQ start	0,22	0,06	0	0,04	0,22	0
UF+MQ end	0,92	0,14	0	0,2	0,04	0

Appendix B – Methodology specifications

Run Limits

10000 events in Ungated Sample

0 Min 0 Sec

50 µL

Only collect events inside

Fluidics

Slow Medium Fast

Flow Rate: 35 µL/min
Core Size: 16 µm

Set Threshold

Delete events on (Minimum: 10)

FL1-H less than 500

- less than 0

SIP Rinse Settings

1 Cycle

Apply Settings Remove Settings

Agitate Plate

1 Cycle every 1 Well(s)

None every 1 Min

Run Horizontally

Run Vertically

SIP Clean

Run SIP Clean After Samples

Figure B1: Flow cytometer software settings.

Table B1: Selected bottles for disinfection and regeneration

<i>Bottle from batch 1</i>	<i>Concentration</i>	<i>Time</i>
1.1	0.1%	30 min
1.2	2.55%	195 min
1.2	2.55%	195 min
1.2	5%	360 min
2.1	2.55%	30 min
2.1	2.55%	360 min
3	0.5%	240 min
3	0.1%	360 min
4	5%	30 min
4	5%	195 min
5.2	0.1%	195 min

Table B2: Sampling and activities of batch bottles.

<i>Date</i>	<i>Bottles</i>	<i>Sample (LP =liquid phase)</i>	<i>Analysis</i>	<i>Shaking and cap opening</i>	<i>Activity (UFP = UF permeate)</i>
2025-01-21	1.1-5.3				Filled with water sources
2025-01-23	5.1	2 x 1 ml LP	FCM	✓	Exchange to UFP
2025-01-28	1.1-5.2	2 x 1 ml LP	FCM	✓	
2025-01-29	1.1-5.3			✓	
2025-01-30	1.1-5.3, 6, 7			✓	Water source fill (6, 7)
2025-02-04	1.1-5.2	2 x 1 ml LP	FCM	✓	
2025-02-05	1.1-4, 5.2-5.3	2 x 1 ml LP	FCM		Exchange to UFP
2025-02-06	1.1-5.3			✓	
2025-02-07	1.1-5.3	2 x 1 ml LP	FCM		UFP refill (before sampling)
2025-02-10	1.1-5.3	2 x 1 ml LP	FCM	✓	
2025-02-11	1.1-4, 5.2-5.3				UFP refill
2025-02-12	1.1-5.3			✓	
2025-02-13	1.1-5.3	1.5 ml LP	FCM	✓	
2025-02-14	1.1-5.3			✓	UFP refill
2025-02-17	1.1-5.3	1.5 ml LP	FCM	✓	
2025-02-18	1.1-5.3			✓	UFP refill
2025-02-20	1.1-5.3	1.5 ml LP	FCM		
2025-03-31	A				UFP fill and autoclaved
2025-04-01	A, B, C	10 ml GAC	Attached cells	✓	UFP fill (not A)
2025-04-03	A, B, C	10 ml GAC	Attached cells	✓	
2025-04-07	A, B, C	10 ml GAC	Attached cells	✓	UFP refill
2025-04-10	A, B, C	10 ml GAC	Attached cells	✓	
2025-04-11	A, B, C				UFP refill
2025-04-14	A, B, C	10 ml GAC	Attached cells	✓	
2025-04-15	A, B, C				UFP refill
2025-04-17	A, B, C	10 ml GAC	Attached cells		

Appendix C – Other results

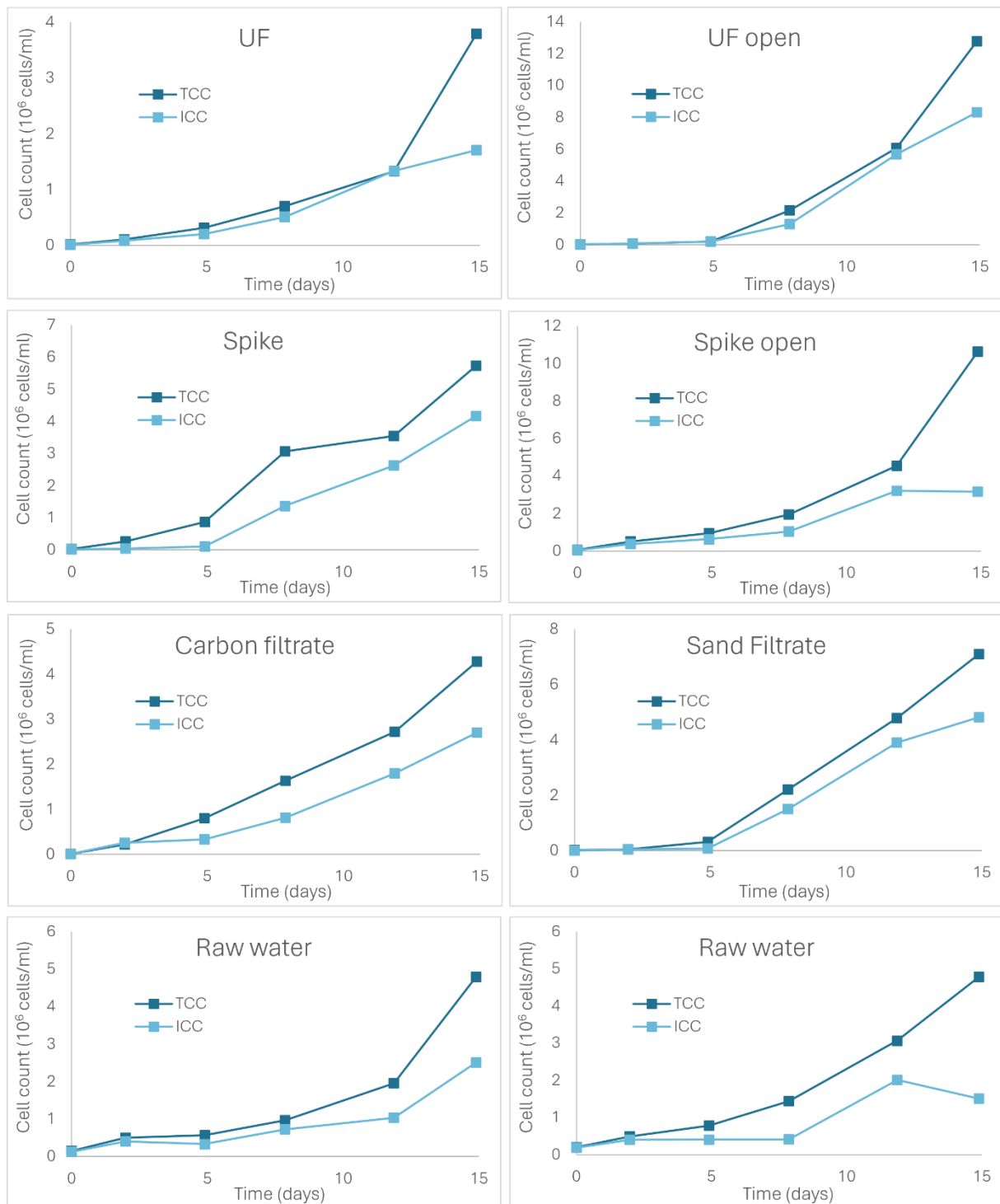


Figure C1: TCC vs ICC in liquid phases of carbons submerged in UF permeate, with refills, that previously had been submerged in water sources (labels in fig). Lines between data points are for illustration.

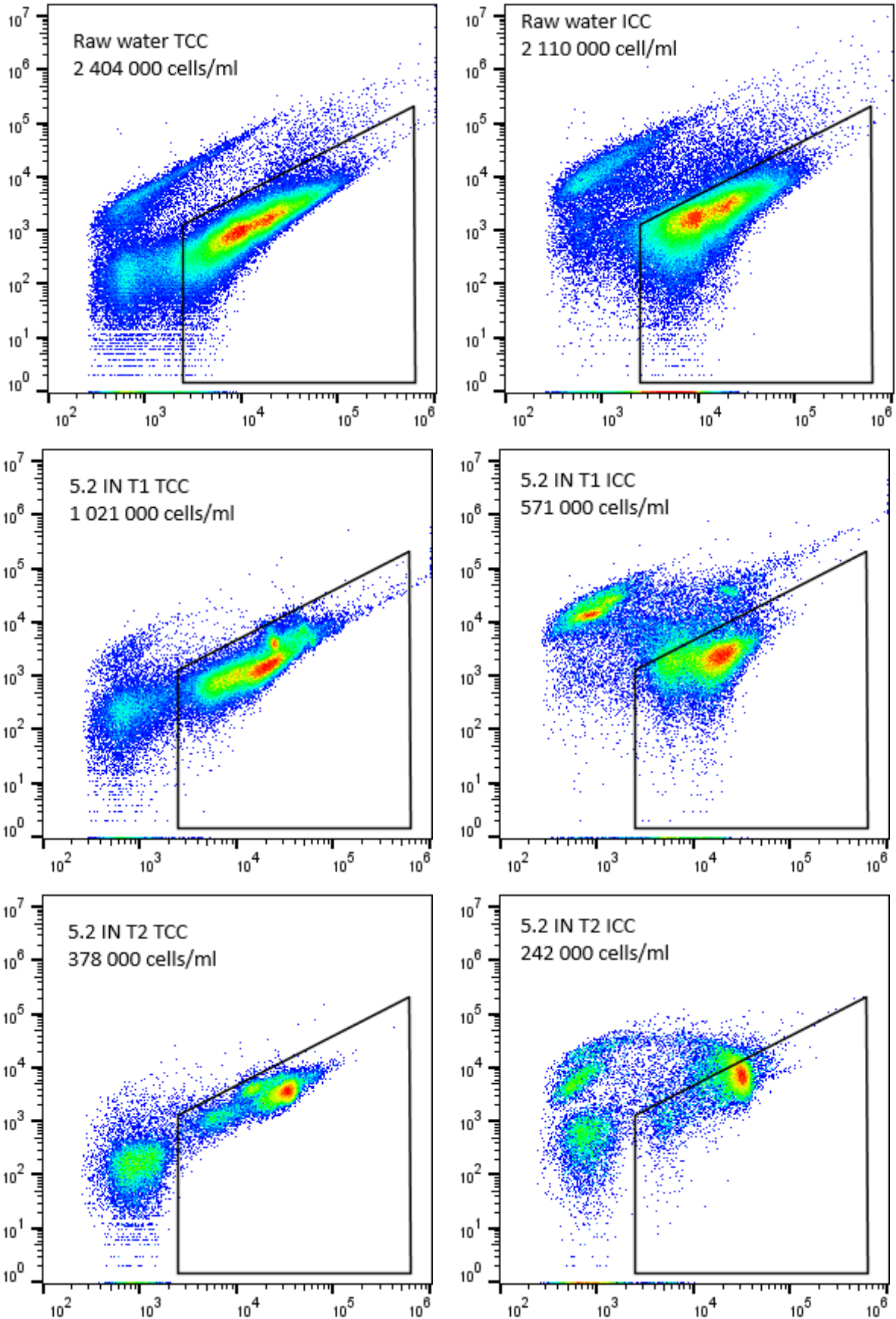


Figure C2: TCC and ICC cytograms of liquid phase samples from bottle 5.2 during attachment and incubation. IN represents samples from attachment phase and UF represent samples from incubation phase. The relative order of samples are given as T with a number.

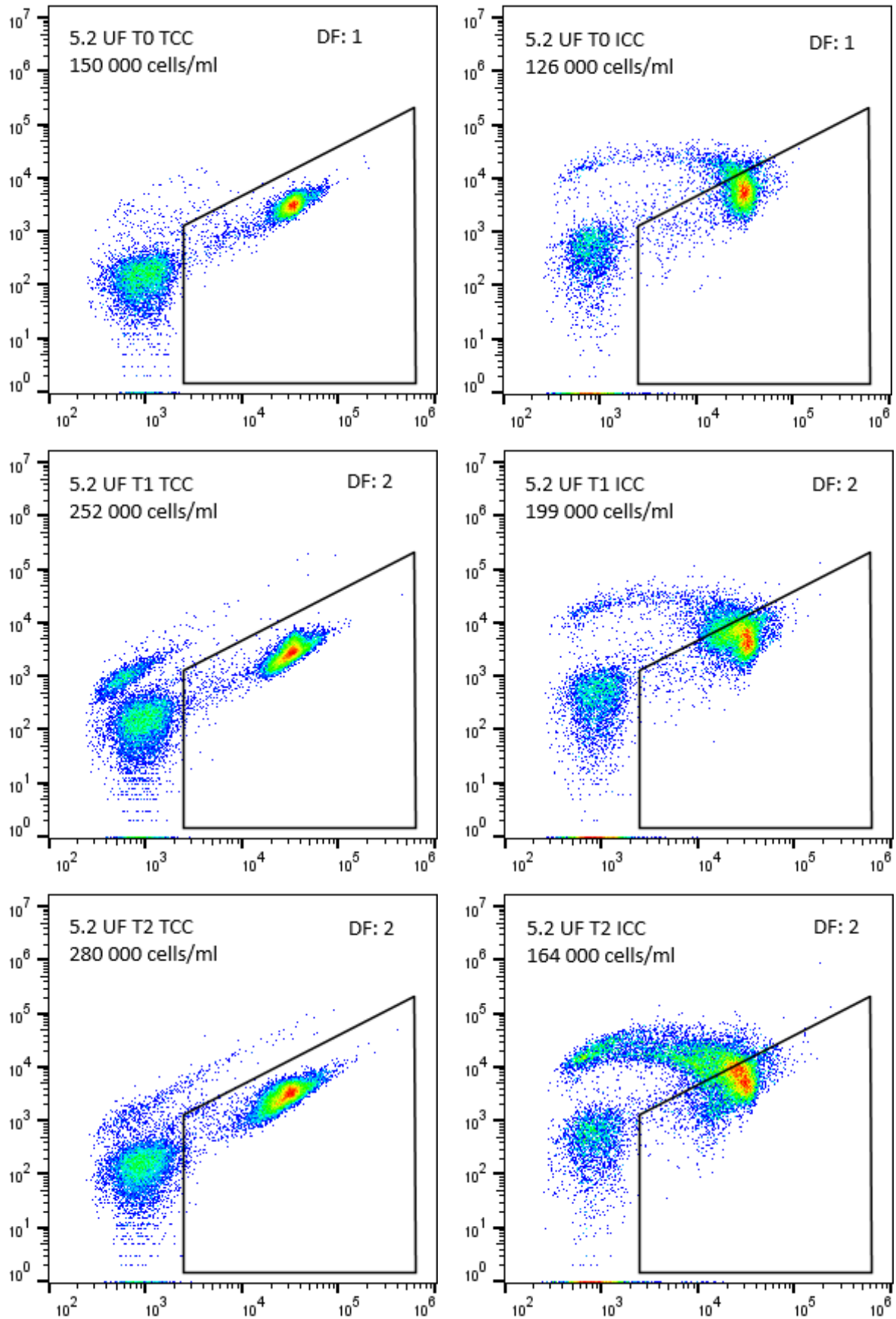


Figure C2: TCC and ICC cytograms of liquid phase samples from bottle 5.2 during attachment and incubation. IN represents samples from attachment phase and UF represent samples from incubation phase. The relative order of samples are given as T with a number.

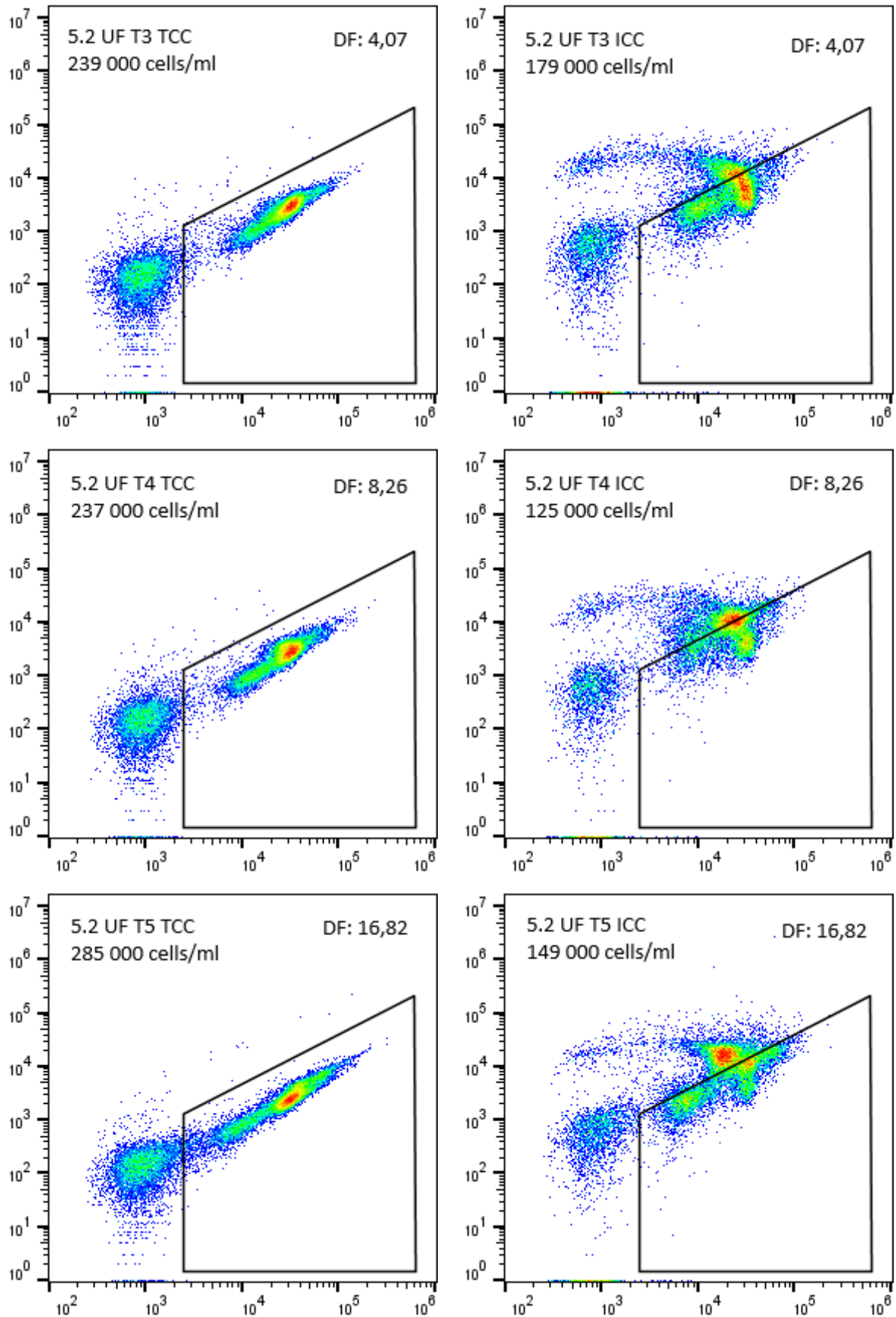


Figure C2: TCC and ICC cytograms of liquid phase samples from bottle 5.2 during attachment and incubation. IN represents samples from attachment phase and UF represent samples from incubation phase. The relative order of samples are given as T with a number.