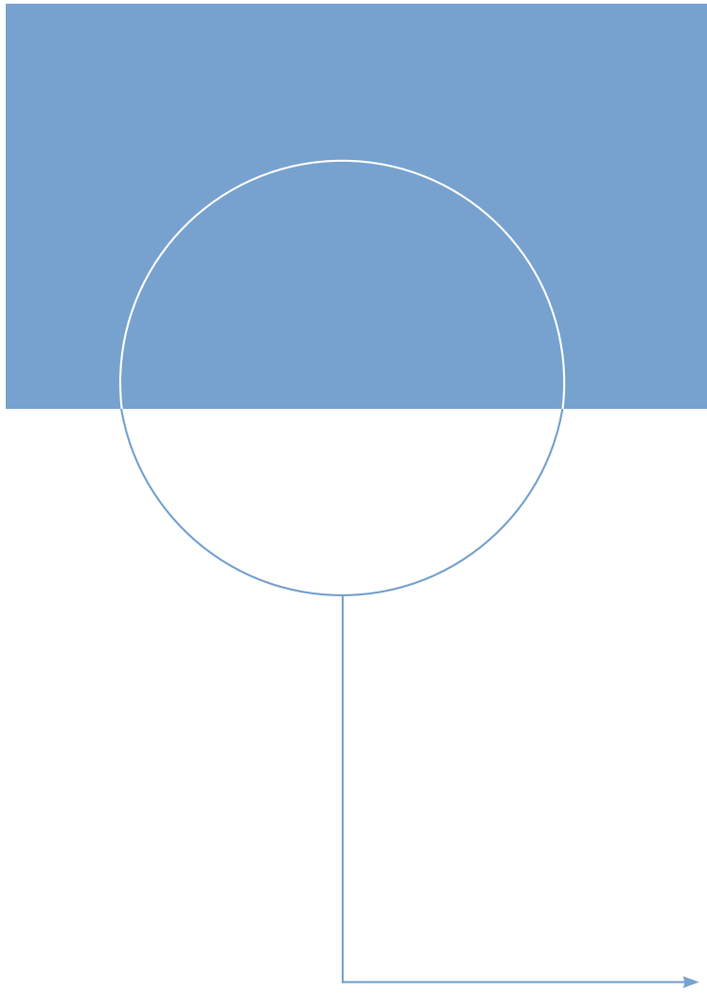

Master's thesis

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Validation of cleaning brushes designs and material properties to improve the biofilm removal for fish industry

Evaluation of cleaning techniques, tools and material properties

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Validation of cleaning brushes designs and material properties to improve the biofilm removal for fish industry

Evaluation of cleaning techniques, tools and material properties

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MSc in Product and System Design
Submission date: June 2019
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Abstract

There is an increased demand of improved cleaning quality in fish factories due to stricter regulations set by the government due to the constant dangers of L. *monocytogenes*. This bacteria is carried by the fish and causes one of the most severe foodborne diseases *listeriosis* which has symptoms such as headache, fever, body ache, sleepiness, neuro-logic disease and has a mortality rate of 30%. In the high risk group of getting this disease are pregnant women and patients with reduced immunity system. This bacteria can survive under extreme environmental conditions and their ability to survive in biofilm constitutes a problem in food production environments, making it hard to remove from otherwise sterile surfaces.

This thesis explores different strategies to fight biofilm formations in the fish factory which grants L. *monocytogenes* a protective barrier against cleaning agents. Two strategies are investigated: The first strategy is to evaluate how the material choice of the equipment used in the fish factory impacts the biofilm, where the surface properties on Stainless steel, Aluminium alloy and Polyethylene High Density (PEHD) were put through different tests such as *water controlled test* and *cleaning tool test*. The test samples were produced by allowing the bacteria P. *fluorescence* and S. *aureus* grow in containers filled with nutrient broth for 2 weeks. As a part of this strategy, a water control test was made to measure how much biofilm is removed without a cleaning tool only rinsed with water, and to observe how biofilm is affected by the material properties.

The last strategy was to introduce cleaning brushes of different designs and investigate how these tools performs against biofilm. The tools were categorized into different design, material type and cleaning methods. Several bacterial samples of each cleaning tool were taken after the experiment was over to investigate the density of bacteria that resides inside the brushes overnight after they have been decontaminated. These tests were kept at 35°C before being analysed at the lab with a photometric machine that measures the absorption level of the samples. The higher absorption, the higher amount of bacteria. The entire objective of introducing tools to fish factories is to measure how effective they are at removing biofilm and if they pose any threat by becoming a highway for the bacteria to spread diseases across the factories.

Sammendrag

Fiskeindustrien har fått strengere krav til vasking opp gjennom årene på grunn av faren for sykdom fra *Listeria monocytogenes* som kommer med fisken. Denne bakterien forårsaker sykdommen *listeriosis* som gir symptomer som feber, verk i kroppen, hodepine, tretthet, nevrologiske symptomer og har en dødelighet på 30%. Risiko gruppen for denne bakterien er gravide og pasienter med nedsatt immunforsvar. I fiskefabrikken kommer bakterien sammen med fisken og sprer seg i fiskeprosesserings anlegget når fiskene blir håndtert. Bakterien vil da spre seg til utstyr som håndterer fisk og forurense fiskene som kommer etter. Denne bakterie typen er overlevelsesdyktig og kan overleve under ekstreme forhold som gjør den vanskelig å eliminere.

I tillegg kan den danne biofilm aleine eller sammen med andre bakterie arter som *Pseudomonas fluorescens* og *Stafylococcus aureus* som gir den ekstra forsvar mot kjemikalier. Til tross for en lav forekomst av listeriosis med 25-30 pr år, forblir det ett stort helseproblem på grunn av dødeligheten av sykdommen som bakterien forårsaker. Dette har fått fiskeindustrien til å se på nye metoder for å forbedre deres vaskemetoder for å øke vaskekvaliteten og dermed senke sjansen for utbrudd av L. *monocytogenes*.

Hovedmålet med denne oppgaven er å utforske to strategier for å bekjempe biofilm som gir bakterien en beskyttende barriere mot kjemikalier. Den første strategien er undersøke hvordan forskjellig material typer påvirker veksten og vasking av biofilm. Eksperimentene ble utført i ett laboratorium hos *Institutt for biologiske fag* i Ålesund der material av typen rustfritt stål, aluminium og polyetylen gjennomgikk tester som *vann kontroll test* og *vaske test*. Vaskeprøvene ble produsert ved å oppbevare test eksemplarene i et vekstbad fylt med bakterier av typen *Pseudomonas fluorescens* og *Stafylococcus aureus* og næringsbuljong. Her ble prøvene oppbevart i 2 uker for å la biofilm vokse på overflaten til materialene. Som en del av strategien blir vann kontroll testen brukt til å måle hvor mye material type kan påvirke biofilm.

Den andre strategien er å introdusere vaskeredskap i forskjellig design inn i vaskeprosessen for å undersøke hvor effektive disse er mot biofilm. Vaskeredskapene ble kategorisert inn i forskjellig design, material type og vaske metode. Som en del av denne strategien ble det tatt en rekke bakterietester av hvert vaskeredskap etter de ble rengjort. Dette for å undersøke bakterieveksten på vaskeredskap etter de har stått over natten med dårlig vedlikehold. Disse bakterie testene ble oppbevart i 35 °C over natten og tatt med til lab for photometric måling av bakterie prøvene for å finne bakterie tettheten. Dette blir gjort siden målet med denne strategien er å undersøke hvor effektive vaskeredskap er mot biofilm og hvis de utgjør en trussel ved å bli en mulig smittekilde for fiskefabrikken.

Preface

The work for this master thesis was carried out at the Department of Ocean Operations and Civil Engineering at the Norwegian University of Science and Technology (NTNU) in Ålesund from the fall semester of 2018 to spring semester of 2019.

I want to thank my supervisor, Professor Ola Jon Mork at the Department of Ocean Operations and Civil Engineering. Ola Jon have a great amount knowledge and a good understanding of how this field works. He expresses this through his enthusiasm and his ability to share his insight in a tangible manner. I am truly grateful to have had the opportunity to work with him.

I also want to express my gratitude to:

Associate Professor Ann-Kristin Tveten at Department of Biological Sciences, for ensuring a well-functioning laboratory. Ann-Kristin also contributed with research materials and the necessary planning to be able to conduct the experiments. She provided the necessary practical training, assistance and guidance throughout the laboratory work. Her key inputs have served as great inspiration to this thesis.

Apprentice Magnus Løkset at the Department of Biological Sciences, for the instructions and assistance at the laboratory.

PhD Candidate Joachim Sebastian Kjesbu at Department of Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) in Trondheim for providing informative counsel for the paper writing.

Head Engineer André Tranvåg at the Department of Ocean Operations and Civil Engineering, for providing the necessary materials needed for the experiments.

Finally, I would like to express my gratitude to my Parents Tomm Arne and Merete, my siblings Silje, and Joakim for their continuous support and encouragement during this tough period.

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Chapter 1

Introduction

The fish industry has played a vital part in Norway's economy over the years with close to 9,5% of Norway's total export in 2018, with Salmon as the main source of income [9]. Over the years Norway's government has been adding stricter regulations regarding the fish industry because of the constant danger with L. *monocytogenes* contamination during production. This created a need for the fish industry to look for new ways to improve their procedures along the entire chain of production from the breeding, until they reach their customers. This has led to an extra cost on daily basis to clean the entire factory thoroughly. The cleaning is done by manual labor, often outsourced to sanitizing companies specialized in this field. These workers clean the factory during the night with strong chemicals and water. This results in a hazardous cleaning environment for the workers when the factory gets covered in a mist of water and toxic fumes from the chemicals they use. One of the suggested solutions to reduce the cleaning cost while also improving the "Health, Environment and Safety" for the companies is to replace human labor with industrial robots for cleaning. There is an on-going PhD researching how to utilize cleaning robots to clean a fish stunner with the use of spray tools.

In this project an investigation on how material properties and cleaning tools can impact the cleaning results will be done. In addition, tests will be made by introducing several designs of cleaning brushes as a new tool into a cleaning process for the fish factory and tested on different material types. A test procedure will be developed for the purpose of testing the cleaning quality with different types of brushes with the support of the bio engineer department. The test procedure will look into how the surface properties impact the formation of biofilm and how effective cleaning tools are to remove the biofilm. Once the cleaning tests have been done, a series of bacteria samples will be taken from each cleaning tool to investigate the dangers these tools carry when they get contaminated by the bacteria. These experiments will then be evaluated and results displayed as % of efficiency for both material type and cleaning tool performance. The contamination level on the tools will be discussed to find the potential dangers it possess and provide suggestions on what should be taken into consideration when creating cleaning procedures to handle the problem in the industry.

1.1 Problem

The Norwegian government has added over the years stricter regulations regarding the fish production due to the dangers with L. *monocytogenes* contamination. This has made the production cost for the fish increase and thus reduced the potential profit from fish sales.

The bacteria contaminates and spreads throughout the factories by cross-contamination from fish to equipment and from equipment to uncontaminated fish. The percentage of the factory contaminated with Listeria will continue to increase during production hours. Therefore, it is important to clean the factory thoroughly so that the total percentage of L. *monocytogenes* contaminated surfaces is as close to 0% as possible before production starts. This is not an easy task since this bacteria can survive extreme environmental conditions, which makes it difficult to eliminate [5]. The bacteria ability to survive inside a biofilm makes it even more difficult to kill with chemicals since the biofilm grants it extra resistance against the cleaning agents. To deal with this the fish industries hire's professional sanitizing companies to thoroughly clean the factory after the production has stopped. This is a costly procedure that takes a lot of time, effort and requires usage of multiple types of strong chemicals. The workers will during this time be exposed to a hazardous environment with toxic fumes which will have a bad impact on their health over time.

A proposed solution to this problem is the usage of advanced robots to clean most of the factory. This has led to research and development of a robot cleaning system for the fish stunner. This system will require further research on cleaning procedures and what impact it will have to implement cleaning brushes as a new possible contamination source into the cleaning processes.

1.2 Motivation

The motivation for choosing this project emerged due to the importance of the health aspect, which main interest is to offer a more reliable method of removing biofilm. This will reduce the necessary amount of chemicals needed to remove the bacteria from the surface and thus improve the work environment for the workers. The cleaning environment consists of a very moist air with toxic fumes from the detergents they use. This hazardous environment will have an effect on the workers overall health when exposed to it on daily basis. Over time, it can cause health problems which can result in a business loss of millions of NOK for the companies. By utilizing cleaning brushes to make the bacteria more vulnerable to chemical cleaning during the decontamination will reduce the necessary amount of chemicals needed and therefore improve the work conditions. It will also increase the cleaning quality and thus reduce the chances for a L. *monocytogenes* outbreak among the population.

1.3 Scope

After evaluating the current cleaning procedure used in a fish process factory, it is evident that there is a need for improvement on both the health issues and cleaning quality issues. The scope of this project is to evaluate tools for cleaning biofilm and how surface properties impacts biofilm formation. To find the best cleaning test procedure an investigation on cleaning technology, biofilm, L. *monocytogenes*, P. *fluorescens*, S. *aureus*, and the current state of the food factory is necessary.

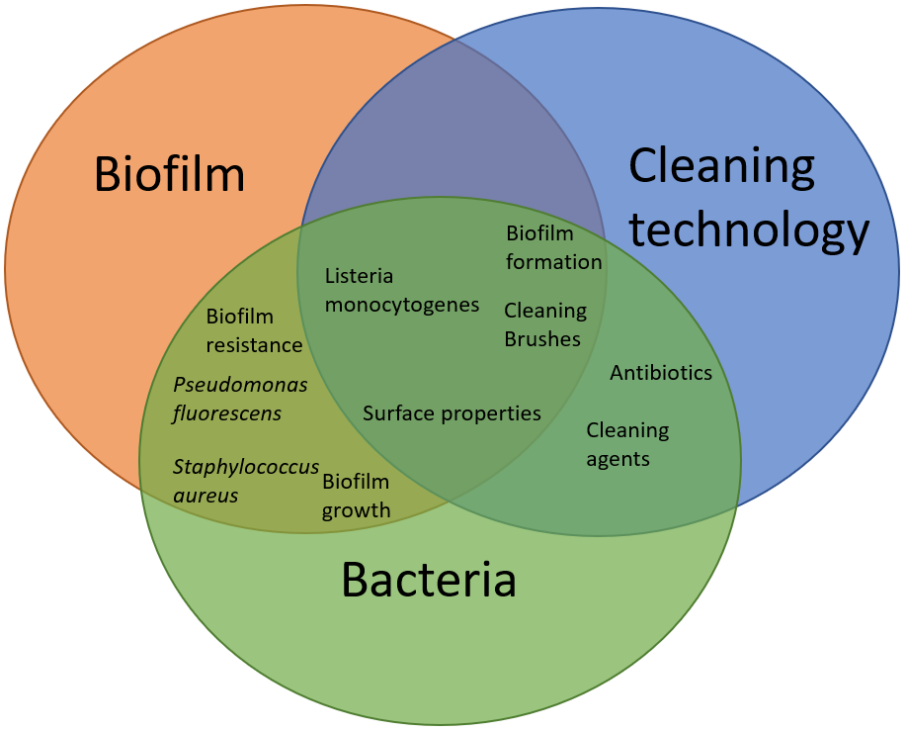

Figure 1.1: The scope of the project is in the *Biofilm*, *Cleaning technology* and *Bacteria*. Biofilm covers different bacteria abilities to create biofilm, how biofilm grows on surfaces and what kind of resistances they have etc. Bacteria scope covers different types of bacteria, their abilities to survive tough environments and the dangers with them, the last scope is the cleaning technology which covers cleaning tools, antibiotics, cleaning agents etc.

Research questions:

1) Are cleaning brushes an effective tool against biofilm?

2) What impact will the cleaning tools have as a possible new contamination source in the fish factories?

3) How can material properties affect the biofilm formation?

1.4 Objectives

The objectives for this thesis is to develop a test procedure that can evaluate cleaning brushes as a new tool to remove biofilm and further investigate the material properties impact on biofilm formation. To finally investigate the dangers cleaning tools possess when they absorb bacteria while cleaning.

A list was made detailing the objectives of the thesis:
1. To develop a test procedure for cleaning experiments in the bio lab.

2. Evaluate the cleaning brushes as a new contamination source and provide suggestions on counter-measures.

3. Investigate the effect material properties have on biofilm formation.

Chapter 2

Theory

2.1 Literature review

In order to determine if this thesis will be a contribution for the knowledge base concerning this field of topic, a throughout literature search is necessary to find out what the current state of the art is. This knowledge is found on CIRP, BIBSYS and NCBI by searching for relevant keywords such as End-Effector, Cleaning, Listeria, Fish factory, Robots, Surface, Nanotechnology, Food industry, Sanitizer, Contamination, Cleaning Robot, Bacteria, biofilm, pseudomonas fluorescens, stafylococcus aureus and MRSA and combining these keywords in different ways to find relevant papers for the project. This thesis is an investigation on what consequences it will have to introduce cleaning brushes as a potential contamination source in the fish factory to remove biofilm from the surface of different material types. A thoroughly research on biofilm, bacteria species and the current state of the fish factory will be necessary to understand the background and the current situation in the fish factories. Especially it is important to research on what *Listeria monocytogenes*, *Staphylococcus aureus* and *Pseudomonas fluorescens* is, what potential threats they provide towards the food industry and how difficult it is to remove these threats. The book *Fundamentals of biofilm research. 2nd ed.* by *Lewandowski, Z. and Beyenal, H* was used along with *Store Norske Leksikon* (SNL.no), *Norsk Helseinformatikk* (NHI.no), *National Center for Biotechnology Information* (NCBI) and *Procedia CIRP* papers to gain basic knowledge.

The first paper found was "Monitoring of contamination sources of L. *monocytogenes* in a poultry slaughterhouse" by Daniela F. Schäfer which covers how the bacteria occurs and spreads rapidly within the factory, points out how and why the cutting machine is the main contamination source for the food production and how ineffective operational cleaning with hot water is. In this paper the Author mentions how this bacteria can resist industrial sanitizers, colonize a large area of the factory and form biofilm to make it even more resistant to cleaning agents. They mention how the design of equipment impacts the growth of bacteria with grooves, hinders, cracks and loose welds which will hinder the cleaners and therefore reduce the cleaning effectiveness. Other sources about this topic were either case studies or research papers which obtained similar results. The research continued by reading *Fundamentals of biofilm research. 2nd ed.* by *Lewandowski, Z. and Beyenal, H* to get a better understanding on how to approach biofilm research, what difficulties this field faces, what biofilm is and why this is a problem for all industries involving microorganisms.

2.2 Fundamentals of Biofilm research

In this book the authors introduce the fundamentals of biofilm research to grant the readers the understanding on how this field has evolved over the years. They mention that the essential components of the research programs are tools and reliable experiment procedures which are developed over time as they gain more knowledge on the topics. The way the bio engineers approach the research is with the use of conceptual, computational, physical and virtual tools. [1, preface] With conceptual tools it is mentioned as an example a conceptual model of biofilms, while physical tools are for example biofilm reactors and microsensors, computational tools are programs to compute/simulate certain states and virtual tools are software to create 3D images using as a source the images taken from laser confocal microscope which creates a 2D-pictures. The authors then mention that science is not driven by the tools, but by the hypotheses. What they mean is that their understanding of the field they research helps to develop tools. An example of this is how microbiology was researched and made good progress worldwide, which allowed the microscope to eventually be invented to assist further research and understanding [1, preface].

Further they talk about how reproducing the same results achieved by other scientists is not easy when trying to do what others did before you. The authors mention the reason for this is how certain factors play in which is difficult to control. One of the factors are the physiology state of microorganisms. A way to deal with this is to standardize the tools and experimental protocols to increase the reproducibility of the results. This includes temperature control, humidity, tool types, quantity, time usage etc [1, preface]. For a biofilm to form it needs planktonic bacteria (free floating) which attaches to the surface. These microorganisms forms micro colonies and starts to excrete*extracellular polymeric substances* (EPS) which for example is slime. At this stage it will need nutrients (proteins) to continue to grow and form a larger biofilm [1,pp. 4-5, pp. 22-28].

The activity of a biofilm is defined as a rate of nutrient utilization per unit of surface area covered (NUR) with the biofilm. The activity can be computed by measurements taken at a macroscale (large surface measurement) or microscale (microscopic scale). The author mentions that if microscale measurements are to be done it is important to find microscale sensors which are available to the substance selected, or else a macroscale measurement will be better. There are also possibilities to use mathematical models for biofilm activity, but the problem with these models is that they assume the microbial activity is uniform which is not the case in real life and thus makes it inaccurate [1, pp.7-9].

The biofilms have different structures which grants them different attributes. The structure is defined on how the biofilm controls the nutrient delivery to every layer in the biofilm [1, pp.7-9]. In biofilm research there are two types of surfaces which are interesting for this particular thesis. The first is the surface of the object the biofilm attaches to, and the second is the surface of the microorganism called microbial cell surface. The surface of the object is called substratum surface and it is what defines the surface properties of the material. There is a lot of research on this field to find the relation between surface properties and biofilm growth, but these results are still unclear [1, pp.10-14].

The process of biofilm formation onto a surface consist of multiple steps. The first step is the absorption state, when gas or liquid of macromolecules such as proteins sticks to the surface of an object and creates a film (a thin layer over the surface). This film can alter the materials' surface properties suppress or enhance the release of toxic metal ions from the surface and act as a nutrient for microorganisms to form biofilms and more. Some materials can resist being covered by a film of water which is known as hydrophobic materials [1, pp.12-14].

There are several tool types available to scan and detect microorganisms on the surfaces and the correct tool selection depends on what the user wants to investigate some of the types used in the field of bio engineering. The first type is the Reflected light microscopy, used to identify microorganism colonizing the surfaces of stainless steel which has been submerged in water [1, pp.15]. Another type is the Scanning electron microscopy (SEM) which scans the sample with a beam of energic electrons that backscatter and form an image of the surface. This is a handy tool for investigating mineral deposits and it is less useful to analyze microorganisms. The reasons are that the machine creates a vacuum which dehydrates the specimen, changing the microorganism during the scan [1, pp.15-16]. Another machine is the Atomic Force Microscopy (AFM) which is a a machine that works as a record player. A cantlever taps into the specimen and detects any changes of position and uses this to create a topography of the sample. This tool works well to create images on microorganisms in the initial state of biofilm formation and it creates high definition pictures compared to the SEM. The most versatile machine for surface analysis is the Time-of-flight Secondary-Ion mass spectroscopy (SIMS). This machine bombards the surface with energetic particles which causes ionized molecules to be emitted from the substances of the surface. These emitted particles are detected and analysed by the machine and it creates high resolution images which allows the study of individual microorganisms [1, pp.19].

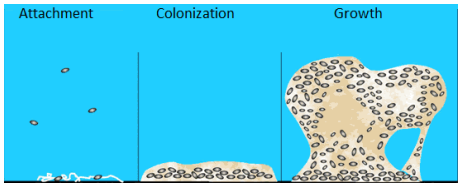

Figure 2.1: Biofilm in three phases, first is attachment where cells sticks to the surface, then colonization phase where cells forms micro colonies, then the growth stage starts where the biofilm is formed and it starts to mature. [1, pp.26].

The formation of biofilm is characterized by 3 phases: the attachment, colonization and growth phase. As mentioned earlier biofilm starts with small planktonic cells attaching to the surface of the material, these cells forms micro colonies. Then the biofilm is formed, and starts to grow. After a while the biofilm has matured enough and it has started to provide a protection layer, making the biofilm more immune. The last stage is when the biofilm reaches critical mass and starts to disperse planktonic bacteria to colonize new areas. It is the material properties which defines how well cells stick to the surface [1, pp.26-27]. When single cells join colonies, they will gain benefits, for example a protective barrier which increases its resistance. This is a problem for the fish industry where L. *monocytogenes* receives protection of biofilm formed by other bacterial strains and thus increases its immunity to cleaning agents.

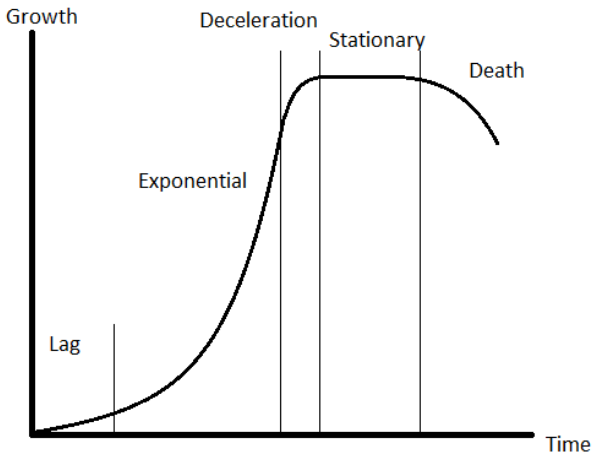

Figure 2.2: Growth curve adapted from [1, pp.23]

The growth curve describes how much microorganism grows over time. There are 5 stages of a growth curve. The first is the lag phase which is a delay in time where microorganisms are adapting to the new environment. The second is the exponential growth phase where the microorganisms are quickly forming. Then they reach the third which is the deceleration phase caused by depletion of nutrients. Once the nutrients reach zero the stationary phase starts, during this period there is no growth until the microorganisms start to decay and we enter the final and fifth stage, the death [1, pp.22-23].

Lastly the author talks about the strategy of biofilm research. The research is based on conceptual models of biofilm processes which is tested continually if the results are consistent. If they are not consistent, the work is converted into further research to detect the reason. If there is an inconsistency between the conceptual model and experimental results then the model will be altered [1, pp.50].

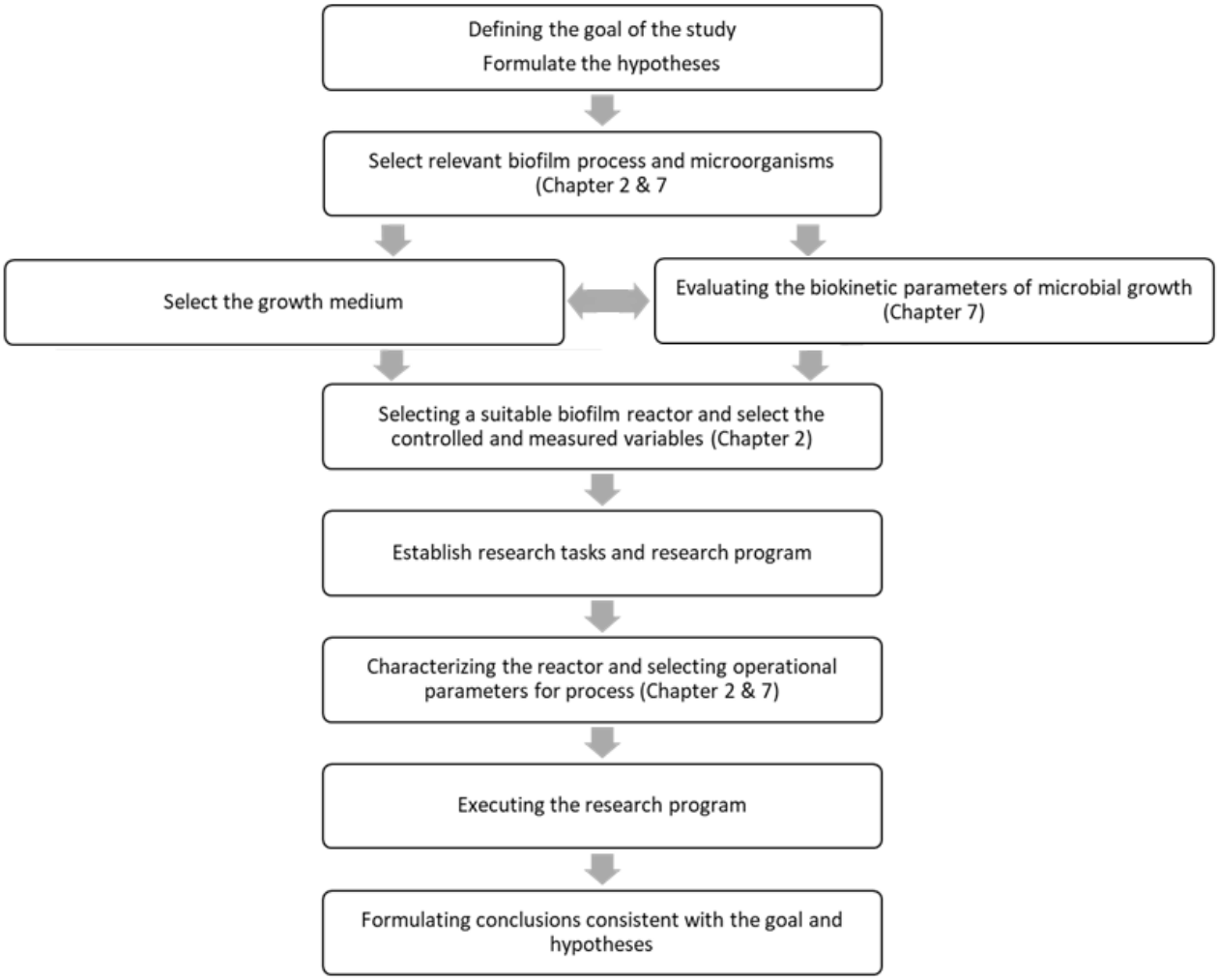

Figure 2.3: The standard sequence of steps for a bio research [1, pp.51].
The goal of the study is defined at the beginning of the research and throughout it is important to check if it is still on track towards the goal.

2.3 Bacterial Species

2.3.1 *Pseudomonas fluorescens*

P. *fluorescence* is a commensal soil bacteria commonly found in the nature. It is a gram-negative bacteria with the ability to produce fluorescent pigment (pyocyanin) which is a yellow colour and is where the name fluorescent derive from [20],[21],[22]. This bacteria has motile polar flagella, hollow tubes hanging out of the main body [22]. These flagella allowing the bacteria to move quickly in water by propelling itself forward to more than 30 um/s [22]. If left alone, the bacteria will end up as a stranded colony on dry surfaces, but it has evolved into concentric growth which will grant it expanding growth rates when cooperating with other types of bacteria, usually due to it's ability to make biofilm [20],[22].

Pathogenecy

P. *fluorescence* can enter the bloodstream and cause septicemia, blood poisoning or infection. Symptoms of this bacteria infections are fever, chills, confusion, nausea, vomiting and rapid heart rate [20],[21],[24].

2.3.2 *Listeria monocytogenes*

L. *monocytogenes* is a pathogenic bacteria which can cause diseases to humans. This bacteria is one of the most dangerous foodborne zoonoses. It is a gram-positive bacteria [3]. It is motile and can therefore move in water by propelling itself forward using the flagella. This bacteria can survive extreme environmental conditions, which makes it difficult to eliminate [5]. Only a heat treatment or strong chemicals can kill the bacteria colony from the surface, but even this is challenging since the bacteria will be protected in a biofilm which makes it extra resistant for cleaning. The bacteria can be found in most food, ranging from milk, cheese, sauces, fish and meat and is therefore a common problem among all food industries [5].

Pathogenecy

This bacteria will cause the disease Listeriosis which has a death rate of 30%. Listeriosis have many symptoms, the most common are headache, fever, body ache, sleepiness and neurologic symptoms. In the high risk group we find the elderly, pregnant women, infants and people with low immune system [3],[5],[10].

2.3.3 *Staphylococcus aureus*

S. *aureus* is a pathogenic bacteria which causes diseases to humans [25],[26],[28]. It is commonly found in hospitals, nature, household dust and human flora. Most humans carry it on their skin, intestines, throat and nose [25],[26],[27]. It is a gram-positive bacteria which grows in clusters and are quite robust. They can live with and without oxygen, can grow at temperatures from 10 to 45°C and live in dry environments[28]. Their ability to survive in biofilm constitutes a problem in hospital environments, making it hard to remove from sterile surfaces.

Pathogenecy

S. *aureus* is one of the most common infections a patient can acquire at the hospital after surgery. The illness can vary from different skin diseases such as abscesses to more serious infections such as pneumonia which is an inflammatory condition on the lungs [25],[26],[27],[28].
This bacteria is also common for eye infections and some strains have developed resistance against antibiotics, *Methicillin Resistant Staphylococcus aureus* (MRSA).

2.4 Monitoring of contamination sources of *Listeria monocytogenes* in a poultry slaughterhouse

Keywords: Sanitizer; Chicken; Contamination sources; Food industry; Listeria;

In the first research paper written by "Daniela F. Schäfer, Juliana Steffens, Juliana Barbosa, Jamile Zeni, Natalia Paroul, Eunice Valduga, Alexander Junges, Geciane T. Backes, Rogério L. Cansian" now called the Authors, evaluates the occurrence of L. *monocytogenes* in a poultry slaughter plant and measures the cleaning effectiveness done by a sanitation company.

The data was collected during production from August to July where the Authors would take samples from both the chicken and the surface of the equipment to measure the percentage of L. *monocytogenes* from Pre-production, during production and post-production. The authors analyzed a total of 920 breast and 774 thigh samples, always at the beginning of the first week every month and detected L. *monocytogenes* on 8% of the breasts and 44-51% for the thighs[8].

The authors found a connection from the data collected that the L. *monocytogenes* occurred more frequent during winter (Winter climate is from May to August in Brazil) and during summer (December to February) had the least occurrence. The authors believe the reason is connected to the temperature. If that is the case, the author believes the lack of temperature control in the factory has an impact of the cleaning results. Further in the research paper, they present their data to show where the bacteria contaminates in the factory by following 80 samples throughout the whole process over the course of 3 days where the samples were taken after cleaning. The 80 chicken thighs/breasts they took samples on had 0% the bacteria before the chiller/cooler machine, and 5% was contaminated after. They then found the occurrence of the bacteria in 33,3% of the samples after the bleeding and plucking machine, and this amount increased to 50% at the cutting mill and up to 76% at the packing phase. The authors mention that these results are similar to what was found in earlier research by "Barbalho, Almeida, Almeida, and Hoper in 2005".

They continue describing how quickly the bacteria spreads during the day along the whole production cycle. The amount of contaminated chicken reached 5% during pre-production and throughout the work shifts these numbers reached as high as 47% which confirms that the contamination percentage increased immediately after production because of cross-contamination between chicken and equipment. The authors also mention that the occurrence of listeria is higher on clean surfaces, the reason for this is that other bacteria strains compete against L. *monocytogenes* when they colonize. That means the listeria bacteria, which is less competitive will thrive in areas with lower amount of competing bacteria species. The authors conclude that the high contamination levels found is caused by a combination of poor cleaning and cross-contamination in the production line after the chiller, especially at the cutting machine [8].

2.5 Assessing *Listeria monocytogenes* presence in Portuguese ready-to-eat meat processing industries based on hygienic and safety audit

Key-words: Food safety; L. *Listeria monocytogenes*; Ready-to-eat meat-based products; Food safety; management system; Processing hygiene indicators;

The aim in the second research paper written by A.R. Henriques, L. Telo da Gama, & M.J. Fraqueza, now called the authors, was to assess the presence of L. *monocytogenes* in Portuguese ready-to-eat meat process industries. The authors studied and analyzed the cleaning procedure of ten different factories [4].

Ready-to-eat meat product is food which comes in an edible form that doesn't need to be cooked such as sausages, salami and ham [4]. This is a problem since the bacteria can only be killed by cooking and not by freezing. The author mentions that earlier research from 2009 and 2012, shares the same understanding on how RTEM with long shelf-lives in refrigeration increases the chance of recontamination. The reason isthat cooked/heat treated meat can receive raw ingredients after it has been cooked and at high risk of being recontaminated. The authors started their research with a survey for all ten factories where they rated their procedures, covering their industrial typology, standard operating procedures, analytical control, personal hygiene program and food-processing technology. This checklist, also known as Good hygiene and manufacturing practices(GHMP) is based on *Codex Alimentarius Commission* and *General Food Law*. In this test it was possible to score a maximum of 82 points. Two companies out of ten scored between 40-49, two more scored in the range of 50-59, three othersscored between 60-69 and the last three scored between 70-79 [4]. The authors took food samples and surface tests on the equipment, where means and standard deviations where calculated and qualitative information was not considered for the analysis. For the factories with the worst score, it was detected a poor implementation of the preventive maintenance plan and no hygienic zoning of critical operation. The company also lacked routines for pathogen tests, raw-material testing and shelf-life determination. They detected some lack in personal hygiene for two of the companies which covers "usage of jewellery, miswearing hair protection, using uniform outside work area, etc" [4].

In their lab results, they found that ca 20% of the samples could be considered to be a potential risk of pathogens such as E. *coli*, L. *monocytogenes*, *Salmonella and Campylobacter*. For the L. *monocytogenes* it was detected on 25% of the samples, which came from cross-contamination from equipment and was found in most of the high GHMP score companies. Another 10% of the total surface on the conveyor belts, benches and cutting boards contained the same bacteria. The author mentions earlier research achieved similar results where they detected the bacteria on 8,3% of the surfaces after sanitation. They mention not only poor cleaning procedures are at fault here, but poor design of equipment. The authors conclude that the result of contamination is caused by inadequate hygiene and improper manufacturing practices. They propose a solution to reduce this problem, such as improvements on the hygienic procedures, equipment design and staffs attitude towards hygiene [4].

2.6 Ready-to-eat Meat Products As a Source of *Listeria monocytogenes*

Keywords: Ready-to-eat meat products; *Listeria monocytogenes*; contamination; food chain;

The authors, Monika Kurpas, Kinga Wieczorek and Jacek Osek wrote a journal in 2018 about L. *monocytogenes* in the ready-to-eat meat products. In this journal they firstly introduce what the bacteria is, and what diseases it causes, their numbers of the occurrence of the disease each year, and the death rate is similar to what Norsk Helseinformatikk and Store Norske leksikon mention [6],[31]. They explain how the bacteria is able to survive in extreme conditions, are resistant to disinfectants, UV light, desiccation, and is therefore difficult to completely get rid of.

They provide information on how to reduce the percentage of L. *monocytogenes* in the end-product by reducing or eliminating the bacteria from certain steps in the production line seen above. The problem is that the human factor plays a large role, especially since employees in the food industry tend to not follow hygienic rules and procedures due to lack of understanding the core problem. There are a few types of sanitizers which can be used within the food environment, such as acidic-anionic, halogen, hydrogen peroxide, quaternary ammonium and chlorine. The cleaning procedures require a full washing and rinsing of the surface to remove all food waste before decontamination. Biofilms can form on all surfaces and is most likely to be found on clean surfaces where there's less competing bacteria. Listeria can form biofilms with other microorganisms which can make the biofilms structure even stronger and more resistant to the majority of cleaning and disinfection agents. The author then talks about the first step in the food production, which is the farm house. From the total of 1962 samples analyzed, only 2,4% contained the bacteria in the carcass, and 9,3% in the intestines, but the meat that went through the grinder to become minced meat contained as high as 50,2%. The authors say this is caused by cross-contamination throughout the production, mostly from the cutting and mincing machine [5].

The next step is the storage of meat and RTE food, which is 15 days for red meat at 7 degrees Celsius and 3 days for poultry meat at 4 degrees Celsius. The authors mention that earlier studies found out that the bacteria can be detected after 16 days in air packing and 23 days in vacuum packing at 5 degrees Celsius. For 25 degrees Celsius it took 6 days in air packing and 18 days for vacuum packing. These results tell us that the higher temperature and air packing decreases the level of Listeria faster than low temperature and vacuum packing. For an RTE (ready to eat) production plant there are multiple exposed areas which can be a source of contamination, these areas are the raw meat warehouse, cutting, packing and final product stockhouse. In each of these areas there is a risk of cross-contamination. For example, can the bacteria spread from water drains on the floor and be carried by the boots of a worker to other parts of the factory, but it is the cutting machine which is the worst source of contamination. Reason for this is that the machines are difficult to clean properly. The authors mention that to maintain a high hygiene standard and sterility in the production facilities an elaboration of

effective cleaning methods is needed and determines which cleaning procedures is the best to use for different cases [5].

The authors continue to write about the next step which is the retail. They mention that a study done in the USA shows that approximately 83% of all listeriosis cases was caused by lunch meat (cold meat) which was cut at the retailers. This means there are a high risk of cross-contamination when retailers use slicing machines to process the meat received from the factory. They detected a higher concentration of bacteria in lunch meat that was sliced separately for each individual customer and it was documented a higher percentage of the bacteria on the meat at the end of the day. It was also discovered that cross-contamination from wood material to clean meat was about 73% and from plastic to clean meat was close to 90%, which means the choice of material used on the equipment matters. The authors conclude that listeriosis is a very dangerous zoonosis, despite its low occurrence, it remains a health concern due to the mortality rate and that RTE food is one of the most effective way to spread this disease among the population. They mention how important it is to pay attention to the entire production chain, standardize food law, procedures for hygiene and sanitation in each country to fight it more effective [5].

2.7 Spectrophotometry

Spectrophotometry is one of the most used methods for quantitative analysis. It is used to measure the amount of light a substance absorbs or transmits.There are two types, the UV-visible spectrophotometer and IR spectrophotometer [29]. The UV-visible utilize ultra violet light while the IR uses light of the infrared spectre. The one used for this experiment is the photometer. The photometer passes light through the sample stored inside a cuvette (Figure 3.32 and Figure 2.4) where the photometer detects the amount of light which passes through it [11].

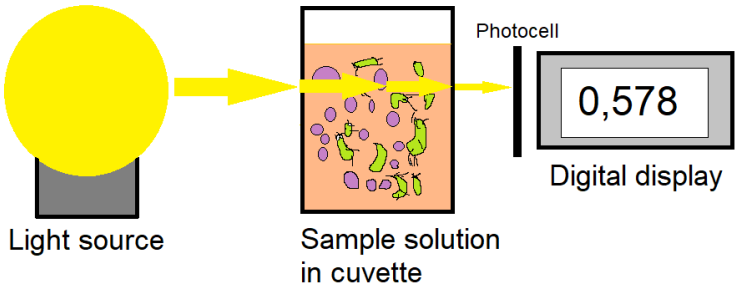

Figure 2.4: A simplified representation of spectrophotometry displaying how a light source is being absorbed by a substance before entering the photocell that register the amount of photons that passes it. Adapted from the illustration by Heesung Shim [30].

The density of bacteria in a sample can be measured by having a known growth medium set as zero which is premeasured on how much light will be absorbed. This substance will act as a background, then we add the bacteria sample into the growth medium for it to be measured by amount of light that is absorbed. The more light absorbed, the more bacteria is inside the sample.

2.8 Fluorescence

Fluorescence is a form of luminescence, a phenomen where a substance emits light. It occurs when the electrons in a fluorophore receives energy in which results in an electronically excited state [12],[13]. The fluorophore will then go through internal conversion which means that the electrons will go down to more stable energy. In this phase all excess energy is released as heat. Energy is emitted in the form of photons while the electron looses further energy and returns back to ground state (Figure 2.5) [13]. Emitted light from fluorescence contains less energy than what was recieved which is why the wavelength is longer which can be seen on (Figure 2.6). Adding a fluorescence dye to the bacterial DNA would give the opportunity to measure bacteria on a surface. SYBR GREEN is a typical dye that binds to double stranded DNA, without impacting the bacteria.

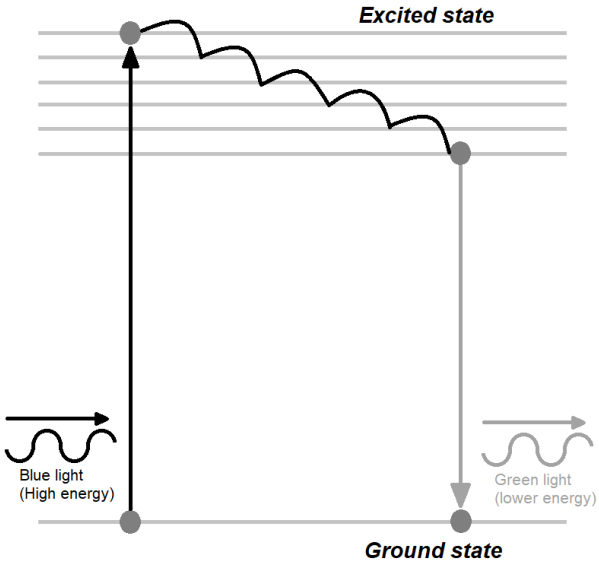

Figure 2.5: A simplified representation of Jablonski diagram showing how energy raises to an excited state, dropping to a stable state before ascending down to ground state releasing energy. Adapted from [13].

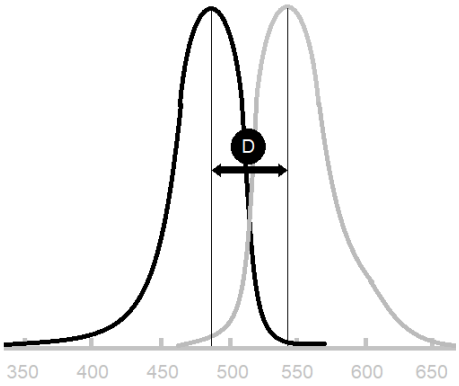

Figure 2.6: A simplified representation adapted from [12] showing how wavelength the energy emitted from the electrons during excited state (black) looses energy and thus getting a longer wavelength (less energy).

2.9 Material

2.9.1 Stainless steel - 304L

The corrosion resistant material used for this project has properties similar to EN 10088-2:2014.This steel type is a widely used stainless steel type for general purpose, including steel in contact with food in factories [18]. It is usually used in sinks, saucepans, cutlery, sanitary ware, tubing, brewery, food and pharmaceutical production equipment. It has excellent corrosion resistant properties to handle thought environments. But this material can fall victim to crevice corrosion caused by environments with chlorides. Crevice corrosion is when the protective layer on the surface of stainless steel are being depleted of oxygen from environmental factors like higher density of chloride, temperature and lower pH values. The oxygen amount will reduce and enriched chloride will start to break down the protective film on the surface, exposing the stainless steel [18].
This material is also weak against stress corrosion cracking at wet environment with temperatures over 60°C. Stress corrosion cracking is when materials break from a combined effect of environment, stress and material properties which together weakens the material. Stainless steel is easy to clean with a low surface roughness [18].

2.9.2 Aluminium alloy - 6082

The Aluminium alloy 6082 has excellent corrosion resistance suitable to handle tough environments[17]. There is a thin oxygen layer on the surface of aluminium which protects it like a barrier. This chemical compound of aluminium and oxygen is Aluminium oxide. When the surface is scratched the barrier gets damaged but will repair itself by reacting with oxygen again. The optimal pH values for Aluminium is between 4,5 and 9. The aluminium has a rougher surface finish than stainless steel which makes it more difficult to clean [17]. Aluminium alloy is typically used in Trusses, bridges, cranes, milk churns, beer barrels and highly stressed application [17].

2.9.3 Polyetylen High Density - PEHD 500

PEHD is a thermoplastic material which means it becomes moldable (soft) when warmed up and hard when cooled down. This material has a really low water absorption properties and easy to clean. It is commonly found in the food industry because of these properties [19]. It is usually found in 3D-printer filament, Chemical-resistant piping, Corrosion protection for steel pipelines, Fuel tanks for vehicles, plastic bottles and equipment for food industry.

Chapter 3

Methodology

The entire objective of introducing tools to fish factories is to measure how effective they are at removing biofilm and if they pose any threat by becoming a highway for the bacteria to spread diseases across the factories.
The goal with this experiment is to investigate two strategies against biofilm. The first is how material can affect the biofilm formation and the second is how effective cleaning tools are in removing biofilm. The test results will be analyzed and used to answer the research questions mentioned at chapter 2:

1) Are cleaning brushes an effective tool against biofilm?

2) What impact will the cleaning tools have as a possible new contamination source in the fish factories?

3) How can material properties affect the biofilm formation?

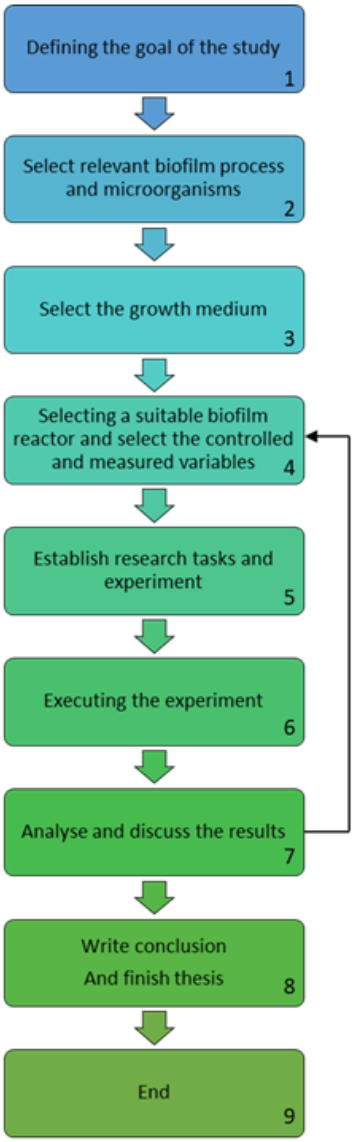

Figure 3.1: Contains the methodology adapted from *Fundamentals of Biofilm research* [1, pp.51].

Each step will be explained below

1) - Defining goals of the study, *research questions*, *objectives* etc.
2) - Choose bacteria relevant for fish factory.
3) - Select a nutrient broth for bacterial growth.
4) - Select a reactor for the experiments, and measurable variables like temperature and growth time.
5) - Carry out research on topics relevant for the defined goals of the study.
6) - Start the cleaning experiment.
7) Analyse and discuss the results.
If results are good, continue to 8), If not go back to 4) and change parameters.
8) Write final conclusion and finish the thesis.
9) Delivery

3.1 Preparation

3.1.1 Planning phase

The experiments requires a lot of time and preparation in advance to make sure the cleaning experiment will work. The preparation consist of a planning phase, building phase, bacteria growth phase and test run to optimize the experiment. The planning phase contains the theory behind what will be investigated along with the strategy on how the experiment will be done. The building phase goes into the details on the samples and tools which are built and bacteria growth phase covers the preparation of bacteria used for the experiment.

A plan for the experiment can be set with a strategy on how the questions asked in the main goal shall be answered with experiments and correct analysis.

1) Cleaning efficiency for cleaning brushes against biofilm.
- By analyzing the results, the efficiency for cleaning brushes based on tool type and cleaning method can be measured.

2) The impact surface properties have on biofilm formation.
- By observation during the experiment and analyzing of the results, the affect material properties have on biofilm can be measured.

3) Contamination risks from the cleaning devices.
- Investigate each cleaning tool by taking bacteria samples to detect the amount of bacteria.

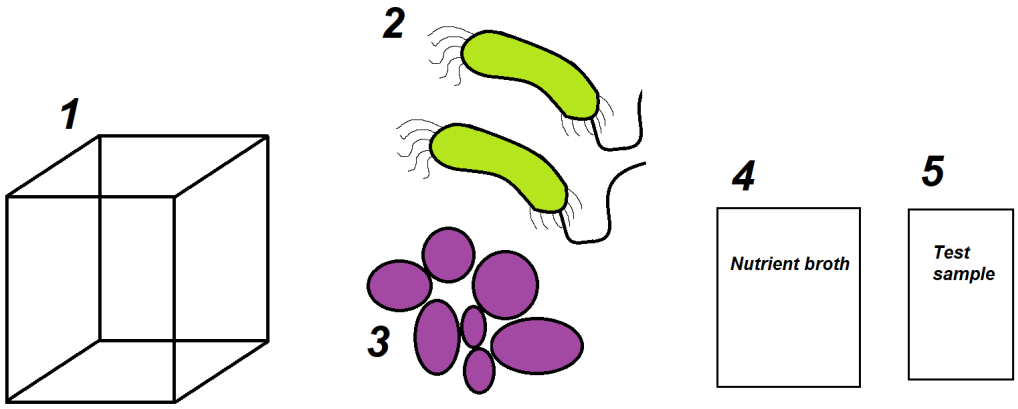

Figure 3.2: The necessary tools needed to prepare the experiment.
1) growth container, 2)*Pseudomonas fluorescens* and *Staphylococcus aureus*
4) Growth medium for bacteria's, 5) Test samples in stainless steel, Aluminium allyo and PEHD-500.

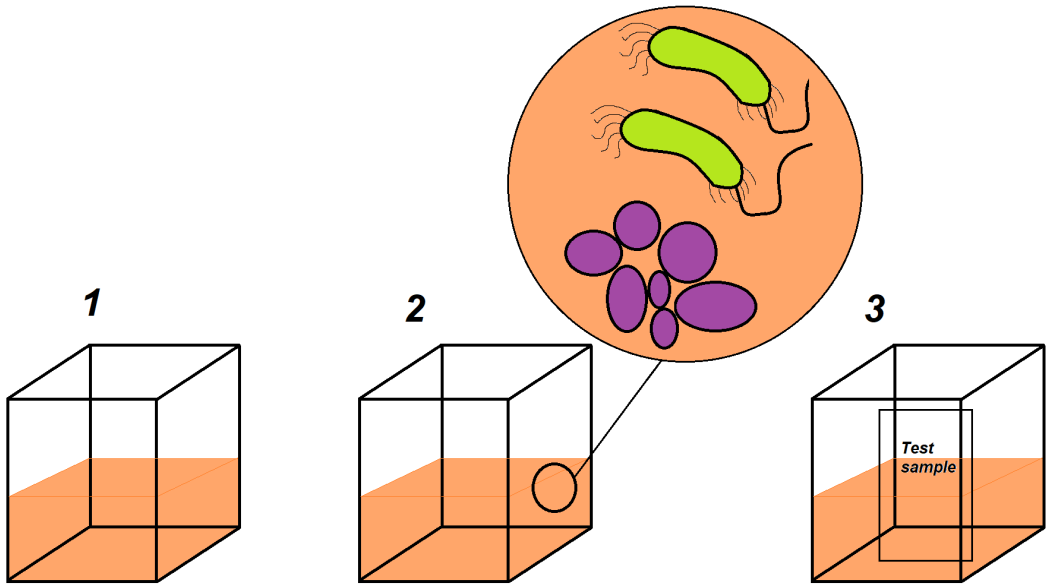

Figure 3.3: Bacteria growth procedure:
1) Fill growth container with growth medium.
2)Add bacteria mix
3) Put samples inside the growth container.

A growth container will be filled with test samples and a a mix of growth nutrients, *Pseudomonas fluorescens* and *Staphylococcus aureus* will be poured in. This mixture will be kept at 25°C for two weeks to allow it to form biofilm on the surfaces of each test samples.

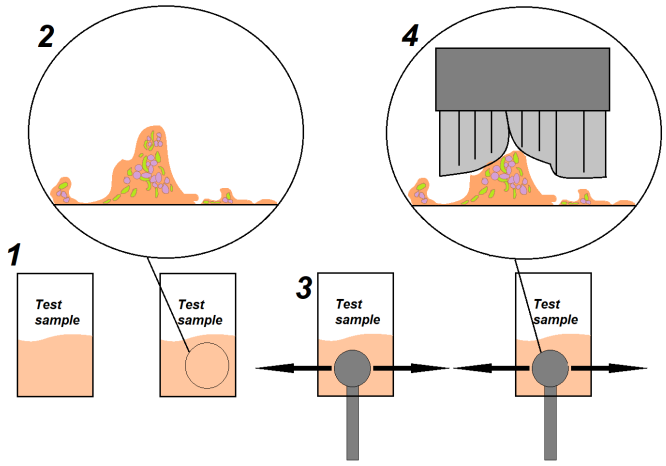

Figure 3.4: Shows how the test samples will be cleaned with tools.
By looking at 2) we can see biofilm formed on the surface, 3) shows the tool going back and forth cleaning and 4) is the cleaning tool removing this biofilm up close.

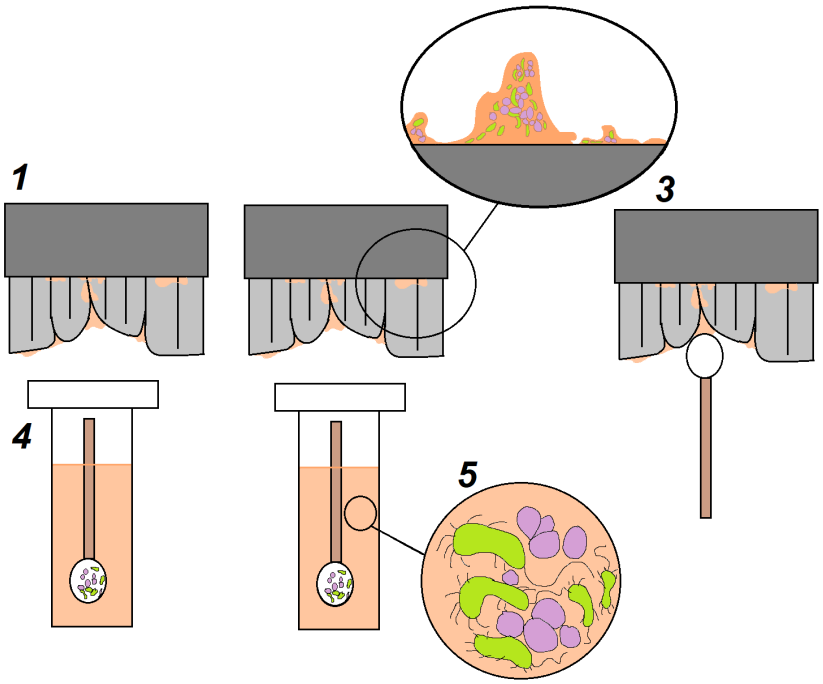

Figure 3.5: Bacteria's have formed on the cleaning brush after cleaning.
3) and 4) Displays how bacteria samples will be taken from the tools.

The cleaning test will start after two weeks of growth period for the biofilm formation. The experiment starts with taking pictures of each test sample before doing a water test. The water test will demonstrate how much biofilm water alone will remove, that way we can tell how much the cleaning brush removes and how much the water removes during the main test. The main test will be with cleaning tools and water (Figure 3.4). The samples will be cleaned for a duration of 10 seconds and the end result will be taken picture of with a Spectrophotometer Figure 3.19. The cleaning tools will be cleaned after each test and at the end of the day test samples will be taken from them at the core and the tip to check the bacteria density (Figure 3.5).

3.1.2 Building phase

The building phase consist of preparing test samples. The test samples used for the test will be of three different kind of materials seen below:
Stainless steel - EN 10088-2:2014
Aluminium alloy - 6082
Polyetylen High Density - PEHD 500

These materials have their pros and cons and in the experiment some of these will be uncovered and pointed out. All three material types can be found in a fish factory and is therefor relevant. To start off this building project a larger amount of pieces needs to be cut. A large sheet plate of stainless steel is cut into 150x100 and 100x100mm plates using a cutting device, the same procedure is done for the aluminium alloy. The PEHD 500 is a thicker block of plastic which is cut by a saw machine into 150x150mm pieces.

A bending machine is used to create 90 degree bends on some of the aluminium alloy pieces. Some plates will be welded together to get a tiny gap, these will consist of two 100x100mm plates which is tack welded together (Figure 3.6) to create some assemblies with a tiny gap between the plates. The same is done for stainless steel which have some 100x100mm plates tack welded together for a tiny gap between the plates, and two larger plates assembled together with an overlap using bolt and nut which can be seen on Figure 3.6. Since plastic is a soft material there was not necessary for special tools to get the job done and only a screw and a hand drill was needed to screw to plates together. There was made a total amount of 20 samples, 8 of stainless steel, 7 of aluminium and 5 of plastic which can be seen on Figure 3.7.

Figure 3.6: To the left: tack welding two aluminium plates together, to the right: Two stainless steel plates assembled with bolt and nut.

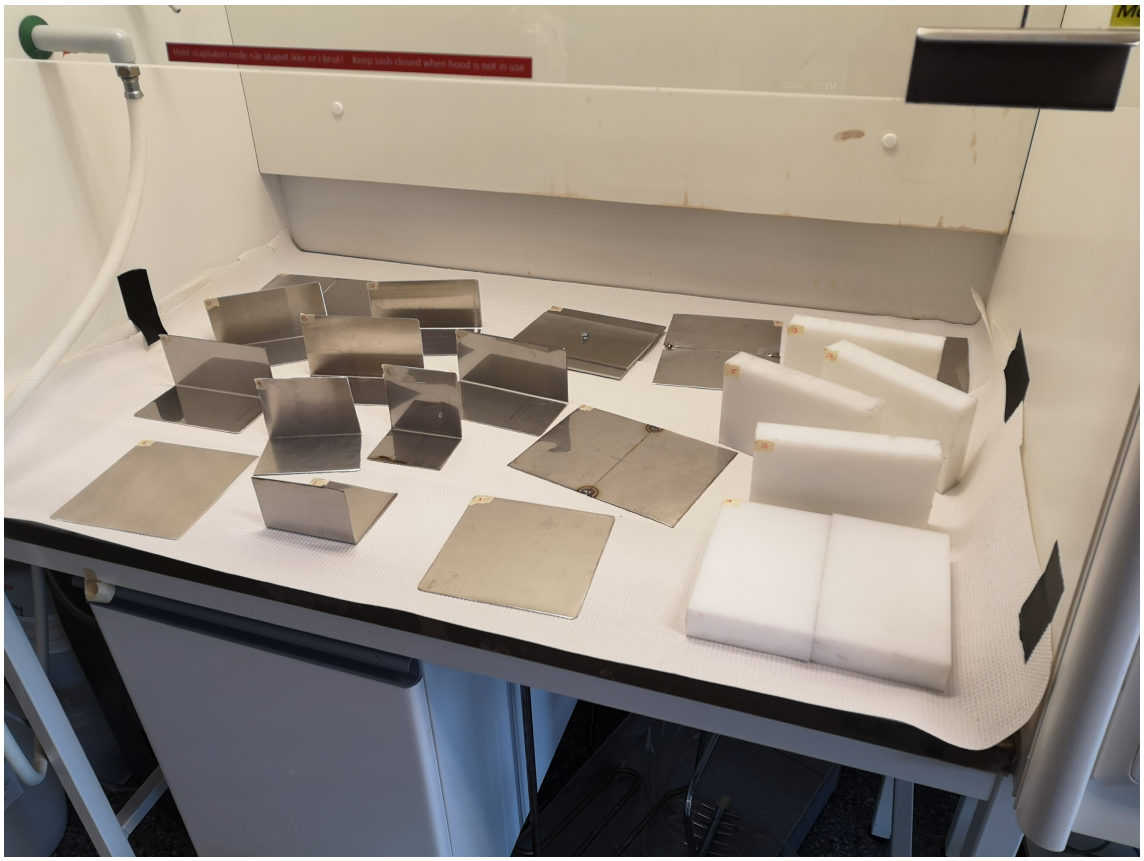

Figure 3.7: The 20 samples ready for experiments. Each of the samples is marked with a number so that the test results can be recorded knowing which test is from which sample.

3.1.3 Tool phase

Traditional cleaning tools with different designs and material hardness. These tools are common in most kitchens and will be tested for both performance during cleaning and contamination level after cleaning. It is important to know if certain design on cleaning tools can help aid bacteria growth while also checking the overall performance. A cleaning tool introduced to the fishing industry needs to be able to do a good job while minimizing risk of cross-contamination, which means it should be easy to decontaminate after use without the risk of bacteria surviving and colonizing the tool and nearby surfaces. Most of the tools did not require any modifications except those attached to a drill. To make these a 3D printed part were made to attached the cleaning tool to the drill which can be seen at Figure 3.9.

Figure 3.8: 1. Hard brush, 2. Medium brush and 3. Soft brush

Figure 3.9: 1. Pipebrush, 2. Rotational brush, 3. Rotational sponge

3.1.4 Bacteria growth phase

Bacteria used for testing were prepared before hand and was a mix of P. *fluorescens* and S. *aureus* which can be seen in a jar on Figure 3.10. The reason P. *fluorescens* and S. *aureus* is used and not L.*monocytogenes* is because of how dangerous this bacteria is to work with, but also since this bactria often joins the bacteria colony with other bacteria like P. *fluorescens*, makes the test still valid. The bacteria will require nutrient broth (Figure 3.11) to be able to grow and develop biofilm over night which we can clean the day after for the test run. The growth nutrient's ingredients can be found in Appendices on page II.

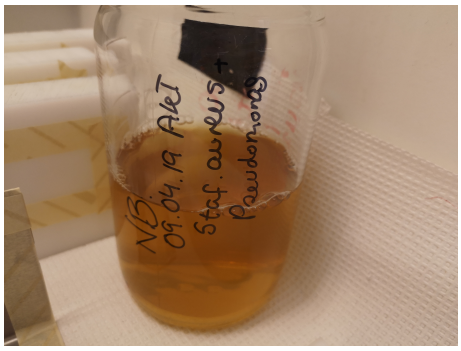

Figure 3.10: Container filled with *P. fluorescens* and *S. aureus*

Figure 3.11: Brewing nutrient broth as a growth medium for the bacteria.

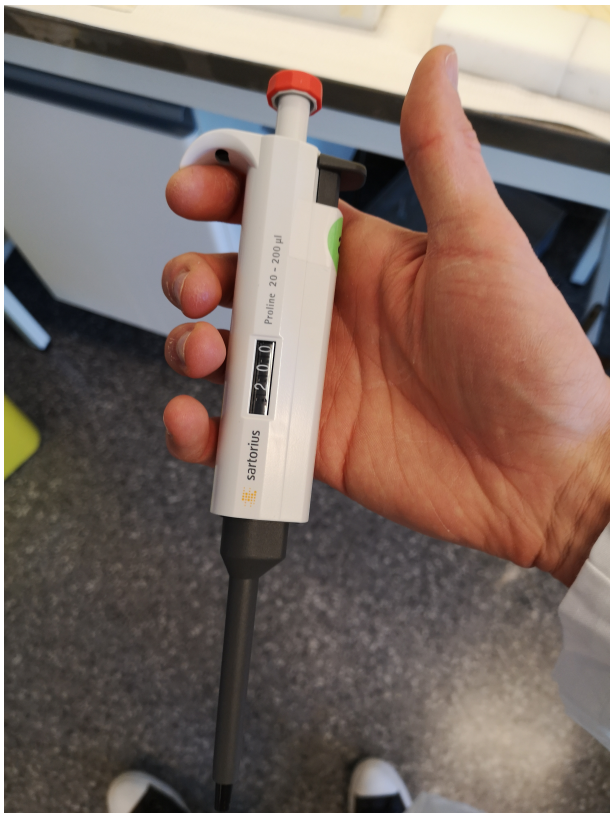

Figure 3.12: A pipette which applies bacteria mix onto the surface.

A pipette is used to extract the bacteria mix and eject it onto the test samples surface as seen on Figure 3.13. This device can be adjusted to eject between 20-200 micro liters, for the test it was adjusted to release 100 micro liter at a time.

3.1.5 Test run

The purpose of the test run is to test if our planned approach will work, at this time we considered to do many small test which was did not require lots of preparation and time. Bacteria was extracted from the container with a pipette and injected onto the surface of the sample. Once every sample has received 500 micro liters onto the surface the samples was brought to the incubator where it would stay over night in 25°C. The day after the cleaning test started where the sample was put under a water test, but because the bacteria had not formed much biofilm (Figure 3.14) all the bacteria washed off. The quick test run was a failiure and only way to get a thoroughly test is to follow the main plan which requires two weeks of preparation (Figure 3.3).

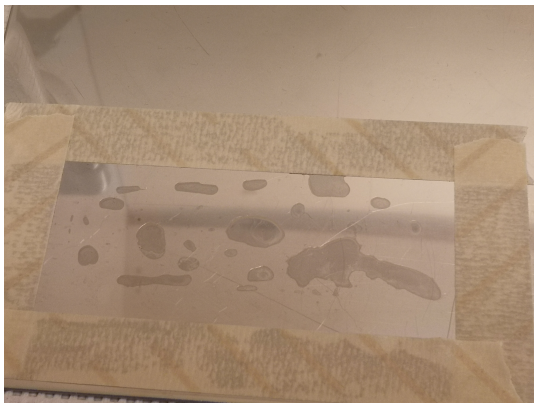

Figure 3.13: Bacteria forming biofilm onto the surface over night.

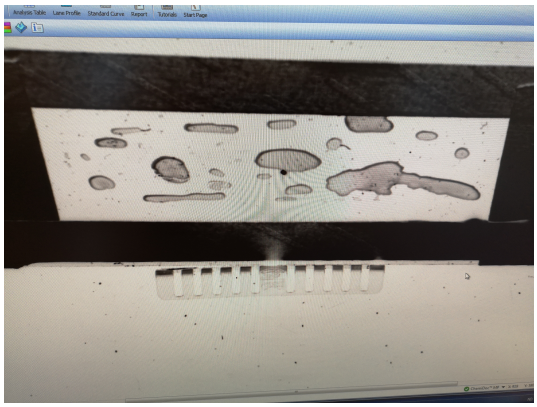

Figure 3.14: ChemiDoc^{TM} Imaging System picture of the biofilm that has formed with the settings for *black and white.*

3.2 Experiments

3.2.1 Biofilm growth phase

The knowledge gained from the test run taught us that the bacteria will need some time to form biofilm which can grow onto the surfaces. We will follow the plan from Figure 3.3 where a container will be filled with growth nutrients and the bacteria mix which can be seen on Figure 3.15 and Figure 3.16. The test samples will be in this bath for two weeks in 25 °Celsius. During this time the bacteria will form biofilm across the surfaces and colonize the test samples.

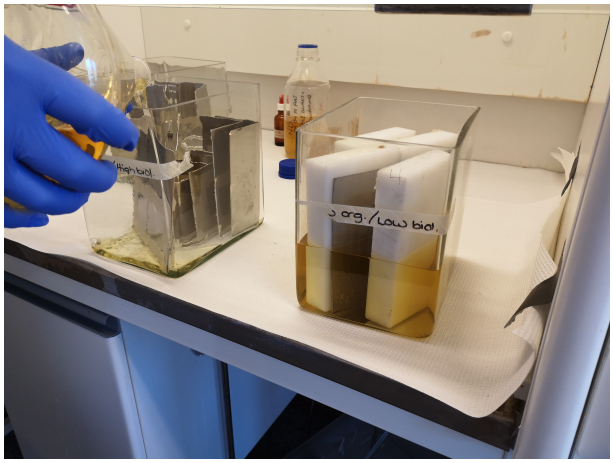

Figure 3.15: Filling up the growth containers with *P. fluorescens* and *S. aureus* and Nutrient broth (Figure 3.11)

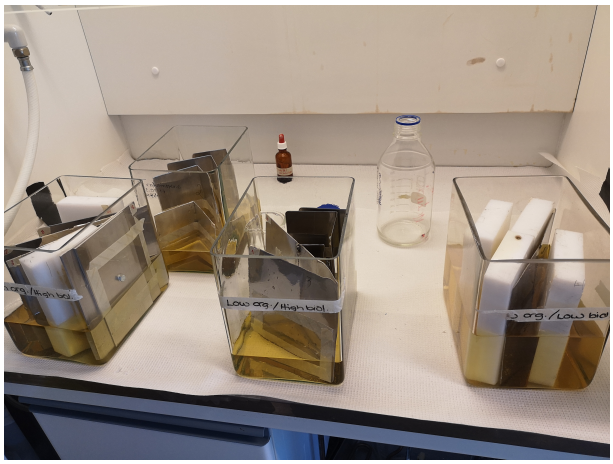

Figure 3.16: Container with samples filled up with bacteria that will be kept at 25 °C for 2 weels.

3.2.2 Cleaning phase

All the test samples were taken out of the containers to dry after being inside the bacteria mix for 2 weeks. After a while we could observe a distinct difference between the 3 material types. A larger amount of biofilm was loosing grip on the surface of the stainless steel plates and fell to the ground (Figure 3.17, Figure 3.18), only a tiny amount fell off aluminium alloy and nothing from plastic. Once the surface is dry the samples will have their surface taken picture of by Biorad's ChemiDoc MP (Figure 3.19). This device is designed to detect fluorescence from substances and make an image of it (Figure 3.20,Figure 3.10).

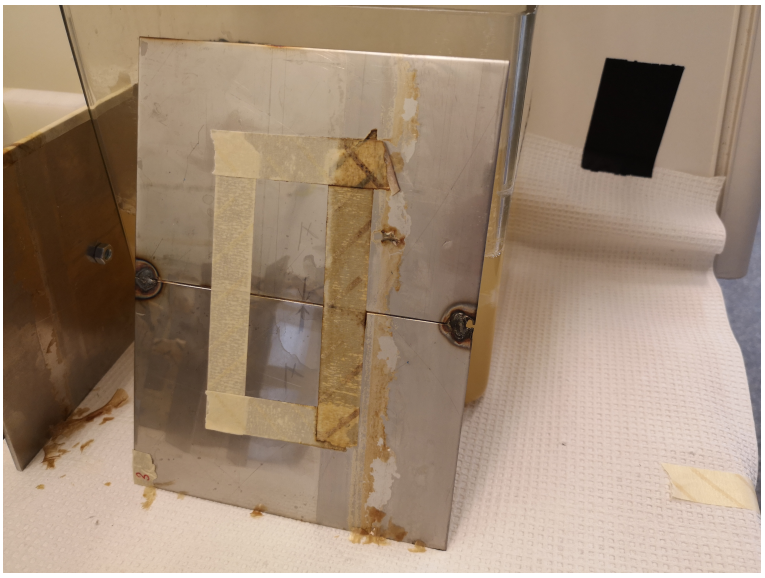

Figure 3.17: Biofilm is loosing the grip and falls off the surface of stainless steel

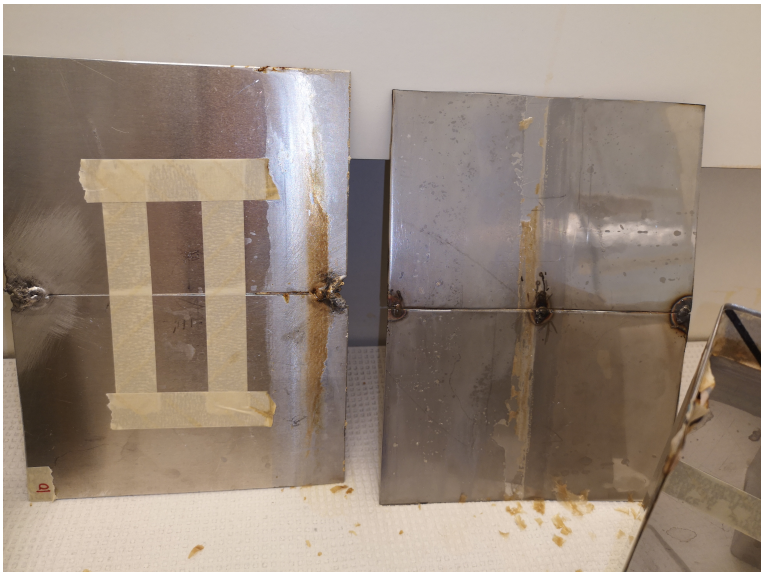

Figure 3.18: Figure shows biofilm falling off the surface of aluminum (left) and stainless steel (right)

Figure 3.19: BIO RAD's ChemiDocMP designed to detect fluorescence.

We start off by taking pictures of each sample to generate a 'before' picture of the surface that will be cleaned (Figure 3.20). In one of the pictures you can see biofilm has been colonizing the sample along the top of the sample (Figure 3.20). Biofilm have formed colonies along the edge of the sample covering both sides. Each sample were observed and the decision of which side of the sample that would be cleaned was based on the amount of biofilm a side contained. Some samples had good amount of biofilm on one side and less on the other which would impact the information gathering from the results. Once all pictures has been taken of each sample we started to prepare for cleaning. There are total of 20 samples which will be cleaned by 7 different tools during a 10 second time frame. Along with investigating the cleaning quality on the biofilm with different tools on different materials, we will also investigate the growth of biofilm between two surfaces which is tightly fixed in place (Figure 3.21).

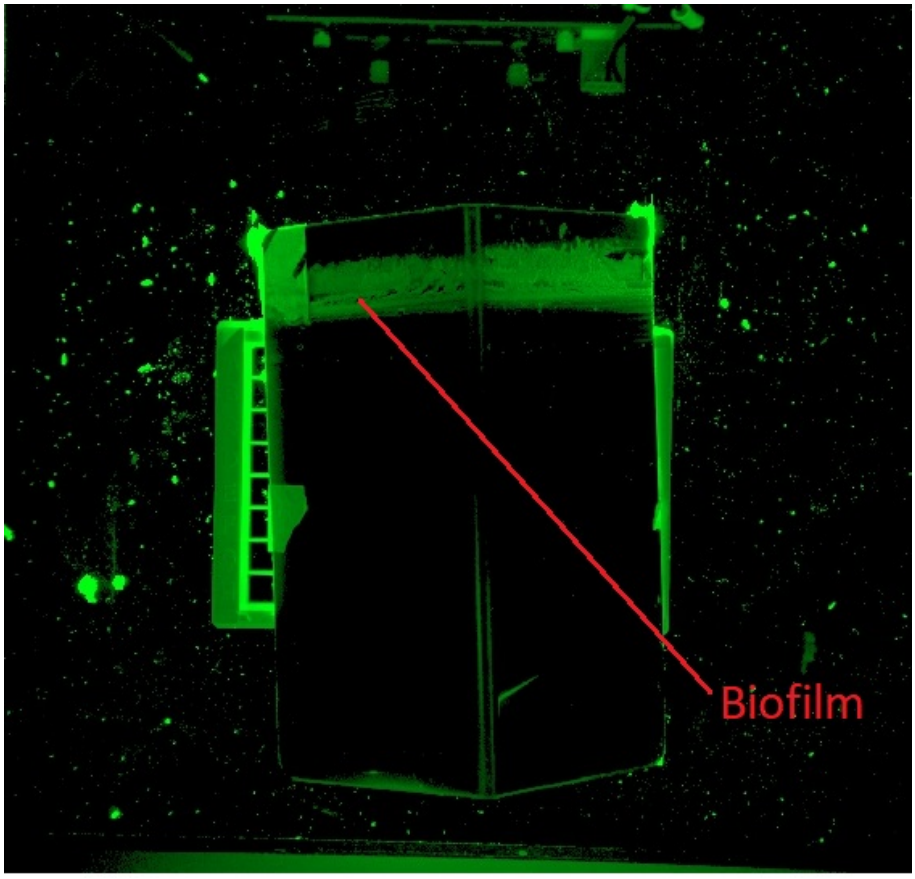

Figure 3.20: A sample with biofilm taken by ChemiDoc MP system before cleaning showing how the biofilm has formed along the topside of the sample. The picture has been taken with the SYBR GREEN settings .

The cleaning test starts with attaching the sample to a stand which holds it in place over a sink. We then bring a container filled with purified water which will be used for the cleaning. The first test is the control test (Figure 3.23) which will measure the amount of biofilm that water alone removes. Once the test is done, we turn the sample around and do a cleaning test with the tools and water combined. (Figure 3.24, Figure 3.25 and Figure 3.26). After each cleaning test, the sample is put to dry, while we continue to clean new samples. Once all samples has been cleaned and dried we can then take a new picture of each surface.

These pictures can be compared to each other to measure the percentage of efficency. The cleaning will be either done with scrubs using linear movement (Figure 3.25) similar to that of a robot and rotational cleaning using a drill (Figure 3.26) . The important part is to perform the same way as a small collaborative robot with not to much force, slow speed and no sudden back and forth movements.

Figure 3.21: The two stainless steel plates attached with a bolt will be used to check how much biofilm can grow between the two plates.

Test No.	Material Type	Cleaning Tool
1	Steel	Rotating Sponge
2	Steel	Soft brush
3	Steel	Medium brush
4	Steel	Pipebrush
5	Steel	Rotating brush
6	Steel	Hard brush
7	Steel	Sponge - Manual
8	Alu	Rotating Sponge
9	Alu	Soft brush
10	Alu	Medium brush
11	Alu	Pipebrush
12	Alu	Rotating brush
13	Alu	Hard brush
14	Alu	Sponge - Manual
15	Plastic	Rotating Sponge
16	Plastic	Soft brush
17	Plastic	Medium brush
18	Plastic	Rotating brush
19	Plastic	Hard brush
20	Steel	Medium brush

Figure 3.22: Shows the planned test in correct order with material type and tool.

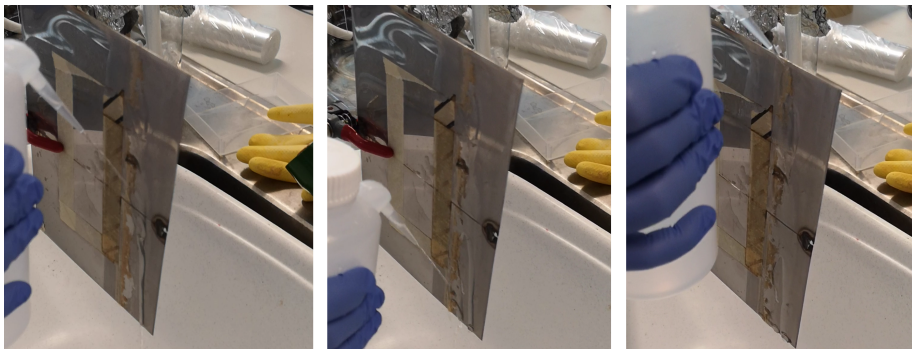

Figure 3.23: The water control test objective is to see how much biofilm is removed by water alone.

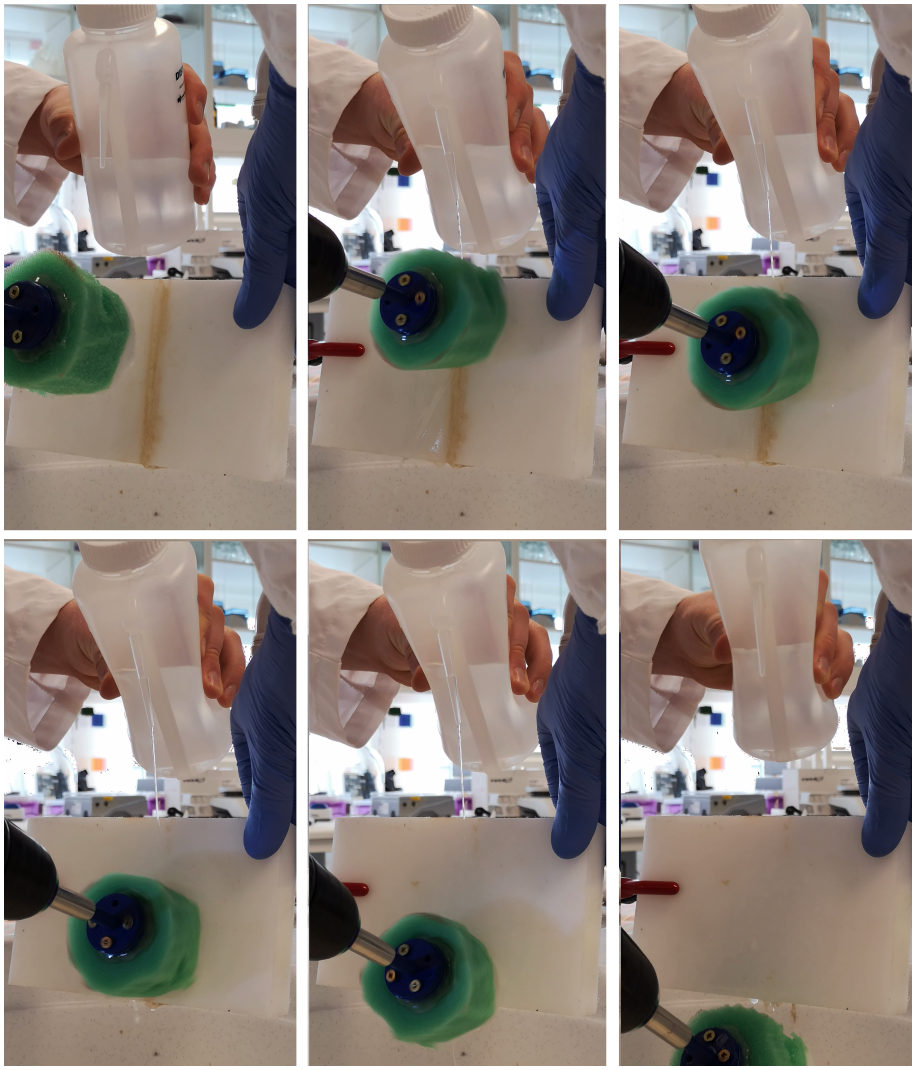

Figure 3.24: The cleaning procedure on plastic with rotational cleaning tool connected to a sponge.

Figure 3.25: The cleaning procedure with cleaning brushes was done in a similar movement pattern as if a robot did the cleaning with slow movements back and forth.

Figure 3.26: The cleaning procedure with a rotational speed close to 240 rpm.

Figure 3.27: Shows a larger amount of biofilm which has lost grip of the surface of stainless steel before the cleaning has started.

3.2.3 Post cleaning phase

The post cleaning phase is the analytical part of the experiment where the cleaning results will be analysed. What will be investigated is the cleaning efficiency of biofilm on different materials with different cleaning tools and the over night growth of bacteria in nutrient broth for control of how much grows in the cleaning brush itself. The tools have been cleaned in a bowl of water after each cleaning test and should contain less bacteria than what they had during the cleaning procedure. We start with taking bacteria samples on each cleaning tool following the plan from Figure 3.5. This test will require cotton swabs (Figure 3.28), sealed containers, growth nutrients (Figure 3.11), pipette (Figure 3.12) and a Spectrophotometer machine.

The process starts with filling a sealed container with 400 micro liter of nutrient broth with a pipette as seen on Figure 3.12. Once the container is full, bacteria from the brush will be collected with a cotton swab (Figure 3.28) by dragging it back and forth through the core of the brush. Once the sample has been collected the cotton swab is placed inside the container and sealed off (Figure 3.29 and Figure 3.30). Then a new container is filled with 400 micro liter of nutrient borth, only that this time the bacteria is collected at the edge of the brush and not at the core. That way we can check how much bacteria is at the edge of the brush compared to the core (Figure 3.5).The process is repeated on every cleaning tool with 2 tests for each cleaning tool except for the sponge which only have one test. The test samples are then stored inside a incubator machine which is set to 35 °C where they will be stored over night to allow the bacteria to grow.

In the morning the samples are carried over to the lab where the photometer machine is. First the photometer is calibrated to the growth medium used. Which means that the Zero point is the amount of light that penetrates the growth medium.. When the test with the bacteria samples are done the difference of light penetration from the bacteria sample and the growth medium sample is measured and will tell us about the quantity of bacteria in the sample. The more bacteria, the less light will penetrate the sample. We will not use the results in calculating the exact amount but rather use percentage answers compared to the other samples, example that one test contains 30 percentage more bacteria than the average. While doing this test it is important that the cuvette is clean and has no fingerprint or any other marks on the surface which can reduce the amount of light beams and thus affect the results.

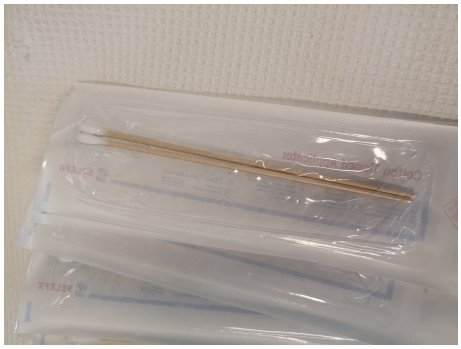

Figure 3.28: Sterile cotton swabs used to gather bacteria samples.

Figure 3.29: Shows test sample being gathered from each tool and stored into a container (Figure 3.30)

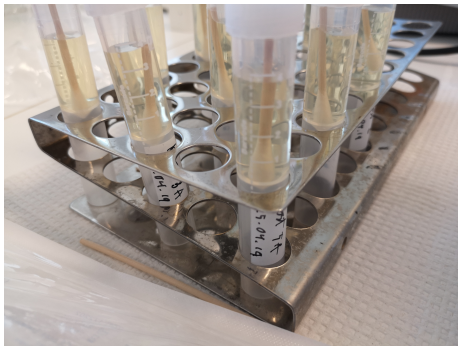

Figure 3.30: Shows the tubes containing cotton swabs with bacteria.

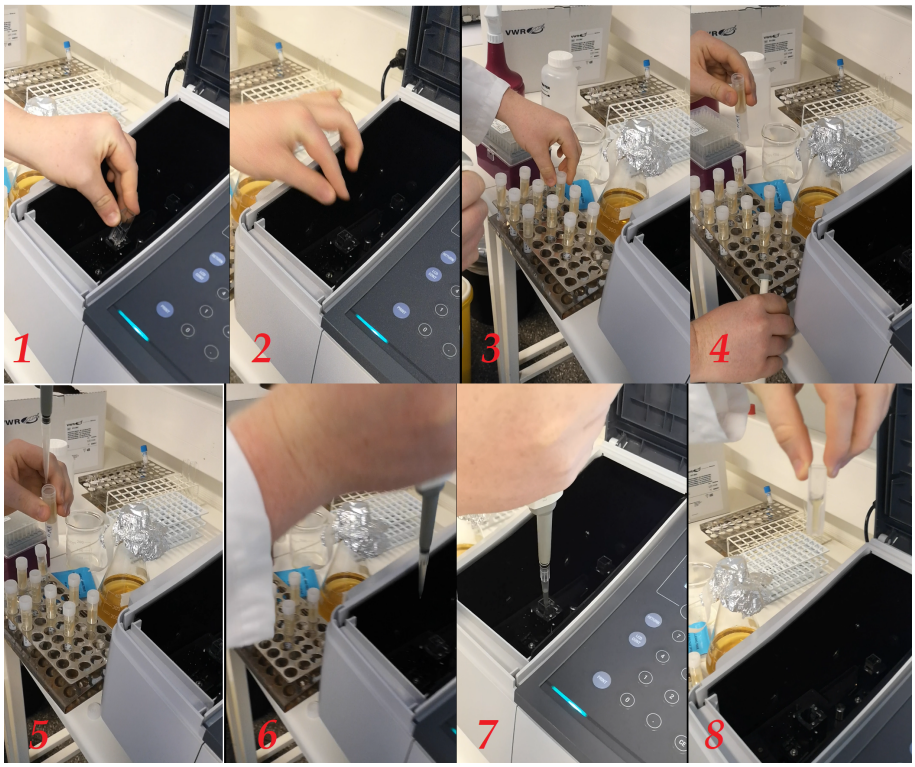

Figure 3.31: Shows the procedure on how the photometry machine measures the absorbance value.

How to do the photometry test:
1) Place the cuvette into the machine
2) Make sure the cuvette is centered.
3) Fill the cuvette with the same medium as the bacteria tests.
4) Calibrate the machine to this medium
5) Clean the cuvette, and place it back into the machine
6) Grab a test sample and unseal it.
7) Use a pipette Figure 3.12 to extract the fluid and fill the cuvette.
8) Run the machine, clean the cuvette and repeat stage 5-8.

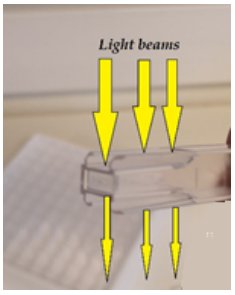

Figure 3.32: Shows how light will penetrate the cuvette which will contain the bacteria test. The more bacteria in the cuvette the more light is absorbed.

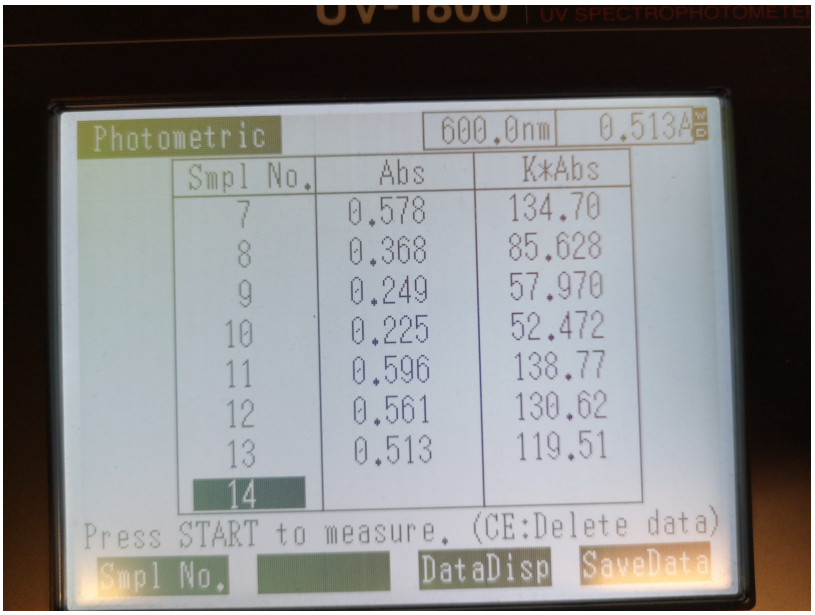

Figure 3.33: A part of the results gained from the photometry test. The full results can be seen on results on Figure 4.4

Once all samples have been cleaned and had their picture taken with the BIO RAD machine (Figure 3.19), calculation on how effective the cleaning tools are can be done. This is done by comparing *before/after* pictures, measure the total biofilm with trigonometry. This kind of analysis is not accurate without the right gear and will have some human errors which needs to be taken into consideration. The equation can be seen below:

$$Efficiency = \frac{New_{Area}}{Old_{Area}} \tag{3.1}$$

Chapter 4

Results and Discussion

There is an increased demand of improved cleaning quality in fish factories due to stricter regulations by the government. It is therefore important to investigate how material types and tool design can impact the results and what dangers the utilization of cleaning tools on biofilm can possess when thinking of cross-contamination. The materials investigated is stainless-steel type similar to NS-EN 10088-2:2014 standard, Aluminium Alloy 6082 and Polyetylen High Density (PEHD 500). All three material types is commonly found in food factories because of their properties to withstand hazardous cleaning environments. Will first look into the material types on how they impacted the experiments results and what observations were made during the tests. The first test is the water control test which enabled two important key knowledge. First, how much biofilm water remove by itself and the other is how the surface properties of each material can impact the results.

Test No.	Material Type	Cleaning Tool	Water test - biofilm areal			Cleaning test - biofilm areal		
			Before	After	% cleaned	Before	After	% cleaned
1	Steel	Rotating Sponge	36	30	16 %	70	36	49 %
2	Steel	Soft brush	10	8	19 %	100	6	94 %
3	Steel	Medium brush	42	31	26 %	16	1	97 %
4	Steel	Pipebrush	50	36	29 %	58	17	70 %
5	Steel	Circular brush	176	138	22 %	12	0	100 %
6	Steel	Hard brush	175	140	20 %	175	73	58 %
7	Steel	Sponge - Manual	39	32	17 %	33	5	85 %
8	Alu	Rotating Sponge	52	42	20 %	105	4	96 %
9	Alu	Soft brush	240	206	14 %	85	16	81 %
10	Alu	Medium brush	100	90	10 %	73	30	59 %
11	Alu	Pipebrush	97	70	28 %	100	4	96 %
12	Alu	Circular brush	100	85	15 %	100	2	98 %
13	Alu	Hard brush	100	95	5 %	44	4	90 %
14	Alu	Sponge - Manual	100	80	20 %	100	14	86 %
15	Plastic	Rotating Sponge	100	80	20 %	100	2	98 %
16	Plastic	Soft brush	100	95	5 %	100	5	95 %
17	Plastic	Medium brush	100	80	20 %	100	25	75 %
18	Plastic	Circular brush	100	85	15 %	100	1	99 %
19	Plastic	Hard brush	100	80	20 %	100	4	96 %
20	Steel	Medium brush	100	76	24 %	100	2	98 %
			Average cleaned		18 %	Average cleaned		86 %

Figure 4.1: Shows the overview of all the results for cleaning tests measured in % efficiency on both water control tests and tool tests. Both Figure 4.2 and Figure 4.3 are extracted from this and presented more detailed.

Total biofilm cleaned	Drying	Water test	Tool tests
Stainless steel	55 %	22 %	95 %
Aluminium	5 %	16 %	90 %
Plastic	1 %	16 %	93 %

Figure 4.2: The average cleaning results categorized by material type. The results are taken from Figure 4.1 and categorized by material type.

The first material tested were the stainless-steel which is known for its higher corrosion resistance compared to other steel types, making it a good candidate to handle the rough environment in a fish factory. The smooth surface makes it easier to remove dirt and biofilm which has colonized the surface after a day of production in the fish factory. This was observed during the experiment when the samples had been put to dry after bathing in the bacterial bath (Figure 3.17 and Figure 3.18). A larger amount of the biofilm which had formed onto the surface started to loose the grip on the surface that dried up. The biofilm started to peel off the surface and an estimate of 50-60% of the total biofilm on the surface of the stainless steel fell off before the cleaning experiment had started. While for Aluminium and PEHD-500 only an estimated amount of 5% or less fell during the drying. The findings confirms how important the material properties are and how much impact the surface can have directly on the biofilm.

It was observed that stainless-steel had a performance of 22% during the water test, while aluminium and PEHD-500 had both similar result of 16% which can be seen at the results categorized by material type (Figure 4.2). These results shows how surface properties can impact the results with additional 6% more biofilm lost from the water tests on stainless-steel which has a more slippery surface compared to the other materials.

The final experiment was done with cleaning tools on each material type. During this test some human errors occurred which impacted the results on some of the tests. These tests that was impacted are not counted into the overall score, but they are test number 1, 4, 6 and 10 on Figure 4.1. During the cleaning experiment the stainless-steel proved its qualities yet again with an average performance of 95% biofilm removed with tools and water combined. The combination of a slippery surface and a cleaning tool made cleaning seem relatively easy where the biofilm vanished without a trace. Similar results happened with aluminium and PEHD-500 as well with a performance of 90% and 93% which shows how effective cleaning tools are against biofilm (Figure 4.2).

Average cleaning by tool	Percentage
Rotating Sponge	97 %
Soft brush	90 %
Medium brush	90 %
Pipebrush	83 %
Circular brush	99 %
Hard brush	93 %
Sponge - Manual	85 %
Average cleaning by movement	
Linear robot movement	90 %
Rotation	93 %
Average cleaning by material type	
Sponge	91 %
Soft	90 %
Medium	94 %
Hard	90 %

Figure 4.3: The average cleaning results categorized by tool type, cleaning motion and material type of the tool.

A total of 7 different tool designs were investigated with brushes that had bristles in different materials, length, density and cleaning method. There was 4 tools which required manual scrubbing and 3 tools which were attached to a drill and cleaned by rotation. The results were put into 3 categories to allow a way to measure their overall score on multiple fields and at the end give them a grade from A to E based on an overall performance. The tools had a relatively high score on all the 7 different types with an average of 86%, or 91% if we ignore the experiments that was impacted by human errors (Figure 4.3).
During the experiment, some tools were standing out of the crowd on either a positive or negative way. The first tool was the circular brush which is a brush with medium bristles connected to a drill that rotates with a speed of 240 rpm. The circular brush made cleaning seem easy with a quick and efficient removal of the biofilm reaching as high as 99% on the performance. The hard, medium and soft brush were the only brushes used to scrub with and they all had an even performance of 90-93%, while the pipebrush had the worst score of 83%. The reason for this is many, but mostly because the pipebrush were making a mess during the experiment forcing us to stop it midways in the cleaning. The pipebrush started to toss biofilm into the air, making it quite contagious and reminded us of the dangers with cross-contamination.

The entire point with introducing tools to the fish factory is to check how effective they are at cleaning and if they possess any threat to the fish industry by becoming a highway for the bacteria to spread diseases. The pipebrush is a perfect example of just that, by its contagious performance which makes it unacceptable for the fish factory. If the cleaning tool is spreading small particles of bacteria to new locations, these bacteria can colonize new surfaces and infect the fish, potentially starting an outbreak, costing the factory millions of NOKs. The last two tools used in the experiment were the sponges, one was used as a rotating tool and the other one with normal scrubbing. The rotating sponge had a high performance of 97% where it basically polished the surface as it was cleaning, while the manual sponge where just scraping the biofilm off the surface and earned a performance of 85%.

Sample name	Sample No.	Abs	Amount compared to average
Soft brush - Bottom	1	0,573	113 %
Soft brush - Top	2	0,577	114 %
Medium brush - Bottom	3	0,71	140 %
Medium brush - Top	4	0,61	121 %
Hard brush - Bottom	5	0,578	114 %
Hard brush - Top	6	0,368	73 %
Pipebrush - Bottom	7	0,249	49 %
Pipebrush - Top	8	0,225	45 %
Circular brush - Bottom	9	0,596	118 %
Circular brush - Top	10	0,561	111 %
Sponge - Surface	11	0,513	101 %
Average	-	0,505	

Figure 4.4: Shows the absorption value received from the spectrophotometry. The higher the values are, the higher density of bacteria.

There was taken bacteria samples from each tool after the cleaning experiments had been done (Figure 3.29). These bacteria samples were then grown in a lab over night so that it could be inspected the day after. The bacteria samples were measured by a machine that sends lights through a sample and registers the absorption value by how much light is absorbed by the bacteria (Figure 2.4 and Figure 3.31). The results from the samples displayed a clear trend in how the bacteria colonized the tools. The first observation was that there was a higher density of bacteria at the core of the tool compared to the tip. This can be explained as an increased difficulty to clean the core of the tool compared to the tip since the core of the brush is less exposed. On average there was 18% higher density of bacteria at the core compared to the tip, but the brush with hard bristles had as high as 57% more bacteria, while medium hardness had 16% more and soft had 1% less bacteria (Figure 4.5).

We do not know why the soft brush had 1% less bacteria at the core compared to the tip but assume that it might have something to do with bad measurements with the photometer machine or if the tool was poorly cleaned before the bacteria samples were taken. The tool that stood out the most was the pipebrush which had between half to third of the bacteria density than the rest of the tools. The pipebrush had long, hard and few bristles compared to the other brushes that had a much higher amount of bristles. Which means there are less surface for the bacteria to attach themselves on making it easier to clean the brush. The medium brush had the highest bacteria density with a number as high as 0,71 at the core and 0,61 at the tip which is really high compared to the average of 0,505. The medium brush had a shorter length on the bristles and much higher density/amount of bristles. This grants the perfect habitat for the bacteria to grow in. The soft brush had quite similar results as medium brush with 14% more bacteria than average as well. The last tool where the sponge which had 1% more bacteria than the average.

Sample name	Difference in percentage
Soft brush	-1 %
Medium brush	16 %
Hard brush	57 %
Pipebrush	11 %
Circular brush	6 %
Average	18 %

Figure 4.5: Shows the % difference of bacteria in the core and tip of each cleaning tool. (Ex. Brush 2 has 16% more bacteria in the core of the brush than at the tip, while soft brush has 1% less bacteria at the core compared to the tip.)

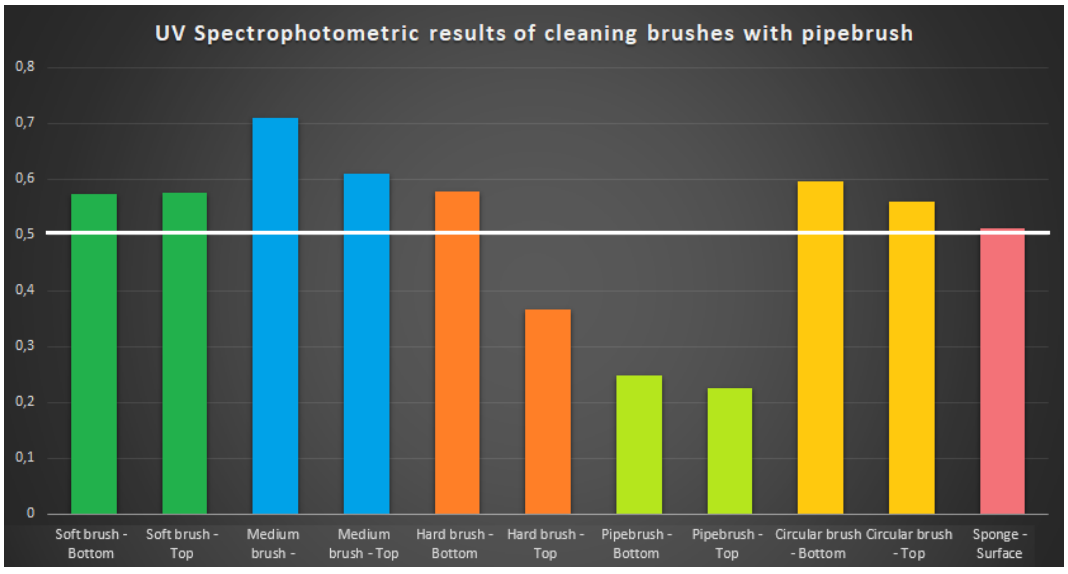

Figure 4.6: The graph shows the visual representation of the results from Figure 4.4.
White line represents the total average, and we can observe that most tools are above average.

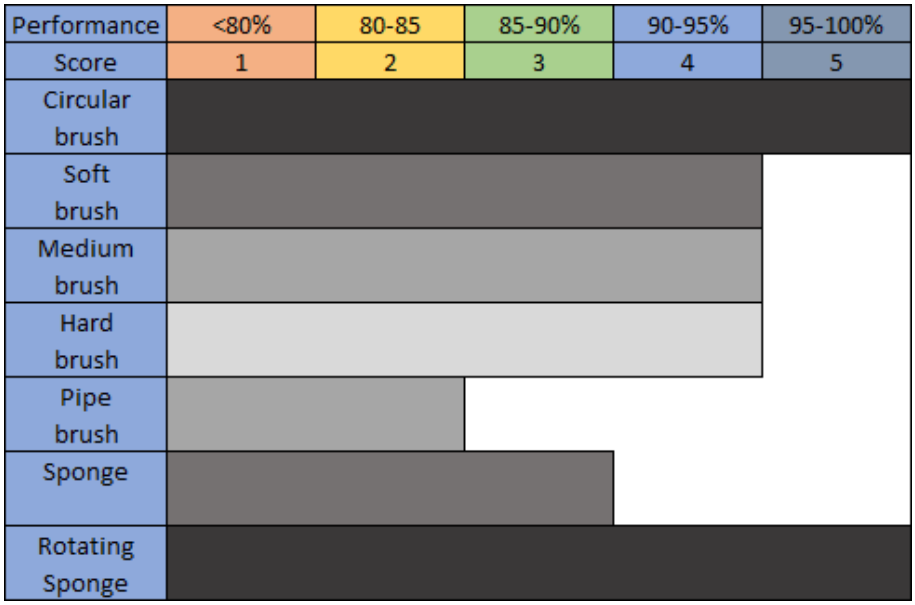

Figure 4.7: The tools are given a score based on performance measured in amount of biofilm removed in %.

A grading system was created to rate the different tools based on performance (Figure 4.7 and bacteria absorption (Figure 4.9). The first grading is for the individual tool performance based on their average score (Figure 4.3). Only two tools made it to top score which was 95% or higher in performance. These tools were the circular brush and rotating sponge which both were attached to a drill. The remaining results can be seen at Figure 4.7. The grading allows for a visual presentation of the performance of the different tools and it might seem strict considering lowest score is 80% or lower. But cleaning in a fish factory is a serious matter and so must the requirements be in order to find the best solutions.

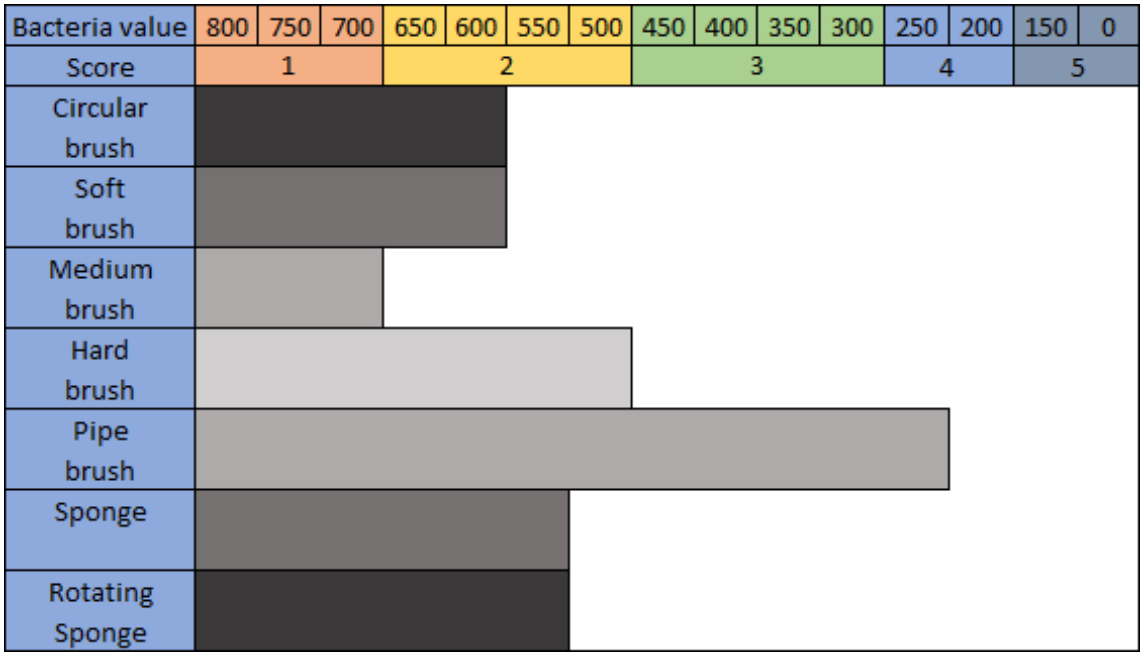

Figure 4.8: The tools are given a score based on the bacteria value measured after the cleaning experiment. The lower the value, the less bacteria.

The next grading is based on the absorption value detected in each tool from the bacteria samples that were taken after the cleaning experiment was over. At this grading most of the tools landed on a weak or strong 3 while the hard brush got a weak 4 and pipebrush got a weak 5 (Figure 4.8. It is important that in this test we do not get a bacteria value represented in quantity, but a value that represents how much light was absorbed by the bacteria. And the exact number of bacteria is not necessary to know either since bacteria can form colonies and spread quickly even when they start in small numbers.

As an observation, the grading of bacteria values might not be the most accurate way to rate tools. Rather than grading the bacteria content in the tool after flushing them clean, its more important to test as many methods to sterilize the brushes after use and rate the different cleaning procedures to actually solve the problem, this kind of research requires a lot of planning, time and effort.

Figure 4.9: The total score based on Figure 4.7 and Figure 4.8.

The total grade is where we add the score earned from each test to grant the tool a grade from A to E which can be seen on Figure 4.9. A total of 7 tools were tested and the average score reached 6 which equals grade C. All the tools except two earned 5-6 points and got grade C while 2 tools earned 7 points and got grade B. It was two of the tools with rotational cleaning which scored the highest on the performance test. The circular brush and rotating sponge displayed how good the usage of a rotation device for cleaning is against biofilm. This kind of tool is also easy to elaborate into a robot which uses cleaning brushes for cleaning. The tools should make it easy for a robot to clean all kinds of surfaces and material shapes with high precision. That means a robot tooling should be designed to meet certain requirements which can be seen on the quality attributes below:

1) Effectiveness
2) Adaptability
3) Easy maintenance

The first attribute is the *effectiveness* which describes the tools capability to produce desired results. This attribute is measured in the tools performance in effective cleaning and should be as close to 100% as possible to avoid leaving any biofilm remains which can protect the *L. monocytogenes* from the decontamination which comes later in the cleaning procedure, there is an increased risk of causing an future outbreak of listeriosis among the population.

The next attribute which will be important is the *adaptability* which is about how well the tool can adapt to new situations. In this case it will be about how well the tool can clean different shapes and how well it can handle obstacles. For example a joint which allows the tool to bend to either sides and clean from angles, or an extension arm which allows the robot to extend the tool further to reach spots it would not otherwise reach. Under this attribute, changeability for the robot is made possible. If the robot requires to change in order to meet certain requirements,

we can grant this by designing certain attributes to the tool. For example make quick-release coupling on the tool that grants the robot the ability to disconnect from the cleaning tool and connect to a different tool. Such as a small tool to spray a certain part of the factory, then it change the tool into a cleaning brush to start scrubbing the surface.

The last and also one of the most important requirements is the *maintenance* of the tool. It is important that the tool is easy to maintain after it has been used. Which means the decontamination of the tools should not be restricted by the tools design. If the tool's design is formed in such a way that it will offer safe haven for bacterial growth it will quickly become a main source of bacterial contamination and make the situation in the factory from bad to worse. It is therefore important that the tool has a clean design with the less difficult spots as possible where bacteria and dirt can gather. Some will be unavoidable like the bristles on the brush will no matter what bring this safe haven for bacterial growth. That only means the tool must be designed not to avoid it, but to make the decontamination of that part easier. For example the cleaning brush can be taken apart and cleaned from both the inside and outside to make sure no bacteria remains among the bristles or that there are some kind of self-flushing system with chemicals to kill off the bacteria before they are able to form biofilm inside the tools.

There is still one topic which needs to be discussed which is about the usage of sponges and the dangers they possess. The sponges used in this experiment are the normal kitchen sponges found in every household. Sponges are known to contain a lot of bacteria because of how they are structured, offering bacteria the perfect habitat to grow in. In a research journal published in 2017 at "The National Center for Biotechnology Information" (NCBI), it was discovered that household sponges could contain up to 54 billion bacteria cells per cubic centimetre and 362 different bacteria species was detected [23]. The scariest part was not the amount of bacteria or the amount of species, but the difference in a well maintained sponge and a dirty sponge. The sponge that was frequently cleaned to kill bacteria had a significantly increase of two of the ten most dominant bacteria granting it a more pathogenic potential compared to a dirty sponge [23]. The reason for this is that the more dominant bacteria are kept in check by the vast amount of weaker bacteria surrounding them, but when these weaker bacteria dies from sanitation it leaves the dominant bacteria a chance to rapidly grow since they won't have any natural enemies to hold them back.

These are important findings for not only the material choice, but also for the decontamination of the tools. If the sanitation of the tool is not good enough, it will kill the weaker bacteria species and allow the more strong and dangerous bacteria species to blossom, making the tool more contagious than it originally was. That means the tool needs to have a design which makes sanitation of it easier and more reliable or else it will grant the more robust bacteria like S.*aureus* and L. *monocytogenes* to thrive and populate the tools after they have been sanitized. These tools will then act as a highway for the bacteria to spread across the factory causing millions of NOKs in losses and a full production stop until the outbreak has been dealt with. In worst case scenario these pathogenic bacteria can infect the workers causing an outbreak of diseases such as the one in Vestnes shipyard [24], were 9 workers were infected by the *Streptococcus pneumonia* bacteria which caused a serious infections and put the entire shipyard in quarantine. This was an outbreak at a shipyard but imagine the consequences of outbreak due to bad cleaning or cleaning procedure which grants the dangerous L. *monocytogenes* the perfect

habitat to spread. The fish that goes through the production line and out to the population won't be detected of L. *monocytogenes* until some customers buy the fish, eats it and gets sick. By this time a larger amount of fish might have been sold across the country infecting mostly customers with weaker immunity system with high risk of fatal consequences resulting in lives lost.

In Norway there has been a total of 479 cases of listeriosis in the last 20 years or an average of 24 each year which can be seen at msis.no (Message system for infectious diseases at Folkehelseinstitutt). Listeriosis is caused by the *Listeria monocytogenes* and is a serious sickness with fatality rate of 30%. In the first quarter of 2019 there has been a total of 9 cases of listeriosis, where the source came from a fish factory which produced a fish we Norwegians eat raw. This fish type was sold from November, but the sickness was not detected until December and it was not contained until January. By this time there was 13 cases of listeriosis where 9 of them were hospitalised in January and the remaining 4 were hospitalised in December. For a company selling fish, having an outbreak like this can cause great harm not only during the outbreak period, but also the aftermath. Not only do they loose money when production stops to decontaminate the factory, but the loss of their reputation can be fatal for the company's survival. That is why one of the main drive for the fish company is to keep improving their cleaning procedures to minimize the risks of such outbreaks. But even if you have great cleaning procedures there will always be a risk of a smaller outbreak. Since the bacteria can be found in the fish, when processing the fish it will contaminate the processing gear. This processing gear will then cross-contaminate fresh fish and the percentage of fish being contaminated by listeria increases during the day as production goes on. That means you can never be certain to stop an outbreak, but by using better cleaning procedures, the correct material type on the equipment and cleaning tools will decrease the chances of it happening.

Future work

The purpose of this thesis was to investigate the possibilities to utilize cleaning brushes as a tool for the removal of biofilm and look into the possible dangers of it becoming a contagious tool. Several solutions were found such as designing the tool with regards on sanitation. The more promising solution found are the cleaning procedures for the brushes, but due to the amount of time remaining there was not enough to investigate hygienic procedures for the tools. For future work it is recommended to look into different ways to sanitize the cleaning brushes and measure each procedure by bacteria sample tests to compare the results. The cleaning procedures will serve as the most important method to decontaminate the cleaning brushes after use. This research can be combined with tool design to allow the tools to fit the cleaning procedures for best possible results. It is important to consider that if the sanitation of tools is not 100% effective, it will only eliminate the weaker bacteria and allow the stronger and more dangerous bacteria to populate the tool, making it more contagious. In addition, the correlation between the material properties and biofilm provided interesting results that could be considered in further development of this project.

Chapter 5

Conclusion

This thesis has investigated different strategies to fight biofilm, which provides L. *monocytogenes* a protective barrier against cleaning agents. Two strategies were investigated: The first one consisted on how the material properties of stainless steel, aluminium alloy and polyethylene (PEHD) affect the formation of biofilm. The test samples were produced by allowing the bacteria P. *fluorescence* and S. *aureus* to grow in containers filled with nutrient broth for 2 weeks. As a part of this strategy, a water control test was made to measure how much biofilm was removed without a cleaning tool, but by only rinsing with water in order to obtain the impact of the material properties in the biofilm. Once removed from the containers, the test samples were put to dry overnight. In this period the stainless steel lost 55% of the total biofilm that had formed while aluminium alloy and PEHD lost 5% or less. This observation confirms how the stainless steel with its smoother surface properties makes it more difficult for the biofilm to establish a strong grip onto the surface. The materials were then tested both with the water control and cleaning experiments, and the same observations of stainless steel having better results were obtained. These findings confirm that the surface properties on the materials do matter on the biofilm formation. These are not new finds since there has been a lot of research on this topic, but the precise relation between surface properties and biofilm growth is still unclear [1,pp.10-14].

The last strategy was to introduce cleaning brushes of different designs and investigate how these tools perform against biofilm. The tools were categorized into different designs, material types and cleaning methods. It was observed during the experiments that the manual cleaning tools had a lower performance than the cleaning tools connected to a drill mainly due to human errors. These tools used a rotational speed of 240 RPM and had a performance of 95% to 99% mainly as a result of no human errors due to how easy the tools are to handle.

It is important not to use similar brushes to the *pipebrush* which tossed bacteria around into the air reminding us of the dangers of tools which can easily spread bacteria. Since the entire point with introducing tools to the fish factory is to evaluate how effective they are at cleaning biofilm and if they provide any threat to the fish industry by becoming a highway for the bacteria to spread diseases, it is very important to see how much bacteria would grow inside the cleaning brush over night if it was badly decontaminated. There was found a higher density of bacteria residing at the core of the brush compared to the tip. The reason is that the core is more difficult to access and thus more difficult to clean, which means that the design of the brush and

the cleaning procedures need to be elaborated together to solve this problem.

To achieve reliable cleaning results, improvements need to be done in the tool design, hygienic procedures, equipment design and the staff's attitude towards hygiene. It all starts with the workers attitude due to the lack of knowledge on hygiene. The first step is therefore to inform the employees and create a common understanding on what they are doing and the reasons for it. Then improvements can be done on cleaning tools and fish processing equipment with a cleaner design and an adequate material type. In addition the hygienic procedures must be developed, containing a detailed approach on how to deal with different tools and equipment, general routines and lastly, the need of continuous improvements on all fields to ensure safe environments.

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Appendix

Nutrient broth

ATCC Medium: 3 Nutrient Agar/Broth

Agar Medium
Nutrient Agar (BD 213000)……..23 g
DI Water……1000 ml

Autoclave at 121ºC.

Broth Medium
Nutrient Broth (BD cat 234000)……8.0 g
DI Water……1000 ml

Autoclave at 121ºC.

*This product is commercially available from BD.

To make medium from scratch, follow formulation below:

Nutrient Agar Composition
Beef Extract……..3.0 g
Peptone……..5.0 g
Agar……15.0 g

Final pH 6.8 +/- 0.2.

*Omit agar for broth medium.

ChemiDoc Imaging System - Picture results

Before cleaning - Test sample 1

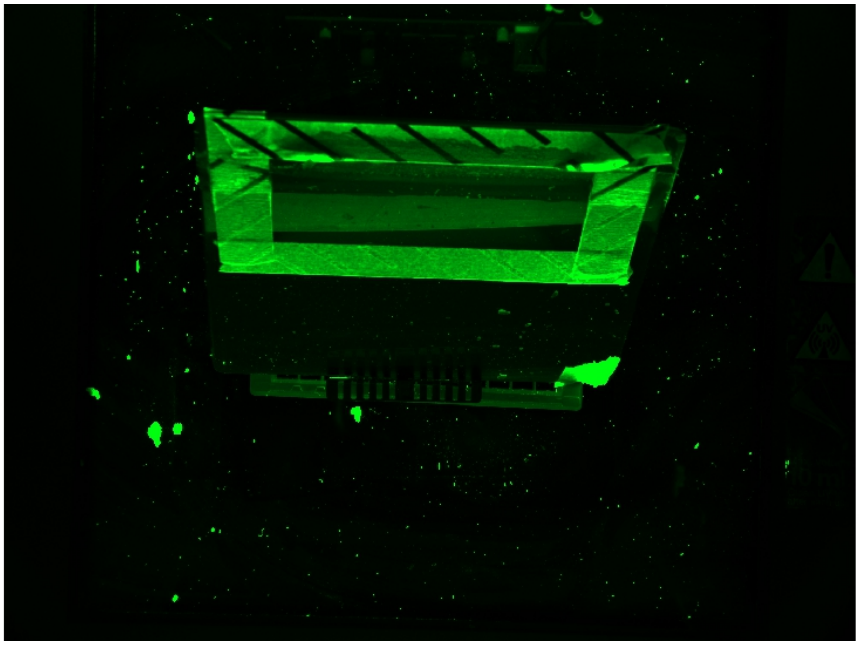

Figure 5.1: Before water control test.

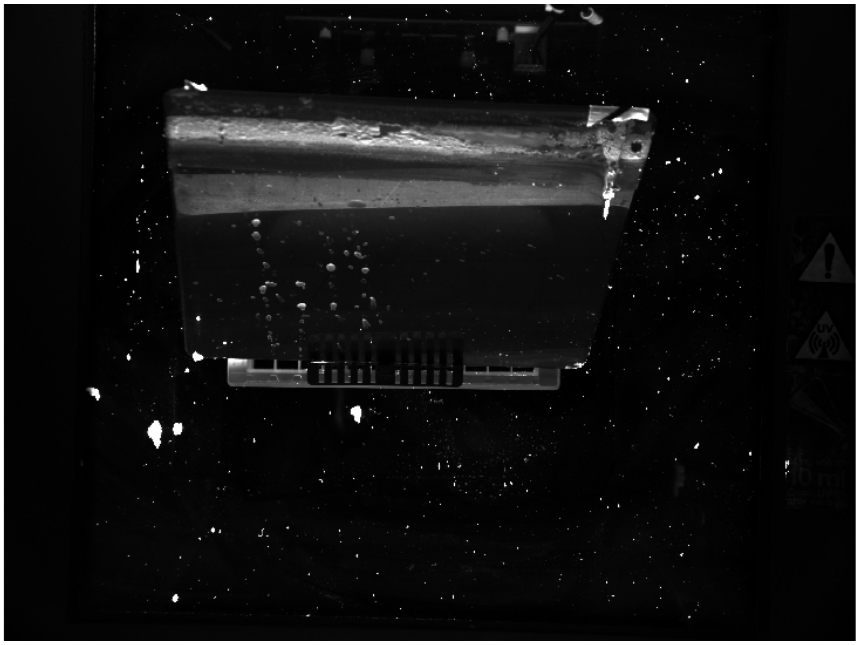

Figure 5.2: Before cleaning test.

After cleaning - Test sample 1

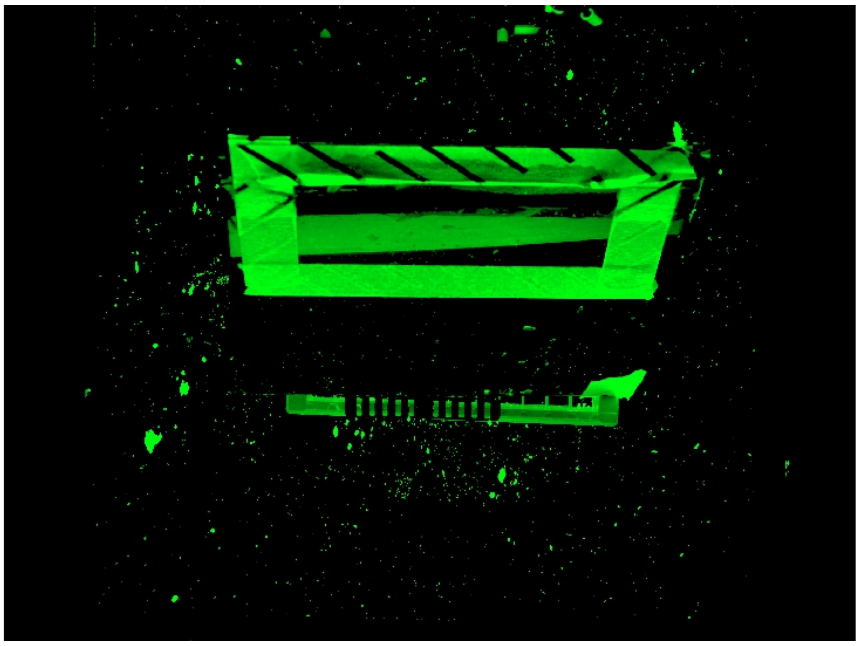

Figure 5.3: After water control test.

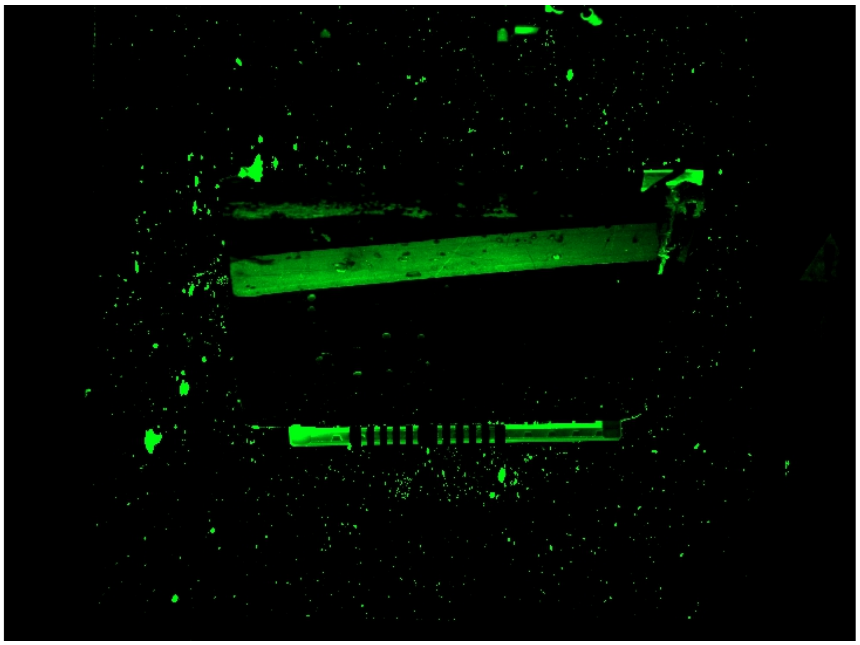

Figure 5.4: After cleaning test.

Before cleaning - Test sample 2

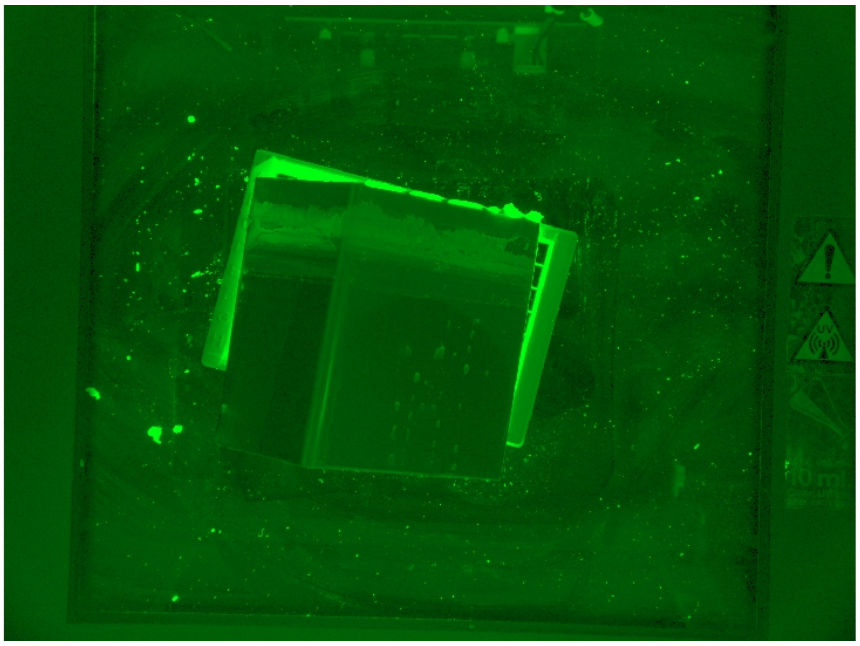

Figure 5.5: Sample 2 before experiment.

After cleaning - Test sample 2

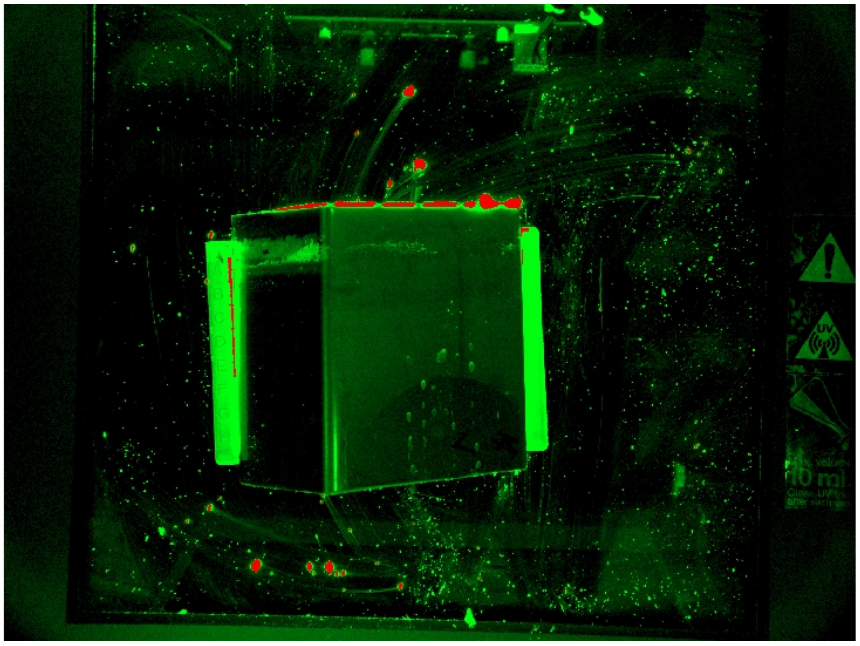

Figure 5.6: Surface on left side was cleaned with water control test, while surface on right side cleaned with tools.

Before cleaning - Test sample 3

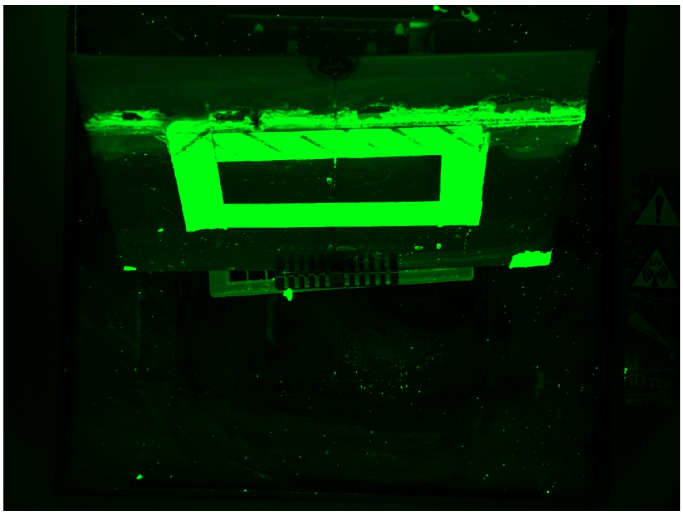

Figure 5.7: Before water control test.

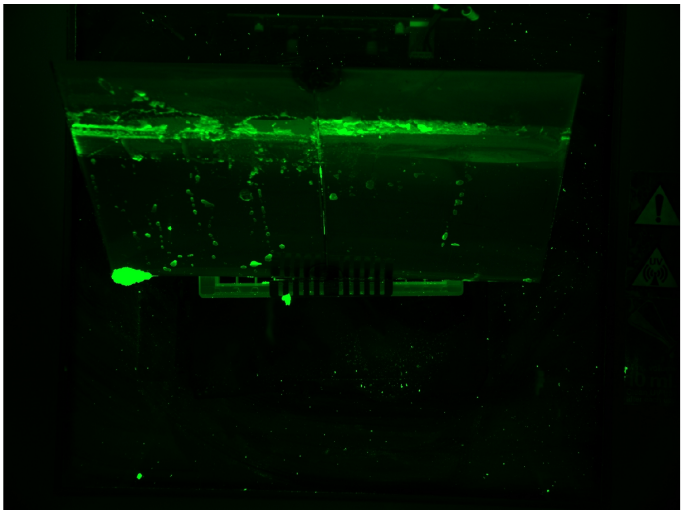

Figure 5.8: Before cleaning test.

After cleaning - Test sample 3

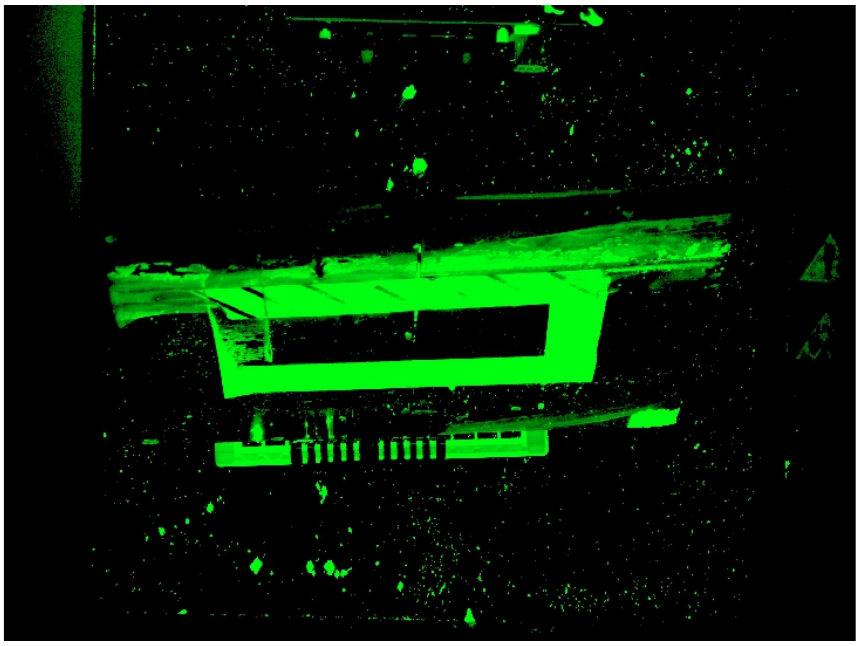

Figure 5.9: After water control test.

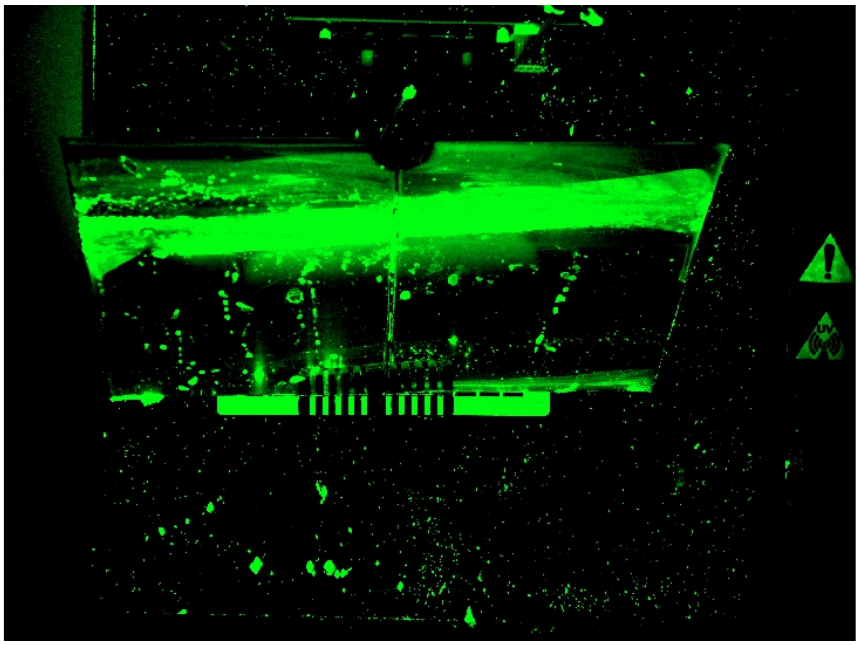

Figure 5.10: After cleaning test.

Before cleaning - Test sample 4

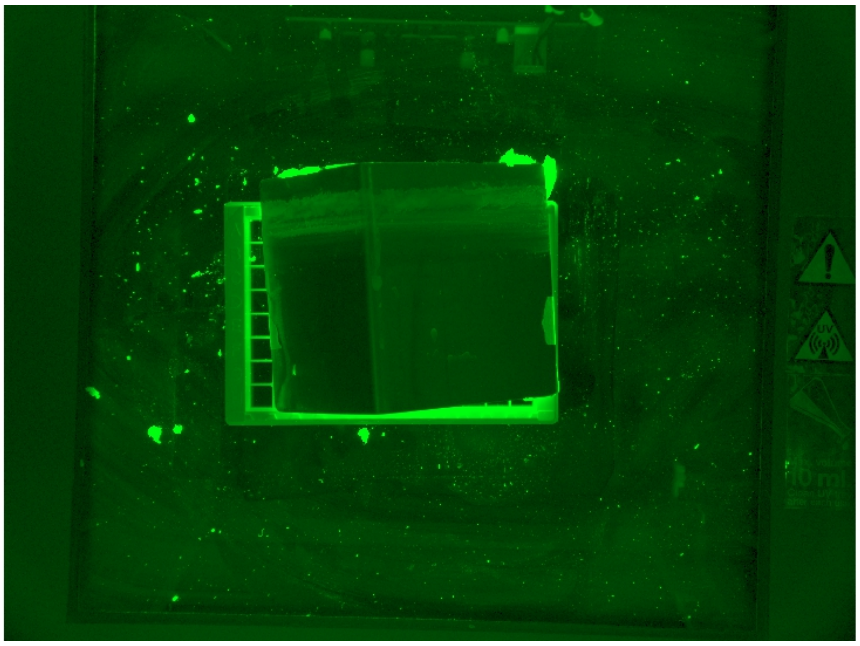

Figure 5.11: Before cleaning experiment.

After cleaning - Test sample 4

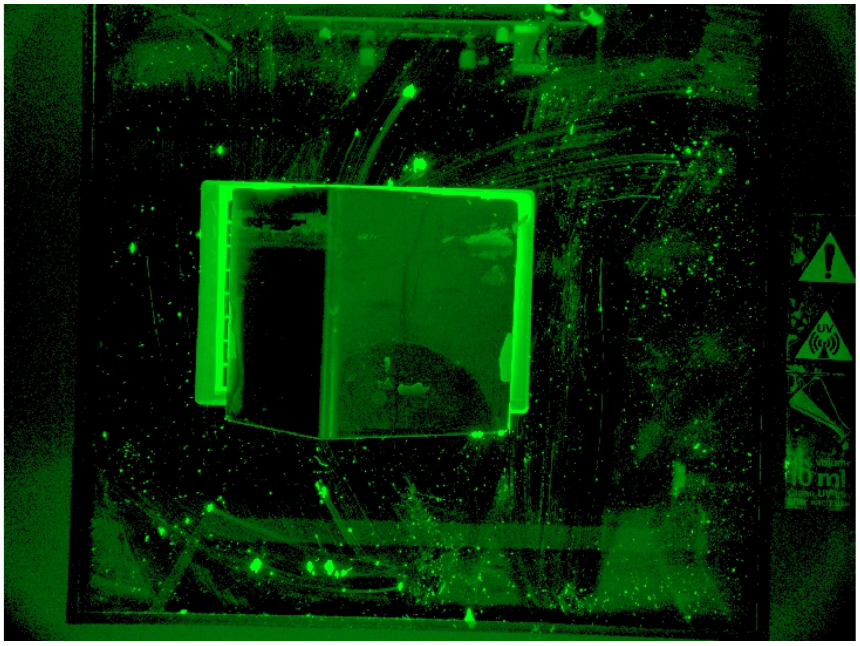

Figure 5.12: Surface on left side was cleaned with water control test, while surface on right side cleaned with tools.

Before cleaning - Test sample 5

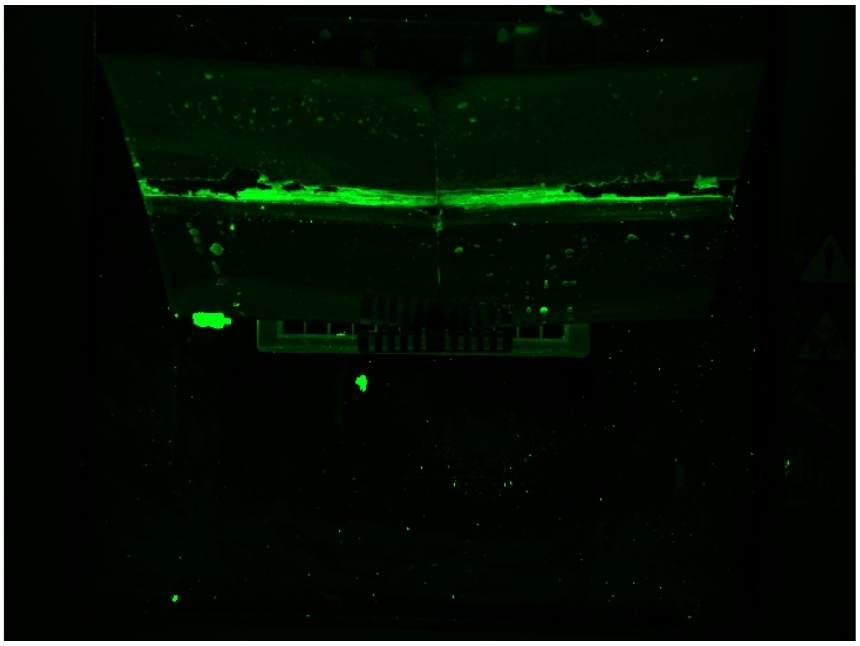

Figure 5.13: Before cleaning experiment.

After cleaning - Test sample 5

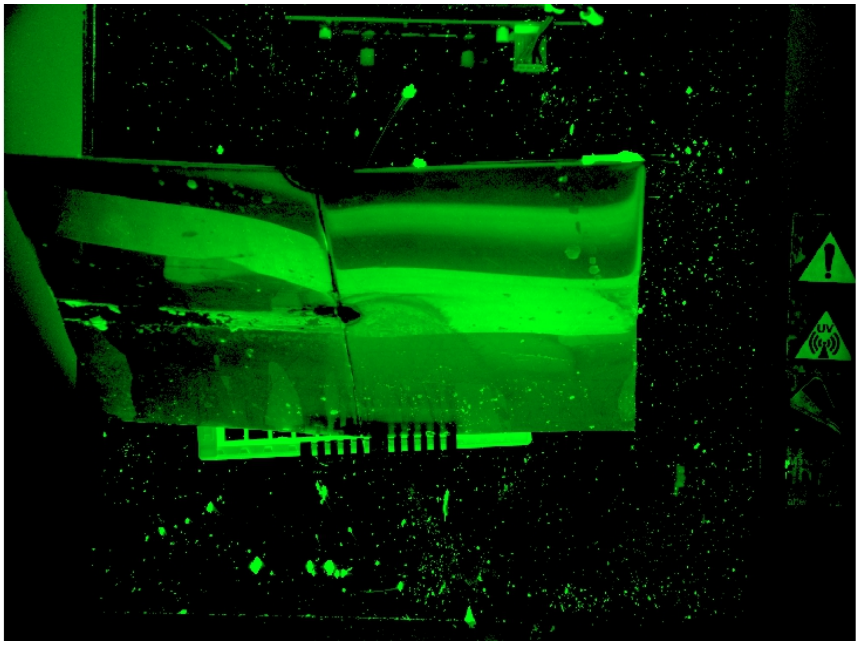

Figure 5.14: Surface on left side was cleaned with water control test, while surface on right side cleaned with tools.

Before cleaning - Test sample 6

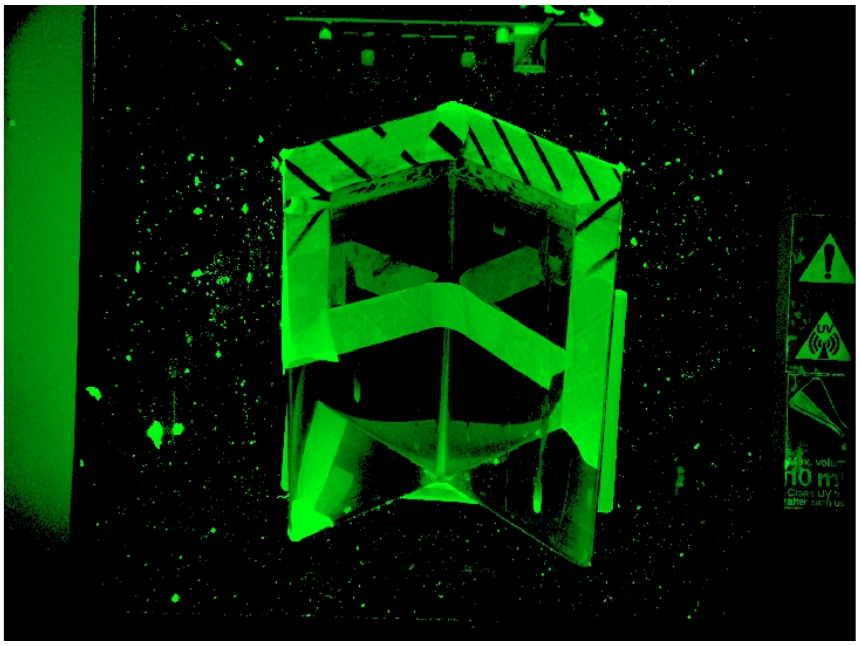

Figure 5.15: Before cleaning experiment.

After cleaning - Test sample 6

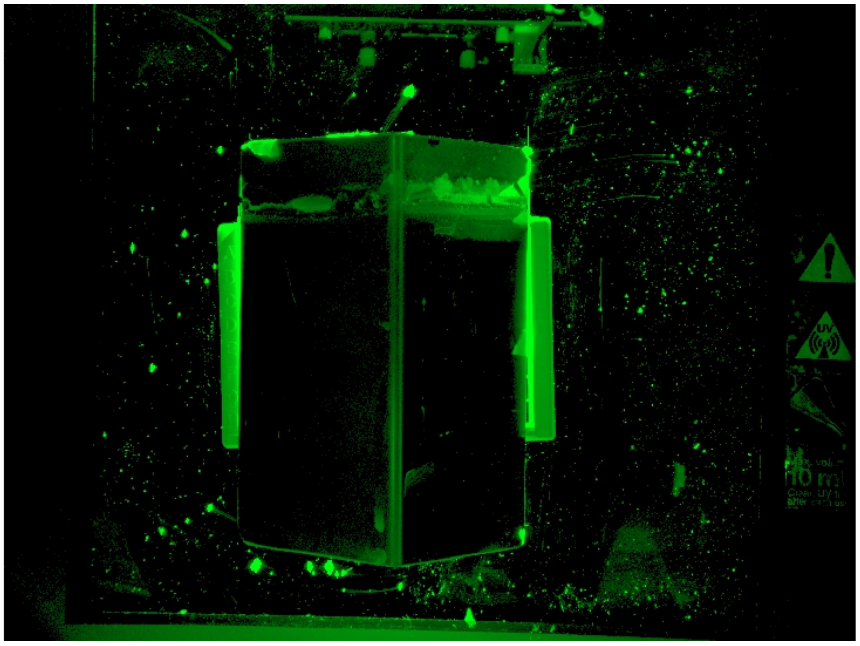

Figure 5.16: Surface on right side was cleaned with water control test, while surface on left side cleaned with tools.

Before cleaning - Test sample 7

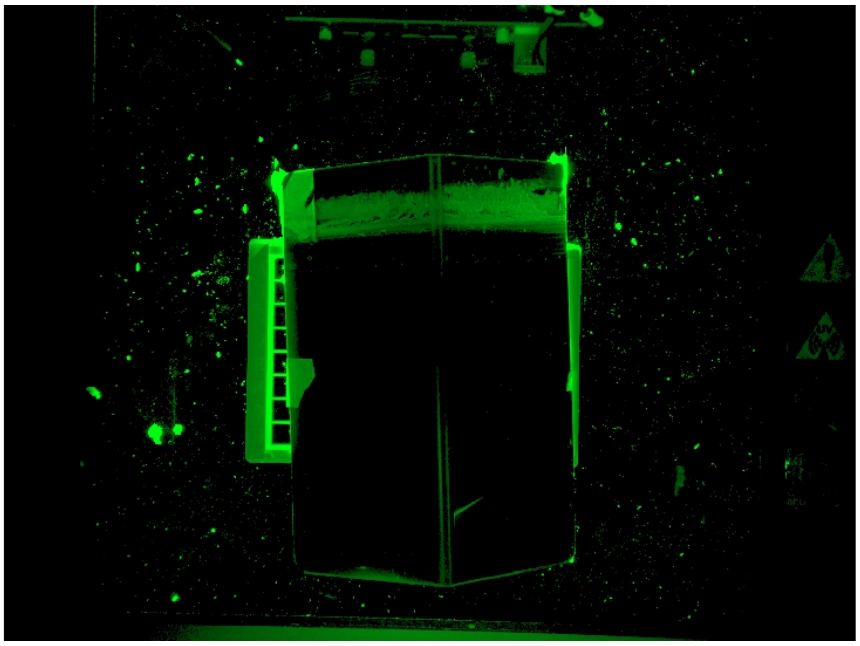

Figure 5.17: Before cleaning experiment.

After cleaning - Test sample 7

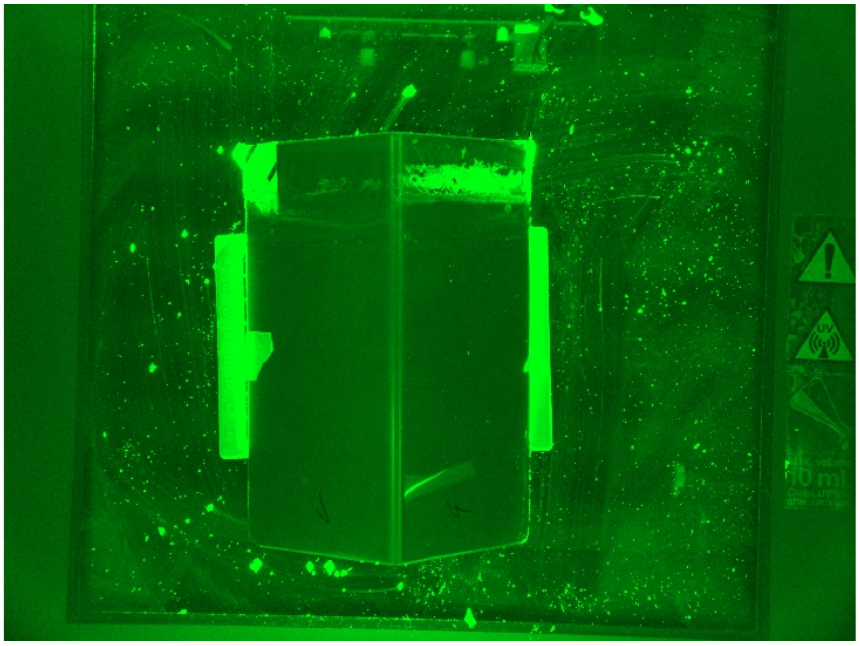

Figure 5.18: Surface on right side was cleaned with water control test, while surface on left side cleaned with tools.

Before cleaning - Test sample 8

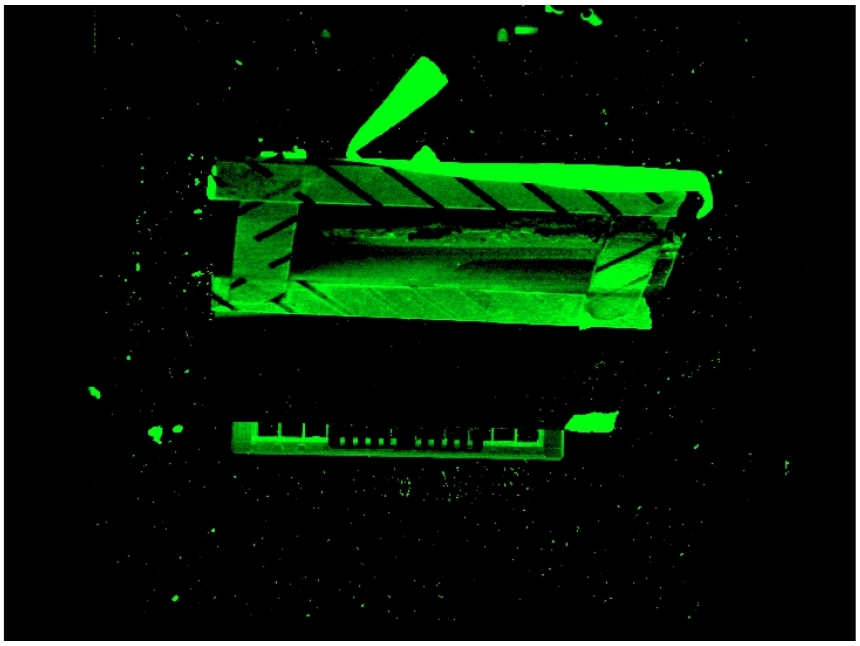

Figure 5.19: Before water control test.

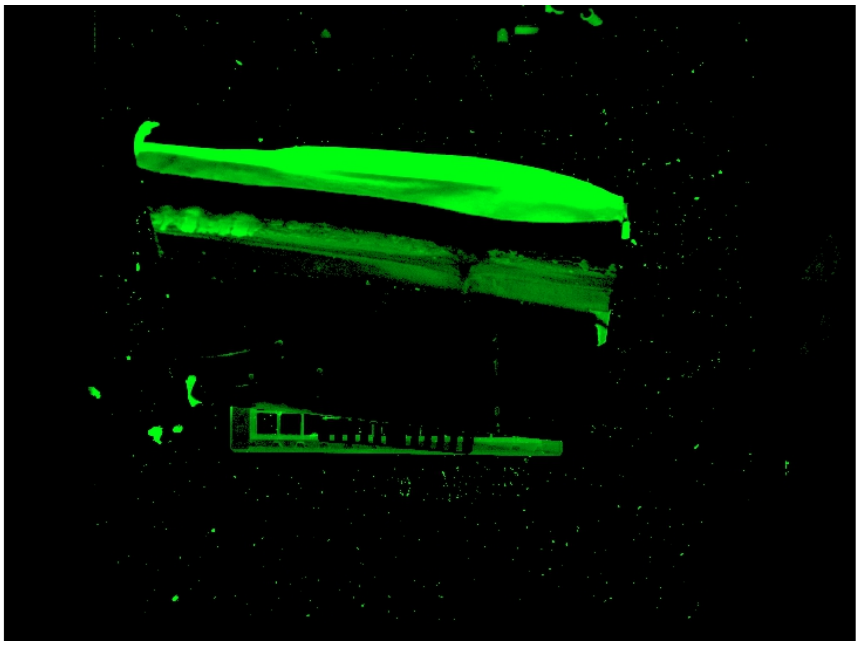

Figure 5.20: Before cleaning test.

After cleaning - Test sample 8

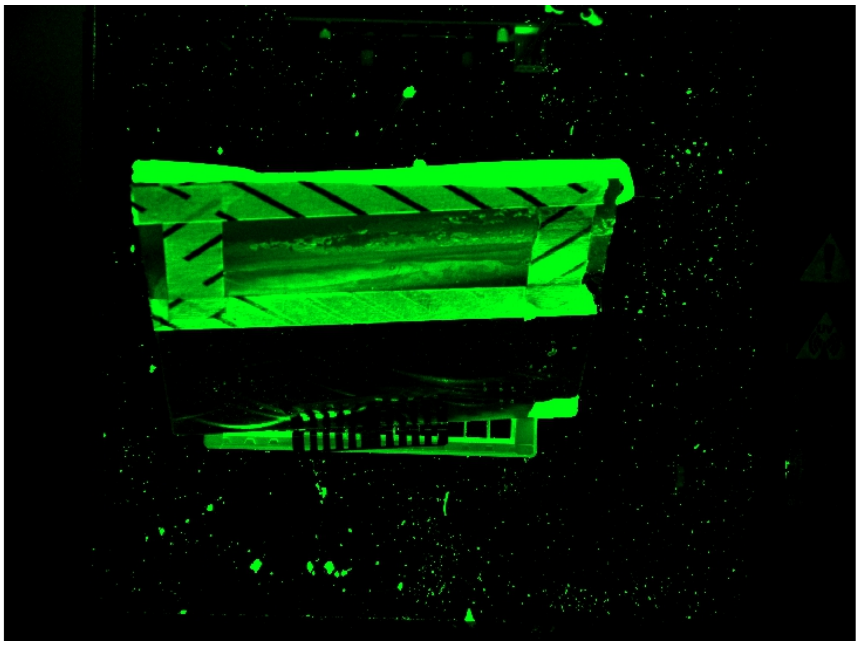

Figure 5.21: After water control test.

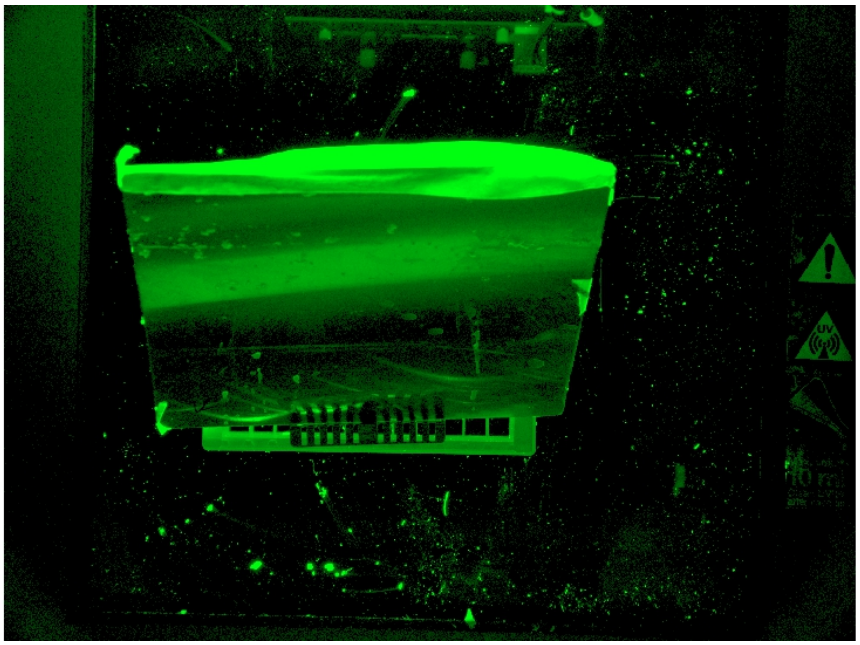

Figure 5.22: After cleaning test.

Before cleaning - Test sample 9

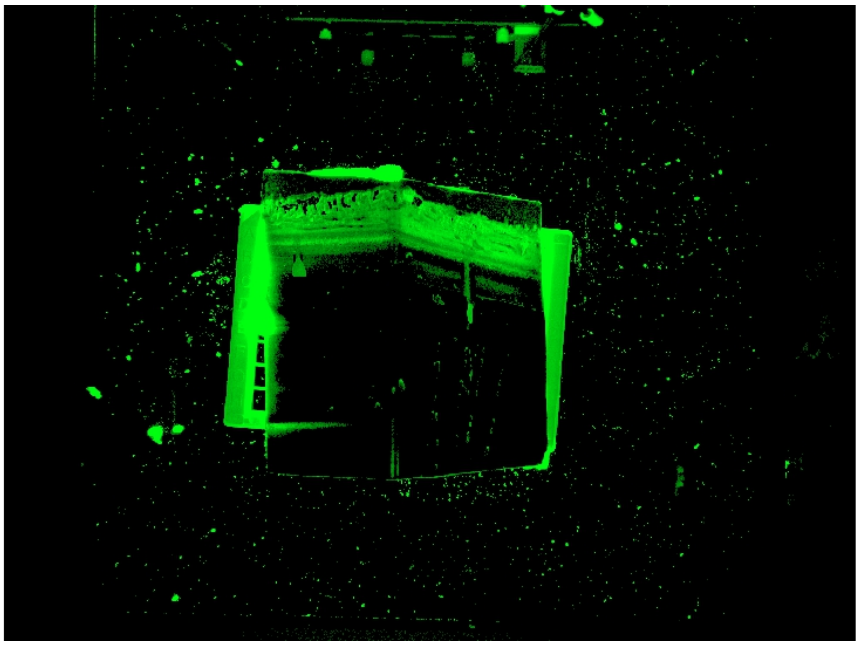

Figure 5.23: Write here.

After cleaning - Test sample 9

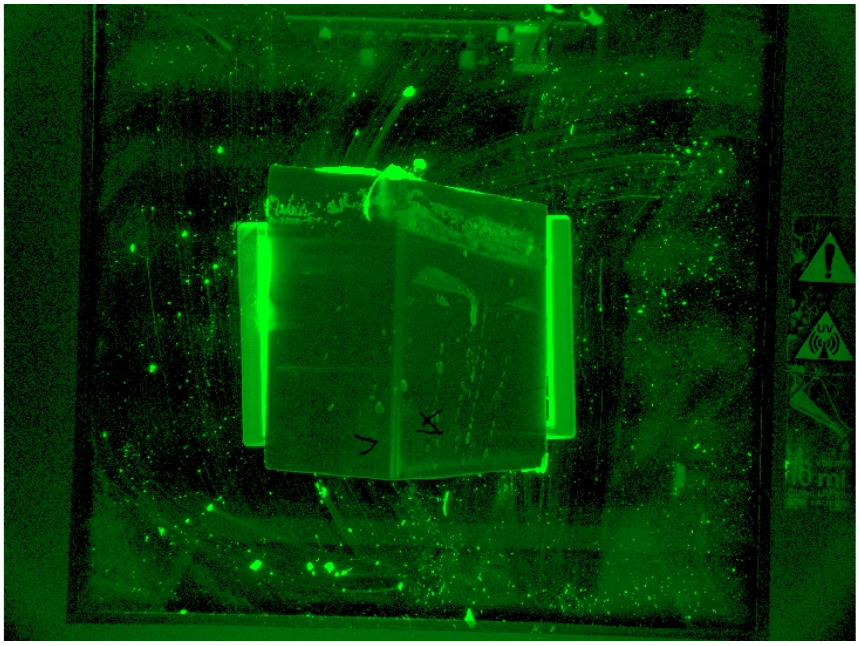

Figure 5.24: Surface on right side was cleaned with water control test, while surface on left side cleaned with tools.

Before cleaning - Test sample 10

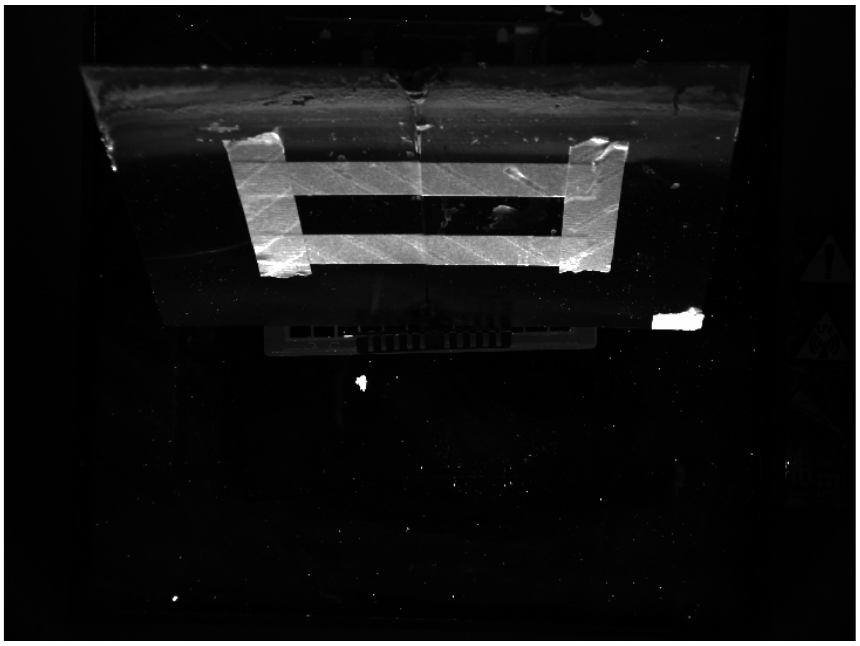

Figure 5.25: Before water control test.

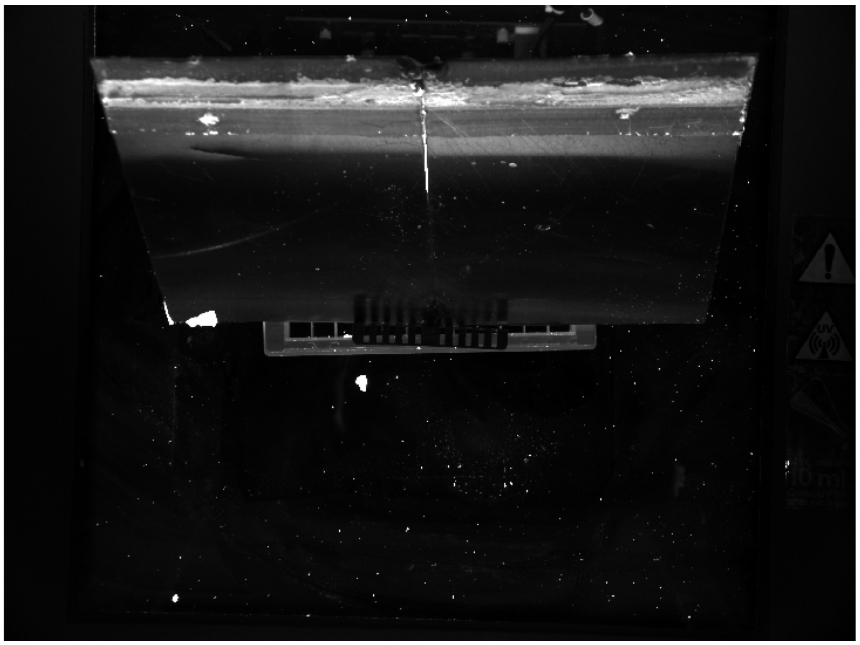

Figure 5.26: Before cleaning test.

After cleaning - Test sample 10

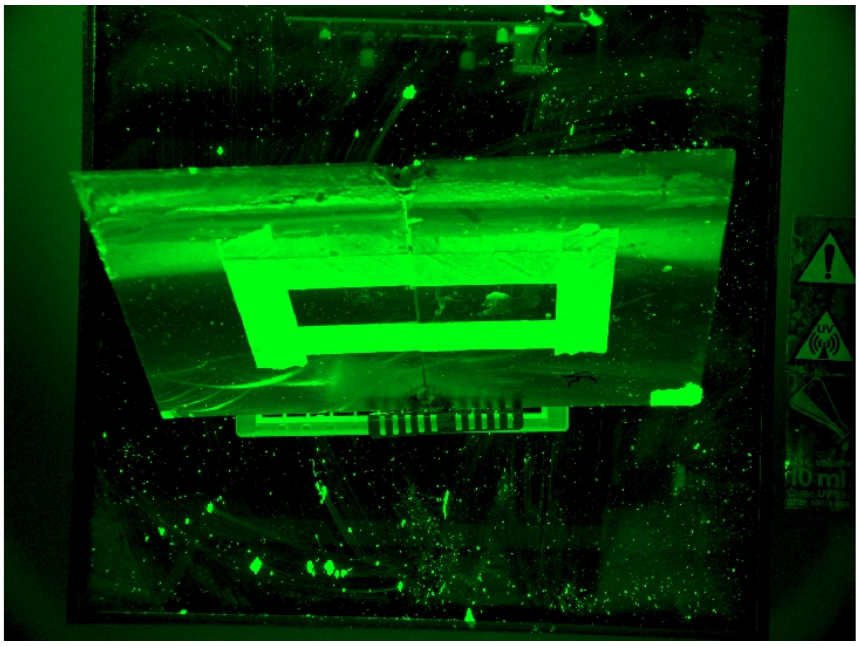

Figure 5.27: After water control test.

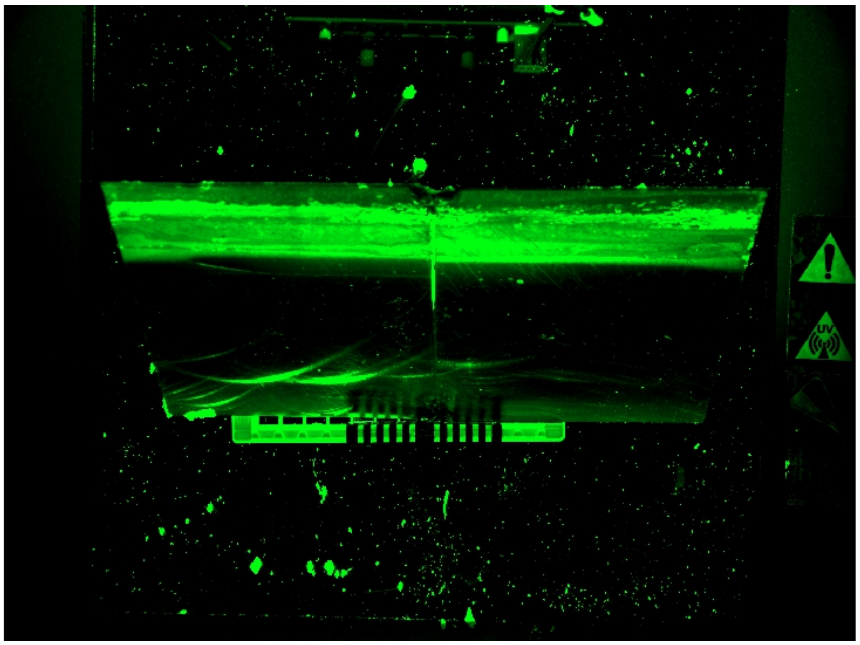

Figure 5.28: After cleaning test.

Before cleaning - Test sample 11

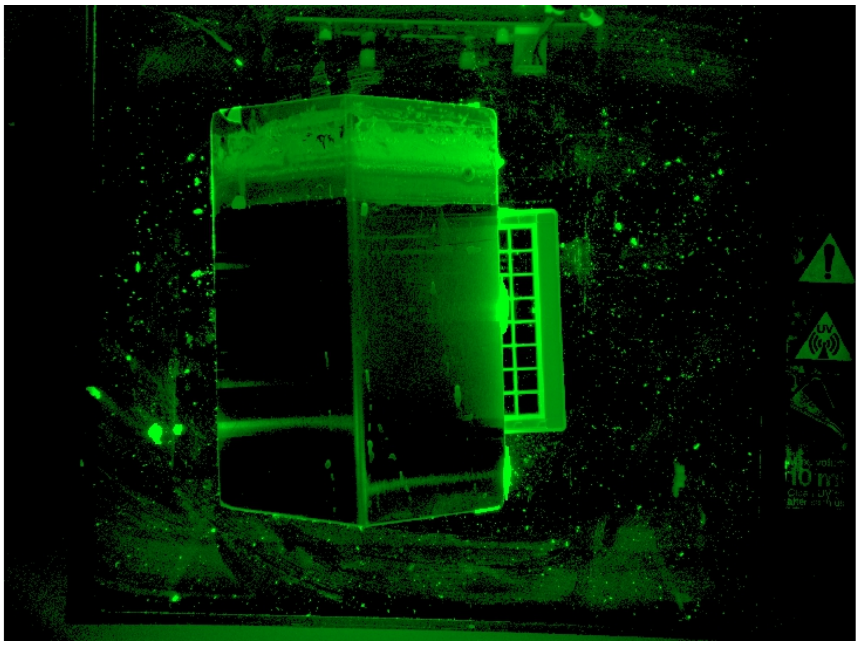

Figure 5.29: Before cleaning experiment.

After cleaning - Test sample 11

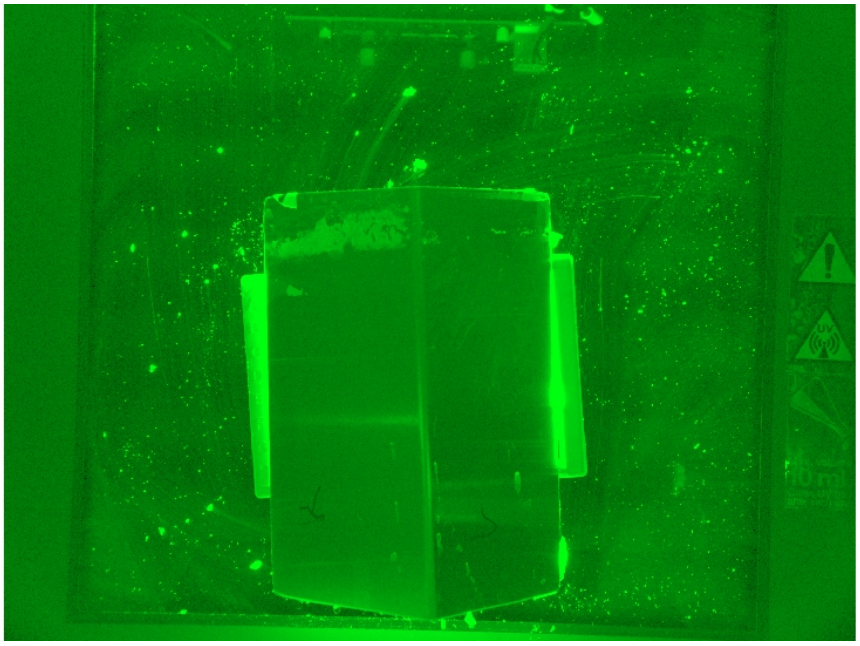

Figure 5.30: Write here.

Before cleaning - Test sample 12

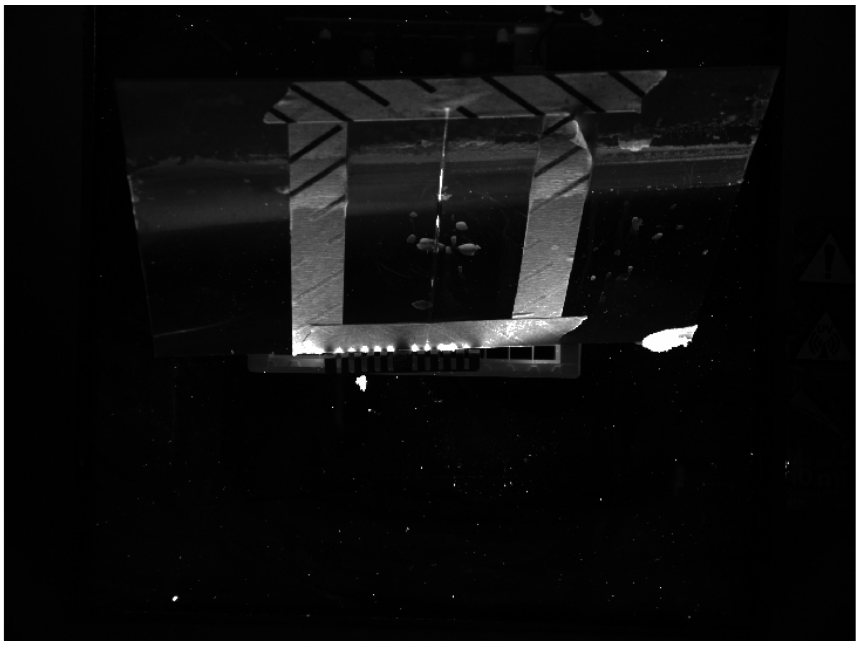

Figure 5.31: Before water control test.

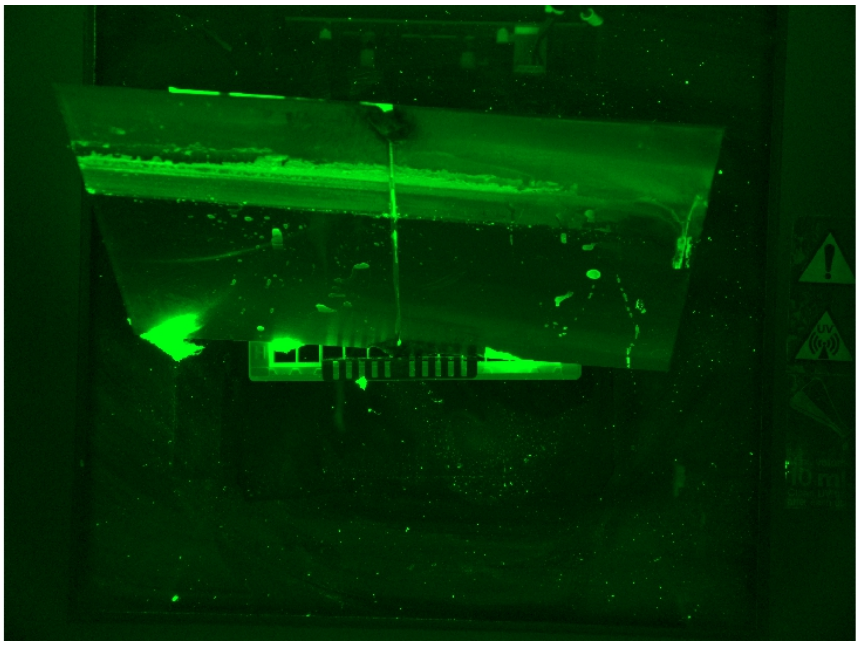

Figure 5.32: Before cleaning test.

After cleaning - Test sample 12

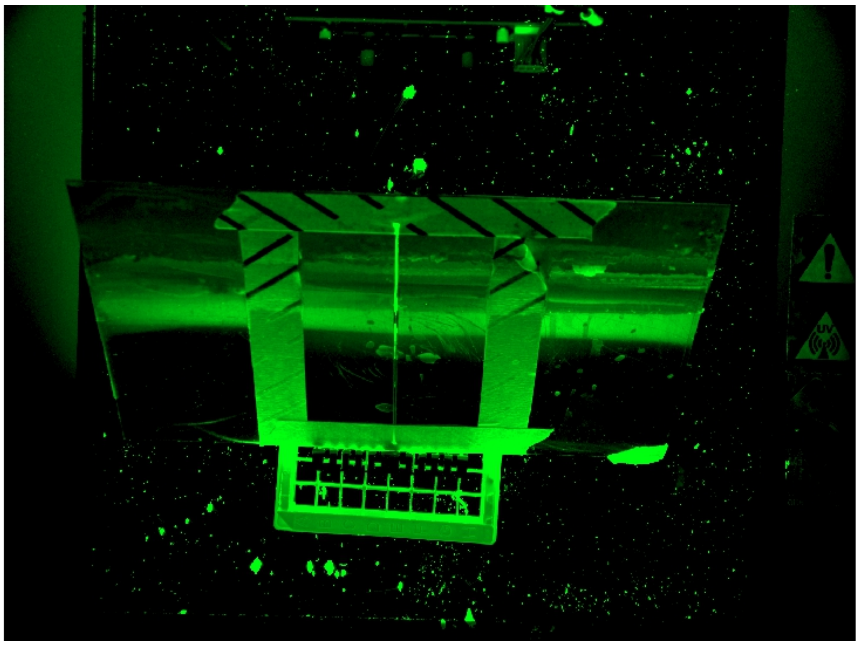

Figure 5.33: After water control test.

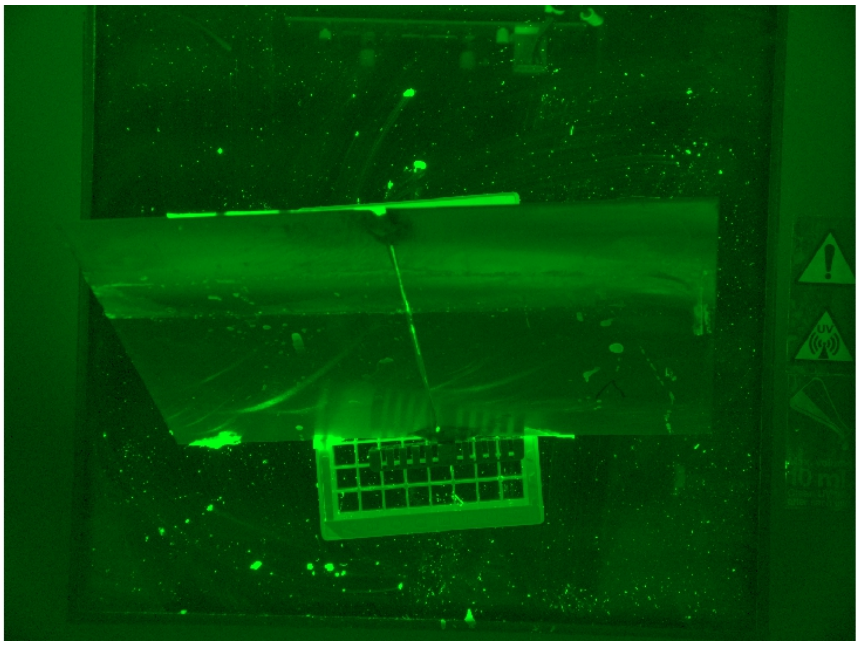

Figure 5.34: After cleaning test.

Before cleaning - Test sample 13

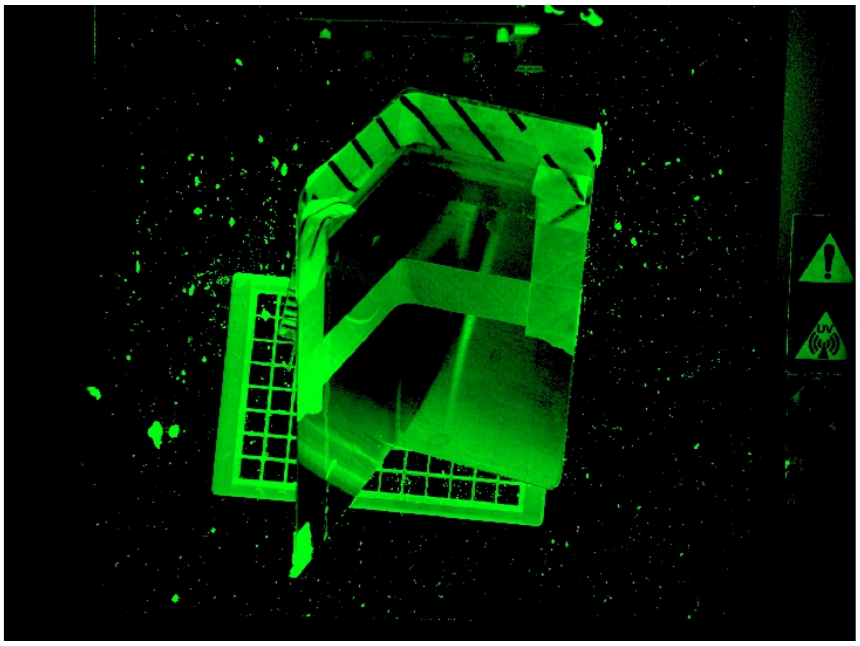

Figure 5.35: Before water control test.

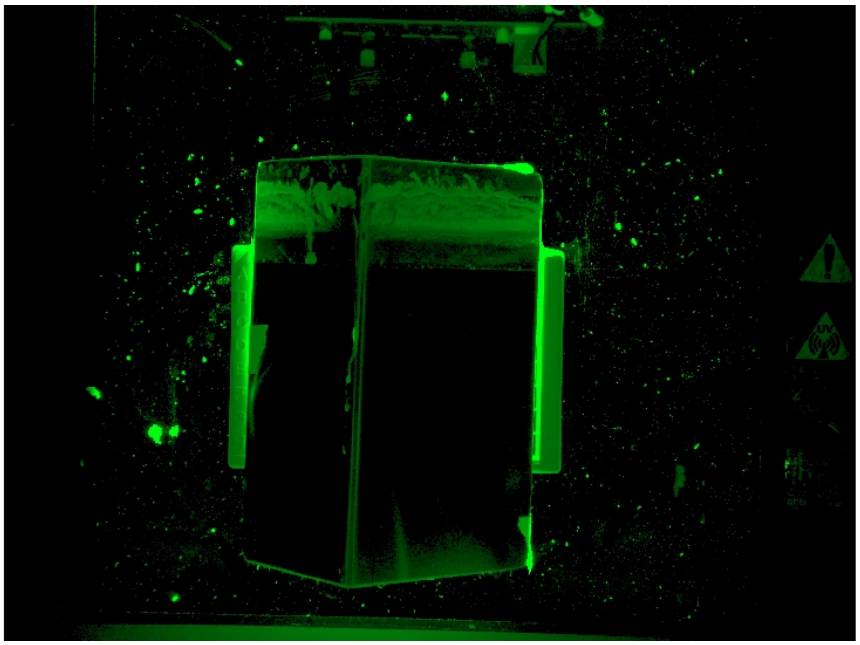

Figure 5.36: Before cleaning test.

After cleaning - Test sample 13

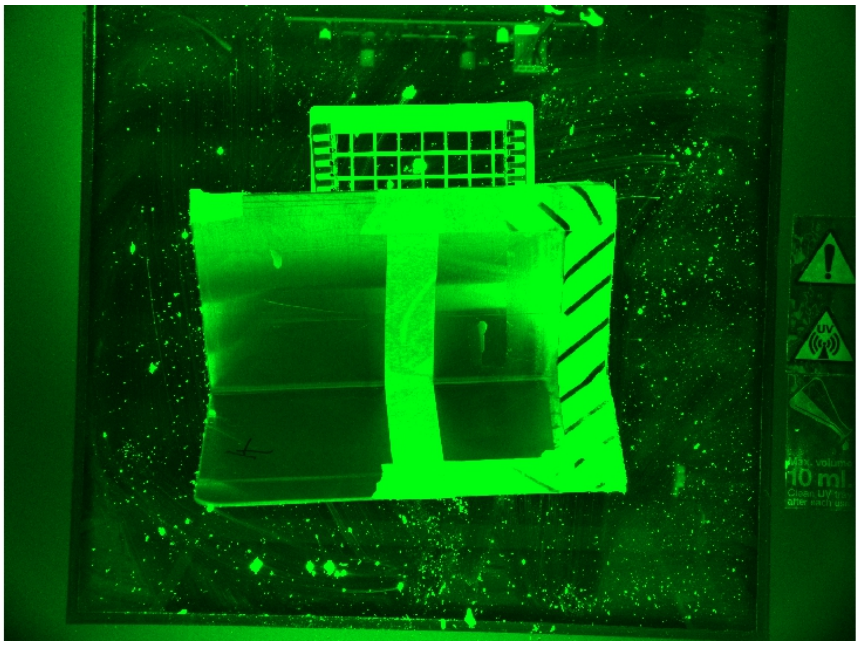

Figure 5.37: After water control test.

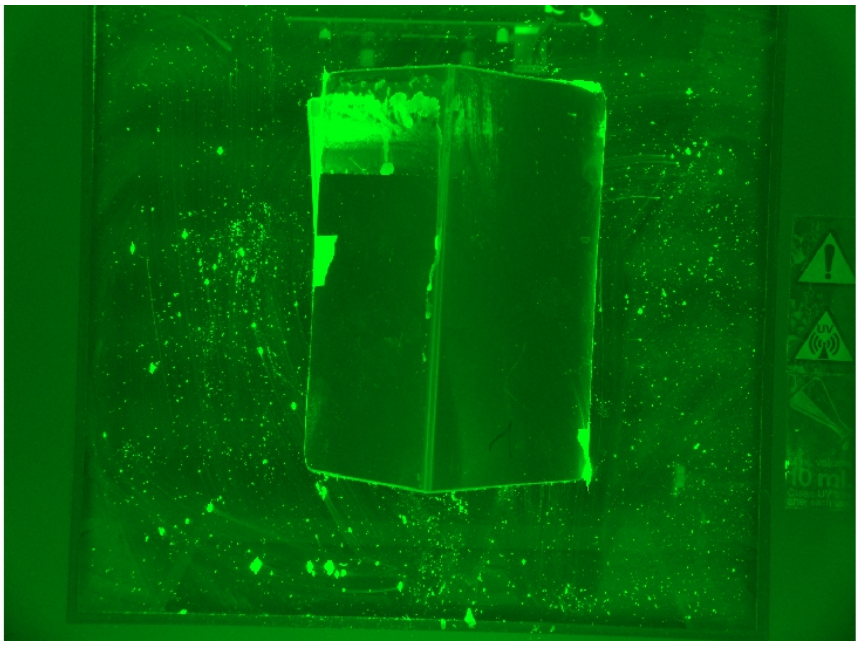

Figure 5.38: After cleaning test.

Before cleaning - Test sample 14

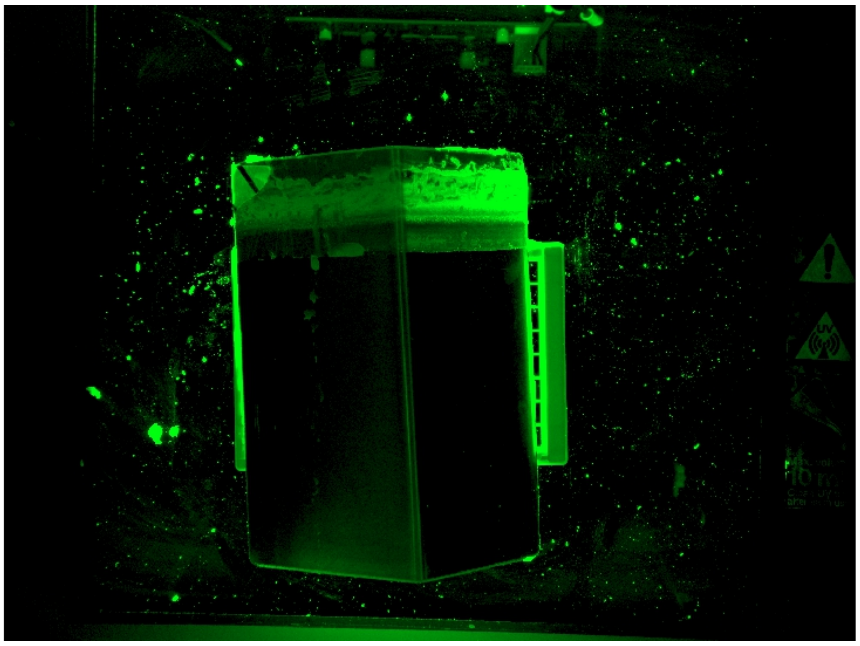

Figure 5.39: Before cleaning experiment.

After cleaning - Test sample 14

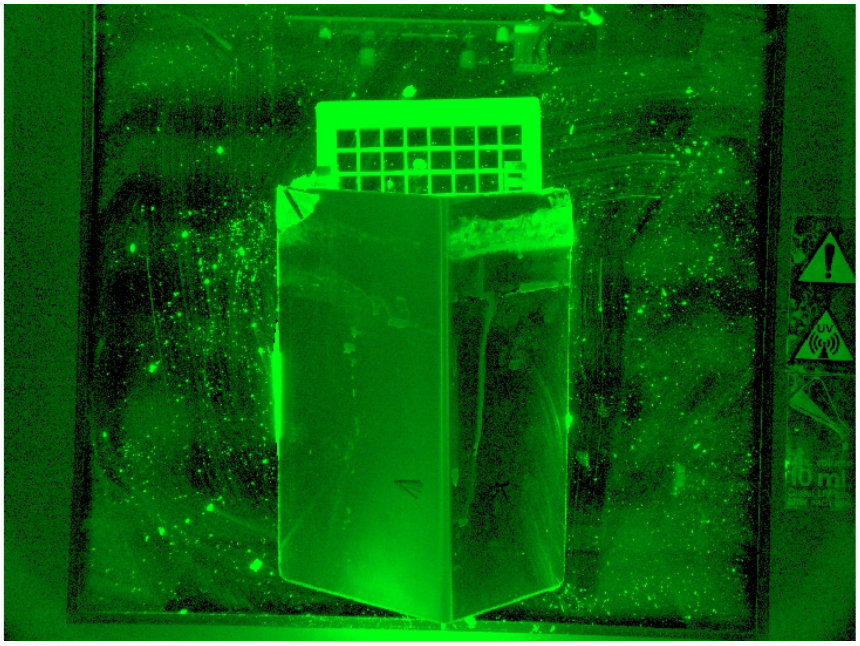

Figure 5.40: Surface on right side was cleaned with water control test, while surface on left side cleaned with tools.

Before cleaning - Test sample 15

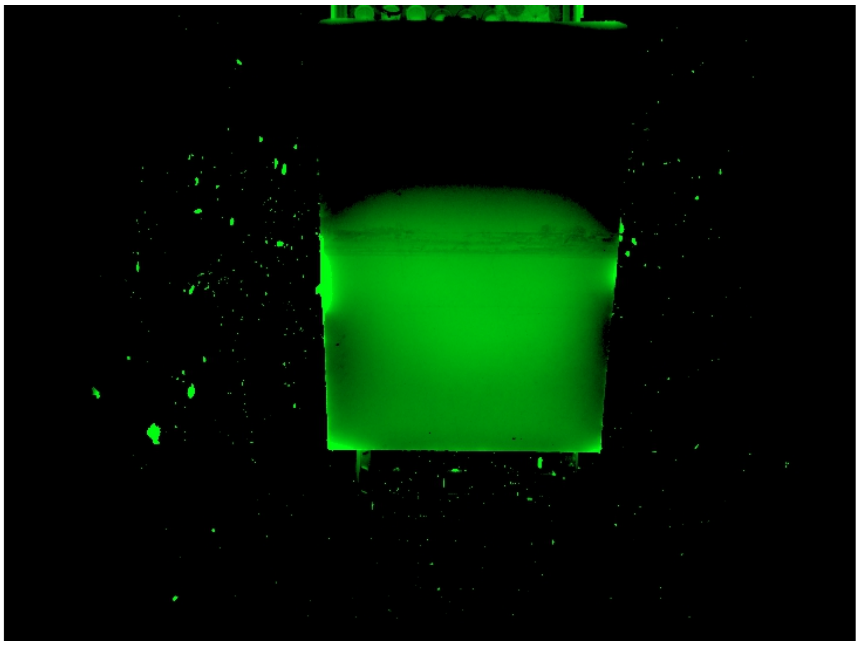

Figure 5.41: Before water control test.

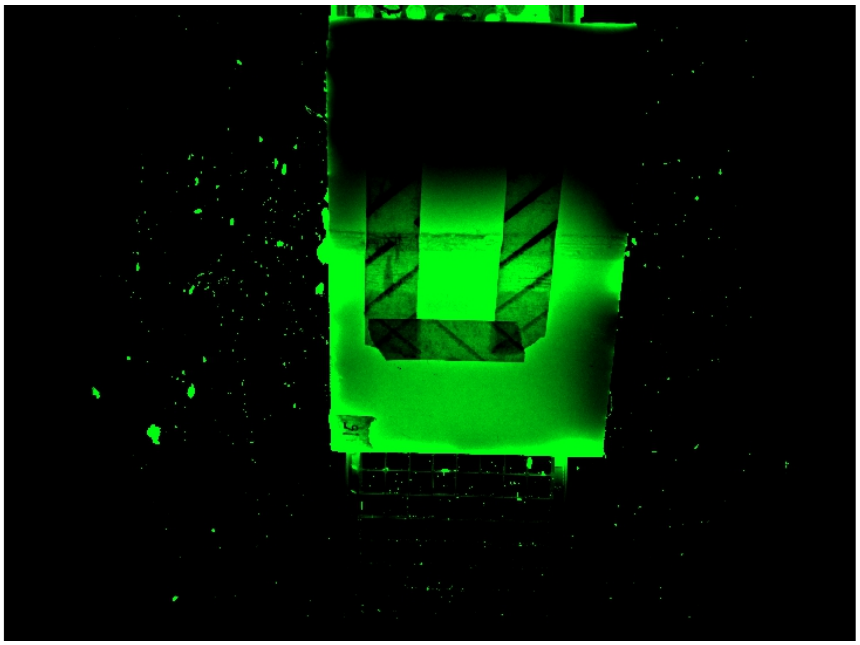

Figure 5.42: Before cleaning test.

After cleaning - Test sample 15

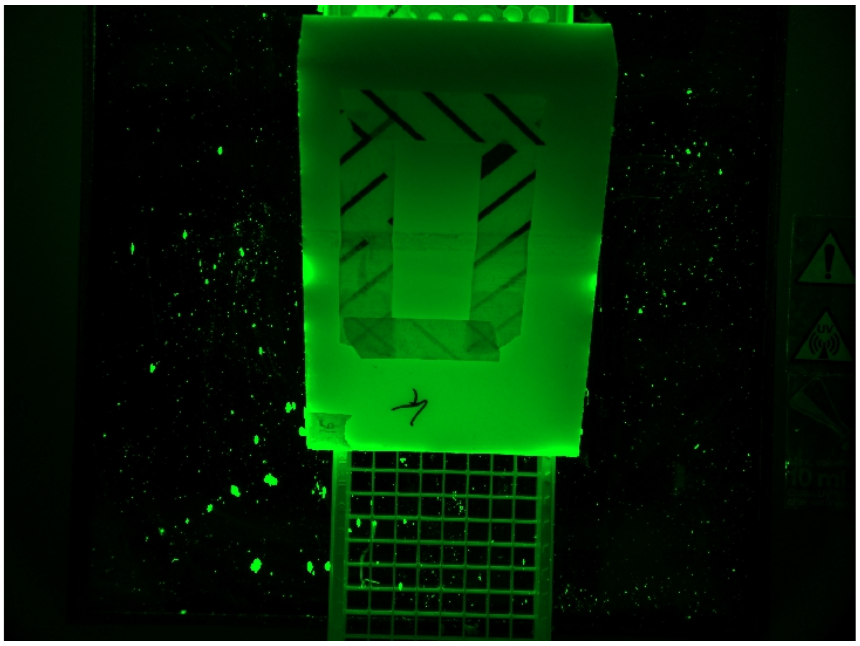

Figure 5.43: After water control test.

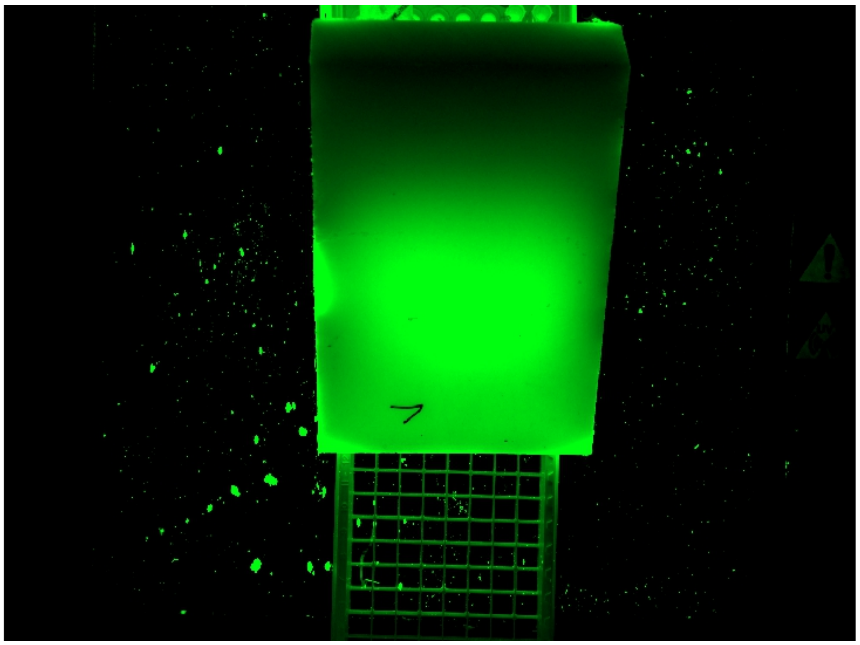

Figure 5.44: After cleaning test.

Before cleaning - Test sample 16

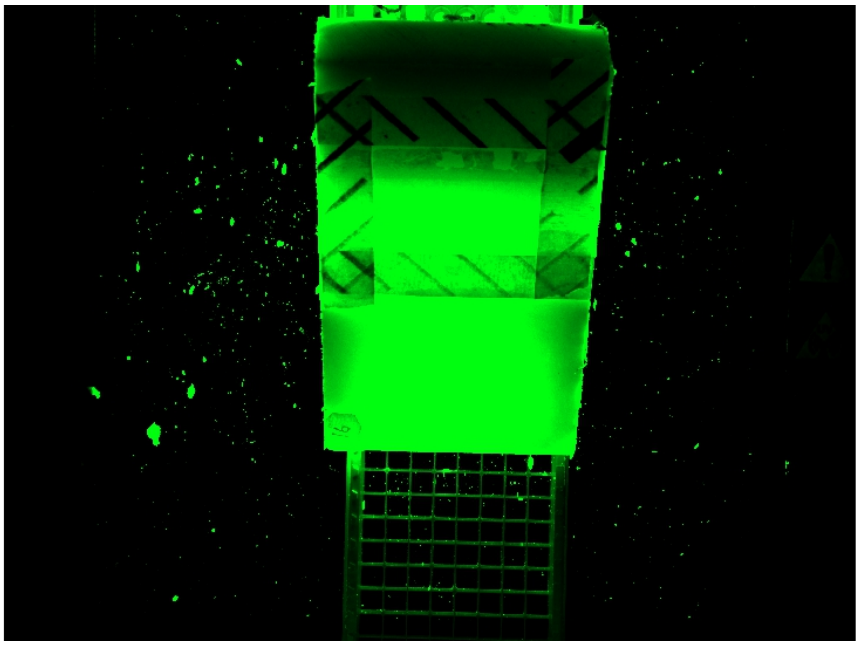

Figure 5.45: Before water control test.

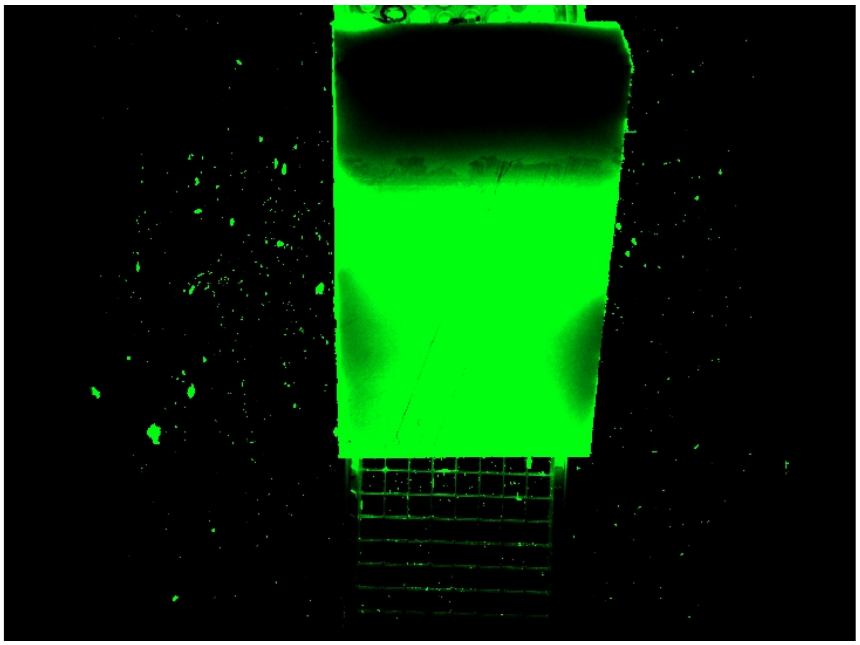

Figure 5.46: Before cleaning test.

After cleaning - Test sample 16

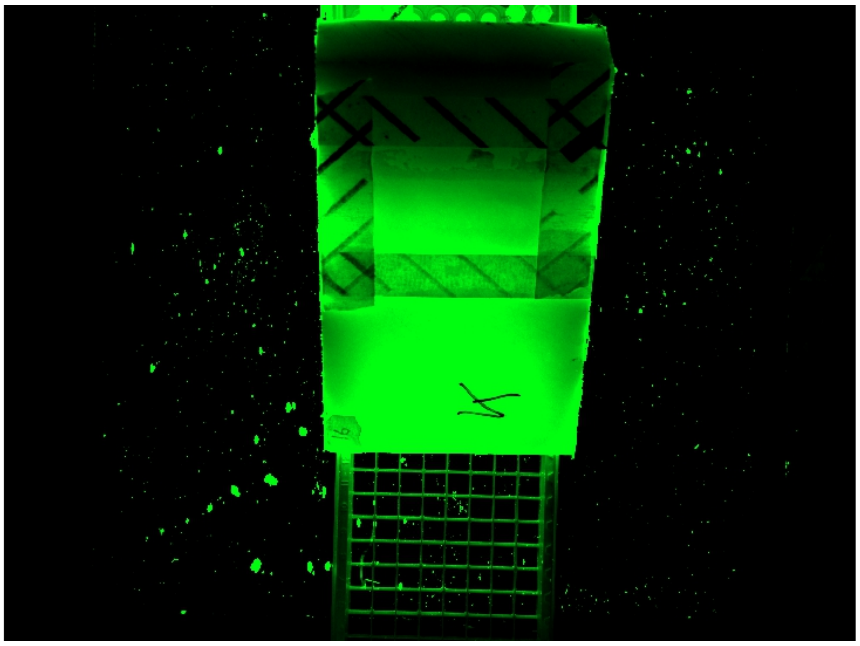

Figure 5.47: After water control test.

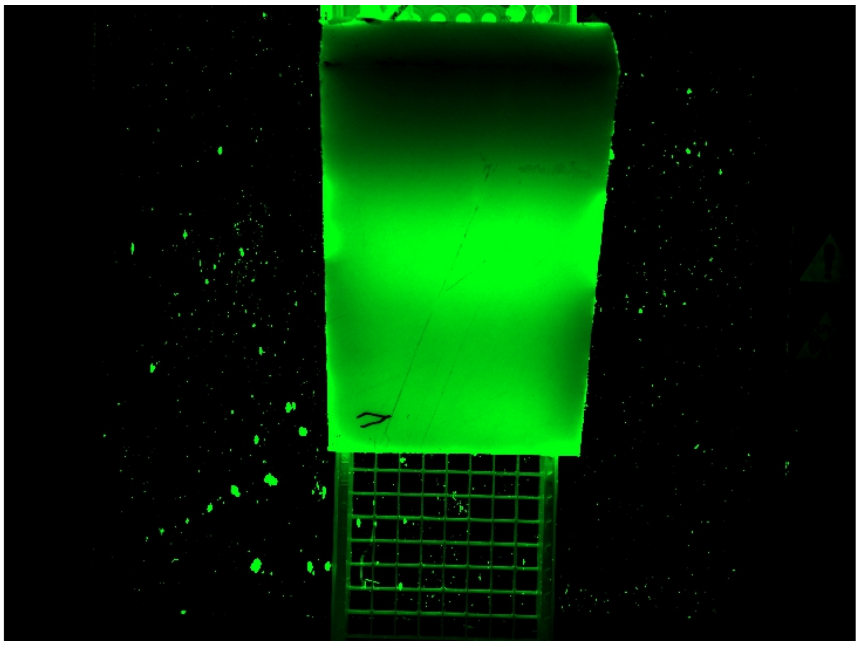

Figure 5.48: After cleaning test.

Before cleaning - Test sample 17

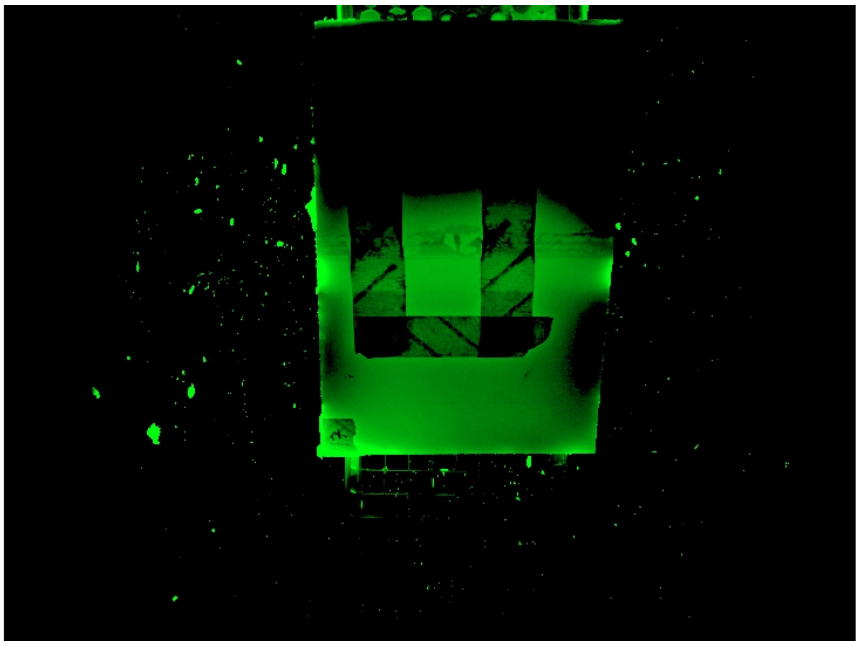

Figure 5.49: Before water control test.

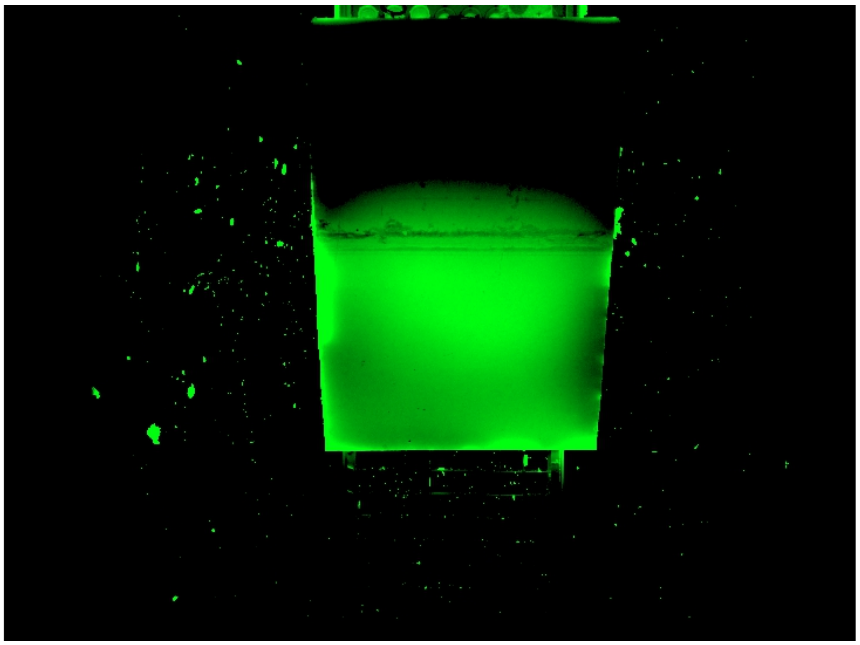

Figure 5.50: Before cleaning test.

After cleaning - Test sample 17

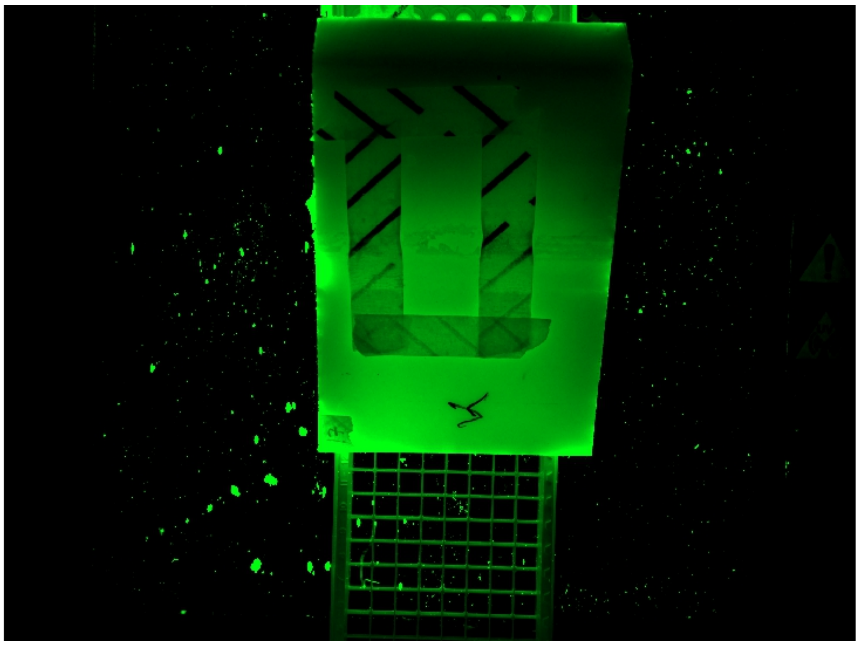

Figure 5.51: After water control test.

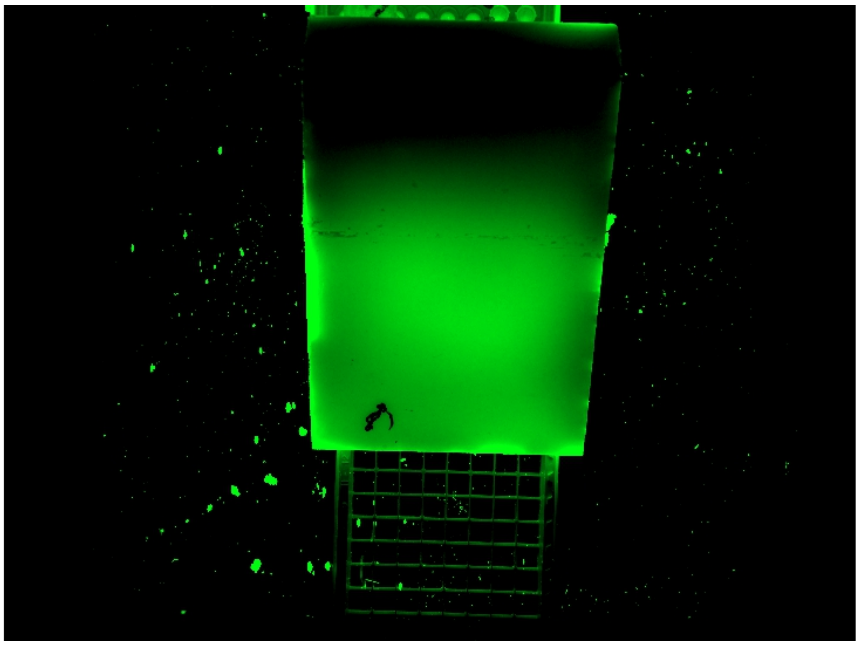

Figure 5.52: After cleaning test.

Before cleaning - Test sample 18

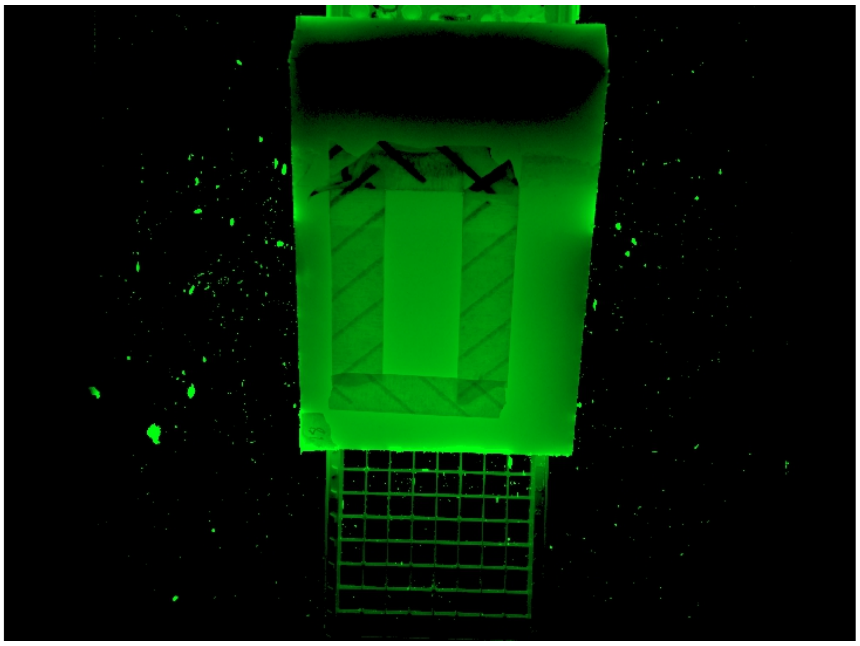

Figure 5.53: Before water control test.

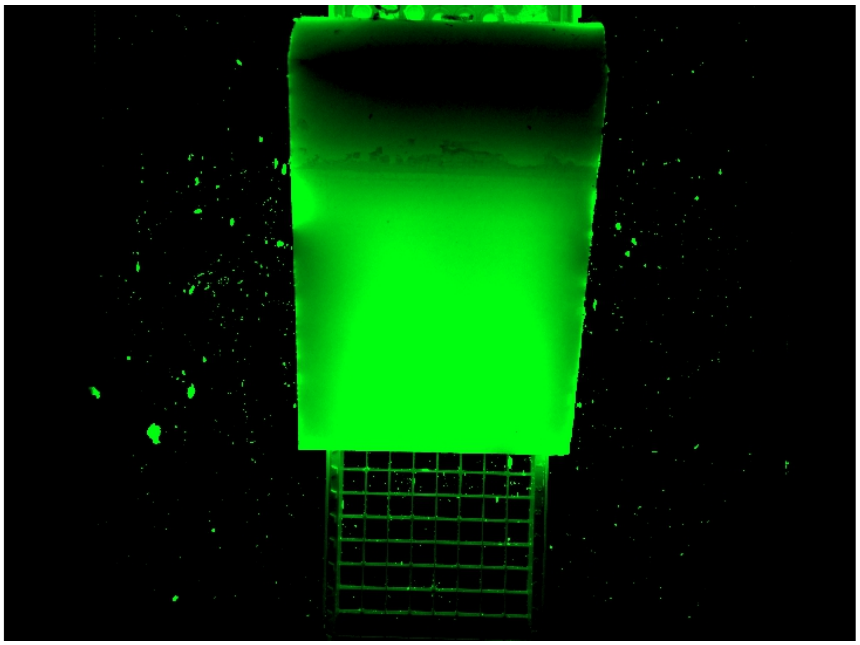

Figure 5.54: Before cleaning test.

After cleaning - Test sample 18

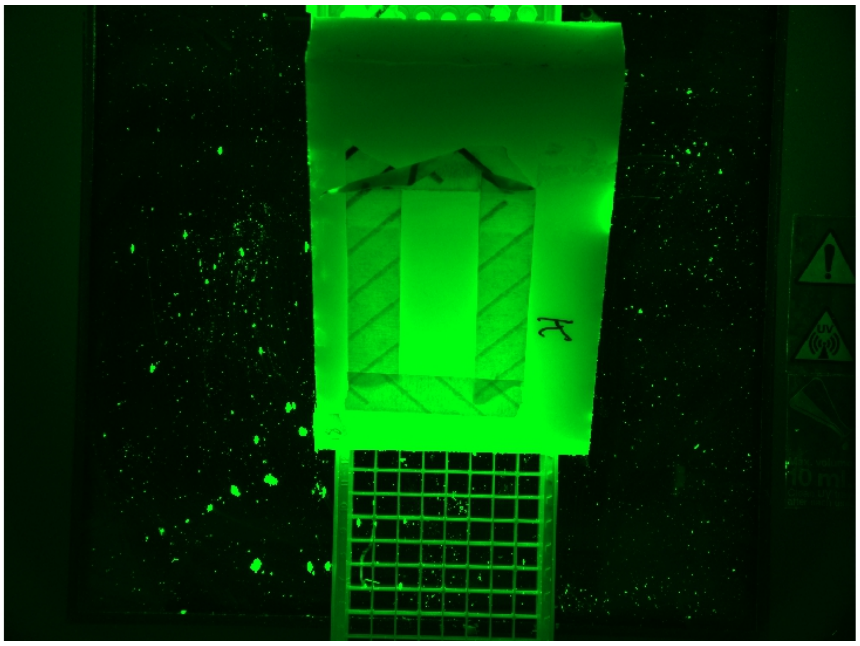

Figure 5.55: After water control test.

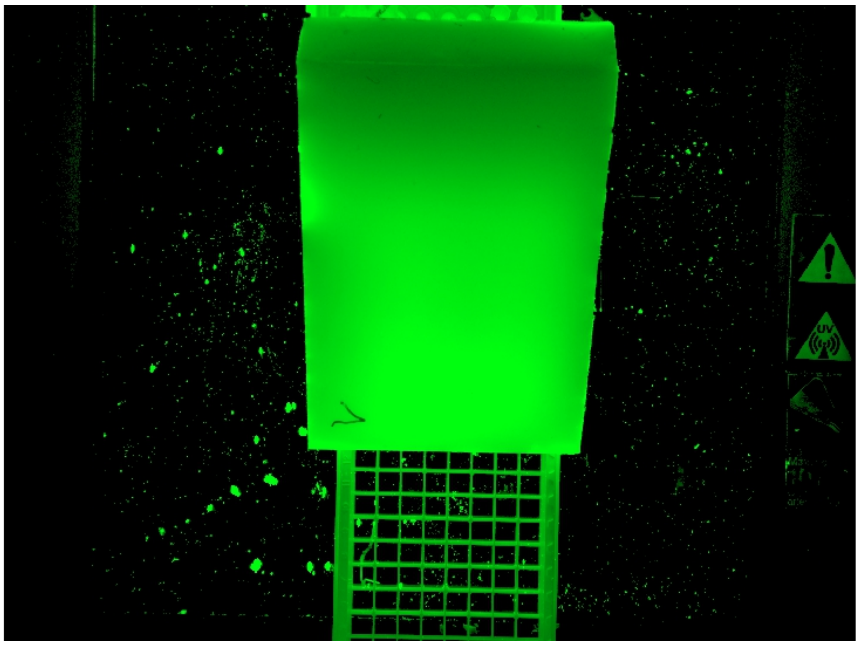

Figure 5.56: After cleaning test.

Before cleaning - Test sample 19

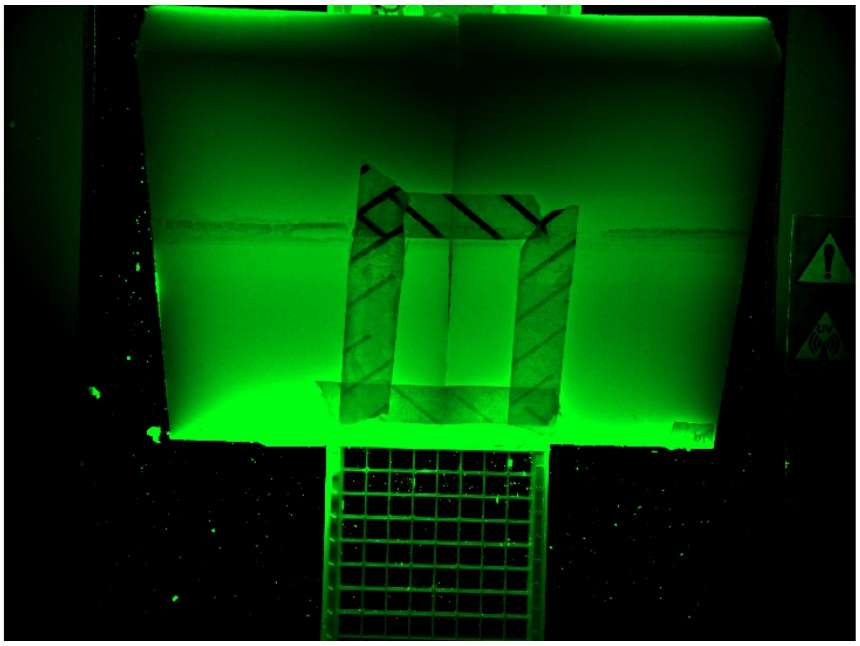

Figure 5.57: Before cleaning experiment.

After cleaning - Test sample 19

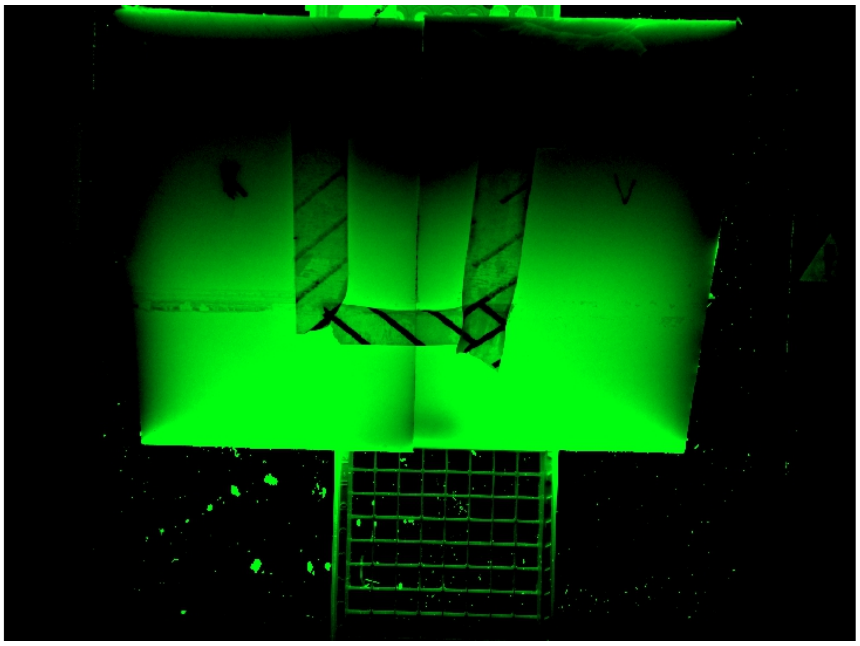

Figure 5.58: Surface on left side was cleaned with water control test, while surface on right side cleaned with tools.

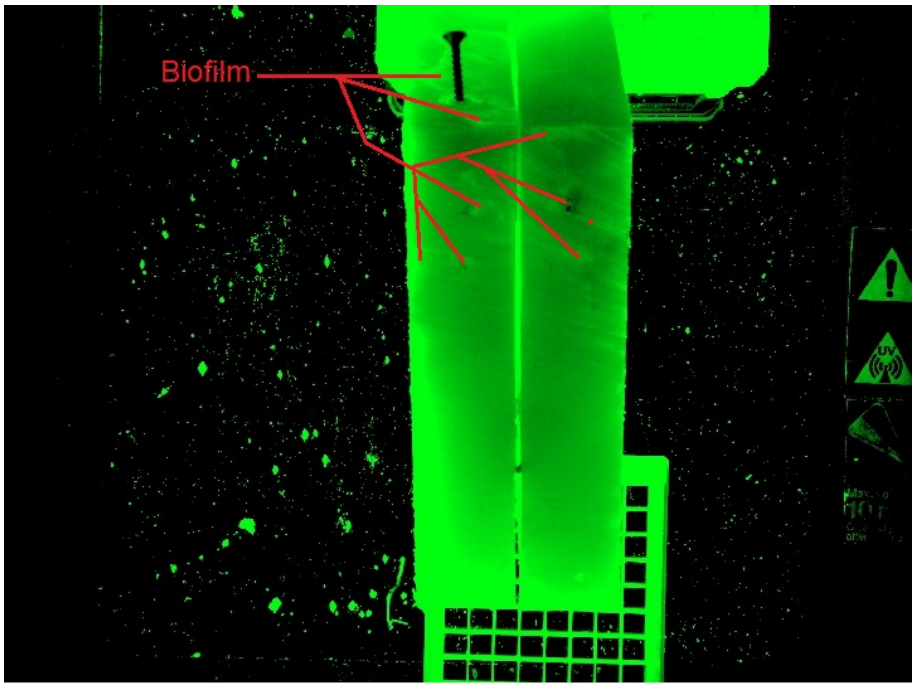

Figure 5.59: Shows the cross section between the two plates where biofilm has grown.

Before cleaning - Test sample 20

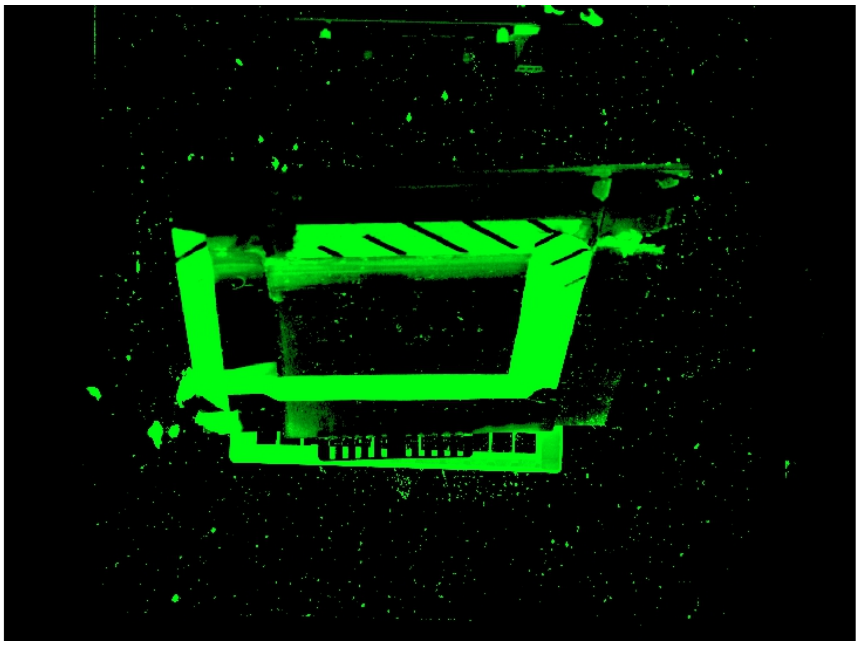

Figure 5.60: Before cleaning experiment.

After cleaning - Test sample 20

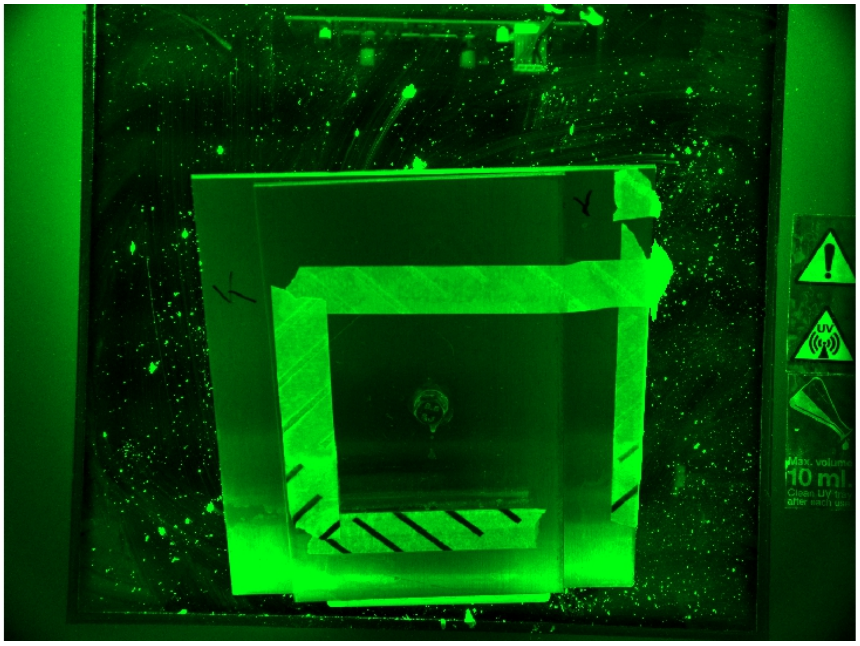

Figure 5.61: Shows the assembled plate after cleaning experiment.Left side with water test and right side with cleaning test..

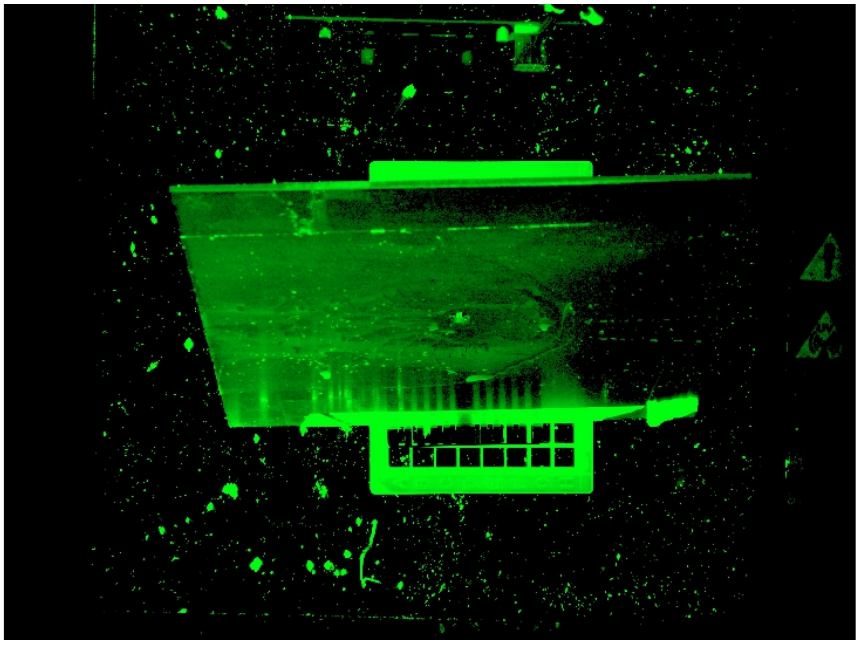

Figure 5.62: Shows the inside of the assembled sample that was connected to another plate with a bolt and nut. Here we can see the whole inside is covered in biofilm.

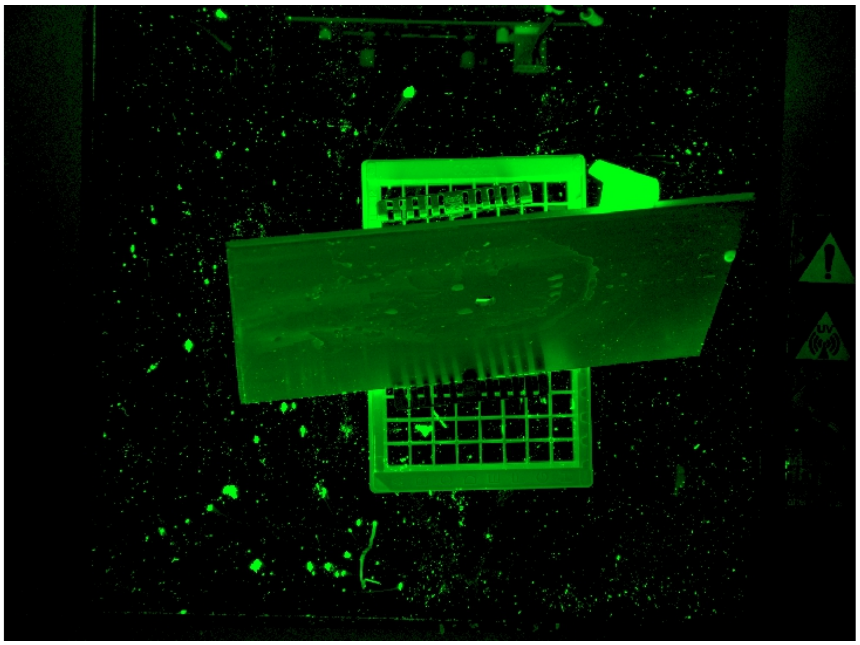

Figure 5.63: Shows the inside of the assembled sample that was connected to another plate with a bolt and nut. Here we can see the whole inside is covered in biofilm.