Detection of the Fish Pathogen *Aeromonas salmonicida* via Quantitative real-time PCR for Aquaculture

Author: Thanuragavi Thamotharaprasath

Bioengineering B.Sc.
27863

Supervisors:

Prof. Dr. Mònica Palmada Fenés (HSRW)
Dr. Florian Borutta (TunaTech GmbH)

Rhine-Waal University of Applied Sciences
Marie-Curie-Straße 1
47533 Kleve, Germany

TunaTech GmbH
Merowingerplatz 1A
40225 Düsseldorf,
Germany

Abstract

PHOTO-SENS is a project that aims to develop photonic biosensing chips as a means of allowing onset detection of pathogenic infections in aquaculture. These chips are meant to be a way for quick and simple routine testing that would enable aquaculture facilities to recognize potential outbreaks and minimize their effects. The chips detect target bacteria using short DNA sequences known as probes which are complementary to target DNA. One of the fish pathogens under consideration for the course of this project is *Aeromonas salmonicida,* that is a causative agent of Furunculosis.

For this work, a primer and probe set specific to *A. salmonicida* was designed. These primers and probes were then tested via qPCR for their selectivity and sensitivity and most importantly, quantification of DNA in samples using two different standard dilution series. Due to *A. salmonicida* being a Gram-negative bacterium, three methods of DNA extraction were used and compared for maximum efficiency. Moreover, experiments were also conducted to mimic capture of eDNA (environmental DNA) from highly diluted water samples.

Upon comparing the three DNA extraction methods, the improved 5% Chelex protocol that included rapid agitation via glass beads, yielded the highest amount of DNA that was closest to expected SQ. The detection of all samples, including very low copy number, was possible via qPCR. The first standard series which was the gBlocks standard series had the lowest detection limit of 1 copy with an efficiency of 105.8% and the second standard series that is the CFU standard series had a detection limit of 10 copies and had an efficiency of 104.2%. The filtration experiment also showed detection in diluted, filtered samples.

Since detection using the designed probe was successful, the probes are therefore considered suitable for detection of *Aeromonas salmonicida* in the PHOTO-SENS project.

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List of Abbreviations

aZMI: asymmetric Mach–Zehnder interferometer
bp: Base pairs
CA-filters: cellulose-acetate filters
CFU: Colony forming unit
Cq-value: Quantification cycle value
CSP: Cell surface protein
ddPCR: drop digital PCR
dNTP: Nucleoside triphosphate
dsDNA: double stranded DNA
eDNA: environmental DNA
FAO: Food and Agriculture Organization of the United Nations
LOD: Limit of Detection
MALDI-TOF-MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
NTC: No template controlOD: Optical Density
PCR: Polymerase Chain Reaction
PFGE: Pulse-field gel electrophoresis
PPP: Pathogens, parasites and pests
qPCR: quantitative real-time Polymerase Chain Reaction
RAPD: Random amplified polymorphic DNA
rpm: Rounds per minuterRNA: Ribosomal RNA
RT-qPCR: reverse transcriptase
qPCRSD: serial dilution
SQ: Starting quantity
ssDNA: single stranded DNA

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1. Introduction

1.1. Significance and Role of Aquaculture in Global Scale

From 1961 onwards, there has been a consistent rise in global food fish consumption. According to the Food and Agriculture Organization of the United Nations, the annual rate of increase until 2017 was nearly double that of the world population growth during the same period (FAO) [2020]. It is one of the fastest-growing animal food production sectors all over the world (Marco Ottinger, 2015) and is continuously increasing to become the main source of aquatic animal food in human consumption, currently accounting for 47% ofthe world's fish supply (Leung & Bates, 2013). Aquaculture is the controlled process of cultivating aquatic organisms, especially for human consumption. It's a similar concept to agriculture, but with fish instead of plants or livestock (gsf). According to the Food and AgricultureOrganization of the United Nations [FAO], 56% of the aquatic animal food productionavailable for human consumption was provided by aquaculture in the year 2020 and by 2030, 62 percent of all seafood produced for human consumption will come from aquaculture (FAO, 2022).

In recent decades, aquatic foods have experienced a surge in popularity for several reasons, including their high accessibility, affordability, and richness in animal protein and micronutrients. This is a major factor contributing to the significant nutritional role fish plays in the diets of people, particularly in developing nations. In certain coastal and island countries like Bangladesh, Indonesia, Senegal, and Sri Lanka, fish accounts for over 50 percent of all consumed animal protein. Additionally, most individuals engaged in aquaculture are found in developing countries such as European Union, the United States of America and Japan (Finegold, 2018), with Asia standing out as the primary region for aquatic farming. Also, China leads in total aquaculture production, with other Asian countries such as India, Indonesia, Vietnam, and Bangladesh following closely, as illustrated in Figure 1 (Marco Ottinger, 2015).

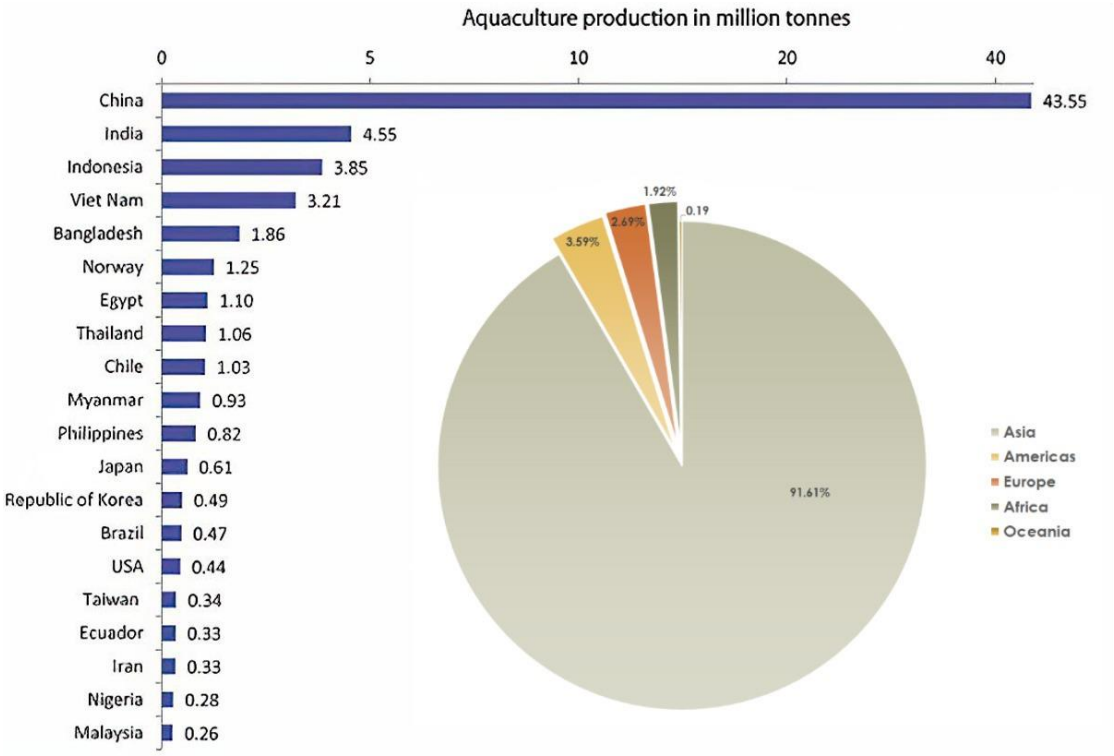


Figure 1: Global total aquaculture production output in 2013. (1) Bar chart: ranking the top 20 countries with highest aquaculture production in 2013. (2) Pie chart: share of total aquaculture production output among continents in 2020. Source: (Marco Ottinger, 2015), (FAO ,2022)

Despite the substantial output of aquatic food items, the persistent increase in demand has led to the over-intensification of production and trade in fish farming. The rapid expansion of the aquaculture industry has rendered it more susceptible to various stressors, including the adverse impacts of pathogens, parasites, and pests (PPP), pollution, and climate change, as highlighted by (Naylor *et al*., 2021).

The hurdles to establishing a sustainable aquacultural industry, while ensuring food security, persist today and have become even more pronounced in recent years.

1.2. Sustainable development in Aquaculture

Numerous initiatives have been undertaken to guide aquaculture and fish farming towards sustainable practices, with the overarching goal of enhancing the aquatic food industry's role in promoting global food security, nutrition, and accessibility to affordable and healthful diets for everyone (FAO, 2022). Considerable focus has been directed towards ecosystem-based management and enhanced system configurations, including sustainable aquacultural systems and offshore aquaculture. Sustainable aquaculture systems play a pivotal role in mitigating the impacts of pollutants and climate change, while offshore aquaculture facilitates high-volume fish production in deep waters without jeopardizing freshwater and coastal environments through issues like nutrient pollution. Additionally, effective governance and policies have emerged as crucial instruments in fostering a sustainable trajectory for aquaculture, aligning with the imperative of meeting the increasing demands of the global population (Naylor *et al*., 2021).

1.3. Fish Pathogens affecting Aquaculture.

With the growth and deepening of aquaculture, the impacts of pathogens, parasites, and pests (PPP) have evolved into chronic issues that are significantly magnified. To address these risks within production systems, various practices are commonly implemented, including but not limited to species rotation, enhanced feed quality, maintenance of pond and cage cleanliness, monitoring and removal of parasites, and the implementation of improved surveillance systems (Naylor *et al*., 2021).

Assessing the complete impact of fish pathogens on aquaculture is challenging due to inadequate data, but economic losses in finfish aquaculture are estimated to range from 1.05 to 9.58 billion US dollars annually (Tavares-Dias and Martins, 2017). Disease outbreaks are facilitated by "predisposing factors," often resulting from suboptimal handling conditions and stressed fish (Plumb and Hanson, 2010).

Nevertheless, infectious diseases induced by pathogens have emerged as the predominant cause of mortality in farmed fish (Leung & Bates, 2013). The occurrence

of these outbreaks can be attributed to diverse stress factors affecting fish, including issues like overfeeding and overcrowding, which contribute to water pollution and degraded water quality. Additionally, climate change is identified as another factor contributing to the regularity of pathogen outbreaks in fish farms, as alterations in temperature and precipitation create favorable conditions for pathogen proliferation (Leung & Bates, 2013).

Table 1 shows a few of the mostimportant bacterial pathogens in aquaculture, their main host fish and the diseases caused.

Table 1: Major bacterial pathogens of economically important fish. Source: (Sudheesh *et al*., 2012)

Causative Agent	Disease	Main host fish
Gram-negatives		
Vibrio anguillarum	Vibriosis	Salmonoids, turbout, seabass, striped bass, eel,cod, and red sea bream
Moritella viscosa	Winter ulcer	Atlantic salmon
Phytobacterium damselae subsp.piscicida (formerly Pasteurella piscicida)	Photobacteriosis (pasteurellosis)	Sea bream, sea bass, sole,striped bass, and yellowtail
Flavobacterium psychrophilum	Coldwater disease	Salmonids, carp, eel,tench, perch, ayu
Flavobacterium columnare	Colimnaris disease	Cyprinids, salmonids, silurids, eel, and sturgeon
Aeromonas salmonicida	Furunculosis	Salmon, trout, goldfish, koi and a

Yersinia ruckeri	Enteric redmouth	variety of other fish species Salmonids, eel, minnows, sturgeon, and crustaceans
Gram-positives		
Lactococcus garvieae (Enterococcus seriolicida)	Streptococcosis/ Lactococcosis	Yellowtail and eel
Streptococcus iniae	Streptococcosis	Yellowtail, flounder, seabass, and barramundi
Renibacterium salmoninarum	Bacterial kidney disease	Salmonoids
Mycobacterium marinum	Mycobateriosis	Sea bass, turbot, and Atlantic salmon
Vagococcus salmoninarum	Coldwater streptococcosis (Vagococcosis)	Salmonoids, rainbow trout,brown trout

The aquaculture sector employs diverse strategies to address instances of pathogen outbreaks, including the utilization of therapeutants and chemical substances for prevention and treatment. However, the application of these methods carries the potential for significant health risks to consumers, workers, fish, and the surrounding ecosystem if not used appropriately. Likewise, the widespread use of antibiotics poses challenges, as their misuse may contribute to the emergence and transfer of antibiotic-resistant bacteria (Naylor *et al*., 2021). This not only increases the severity of pathogen outbreaks but is also a hazard for humans as the transfer of antibiotic-resistant bacteria to humans may also occur (Adams & Gunn, 2017).

Pathogen outbreaks in the aquaculture industry can result in significant financial

ramifications. Substantial losses often ensue due to elevated mortality rates, amplifying the financial burden associated with disease control efforts (Adams & Gunn, 2017). While alternatives such as vaccines and selective breeding have been identified, these approaches tend to be relatively costly and challenging to replicate across different species (Adams & Gunn, 2017; Naylor *et al*., 2021).

Due to this, disease outbreak is one of the major barriers towards a sustainable growth of aquaculture and has therefore been deemed as the 'global aquaculture disease crisis' (Bouwmeester *et al.*, 2020).

1.4. Bacterium – *Aeromonas salmonicida*

Aeromonas salmonicida is the oldest known infectious agent to be linked to fish disease and constitutes a major bacterial pathogen of fish, in particular of salmonids. This bacterium can be found almost worldwide in both marine and freshwater environments and has been divided into several sub-species (Menanteau *et al.*, 2016). *Aeromonas salmonicida* subsp. salmonicida is an important bacterial fish pathogen, which was originally isolated at a German freshwater farm by Emmerich and Weibel (1894) and was given the name Bacterium salmonicida. Subsequently it was proposed by Griffin *et al*. (1953) to place the bacterium in the genus Aeromonas and re-classify the name of the species as *Aeromonas salmonicida* (Snieszko, 1957). The genus of Aeromonas has also gone through many taxonomic re-classifications and was eventually placed in the family Aeromonadaceae by Colwell *et al*. (1986). Although the species of *Aeromonas salmonicida* was first thought to be homogenous, by use of biochemical and molecular methods it has thus far been divided into five subspecies: salmonicida, masoucida, achromogenes, smithia, and pectinolytica (Austin, 1993; Wiklund and Dalsgaard, 1998; Kozinska *et al*., 2002; Beaz-Hidalgo *et al*., 2008; Studer *et al*., 2013). The four latter subspecies all belong to the so called "atypical" group, while subspecies salmonicida is the only *Aeromonas salmonicida* known as "typical" and is the causative agent of furunculosis. This genus consists of Gram-negative, rod-shaped, oxidase-positive, facultative anaerobic bacteria that are widely distributed in the aquatic environment (Abhishek *et al.*, 2017).

1.5. Furunculosis and its Historical background

Furunculosis is now spread worldwide, though the first time furunculosis was observed and documented among fish was in 1894 by Emmerich and Weibel. They observed swellings resembling boils as well as ulcerative lesions in brown trout (Salmo trutta) at a German freshwater hatchery. After the initial description by Emmerich and Weibel (1894), furunculosis was believed to be a hatchery associated infection until the studies of Plehn (1911) showed that furunculosis was also present among wild trout in Germany and others also observed the infection in several countries all over the world, including Great Britain who suffered great losses (Fuhrman, 1909; Pittet, 1910; Surbeck, 1911; Arkwright, 1912; Mettam, 1915; Christensen, 1980). In the United States, furunculosis was first described by Marsh (1902) at hatcheries in Michigan. Shortly thereafter, the infection was found in numerous salmon and trout hatcheries throughout the United States (Fish, 1937; Smith, 1942).

The origin of furunculosis in the United States is uncertain, though the general theory is that either it was brought along with brown trout from Germany, or it spread from rainbow trout farmed in the Western part of the United States (Fish, 1937). Signs of furunculosis were also seen in several fish species in Canada by Duff and Stewart (1933) and various trout farms in Japan (Furunculosis committee, 1933). In Denmark furunculosis was first described in the 1950s at freshwater rainbow trout farms by Rasmussen (1964).

In parallel with this discovery, a massive expansion in rainbow trout production started that continued its growth even further as production became established in seawater in the 1970s (Christensen, 1980). Moreover, a Bayesian temporal tree based on SNP analysis of 101 sequenced *A. salmonicida* showed that there have been four main introductions of *A. salmonicida* in Denmark, two of which occurred approximately the same time as the first expansion in rainbow trout production (~ 1950) and the other two during the second expansion in seawater (~ 1970) (Manuscript III). At present, it is in the seawater production during elevated temperatures that furunculosis is of great concern and causes huge financial losses (Larsen and Mellergaard, 1981; Dalsgaard and Madsen, 2000; Pedersen *et al*., 2008).

1.6. Clinical signs of disease

Fish infected with *A. salmonicida* do not necessarily show any clinical signs of disease; however, when fish become stressed or are compromised in some way, such that their immune system is lowered and a favorable condition within the fish is created for the pathogen, the infection can spread throughout the body and clinical signs can become visible (Cipriano *et al*. 1997; Hiney *et al*., 1997; 19 Hiney and Olivier, 1999; Austin and Austin, 2007; Noga, 2010). Typical clinical signs of the infection can include lethargy, lack of appetite, skin hyperpigmentation, boils and/or ulcers on the skin, lesions, internal hemorrhaging, enlargement of the spleen, septicemia and anemia (Fig. 2) (McCarthy, 1977; Ferguson and McCarthy, 1978; McCarthy and Roberts, 1980; Hiney *et al*., 1997; Hiney and Olivier, 1999; Austin and Austin, 2007; Noga, 2010).

Figure 2. Rainbow trout with signs of furunculosis.
At the top: Boil and ulcer on the skin (photo by Morten Sichlau Bruun). To the left: Ulcer on the skin (Christensen, 1980). To the right: Enlargement of the spleen and hemorrhaging from internal organs (Photo by Morten Sichlau Bruun).

1.7. Antibiotic treatment

In Denmark the antibiotics used in aquaculture have been sulfadiazine, trimethoprim, oxytetracycline and furazolidone (Dalsgaard *et al*., 1994) and since 1986, the only antibiotics licensed for use in aquaculture have been sulfadiazine/trimethoprim and oxolinic acid. The prevalence of antibiotic resistance genes (ARGs) among *A. salmonicida* in Denmark has been low, 5% in the study by Dalsgaard *et al*. (1994) and 9% in Manuscript III. Nevertheless, repeated treatment with antibiotics has proven to have many drawbacks, including induction of drug resistance in microorganisms, suppression of the immune system in fish, accumulation of residues in the fish, sediment and surrounding environment of the fish farms (Rijkers *et al*., 1981; Jacobsen and Berglind, 1988; Björklund *et al*., 1990; Aoki, 1997; Sørum, 1998; Sørum, 1999; Muziasari *et al*., 2014). One major threat posed regarding antibiotic treatment is the ability of various genetic elements such as ARG 20 carrying plasmids (R plasmids) and integrons to disseminate multiple transferable ARGs (Aoki, 1997; L'Abée-Lund and Sørum, 2001; Berglund, 2015).

In the study by L'Abée-Lund and Sørum (2001), *A. salmonicida* and other bacteria originating from different locations around the world were investigated for the presence of a class 1 integron. Along with the integron, several ARGs were found: aadA2, dfr16, aadA1, dfrIIc, qacG, orfD, tetA and tetE, indicating that not only do class 1 integrons facilitate antibiotic resistance in marine environments, but also that ARGs can be transmitted between bacteria in various environments, since the found ARGs cassettes have also been associated with humans (L'Abée-Lund and Sørum, 2001). In agreement, Muziasari *et al*. (2014) found class 1 integrons and ARGs sul1, sul2 and dfrA1 in the sediment from farms located in the northern Baltic Sea and these same three ARGs, along with aadA2 and aadA1, were also found in Danish *A. salmonicida* isolated from furunculosis outbreaks (Manuscript III). Kadlec *et al*. (2011) moreover found both class 1 integrons and ARGs against sulfonamide, trimethoprim and other antibiotics among Aeromonas species from Germany, where the only antibiotic therapy of fish is a combination of the two mentioned antibiotics.

The greatest concern with broad host range conjugative plasmids is that they can transfer ARGs across different bacterial genera and similar R plasmids have been

isolated from separate ecological niches and across different environments (Sørum, 1998; L'Abée-Lund and Sørum, 2000; Sørum *et al*., 2003; Smillie *et al*., 2010). Sørum (1998) reported that after only 24 hours of mating between a fish pathogenic atypical Aeromonas carrying an R plasmid and Escherichia coli, the plasmid was directly transferred to every second E. coli cell. Direct transfer of the R plasmid from the atypical Aeromonas to human pathogens like Salmonella enteritidis and Salmonella typhimurium was also possible (Sørum, 1998). The atypical Aeromonas was also believed to be the origin of an R plasmid in *A. salmonicida* from a furunculosis outbreak (Sørum, 1998). Direct transfer of ARGs from pathogenic *A. salmonicida* to E. coli cells was also reported in the study by Aoki *et al*., (1971). In the whole genome sequencing (WGS) study, none of the 101 *A. salmonicida* that were sequenced harbored any of the five investigated R plasmids (Manuscript III). However, eight *A. salmonicida* that also harbored multiple ARGs did show coverage (< 60%) of at least one of the R plasmids, indicating they could have acquired ARGs from the plasmids in the past through horizontal gene transfer and then subsequently lost the plasmid.

1.8. Vaccination

Unlike treatment with antibiotics, one does not have to worry about the bacterial pathogens developing resistance against vaccinations (Vinitnantharat *et al*., 1999), who provide a better alternative for future control of furunculosis. Immunization of fish against furunculosis by vaccine administration was already introduced experimentally in 1937, however, not until the early 1990's successful implementation of oil-adjuvanted vaccines in salmon aquaculture has there been made great advances in this field of research (Midtlyng, 1997). Fish can be immunized orally or by immersion or injection, though oral and immersion vaccines are less stressful for the fish than injection and would be preferred if their protection level would equal the one produced by injection (Vinitnantharat *et al*., 1999).

Over time, handling techniques improved for injection vaccines and automatic equipment, manuals and instruction videos for training became available, making it possible for injection vaccines to be administered on a large scale (Eithum, 1993; Midtlyng, 1997).

Even though numerous side effects for oil adjuvants have been observed, including lesions, pigmentation, granulomatous inflammation in the liver, autoimmune reactions and intra-abdominal adherences, it is still recommended to use this administrative method to minimalize loss of fish due to disease (Midtlyng, 1996; Midtlyng *et al*., 1996; Midtlyng, 1997; Håstein *et al*., 2005; Koppang *et al*., 2008; Satoh *et al*., 2011). Research involving *A. salmonicida* IROMPs has shown that they also could have a potential as antigens included in vaccines, due to their in vitro bactericidal effect on both A-layer negative and positive *A. salmonicida* strains and in vivo protection of Atlantic salmon (Bricknell *et al*., 1999; O'Dowd *et al*., 1999). ECPs of *A. salmonicida* are already part of oil adjuvant vaccines against furunculosis, however, their contribution to the vaccine protection remains uncertain as studies using ECPs or their extracts as antigen showed varying results (Cipriano, 1982; Cipriano and Pyle, 1985; Prost, 2001).

Carrier fish have been recognized to play a significant role in the transmission of *A. salmonicida*, due to the ability of fish being able to shed bacteria in their surroundings and *A. salmonicida* being able to survive in water without a host (McCarthy, 1980; Rose *et al*., 1989b; Rose *et al*., 1990; Hastein and Lindstad, 1991; Nomura *et al*., 1992; Smith, 1992; Morgan *et al*., 1993; Nomura *et al*., 1993; Ogut and Reno, 2005).

1.9. Susceptibility of fish species

Initially furunculosis was believed to be an exclusive disease of salmonids. Since then it has become known that *A. salmonicida* can also infect other fish species and other aquatic animals in freshwater and seawater e.g. catfish, carp, turbot, American eel, goby and wrasse (Bernoth *et al*., 1997).

It has also become apparent that susceptibility to furunculosis varies among the host species (e.g. Plehn, 1911; Fish, 1937; McCarthy, 1977; Ellis and Stapleton, 1988; Perez *et al*., 1996). In general, fish belonging to the family Salmonidae are thought to be the most susceptible to furunculosis (McCarthy, 1977). Especially brown trout, brook trout (Salvelinus fontinalis) and Atlantic salmon have shown to be highly susceptible, while rainbow trout seemed to be more resistant as they needed to be wounded in a bath experiment before showing any signs of disease (McCarthy, 1977).

Difference in susceptibility to furunculosis has been related to their immune system activity and especially their varying mucosal activity that is one of the main physical barriers and contains bioactive molecules such as lysosomes and other bacteriolytic enzymes (e.g. Cipriano and Heartwell, 1986; Cipriano *et al*., 1992; Cipriano *et al*., 1994a; Svendsen and Bøgwald, 1997). Teleost (bony) fish in general do exhibit a variation in their immune system wherein mucosal activity against pathogens is included (Dickerson, 2009). In agreement, a study by Cipriano and Heartwell (1986) showed that the fish species' mucus antibacterial activity directly correlated with their resistance towards furunculosis.

1.10. Aim of the work

This research endeavors to utilize quantitative polymerase chain reaction (qPCR) for the identification of *Aeromonas salmonicida* within the scope of the PHOTO-SENS project. The target molecular marker for this real-time PCR analysis is the 16S gene of *A. salmonicida*. The methodology involves the design and subsequent validation of a probe along with a specific set of primers tailored for the precise detection of the pathogen. Concurrently, standard series will be generated and employed to quantify unknown samples following bacterial cultivation. This systematic approach will enable an evaluation of the designed primer-probe assay's specificity and sensitivity. Furthermore, additional investigations are slated to assess the effectiveness of DNA extraction techniques for Gram-negative with a different approach of quantifying *Aeromonas salmonicida*, incorporating a simulated e-DNA filtration process. This simulation is designed to emulate the extraction of environmental DNA from water samples, mirroring common practices in aquaculture facilities and fish hatcheries.

1.10.1. About PHOTO-SENS Project

The PHOTO-SENS initiative is supported and financed by the European Union's Horizon 2020 Research and Innovation program, aligning with the Zero Hunger objective of the UN's 2030 Agenda. The project is scheduled to run for a duration of 3.5 years, concluding in May 2024, with a total EU contribution of 3 million euros (CORDIS, 2020). The primary objective of this undertaking is the development of a

photonic biosensing chip designed for the identification of salmon pathogens. The collaboration involves five multinational companies, each specializing in a specific aspect of the value chain. Surfix B.V., NL, is responsible for the production of photonic biochips, PHIX B.V., NL, oversees photonics assembly and packaging, CSEM, CH, handles microfluidics integration, LRE Medical GmbH, D, manages equipment manufacturing, and Tunatech GmbH, D, focuses on diagnostic services in aquaculture (CORDIS, 2020).

TunaTech GmbH assumes a critical role in the project, concentrating on the development and functional validation of innovative genetic biomarkers for the three pathogens involved, including *Aeromonas salmonicida*. The DNA chips utilized in this context consist of probes—short DNA sequences spanning 20-30 base pairs—that are specific to their target DNA sequence within a selective pathogen. These probes serve as a surface coating on biosensor chips. Consequently, when pathogenic DNA interacts with these biosensor chips, a short double-stranded DNA fragment form. This binding induces a phase shift in the output of the asymmetric Mach–Zehnder interferometer (aZMI), enabling accurate monitoring and detection (PHOTO-SENS). Figure 3 provides a visual representation to enhance comprehension of the PHOTO-SENS project's approach. These chips are expected to be a fast and cost-effective method of detecting bacterial pathogens from aquaculture samples which would help prevent severe infection outbreaks.

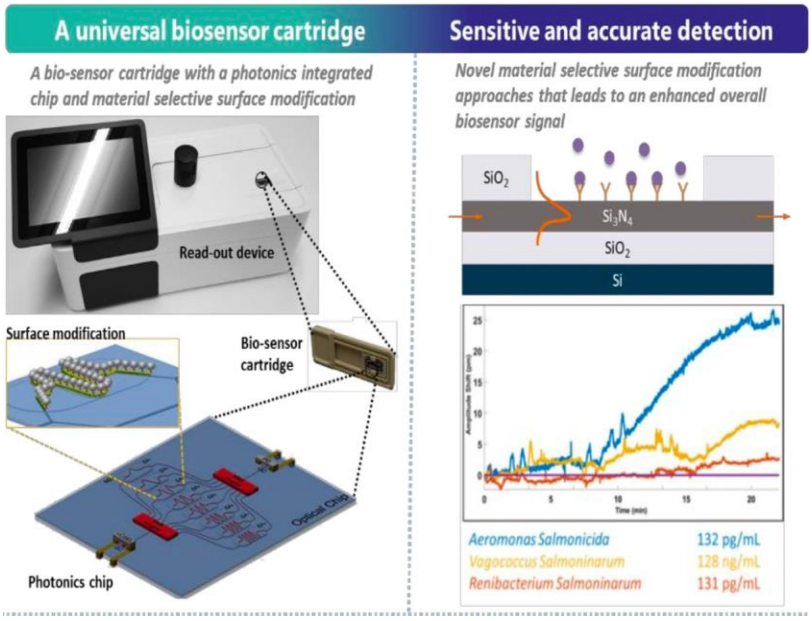


Figure 3: The novel approach of the PHOTO–SENS project with eDNA results for 3 salmon pathogens fromwater samples. Adapted from: (PHOTO-SENS)

1.10.2. PCR and Real Time qPCR for the detection of *A. salmonicida*

Various molecular techniques are currently employed for the detection, quantification, and examination of microbial populations. One of the most widely utilized methods is quantitative real-time PCR (qPCR), a PCR-based technique that concurrently monitors the amplification product as the reaction progresses in real-time (BIO-RAD; Postollec *et al*., 2011).

qPCR is a method preferred by many scientists for it is faster than other conventional methods as it decreases post-process steps as well asminimizes experimental errors (Torres-Corral & Santos, 2019). It is also quite sensitiveand throughput (Čepin, 2017a). The basic principle of qPCR is the amplification of a short specific part of target fluorescently labelled DNA in cycles with each cycling exponentially amplifying the target DNA template (Čepin, 2017a).

Before delving into the intricacies of qPCR, it is essential to comprehend the fundamental mechanisms of basic PCR (Polymerase Chain Reaction). PCR necessitates a DNA template (Figure 4; green), a short initial sequence complementary to the target DNA (primer; Figure 4; red), the polymerase enzyme serving as a "copy machine" for DNA (not depicted), and the presence of small DNA components known as nucleotides (Figure 4; blue). A buffer is employed to stabilize reaction conditions (Mülhardt, 2009).

A schematic representation of the Polymerase Chain Reaction can be found in Figure 4 below. 1) Denaturation of DNA double-strand into single-stranded DNA. 2) Primer annealing at single strands. 3) Elongation of double-stranded sequence by complementary addition of nucleotides. DNA undergoes amplification through multiple repetitions of these steps, with the DNA amount doubling in each cycle.

In the initial phase, DNA is separated into single strands through denaturation at elevated temperatures (Figure 4; 1). Subsequent to a temperature reduction, primers bind to the single strands during an annealing step (Figure 4; 2). The polymerase then attaches to the short double-stranded DNA section and extends it by incorporating

nucleotides complementary to the target DNA (Figure 4; 3) (Mülhardt, 2009). The DNA amount doubles in each PCR cycle, leading to an exponential rise in DNA. After several cycles, enough DNA is present and can be stained and visualized, for instance, on an agarose gel. Amplification exclusively occurs when the DNA sample possesses a sequence complementary to the primer sequence. Therefore, employing specific primers enables the assessment of an unknown sample for the presence or absence of specific DNA, such as that of a fish pathogen like *Aeromonas salmonicida*.

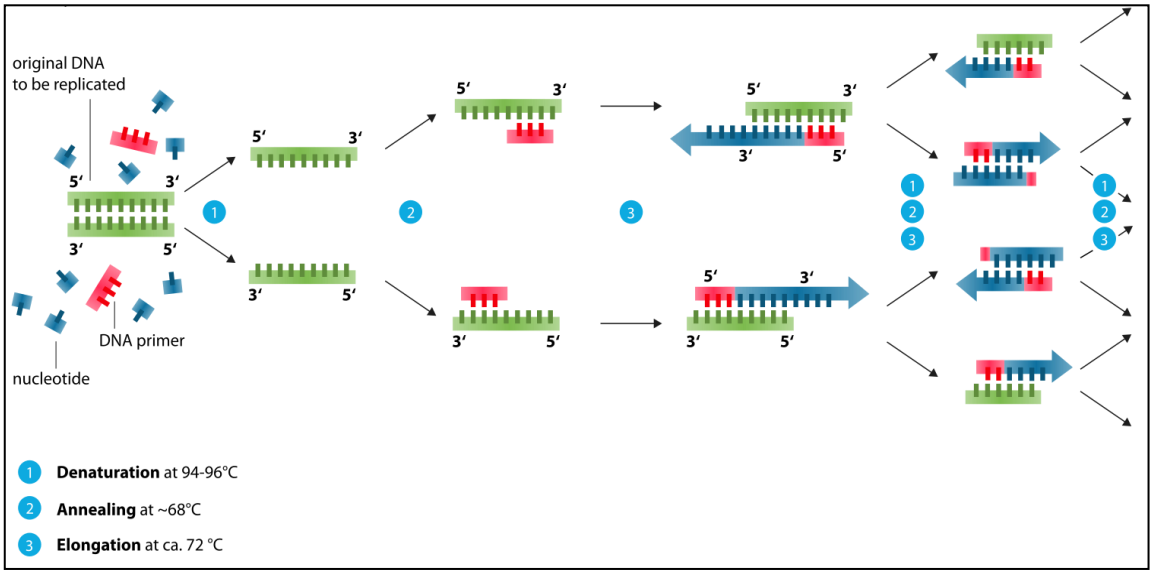


Figure 4: Polymerase chain reaction schematic. 1) Denaturation of DNA double-strand into single-stranded DNA. **2)** Primer annealing at single strands. **3)** Elongation of double-stranded sequence by complementary addition of nucleotides. DNA is amplified through multiple repetitions of these steps, with the amount of DNA doubling in each cycle. Source: https://upload.wikimedia.org/wikipedia/commons/9/96/Polymerase_chain_reaction.svg.

Most quantitative polymerase chain reactions (qPCRs) involve a fluorescently labelled probe for the detection of target DNA. These probes consist of short DNA sequences specific to the target DNA template, typically combined with one or more fluorescent dyes and a quencher that suppresses fluorescence to prevent premature signalling. Ensuring the proper placement of the probe between the forward and reverse primers is essential. The qPCR process comprises three main steps: denaturation, where double-stranded DNA (dsDNA) is cleaved and separated into two single-stranded DNA strands (ssDNA); annealing, facilitating the binding of the probe and primer to the ssDNA template; and primer extension/elongation, involving the addition of complementary bases by DNA polymerase to produce a complimentary copy, forming

dsDNA once again. As the qPCR progresses and DNA is amplified, the probe is cleaved, and the quencher is separated, leading to the emission of a fluorescent signal that is measured. The fluorescence increases faster during qPCR cycles when the initial template DNA quantity in the sample is higher. This entire process is illustrated in Fig. 5A for visual clarity.

The term used for cycles in which fluorescence is detected is the quantification cycle value (Cq-value). A low Cq-value indicates a high copy number of the target DNA in the sample. Fig. 5B depicts an amplification plot obtained from qPCR cycles, showing an exponential phase followed by a non-exponential phase. The exponential phase sees a rapid increase in PCR product amount, resulting in a detectable fluorescence signal. However, as the reaction progresses and components such as buffer and primers are consumed, the reaction slows down, transitioning to the plateau non-exponential phase—typically after cycle 28, as per Fig. 5B (BIO-RAD).

Towards the conclusion of the process, standard curve plots are generated, correlating Cq-values of samples with known concentrations against the initial sample DNA amount. This allows the estimation of starting quantities for unknown samples.

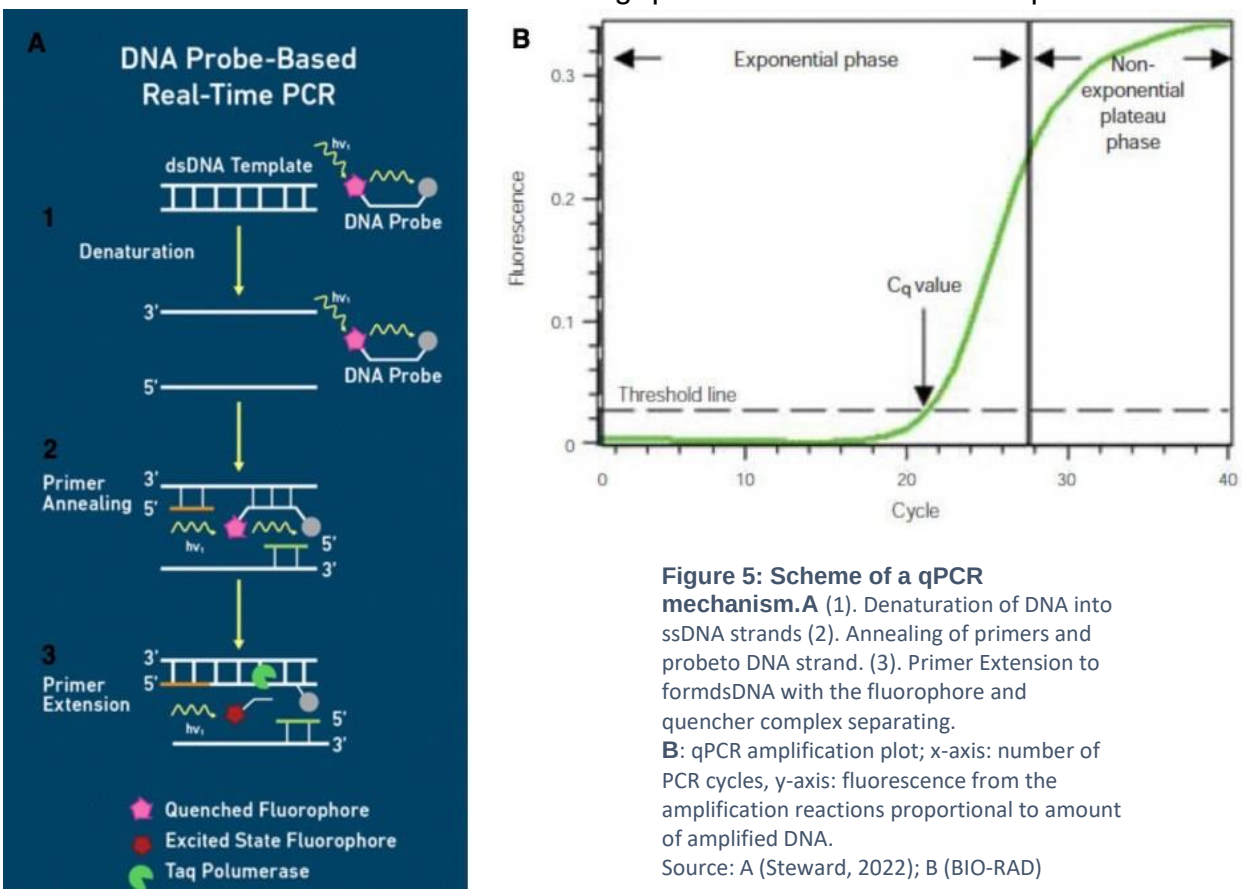


Figure 5: Scheme of a qPCR mechanism.A (1). Denaturation of DNA into ssDNA strands (2). Annealing of primers and probeto DNA strand. (3). Primer Extension to formdsDNA with the fluorophore and quencher complex separating.
B: qPCR amplification plot; x-axis: number of PCR cycles, y-axis: fluorescence from the amplification reactions proportional to amount of amplified DNA.
Source: A (Steward, 2022); B (BIO-RAD)

2. Materials and Methods

2.1. Cultivation of Bacteria

2.1.1. Preparation of M1 Meda and Agar Plates

The Nutrient Agar/Broth Medium or DSMZ Medium M1, specified in The Bacterial Diversity Metadatabase – BacDive (Strain ID: DSM 19634), serves as the medium for cultivating *A. salmonarium*. To prepare 500 mL of the liquid culture medium, 2.5 grams of Peptone and 1.5 grams of Meat extract were dissolved in 500 mL of distilled water in a DURAN glass bottle. The mixture underwent autoclaving at 121°C for 20 minutes. After autoclaving, the sealed DURAN bottle was left on the lab bench for later use.To create agar plates intended for cultivation on solid media, a 500 mL agar-based medium was formulated by combining 2.5 grams of Peptone, 1.5 grams of Meat extract, and 7.5 grams of agar were added into 500 mL of distilled water. The mixture was then autoclaved at 121°C for 20 minutes. After the autoclaving cycle, the liquid agar medium was poured into Petri dishes and left to solidify at room temperature. After solidification, the labeled plates were inverted and stored in the refrigerator at 4°C until needed.

2.1.2. Bacterial Cultures and Crycultures

To initiate cultivation in a liquid medium, a frozen pellet of *A. salmonicida* stored at -20°C was utilized. The pellet was first allowed to thaw and then resuspended in 200µL of liquid M1. This culture was then used to inoculate approximately 75 mL of liquid M1 in a flask. It was made sure that the inoculation was done near a lit Bunsen Burner to maintain heat sterilization and prevent any possible contaminations. These newly inoculated flasks were sealed shut with a cork stopper/aluminum foil and then kept on a VWR Incubating Orbital Shaker at 28°C and with a shaking speed of 80 rpm.

Once growth was seen in the flask (in the form of a hazy, translucent liquid), plating was performed for cultivation on solid media. This plating involved using pre-prepared agar plates, stored in the fridge at 4-8°C. The cultures were diluted first, usually in

1:1,000,000 and 1:5,000,000 dilutions using the M1 media. 100µL of the diluted cultures were then pipetted onto the agar plates and evenly spread with a reusable Drigalski spatula. It was ensured that the plating of bacteria was performed close to the flame of a Bunsen Burner to avoid contamination.

The plates were then sealed shut with Parafilm and allowed to settle for a while before inverting to be placed in an incubator at about 27°C. The culture was allowed to grow for at least 24 hours before bacterial colonies were visible in the form of colony forming units (CFU) on the agar plates. Plating for the dilution was performed five times in order to enhance the accuracy of CFU counting, ensuring a more reliable average and greater confidence in the results.

Bacterial glycerol stocks, referred to as cryocultures, play a vital role in the long-term preservation and storage of frozen bacterial cultures due to the stabilizing properties of glycerin. This is because glycerin helps stabilize the frozen bacteria and helps keep the cells alive, preventing damage to the cell membranes (AddGene). These cryocultures are a valuable resource when initiating new bacterial cultures. To create these cryocultures, 500 µL of bacterial culture and 500 µL of 50% Glycerin were pipetted into 1.5 mL Eppendorf tubes. Furthermore, 5 aliquots of 1 mL of pure liquid culture were also taken for short-term preservation. All these aliquots were stored in the freezer at -20°C.

2.1.3. Growth Measurement

For monitoring and measuring the growth of *A. salmonicida,* 1000µL of culture from the original flasks was taken as carry-over medium to inoculate new flasks. These freshly inoculated flasks were allowed to shake on the VWR Incubating Orbital Shaker for an hour after which 250 µL of the culture was transferred to Falcon® Tissue Culture 96 Well Flat Bottom Plates. 10 wells in total were filled with the bacterial culture along with 4 wells filled with M1 media to be used as blank. Growth of the bacterial cultures was then measured using a Tecan Sunrise Absorbance Microplate Reader with the absorbance of the samples set at 600 nm. Over a 24-hour period, measurements were taken at 15-minute intervals, with shaking before each reading. The software used for this growth measurement experiment was XFluor4 Version 4.51.

2.2. Designing *A. salmonicida* Specific Primers and Probe

2.2.1. Identification of Target 16S Gene

The 16s ribosomal RNA (rRNA) gene is a highly conserved gene in bacteria and has long been used for bacterial identification (Clarridge, 2004). This is due to the gene's function as a molecular chronometer, which has remained unchanged over time. Additionally, its widespread presence in almost all bacteria contributes to its reliability. Not only this, the 16s rRNA gene, with a length of approximately 1,500 bp, proves suitable for informatics applications (Janda & Abbott, 2007). Despite its substantial size, the gene exhibits interspecific polymorphisms, enabling accurate identification of distinct bacterial species and facilitating the discernment of close relationships at both genus and species levels (Clarridge, 2004). Usually, universal primers used for the 16s rRNA gene are conserved at the beginning of the gene (~540 bp) or towards the end of the whole sequence (~1,550 bp region) (Clarridge, 2004). Similarly, for the purpose of this thesis, the 16s rRNA gene was chosen for the designing of the primer and probe specific to *A. salmonicida.* The 16s gene sequences chosen for this purpose were targeted to be within the range of 1,000 bp to somewhere between 1,300 and 1,450 bp.

Nevertheless, it is crucial to note that the 16s primers and probes discussed were only designed to illustrate the workflow within this thesis and were not applied in any of the conducted work or presented results. Due to contractual confidentiality, the original gene locus and primer sequences utilized in the experiments detailed in this thesis cannot be disclosed. However, the primer and probe design details provided in the subsequent section adhere to a comparable approach, aiming to develop species-specific primers and probes for detection using qPCR.

2.2.2. Comparison of Selected Gene with Other Fish Bacterial Strains

To develop a primer and probe specific to *A. salmonicida* and avoid the amplification of DNA from other bacteria or pathogens, it was imperative to identify a distinct region on the 16s gene that sets *A. salmonicida* apart from other fish pathogens.

This consideration held significant relevance both in the context of conducting qPCRs and within the framework of the PHOTO-SENS project. Given that samples for the project would be sourced from authentic environments like rivers, where multiple fish pathogens might coexist, it was crucial to ensure the specificity of the probe on the biosensor chips. The probe needed to be precise enough to selectively identify the target bacteria when these samples were subjected to testing on the biosensor chips.

To achieve this objective, the 16s gene sequences of ten different bacteria were examined, comprising five Gram-positive and five Gram-negative species. The selected Gram-positive bacteria were *Vagococcus salmonarium*, Mycobacterium *marinum, Renibacterium salmoninarum, Streptococcus iniae, and Streptococcus phocae, while the selected Gram-negative bacteria included were Yersinia ruckei, Flavobacterium columnare, Moritella viscosa, and Flavobacterium branchiophilum*. Notably, all these bacteria are recognized as significant fish pathogens, as outlined by Sudheesh *et al*. (2012) in a table. To conduct the analysis, approximately six 16s gene sequences were compared and aligned for each bacterium. These sequences were sourced from the NCBI Nucleotide Database, with a specified target length, resulting in the analysis of a total of 60 gene sequences.

2.2.3. Designing the Primer and Probe using Unipro UGENE

The sequences, acquired in FASTA format, were imported into Unipro UGENE Version 40.0, an open-source software utilized for DNA and protein sequence visualization, alignment, assembly, and annotation (Konstantin Okonechnikov *et al*., 2012). ClustalW multiple sequence alignment tool was used to align the 60 sequences. Notably, these sequences exhibited varying levels of similarity, such as in the case of Streptococcus, where the similarity ranged from 82% to 84% (Torres-Corral & Santos, 2019), when compared to the 16s rRNA gene of *A. salmonicida*. Therefore, to enhance the analysis, a variable region within the 16s rRNA sequence, distinct from *A. salmonicida* and other genetically related bacterial species under examination, was selected.

For the alignment and primer-probe design, a variable region spanning from 107 bp to 190 bp was chosen due to its significant variance from other bacterial strains. The 16s

rRNA sequence of A. salmoninarum was uploaded into the IDT PrimerQuestÔ Tool to generate a forward and reverse primer, along with a fluorescently labeled probe intended for use in qPCR. Similarly, PCR primers were also designed in this way that spanned the entire qPCR amplicon region that is, starting from 89 bp to 390 bp.

Figure 6 shows a screenshot of the workflow from Unipro UGENE, depicting the *A. salmonicida* specific primers and probe for qPCR. It shows the sequence of each bacterial strain used with the sequence for *A. salmonicida* marked as reference. Similarities within the sequences are indicated by nucleotides of the same color. The final primer pair and probe were derived after adjusting specific parameters in the process.

The forward primer for qPCR is represented by a navy-blue box, spanning the 402-423 bp region, while the reverse primer, also in a navy-blue box, covers the 460-479 bp region. Positioned between the forward and reverse primers, the qPCR probe is illustrated in a light blue box, ranging from 427 to 450 bp in the image.

It is crucial to note that the base pair (bp) location and the alignment illustrated in Figure 6 differ from the original primers and probes designed due to gaps in the alignment.

Figure 6: *A. Salmoninarum* primer and probes designed compared with the other reference bacterial strains.
Please note: Pictures for bp from 300- 449 have been skipped because this region held no relevance to the primer design and was highly similar to the other bacterial strains.

Therefore, as the result, the forward primer (5'→3'), spanning from position 107- 125 and the reverse primer (5'→ 3') from position 172-190 on the *A. salmonicida* 16s rRNA gene sequence was obtained.

A detailed overview of the primers for PCR and qPCR as well as the probe designed are shown below in Table 2.

Table 2: Length and sequence details of the qPCR primer set and probe. The total length and GC% contentof the primer sequence is also shown. Source: IDT PrimerQuest Tool.

	Coding Strand (5’□ 3’)	Length	Temp	GC%
qPCR PrimerF	TGTAAAGCACTTTCAGTGAGGA	22	62	40.9
qPCR Probe	AAGGTTGGCGCCTAATACGTGTCA	24	68	50
qPCR PrimerR	GGTGCTTCTTCTGCGAGTAA	20	62	50

2.3. DNA Extraction

DNA extraction is a process aimed at purifying DNA from a sample and isolating it from cellular components through a combination of physical and chemical methods. Typically, DNA extraction involves cell lysis, DNA solubilization, and subsequent enzymatic actions to remove macromolecules like proteins and RNA (Gupta, 2019). Various techniques, including both manual methods and commercial DNA kits, are available for DNA extraction (Gupta, 2019). However, it is dependent on the DNA extraction techniques used that determine the efficiency of the extraction in terms of DNA quantity and quality i.e. purity of contaminants. Due to *A. salmonicida* being a Gram-negative bacterium, various methods can be used to break its peptidoglycan layer around it. As a result, it is imperative to employ a DNA extraction method capable of disrupting this protective layer and releasing intracellular molecules (Andreas Otto *et al*., 2012). In the context of this thesis, three different methods were used for *A. salmonicida* DNA extraction, and each of these methods is detailed below.

2.3.1. DNA Extraction 5% Chelex DNA Extraction

DNA extraction using 5% Chelex solution is one of the most used methods for DNA extraction and the first protocol was developed by Walsh *et al*. (1991) This technique is favored over alternative methods due to its effectiveness, time efficiency, and reduced labor intensity according to BIO-RAD. The fundamental principle behind using Chelex for DNA extraction lies in its ability to chelate polyvalent metal ions, such as Mg2+, which serve as cofactors for DNAases (Utkarsha A. Singh *et al*., 2018).

By being present during boiling, Chelex prevents the degradation of DNA by these metal ions at high temperatures and facilitates the release of DNA into the solution through enzymatic digestion (BIO-RAD; Walsh *et al*., 1991). To prepare the Chelex solution, 1.25 grams of Chelex 100 Resin pellet was dissolved in 25 mL of TE Buffer or water to achieve a 5% Chelex w/v (weight per volume) concentration. In the DNA extraction process, each sample underwent centrifugation at 15,000 rpm for 5 minutes, and the supernatant was discarded. To each sample, 98µL of 5% Chelex solution was added to resuspend the bacterial pellet before adding 2µL of Proteinase K as well.

However, prior to pipetting the Chelex solution into the sample, it was made sure that the resin beads are evenly distributed in the solution and therefore was vortexed at highspeed. The samples were then placed in the Thermomixer for 3 heating steps. The first two steps involved 15-minute periods of shaking at 1,300 rpm and a temperature of 56°C, with a brief vortexing interval in between. For the final step, the temperature was elevated to 99°C, and the samples were shaken at 1,300 rpm for 20 minutes. After completing the heating steps, the samples were centrifuged once more at 15,000 rpm for 5 minutes to facilitate the settling of cell debris at the bottom, allowing the supernatant containing the free DNA to be collected and transferred into new tubes. To mitigate the risk of sample evaporation and lid opening due to pressure buildup, Safe-Lock tubes were consistently used for each sample. The extracted DNA was then ready for further downstream steps.

2.3.2. Improved Chelex DNA Extraction via Rapid Agitation with Glass Beads

The results obtained from DNA extraction via regular Chelex extraction and kit extraction were below satisfactory. It was believed this was because, as mentioned before, the cell walls of *A. salmonicida* bacteria are rigid due to their stabilizing surrounding layers of peptidoglycan. Consequently, it was deemed necessary to employ more potent cell lysis techniques to disrupt the cell walls effectively, thereby enabling Proteinase K to enhance its functionality in DNA extraction. The breakthrough idea then occurred to combine the two already used DNA extraction protocols and optimize the 5% Chelex DNA extraction method by adding a mechanical cell disruption

step using glass beads as described by the Macherey- Nagel™ NucleoSpin™ Microbial DNA Kit Extraction User Manual.

For this method of DNA extraction, a sample was prepared similar to in 5% Chelex protocol that is centrifuging each sample for 5 minutes at 15,000 rpm. The supernatant was discarded, and the pellet was resuspended by adding 98 µL of 5% Chelex solution as well as 0.5 mm diameter glass beads. Approximately 50% of the bead load volume was utilized for each sample, aligning with the optimal parameters suggested by Butler and Guimarães (2021). According to their recommendations, a bead diameter of 0.5 mm and a 60% bead loading volume are considered optimal, as factors such as bead filling, bead diameter, agitation speed, and time collectively influence the efficiency of the overall process. Additionally, the use of smaller beads leads to quicker disruption, as highlighted by Geciova *et al*. (2001). Each sample was then subjected to the agitation device, depicted in Figure 7, and agitated at a high speed of 2,400 rpm for 15 minutes.

The following steps were similar to that described in the 5% Chelex protocol, that is addition of 2µL of Proteinase K followed by 3 heating steps on the Thermomixer. For the first two steps, the samples were heated to 56°C and shaken for 15 minutes with intermittent vortexing between each heating cycle. The final step included increasing the heating temperature to 99°C and the samples shaken for 20 minutes. To collect the extracted DNA, the sample tubes were centrifuged for 5 minutes at 15,000 rpm to settle the cell debris and allow the supernatant containing DNA to rise to the top to be transferred into new, clean tubes.

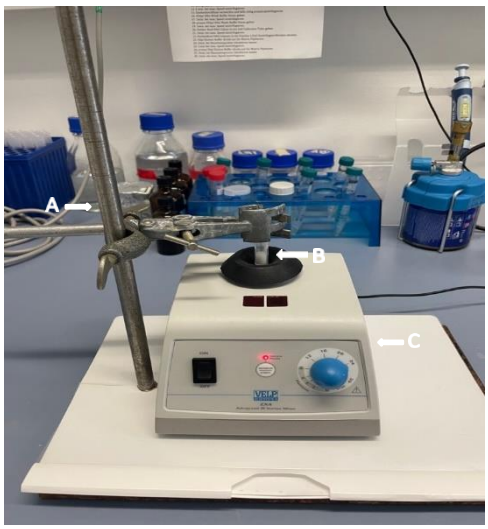


Figure 7: Improvised agitation device. Bead tube containing sample (B) held bythe retort stand (A) on top of avortex (C) at 2,400 rpm speedfor 12 minutes for cell disruption of Gram-positive *V. salmoninarum* cells.

A: Retort Stand
B: Bead Tube containing sample
C: Vortex

2.4. PCR

Polymerase Chain Reaction (PCR) is a common technique to amplify and make multiple copies of a particular section of DNA. While PCR swiftly confirms the presence of target species, it falls short in verifying a pure culture. The first PCR for this thesis was conducted with general 16s primers that were not specific to any species but would amplify all bacterial DNA present in a sample. This was done for the very first samples of the bacterial culture. The resultant PCR product was then sequenced in order to check for presence of *A. salmonicida* or any other bacterial contamination. All samples were confirmed as pure *A. salmonicida* and therefore, the culture was verified to be pure for further experimentation.

A second PCR was conducted to obtain an electrophoresis gel with fading band intensities with decreasing copy number in a CFU serial dilution series. Further elaboration on this process is detailed in Section 3.5.2. This particular PCR employed the *A. salmonicida*-specific primer set designed in Section 2 and depicted in Figure 5.

For every PCR, a master mix needs to be prepared with a buffer, primers, DNA nucleotides (dNTPS) and the enzyme Taq Polymerase. The buffer is needed to ensure the optimal conditions for the reaction, primers to initiate PCR reaction and bind to either side of target DNA sequence, dNTPS that are DNA bases (A, C, G and T) are needed to form a new DNA strand and the Taq Polymerase enzyme enables the addition of new bases (yourgenome).

The master mix for a single sample was prepared by adding 1.25 µL of 10x PCR Buffer S with 15 mM MgCl2 (Genaxxon Bioscience), 0.25µL of Bioline dNTPs, 0.25µL of forward primer (10µM), 0.25µL of reverse primer (10 µM), 0.0625µL of Taq DNA-Polymerase 250 units – 5U/µL (Genaxxon Bioscience) and 9.45µL of molecular grade water. When handling multiple samples, this master mix composition was scaled by the number of samples, with an additional 10% prepared to accommodate pipetting errors.

Each individual sample required 11.5 µL of the master mix, followed by the addition of 1 µL of the sample's DNA template, resulting in a total volume of 12.5 µL per sample.

This was done in 8-tube PCR strips and the PCR itself carried out in SensoQuest Labcycler Gradient (Thermoblock 96 wells). The PCR program selected comprised of annealing temperature of 54°C.

The first step of the PCR program consisted of initial denaturation at 94°C for 1 minute. The next steps were repeated for 35 cycles as follows: denaturation at 94°C for 30 seconds, primer annealing at 54°C for 30 seconds, and primer extension at 68°C for 30 seconds. The final step consisted of primer extension for 5 minutes at 68°C before ending the PCR program. The PCR machine maintained the sample at 10°C indefinitely until removal.

2.4.1. Gel Electrophoresis

Agarose Gel Electrophoresis was the method used to visualize the PCR product. This technique is widely utilized to separate DNA fragments based on their varying sizes. Its basic principle lies in the migration of DNA fragments within the agarose gel when placed in an electric field. The negatively charged phosphate backbone of DNA moves towards the positively charged anode due to the mass/charge ratio of DNA molecules, thereby separating them by size (Lee *et al*., 2012).

Gels of 60 mL volume were prepared, containing either 1% or 2% agarose concentrations. To create these gels, 0.6 grams or 1.2 grams of agarose, respectively, were added to 60 mL of TAE Buffer. This mixture was heated in the microwave until the solution became clear to dissolve the agarose. Next, 6 µL of Roti Gel Stain was added to the solution before it was poured onto a gel casting tray that serves as a mold. A well-forming template (commonly called a comb) was positioned across the casting tray to let wells form once the gel solution solidifies. The gel was allowed to cool for 30 mins before being placed into an electrophoretic chamber containing TAE Buffer.

The gel was then loaded with 2.5 µL of a size marker DNA ladder of 100 bp, along with 5 µL of PCR product mixed with 1 µL of 6x concentrated loading dye was also pipetted into the formed wells. The gel was allowed to run at 100 V for 30 mins before being

placed onto the UV table for analysis. For this step, it was ensured that protective face shield was worn.

2.4.2. Sequencing

Sequencing was performed in order to confirm the identity of the bacterial extracts and make sure further work is carried out with a pure *A. salmonicida* culture. For this, PCR product from the PCR done for DNA extracts with non-*A. salmonicida* specific 16s rRNA primers were used.

1 µL of this PCR product was added to an Eppendorf Tube along with 2.5 µL of either forward or reverse 16s rRNA primer and 6.5µL of molecular grade water, to reach a total volume of 10 µL. These tubes were then labeled and sent to Macrogen Europe (https://www.macrogen-europe.com) for sequencing. The sequencing result chromatograms were then checked for cleanliness by looking for overlaps and confirmed for identification, the sequence was copied from Unipro UGENE and added to BLASTN tool from NCBI (National Center for Biotechnology Information)

2.5. qPCR

For qPCRs, only *A. salmonicida* specific primers and probes as described in Section. 2 were used. A master mix was prepared using 5 µL of Probe Master Mix – Low ROX (Geneaxxon Bisoscience qPCR 2x Master mix with 50 nM ROX), 1 µL of *A. salmonicida* primer probe mix and 3µL of molecular grade H2O. This composition was suited for 1 sample and therefore, for more samples was simply multiplied with the number of samples, while also making an additional 10% buffer to accommodate potential pipetting errors.

Subsequently, 9 µL of the master mix was dispensed into Bio-Rad Hard Shell 96 microplates—filling wells equivalent to the number of samples—and 1 µL of DNA template was added to each well. The qPCR microplate was then sealed with a Bio-Rad Microseal® B Adhesive Sealer before being put into the qPCR machine. The qPCRs were done in Bio-Rad CFX96 TouchÔ Real-Time PCR Detection System. The programmed procedure involved two steps: an initial 15-minute denaturation at 95°C

to ensure complete removal of chemical inhibition from the chemically modified DNA polymerase in the buffer, followed by 40 cycles of DNA denaturation at 95°C for 10 seconds and primer annealing at 60°C for an additional 10 seconds. Measurements were taken after each cycle with the VIC fluorophore channel that has a stimulation maximum at 526 nm and an emission maximum at 543 nm (Lumiprobe). The results were then analyzed using the software CFX MaestroÔ Version 2.2 for further evaluation and interpretation.

2.6. Quantification of Samples

2.6.1. Quantification of DNA Extracts with Standard Series

By performing qPCRs, the aim was to estimate a target copy number of a reference sample against a defined standard curve of absolute concentrations known as standard dilution series. Samples to be quantified were prepared from the bacterial liquid culture aliquots stored during plating of the culture. Upon the identification of growth and CFU count was done, an anticipated value for the number of bacteria within the original, undiluted culture was calculated. Starting from 10 million expected number of copies down to 1 expected number of copies in a sample was extracted for DNA and placed in the qPCR along with a standard dilution series. There were 2 standard dilution series prepared and are explained later in this section. The qPCR measured and provided with a starting quantity (SQ) relative to the standard dilution series used for these samples.

This value was then compared to the expected copy number for the sample in order to quantify as well as to assess the performance of the DNA extraction method used.

2.6.2. Quantification of DNA Extracts with Standard Series

gBlocks are double-stranded, sequence-verified long DNA fragments ranging from 125 – 1000 bp (IDT). A gBlocks Gene Fragment specific to *A. salmonicida* was ordered from Integrated DNA Technologies Inc. (IDT) and comprised of a total of 224 bp. This gBlocks Gene Fragment covered the entire qPCR amplicon region. The gBlocks Gene Fragment was delivered freeze-dried and required resuspending to reach a final

concentration of 10 ng/µL. The information provided by the supplier was used to calculate the copies/ µL in the resuspended gBlocks Gene Fragment solution. This was done using the following formula.

$$\frac{(10\ ng/\mu L * 7.23\ fmol/ng * (6.022 \times 10^{23}\ copies/mol))}{10^{15}\ fmol/mol} = 4.35\ \times 10^{10}\ copies/\mu L$$

Therefore, in a single microliter of undiluted gBlocks Gene Fragment solution, there were 4.69 x 10^{10} copies of the *A. salmonicida* gene fragment. In order to obtain a starting dilution of 100 million copies/ µL, 1 µL of gBlocks Gene Fragment was added to 468 µL of molecular grade water in an Eppendorf Tube. This tube was then used to create a dilution series, progressing in decrements from 10 to 1 copy per microliter, with an additional dilution set at 5 copies per microliter.

To enhance data reliability, a standard curve with average Cqs was also plotted for the gBlocks standard dilution series. This involved conducting qPCR across 6 separate runs and computing the mean SQ for each run. A standard deviation was also calculated to show the dispersion of data relative to the average. Both parameters were calculated using Microsoft Excel.

2.6.3. Standard Dilution Series from CFU counts

A second standard dilution series was also created using the CFU counts obtained from the plated bacterial culture. To do so, simply an aliquot corresponding to 10 million CFUs was used to extract DNA using the improved Chelex DNA extraction method. This was then used to perform a 1:10 serial dilution to go down to 1 copy/ µL. A 5 copy/ µL was also created with a 1:2 dilution.

Both standard dilution series were used to generate a standard curve. This was done automatically by the CFX MaestroTM Software when the samples were labelled as standards. These standard curves were then used to estimate the SQ of the quantification samples with respect to the standards. Efficiency as well as correlation

coefficients ($R^{2'}$) was also calculated. The formula used for calculating % efficiency is as follows (Ma *et al*., 2021)

$$E = ((10^{\frac{1}{m}}) - 1) * 100$$

For an assay, an efficiency nearing 100% and an R^2 value surpassing 0.98 are considered indicative of a robust correlation between data points, ensuring high confidence in the results' consistency (BiteSizeBio, 2022). The y-intercept of the best fit line provides a theoretical sensitivity of the assay.

Similar to the approach undertaken with the gBlocks standard dilution series, an averaged standard curve was made for the CFU standard series. For this, the experiment was repeated using qPCR 5 times, the resultant Cqs then averaged for each SQ as well a standard deviation also calculated using Microsoft Excel.

2.7. Filtration Experiment using Cellulose-Acetate Filters

To simulate eDNA extraction as it would be conducted in aquaculture facilities for the PHOTO-SENS project's biosensor photonic chips, a replication of a comparable filtration process was conducted. This involved an experiment with bacterially spiked water. eDNA refers to DNA from microscopic organisms, detached cells and free DNA that is released from living cells (Majaneva *et al*., 2018).

The procedure entailed spiking 1 liter of distilled water with bacterial culture, ranging from 10 million cells down to 1 cell based on the CFU count. The experiment was done using a filtration setup illustrated in Figure 8, the bacterially spiked water was poured onto the glass holder (C) before the vacuum pump (A) was turned on. As the water gradually filtered through, the eDNA collected onto the filter (D), while the residual water was gathered in the collection vessel (H). Once all the water had filtered through, the filter was picked up with previously disinfected tweezers and placed into Petri dishes and allowed to dry on a heating plate set at 30°C for 15 minutes.

The filters were then carefully picked up and cut into small pieces into Safe-Lock tubes before carrying out DNA extraction. For this, 990 µL of 5% Chelex solution was used

along with 10 µL of Proteinase K. The extraction duration was doubled compared to the standard protocol, encompassing a first heating step at 56°C for 30 minutes, thorough vortexing, followed by a similar second heating step at 56°C for 30 minutes, another vortexing, and concluding with a final step at 99°C for 40 minutes. Subsequently, the tubes underwent centrifugation at 15,000 rpm for 5 minutes, with 200 µL of supernatant collected and transferred into fresh Eppendorf tubes. This supernatant containing the DNA was then used for qPCR for quantification.

Throughout the filtration process, special attention was paid to disinfecting the glass holder (C), the sieve (E), the glass funnel (F) and tweezers after each use for every sample using 70% ethanol and igniting briefly. Also, for this experiment, cellulose-acetate filters with a pore size of 0.45 µm from Sartorius Stedim Biotech were used.

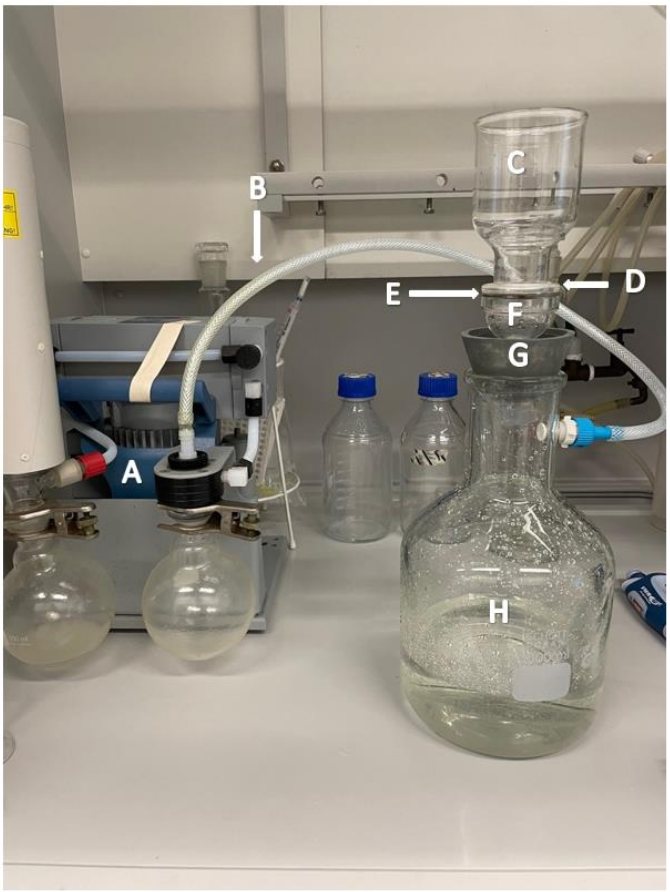


Figure 8: Filtration Equipment Setup. Spiked water is poured into holder (C) and vacuum pump (A) turned on. The water gets filtered through filter (D) and metal sieve (E) before getting collected incollecting vessel (G).

A: Vacuum PumpB: Tube
C: Glass Holder
D: Cellulose Filter (white sheet in picture)E: Metal sieve (silver metallic ring in picture)
F: Glass funnel G: Rubber seals
H: Collecting Vessel

3. Results

3.1. Inoculation and growth of *A. salmonicida*

The attempt to cultivate *A. salmonicida* under the previously specified conditions proved successful. Visible growth was observed within 24 hours in the two inoculated flasks labeled A. Sal 1 and A. Sal 2. The flasks exhibited a consistently murky dark yellowish colour appearance, indicative of a pristine culture. Nonetheless, to confirm the purity, sequencing was conducted using the PCR product obtained, and the outcomes are detailed in Section 3.3.

3.1.1. Photometric Growth Measurement

To assess and monitor the growth time of the *A. salmonicida* culture, an experiment measuring the optical density (OD) of the culture was performed. As described in Section. 2.1.3, the growth of the culture was measured over a period of 24 hours with measurements taken every 15 minutes. Figure 9 shows a graph of the optical density measurement of A. Sal P and A. Sal 2P culture against time. This measurement and calculation were done using Microsoft Excel where the OD of the culture was subtracted from the OD of the blank.

The red and blue dots represent the data points of the OD measurement taken every 15 minutes. At time 0 OD already shows the value at 0.4 so a higher dilution would have resulted in a lower starting value. The graph also shows error bars that depict the standard deviation. It can be seen the OD values are quite precise in the exponential phase of the bacterial growth, but the error bars are larger from the latter part of stationary phase and death phase.

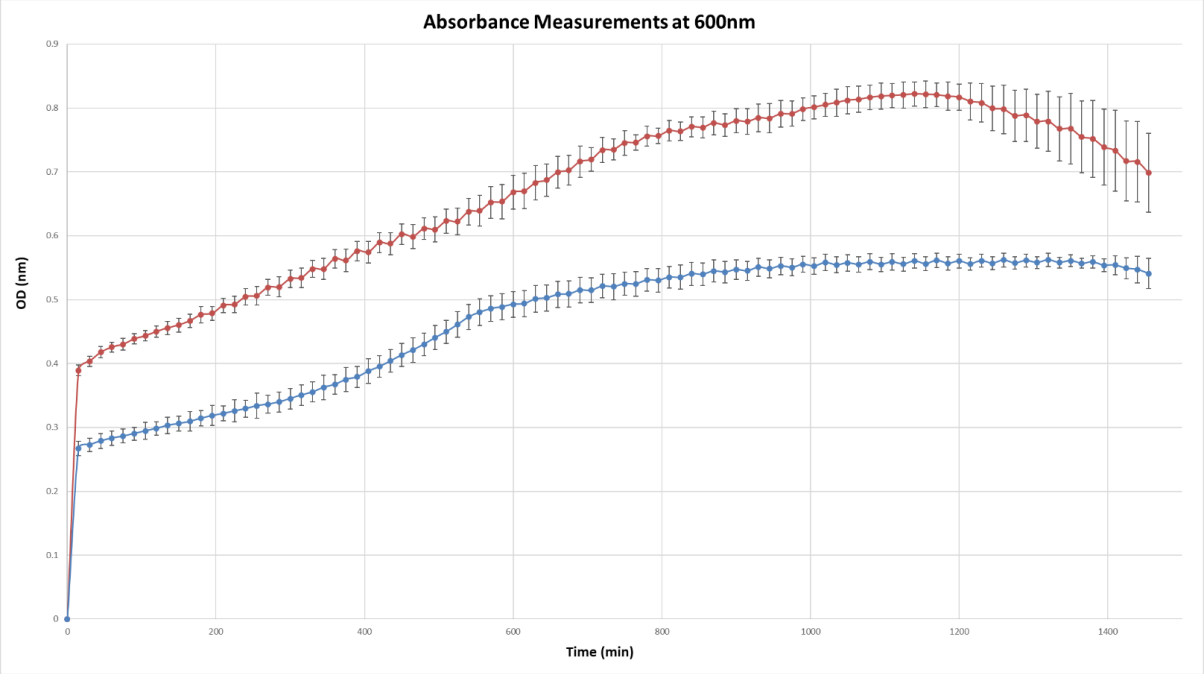


Figure 9: Optical Density (OD) measurement of culture A. sal P(red) and A. Sal 2P(blue). The red and blue dots represent the data points, and the error bars represent the range of uncertainty in a certain data point. The depicted data points are the mean values of 10 single measurements.

3.1.2. CFU Count

The plating of the liquid culture served the dual purpose of monitoring the uniformity of bacterial colonies and quantifying aliquots for subsequent qPCRs. The plating process was conducted approximately 24 hours post-inoculation for both flasks, and both exhibited similar appearances. A 1:5,000,000 dilution was applied for plating, with each culture plated three times to enhance result precision.

Despite both cultures being grown and planted under identical conditions simultaneously, there were very prominent incidences of contamination, in *Aeromonas salmonicida* cultures. In order to get rid of these contaminations and get a clean culture, several attempts were made where picks were taken from the plated colonies and used to inoculate new flasks.

The colony-forming unit (CFU) count data, including average CFU and average cells/µL, is presented in Table 4. Values for A. sal 1 are denoted in red to highlight inaccuracies, and similarly, for A. sal 2P, one inconsistent value is marked in grey.

Table 3: CFU counts of plating experiments of culture V. Sal 1 and V. Sal 2P.

Culture	CFU/ plate of 1:5 mil dilution	Average cells/ 100µL	Average cells/ µL	Standard Deviation
A. Sal 1	0	0.6	3,000	1.34
	3			
	0			
	0			
	0			
A. Sal 2P	25	79.25	396,250	8.655
	68			
	87			
	77			
	85			

Figure 10 shows the cultural plates with the *Aeromonas salmonicida* bacterial colonies.

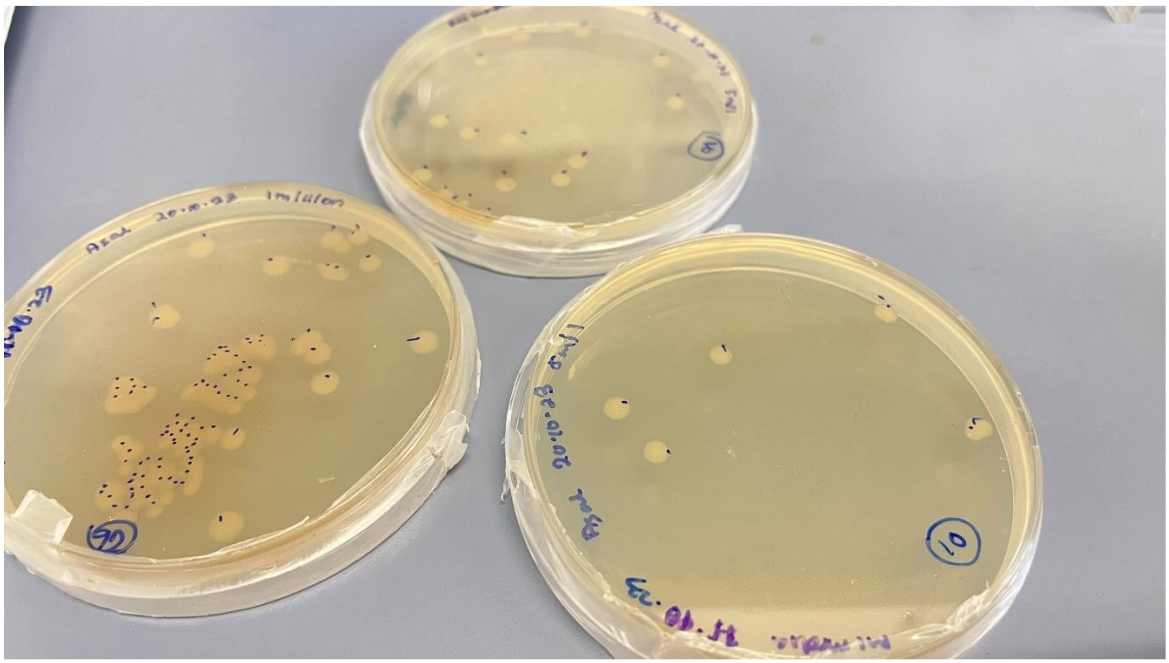
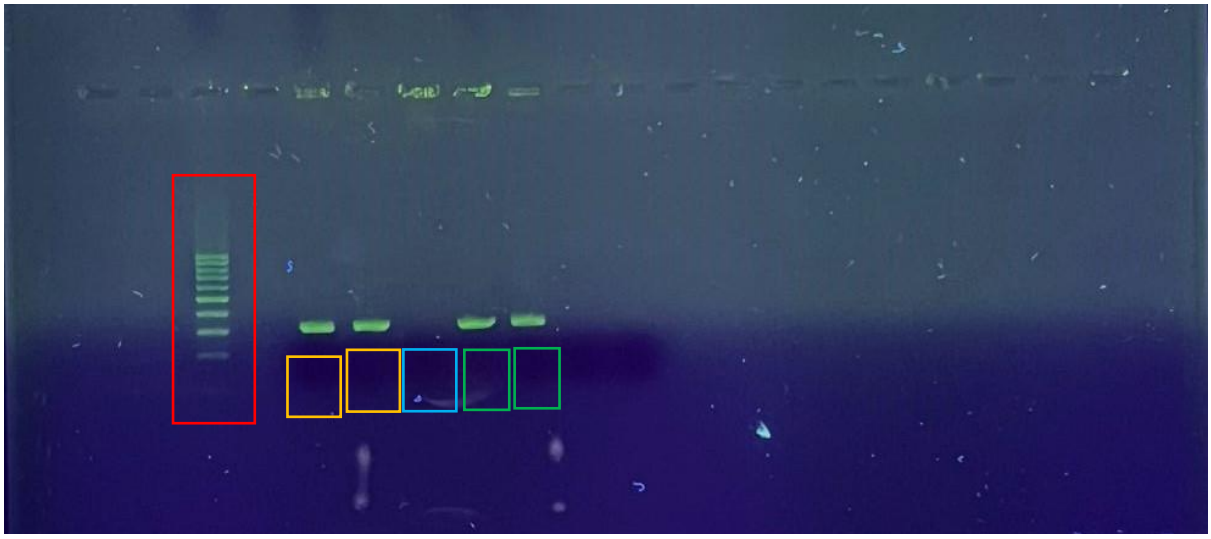


Figure 10: CFU on 3 plates displayed on a table.

3.2. Gel Electrophoresis followed by PCR

To identify the species of fish pathogens, DNA extraction followed by PCR and gel electrophoresis was conducted. The PCR product derived from specific primers for *A. salmonicida* was utilized to generate an agarose gel, serving the dual purpose of confirming the success of PCR amplification and exhibiting consistent bands for all utilized samples. This outcome provided validation for the presence of *A. salmonicida* bacteria in the culture. Figure 11 displays this specific agarose gel, using Two PCR product samples obtained from 5% Chelex DNA Extraction. Two previous *A. salmonicida* kit extracts (indicated by K) along with a no template control (NTC) with water. Figure 11 illustrates that the distinct bands were evident for the 5%Chelex extraction and the NTC remained empty, as expected.

Figure 11: Agarose gel showing bands for *A. salmonicida.* First well (red box): 100 bp DNA Ladder. **1K and 2K (yellow box)**: old *A. salmonicida* Chelex extracts; **S1 and S2 (green box) :** DNA ; **NTC (blue box) :** H_2O.



3.3. Sequencing

The sequencing outcomes are illustrated in Figure 12, revealing well-defined and distinct peaks. Notably, each distinct color corresponds to a single peak, and these peaks exhibit even separation without any background signals or baseline noise in the chromatograms. These features are indicative of high-purity DNA and an effective primer design, as outlined by Eurofins Genomics. Initial poor resolutions of peaks, observed at the outset, are a common characteristic in sequencing chromatograms.

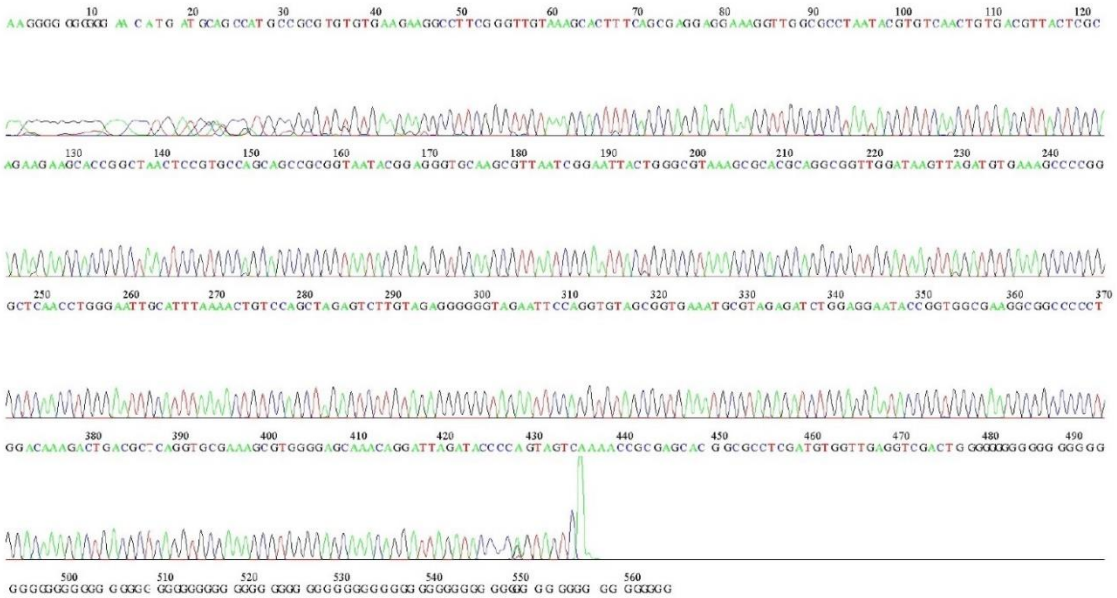


Figure 12: Sequencing chromatograms for *A. salmonicida,* chromatogram with forward primer

3.4. Comparison of the various DNA Extraction Methods

Two samples were used to compare the DNA extraction method used. one set with the 5% regular chelex extraction method and the other three sets with improvised chelex extraction method with continuous vortex mix for 10 minutes, 15 minutes and 20 minutes respectfully. Based on the Cq values of the qPCR we can conclude that they were almost the same. type of the DNA extraction method did not make a difference because *Aeromonas salmonicida* is a Gram-negative bacterium therefore lysis of the cell wall is very easier compared to a Gram-positive bacterium which consist of a thick peptidoglycan layer and *A. salmonicida* can be lysed easily with a simple 5% chelex extraction method. We continued to do our project only with chelex extraction afterwards.

Figure below shows the CQ values of Sample 1 and Sample 2.

	Sample 1	Sample 2
Regular Chelex Extraction	27.90	30.82
10mins of Improved Chelex Extraction	27.51	30.83
15mins of Improved Chelex Extraction	27.22	30.05
20mins of Improved Chelex Extraction	27.47	30.73

Figure 13: Comparison of the different kind of DNA extraction methods used.

3.5. Comparison of Quantification experiment with gblock Standard Series

To quantify the bacterial aliquots, a gBlocks Standard Series was created. Table 5 shows the results of 5qPCR attempts with gBlocks Standard Series. The table contains the Cq values obtained for each SQ in each attempt as well as the average of each of the 5 runs and the standard deviation for each SQ throughout all runs. As can be seen, even 1 copy was detected throughout all runs.

gBlocks SQ	LOG SQ	29.9.23	16.10.23	23.10.23	02.11.23	21.11.23	AVG	ST. DEV
10,000,000	7	14.71	15.23	15.82	18.2	14.58	15.71	1.48
1,000,000	6	18.42	19.74	19.77	22.63	19.14	19.94	1.60
100,000	5	22.11	23.2	23.41	27.13	22.76	23.72	1.97
10,000	4	26.12	27.07	27.24	29.04	25.18	26.93	1.44
1,000	3	29.36	31.93	31.10	31.98	28.17	30.51	1.68
100	2	33.32	35.55	34.42	32.99	30.23	33.30	1.99
10	1	35.91	38.72	38.93	33.35	31.49	35.68	3.27
1	0	37.96	39.16	38.99	34.84	32.87	36.76	2.78
NTC	-	35.86	-	-	36.8	35.83	-	-

Table 4: Performance of gBlocks Standard Series. The values not detected in a run as well as positive NTCs are shown in red. The average as well as standard deviation calculated for each SQ is also shown.

3.5.1. gBlock Standard Curve

This data was then used to generate a gBlocks Standard Curve with the mean Cq values plotted against the LOG starting quantity as shown in Figure 12. The standard curve has an efficiency (E) of 111.38% and a R^2 value of 0.9804.

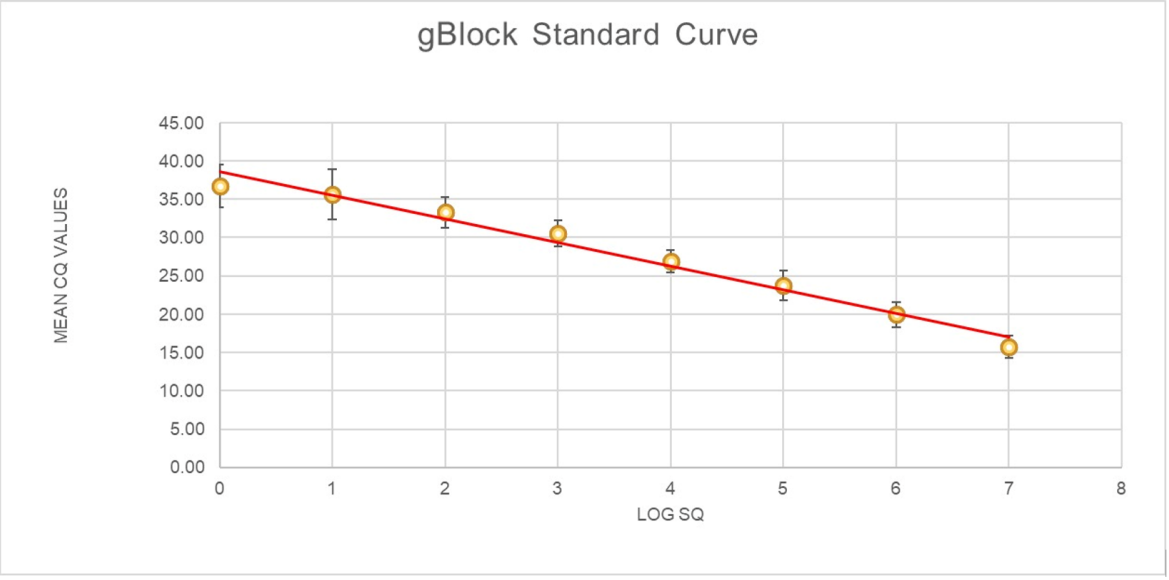


Figure 14: Standard curve for gBlocks Standard Series used for qPCR. Mean Cq values plotted against the LOG of SQ. Equation of the regression line and error bars showing standard deviation are also shown.

3.6. Quantification experiment using CFU Standard Series

gBlocks represent highly pure short DNA fragments, offering efficiency in quantification. However, the quantification results provided by gBlocks Standard Series Dilutions may not necessarily reflect the reality of the bacterial culture. To obtain quantification results that closely correspond to the bacterial culture aliquots, a second serial dilution was created using the CFU count obtained, as shown in Table 5 and Figure 8. Comparing these two serial dilution series allows for the determination of a specific 'factor' that compensates for factors such as dead cells, dormant cells, free DNA, etc. This calculated 'factor' can then be applied to estimate the actual amounts of bacteria.

To assess the newly created CFU standard dilution series and determine its acceptability in relation to expected starting quantities of the *A. salmonicida* culture, replicate qPCRs were conducted. Table 5 presents the results obtained from these

qPCRs, including the quantification cycle (Cq) values for each starting quantity in every run, along with the calculated average and standard deviation. It is observed that the Cq values for this CFU serial dilution are slightly delayed compared to the gBlocks Standard Series Cqs, as shown in Table 6.

CFU SQ	LOG SQ	15.9.23	19.10.23	22.10.23	23.10.23	AVG	ST. DEV
10,000,000	7	29.37	23.57	26.19	32.69	27.96	3.95
1,000,000	6	31.49	27.01	28.86	34.28	30.41	3.17
100,000	5	35.38	29.76	25.33	38.22	32.17	5.76
10,000	4	39.3	34.83	31.74	-	35.29	3.80
1,000	3	-	35.12	37.52	39.46	37.37	2.17
100	2	-	36.06	37.27	-	36.67	0.86
10	1	-	35.60	38.22	38.90	37.57	1.74
1	0	-	37.59	36.30	38.84	37.58	1.27
NTC	-	-	-	36.21	39.83	-	-

Table 5: Performance of CFU Standard Series. The values not detected in a run as well as positive NTCs are shown in red. The average as well as standard deviation calculated for each SQ is also shown.

3.6.1. CFU Standard Curve

Using the data in Table 5, a standard curve for CFU standard series was generated as is shown by Figure 15. It is a plot of the average Cqs obtained by the CFU Serial Dilution Series against the LOG of starting quantities. The R^2 is equal to 0.8623.

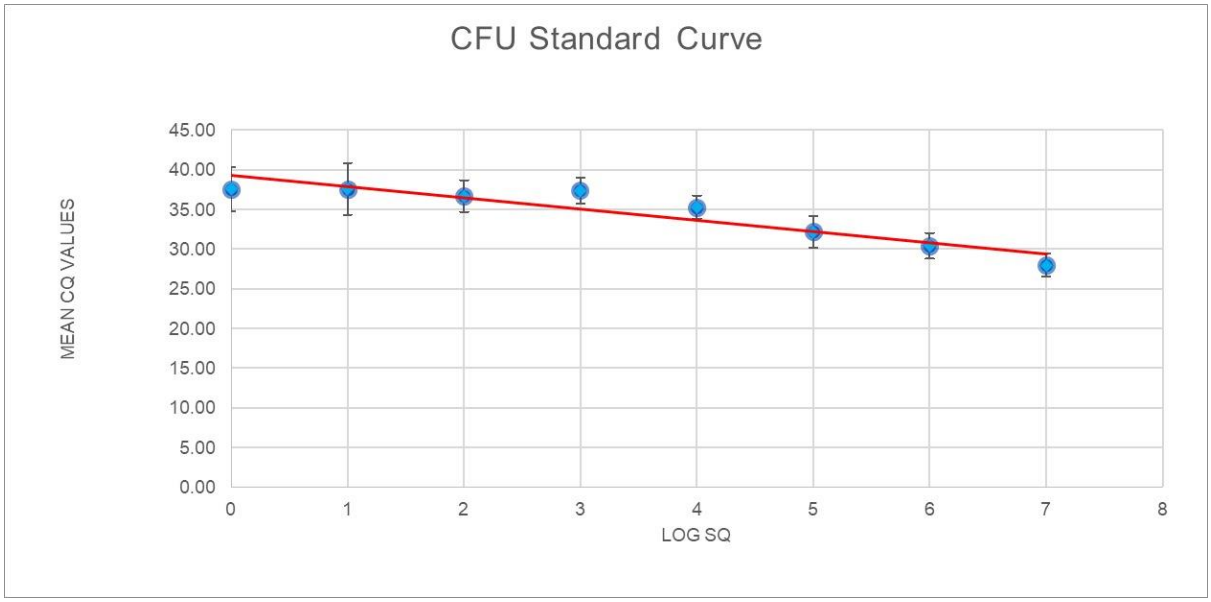


Figure 15: **Standard curve for CFU Standard Series used for qPCR.** Mean Cq values plotted against the LOG of SQ. Equation of the regression line as well as the error bars depicting standard deviation are also shown.

Since the values obtained on both gBlock and CFU serial dilutions had very low values of *A. sal* count, quantifying *Aeromonas salmonicida* through this analysis did not work well in this project. Therefore, did not continue to eDNA filtration in this method and to quantify *Aeromonas salmonicida* a slightly different approach was made.
In this new approach instead quantifying the whole set of serial dilution based on CFU count, initially stored media aliquots were directly quantified with three sets of volumes (1000 ul, 100 ul, 10 ul) and then eDNA filtration was done but this time based on volume measurements which was used before using the same media aliquots followed by a qPCR quantification.

The table below shows the new media aliquot CFU qPCR quantification results and respective standard curve obtained using the mean Cq values vs Starting volume values.

CFU results Starting Volume (µL)	26.10.23	20.10.23	29.10.23	AVG	ST. DEV
1,000	20.67	20.64	18.12	19.81	1.46
100	22.79	24.18	21.70	22.89	1.24
10	27.12	28.05	23.54	26.24	2.38
NTC	39.94	35.81	35.12	-	-

Table 6: Performance of CFU Standard Series VS Starting volume. The values not detected in a run as well as positive NTCs are shown in red. The average as well as standard deviation calculated for each SQ is also shown.

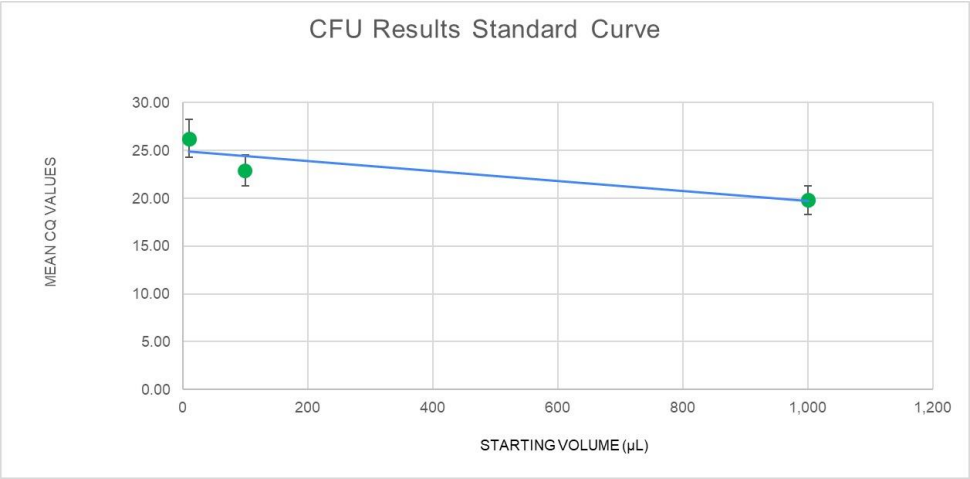


Figure 14: CFU Filtration Standard Curve with Cq mean and Starting volume.

3.7. Quantification of both Media Aliquot (CFU) and eDNA Filtration

A filtration experiment was conducted by introducing known quantities of bacterial culture into 1 L of distilled water, followed by filtration using the setup outlined in Figure 7 and detailed in Section 2.7. Cellulose-acetate filters were employed in this process. After DNA extraction from the filters, quantification was performed using Consistent with previous quantification experiments, the qPCRs were repeated three times. expecting that the quantified cell amounts would be lower than the expected number of cells in a sample due to losses during filtration and the subsequent extraction step, the results, as indicated in Table 7, confirmed this expectation. The samples utilized had a starting volume of 1000 ul, 100 ul and 10 ul.

eDNA CFU Starting Volume (µL)	26.10.23	20.10.23	29.10.23	AVG	ST. DEV
1,000	26.57	17.34	18.28	20.73	5.08
100	27.68	20.63	21.51	23.27	3.84
10	30.28	24.06	25.14	26.49	3.32
NTC	36.31	33.83	34.41	-	-

Table 7: Starting volume measured filtration experiment.

Positive NTC was detected throughout this experiment. It can be avoided in future by repeating the same experiment multiple times. Using the Cq mean, the standard curve was plotted against the Starting volume.

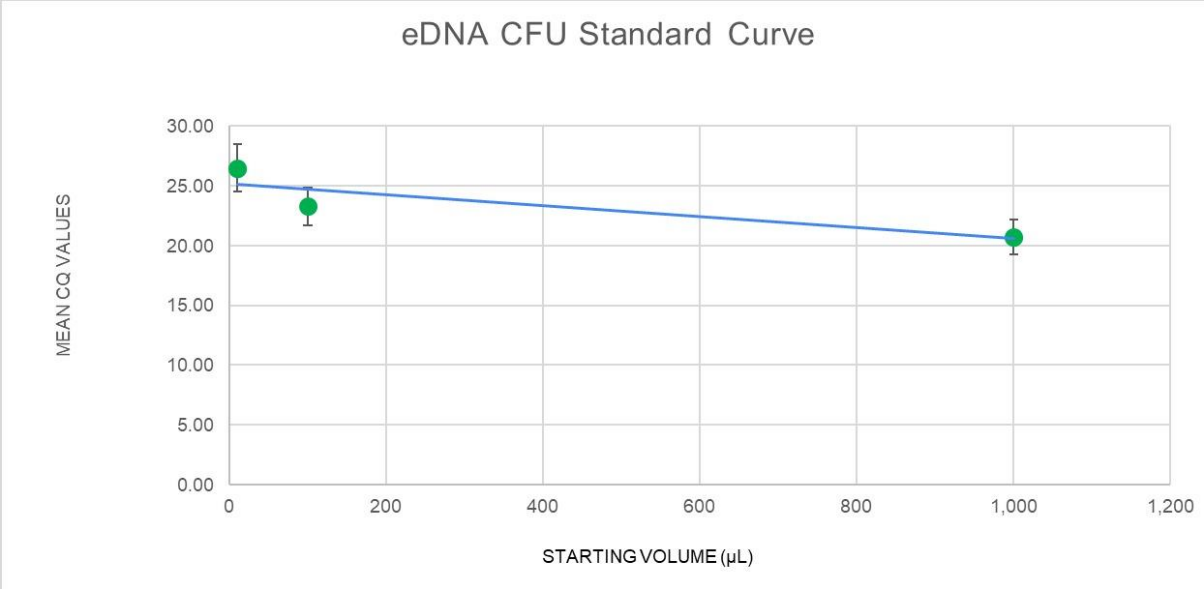


Figure 16: eDNA Filtration Standard Curve with Cq mean and Starting volume.

With the quantification results obtained, recovery rates of *Aeromonas salmonicida* samples were calculated. A closer comparison is shown by the table below to provide an overview of the quantification comparison.

	26.10.23			20.10.23			29.10.23		
Starting Volume (µL)	eDNA	CFU	Recovery Rate	eDNA	CFU	Recovery Rate	eDNA	CFU	Recovery Rate
1,000	8.34E+04	6.83E+06	1.22%	3.87E+05	2.91E+06	13.30%	1.73E+06	7.15E+07	2.42%
100	7.22E+03	8.79E+05	0.82%	2.36E+04	5.70E+05	4.14%	1.11E+06	4.22E+07	0.26%
10	1.16E+03	3.71E+04	3.13%	1.28E+03	9.65E+04	1.33%	5.12E+04	7.96E+06	0.64%

Table 8: Comparison of eDNA results versus CFU series in Cq values and their recovery rate percentages.

4. Discussion

4.1. Growth of *A. salmonicida*

A pristine bacterial culture forms the foundation of any study focused on a specific microorganism. Similarly, the initial phase of this research involved cultivating a pure culture of *A. salmonicida*. Numerous attempts were made to inoculate flasks using stored *A. salmonicida* cultures, but these were found to be highly contaminated. Even inoculations from a freshly prepared batch of *Aeromonas salmonicida* resulted in contamination after a few attempts. Eventually, a selection from a previous *A. salmonicida* culture plate was successfully used to inoculate and establish a culture that remained uncontaminated throughout the thesis work, displaying uniform growth within a 24-hour period.

A portion of the original flask's medium was transferred to inoculate a new flask, and the optical density (OD) of the culture was measured using a photometer, as outlined in Section 2.1.3. OD measurement, a widely adopted method, provides a quick, simple, cost-effective, and minimally disruptive means of estimating cell numbers in a liquid suspension (Beal *et al*., 2020). The results of the OD600 experiment are presented in Section 3.1.2, Figure 9, depicting a growth curve.

The growth curve encompasses four phases: the lag phase, occurring immediately after culture inoculation with no cell growth; the log or exponential phase, characterized by exponential microbial growth; the stationary phase, where cell density remains constant; and the death phase, marked by a decline in the curve as the total cell number decreases. Figure 9 reveals a similar pattern, indicating exponential bacterial growth from approximately 400 to 800 minutes, a slightly flattened peak from 800 to 900 minutes, and a subsequent sharp drop in measured OD.

As discussed earlier, Figure 9 demonstrates that measured OD values are relatively precise during the exponential phase, but this precision diminishes in the stationary and death phases, as evident from comparatively larger error bars. This phenomenon can be attributed to the equilibrium between growth and death in the stationary phase, where the bacterial population remains constant, with numerous cells continuing to

divide and others beginning to die. However, this also highlights one of the reasons why OD600 measurements are deemed unreliable, as they also account for dead cells, resulting in an inaccurate estimation of the culture's growth and metabolic activity.

Additionally, it is important to note that the starting OD measurement is recorded as 0.4, not 0, due to the consideration that a higher dilution would have resulted in a lower starting value. Furthermore, the OD600 measurement acknowledges air bubbles in the culture as living cells, further contributing to unreliable results (LAMBDA).

4.2. Chelex DNA Extraction

Chelex protocols have gained popularity for expedient DNA extractions, particularly in cases of low DNA concentrations (Singh *et al*., 2018). Initially developed by Walsh *et al*. in 1991, the Chelex DNA extraction protocol has since undergone adaptations and optimizations for various applications (Simon *et al*., 2020; Singh *et al*., 2018; Walsh *et al*., 2013). Despite certain drawbacks pertaining to the purity and stability of extracted DNA, the simplicity, cost-effectiveness, and speed of Chelex extraction methods often outweigh these concerns (Singh *et al*., 2018). In comparison to extraction kits, Chelex protocols typically involve fewer steps and fewer chemicals (Walsh *et al*., 2013).

Within the context of this thesis, target DNA was successfully detected through qPCR in almost all Chelex-extracted samples. Notably, a Chelex No Template Control (NTC) remained empty (Table 15, p.43), supporting the assumption that Chelex itself does not interfere with qPCR detection. Anticipated recovery rates of Chelex extracts were expected to be less than 100%, considering the likelihood that not all cells in a sample are successfully lysed, and some DNA may be lost during the transfer of supernatant to new tubes. However, in initial quantification experiments, more DNA was detected than expected to be present in the samples. These discrepancies were likely associated with inconsistencies between the different standard series applied, as previously discussed in Section 4.3 on Quantification and Factors. Consequently, estimating recovery rates for the Chelex extraction method proves challenging.

Still based on the results of proceeding the DNA extraction via all three methods listed above it was quite evident to assume that all are nearly same because the *A.*

salmonicida is a Gram-negative bacterium and it was easier to lyse compared to a Gram-positive bacterium therefore Chelex was an optimal choice for this thesis work.

4.3. qPCR Performance

The efficiency value in qPCR provides insight into how many times a single DNA copy is amplified in a qPCR cycle. An efficiency of 100% signifies that the amount of DNA precisely doubled in each cycle (Kralik and Ricchi, 2017). However, apparent efficiency can be influenced by factors such as the presence of inhibitors, inaccurate pipetting, and DNA template concentration (Johnson *et al*., 2013). In practical terms, qPCR efficiencies typically fall within the range of 90–105% (Johnson *et al*., 2013). The Bio-Rad qPCR assay guide recommends an efficiency between 90–110% for assay validation (Bio-Rad Laboratories, 2019). A robust qPCR assay should cover at least six logarithmic steps with a linearity of R2 > 0.98 for three technical replicates (Johnson *et al*., 2013). Additionally, it is advisable to conduct experiments with technical replicates, where the standard deviations for each set of technical replicates should be <0.2 (Bio-Rad Laboratories, 2019).

In this study, all tested standard series meet the stability criteria concerning linearity and a range of at least six logarithmic steps. However, the calculated qPCR efficiencies varied among the different standard series. For the gblock standard curve, plotted from the average Cq values, resulted with a linearity coefficient of 0.9804 and efficiency of 11.38%. The lower higher suggests that can be pipetting errors, polymerase enzyme activators, inhibition by reverse transcriptase, inaccurate dilution series and unspecific products and primer dimers when using intercalating dyes.

4.4. Analytical specificity and sensitivity

Ensuring assay specificity is paramount for the accurate detection of pathogens. Assay specificity refers to the extent to which samples containing undesired DNA sequences are correctly identified and indicated as positive results. A well-crafted assay ideally exhibits zero occurrences of false positives (Johnson *et al*., 2013). Similarly, the primers and probes designed for this study demonstrated high specificity, as evidenced by the absence of positive results when attempting to identify contaminated cultures

through PCR and gel electrophoresis.

Positive results in qPCRs are frequently observed due to various factors such as limited lab conditions for incubation, product carryover, and cross-contamination between different samples, among others. This is evident in the results presented in Tables 6 and 8, where a few positive No Template Controls (NTCs) were recorded. NTCs, typically prepared with molecular-grade water, serve as negative controls in qPCRs and are essential for identifying setup contamination and primer-dimer product amplification. These controls include all PCR reagents except for a DNA template (Nolan *et al*., 2013). Table 9 provides a summary of the quantification cycle (Cq) values for all the NTCs used in various qPCRs.

Table 9: Cq values of NTCs detected in various qPCRs. The positive NTCs are indicated with numerical values. An averageCq was also calculated.

	SQ Value for NTC							
	29.09.23	15.09.23	16.10.23	19.10.23	22.10.23	23.10.23	02.11.23	21.11.23
NTC	35.86	N/A	N/A	N/A	36.21	39.83	36.8	35.83

As seen above, out of the 8 runs, 5 of them had positive NTCs. Potential random contamination may have occurred during the loading of DNA into the qPCR plates. Due to shortages in supplies, loose gloves were utilized during this process, raising the possibility that small amounts of sample DNA template might have come into contact with the gloves, subsequently transferring to the qPCR plates. Additionally, both pre-PCR and post-PCR procedures were conducted within the same laboratory space, introducing the potential for contamination, where a PCR product carryover from a previous reaction could contaminate a new qPCR plate or DNA extract, leading to a positive result. It is noteworthy, however, that the majority of No Template Control (NTC) quantification cycle (Cq) values were observed late in the reaction towards its conclusion. According to D'haene and Hellemans (2010), such late positive signals can be disregarded if the difference between the highest Cq value of the run and the Cq value obtained for the NTC is sufficiently large. Considering that the highest average Cq value was 11.97 with the gBlocks Standard series (Table 6) and the average Cq

value for all NTCs was 38.96 (Table 9), the difference is substantial enough to deem the positive NTCs negligible.

Assay sensitivity, or the Limit of Detection (LOD), is defined as the lowest amount of cells, or at least 95% of the positive samples, that can be detected to yield a positive result. However, for increased confidence in the LOD, replicates need to be conducted (Kralik & Ricchi, 2017).

4.5. Standard Series Performance

A robust and precise quantitative polymerase chain reaction (qPCR) assay is associated with a high qPCR efficiency. Ideally, in a perfect qPCR, the number of initial DNA molecules should double every cycle, resulting in 100% efficiency. However, real-life reactions are seldom flawless, and efficiency typically ranges between 90% and 110%, with gradients falling between -3.2 and -3.5 (Bustin *et al*., 2009). An efficiency of 110% may indicate polymerase inhibition, which is often most pronounced in the least diluted sample, leading to deviations from linearity (Svec *et al*., 2015). Polymerase inhibition occurs when excessive amounts of DNA/RNA or carry-over material are present in a sample (Čepin, 2017b). Conversely, an efficiency value below 90% may signal inhibitor contamination, poor primer efficiency, or inaccurate pipetting (BiteSizeBio, 2022). Furthermore, a stable qPCR assay should exhibit an R2 value greater than 0.98 over at least 6 logs and three replicates (Bustin *et al*., 2009).

For this study, two standard dilution series were employed, and both met the criteria for a stable qPCR in terms of linearity coefficient, slope of the standard curve, and efficiency. The R2 value for the gBlocks standard curve was 0.9804, while for the CFU standard curve, the R2 value was found to be 0.8623. Similarly, the gradient for the gBlocks standard curve was -3.08, and for the CFU standard curve, it was -1.41, both falling within the determined range. The efficiency of the gBlocks standard curves was calculated to be 111.38%. Figure 17 provides a side-by-side comparison of the two standard curves.

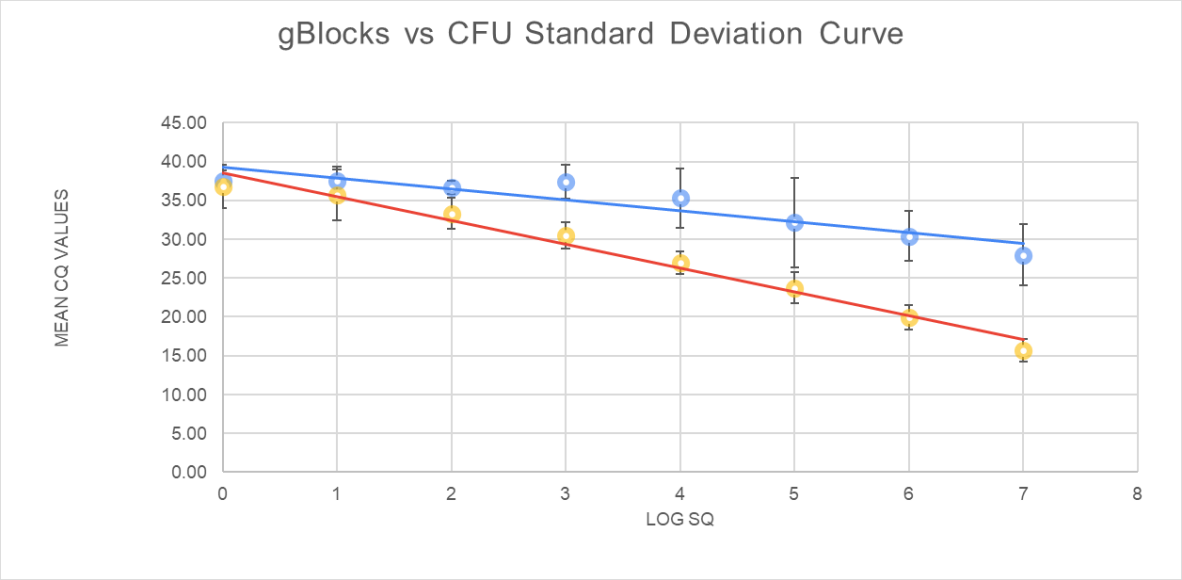


Figure 17: Comparison of the gBlocks and CFU standard curves.

4.6. qPCR Results

The detection of *A. salmonicida* was possible throughout all samples, regardless of the DNA extraction method or Standard Series, we continued to use 5% regular chelex extraction method throughout the experiment because lysing the Gram-negative bacterial layer was not hard as gram positive bacteria. Chelex extraction method where the recovery rates were close to a 100% or even over for certain samples which can be explained by, as mentioned for gBlocks quantification for 1 copy number, an incorrect expected SQ value. This is because CFU counts only account for living cells that are able to proliferate and does not take into account dead or dormant cells or free DNA. Similarly, a colony on a plate that may be counted as a single colony may actually consist of cluster of cells that are not seen by the naked eye. This means CFU counts have low accuracy and the counts are often affected by cell adhesion and clumping (Beal *et al.*, 2020).

However, through qPCR each DNA template present in a sample is detected and counted, resulting in a higher detected SQ than expected.

Comparatively, the % recovery rates obtained were better for CFU standard series than for gBlocks standard series. This is because with the CFU serial dilution, a higher recovery rate was obtained for both regular 5% Chelex extracts and the kit extracts,

ranging from approximately 2.5-4.9 times higher in 5% Chelex extracts and about 4-fold to 25-fold higher in kit extracts as shown by Table 10. For the improved Chelex extraction, however, CFU serial dilution had over 100% recovery rates and the detection factor were about 2.50-9.60 times higher. This again can be explained by the improper CFU counts that do not account for non-living cells.

In this project initially the expected qPCR results were very low which led to a point of it was difficult to trace when the number of DNA copy reduces in serial dilution. Having a positive NTC was also another hindrance in this quantification process. To rectify this starting volume-based quantification was done. Although the recovery rate is low in general, we were still able to detect *Aeromonas salmonicida* and quantify it. The table below shows the Cq values of the media aliquots used in the new approach handled in this quantification.

	SQ Mean					
	eDNA Filtration			CFU results		
Starting Volume (µL)	26.10.23	20.10.23	29.10.23	26.10.23	20.10.23	29.10.23
1,000	8.34E+04	3.87E+05	1.73E+06	6.83E+06	2.91E+06	7.15E+07
100	7.22E+03	2.36E+04	1.11E+06	8.79E+05	5.70E+05	4.22E+07
10	1.16E+03	1.28E+03	5.12E+04	3.71E+04	9.65E+04	7.96E+06

Table 10: Quantification results of both eDNA filtration and CFU series.

4.7. Filtration experiment for PHOTO-SENS

The primary objective of the filtration experiments was to replicate the filtration process for environmental DNA (eDNA) from highly diluted water samples, mirroring the procedures intended for PHOTO-SENS biosensor chips. As previously outlined, these biosensor chips aim to identify the presence of eDNA from fish pathogens, including *Aeromonas salmonicida*, in water samples. Conventionally, eDNA capture methods involve filtration or centrifugation, with filtration demonstrating higher efficiency (Majaneva *et al*., 2018). The experimental design involved spiking 1 L of distilled water with an *A. Salmonicida* culture, followed by filtration through cellulose acetate (CA)

filters. Subsequently, DNA was extracted from these filters for detection and quantification using quantitative polymerase chain reaction (qPCR). It was expected that there would be a loss of detected DNA during the qPCR process due to the filtration and DNA extraction steps, despite the initial estimated starting quantity. This was the case as seen in the results shown by Table 11 also showed the % recovery rate calculated for expected SQ for 1000ul ,100 ul and 10ul.

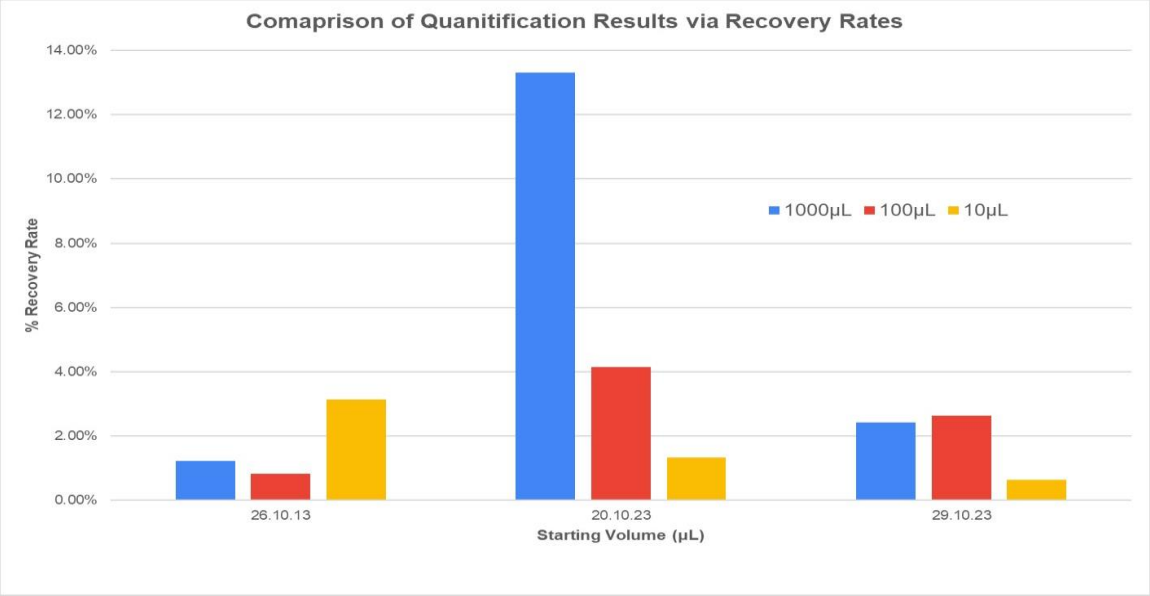


Figure 18: Comparison of the % recovery rate for each media aliquot samples used.

Although, this experiment provided results that were considered satisfactory for the scope of this work where all samples including the small amounts of *A. salmonicida were* detected and quantified using qPCR. However, to gain an increased confidence in the results, the filtration experiment would need to be repeated several times.

4.8. Findings of similar research

The primers formulated for this study exhibited specificity solely to A. salmoninarum and did not yield positive signals with any other bacterial species. This specificity was achieved by selecting a primer region during the primer and probe design process that was unique to *A. salmonicida*. Notably, this chosen primer region bears similarity to the region selected for primer design by Torres-Corral and Santos (2019).

Since the project PHOTO-SENS focuses on 3 main bacteria namely, *Vagococcus*

salmoninarum, Aeromonas salmonicida, and *Yersinia ruckeri,* it is also valuable to mention the progress being made in the context of detection of these bacteria in fish samples. Bartkova *et al.* (2017) conducted experiments to detect and quantify *Aeromonas salmonicida* in fish tissue. They followed a similar course of work by designing primers specific to *A. salmonicida* and testing them successfully for specificity and sensitivity via qPCR with their LOD to be 40 target copies/ reaction. Bastardo *et al.* (2012) designed specific primers for detection and quantification of *Yersinia ruckeri* in fish samples and was able to detect an LOD of 1.7 CFU.

The bacterium *A. salmonicida* was discovered over 100 years ago, however, there are still many questions regarding this pathogen and the disease furunculosis that remain unanswered.

The best way to gain more knowledge on the host-pathogen relationship of *A. salmonicida* is through in vivo imaging, a valuable approach that is rapidly advancing. Although fluorescent and bioluminescent reporters are still the most frequently used reporters for imaging, their limitations of resolution and range of depth prevent the possibility of obtaining a detailed picture of the host pathogen relationship. Notably, one emerging method that promises to revolutionize imaging and surpasses both of the above methods in resolution and range of depth is photoacoustic imaging, which uses ultrasound waves for imaging (Xu and Wang, 2006). Thus far this technology has only been used for human biomedical research; however, in the near future this imaging method could become available in the veterinary field.

Another detection method for *A. salmonicida* that would have the same high sensitivity as the real-time PCR assay, but would enable 100% detection of all *A. salmonicida*, could be developed by changing the target of the present assay. One possible target could be the high-copy number plasmid pAsa1 that thus far seems to be universally present and stable in all *A. salmonicida* (Attéré *et al*., 2015). Much data has been obtained by WGS of the 101 *A. salmonicida* isolates and only a fraction of this data has been utilized for analysis thus far. Indeed, much more valuable and in-depth knowledge could be found by applying some of the available bioinformatics tools, a promising goal for which the dataset created by our WGS analyses can provide a solid foundation.

4.9. Outlook

Although the results obtained throughout this work were satisfactory and relevant in context of the PHOTO-SENS project, the experiments still had room for optimization and refining.

Primarily, the inability of qPCR to distinguish between viable and non-living cells, identified as the foremost drawback by Kralik and Ricchi (2017), poses a significant challenge for accurate quantification. This limitation results in the quantification of dead cells alongside living cells, leading to inaccurate results regarding the number of viable cells. However, it is widely acknowledged that this issue with DNA measurement does not extend to RNA, as RNA has low stability and disintegrates within minutes in dead cells. Consequently, reverse transcriptase qPCR (RT-qPCR) may present a more suitable approach for bacterial cell quantification (Kralik & Ricchi, 2017).

Similarly, the PHOTO-SENS project involves the detection of exceedingly low quantities of bacterial cells in highly diluted water samples, presenting a formidable challenge. The lowest copy number detected in this study's filtration experiments was 10 copies in 1 L of distilled water. Therefore, an alternative approach for extremely low-level detection could involve the utilization of droplet digital PCR (ddPCR). ddPCR, a recent advancement in PCR methods, allows the random allocation of target DNA into discrete droplets through microfluidics. These droplets are individually thermally cycled and screened using fluorescence measurement for the detection of target DNA. Quantification of DNA using this method has been found to be highly accurate (Zaiko *et al*., 2018).

To enhance the accuracy and detection limit of filtration experiments, the use of filters with a small pore size, such as 0.20 µm, could be beneficial. According to Majaneva *et al*. (2018), cellulose-nitrate filters yield the highest amount of DNA compared to other filters employed for environmental DNA (eDNA) capture.

Although the experiment was intended to simulate conditions for the PHOTO-SENS biosensor chips, it did not account for nutrient and chemical contamination, as well as

other microbial cell components likely present in water samples from hatcheries and rivers, potentially causing hindrance. Therefore, future adaptations of the experiment should aim to closely resemble water samples from the specified sites.

Similar to the approach of Standish *et al*. (2022) where a successful duplex qPCR was attempted for detection and quantification of *Aeromonas salmonicida* and *Carnobacterium maltaromaticum,* a multiplex qPCR could be done for the detection of all 3 salmon pathogen that are involved in the PHOTO-SENS project. This could be done by using a mix of primers and differently fluorescent-labelled probes that are specific to the bacterial pathogens in question.

5. Summary

Aquaculture has gained immense popularity in recent years and is one of the fastest growing animal food production sectors. However, a sustainable future for aquaculture is severely threatened due to various factors, one of them being bacterial outbreaks from common fish pathogens. *Aeromonas salmonicida* is a Gram-negative fish pathogen that is known to cause Furnoculosis and the detection methods include culturing, serology and molecular biology techniques. Controversy surrounds its possible independent existence in water; enzyme-linked immunosorbent assay and the polymerase chain reaction have detected *A. salmonicida* in the absence of colony-forming units, but cells that are nonculturable may be significant to fish pathology. Furunculosis is probably transmitted by the pathogen's entry into gills, mouth, anus and/or surface injury of fish through contact with infected fish or contaminated water. Disease-control is possible by good husbandry practices, disease-resistant stock, improved diets, nonspecific immunostimulants, antimicrobial compounds and vaccines.

This work was done as a part of the PHOTO-SENS project that aims to develop photonic biosensing chips for the detection of salmon pathogens. These chips ought to make use of short DNA sequences known as probes that are specific to target DNA of a pathogen. The binding of the two results in a detectable signal. Therefore*,*
A. salmonicida specific primers and probes were designed for the detection of *A.*

salmonicida in samples using qPCR. The quantification was done using a standard curve generated from two different standard dilution series used. Moreover, due to low values of Quantification results a slightly different approach was done to quantify *Aeromonas salmonicida.*

Most of the experiments conducted during this work can still be finetuned for further work, the foremost being able to distinguish between living and non-living cells and determining how relevant this distinguishment is to the PHOTO-SENS project. The filtration experiment can also be repeated several times and further adapted for improved results. In conclusion, although there is still potential for further research and enhancement, this work regarding the testing of *A. salmonicida*-specific primer and probes for the detection of quantification of the pathogen was successful and therefore, is suitable for use in the PHOTO-SENS project.

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