RHINE-WAAL UNIVERSITY OF APPLIED SCIENCES

Detection of the Fish Pathogen *Vagococcus salmoninarum* via Quantitative real-time PCR for Aquaculture

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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 *Vagococcus salmoninarum*, that is a causative agent of Vagococcosis.

For this work, a primer and probe set specific to *V. salmoninarum* 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 *V. salmoninarum* being a Gram-positive bacteria, 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 *Vagococcus salmoninarum* 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 control
OD: 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 minute
rRNA: Ribosomal RNA
RT-qPCR: reverse transcriptase qPCR
SD: serial dilution
SQ: Starting quantity
ssDNA: single stranded DNA

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1. Introduction

1.1. Value and Importance of Aquaculture

Aquaculture 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% of the world's fish supply (Leung & Bates, 2013). According to the Food and Agriculture Organization of the United Nations [FAO], 56% of the aquatic animal food production available for human consumption was provided by aquaculture in the year 2020 (FAO, 2022).

Aquatic foods have gained immense popularity in the last few decades due to various reasons such as being highly accessible, affordable as well as being a rich source of animal protein and micronutrients. This is largely the reason why fish comprises a nutritionally vital part of people's diet specially in developing countries. In some coastal and island countries such as Bangladesh, Indonesia, Senegal, and Sri Lanka, it contributes over 50 percent of all animal protein consumed. Moreover, the number of people employed in aquaculture is also the highest in developing countries (Finegold, 2018) with Asia being the most important region for aquatic farming. Similarly, China dominates the total aquaculture production output followed by other Asian countries namely India, Indonesia, Vietnam and Bangladesh as shown in Figure 1. (Marco Ottinger, 2015).

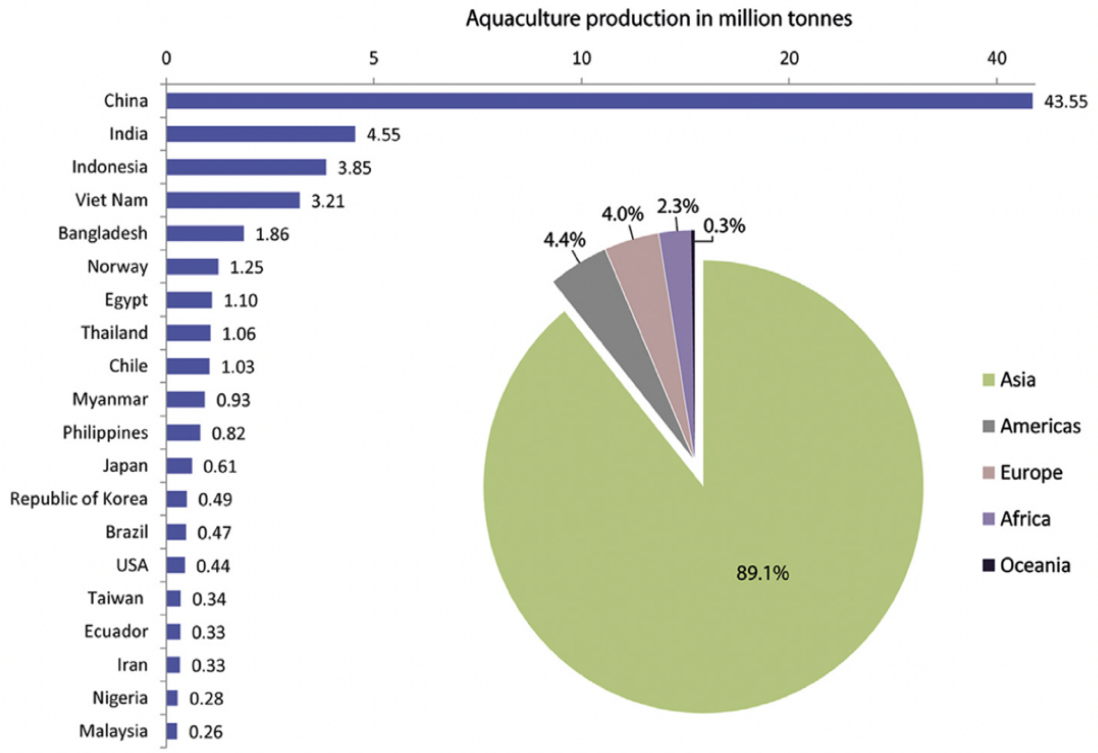


Figure 1: Global total aquaculture production output in 2013. (1) Bar chart: ranking the top 20 countries with highest aquaculture production in 2013. (2) Pie chart: share of total aquaculture production output among continents in 2013. Source: (Marco Ottinger, 2015)

Despite the high production of aquatic food products, the demand for these products continues to grow and dealing with this growing demand has resulted in over-intensification of production and trade in fish farming.

The aquaculture industry has become increasingly vulnerable to stressors due to its rapid expansion. These stressors include the harmful effects of pathogens, parasites and pests (PPP), pollution and climate change, to name a few (Naylor *et al.*, 2021). These challenges to a sustainable aquacultural industry while maintaining food security persist today and have only gotten much stronger in the recent years.

1.2. Global Approach to Sustainable Aquaculture

Much efforts have already been made to steer aquaculture and fish farming towards sustainability. These efforts are also meant to optimize the contribution of the aquatic food industry towards global food security, nutrition and ensure affordable healthy diets for all (FAO, 2022). Immense amount of attention has been paid to ecosystem-based management and improved system designs such as recirculating aquacultural systems and offshore aquaculture. Recirculating aquaculture systems help minimize

the effects of PPP and climate change while offshore aquaculture promotes a production of high volume of fish in deep waters without risking negative impacts such as nutrient pollution on freshwater and coastal environments. Similarly, improved governance and policies have also been an essential tool in promoting a sustainable future for aquaculture while being able to meet the needs of the growing population of the world (Naylor *et al*., 2021).

A similar initiative is the 'Blue Transformation' project by FAO. The Blue Transformation project aims to promote a sustainable aquaculture expansion and intensification as well as the effective management of fisheries to ensure healthy stocks while securing livelihoods of people that heavily depend on aquaculture. In addition, one of the objectives is also to upgrade value chains i.e. make sure an all-rounded viability of aquatic food systems and secure nutritional outcomes. FAO believes that if successfully implemented, the Blue Transformation can help the aquacultural industry sustainably provide about a 25% growth in per capita aquatic food consumption by 2050 when the world population is expected to reach about 10 billion (FAO, 2022).

1.3. Pathogens affecting Aquaculture

Since the expansion and intensification of aquaculture, the effects of PPP have only become chronic and severely amplified. In order to combat these risks throughout production systems, a variety of practices are often adopted such as species rotation, improved feed quality, pond and cage cleanliness, parasite monitoring and removal, enhanced surveillance systems, etc. (Naylor *et al*., 2021).

However, infectious disease caused by pathogens have been, by far, the biggest killer of farmed fish (Leung & Bates, 2013). Such outbreaks may be caused by various stress factors for fish such as overfeeding and overcrowding resulting in pollution and degraded quality of water. Similarly, climate change is another reason for frequent pathogen outbreaks in fish farms as changes in temperature and precipitation favor pathogen proliferation (Leung & Bates, 2013). Table 1. shows few of the most important 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 variety of other fish species
Yersinia ruckeri	Enteric redmouth	Salmonids, eel, minnows, sturgeon, and crustaceans
Gram-positives		
Lactococcus garvieae (Enterococcus seriolicida)	Streptococcosis/ Lactococcosis	Yellowtail and eel
Streptococcus iniae	Streptococcosis	Yellowtail, flounder, sea bass, 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 industry uses a variety of approaches to respond to such pathogen outbreaks. One of them being the use of therapeutants and chemical substances to prevent and treat pathogens. However, this does not come without the risk of posing serious health risk for consumers, workers, fish as well as the surrounding ecosystem if used improperly. Similarly, antibiotics are also widely used but this can be problematic if misused for it can lead 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 breakouts can also have serious financial implications on the aquaculture industry. Enormous losses tend to occur due to increased mortality which in turn lead to further financial burden when attempted to control the disease (Adams & Gunn, 2017). Although better alternatives such as the use of vaccines and selective breeding have been found, these methods tend to be fairly expensive and are difficult to replicate between 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 – *Vagococcus salmoninarum*

Vagococcus salmoninarum (V. salmnoninarum) is an uncommon bacterium that is associated with cold water disease outbreaks in aquaculture facilities. It is the causative agent of Cold Water Streptococcosis as it occurs in water temperatures below 10-12°C. The disease is also commonly called Vagococcosis (Torres-Corral & Santos, 2019). *V. salmoninarum* is a coccoid-shaped (Michel *et al*., 1997), gram-positive bacteria and is member of the *Enterococcaceae* family (Saticioglu *et al*., 2021). *Vagococcus salmoninarum* was first isolated from adult rainbow trout *(Oncorhynchus mykiss)* at hatcheries in Idaho and Oregon, USA. Since then, it has been associated with disease breakouts in rainbow trout in Spain, France, Italy and Turkey and therefore is an important bacterium in the European adult and sub-adult rainbow trout industry (Metin & Biçer, 2020). *V. salmoninarum* was also found in brown trout *(Salmo trutta)* and Atlantic salmon *(Salmo salar)* in Australia and Norway (Standish, Erickson, *et al*., 2020) as well as is quite widespread throughout the world in salmonid species.

V. salmoninarum has two distinct but troubling characteristics that make it a serious threat to aquaculture. First, disease outbreaks from *Vagococcus salmoninarum* have ≥50% mortality rates in broodstock. This is so particularly when the infection is associated with post-spawn stress and handling. Secondly, in-vitro testing has shown that despite *V. salmoninarum* being sensitive to antibiotics, field treatments largely

remain ineffective (Standish, Erickson, *et al*., 2020). *V. salmoninarum* is found to be quite similar to other Streptococcus bacteria. However, a distinctive property of the pathogen is that it is characterized by α-hemolytic activity and does not grow at temperatures higher than 42°C. Another specific characteristic to *V. salmoninarum* is that it can grow in 6.5% NaCl concentration which has been used to explained its severe infection and high mortality rates (Saticioglu *et al*., 2021).

1.4.1. Signs and symptoms of *V. Salmoninarum*

The diagnosis of diseased fish in aquaculture farms is generally based on the signs and lesions occurring on the body surface of the affected fish. Most of the symptoms of infection with *V. salmoninarum* are similar to that of other Gram-positive cocci that usually occur as persistent infections with repeated outbreaks due to the presence of a stressor (Michel *et al*., 1997). Effected fish most commonly show symptoms such as lethargy, swimming difficulty, paleness, etc. Clinical examination of diseased fish also demonstrate darkening of the skin and swelling of the abdomen (Tanrikull *et al*., 2014). Mono and bilateral exophthalmos, prolapsed anus, eyeball disruption and melanosis are also common signs of Vagococcosis. Often external hemorrhage in the jaw, eye, abdomen, mouth and ventral fins are also seen. Furnacles and erosive lesions on the side of the body as shown by Fig. 2a is another common sign of the disease. The most noteworthy internal symptoms include accumulation of bloody ascitic fluid in the cavity of the body. There may also be hemorrhage in the stomach and the ovaries as shown in Fig. 2b. Other signs include a yellow and bloody gelatinous exudate in the intestine of the affected fish. However, the most typical symptom is the fibrous deposit on the heart, spleen, liver and other organs of the body as shown by Fig. 2c and d (Didinen *et al*., 2011).

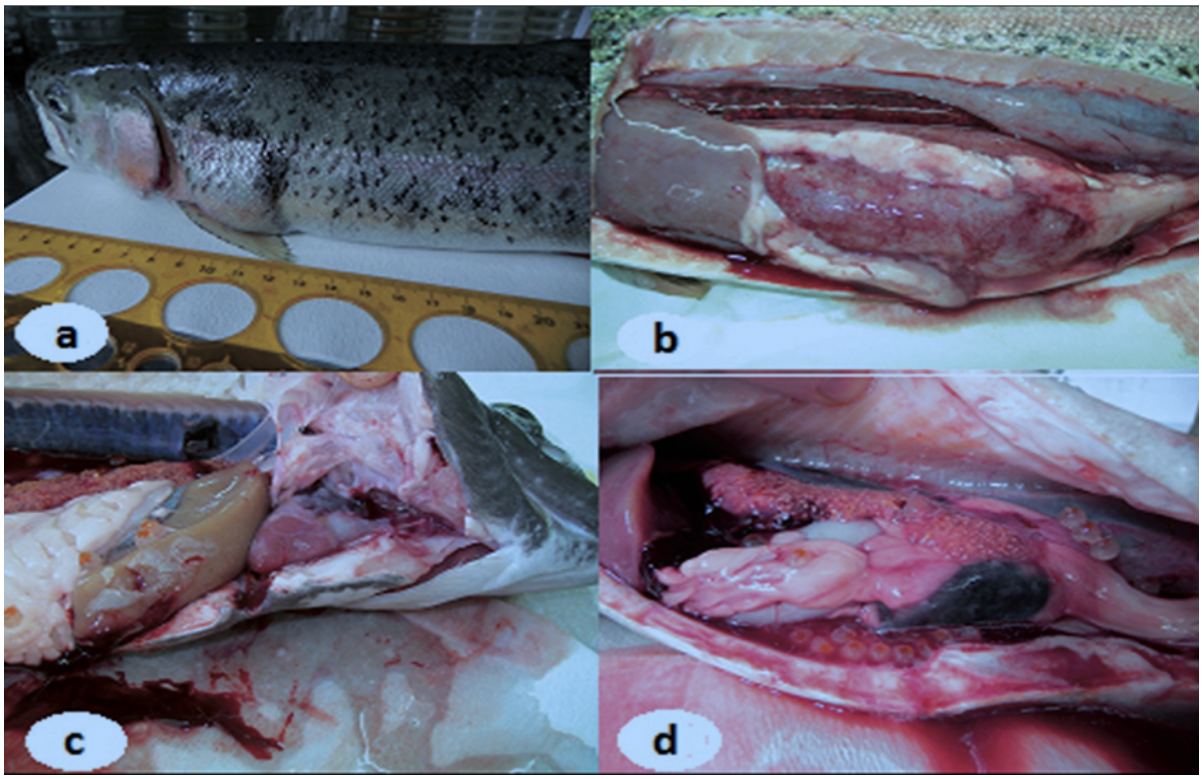


Figure 2: Rainbow trout brood infected with *V. salmoninarum.* (a) Furnacle on the side of the body. (b) Hyperemia and hemorrhage in the ovary and stomach. (c) Fibrinous deposits on the heart and paleness of the liver. (d) Fibrinous deposits on the spleen. Adapted from: (Didinen *et al*., 2011)

Similarly, as mentioned before, *V. salmoninarum* also causes serious infections in salmonoid broodstock and has symptoms such as egg putrefaction and gonadal dysfunction (Saticioglu *et al*., 2021). It has also been observed that infection caused by *V. salmoninarum* effects male and female fish differently, resulting in female fish having disproportionately higher morbidity and mortality rates as compared to males (Standish, Erickson, *et al*., 2020).

1.4.2. Detection of *V. salmoninarum*

New outbreaks are usually reported and confirmed on the basis of phenotypic and histopathological features. However, because these phenotypic characteristics are similar to streptococci, they cannot be relied upon for the correct identification of the bacteria (Torres-Corral & Santos, 2019). Therefore, improved analytical tools are needed for the apt and accurate identification of bacteria that can also discriminate between related strains.

One of the traditional ways is the serotyping of bacterial isolates but this method has a limited applicability and obtaining antisera from the animal is not the most convenient option. Identification via polymerase chain reactions (PCR) and quantitative PCR

(qPCR) are other popular medical and biological research approach that provides with a large variety of information such as specie identification, genetic relatedness of strains, virulence of strain, etc. Other techniques such as pulse-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), cell surface protein (CSP), etc. have been found to be extremely effective in the characterization of Gram-positive bacteria. A recent approach, known as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), for the diagnosis and discrimination between Streptococci and *V. salmoninarum* has been found to be simple, quick and cost-effective (Torres-Corral & Santos, 2019).

1.4.3. Current and Prospective Treatments of *V. salmoninarum*

As mentioned before, *V. salmoninarum* appears to be sensitive to antibiotics in-vitro but the use of antibiotics in field to treat vagococcosis has proved ineffective.

It has been found that *V. salmoninarum* is susceptible to several drugs namely, ampicillin, amoxycillin, erythromycin, oxytetracycline and doxycycline but such treatments were found to be effective only for short period of time (5-7 days) (Metin & Biçer, 2020). Standish *et al*., 2020 also reported a similar case where *V. salmoninarum* had strong and consistent sensitivity to florfenicol and tulathromycin initially. The fish responded well to the first 10-day course of the antibiotic and infection was reduced temporarily before the antibiotic rendered ineffective towards the end of epizootic in a second 10-day course and the chronic mortalities continued. However, the heavy use of antibiotics can lead to the emergence of antibiotic-resistant fish pathogen as well as resistant zoonotic fish bacteria that may prove harmful to humans.

There have also been attempts to use vaccines using an injectable bacterin with a combination of *Aeromonas salmonicida* and *V. salmoninarum* but similar results were obtained. The fish responded well initially but following the second treatment, mortalities increased to ≥50%, resulting in the culling of the remaining broodstock (Standish, Erickson, *et al.*, 2020). These finding were analogous to vaccination experiment on rainbow trout conducted by Michel *et al*. (1997).

A new alternate method of treating bacterial fish diseases is under consideration. It is the use of herbal products such as essential oils to control fish pathogen as they have anti-microbial properties as well as are natural and safe (Citarasu, 2008). In a study conducted by (Metin & Biçer, 2020), antimicrobial effects of thyme, black cumin,

ginger, St. John's Wort and rosemary oil against *V. salmoninarum* were investigated. Strong antimicrobial activity in the form of large inhibition zones was found at different concentrations of thyme and clove oil against the pathogen, *Vagococcus salmoninarum.* This proves that although further research is required, there is serious potential in using essential oils against treating bacterial fish pathogen for they are cheap, and natural and would help avoid overuse of antibiotics and therefore, reduce the risk of emergence of antibiotic-resistant bacteria.

1.5. Aim of the work

This work aims to be able to detect *Vagococcus salmoninarum* using qPCR for the PHOTO-SENS project. The 16-S gene of *V. salmoninarum* is to be used as target molecule for the development of a real-time PCR. It involves the designing and thereafter, testing of a probe along with specific set of primers for the detection of the pathogen. Furthermore, standard series are to be created and utilized in order to quantify unknown samples once the bacteria is cultivated. Doing so will help analyze the specificity and the sensitivity of the designed primer probe assay. Further experimentation is also to be carried out that compare the efficiency of different DNA extraction methods for the gram-positive *Vagococcus salmoninarum* as well as a mock e-DNA filtration. This would mimic extraction of eDNA from water samples as it is to be done in aquaculture facilities and fish hatcheries.

1.5.1. About PHOTO-SENS Project

The PHOTO-SENS project is founded and funded by the European Union's Horizon 2020 Research and Innovation program as a part of the Zero Hunger goal of the UN's 2030 Agenda. The project is ought to run for 3 years, ending in November '2023 (CORDIS, 2020) receiving a total EU contribution of 3 million euros. The main aim of the project is to develop photonic biosensing chip for the detection of salmon pathogen. There are 5 multinational companies working together on this project with each specialized on a certain task along the value chain i.e. production of photonic biochips by Surfix B.V., NL, photonics assembly and packaging by PHIX B.V., NL, microfluidics integration by CSEM, CH, equipment manufacturing by LRE Medical

GmbH, D, and diagnostic services in aquaculture by Tunatech GmbH, D (CORDIS, 2020).

TunaTech GmbH is responsible for the development and functional validation of novel genetic biomarkers for the 3 pathogens involved in this project, one of them being *Vagococcus salmoninarum*. These DNA chips are based on the use of probes that are short DNA sequences - 20-30 bp (base pairs) and are specific to their target DNA sequence in a selective pathogen. These probes are used as surface coating on the biosensor chips. Hence, when pathogenic DNA comes into contact with these biosensor chips, a short double stranded DNA fragment is formed. This binding causes a phase shift of the output produced on the asymmetric Mach–Zehnder interferometer (aZMI) which can then be monitored and detected with high accuracy (PHOTO-SENS). This is further summarized in Figure 3 to help better understand the approach of the PHOTO-SENS project.

These chips are expected to be a fast and cost-effective method of detecting bacterial pathogen from aquaculture samples which would help prevent severe infection outbreaks.

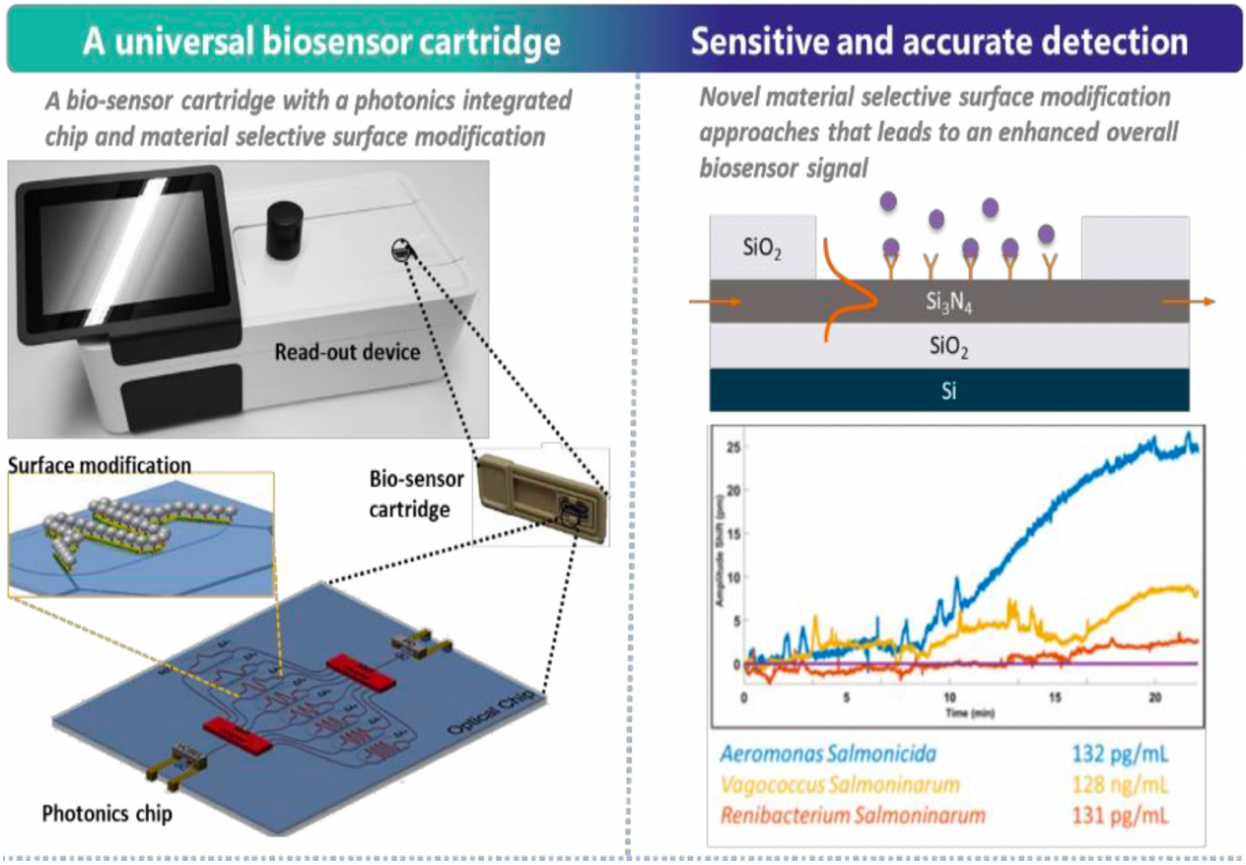


Figure 3: The novel approach of the PHOTO–SENS project with eDNA result for 3 salmon pathogens from water samples. Adapted from: (PHOTO-SENS)

1.5.2. qPCR for the detection of *V. salmoninarum*

Multiple molecular methods are currently in use to detect, quantify and study microbial populations. Amongst them, of the most popular methods is a PCR-based technique known as, quantitative real-time PCR (qPCR), so called because it simultaneously measures the amplification product as the reactions progresses i.e. 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 as minimizes experimental errors (Torres-Corral & Santos, 2019). It is also quite sensitive and 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).

Most qPCRs make use of a fluorescently labeled probe that allows the detection of target DNA. These probes are short DNA sequences that are specific to the target DNA template and are usually combined with one or more fluorescent dye as well as a quencher that suppresses the fluorescence to prevent premature signaling. It is also important to make sure that the probe is located between the forward and reverse primers. qPCR consist of 3 main steps: first being denaturation, where double stranded DNA (dsDNA) is cleaved and separated into 2 single stranded DNA strands (ssDNA), the second step is annealing that promotes the binding of the probe and the primer to the ssDNA template and, the final step is primer extension/elongation which involves the addition of complementary bases by DNA polymerase to produce a complimentary copy, forming a dsDNA again. As the qPCR runs and the DNA is amplified, the probe is cleaved and the quencher is separated, resulting in the emission of a fluorescent signal that is measured. The higher the initial number of template DNA in the sample, the faster the fluorescence will increase during the qPCR cycles (BIO-RAD; Čepin, 2017a; MERCK; Steward, 2022). This process is also depicted in Fig. 4A to help visualize the process.

The term used for the cycles in which fluorescence is detected is called quantification cycle value (Cq-value). A low Cq-value is equivalent of a high copy number of the target DNA present in the sample (Čepin, 2017a). Fig. 4B shows an amplification plot that is usually obtained from qPCR cycles. The plot shows an exponential phase that is followed by a non-exponential phase. This is because during the exponential phase, the amount of PCR product increases exponentially and accumulates as the cycles

increase and eventually, yields a detectable fluorescence signal. However, as the reaction progresses, reaction components e.g. buffer, primers, etc. are consumed and one or more components become limiting. This is when the reaction slows down and shifts towards the plateau non-exponential phase – that is cycle 28 onwards according to Fig. 4B (BIO-RAD).

At the end of the process, standard curve plots with Cq-values of samples of known concentrations against the amount of DNA in initial samples are generated. This way starting quantities of unknown samples can be estimated.

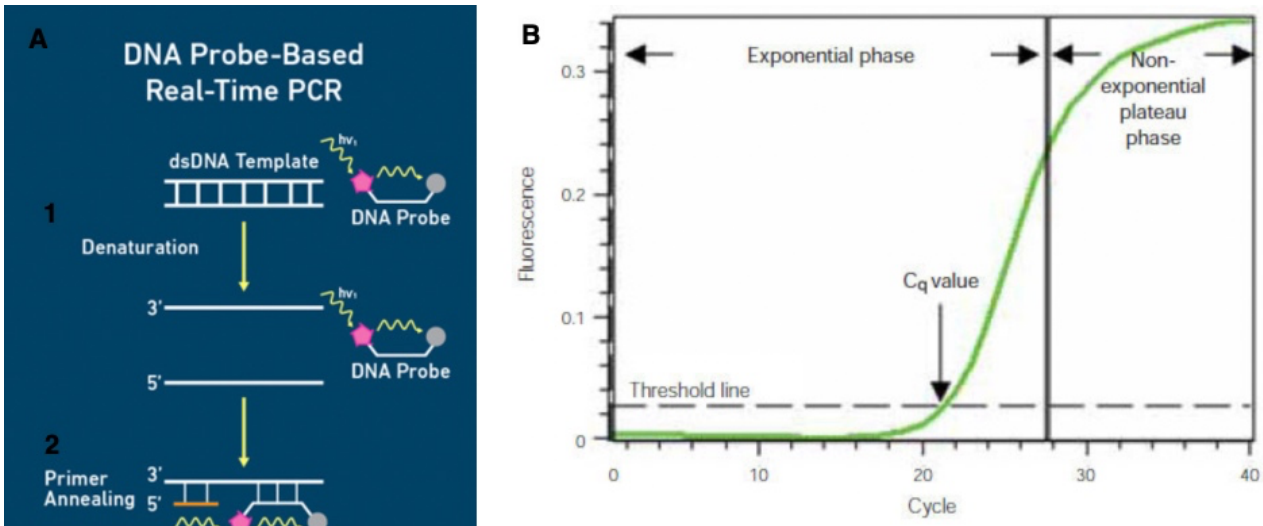


Figure 4: Scheme of a qPCR mechanism.
A (1). Denaturation of DNA into ssDNA strands (2). Annealing of primers and probe to DNA strand. (3). Primer Extension to form dsDNA with the fluorophore and quencher complex separating.
B: qPCR amplification plot; x-axis: number of PCR cycles, y-axis: fluorescence 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 M92 Media and Agar Plates

Medium used for the cultivation of *V. salmoninarum* is the Trypticase Soy Yeast Extract Medium or simply, DSMZ Medium M92 as mentioned on The Bacterial Diversity Metadatabase – BacDive ((BacDive) – Strain ID: 5261). To prepare 500 mL of the medium for the cultivation of bacteria in liquid culture, 15 grams of Tryptic Soy Broth along with 1.5 grams of Yeast extract were dissolved in 500 mL of distilled water in a DURAN® glass bottle. This mixture was then autoclaved at 121°C for 20 minutes. Once the autoclave cycle was completed, the DURAN® bottle was closed tightly and kept on the lab bench until use.

For the preparation of agar plates for cultivation on solid media, a 500 mL agar medium was prepared using 15 grams of Tryptic Soy Broth, 1.5 grams of Yeast extract, and 7.5 grams of agar that were added to 500 mL of distilled water. The mixture was then autoclaved at 121°C for 20 minutes. Upon the end of the autoclaving cycle, the agar media while still liquid was poured into Petri dishes and allowed to solidify at room temperature. Once solid, the plates were inverted, labeled, and stored in the refrigerator at 4°C until needed.

2.1.2. Bacterial Cultures and Cryocultures

For cultivation in a liquid medium, a frozen pellet of *V. salmoninarum* at -20° was used. The pellet was first allowed to thaw and then resuspended in 200 µL of liquid M92. This culture was then used to inoculate approximately 50 mL of liquid M92 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 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. For this, aliquots from the liquid culture were diluted with M92 to obtain a 1:5,000,000 dilution. 100 µL of the diluted cultures were then pipetted onto the agar

plates and spread evenly 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 ensure that a better average for the CFUs is achieved upon counting to have an increased confidence in the results.

Bacterial glycerol stocks, also known as cryocultures, are often made as they are very important for the long-term preservation and storage of frozen bacterial culture. 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 helpful when a new bacterial culture is needed to be started. Therefore, cryocultures were prepared by pipetting 500 µL of bacterial culture as well as 500 µL of 50% Glycerin into 1.5 mL Eppendorf tubes. Furthermore, 5 aliquots of 1 mL of pure liquid culture were also taken for short-term preservation. All of these aliquots were stored in the freezer at -20°C.

2.1.3. Growth Measurement

For monitoring and measuring the growth of *V. salmoninarum*, 200 µL 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 before 250 µL of the culture was pipetted into Falcon® Tissue Culture 96 Well Flat Bottom Plate. 8 wells in total were filled with the bacterial culture along with 4 wells filled with M92 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. The growth was measured over the span of 24 hours with 15-minute intervals and shaking prior each measurement was taken. The software used for this growth measurement experiment was the XFluor4 Version 4.51.

2.2. Designing *V. salmoninarum* 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). The reason for that is that the function of the gene has not changed over time and is basically a molecular chronometer. Moreover, it is present in almost all bacteria. Not only this, the 16s rRNA gene is about 1,500 bp long which is a length sufficient for informatics purposes (Janda & Abbott, 2007). Even though the gene is fairly large, it has interspecific polymorphisms that allows correct identification of different bacterial specie and even assignment of close relationships at both genus and species level (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 *V. salmoninarum.* The 16s gene sequences used for this purpose were all targeted to be around 1,000 bp to somewhere between 1300 to 1450 bp.

However, it is important to mention that these 16s primers and probes were only designed for the sake of showing the workflow through the thesis and, were not used for any of the work and results shown in this thesis. Due to contract confidentiality, the original gene locus and primer sequences tested throughout the experiments in this thesis cannot be mentioned. However, all the details regarding primer and probe designing mentioned in the following section are based on a similar idea of developing a specie-specific primer and probe for detection with qPCR.

2.2.2. Comparison of Selected Gene with Other Fish Bacterial Strains

In order to design a primer and probe specific to *V. salmoninarum* so that it does not amplify the DNA of other bacteria or pathogen, it was necessary to find a region on the 16s gene that differs in *V. salmoninarum* as compared to other fish pathogens. This was important in the context of conducting qPCRs as well as in terms of the PHOTO-SENS project as samples will be taken from a realistic environment such as

rivers where more than one fish pathogen must be present. When these samples are to be tested on the biosensor chips, the probe on the chip must be specific enough to detect the bacteria of interest.

For this purpose, 16s gene sequences of 10 different bacteria were compared. 5 of them being Gram-positive and the remaining 5 being Gram-negative. The selected Gram-positive bacteria were *Mycobacterium marinum, Renibacterium salmoninarum, Streptococcus iniae, and Streptococcus phocae, while the selected Gram-negative bacteria included were Aeromonas salmonicida, Yersinia ruckei, Flavobacterium columnare, Moritella viscosa, and Flavobacterium branchiophilum*. All of these bacteria are also some of the most fish pathogen bacteria as described by Sudheesh *et al*. (2012) in a table. For each bacterium, about six 16s gene sequences were compared and aligned. These sequences were downloaded from NCBI Nucleotide Database with a specified target length. A total of 60 gene sequences were analyzed this way.

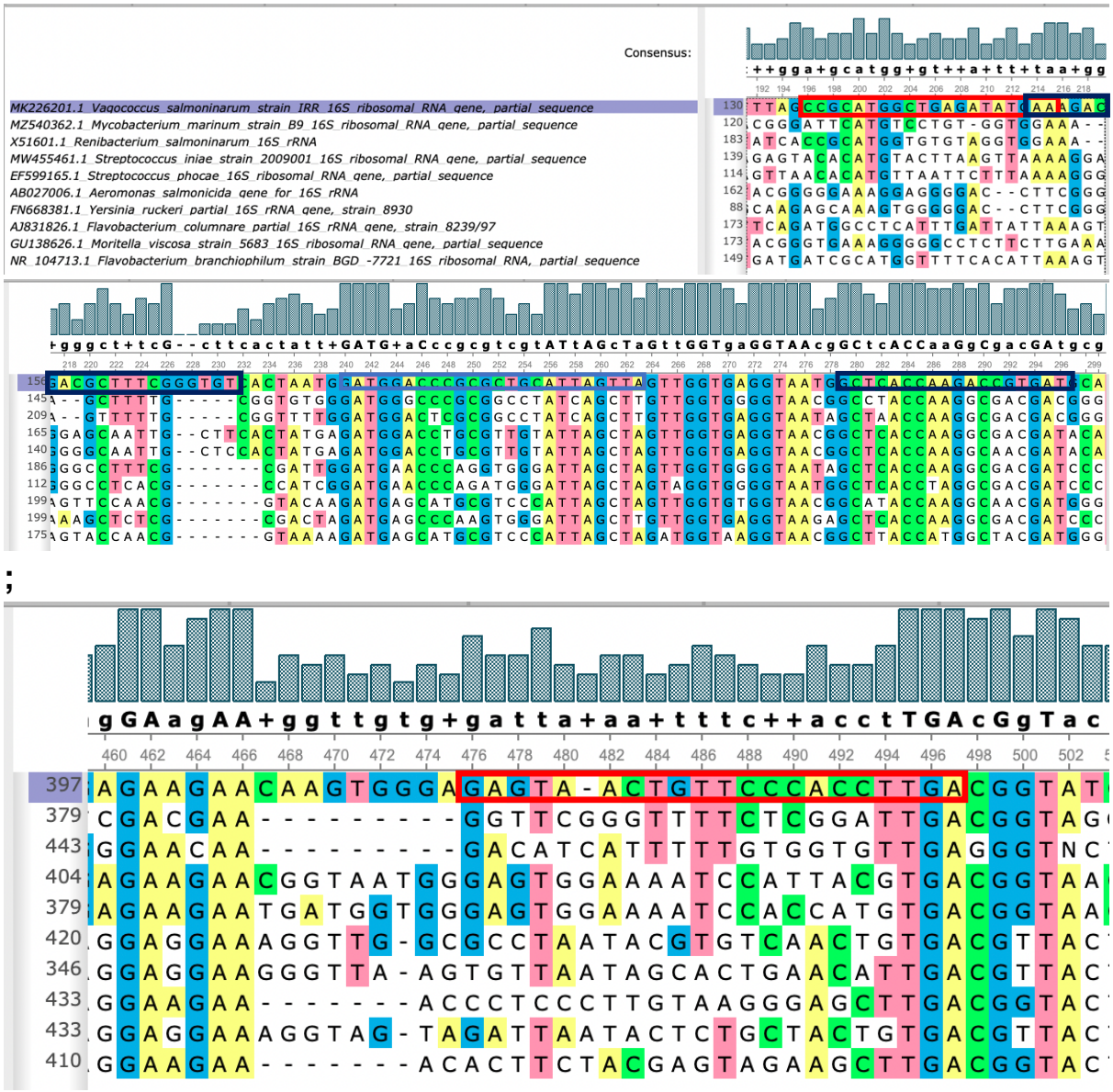
2.2.3. Designing the Primer and Probe using Unipro UGENE

The sequences were downloaded in FASTA format and imported to Unipro UGENE Version 40.0. Unipro UGENE is an open-source software used 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. The sequences showed variable levels of similarity (E.g. Streptococcus: similarlity ranging from 82% to 84% (Torres-Corral & Santos, 2019)) when compared to the 16s rRNA gene of *V. salmoninarum*. Therefore, for better analysis, a variable region in the 16s rRNA sequence was selected that was completely different to the specie *V. salmoninarum* from other genetically related bacterial specie being examined in this study.

For alignment and designing of the primers and probe, a variable region spanning from 107 bp to 190 bp was chosen due to its high variance from other bacterial strains. The 16s rRNA sequence of *V. salmoninarum* was uploaded to IDT PrimerQuest™ Tool in order to generate a forward and reverse primer as well as a fluorescently-labeled probe 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 5 shows a screenshot of the workflow from Unipro UGENE, depicting the *V. salmoninarum* specific primers and probe for qPCR. It shows the sequence of each bacterial strain used with the sequence for *V. Salmoninarum* marked as reference. Any similarities within the sequence are shown by nucleotide of the same color. A final primer pair and probe were obtained after modifying certain parameter.
PCR forward and reverse primer are shown by red boxes within the 196-215 bp and 476-497 range on the picture, respectively. qPCR forward primer is depicted in navy blue box ranging from 214-231 bp while the reverse primer is also in a navy blue box, spanning from 279-296 bp region. The qPCR probe can be found in the middle of both the forward and reverse primer, in a light blue box ranging from 240-261 bp on the picture.

It is important to mention that the bp location and the alignment depicted by Figure 5 is different from the original primers and probes designed because of gaps in the alignment.



;

Figure 5: *V. 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 final result, the forward primer (5'→ 3'), spanning from position 107-125 and the reverse primer (5'→ 3') from position 172-190 on the *V. salmoninarum* 16s rRNA gene sequence was obtained.

A detailed overview on the primers for PCR and qPCR as well as the probe designed are shown below in Table 2 and 3 respectively.

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

	Coding Strand (5'→ 3')	Length	Temp.	GC%	Amplicon Length
PCR Primer F	CCGCATGGCTGAGATATGAA	20	62	50	301
PCR Primer R	GAGTAACTGTTCCCACCTTGAC	22	62	50	

Table 3: Length and sequence details of the qPCR primer set and probe. The total length and GC% content of the primer sequence is also shown. Source: IDT PrimerQuest Tool.

	Coding Strand (5'→ 3')	Length	Temp	GC%	Amplicon Length
qPCR Primer F	AAAGACGCTTTCGGGTGT	18	62	50	83
qPCR Probe	GATGGACCCGCGTGCATTAGTTA	24	68	54	
qPCR Primer R	GCTCACCAAGACCGTGAT	18	62	55.6	

2.3. DNA Extraction

DNA extraction is a method to purify DNA from a sample and separate it from its cell components using physical as well as chemical methods. DNA extraction usually involves the lysing of cells and solubilization of DNA, followed by enzymatic action to remove macromolecules such as proteins, RNA, etc. There are a wide variety of methods for DNA extraction such as manual ones as well as commercial DNA Kits (Gupta, 2019). However, it is dependent on the DNA extraction techniques used that determines the efficiency of the extraction in terms of DNA quantity and quality i.e. pure of contaminants. Due to *V. salmoninarum* being a Gram-positive bacterium, it has a tough peptidoglycan layer around it. Therefore, it is necessary to use a DNA extraction technique that would disrupt this thick protective layer and release intracellular molecules (AndreasOtto *et al*., 2012). For the work through this thesis, 3 different methods were used for *V. salmoninarum* DNA Extraction. Each of them is described below.

2.3.1. 5% Chelex DNA Extraction

DNA extraction using 5% Chelex solution is one of the most commonly used methods for DNA extraction and the first protocol was developed by Walsh *et al*. (1991) This method is preferred over others for it is effective, time-efficient and is less labor-

intensive (BIO-RAD). The basic principle of using Chelex for this purpose is that Chelex resin chelates to polyvalent metal ions such as Mg^{2+} that act as cofactors for DNAases (Utkarsha A. Singh *et al*., 2018). Therefore, its presence during boiling prevents the degradation of DNA by these metal ions at high temperatures and instead allow release of DNA into the solution via enzymatic digestion (BIO-RAD; Walsh *et al*., 1991). Chelex solution was prepared by dissolving 1.25 grams of Chelex 100 Resin pellet in 25 mL of TE Buffer or water in order to reach a 5% Chelex w/v (weight per volume) concentration. Every sample for DNA extraction was first centrifuged at 15,000 rpm for 5 mins and the supernatant discarded. To each sample, 98 µL of 5% Chelex solution was added to resuspend the bacterial pellet before adding 2 µL 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 are 15-minute steps of shaking at 1,300 rpm and at a temperature of 56°C, with a brief vortexing step in between. For the last step, the temperature was raised to 99°C and the samples allowed to shake at 1,300 rpm for 20 minutes. Once the heating steps were done, the samples were centrifuged again at 15,000 rpm for 5 minutes to allow the cell debris to settle at the bottom and the supernatant containing the free DNA to be collected and transferred into new tubes. It was also ensured that for every sample, Safe-Lock tubes were used to reduce the risk of sample evaporation and the lid opening up due to buildup of pressure. The extracted DNA was then ready for further downstream steps.

2.3.2. Macherey-Nagel™ NucleoSpin™ Microbial DNA Kit Extraction

For this, a commercial DNA Extraction Kit supplied by Macherey-Nagel™ was used and the manufacturer's protocol followed. The sample was prepared by first extracting a microbial pellet via centrifuging for 5 minutes at 15,000 rpm and discarding the supernatant. The pellet was then resuspended by the provided Elution Buffer before transferring this cell suspension into a Bead Tube provided that contained 40–400 µm diameter glass beads. 40 µL of Proteinase K was next added to this cell suspension prior to the following agitation step on a disruptive device. It was prescribed by the user manual to adjust the time and frequency of disruption according to the type of

bacteria. For gram-positive bacteria, the recommended time was 12 minutes. An agitation device was made by combining a retort stand to hold the sample tube on a vortex as shown by Fig. 6. Once the agitation step was finished, the protocol included 2 centrifuging and DNA binding steps in a provided NucleoSpin™ Microbial DNA Column. A NucleoSpin™ Microbial DNA Column contains a silica membrane to capture DNA on its surface, allowing any impurities such as salts and buffer to flowthrough. The silica membrane was then washed twice before the column was dried and the DNA finally eluted in an Elution Buffer to yield highly pure DNA.

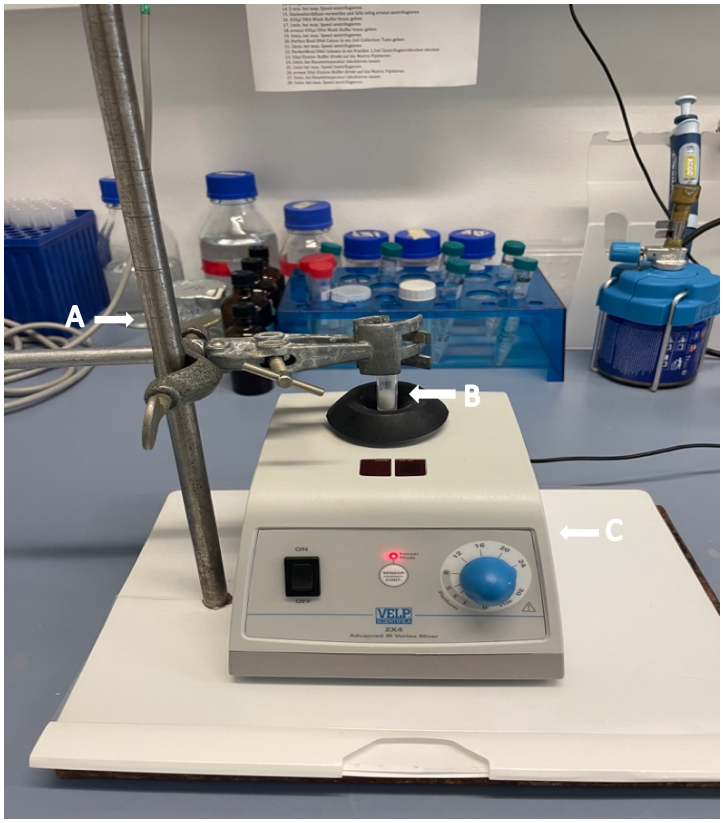


Figure 6: Improvised agitation device. Bead tube containing sample (B) held by the retort stand (A) on top of a vortex (C) at 2,400 rpm speed for 12 minutes for cell disruption of gram-positive *V. Salmoninarum* cells.

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

2.3.3. 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 Gram-positive bacteria are rigid due to their stabilizing surrounding layers of peptidoglycan. Therefore, stronger cell lysis methods were needed to disrupt the cell walls and allow Proteinase K to function better for 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, 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. An approximate 50% of bead load volume was used for each sample. This is because according to Butler and Guimarães (2021), a bead diameter of 0.5 mm and 60% bead loading volume is considered to be optimal as parameters such as bead filling, bead diameter along with agitation speed and time affect the efficiency of the overall process. Moreover, using smaller beads results in more rapid disruption (Geciova *et al*., 2001). Each sample was then placed onto the agitation device as shown in Figure 6 and allowed to shake 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 2 µL 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 a brief vortexing step between each. 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.

2.4. PCR

Polymerase Chain Reaction (PCR) is a common technique to amplify and make multiple copies of a particular section of DNA. PCRs are a fast and a quick way to confirm the presence of target specie however, PCRs cannot confirm a pure culture. The first PCR for this thesis was conducted with general 16s primers that were not specific to any specie 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 *V. salmoninarum* or any other bacterial contamination. All samples were confirmed as pure *V. salmoninarum* 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. This is explained more in Section 3.5.2. For this PCR, the *V. salmoninarum* specific PCR primer set designed in Section 2 and showed in Figure 5 were used. For every PCR, a mastermix needs to be prepared with a buffer, primers, DNA nucleotides (dNTPS) and the enzyme Taq Polymerase. The buffer is needed to ensure right 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 mastermix for a single sample was prepared by adding 1.25 µL of 10x PCR Buffer S with 15 mM $MgCl_2$ (Genaxxon Bioscience), 0.25 µL of Bioline dNTPs, 0.25 µL of forward primer (10 µM), 0.25 µL of reverse primer (10 µM), 0.0625 µL of Taq DNA-Polymerase 250 units – 5U/ µL (Genaxxon Bioscience) and 9.45 µL of molecular grade water. For more samples, this mastermix composition was simply multiplied with the number of samples. However, for every mastermix prepared, circa. 10% extra was prepared in order to account for pipetting errors. 11.5 µL of mastermix was used for a single sample with 1 µL further added of the DNA template of the sample, reaching 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 of denaturation for 30 seconds; primer annealing at 54°C for 30 seconds; followed by primer extension for 30 seconds at 68°C. The final step consisted of primer extension for 5 minutes at 68°C before ending the PCR program. The PCR machine kept the sample at 10°C for infinity until removed.

2.4.1. Gel Electrophoresis

Agarose Gel Electrophoresis was used to visualize the PCR product. Agarose gel electrophoresis is a popular technique to separate DNA fragments of varying sizes. The basic principle of this method is that when the agarose gel is placed in an electric field, the negatively charged phosphate backbone of DNA migrates to the positively

charged anode and because DNA has a mass/charge ratio, DNA molecules are separated by size (Lee *et al*., 2012).

60 mL gels with 1% or 2% agarose concentrations were used. 0.6 grams or 1.2 grams of agarose, respectively, was added to 60 mL of TAE Buffer. To dissolve the agarose in the liquid, this suspension was then heated in the microwave for about 1 minute or until the solution was clear. 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-former template, or commonly known as a comb, was placed 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. 5 µL of PCR product mixed with 1 µL of 6x concentrated loading dye was also pipetted into the 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 *V. salmoninarum* culture. For this, PCR product from the PCR done for DNA extracts with non-*V. salmoninarum* 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 6.5 µL 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 *V. salmoninarum* specific primers and probes as described in Section. 2 were used. A mastermix was prepared using 5 µL of Probe MasterMix – Low ROX (Geneaxxon Bisoscience qPCR 2x Mastermix with 50 nM ROX), 1 µL of *V. salmoninarum* primer probe mix and 3 µL of molecular grade H_2O. This composition was suited for 1 sample and therefore, for more samples was simply multiplied with the number of samples while also making 10% extra to account for pipetting errors. 9 µL of mastermix was then pipetted into a Bio-Rad Hard Shell 96 microplates into as many wellsg as the number of samples along with 1 µL of DNA template. 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 Touch™ Real-Time PCR Detection System. The program used comprises of 2 steps, the first step was the initial denaturation at 95°C for 15 mins to completely remove the chemical inhibition of the chemically modified DNA polymerase in the buffer. This was then followed by 40 cycles of denaturation of DNA at 95°C for 10 seconds and primer annealing at 60°C for another 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 Maestro™ Version 2.2.

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. Once there was growth and a CFU count was done, an expected value was calculated for the number of bacteria in the original undiluted culture. Starting from 10 million expected number of copies down to 1 expected number of copy 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 explain 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. Standard Dilution Series from gBlocks Gene Fragments

gBlocks are double-stranded, sequence-verified long DNA fragments ranging from 125 – 1000 bp (IDT). A gBlocks Gene Fragment specific to *V. salmoninarum* 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.

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

Therefore, in a µL of undiluted gBlocks Gene Fragment solution, there were 4.35 x 10^{10} copies of the *V. salmoninarum* gene fragment. In order to obtain a starting dilution of 100 million copies/ µL, 1 µL of gBlocks Gene Fragment was added to 434 µL of molecular grade water in an Eppendorf Tube. This tube was then used to create dilution series going down into steps of 10 to 1 copy/ µL. A 5 copies/ µL dilution was also created.

A standard curve with average Cqs was also plotted for the gBlocks standard dilution series to increase the reliability of the data. This was done by performing qPCR for a total of 6 times and calculating a mean for each SQ throughout the 6 runs. A standard deviation was also calculated to show the dispersion of data relative to the average. Both of these 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).

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

The efficiency of an assay should be close to 100% while the R^2 value should be >0.98 to have a good confidence with the correlation between the data points (BiteSizeBio, 2022). The y-intercept of the best fit line provides a theoretical sensitivity of the assay.

Similar to gBlocks standard dilution series, an average standard curve for CFU standard series was also made. 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

In order to mimic eDNA extraction as it would be done from aquaculture facilities for use on the PHOTO-SENS project biosensor photonic chips, an imitation of a similar filtration was performed via conducting 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). To do so, 1 L of distilled water was spiked with bacterial culture corresponding to 10 million cells down to 1 cell according to the CFU count. The experiment was done using the filtration set up as depicted in Figure 7. The bacterially spiked water was poured onto the glass holder (C) before the vacuum pump (A) was turned on. As the water slowly filtered through, the eDNA was collected onto the filter (D) with the residue water being collected 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 time was doubled from the regular extraction protocol with the first heating step being at 56°C for 30 minutes, followed by a thorough vortexing and then a similar second heating step at 56°C for 30 minutes and vortexing again after. The final step was done at 99°C for 40 minutes before the tubes were centrifuged at 15,000 rpm for 5 mins. 200 µL of supernatant was then 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.

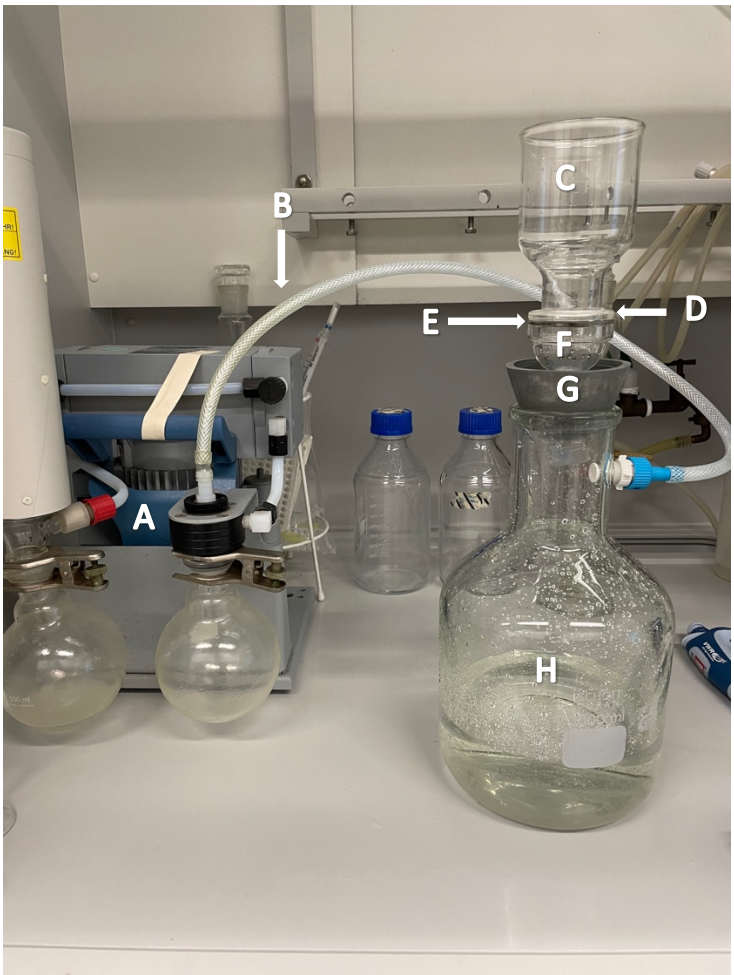


Figure 7: Filtration Equipment Setup. Spiked water is poured into holder (C) and vacuum pump (A) turned on. The water gets filtered through filter (D) and metal sieve (E) before getting collected in collecting vessel (G).

A: Vacuum Pump
B: 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. Growth of *V. salmoninarum*

The attempt to grow *V. salmoninarum* in the set conditions as described earlier was successful. Within 24 hours, there was growth in the two inoculated flasks and labelled V. Sal 1 and V. Sal 2P. The flasks had a uniform translucent brown appearance that was considered to be a clean culture. However, for complete confirmation, sequencing from the PCR product obtained was still done. The results of which are shown in Section. 3.3.

3.1.1. CFU Count

The plating of the liquid culture was performed not only to monitor uniform bacterial colonies but also to obtain a quantification for the aliquots that were to be used later for qPCRs. Plating was performed almost 24 hours after inoculation for both flasks, with both of them more or less the same in appearance. For both cultures, plating was done with a 1:5,000,000 dilution and each culture plated 5 times to increase the precision of the results. Despite both cultures grown and plated under same conditions and at same times, V. Sal 1 resulted in only 3 colonies in one plate with the remaining 4 staying empty. However, each of V. Sal 2P plates had colonies that were counted. However, only four plates had values within a similar range and the fifth plate of V. Sal 2P culture was considered as an anomaly. This was because the CFU value obtained for this plate was too far off from the rest of the plates. Therefore, it was excluded from average calculation. The CFU count data obtained is shown in Table 4. It also shows the average CFU as well as the average cells/µL calculated. The values for V. Sal 1 are marked in red as they are incorrect. Similarly, for V. Sal 2P, one of the values that was inconsistent with the rest of the values is marked in grey.

Table 4: CFU counts of plating experiments of culture V. Sal 1 and V. Sal 2P.

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

Figure 8 shows the 4 plates of V. Sal 2P with the bacterial colonies as well as the total CFU count number. Due to this, all further work done was based on the V. Sal 2P culture and the V. Sal 1 culture was discarded. Secondly, the average obtained for V. Sal 2P (79.25) was rounded off to a complete 80 to make further calculations easier.

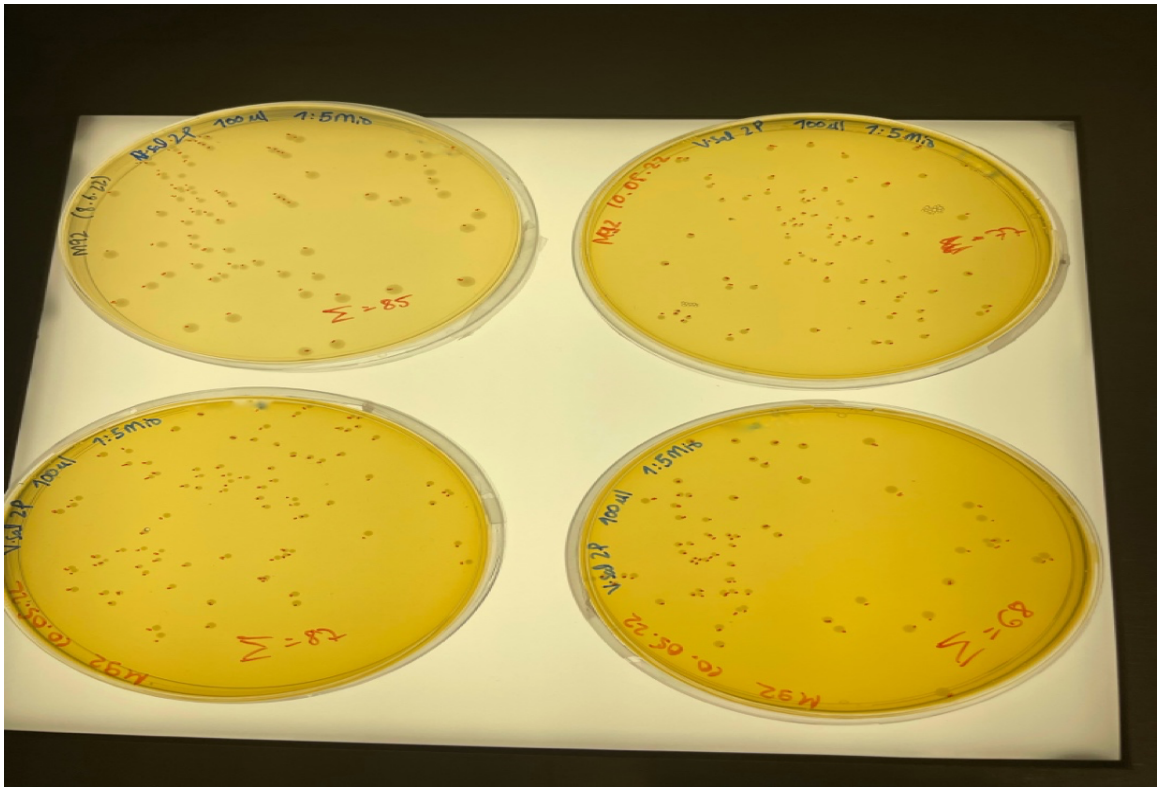


Figure 8: V. Sal 2P CFU on 4 plates displayed on a UV table. CFU Count: Top left: 85; Bottom left: 87; Top right: 77; Bottom right: 68. 5th plate is not shown in the picture because it was an outlier.

3.1.2. Photometric Growth Measurement

To assess and monitor the growth time of the *V. salmoninarum* 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 V. 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 blue dots represent the data points of the OD measurement taken every 15 minutes. 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.

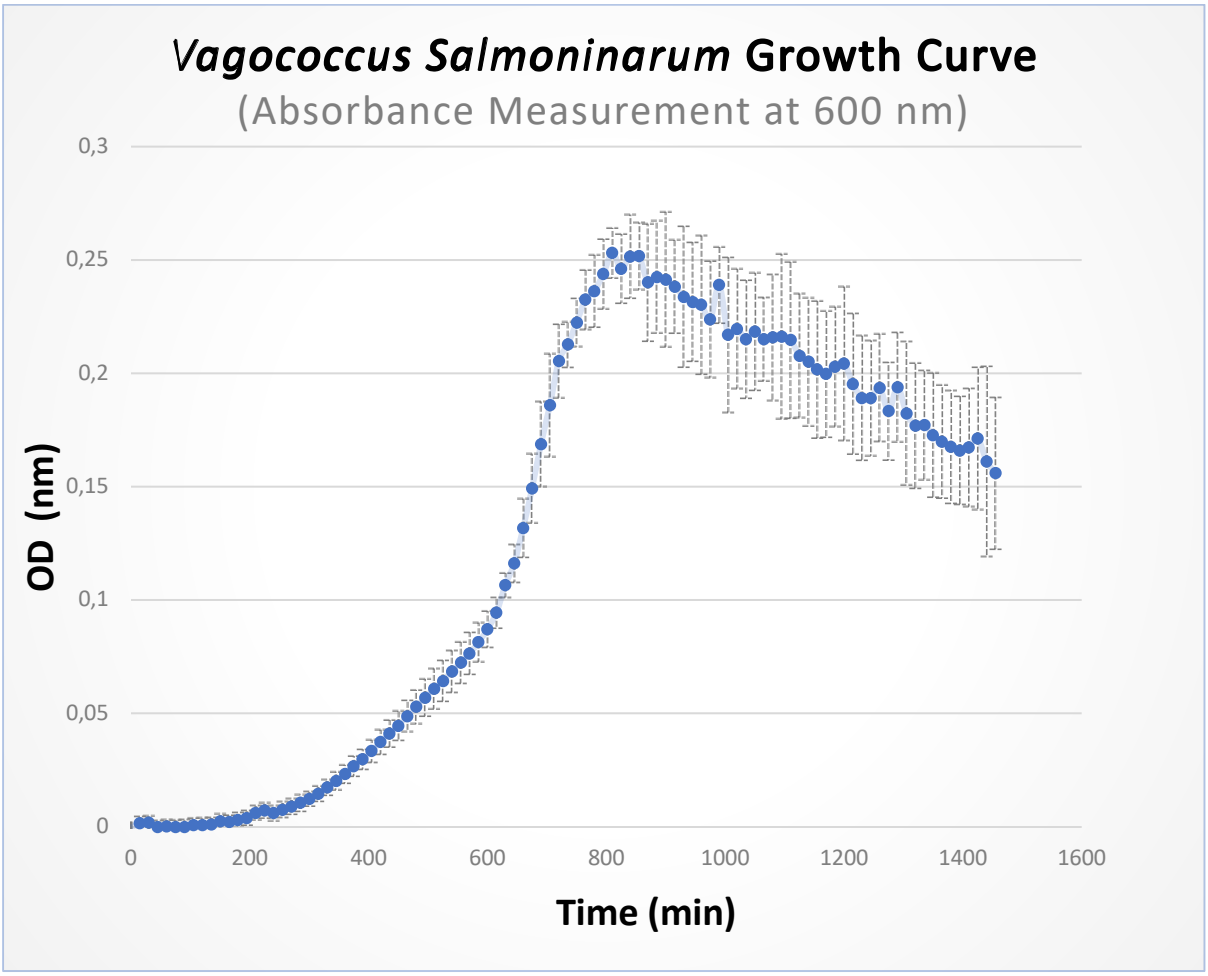


Figure 9: Optical Density (OD) measurement of culture V. Sal 2P. Blue dots represent the data points and the error bars represent the range of uncertainty in a certain data point.

3.2. PCR and Gel Electrophoresis

The PCR product obtained from the *V. salmoninarum* specific primers was used to produce an agarose gel that not only showed that the PCR amplification was successful but also to show steady bands for all the samples used. This would confirm the presence of *V. salmoninarum* bacteria in the culture. For this specific agarose gel, shown by Figure 10, 4 samples of the PCR product from Improved Chelex DNA Extraction via Rapid Agitation with Glass Beads (denoted by S) were used. 2 old *V. salmoninarum* kit extracts were also used (denoted by K) along with salmon DNA as negative control (denoted by -C). A no template control (NTC) with water was also included. As seen in Figure 10, the 2 kit extracts had no signal but distinct bands for the 4 samples from improved Chelex extraction can be seen clearly. A DNA cloud formed for the negative control which was expected for the big salmon DNA while NTC remain empty as expected.

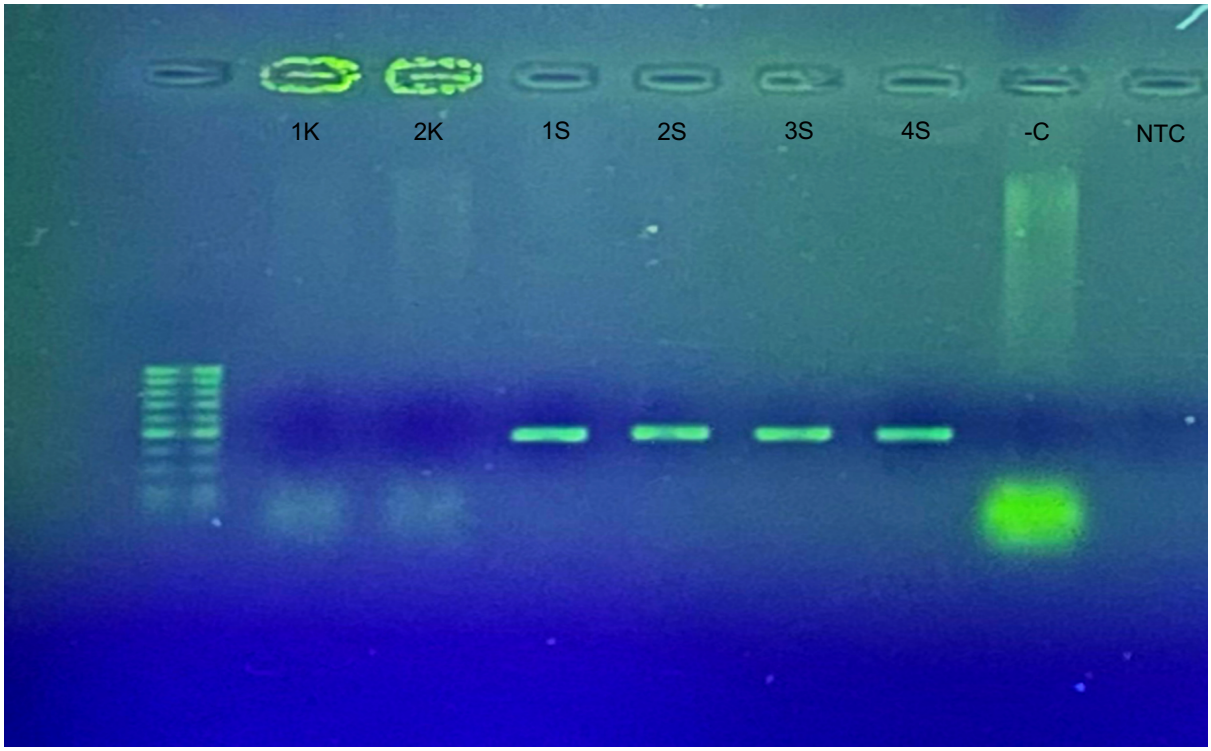
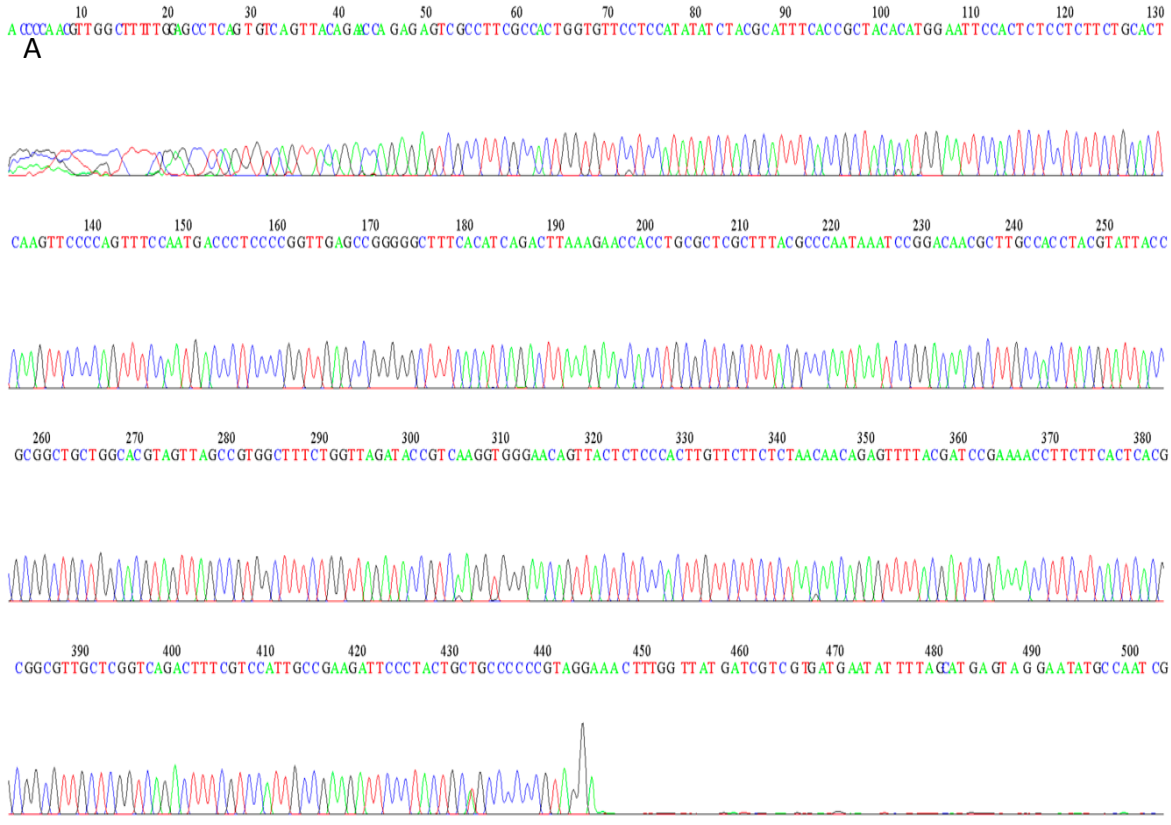


Figure 10: Agarose gel showing bands for *V. Salmoninarum.* First well: 100 bp DNA Ladder. **1K and 2K**: old *V. Salmoninarum* kit extracts; **1S, 2S, 3S, 4S**: *V. Salmoninarum* via improved Chelex extraction; **-C:** Salmon DNA; **NTC:** H_2O.

3.3. Sequencing

The sequencing results received are shown by Figure 11A and B. Well-formed and distinctive peaks can be seen. Moreover, there is a single peak for each distinctive color. The peaks are also evenly separated and there are no background signals or baseline noise recorded on the chromatograms. These characteristics are that of DNA of high purity and an optimal primer design (EurofinsGenomics). Poor resolutions of peaks can be seen in the beginning which is a common trait for sequencing chromatograms.

Figure 11A is the sample that was prepared with the reverse primer while Figure 11B is the chromatogram from the sample that contained the forward primer.



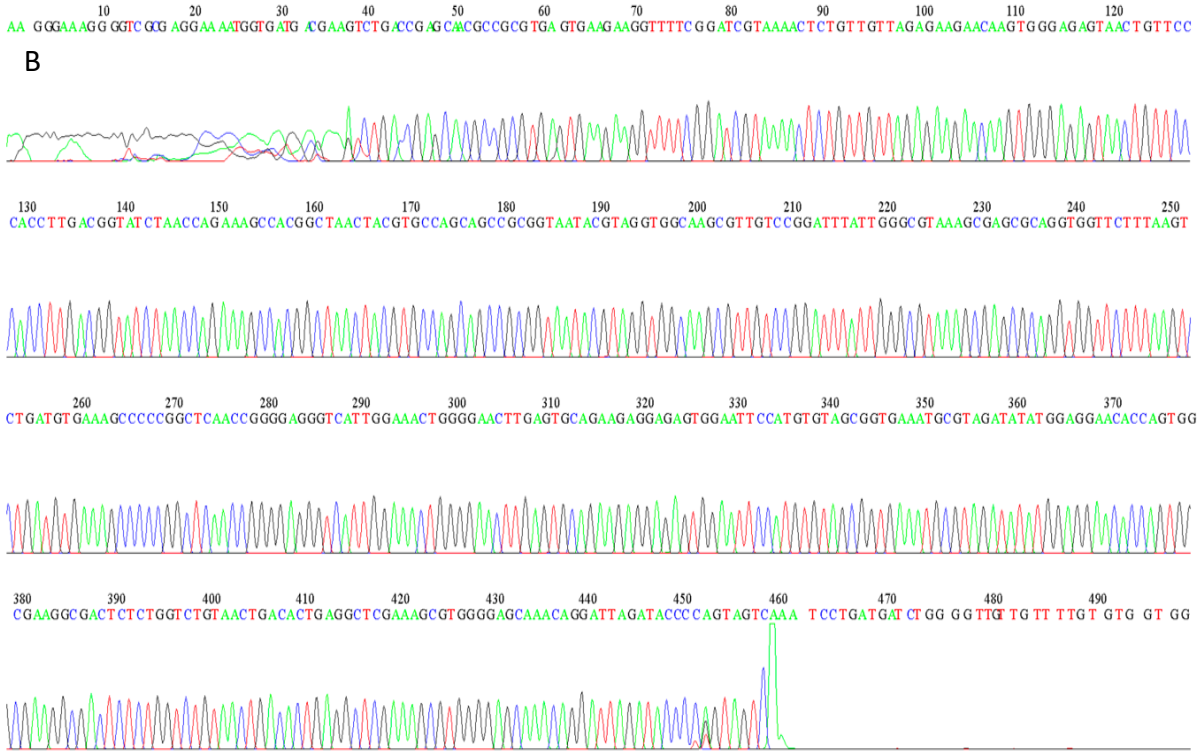


Figure 11: Sequencing chromatograms for *V. Salmoninarum*. A: chromatogram with reverse primer; **B:** chromatogram with forward primer.

3.4. Comparison of the 3 DNA Extraction Methods

The 3 methods of extraction used for this work were compared to determine the performance of each. For this purpose, the extracts from all 3 methods ranging from 10 million copies to 1,000 copies were run in a qPCR. Each of them was set as standards in the CFX Maestro™ software to yield standard curves. The Cq values obtained for this qPCR attempt are shown by Table 5.

Table 5: Cq values comparing the 3 different type of DNA extraction method used.

Cq values			
SQ	**5% Chelex DNA extraction**	**Kit DNA extraction**	**Improved Chelex DNA extraction**
10,000,000	24.18	20.27	17.89
1,000,000	27.24	29.79	21.40
100,000	30.30	35.25	24.97
10,000	33.65	38.02	28.10
1,000	36.82	38.91	31.66

As seen in the table, kit extracted DNA had the latest Cq followed by the regular 5% Chelex DNA extracts. This can be explained by inefficient extraction done by these protocols where the thick peptidoglycan layer of *V. Salmoninarum* was not lysed properly to extract the DNA within. However, the improved Chelex DNA extracts showed fairly early Cq values where it can be deduced that the extraction protocol with rapid agitation with the glass beads worked. Hence, for the same amount of SQ for all 3 extraction methods, more DNA was detected from improved Chelex DNA extraction, then followed by regular 5% Chelex extraction and lowest DNA detected from DNA extracts form kit. The data in Table 5 is also visualized in Figure 12, that shows plot comparisons of the 3 types of extracts. Figure 12A shows curves from all 3 extraction methods while Figure 12B, C, and D show individual curves for each method of extraction.

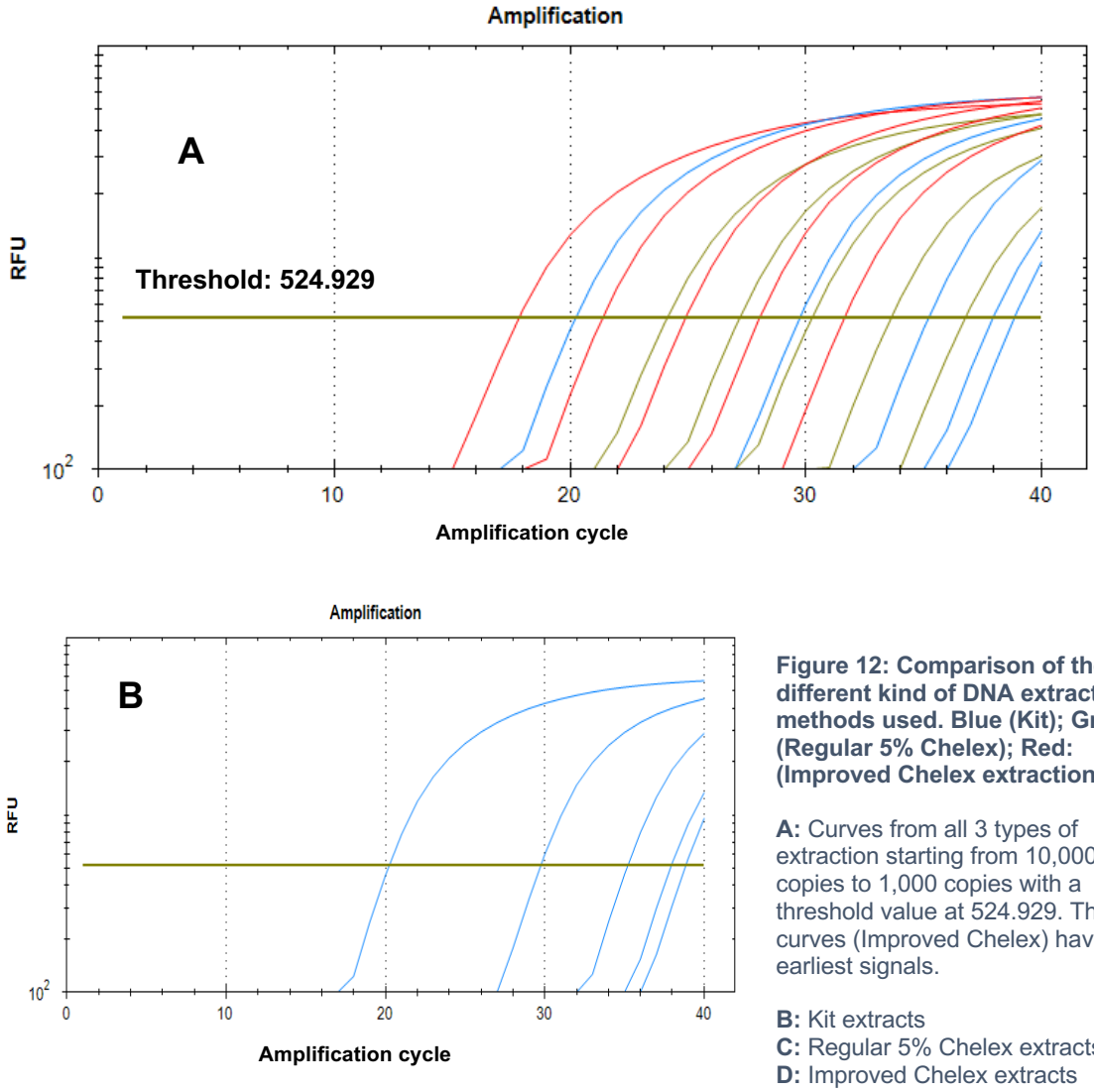
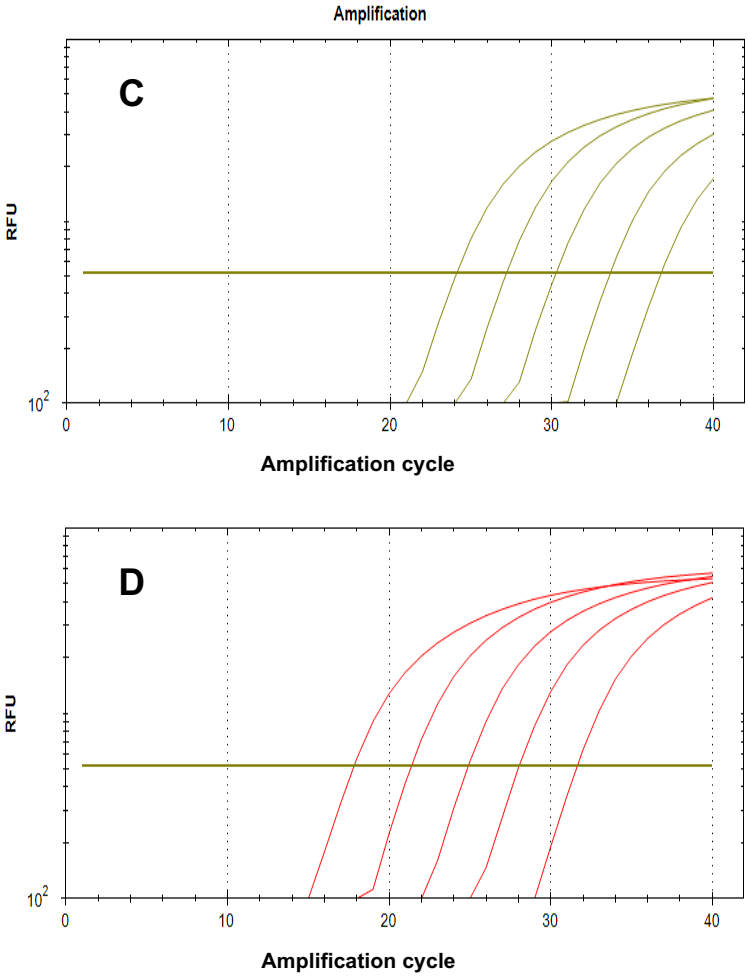


Figure 12: Comparison of the 3 different kind of DNA extraction methods used. Blue (Kit); Green (Regular 5% Chelex); Red: (Improved Chelex extraction)

A: Curves from all 3 types of extraction starting from 10,000,000 copies to 1,000 copies with a threshold value at 524.929. The red curves (Improved Chelex) have the earliest signals.

B: Kit extracts
C: Regular 5% Chelex extracts
D: Improved Chelex extracts



As seen in the individual curves shown by Figure 12D, the improved Chelex extraction method had the earliest signals with curves that are evenly-spaced with respect to copy number from 10,000,000 to 1,000. Similarly, Figure 12C also shows evenly-spread curves for regular Chelex extraction but the curves were considerably late as compared to improved Chelex extraction method which means a lower detection limit. However, the kit extracts curves shown by Figure 12B are neither spaced uniformly nor are the signals consistent to the copy number which makes these extracts unreliable.

3.5. Quantification experiment with gBlocks Standard Series

In order to quantify the bacterial aliquots, a gBlocks Standard Series was created. Table 5 shows the results of 6 qPCR attempts with gBlocks Standard Series. The table contains the Cq values obtained for each SQ in each attempt as well as the averages

of each of the 6 runs and the standard deviation for each SQ throughout all runs. As it can be seen, even 1 copy was detected throughout all runs.

Table 6: 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.

gBlocks SQ	LOG SQ	12.7.22	13.7.22	20.7.22	21.7.22	10.8.22	31.8.22	AVG	ST. DEV
100,000,000	8.00	-	11.12	-	12.48	11.88	12.41	11.97	0.682
10,000,000	7.00	N/A	14.39	15.61	15.51	15.83	16.07	15.48	0.647
1,000,000	6.00	19.10	17.83	19.47	20.05	19.67	20.27	19.40	0.873
100,000	5.00	22.42	21.17	23.18	25.06	24.12	25.21	23.53	1.577
10,000	4.00	25.60	24.66	26.21	26.68	27.05	27.49	26.28	1.030
1,000	3.00	28.45	28.02	29.40	29.42	29.46	30.24	29.17	0.798
100	2.00	32.25	31.54	33.01	33.48	33.23	33.95	32.91	0.875
10	1.00	33.99	33.31	34.69	34.62	35.13	34.16	34.32	0.638
5	0.70	35.47	34.48	35.43	35.15	36.13	35.03	35.28	0.548
1	0.00	37.98	36.97	38.34	38.61	37.95	37.57	37.90	0.718
NTC	-	N/A	37.49	38.00	N/A	N/A	39.65	-	-

3.5.1. gBlocks 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 105.8% and a R^2 value of 0.9926.

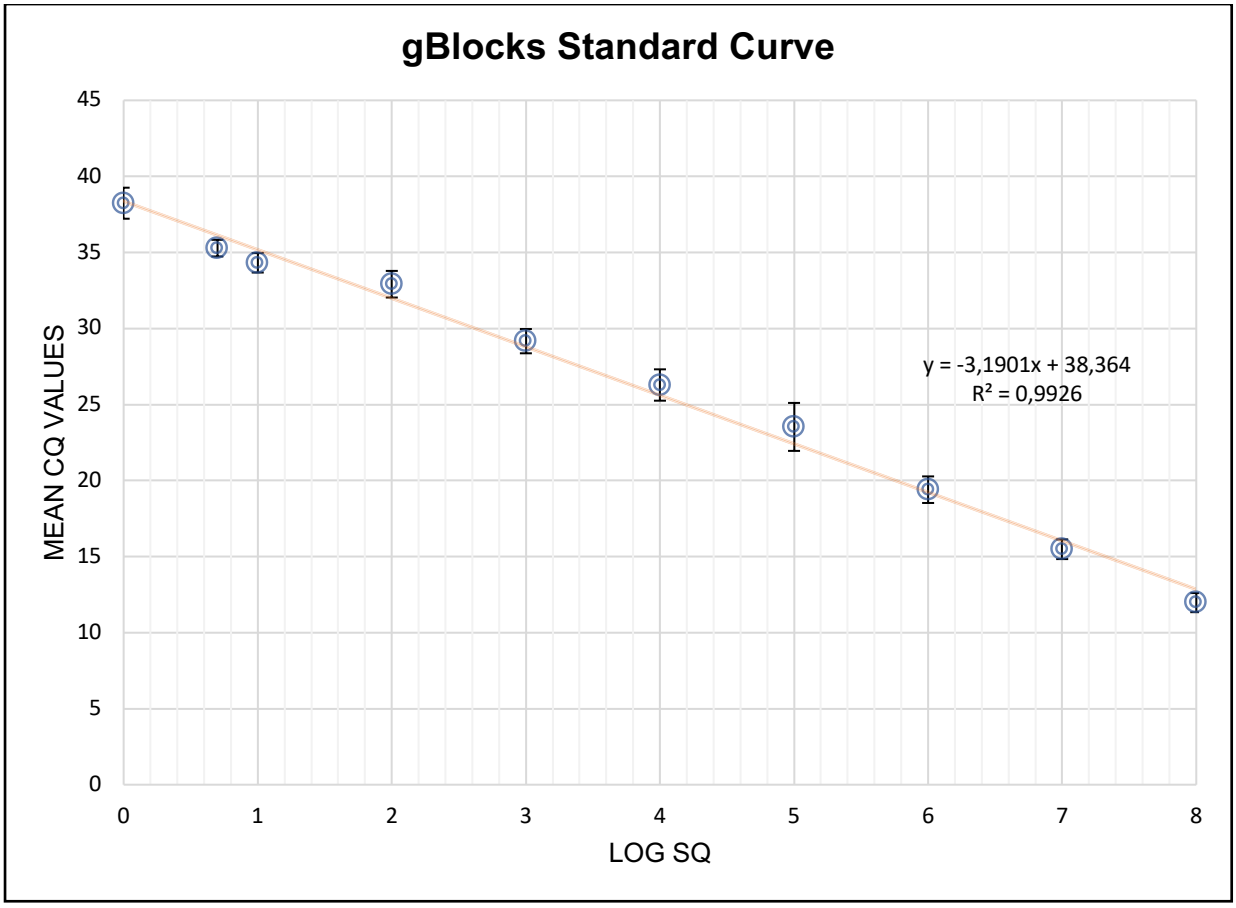


Figure 13: Standard curve for gBlocks Standard Series used for qPCR. Mean Cq values plotted against the LOG of SQ. Equation of the regression line and error bars showing standard deviation are also shown.

3.5.2. Quantification results with 3 different kinds of DNA Extracts

In order to quantify the bacterial load and the losses from each extraction method, quantification qPCRs were performed for each with gBlocks Standard Dilution Series. Each extraction sample set was run in triplicate with gBlocks Standard and the SQ for each then averaged. The raw data of the SQ obtained for triplicates runs for each extraction method are shown in Supplementary Table 1A, B and C in the Annex. The mean SQs calculated are shown in Table 7 that compare the quantification of the 3 different extraction methods with the gBlocks Standard Dilution Series.

Table 7: Quantification run with gBlocks Standard Series. Recovery rate that shows the difference between the obtained quantification from the expected values is also shown for all three DNA extraction methods. Undetected samples are marked in red.

Expected starting Quantity (SQ)								
Sample	**10,000,000**	**1,000,000**	**100,000**	**10,000**	**1,000**	**100**	**10**	**1**
5% Chelex extraction	56,440.00	6,466.67	643.83	41.33	4.19	13.56	0.55	0.67
Recovery Rate	0.56%	0.65%	0.64%	0.41%	0.42%	13.56%	5.5%	67%
Kit Extraction	648,866.67	831.80	8.89	1.22	0.43	0.78	0.29	N/A
Recovery Rate	6.48%	0.08%	0.008%	0.01%	0.04%	0.78%	2.9%	N/A
Improved Chelex Extraction	8,516,000.00	420,166.67	51,490.00	2,244.00	182.73	1.34	1.70	2.51
Recovery Rate	85.16%	42%	51.49%	22.44%	18.27%	1.34%	17%	251%

A % recovery rate was also calculated to compare the detection limit of each DNA extraction method. As seen, improved Chelex DNA extraction had the highest % recovery rate (at least around 50% for almost all samples) with minimal losses. The greatest losses were seen with Kit DNA Extraction where the detection limit was extremely low (lower than 1) or zero. The regular Chelex extraction method also showed fairly poor results. This means both these methods showed a cell quantity that was less than what was added to the sample.

3.6. Qauntification experiment using CFU Standard Series

gBlocks are highly pure short fragments of DNA and while they are quite efficient for quantification, it is not necessary that the quantification results provided by gBlocks Standard Series Dilutions is realistic in regards to the bacterial culture. It was decided that in order to yield a quantification result that closely corresponds to the bacterial culture aliquots to be quantified, a second serial dilution is to be created using the CFU count obtained as shown in Table 4 and Figure 8. These two serial dilutions series can then be compared to determine a certain 'factor' that compensates for dead cells, dormant cells, free DNA, etc. This calculated 'factor' can then be used to estimate the actual amounts of bacteria.

Therefore, in order to test this newly created CFU standard dilution series and to see if it would yield results that would be acceptable in regards to expected starting

quantities of *V. salmoninarum* culture, replicate qPCRs were attempted. Table 8 shows the results obtained with these qPCRs i.e. the Cq values of each starting quantity for every run, a calculated average as well as standard deviation. As seen, the Cq values for this CFU serial dilution are slightly late as compared to gBlocks Standard Series Cqs as shown in Table 6. Similarly, all samples were detected in all runs with the lowest starting quantity being 1 except for expected starting quantity of 10 in two runs (on 9.8.22 and 25.8.22) as well as CFU starting quantity of 5 (on 26.7.22).

Table 8: 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.

CFU SQ	LOG SQ	20.7.22	26.7.22	9.8.22	15.8.22	30.8.22	AVG	ST. DEV
10,000,000	7.00	17,35	16,90	17,48	17,29	16,83	17,17	0,288
1,000,000	6.00	20,54	20,01	20,98	20,17	20,32	20,40	0,377
100,000	5.00	23,93	23,50	24,93	23,92	24,28	24,11	0,534
10,000	4.00	27,38	27,07	28,06	27,06	27,08	27,33	0,430
1,000	3.00	30,98	31,56	32,98	32,44	32,40	32,07	0,794
100	2.00	34,81	33,90	35,83	36,58	35,86	35,40	1,047
10	1.00	37,40	37,03	N/A	N/A	38,14	37,52	0,565
5	0.70	37,59	N/A	39,35	37,39	36,66	37,75	1,141
1	0.00	37,46	39,34	38,77	38,18	38,15	38,38	0,709
NTC	-	38,00	39,86	39,80	N/A	38,52	-	-

3.6.1. CFU Standard Curve

Using the data in Table 8, a standard curve for CFU standard series was generated as is shown by Figure 14. It is a plot of the average Cqs obtained by the CFU Serial Dilution Series against the LOG of starting quantities. The efficiency is calculated to be 104.2% and the R^2 is equal to 0.9825.

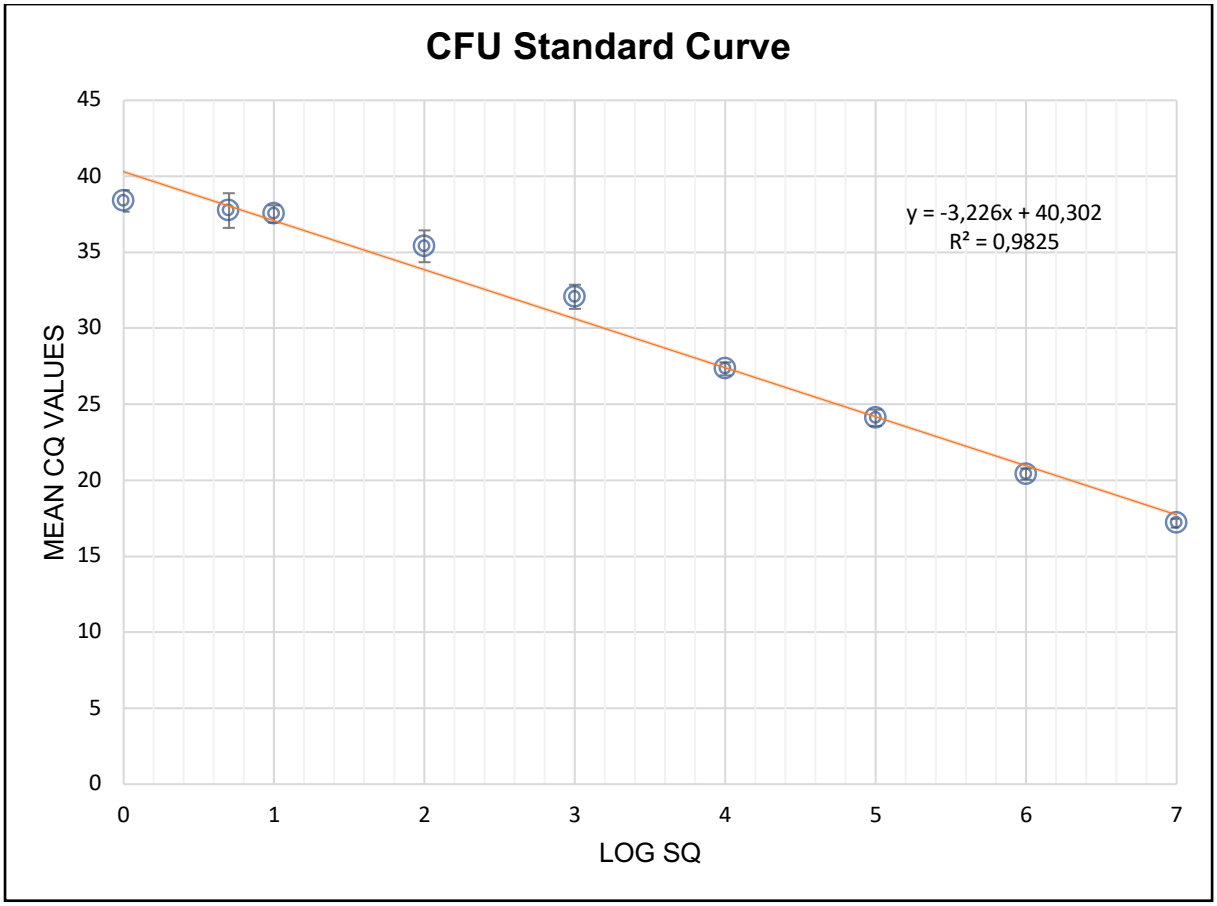


Figure 14: **Standard curve for CFU Standard Series used for qPCR.** Mean Cq values plotted against the LOG of SQ. Equation of the regression line as well as the error bars depicting standard deviation are also shown.

3.6.2. Electrophoresis Gel for Band Intensities correlating to copy number

A PCR was done with *V. salmoninarum*-specific primers and the PCR product obtained used to make an agarose gel. CFU Serial Dilution Series ranging from 100,000 copies to 1 copy were used as template. The aim of this experiment was to generate an agarose gel that would depict the decreasing band intensities as the copy number in the sample decreases as shown by Figure 15. The experiment was inspired by the work of Torres-Corral and Santos (2019). The experiment proved successful as decreasing band intensity can be seen with the first glance at Figure 15. Clear bands can be seen for 100,000 copies to 100, band for 10 copy number is also barely visible however after that, for copy 5 and 1, the bands completely fade out. A no template control with water was also used and no band was seen for it as expected.

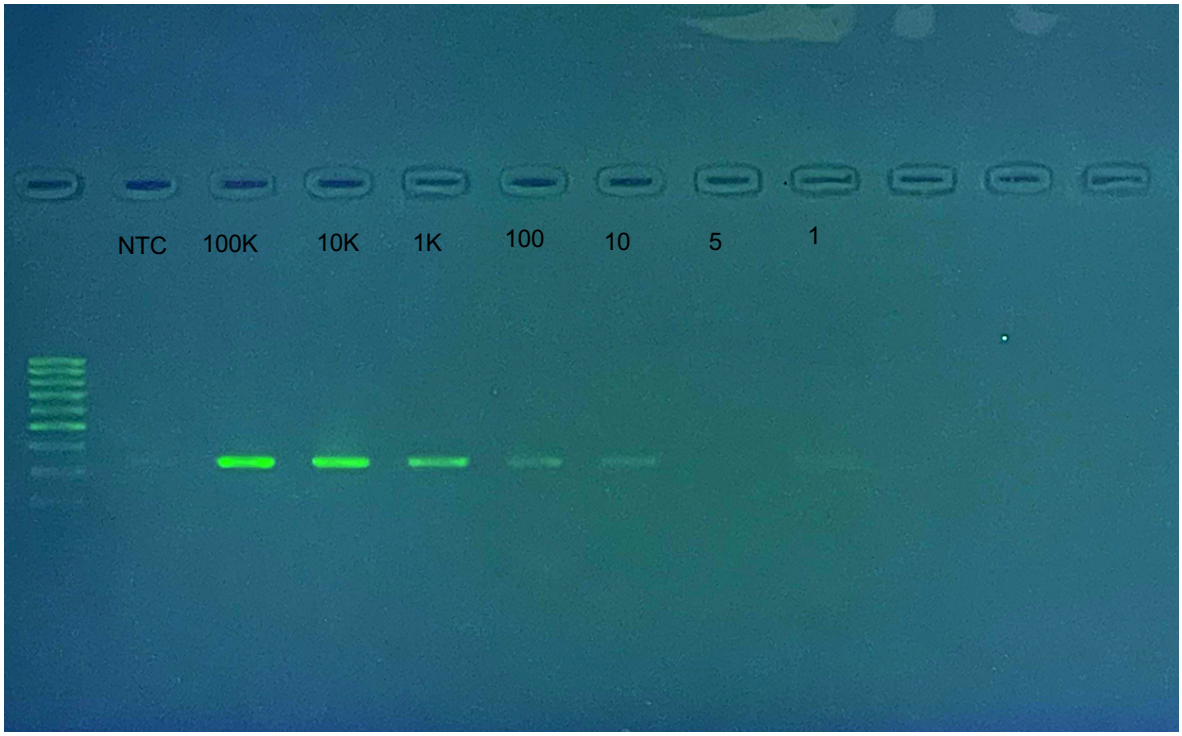


Figure 15: Agarose gel showing bands for *V. salmoninarum CFU Serial Dilutiom..* First well: 100 bp DNA Ladder; NTC: no template control; 100K band down to 1.

3.6.3. Quantification with 3 different kinds of DNA Extract

A second quantification experiment was done, this time however with the CFU Serial Dilution. The samples to be quantified were similar to the ones used for gBlocks serial dilution quantification attempt that is the DNA extracts ranging from 10,000,000 estimated starting quantity down to 10 starting quantities for the 3 different DNA extraction methods used. To make sure the experiment methodology remained same, each DNA extract method was quantified in a qPCR with the CFU serial dilution thrice. This raw data obtained is shown by Supplementary Table 2A, B, and C in the Annex. The mean of each method's triplicate run was calculated and shown below in Table 9. The table also shows the % recovery rate of each DNA extraction method to provide an overview of the quantification comparison for each method.

Table 9: Quantification run with CFU Standard Series. % Recovery rate that shows the difference between the obtained quantification from the expected values is also shown for all three DNA extraction methods.

Expected starting Quantity (SQ)							
Sample	**10,000,000**	**1,000,000**	**100,000**	**10,000**	**1,000**	**100**	**10**
5% Chelex extraction	140,733.33	19,210.00	1,921.00	137.13	18.77	44.33	2.70
Recovery Rate	1.41%	1.92%	1.92%	1.37%	1.87%	44.33%	27.03%
Kit Extraction	2,669,000.00	2,483.67	23.62	6.49	5.94	19.26	3.40
Recovery Rate	26.69%	0.25%	0.02%	0.06%	0.59%	19.25%	34%
Improved Chelex Extraction	21,770,000.00	1,250,933.33	169,333.33	8165.33	799.70	12.86	9.55
Recovery Rate	217.70%	125.09%	169.33%	81.65%	79.97%	12.86%	95.47%

In general, the quantification obtained from the CFU Standard Series is comparatively better, throughout all 3 DNA extraction methods, than shown by the gBlocks Standard Series. Through each extraction method, when compared between the two serial dilution series, performed better with CFU Serial Dilution series and gave quantified starting quantities closer to the expected started quantities. A closer comparison is shown by Table 10 with the % recovery rates for each serial dilution shown side by side.

Table 10: Comparison of % Recovery Rate of aliquots with the CFU and gBlocks Standard Dilution Series. A factor showing the difference between the two serial dilutions' quantification was also measured and shown.

% Recovery Rate Comparison								
Sample		**10,000,000**	**1,000,000**	**100,000**	**10,000**	**1,000**	**100**	**10**
5% Chelex	**gBlocks SD**	0.56%	0.65%	0.64%	0.41%	0.42%	13.56%	5.5%
	CFU SD	1.41%	1.92%	1.92%	1.37%	1.87%	44.33%	27.03%
	Factor	2.51	2.95	3	3.34	4.45	3.26	4.91
Kit	**gBlocks SD**	6.48%	0.08%	0.008%	0.01%	0.04%	0.78%	2.9%
	CFU SD	26.69%	0.25%	0.02%	0.06%	0.59%	19.25%	34%
	Factor	4.11	3,13	2,50	6,00	14,75	24,68	11,72
Improved Chelex	**gBlocks SD**	85.16%	42%	51.49%	22.44%	18.27%	1.34%	17%
	CFU SD	217.70%	125.09%	169.33%	81.65%	79.97%	12.86%	95.47%
	Factor	2,56	2,98	3,29	3,64	4,38	9,60	5,62

3.7. Quantification of eDNA Filtrates with both Standard Dilution Series

A filtration experiment was carried out where known amounts of bacterial culture was added to 1 L of distilled of water and filtered using the filtration setup shown in Figure 7 and described in Section. 2.7. Cellulose-acetate filters were used for this. Once the DNA was extracted from the filters, it was then quantified with qPCR using both Serial Dilution Series. Similar to prior quantification experiments, the qPCRs were done thrice. The raw data for each run obtained is shown in Supplementary Table 3A and B in the Annex. Even before running the qPCR, it was already expected that the quantified amounts of cells will be less than the expected number of cells in a sample because of losses occurring due to filtration and the subsequent extraction step. This was indeed the case and the results are shown by Table 11. The samples used had an estimated starting quantity from 10,000,000 cells going down to 10 cells.

Table 11: Starting quantities measured filtration experiment with gBlocks and CFU Serial Dilution Series. The ration of measured SQ gBlocks to CFU is also shown.

Measured SQ				
Expected SQ (CFU)	**gBlocks quantification**	**% recovery rate**	**CFU quantification**	**% recovery rate**
10,000,000	151,166.7	1.51	553,033.3	5.33
1,000,000	15,713.3	1.57	47,116.7	4.71
100,000	918.2	0.91	3,128.7	3.12
10,000	577.5	5.77	2,072.3	20.72
1,000	48.1	4.81	179.7	17.97
100	4.5	4.50	20.4	20.4
10	3.2	32.0	11.8	118

As shown, there are losses recorded for aliquots with both serial dilution series. However, the losses seem to be higher for gBlocks quantification as for CFU quantification. % Recovery rate was also calculated and is shown in Table 11. These results are also visualized in the bar chart shown by Fig. 16.

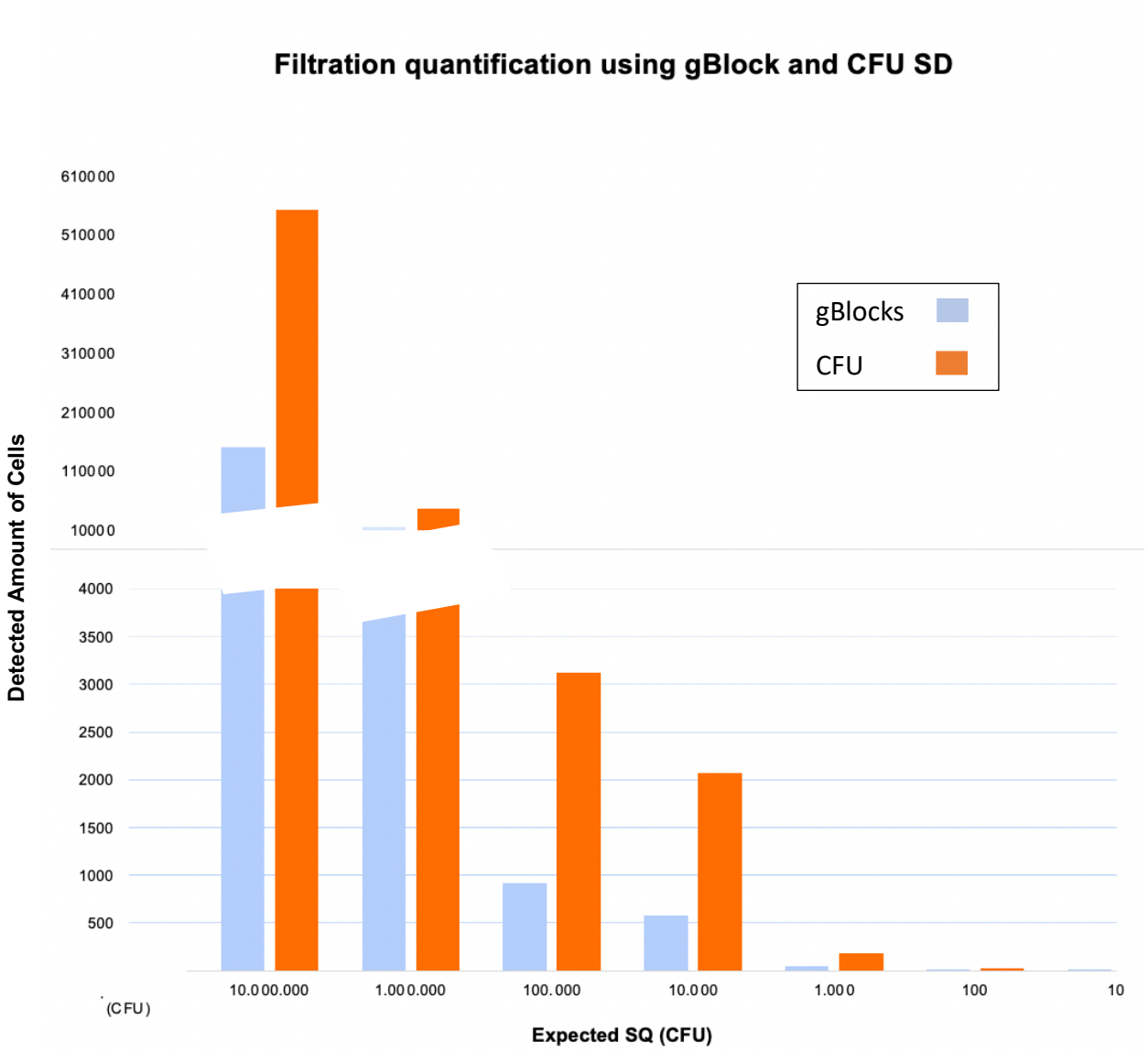


Figure 16: Comparison of filtration quantification results via gBlocks and CFU serial dilution (SD) series. There is a y-axis break to show data bars for higher quantification values.

Through all experimentation, it was clearly seen that the CFU serial dilution series provided a better result as compared to gBlocks Serial dilution. This can be explained by the fact that the CFU serial dilution was made using the same bacterial culture that was used for quantification aliquots and therefore, provided relatively realistic and comparable results.

4. Discussion

4.1. Growth of *V. salmoninarum*

A clean bacterial culture is the basis of any study that is conducted on a certain microorganism. Similarly, the cultivation of a pure *V. salmoninarum* culture was the first step to this work. Several inoculations were done with stored *V. salmoninarum* culture however, these stored cultures were highly contaminated. Finally, a freeze-dried *V. salmoninarum* pellet was used to inoculate and obtain the culture that was used throughout the work of this thesis. This culture showed a uniform growth within a span of 24 hours.

Carry over medium from the original flask was taken to inoculate a new flask which was then used to measure the OD of the culture on a photometer as described in Section. 2.1.3. OD measurement is one of the most commonly used method to estimate the number of cells in a liquid suspension for it is fast, simple, inexpensive, and fairly non-disruptive (Beal *et al*., 2020). The results of the OD600 experiment are shown in Section. 3.1.2, Figure 9 with a growth curve. A growth curve is divided into 4 parts; first being the lag phase that is right after inoculation of a culture and no cell growth takes place. This is followed by a log or exponential phase where there is exponential microbial growth. The third part is the stationary phase where the cell density in the culture remains stagnant and then finally the drop in the curve with the death phase where total cell number begins to decrease. A similar curve is seen in Figure 9. Where there is exponential growth of bacteria from about 400 minutes to 800 minutes, a slightly flat peak from 800 to 900 minutes which is then followed by a sharp drop in measured OD. As mentioned before, Figure 9 shows that the measured OD values are quite specific in the exponential phase but this is not the case in the stationary and death phase as is shown by comparatively larger error bars. This can be explained by the growth and death balance of the stationary phase where the bacterial population remains constant but plenty of cells are continuing to divide while many are beginning to die. However, this also accounts for one of the reasons why OD600 measurement are not considered reliable as it also measures dead cells. This results in an incorrect estimation of growth and metabolic activity of the culture.

Similarly, OD600 measurement also accounts air bubbles in the culture as living cells which again leads to unreliable results (LAMBDA).

4.2. Analytical specificity and sensitivity

Assay specificity is of utmost importance for the reliable detection of pathogen. Assay specificity refers to the degree to which samples containing unwanted DNA sequences are identified and shown as positive results. A well-designed assay has this as zero (Johnson *et al*., 2013). Similarly, for the primers and probes designed for this work were highly specific as none of the tests with the primers had a positive result when attempted to identify the contaminated cultures with PCR and gel electrophoresis.

Positives are often seen in qPCRs due to product carryover, cross-contamination between different samples, etc. It can be seen through results shown in Table 6 and 8. That a few positive NTCs were recorded. An NTC is made with molecular grade water is often included in qPCRs as negative control and is used to identify the set-up contamination and primer-dimer product amplification. It consists of all PCR reagents except for a DNA template (Nolan *et al*., 2013). Table 12 summarizes the Cq values for all the NTCs that were used for various qPCRs.

Table 12: Cq values of NTCs detected in various qPCRs. The positive NTCs are marked by red. An average Cq was also calculated.

Cq values for NTC										
	12.7.22	**13.7.22**	**20.7.22**	**21.7.22**	**26.7.22**	**9.8.22**	**10.8.22**	**15.8.22**	**30.8.22**	**Average**
NTC	N/A	37.49	38.00	N/A	39.86	39.80	N/A	N/A	39.65	38.96

As it can be seen that of the 9 runs, 5 of them had positive NTCs. This can be explained by either random or reagent contamination. Random contamination may have occurred when loading the DNA into the qPCR plates. Due to delivery shortages, loose gloves were available while this work was being done and its highly likely that small amounts of sample DNA template came into contact with the gloves which was carried over into the qPCR plates. Similarly, all pre-PCR and post-PCR procedures were done in the same laboratory space. This can be another potential source of contamination

that a PCR product carryover from a previous reaction was carried over to contaminate a new qPCR plate or DNA extract, resulting in a positive result. However, it should also be noticed that most NTC Cq values were quite late that is towards the end of the reaction. According to D'haene and Hellemans (2010), such positive signals can be ignored if the difference between the highest Cq value of the run and the Cq value obtained for NTC is sufficiently large. As the highest average Cq value was 11.97 with the gBlocks Standard series (Table 6) and the average Cq value for all NTCs is 38.96 (Table 12), the difference being great enough to deem the positive NTCs negligible.

Assay sensitivity or Limit of Detection (LOD) is described as the lowest amounts of cells or at least 95% of the positive samples that can be detected and give a positive result. However, to have an increased confidence in the LOD, replicates needs to be done (Kralik & Ricchi, 2017). For quantifications based on gBlocks Standard Dilution Series, the lowest cell amount detected was 1 in all samples except for the ones that had DNA extraction done with kit. As for with CFU Standard Dilution Series, 10 was the LOD across all samples, regardless of what DNA extraction method was used.

4.3. Standard Series performance

A high qPCR efficiency is synonymous to a robust and precise qPCR assay. In an ideal qPCR, the number of initial DNA molecules should double every cycle resulting in a 100% efficiency. However, these reactions are rarely perfect in real life and the efficiency usually ranges between a 90 to 110% with gradients between -3.2 and -3.5. (Bustin *et al.*, 2009). A 110% efficiency is possible and usually tends to indicate polymerase inhibition, which is usually strongest in the least diluted sample resulting in deviation from linearity (Svec *et al*., 2015). Polymerase inhibition occurs when excessive amounts of DNA/RNA or carry-over material is present in a sample. (Čepin, 2017b). However, an efficiency value below 90% inhibitor contamination, poor primer efficiency, or inaccurate pipetting (BiteSizeBio, 2022). Moreover, a stable qPCR assay should demonstrate an R^2 >0.98 over at least 6 logs and three replicates (Bustin *et al*., 2009).

There were two standard dilution series used for this work and both of them meet the benchmark of a stable qPCR in regards to linearity coefficient, slope of the standard curve and efficiency. The R^2 value for the gBlocks standard curve plotted was 0.9926

while for CFU standard curve, the R^2 value was found to be 0.9825. Similarly, the gradient for the gBlocks standard curve was -3.19 and for CFU standard curve was -3.22 which are both within the range determined. The efficiency of the gBlocks standard curves was calculated to be 105.8% with 104.2% efficiency calculated for CFU standard curve. A side-by-side view of the two standard curves is shown in Figure 17.

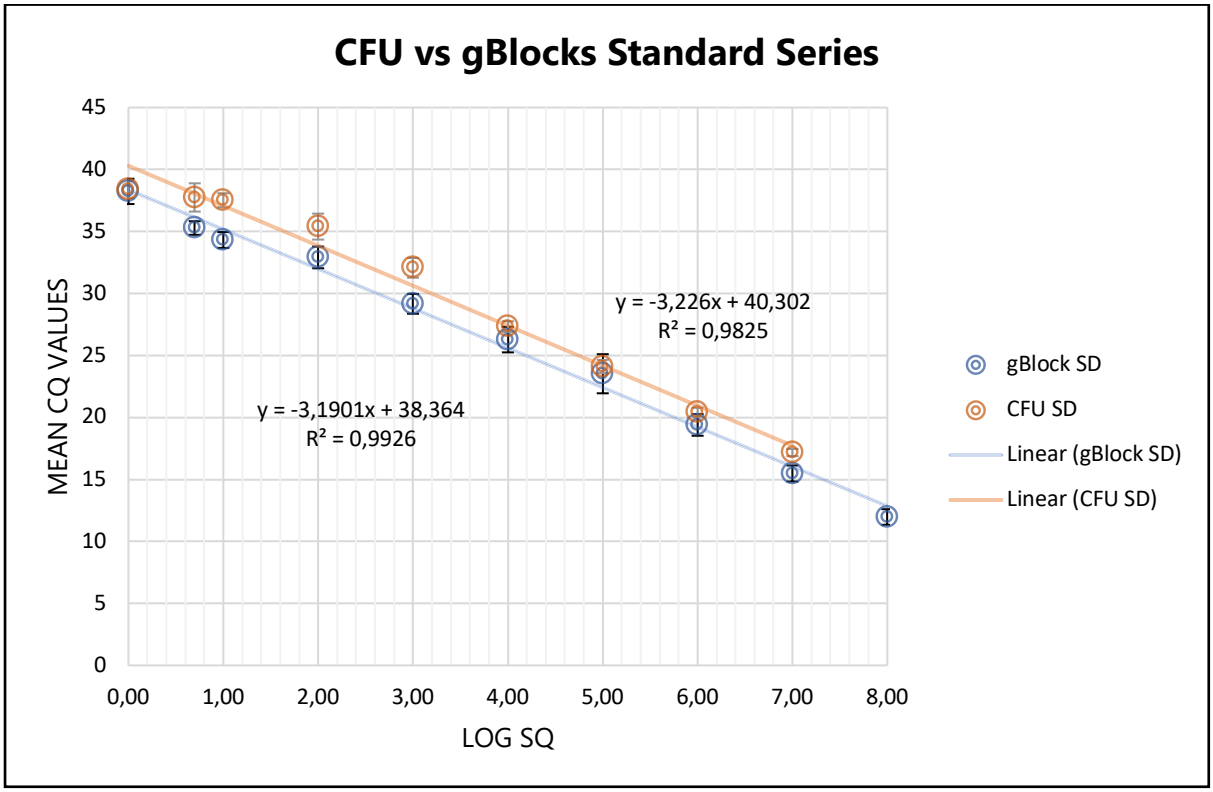


Figure 17: Comparison of the gBlocks and CFU standard curves. The equation for the regression line as well as the R^2 value for each curve is also shown.

4.4. qPCR results

The detection of *V. salmoninarum* was possible throughout all samples, regardless of the DNA extraction method or Standard Series used. However, the quantification of these samples was fairly variable.

Through quantification via gBlocks, the % recovery rates measured for the different DNA extraction methods were largely variable. The % recovery rates were found to be over a large range for kit extracts, ranging from 0.008% to 6.48% with 1 copy number not being detected at all. Similarly, for the regular 5% Chelex extraction, the lowest %

recovery rate recorded was 0.41% going up to a high of 67%. This quantification, although not reliable, still provided with a better quantification result than the kit extracts. The best quantification using gBlocks Standard Dilution Series was obtained with the extracts that were made using the improved Chelex protocol. They had a % recovery rate ranging from 1.34% going very close to the expected starting quantity with a percentage of 85.16%. However, for 1 copy, a % recovery rate of 251% was seen which is either an anomaly or a result of inaccurate estimate of expected SQ.

A second CFU standard series was made with the expectation to improve the quantification result as this standard series was created with the same bacterial culture. In terms of quantification through each extraction method, results are similar to that seen with quantification experiment with gBlocks. This means there were heavy losses recorded with Kit extraction and a very low % recovery rate can be seen, within the ranges of 0.02%% and 26.69%. This is slightly better with regular 5% Chelex extraction but still not satisfactory results where the recovery rates ranged from a low 1.37% and reaching a 44.33%, but only for one sample. The best quantification can be seen with the Improved 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.

Since the CFU serial dilution was also made from the improved DNA extraction method i.e. it also started with a higher amount of cells than expected, the quantification for the DNA extracts from improved Chelex DNA protocol is also higher. Regular Chelex protocol and the kit DNA extraction method were not optimal methods of DNA extraction for gram-positive *V. salmoninarum* and the unknown aliquots were assumed to have less DNA than expected in the first place. As shown with the results in Section 3.4, it was seen that of all the DNA extraction methods used, the improved Chelex DNA Extraction method yielded the best results for the Gram-positive *V. salmoninarum* bacteria. Figure 18A, B, and C show a comparison between the % recovery rates for each extraction method using gBlocks and CFU serial dilution that shows a clear difference in the efficiency of each DNA extraction method as well as the two serial dilution series.

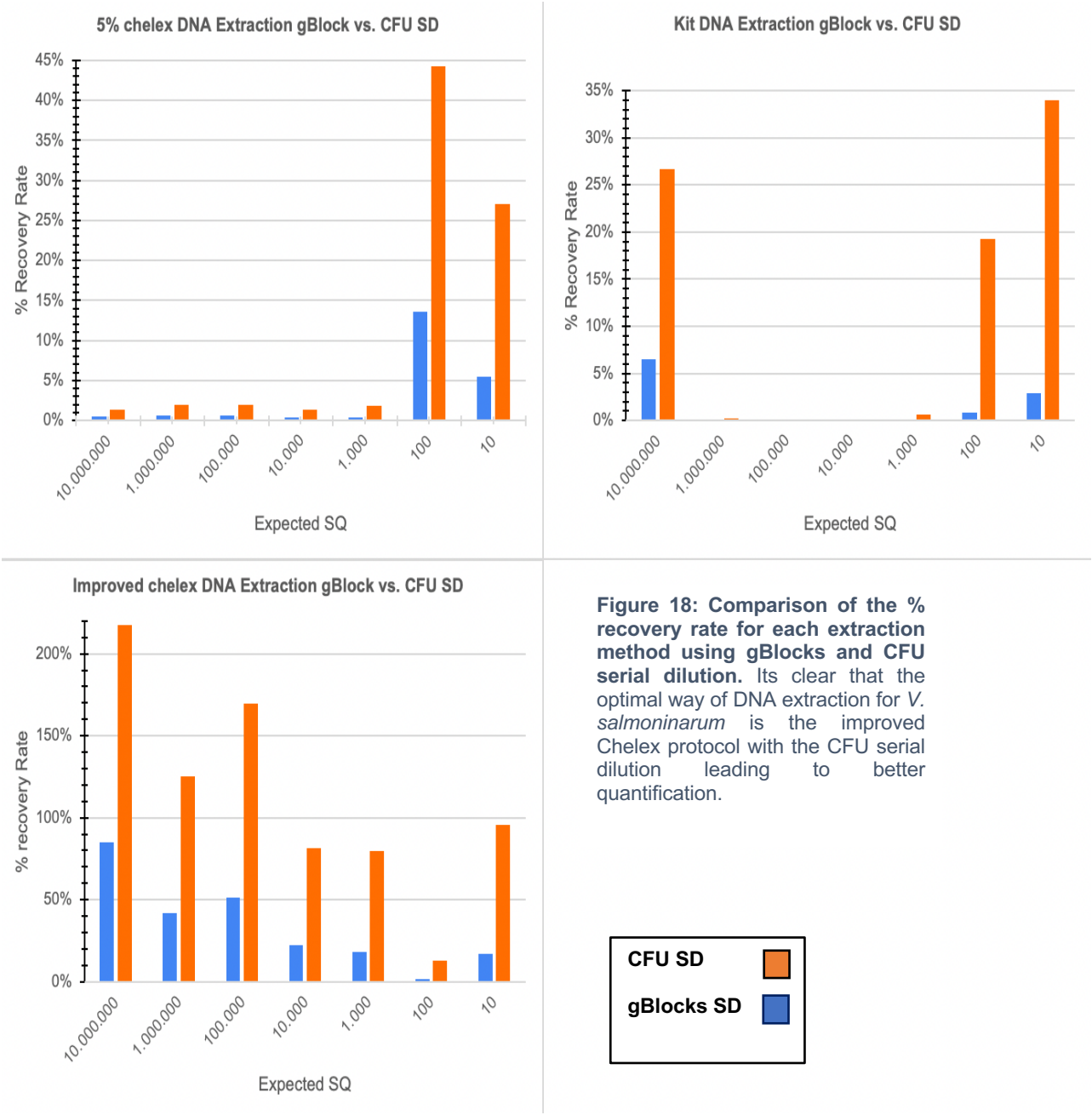


Figure 18: Comparison of the % recovery rate for each extraction method using gBlocks and CFU serial dilution. Its clear that the optimal way of DNA extraction for *V. salmoninarum* is the improved Chelex protocol with the CFU serial dilution leading to better quantification.

4.5. Filtration experiment for PHOTO-SENS

The main aim of the filtration experiments was to mimic the filtration of eDNA from highly diluted water samples as it is supposed to be done for the PHOTO-SENS biosensor chips. As described earlier, the biosensor chips are to be used to detect the presence of fish pathogen eDNA, one of which being *Vagococcus salmoninarum* in water samples. eDNA is usually captured using filtration or centrifugation, however capture of eDNA on filters has been found to be more efficient. (Majaneva *et al.*, 2018) The experimental design consisted of spiking 1 L of distilled water with *V.*

Salmoninarum culture, filtering this water through CA-filters and extracting DNA from these filters to be detected and quantified on qPCR.
Although expected starting quantity was estimated, a loss on DNA detected on the qPCR was already expected due to filtration and DNA extraction step. This was definitely the case as seen in the results shown by Table 11 and visualized by Figure 16. Table 11 also showed the % recovery rate calculated for expected SQ ranging from 10,000,000 to 10 for both serial dilution series. The % recovery rate calculated for CFU standard series was about 3.5 times higher than that of gBlocks standard series for each respective expected SQ.

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

4.6. Findings of similar Research

The primers designed for this work were specific to *V. salmoninarum* and did not provide positive signals with any other bacterial specie. This was because the primer region selected during designing of the primers and probes was exclusive to *V. salmoninarum.* This primer region was similar to the region selected for designing primers by Torres-Corral and Santos (2019). Although this study was based on detection and quantification of *V. salmoninarum* using SYBR Green I-based-real-time PCR and included a melt curve, the lowest detection limit recorded was 0.034 amplicon copies per assay. The detection limit of this work was 1 copy when quantified with gBlocks standard series and 10 when the CFU standard series were used for the qPCR. Similarly, from the work of Standish, Leis, *et al*. (2020), it was also seen that the LOD was measured was as 10 copies when they used the gBlocks standard curve. This concludes that the results of this work were synonymous to work done prior by other researchers.
The work of Torres-Corral and Santos (2019) also focused on the detection of *V. salmoninarum* in artificially inoculated fish tissue homogenates and also testing the applicability of the assay in fish suffering from vagococcosis. Through artificial spiking

of bacteria into fish samples, a higher range of 16s rRNA gene was recorded, from about 358 to 0.07 copies per microliter. The experiment conducted in clinical fish samples showed that the primers designed were useful and a bacterial DNA load of 0.18 to 0.02 copy number was recorded in samples from the eye, spleen, kidney, skin and intestine of *V. salmoninarum* infected fish. Therefore, although detection and quantification of the bacteria directly extracted from fish was not done, there's a high chance that the assay designed would be applicable for such an experiment since the primer region was similar to that of 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.

4.7. 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.

Foremost, the fact that the qPCR is unable to distinguish between viable and non-living cells, which is unarguable the biggest disadvantage of qPCR (Kralik & Ricchi, 2017), posed a problem for correct quantification. This means dead cells are also quantified along with living cells and therefore, results are erroneous in regards of number of living cells. However, many researchers agree that although this problem exists with measuring DNA, it is not the case with RNA. This is because RNA is known to have low stability and disintegrates within minutes in dead cells. Hence, reverse transcriptase qPCR (RT-qPCR) could prove to be a better approach for bacterial cell quantification (Kralik & Ricchi, 2017).

Similarly, the PHOTO-SENS project involves the detection of extremely low amounts of bacterial cells in highly diluted water samples which can prove to be a rather difficult task. The lowest copy number detected through this work in the filtration experiments was 10 copies in 1 L of distilled water. Therefore, another approach for extremely low-level detection can be the use of droplet digital PCR (ddPCR). ddPCR is a recent advancement in PCR methods that allows the random allocation of target DNA into discrete droplets via microfluidics. These droplets are then thermally cycles and screened individually using fluorescence measurement for the detection of target DNA. Quantification of DNA using this method have been found to be quite accurate. (Zaiko *et al*., 2018).

To further improve the accuracy and detection limit of filtration experiments, it can also be valuable to make use of filters with a small pore size such as 0.20 µm. According to Majaneva *et al*. (2018), cellulose-nitrate filters found to yield the highest amount of DNA as compared to other filters used for eDNA capture.
Although, this was supposed to be an imitation of the experiments to be conducted for the PHOTO-SENS biosensor chips, the experimental setup did not account for nutrient and chemical contamination as well other microbial cell components that are likely to be present in water samples from hatcheries and rivers and could cause potential hindrance. Therefore, the experiment can be adapted in the future to closely resemble water from sample sites.

Similar to the approach of Standish *et al*. (2022) where a successful duplex qPCR was attempted for detection and quantification of *Vagococcus salmoninarum* 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 the 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. *Vagococcus salmoninarum* is a Gram-positive fish pathogen that is known to cause Vagococcosis or 'cold water streptococcosis', due to only occurring in water temperatures below 10-12°C. *V. salmoninarum* outbreaks tend to be extremely troubling for they have ≥50% mortality rates in broodstock and field-treatments have also shown them to be resistant to antibiotics. Therefore, it is of extreme importance to recognize and aptly address the presence of the bacteria early-on.

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 are 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, *V. salmoninarum* specific primers and probes were designed for the detection of *V. salmoninarum* in samples using qPCR. The quantification was done using a standard curve generated from two different standard dilution series used. Moreover, due to *V. salmoninarum* being a Gram-positive bacteria, 3 different ways of DNA extraction were utilized.

The improved 5% Chelex protocol proved to be the most efficient way of DNA extraction for *V. salmoninarum* as highest amounts of DNA were detected in those samples when compared to the samples that were prepared using the regular 5% Chelex protocol and a commercial DNA extraction kit.
Similarly, both the standard series showed the highest recovery rates for the samples that has undergone the improved 5% Chelex DNA extraction.

However, between the 2 standard dilution series, there was a difference in quantification obtained which was assumed to stem from the inaccurate CFU counts that tend to underestimate total cell counts due to formation of clusters and not accounting for dead cells. With the gBlocks standard series, 1 copy in a sample was

successfully detected while for the CFU standard series, the lowest number of copies detected successfully was 10. The gBlocks standard series was reliable for quantification comparison between the 3 DNA extraction methods, the CFU standard series provided a better perception on the total number of cells present in a sample. Filtration experiments done to imitate filtration of eDNA from water samples also displayed similar results that is a higher % recovery rate with the CFU serial dilution.

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 *V. salmoninarum*-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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7. Statutory Declaration

I hereby declare that I wrote the present dissertation with the topic: “Detection of the Fish Pathogen *Vagococcus salmoninarum* via Quantitative real-time PCR for Aquaculture” independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice.

Duesseldorf, 24/10/22. Hafsa Kanwal, 

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9. Annex

Supplementary Table 1A: Raw data for the qPCR quantification runs for the DNA aliquots prepared with improved Chelex DNA extraction protocol with gBlocks standard series.

Improved Chelex Extraction – gBlocks quantification			
Expected SQ	12.7.22	21.7.22	31.8.22
10,000,000	N/A	9487000.00	7545000.00
1,000,000	270100.00	493700.00	496700.00
100,000	35090.00	67350.00	52030.00
10,000	1313.00	3046.00	2373.00
1,000	101.50	257.00	189.70
100	0.64	2.43	0.95
10	0.51	2.11	2.49
1	0.88	3.58	3.06

Supplementary Table 1B: Raw data for the qPCR quantification runs for the DNA aliquots prepared with regular Chelex DNA extraction protocol with gBlocks standard series.

Regular Chelex Extraction – gBlocks quantification			
Expected SQ	21.7.22	10.8.22	31.8.22
10,000,000	78620.00	35530.00	55170.00
1,000,000	6371.00	6369.00	6660.00
100,000	664.80	697.20	569.50
10,000	45.70	45.60	32.70
1,000	3.98	4.92	3.66
100	14.10	12.80	13.78
10	0.43	0.51	0.72
1	0.24	N/A	1.10

Supplementary Table 1C: Raw data for the qPCR quantification runs for the DNA aliquots prepared with kit DNA extraction with gBlocks standard series.

Kit Extraction – gBlocks quantification			
Expected SQ	13.7.22	21.7.22	31.8.22
10,000,000	330500.00	429100.00	1187000.00
1,000,000	520.20	883.20	1092.00
100,000	6.86	16.20	3.60
10,000	0.48	2.20	0.99
1,000	0.17	0.30	0.82
100	0.17	N/A	1.39
10	N/A	0.24	0.34
1	N/A	N/A	N/A

Supplementary Table 2A: Raw data for the qPCR quantification runs for the DNA aliquots prepared with improve Chelex DNA extraction protocol with CFU standard series.

Improved Chelex Extraction - CFU SD quantification			
Expected SQ	26.7.22	9.8.22	30.8.22
10,000,000	11100000.00	N/A	32440000.00
1,000,000	678800.00	1639000.00	1435000.00
100,000	120700.00	197100.00	190200.00
10,000	6090.00	9884.00	8522.00
1,000	594.80	913.20	891.10
100	16.12	8.98	13.50
10	9.13	10.50	9.04

Supplementary Table 2B: Raw data for the qPCR quantification runs for the DNA aliquots prepared with regular Chelex DNA extraction protocol with CFU standard series.

Regular Extraction - CFU SD quantification			
Expected SQ	26.7.22	15.8.22	30.8.22
10,000,000	124900.00	148300.00	149000.00
1,000,000	18610.00	19200.00	19820.00
100,000	1901.00	1929.00	1933.00
10,000	142.90	146.20	122.30
1,000	25.52	10.80	20.02
100	41.40	46.80	44.80
10	2.70	1.93	3.48

Supplementary Table 2C: Raw data for the qPCR quantification runs for the DNA aliquots prepared with kit DNA extraction with CFU standard series.

KIT Extraction - CFU SD quantification			
Expected SQ	26.7.22	15.8.22	30.8.22
10,000,000	1736000.00	3194000.00	3077000.00
1,000,000	2194.00	2390.00	2867.00
100,000	31.70	14.30	24.86
10,000	7.05	2.26	10.15
1,000	9.20	3.88	4.74
100	2.30	N/A	36.21
10	N/A	N/A	3.40

Supplementary Table 3A: Raw data for the qPCR quantification runs for the filtrates with gBlocks standard dilution series.

G block and filtrates.			
expected SQ	22.8.22	23.8.22	23.8.22
100,000,000	-	-	-
10,000,000	112,800.0	168,600	172,100
1,000,000	14,210.0	15,910	17,020
100,000	846.7	892.9	1015
10,000	542.2	490.4	699.9
1,000	43.5	46.2	54.5
100	6.4	4.84	2.26
10	4.7	1.25	3.79

Supplementary Table 3B: Raw data for the qPCR quantification runs for the filtrates with CFU standard dilution series.

CFU SD and filtrates			
Expected SQ	23.8.22	24.8.22	24.8.22
10,000,000	765,300.0	517,600	376,200
1,000,000	48,570.0	47,930	44,850
100,000	2,313.0	3525	3548
10,000	1,476.0	2472	2269
1,000	96.5	246.2	196.3
100	13.2	25.1	22.9
10	7.4	14.8	13.2