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Quantitative real-time PCR Detection of *Yersinia ruckeri* for Application in Aquaculture

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Abstract

To detect pathogen infections in aquaculture early and reduce their impact, a photonic biosensing chip is being developed in the PHOTO-SENS project, to enable fast and easy routine testing in aquaculture facilities. One target bacterial pathogen is *Yersinia ruckeri*, the causative agent of enteric redmouth disease. Detection is based on short DNA sequences (probes), that are complementary to the DNA of their target pathogen.

In this work, a *Yersinia ruckeri*-specific probe was designed together with a set of qPCR primers. The probe and primers were tested in qPCR experiments, including quantification of extracted DNA, specificity and sensitivity testing, and detection of bacterial DNA from highly diluted samples to mimic the detection of environmental DNA. Three different standard series were created for the quantification of unknown samples. The influence of fish tissue on detection was tested, and the efficiency of Chelex-DNA extractions were compared to peqGOLD Tissue DNA Mini Kit extractions.

During quantification experiments with a first standard series (Y1P), the quantification output of most samples was 12–175 times higher than expected. The lower limit of detection was at 100 copies based on that standard, with an efficiency of 88%. The discrepancy was reduced with a gBlocks standard (limit of detection: 5 copies; efficiency: 92%). A CFU standard provided quantification results in reasonable agreement with expected CFU starting quantities (limit of detection: 1 CFU; efficiency: 106%). Detection of diluted samples after filtration was possible. Chelex DNA extractions yielded higher amounts of detectable DNA compared to a DNA extraction kit. Less bacterial DNA was extracted in bacterial culture samples containing fish tissue than in samples that did not contain fish tissue.

Higher-than-expected quantification outputs were attributed to flaws in the creation of a first standard series as well as detectable DNA from dead cells or clusters that were not accounted for in the expected starting quantities. The gBlocks standard was assumed to be most reliable for quantification of exact copy numbers, while the CFU standard produced results that could be accounted for in CFU counts. As these challenges concerned the standard series' and not the probe itself, it was concluded that the probe was suitable for application in the PHOTO-SENS project for detection of *Yersinia ruckeri*.

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List of Abbreviations

bp	Basepairs; measure of length of a gene or DNA fragment
CA	Abbreviation used for filtrations with cellulose acetate filter
CFU	Colony forming units; colonies of bacteria after growth on agar plates
CPA	Cryoprotective additive; added to samples to protect them from freezing damage
Cq	Quantification cycle in which fluorescence signal exceeds a baseline threshold value in qPCR
CV%	Coefficient of variation (percentage), also called relative standard deviation; standard deviation of a group of samples divided by their respective mean
dNTP	Nucleoside triphosphate: DNA building block containing DNA backbone structure and a nucleic acid; dATP, dGTP, dTTP or dCTP
DSMZ	Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH
EO	Abbreviation used for filtrations with ethylene-oxide-treated (EO-treated) glass fiber filters
ERM	Enteric redmouth disease; caused by *Yersinia ruckeri*
NTC	No template control; sample consisting of e.g. water to test reaction components for a signal
OD	Optical density; absorbance measurement used to determine growth of bacterial cell cultures
PCR	Polymerase chain reaction
qPCR	Quantitative / real-time polymerase chain reaction; used to amplify, detect and quantify DNA
RFU	Relative fluorescence units; unit for fluorescence measurements in qPCR
rpm	Rounds per minute; specification of rotational shaking and centrifugation speed
SQ	Starting quantity; amount of target DNA in a qPCR sample before amplification

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1. Introduction

1.1 Global Relevance of Fish and Aquaculture

Since 1961, global food fish consumption has increased steadily. The annual rate of increase until 2017 was almost twice as high as the world population growth in that time period, according to the Food and Agriculture Organization of the United Nations [FAO] (2020). Among animal protein foods, fish has seen the highest increase, accounting for 17% of global animal protein intake and 7% of all proteins in 2017. In countries such as Bangladesh, Cambodia, the Gambia, Ghana, Indonesia, Sierra Leone, Sri Lanka and several Small Island Developing States, the percentage of fish protein reached 50% or more of animal protein intake per capita. In 2018, per capita fish consumption was at 20.5 kg (live weight equivalent), compared to 9.0 kg in 1961. 52% of fish for human consumption stemmed from aquaculture sources, including inland, marine, and coastal aquaculture. Of the global fish production, including non-food purposes like fishmeal and fish oil, which are used as feed in aquaculture and livestock, aquaculture contributed 46%. Fish and fishery products are closely linked to global trade, as 38% of all fishery and aquaculture products in 2018 were traded internationally, with the largest fish importing markets being the European Union, the United States of America and Japan. Main exporters were China, Norway, and Vietnam, followed by India, Chile, and Thailand. Of internationally traded fish, 54% of global export value and 60% of total volumes were produced by developing countries. However, fish consumption in developing and least developed countries has also steadily increased in the last 20 years, with developing countries having imported 31% of global fish and fish products by value and 49% in quantity, in 2018 (FAO, 2020).

1.2 UN Sustainable Development Goals and Planetary Health Diet

Its importance as animal protein source for global nutrition and the direct connection to aquatic life makes fish a considerable factor in two of the 17 Sustainable Development Goals adopted by all member states of the United Nations in 2015 (Division for Sustainable Development Goals [DSDG]). The Zero Hunger goal aims to end hunger and ensure permanent access to safe, sufficient and nutritious food for all people, especially those in financial or otherwise vulnerable situations (DSDG, Goal 2). A sustainable and balanced diet is also the subject of the Planetary Health Diet that was

developed and published by the EAT-Lancet Commission in 2019. It suggests a dietary framework for healthy nutrition within the sustainable limits of our planet, meant to reduce nutrition-influenced diseases and mortalities while also respecting planetary boundaries (Kirk-Mechtel, 2020). The suggested diet consists mainly of fruits, vegetables, wholemeal products, pulses, nuts, and unsaturated fats, with additions of moderate amounts of fish, seafood, and poultry (Kirk-Mechtel, 2020). While fish can be omitted from the diet, a daily intake recommendation is set at 28 g, with a range of up to 100 g. This is four times higher than the recommendation for red meat and pork (each at 7 g), and has a higher possible range than most of the other protein sources (Willett et al., 2019, Table 1). The importance of aquaculture is highlighted in the report of the EAT-Lancet Commission for its rapid growth and potential to provide nutritional value to many, steering production of proteins from animal sources in the direction of reduced environmental impact and enhanced health benefits (Willett et al., 2019).

“Life below water” is goal number 14 of the 17 Sustainable Development Goals (DSDG, Goal 14). Its aim is to sustainably manage and protect marine and coastal ecosystems, regulate harvesting, and end destructive fishing practices and general overfishing. Aquaculture is mentioned specifically in the context of Small Island developing States and least developing countries, to establish a sustainable use of marine resources (DSDG, Target 14.7).

1.3 Fish Pathogens in Aquaculture

Disease-causing fish pathogens are a threat to the safe and reliable production of fish from aquaculture sources. While it is difficult to fully assess the impact of fish pathogens on aquaculture due to a lack of suitable data, economic losses in finfish aquaculture have been estimated to reach 1.05 to 9.58 billion US dollar every year (Tavares-Dias and Martins, 2017). Disease outbreaks are favored by so called “predisposing factors” that are often the consequence of sub-optimal handling conditions and stressed fish (Plumb and Hanson, 2010). Stressors include high stocking densities, poor water quality, inappropriate temperatures, and transport or handling of fish (Plumb and Hanson, 2010). In the context of climate change, stress caused by increasing water temperatures and CO_2-induced ocean acidification may further worsen the situation (Austin and Austin, 2016; Raven et al., 2005).

Losses are caused by costs of disease control, reduced productivity, increased mortality and price reduction due to disease lesions (Adam and Gunn, 2017; Maldonado-Miranda et al., 2022). Furthermore, a negative perception of the public connected to disease outbreaks may lessen the demand specifically of aquaculture products and negatively impact the market (Adam and Gunn, 2017). Table 1 shows an overview of a few documented economic losses with their value estimates and causative agents. However, actual losses are likely much higher.

Table 1: Economic losses due to bacterial pathogens in aquaculture industry. Adapted from Maldonado-Miranda et al., 2022.

Host species	Bacterium	Estimated loss (in million USD)	References
Hypophthalmichthys molitrix, *Hypophthalmichthys nobilis* and *Carassius carassius*	*Aeromonas hydrophila*, *Yersinia ruckeri*, and *Vibrio fluvialis*	120	Wei (2002)
Ctenopharyngodon idella	*Vibrio anguillarum*	0.5	Thi-Van et al. (2002)
Oreochromis niloticus	*Aeromonas* spp., *Pseudomonas fluorescens*, *Flavobacterium columnare*, and *Streptococcus* sp.	4	Kubitza et al. (2013)
Oreochromis niloticus	*Aeromonas caviae*	0.013	Martins et al. (2008)
Hybrid surubim	*Aeromonas hydrophila*	0.16	Silva et al. (2012)
Pseudoplatystoma reticulatum	*Citrobacter freundii*	Not reported	Pádua et al. (2014)
Seriola spp.	*V. anguillarum*	200	Gracía-Mendoza (2017)
Ictalurus punctatus	*A. hydrophila*	10	USDA (2018)
I. punctatus, *Oncorhynchus mykiss*, sport fish, bait fish, and ornamental fish	*Flavobacterium columnare*	40–50	USDA (2018)
Catfish *I. punctatus* and Hybrid catfish	*Aeromonas hydrophila* virulentas, *Edwardsiella ictaluri*, and *F. columnare*	16.9	Peterman (2019)
Oncorhynchus kisutch, *Oncorhynchus mykiss*, and *Salmo salar*	*Piscirickettsia salmonis*	700	Flores-Kossack et al. (2020)
Oreochromis niloticus	*Streptococcus agalactinae* and *Franciella noatunesis* sub. *Orientalis*	0.219	Junior et al. (2020)

Other than economically, aquatic animal health is also of relevance considering nutrition, human health, and employment. Especially in countries like Bangladesh, in which aquaculture provides 63% of animal protein consumption, a failure to meet market demands might negatively impact nutrition, household food security and income (Adam and Gunn, 2017). Furthermore, the application of antibiotics to handle infections in aquaculture bears the risk of the development of resistant bacteria, increasing the severity of disease outbreaks for the fish and aquaculture sector and additionally leading to resistant bacteria in humans (Adam and Gunn, 2017). Environmentally, the risk of

spreading diseases from aquaculture to wild stock should be considered as well, especially in the context of restocking of threatened fish populations.

1.4 *Yersinia ruckeri*

Yersinia ruckeri is the causative agent of enteric redmouth disease (ERM), also called yersiniosis, one of the most important diseases in salmonid fish according to Kumar et al. (2015). It is a Gram-negative, rod-shaped enterobacterium with a diameter of around 0.75 µm and a length of 1–3 µm. First identified in the USA in the 1950s, *Yersinia ruckeri* has since been found in North and South America, Europe, Australia, South Africa, the Middle East, and China. The species has been divided into several strains with different surface antigens (serotypes) and different biological behavior (biotypes). Outbreaks of ERM in salmonids are mainly caused by serotype O1a, which has been found to be highly genetically homogeneous. While enteric redmouth disease is most commonly associated with rainbow trout (*Oncorhynchus mykiss*), it has also been reported in other fish species like the Atlantic salmon (*Salmo salar)* (Austin and Austin, 2016; Kumar et al., 2015).

1.4.1 Severity of Enteric Redmouth Disease

Despite the low levels of mortalities of ERM outbreaks, a sustained and ongoing infection can result in high losses over time. In an assessment from 1976 by Klontz and Huddleston, it was estimated that enteric redmouth disease may cause cumulative losses of 30-35% of the rainbow trout population (Austin and Austin, 2016). The disease is most acute in young fish, whereas older or larger fish may develop it as a more chronic condition (Kumar et al., 2015). At water temperatures of 15-18°C, the severity of the disease reaches a peak, but decreases at temperatures below 10°C (Austin and Austin, 2016). Another relevant factor for severity is water salinity, with significantly reduced mortalities at increased salinity (Austin and Austin, 2016). Lethality of the disease depends on the different subtypes of *Yersinia ruckeri.* The LD_{50} dose that was lethal for 50% of test subjects has been found to be 3.0×10^5 cells/mL and 1.0×10^7 cells/mL for two serotypes of the bacterium. At a dose of 10^5 cells/fish, biotype 2 was found to kill rainbow trout within 4 days (Austin and Austin, 2016).

1.4.2 Route of Infection

There are several possible modes of entry of *Yersinia ruckeri* into the host fish, including entry through injured skin, the lateral line canal, which is a sensory system on the

lateral side of fish used for orientation, and entry by invading the intestinal mucus in the fish stomach after swallowing (Otani et al., 2014). However, as fastest and most probable route of entry, it has been suggested that the pathogens enter the host organism through the secondary lamellae of the gills and are then spread through the blood system to the internal organs (Kumar et al., 2015; Otani et al., 2014). *Yersinia ruckeri* could be detected in the blood as early as one minute post infection, with significant amounts at 40 and 60 minutes post infection compared to samples before infection ($p = 0.0279$ and $p = 0.0076$, respectively) (Otani et al., 2014). It was furthermore measured in the lumen of the intestine 30 minutes after infection, in the kidney after three days and the liver, spleen, brain and heart seven days after infection (Kumar et al., 2015).

From infected fish, *Yersinia ruckeri* can be directly transmitted to non-infected fish. Other possible ways include transmission through feces, as *Yersinia ruckeri* can survive at last 4 months without a host organism (Kumar et al., 2015). Coquet et al. (2002) observed the ability of *Yersinia ruckeri* to form biofilms on solid supports that are often found in fish farm tanks (Kumar et al., 2015). *Yersinia ruckeri* biofilms were found to consist of several dispersed but interconnected microcolonies of the bacteria (Wrobel et al., 2020). Biofilms facilitate recurrent infections in fish farms and constitute a method of transmission that remains undetected until first symptoms or casualties appear (Kumar et al., 2015). In addition, an asymptomatic carrier state has been determined in which the fish can survive for around two months. In this state, the fish are infectious without showing symptoms themselves (Kumar et al., 2015). Transmission of the bacteria from carriers to healthy fish only occurred when fish were stressed, for instance by an increase in water temperature (Austin and Austin, 2016; Hunter et al., 1980; Kumar et al., 2015). Other possible stressors leading to outbreaks in seemingly healthy fish include handling of fish and high stock densities, resulting in lower water quality with more metabolic waste products and lower oxygen levels (Austin and Austin, 2016). Furthermore, aquatic invertebrates and birds have been suspected to act as transmission vectors, and the pathogen could possibly be transmitted vertically from mother to progeny (Kumar et al., 2015).

1.4.3 Clinical Signs of Enteric Redmouth Disease

The name “enteric redmouth disease” stems from its most characteristic symptom: bleedings in the tissue under the skin (subcutaneous hemorrhages) that appear in and

around the mouth and throat of the fish (Figure 1B). Other visible signs of the disease include the darkening of skin or the protrusion of one or both eyeballs (exophthalmia). Signs of the disease can also be found when examining the internal organs of infected fish. Typical are an enlarged and blackened spleen (Figure 1C, white arrow), a reddened lower intestine filled with an opaque, yellowish fluid and pin-point red spots (petechial hemorrhages) on the surfaces of liver, pancreas, pyloric caeca, swim bladder and the lateral muscles. ERM disease has also been associated with behavioral changes like swimming close to the water surface, lethargic movement, and appetite loss. However, fish may also be infected as asymptomatic carriers without showing any noticeable symptoms (Kumar et al., 2015).

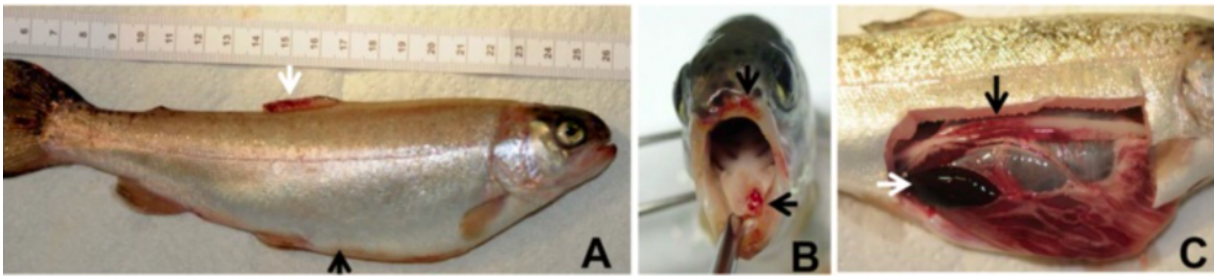

Figure 1: Clinical signs of enteric redmouth disease. Shown on a rainbow trout. **A:** darkend skin, hemorrhages in the dorsal fin (white arrow) and enlarged abdominal valley (black arrow). **B:** hemorrhages in and around the mouth. **C:** reddened intestine (black arrow) and enlarged, black spleen (white arrow). Adapted from Kumar et al., 2015.

1.4.4 Detection of *Yersinia ruckeri*

Other than diagnosis by physical symptoms of the fish or culturing of bacteria from aquaculture samples, several diagnostic assays have been developed for the detection of *Yersinia ruckeri*. Molecular methods are based on immunology and include assays with ELISA, agglutination, and immunofluorescent labeled antibodies. Other detection methods target the genome of the bacterium, with restriction fragment length polymorphism assays and amplification through polymerase chain reaction (PCR), quantitative real-time PCR (qPCR) or loop-mediated isothermal amplification (LAMP). While the different strategies each possess certain advantages, PCR has been successfully applied to detect low quantities of *Yersinia ruckeri*. This is crucial for the early detection of outbreaks and may allow the detection of asymptomatic carriers. With appropriate measures, the spreading of enteric redmouth disease could potentially be prevented or stopped in an early stage, to reduce economic losses and associated risks (Kumar et al., 2015).

1.4.5 Treatment

For the prevention of ERM outbreaks, a vaccine has been developed using inactivated whole cells of *Yersinia ruckeri*. Administration is possible by injection or oral vaccine. Another possibility is the immersion of the fish in a solution containing the vaccine, which is taken up through the skin, gills, or gut (Bøgwald, 2019; Kumar et al., 2015). However, immersion vaccines with inactivated cells generally have a low vaccination efficacy (Bøgwald, 2019). In addition, the two different identified biotypes of *Yersinia ruckeri* require different vaccines. Austin and Austin (2016) provide an overview of different vaccination strategies tested since the 1960s.

After the onset of the disease, standard treatment of *Yersinia ruckeri* outbreaks is performed using antibiotics. While still effective, concerns have been raised about the development of antibiotic resistances, especially since only few antibiotics are commonly used. *In vitro* experiments showed a certain readiness of *Yersinia ruckeri* to develop a resistance against some of these, namely oxolinic acid, oxytetracycline and potentiated sulphonamide. Alternative treatments include the oral admission or injection of probiotic bacteria like *Aeromonas sobria, Bacillus licheniformis, Bacillus mojavensis, Bacillus subtilis, Carnobacterium maltaromaticum, Carnobacterium divergens, Entobactere cloacae*, *Lactobacillus lactis* and *Lactobacillus fermentum.* While the *Lactobacillus* species are antagonists of *Yersinia ruckeri* and significantly reduced their adhesion to the fish mucus, most of these probiotics are suspected to obtain their efficacy from their general immuno-stimulating effects (Kumar et al., 2015).

1.5 PHOTO-SENS Project

The PHOTO-SENS project is a multinational innovation project funded under the Horizon 2020 Framework Programme of the European Union (Community Research and Development Information Service (CORDIS), 2020). The project has a duration of three years until December 2023 and receives EU contributions of nearly 3 million euros. The cooperation partners are five companies from the Netherlands, Germany, and Switzerland, namely: Surfix B.V., PHIX B.V., TunaTech GmbH, LRE Medical and the Swiss Center for Electronics and Microtechnology (Surfix BV, 2020). Aim of the project is the development of a photonic biosensing chip and readout device for the detection of salmon pathogen biomarkers (Surfix BV, 2020). With the chip, the fast and

inexpensive detection of DNA of selected disease-causing bacteria, viruses and water molds from water-based aquaculture samples shall be implemented (Surfix BV, 2020).

TunaTech GmbH is responsible for the selection and testing of biomarkers for a number of pathogens, including *Yersinia ruckeri* (Surfix BV, 2020). For this purpose, a short, single-stranded DNA sequence is being designed that is specific to its respective target pathogen. This DNA sequence, called probe, is complementary to the DNA of the pathogen. It is used as surface coating on a biosensor chip, represented by the light blue molecule chains in Figure 2A or the blue, y-shaped molecules in Figure 2B. When the pathogenic DNA (represented by green shapes in Figure 2B) binds to the probe on the chip surface, a short double stranded section is formed. This double stranded section results in a wavelength shift of laser light that is directed at the biosensor (Figure 2B). Thus, a change in the wavelength detected by the biosensor indicates the presence of a specific pathogen. Figure 2A shows designs and the implemented and functional biosensor developed by Surfix B.V.

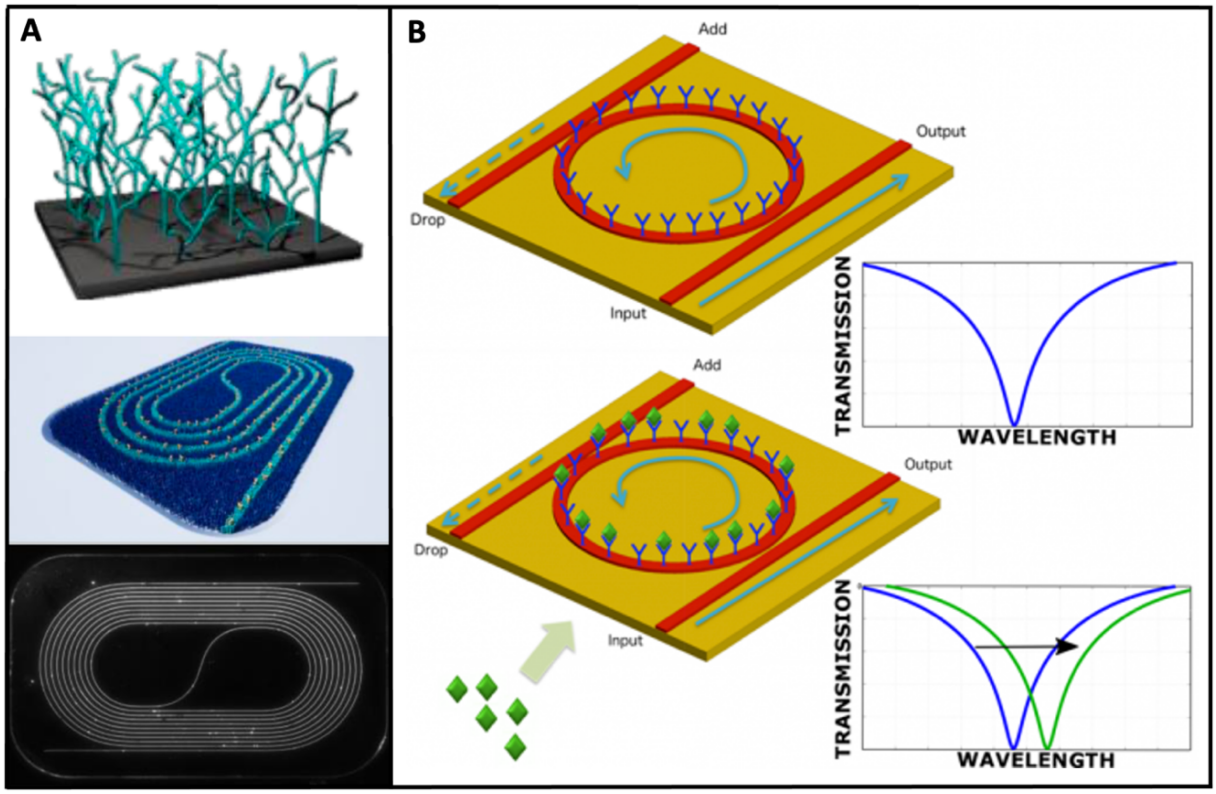

Figure 2: Structure and working mechanism of a photonic biosensor. A) Surface chemistry, prototype design and functional biosensor, adapted from Surfix BV, 2020. **B)** Working mechanism of a photonic biosensor. Binding of target molecules (green) to ligands on chip surface (blue) results in detectable wavelength shift of light. Source: https://photonics.deib.polimi.it/biosensing/.

1.6 PCR and Quantitative Real-time PCR Detection of DNA

Quantitative real-time PCR (qPCR) is a laboratory technique that can be used for the detection and quantification of DNA. Further developed from the basic polymerase chain reaction (PCR), it has become a well-established tool in research and diagnostics (Kralik and Ricchi, 2017). Before advancing to the specifics of qPCR, the underlying mechanisms of basic PCR must be understood. PCR requires a DNA template (Figure 3; green), a short starting sequence that is complementary to the target DNA (primer; Figure 3; red), the enzyme polymerase as a "copy machine" for DNA (not depicted), and the presence of small DNA components called nucleotides (Figure 3; blue). A buffer is used to stabilize reaction conditions (Mülhardt, 2009).

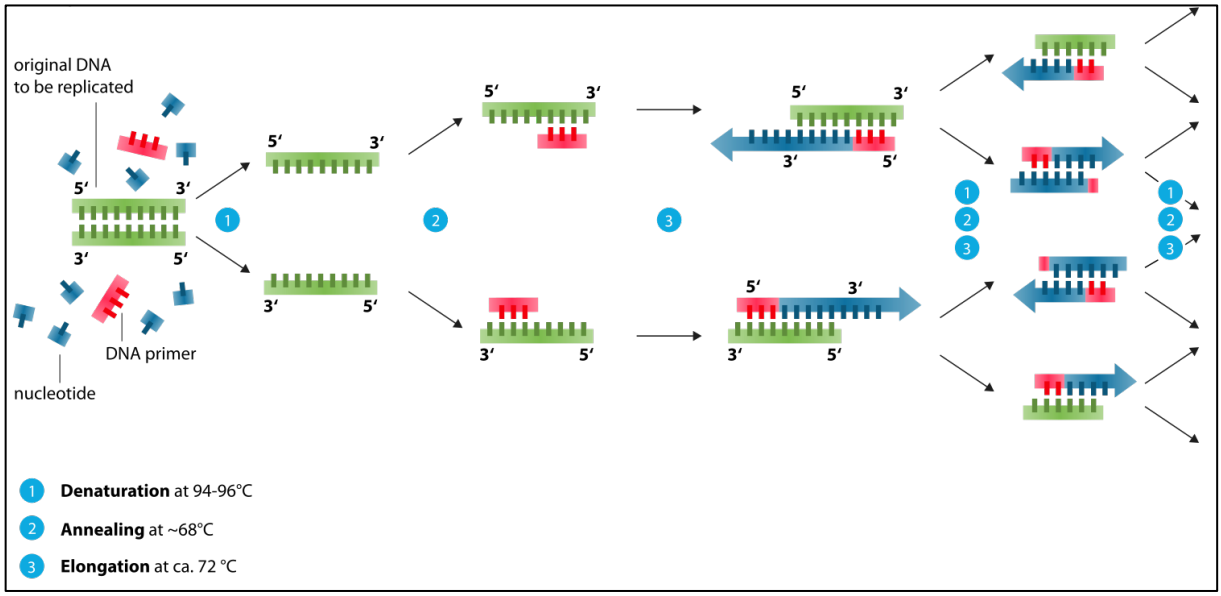

Figure 3: Polymerase chain reaction schematic. 1) Denaturation of DNA double-strand into single-stranded DNA. **2)** Primer annealing at single strands. **3)** Elongation of double-stranded sequence by complementary addition of nucleotides. DNA is amplified through multiple repetitions of these steps,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

In a first step, the DNA is split into single strands by denaturation at high temperatures (Figure 3; 1). When the temperature decreases, primers bind to the single strands in an annealing step (Figure 3; 2). The polymerase can then attach to the short double-stranded section of DNA and elongate it by addition of nucleotides that are complementary to the target DNA (Figure 3; 3) (Mülhardt, 2009). The amount of DNA can be doubled in each PCR cycle, resulting in an exponential increase of DNA. After several cycles, enough DNA is present that can be stained and visualized, for example on an agarose gel. Amplification only occurs when the DNA sample has a sequence that is

complementary to the primer sequence. Thus, with the application of selected primers, an unknown sample can be checked for the presence or absence of specific DNA, for example of a fish pathogen like *Yersinia ruckeri.*

In contrast to basic PCR, in which the visualization of the amplification outcome can only happened after completion of the full PCR program, quantitative real-time PCR allows real-time assessments of the amplification process based on fluorescence measurements. One of several methods is the qPCR with a fluorescently labeled hydrolysis probe, that allows the detection and quantification of specific target DNA. Like the primers, the probe is a short DNA sequence that is selected specifically for a target organism. The probe is linked to at least one fluorescent dye, and a quencher that suppresses its fluorescence, so that there is no signal in the base state of the probe (Figure 4; 1). The probe sequence must be positioned between the sequences of the forward and reverse primer. During the elongation step, the polymerase cleaves the probe from the template DNA strand, separating the fluorophore and the quencher (Figure 4; 3). Without the quencher in proximity, the fluorescence of the fluorophore is no longer inhibited and can be recorded (Mülhardt, 2009). The fluorescence signal increases exponentially with every cycle until it reaches a plateau, resulting in a sigmoidal curve. After several cycles, the signal exceeds a threshold value that is set to blend out background radiation. The specific quantification cycle in which the threshold is exceeded is noted down as Cq value. The more DNA was present in the original sample, the less cycles are needed to reach the necessary fluorescence level and the lower is the respective Cq value. With the help of standard curves in which the Cq values of samples with known concentrations are plotted against the amount of DNA that was originally present in these samples, the starting quantities (SQs) of unknown samples can be calculated (Mülhardt, 2009).

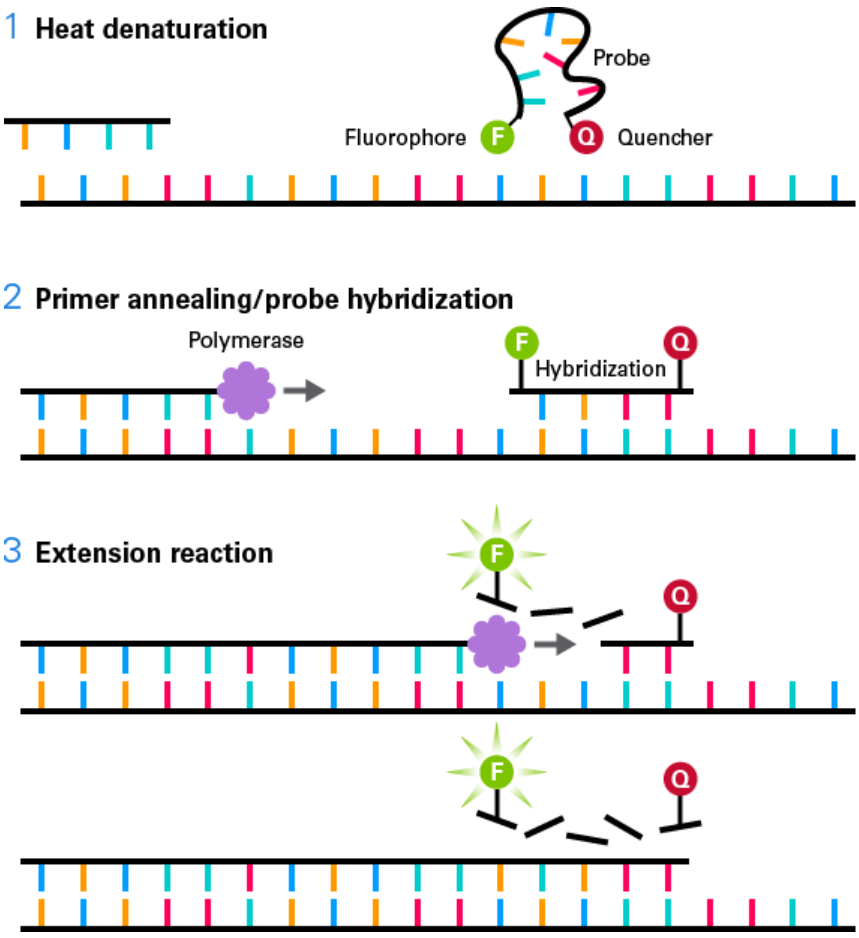

Figure 4: Schematic of qPCR with fluorescently labeled hydrolysis probe. 1) Denaturation of DNA into single strands. **2)** Annealing of primer and probe to DNA single strands. The probe is linked to a fluorescent marker (F) and a quencher (Q) that suppresses the fluorescence. **3)** The polymerase cleaves the probe, separating the fluorophore from the quencher and resulting in a fluorescence signal. Source: https://www.takarabio.com/learning-centers/real-time-pcr/overview/one-step-rt-qpcr-kits.

1.7 Aim of this Work

The main aim of this work is to design and test a probe for the detection of *Yersinia ruckeri*, to be used in the PHOTO-SENS project. The performance of the probe for detection of the bacterium is evaluated in several qPCR experiments. In addition to the probe, a set of primers is required for qPCR, which is designed and tested together with the probe. A standard series shall be created and validated for the quantification of unknown samples. The specificity and sensitivity of the assay shall be determined. Additional experiments include evaluation of the DNA extraction method, the spiking of fish tissues with bacterial culture, and the imitation of experiments for the extraction of environmental DNA (eDNA) from water-based samples.

2. Materials and Methods

2.1 Cultivation Methods

2.1.1 Media Preparation

Cultivation of *Yersinia ruckeri* was performed using the medium DSMZ M535 Trypticase Soy Broth Agar, as recommended in the BacDive Bacterial Diversity Metadatabase by the Leibniz-Institut German Collection of Microorganisms and Cell Cultures GmbH (German abbreviation: DSMZ; (Yersinia ruckeri | BacDiveID:5223.). For cultivation in liquid culture, 15 grams of Trypticase Soy Broth were dissolved in 500 mL of distilled water and autoclaved at 121°C for 20 minutes. For cultivation on agar plates, 7.5 grams of agar were added to the medium composition before autoclaving (Medium 535. Trypticase Soy Broth Agar, 2007). Liquid medium was kept in closed DURAN® glass bottles on the lab bench. Agar medium was poured into Petri dishes while still liquid and left to solidify at room temperature. Plates were then inverted and stored in a sleeve in the refrigerator at 4°C until use.

2.1.2 Bacterial Cultures

For liquid cultures, between 100 and 600 µL of frozen *Yersinia ruckeri* culture (-20°C) were inoculated in around 50 mL of liquid M535. Flasks were kept on a VWR Incubating Orbital Shaker at 28°C and a shaking speed of 80 rpm. For plating of bacteria, aliquots of liquid culture were diluted with M535. Of the desired dilution steps, 50 µL were pipetted onto Agar plates. The liquid was spread evenly with a reusable Drigalski spatula. Plating of bacteria was performed close to a Bunsen burner flame and the Petri dish lids were only partly opened and not entirely removed. Afterwards, the plates were sealed with Parafilm and left to dry for a while before inverting. Inverted plates were placed in the incubator oven at ~27°C and left to grow for at least 24 h. After growth, bacterial colonies were optically enumerated in a colony forming units (CFU) count. Platings were performed in triplicates, resulting in three plates per dilution step.

2.1.3 Preservation of Cultures

Glycerin is a commonly used cryoprotective additive (CPA) for the freezing of microorganisms. While it has been proven to be effective for preservation, toxic effects have been observed on multiple microorganisms (Hubálek, 2003). Thus, the addition of

Glycerin as CPA was only applied to aliquots of bacterial cultures intended for long-term storage and omitted for short-term storage. For short-term preservation, 1 mL aliquots of liquid culture were stored in 1.5 mL Eppendorf tubes at -20°C. For long-term preservation, cryocultures were prepared by mixing 500 µL of bacterial culture with 500 µL of 50% Glycerin, resulting in a 25% Glycerin concentration.

2.1.4 Growth Measurements

Growth measurements were performed on flasks inoculated with 200 µL of four different *Yersinia ruckeri* frozen cultures. After 15 minutes, 4 x 250 µL of each flask were pipetted into a Falcon® Tissue Culture 96 Well Flat Bottom Plate. As blank, four wells were filled with 250 µL of M535 medium. In a Tecan Sunrise absorbance microplate reader, the absorbance of the samples at 600 nm was measured over 24h in 15-minute intervals with shaking immediately before each measurement. Measurements were performed with the XFluor4 Version 4.51 software.

2.2 Primer and Probe Design

For reasons of confidentiality, the gene locus and sequence of the primer sets used in this thesis will not be disclosed. The primer and probe design will instead be demonstrated with a placeholder design. Please note that the primers and probe designed in this following section are not the ones that were tested in the scope of this thesis. All results and pictures included in this work were obtained with a similarly designed primer and probe set with a different gene locus, based on the same theoretical background of species-specific primers and probe for qPCR detection.

2.2.1 Target Gene and Region

The 16S rRNA gene is the most used target gene for identification and phylogenetic studies of bacteria. Its structural role and crucial involvement in protein synthesis make it ubiquitous in microorganisms, highly conserved and slow to evolve. Despite the general stability of the gene caused by the necessity of its functionality, multiple variable and hypervariable regions exist that allow distinction between different bacterial genera, species, and subspecies. While the 16S rRNA gene is on average around 1,500 basepairs (bp) long, microbiological laboratory practice usually targets only the first 500 bp of the gene (Church et al., 2020). For more definitive differentiation, the use of longer gene sequences or several gene regions is suggested by Church et al. (2020) in their review paper on the performance and application of the 16S rRNA gene for

routine identification of bacteria. Thus, the 16S gene sequences used for primer design in this section were all 1,030 bp or longer, with a large majority being between 1,306 and 1,512 bp long.

2.2.2 Reference Strains

A primer and probe set for qPCR and a PCR primer set were developed that should be specific to *Yersinia ruckeri* and should not amplify the DNA of other organisms like other pathogenic fish bacteria. To achieve that, 16S gene sequences of ten different bacterial strains were compared in an alignment. The bacterial strains were chosen from a table containing major bacterial pathogens of economically important fish by Sudheesh et al. (2012), with the addition of *Vagococcus salmoninarum* from Mishra et al. (2018). The following bacteria were considered in this primer design: *Aeromonas salmonicida, Flavobacterium branchiophila, Flavobacterium columnare, Pasteurella skyensis, Piscirickettsia salmonis, Renibacterium salmoninarum, Streptococcus phocae, Vagococcus salmoninarum, Vibrio anguillarum,* and *Yersinia ruckeri* as target organism. An adapted table with an overview of the bacteria, the diseases they cause, and their main host fish can be found in the Annex (Supplementary Table 1). Per selected strain, seven or more 16S gene sequences were downloaded from the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/), with a target length as described above. A total of 114 sequences were evaluated this way. Supplementary Table 2 contains a list of the NCBI accession numbers of the gene sequences that were used in this primer design.

2.2.3 Alignment

Sequences were imported to Unipro UGENE Version 38.1 and aligned in a ClustalW multiple sequence alignment. To avoid a gap-heavy nonsense alignment, the sequences of the different bacterial strains were added and aligned stepwise to a pre-alignment of the *Yersinia ruckeri* sequences. The alignment was further improved by cutting gaps automatically and manually.

2.2.4 Designing the Primers and Probe

From the final alignment, the 142–240 bp region was selected due to its high variability between different strains. The *Yersinia ruckeri* sequence in that region was entered into the IDT PrimerQuest™ Tool (https://eu.idtdna.com/PrimerQuest) to generate two primers and a double-quenched fluorescence probe for qPCR. Search parameters

were slightly adjusted to yield a resulting primer-probe design that was very similar to a manual prototype design. In addition to the primer and probe design for qPCR, a primer for regular PCR was designed spanning the qPCR amplicon region. It was again verified that the designed primers were in a region of high variability. An overview of the primers and probe designed in this section can be found in Table 2. Note that the table does not show the fluorescence dye and double quenchers that are attached to the qPCR probe sequence.

Table 2: Sequences and specifications of two PCR primers and a set of qPCR primers with a hydrolysis probe. Melting temperature (Tm) and Guanine-Cytosine content (GC%) taken from the IDT PrimerQuest tool.

	Sequence (5' → 3'), coding strand	Length	Tm	GC%	Amplicon Length
PCR Primer F	ATG TCT GGG GAT CTG CCT GAT GG	23 bp	68°C	56.5	198 bp
PCR Primer R	TGA GAG GAT GAC CAG CCA CA	20 bp	65°C	55	
qPCR Primer F	GGG ATA ACT ACT GGA AAC GGT A	22 bp	61°C	45.5	89 bp
qPCR Probe	CGC ATA ACC GTC GCA AGA GCA AAG	24 bp	68°C	54	
qPCR Primer R	CTC ACG CCA TCG GAT GAA	18 bp	62°C	55.6	

Figure 5 shows one sequence of each strain used in primer design in the region of the PCR amplicon. *Yersinia ruckeri* was marked as reference sequence. Differences with this sequence are highlighted in color. The depicted alignment is 202 bp long due to a few gaps caused by the alignment. The qPCR probe sequence can be found between base pairs 59–82 in this alignment (black box), a region with high interspecies variation. The pink and blue boxes highlight the PCR and qPCR primers, respectively.

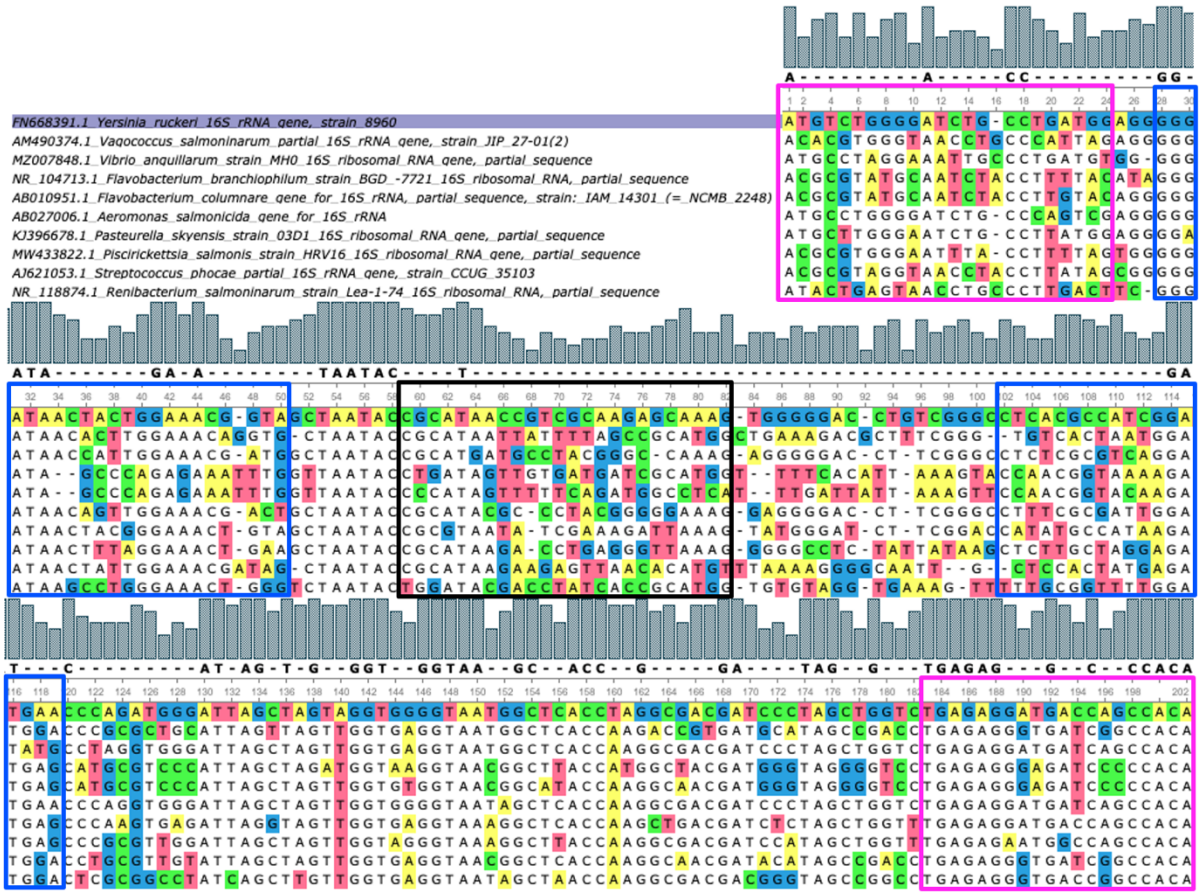

Figure 5: PCR amplicon region of the ten bacterial strains used for primer design. One exemplary sequence is shown for each strain that was used in primer design. *Yersinia ruckeri* was marked as reference strain (fully colored). Nucleotides in which other strains differed from the reference sequence are highlighted in color. The primers were designed as follows, in order: PCR forward primer (pink box), qPCR forward primer (blue box), qPCR probe (black box), qPCR reverse primer (blue box), PCR reverse primer (pink box).

2.3 DNA Extraction

Most DNA extractions were performed using a 5% Chelex solution. Chelex protocols make use of high temperatures to denature cells and release their DNA into the solution (Singh et al., 2018). The Chelex resin binds polyvalent metal ions like magnesium 2+, which is a cofactor of DNases, thus preventing DNA degradation during or after boiling (Bio-Rad Laboratories, 2021; Singh et al., 2018). Extractions based on Chelex are simple, fast and effective (Walsh et al., 2013).

Chelex solutions were prepared by dissolving 0.5 g of Chelex 100 Resin in 10 mL of TE Buffer at pH of 8.0 or in water, to yield a 5% Chelex w/v (weight per volume) concentration. DNA extractions were performed with Safe-Lock tubes to minimize the risk of opening of the lid and sample evaporation due to heat and pressure build-up. Liquid

bacterial aliquots were centrifuged at 15,000 rpm for 5 minutes. The supernatant was discarded, and the bacterial pellet was resuspended with 98 µL of Chelex 5% solution. 2 µL of proteinase K (20 mg/mL) were added. Samples were placed in a Thermomixer at 56°C and 1,300 rpm shaking for 30 minutes, interrupted by a short vortexing step at 2,400 rpm after 15 minutes. Temperature was then increased to 99°C for 20 minutes. After heating, the samples were again centrifuged at 15,000 rpm for 5 minutes to precipitate cell debris and Chelex beads. The supernatant, containing the extracted DNA, was transferred to a new tube.

DNA extractions using an extraction kit were performed with the peqGOLD Tissue DNA Mini Kit from PEQLAB, following the manufacturer's instructions.

2.4 PCR

For PCR, a non-species-specific 16S rRNA primer set and the *Yersinia*-specific PCR primer were used. The general 16S rRNA primer was used to amplify all bacterial DNA in the first culture samples. By sequencing of the PCR products, the samples were checked for presence of *Yersinia ruckeri* and possible contaminations. For creation of a standard series as explained later in 2.8.1 Creation of a Standard Dilution Series, PCR was also performed with the *Yersinia ruckeri*-specific PCR primer set, which was designed to span the region of the qPCR amplicon. The positioning of this "external" primer set relative to the qPCR primers and probe is visualized in Figure 5, where the PCR primer set is highlighted by a pink box.

A PCR mastermix was prepared with the following composition: 2.5 µL of OneTaq Standard Reaction Buffer B9022S (clear, 5x concentrated; New England Biolabs® GmbH), 0.25 µL of dNTPs (10 mM; Bioline), 0.25 µL forward primer (10 µM), 0.25 µL reverse primer (10 µM), 0.0625 µL OneTaq DNA Polymerase M0480G (New England Biolabs® GmbH) and 8.2 µL of molecular grade water. This mastermix composition was adapted from the manufacturer's recommendations for a 12.5 µL reaction (PCR Protocol for OneTaq® DNA Polymerase (M0480)). Amounts are given for one sample and were multiplied by the number of samples given into the PCR, with 10% added extra to account for pipetting error.

For each sample, 11.5 µL of the mastermix were pipetted into 8-tube PCR strips. 1 µL of the respective PCR template was added. PCR was performed in a SensoQuest Labcycler Gradient (Thermoblock 96 wells). The PCR program was adapted from a 16S rRNA program by Klindworth et al. (2012). The program was prolonged to 30 cycles and an annealing temperature of 55°C was chosen. The program included the following steps: initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 40 seconds, primer annealing at 55°C for 2 minutes and extension at 72°C for 1 minute; final extension step at 72°C for 7 minutes. The PCR cycler then kept the samples at 8°C until they were removed from the machine.

2.5 Gel Electrophoresis

PCR results were visualized via agarose gel electrophoresis. Electrophoresis was performed with 60 mL gels and a 1.5% or 2% agarose concentration. 0.9 g or 1.2 g of agarose were dissolved in 60 mL of TAE Buffer (1x concentrated) and heated in a microwave until the agarose was dissolved and the solution was clear. After 4.5 µL of Roti Gel Stain were added, the solution was cast onto the gel sledge and left to cool for 30 minutes. Upon placing the gel into the gel chamber and removing the combs, the gel was loaded with 2.5 µL of 100 bp DNA ladder and 5 µL PCR product, mixed with 1 µL Loading Dye (6x concentrated) when a clear buffer was used for PCR. The gel was run at 100 V for 30–35 minutes. The gel was then placed on a UV table to observe and document results.

2.6 Sequencing

To ensure that the bacterial cultures were indeed *Yersinia ruckeri*, and to check for contamination with other bacteria, extracted DNA of the first batch of culture was sequenced. The DNA extracts were first used in a PCR with the non-species-specific 16S rRNA primer set as well as the outer primer set specific to *Yersinia ruckeri* to amplify DNA. 1 µL of the PCR product was transferred to a tube containing 6.5 µL molecular grade water and 2.5 µL of the forward or reverse primer used for the PCR of the specific sample. These tubes were then sent to Macrogen Europe (https://dna.macrogen-europe.com/eng/) for sequencing. Upon receiving the sequencing results, the cleanliness of the run was checked by looking for overlaps in the sequencing chromatogram and the sequences were entered into the NCBI BLASTN tool for identification of the organism (Madden, 2002).

2.7 qPCR

For qPCR, a mix containing the *Yersinia ruckeri*-specific qPCR primers and hydrolysis probe was used. A mastermix was prepared with 5 µL of Sso Advanced Universal Supermix (Bio-Rad), 1 µL of the mixture containing the qPCR primers and hydrolysis probe, and 3 µL of molecular grade water. Amounts are given for one sample and were multiplied by the number of samples given into the qPCR, with 10% extra to account for pipetting error. According to the manufacturer's specifications, the resuspended primer probe mixture contained 250 nM of probe and 500 nM of primers (1x concentrated).

9 µL of the mastermix were distributed to 0.1 mL 8-tube qPCR strips or a Bio-Rad Hard Shell 96 microplate, depending on the number of samples. 1 µL of the respective qPCR template was added. The qPCR strips were closed with their integrated lids and the microplates were sealed with Microseal® B Adhesive Sealer from Bio-Rad. qPCR was performed with a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System, using a simple two-step program. An initial denaturation step at 95°C for 3 minutes was followed by 40 cycles of DNA denaturation at 95°C for 10 seconds and primer annealing at 60°C for another 10 seconds. Measurements were performed after each cycle using the HEX channel at 554 nm, since the hydrolysis probe was fluorescently labelled with HEX at the 5' end and double-quenched with ZEN™ internally and Iowa Black® FQ at the 3' end. Result documentation and analysis was performed using the CFX Maestro™ Software Version 2.2.

2.8 Quantification

2.8.1 Creation of a Standard Dilution Series

For the creation of a standard dilution series, a PCR of extracted DNA from *Yersinia ruckeri* was performed with the PCR primer set (positioning similar to PCR primers in Figure 5, pink boxes). The PCR products were used for gel electrophoresis on a 1.5% agarose gel. After gel electrophoresis, the DNA bands were cut out from the agarose gel and transferred to 2 mL Safe-Lock tubes. The DNA was then extracted from the gel slices with the QIAEX® II Gel Extraction Kit, following the manufacturer's quick-start protocol. The extraction method makes use of QIAEX II solution, incubated with the sample at 50°C, to solubilize the agarose and bind DNA. After centrifugation, the DNA containing pellet was washed three times with QX1 and PE buffer to remove agarose

traces and other contaminants. DNA was eluted into water twice and the two eluates were combined in one tube. The gel extracted DNA was then measured on a NanoDrop™ One from Thermo Scientific™. Each sample was measured three times. Two extracts were chosen for the creation of a standard series: the extract with the highest DNA concentration and the extract with the highest A260/280 purity ratio. The following formula was applied to calculate the copies/µL in the DNA extracts:

$$\frac{\bar{c}_{NanoDrop}(ng/\mu L) \times 10^{-9} g/ng \times N_A(copies/mol)}{MW(mol/\mu L)} = c_{calculated}\ (copies/\mu L)$$

$\bar{c}_{NanoDrop}$: mean of three DNA concentration measurements performed with NanoDrop™ One

N_A: Avogadro's constant (6.022*10^{23} molecules/mol); 1 molecule ≙ 1 DNA copy

MW: molecular weight of the PCR amplicon

The molecular weight of the PCR amplicon was obtained by entering the sequence of the PCR product into an online calculator for the molecular weight of DNA and choosing dsDNA as output (https://www.bioinformatics.org/sms2/dna_mw.html).

Based on the calculated copies/µL in the measured extracts, dilutions were performed to reach a concentration of 100 million copies/µL. From this starting concentration, a dilution series was prepared in steps of 10, down to 1 copy/µL. Additional dilutions at 50 copies/µL and 25 copies/µL were prepared later. These samples with known concentrations were then used as standard series for quantification of unknown samples.

2.8.2 Creation of a Standard Dilution Series from gBlocks Gene Fragment

To check the accuracy of the self-made standard dilution series, a second standard dilution series was created from a gBlocks Gene Fragment that was ordered from IDT. gBlocks Gene Fragments are high-fidelity double-stranded DNA fragments that undergo a post-synthesis quality control including size verification by capillary electrophoresis and sequence identification by mass spectronomy (Integrated DNA Technologies Inc.). The gBlocks fragments were designed to span the qPCR amplicon region, framed on both sides by ~60 protective bases, with a total length of 227 bp. The freeze-dried gBlocks Gene Fragments were resuspended following the

manufacturer's instructions to yield a concentration of 10 ng/µL. Calculations to obtain the copies/µL in the resuspended solution were performed based on concentration information given on the manufacturer's specification sheets. The calculation was as follows:

$$\frac{10\ ng/\mu L\ \times\ 7.14\ fmol/ng\ \times\ 6.022 \times 10^{23}\ copies/mol}{10^{15}\ fmol/mol} = 4.2997 \times 10^{10}\ copies/\mu L$$

To reach a starting dilution of 100 million copies/µL, 1 µL of the gBlocks solution were added to a tube with 429 µL of molecular grade water. From this tube, a standard dilution series was performed in steps of 10 down to 10 copies/µL, with some additional intermediate steps in the lower concentration range for 50 copies/µL, 25 copies/µL and 5 copies/µL.

2.8.3 Creation of a CFU Standard Dilution Series

A third standard dilution series was created based on the CFU counts that were performed with plated bacterial culture. For this standard series, DNA from liquid aliquots corresponding to 10 million colony forming units was extracted. From this starting concentration, a series of dilution steps was performed as described for the other standard series.

2.8.4 Standard Curve for Quantification of DNA

For quantification of bacterial DNA via qPCR, a standard series must be included in the qPCR run next to the samples to be analyzed. By defining the standard series as such in the qPCR software (CFX Maestro™ Software Version 2.2), a standard curve is created automatically, and the starting concentrations of unknown samples are determined based on their position on the standard curve. This process can also be performed manually by plotting the Cq values of the standard against the logarithmic starting quantity. Unknown samples (x) can then be quantified based on their Cq value, using the y-intercept and slope of the standard curve:

$$SQ_x = 10^{\left(\frac{Cq_x - (y-intercept)}{slope}\right)}$$

2.8.5 Checking Efficacy of DNA Extractions and Quantification

Samples to be quantified by the different standard series were obtained from liquid bacterial culture after a CFU count. The aliquots that were used had been frozen at the

same time that the cultures were plated. From the CFU count, an expected value of the number of bacteria in the undiluted culture was calculated. DNA was extracted for different expected starting quantities and added to a qPCR with at least one of the different standard series. The measured SQ, quantified by the respective standard series, was then compared to the expected value, to evaluate the performance of DNA extractions and quantification.

2.9 Filtration Experiments

To imitate the extraction and analysis of environmental DNA (eDNA), a filtration experiment with bacterially spiked water was conducted. 1 L of distilled water was spiked with liquid bacterial culture from 1 billion cells down to 10,000 cells, based on CFU counts. The filtration setup, demonstrated in Figure 6, included a collection vessel (C) for the filtered water, connected to a vacuum pump (A). The opening of the collection vessel was closed with a glass filter cone (E) and sealed by air-tight rubber seals (D). A metal sieve (G) was placed on the filter cone and the filter paper was put on top. A glass top piece (F) was placed on the very top.

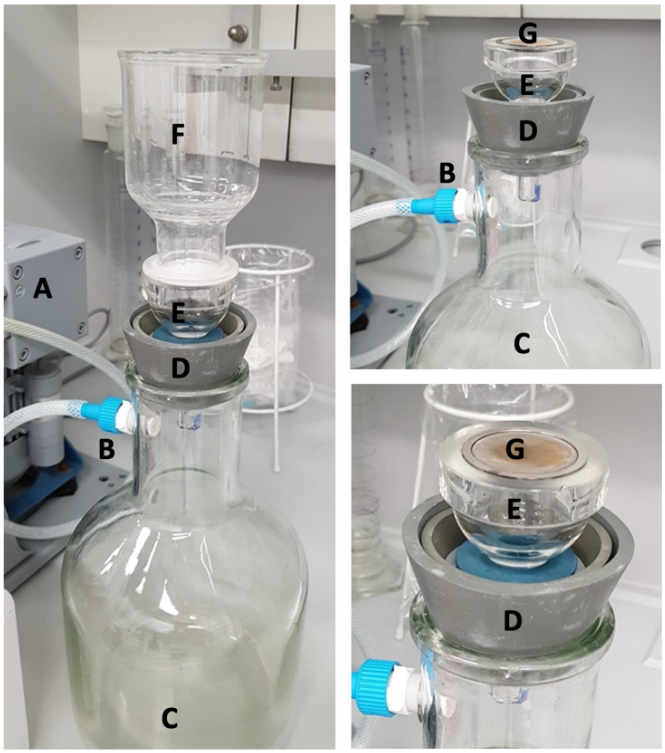

Figure 6: Filtration setup. Left: Full setup, including filter paper. **Right top:** Setup without glass top piece. **Right bottom:** Close-up of rubber seals (D) and metal sieve (G) on filer cone (E).

A: vacuum pump
B: tubing
C: collection vessel
D: rubber seals
E: glass filter cone
F: glass top piece
G: metal filter sieve

Filter paper is placed on metal filter sieve (G). Glass top piece (F) is placed on top. Spiked water is decantered into glass top piece (F). Vacuum pump (A) is turned on. Water is sucked through filter paper on filter sieve (G) into collection vessel (C). Bacterial cells are retained on filter paper.

The glass top piece, metal filter sieve and glass filter cone as well as tweezers that were used for handling of the filter paper were disinfected with 70% ethanol after each use and briefly ignited. The filtration experiment was performed once with cellulose

acetate filters with a pore size of 0.45 µm and once with EO-treated glass fiber filters from Macherey-Nagel with retention capacity of 0.4 µm.

After filtration, filter papers were placed in slightly opened Petri dishes on a heating plate at 36°C for 20 minutes. Filters were then rolled up with two tweezers without touching the filter area that contained the bacterial cells. The filters were cut in small pieces into 2 mL Safe-Lock tubes. DNA extraction was performed with 990 µL Chelex 5% solution and 10 µL proteinase K. The extraction protocol was doubled in time (60 minutes at 56°C and 1,300 rpm shaking; 40 minutes at 99°C and 1,300 rpm shaking) and samples were vortexed every 10 minutes. After centrifugation, the supernatant was transferred to a fresh tube and centrifuged again. After the second centrifugation, 200 µL of supernatant were again transferred to a fresh tube as the final DNA extract. DNA extracts were then included in several qPCR runs for quantification.

2.10 Fish Matrix Experiments

Two experiments were performed to assess the influence of fish tissue on the extraction and detection of bacterial DNA. DNA of bacterial culture was extracted with and without 20 mg of fish tissue. Chelex solution was added to a total volume of 98 µL depending on the volume of bacterial culture used for extraction (range: 81–96 µL). 2 µL of proteinase K were added. The Chelex-extracted measurements were compared with a Wilcoxon sign-rank test to check whether the presence of a fish matrix significantly influenced the extraction and detection of bacterial DNA. The extraction was also performed using the peqGOLD Tissue DNA Mini Kit from PEQLAB for eight samples, to compare the two extraction methods.

3. Results

3.1 Growth of *Yersinia ruckeri*

Yersinia ruckeri was successfully grown under the selected culturing conditions. Plated cultures were uniform in appearance and were thus considered to be clean. PCR products sent for sequencing were identified as *Yersinia ruckeri* using the NCBI BLASTN tool. To assess growth times, the optical density (OD) of four culture samples was measured in quadruplicate, every 15 minutes over a course of 24 hours. Four wells with M535 medium were used as blank. The average OD for the quadruplicate measurements was calculated for each measurement time and the OD of the blank was subtracted. Figure 7 shows the average optical density for each sample plotted against time. The red line at 15h indicates a time where all four samples were close to their maximum value, after the exponential phase and before the death phase. A graph including the measurements of M535, before blanking the sample measurements, can be found in the Annex (Supplementary Figure 1).

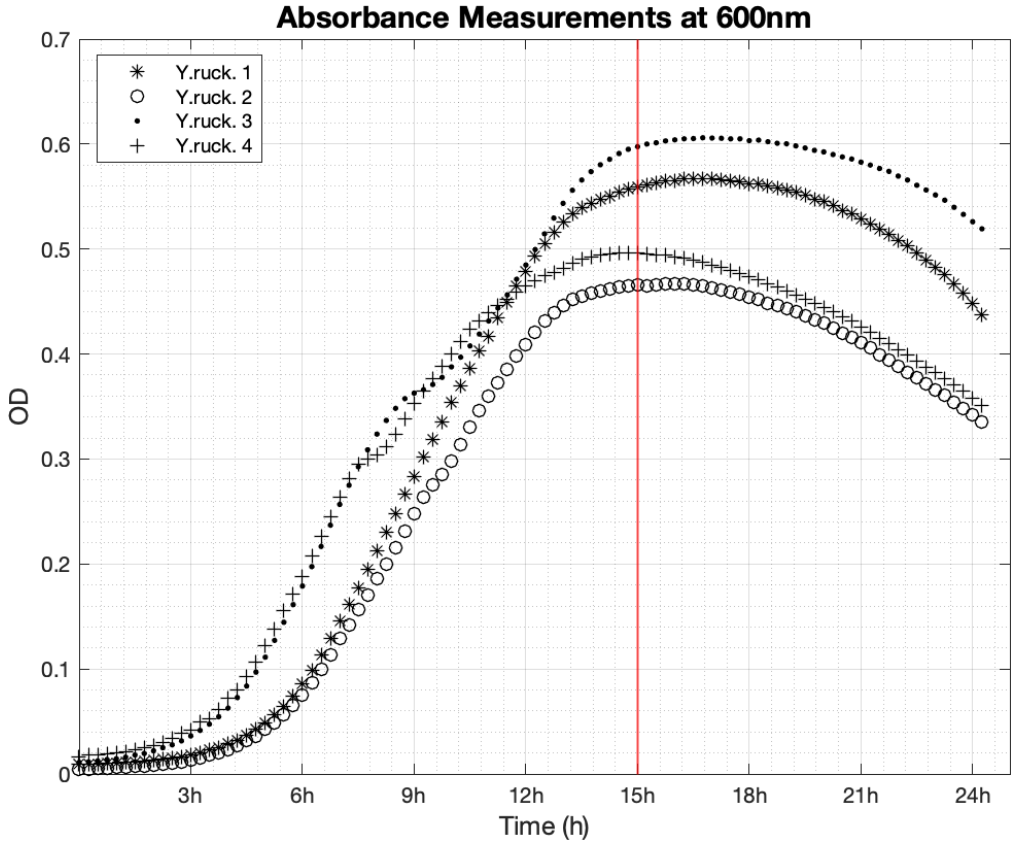

Figure 7: Average optical density (OD) measurements of four *Yersinia ruckeri* samples. OD of blank (M535) was subtracted. After 15h (red line), all samples were close to their maximum value.

The effect of growth time could also be observed through plating experiments that were performed to obtain a quantification estimate for aliquots of bacterial cultures later used in qPCR. Platings were performed in triplicates for three dilution steps and the colony forming units (CFU) were counted. The average cell number per µL of original culture was calculated for each plate and on average. An overview can be seen in Table 3. One culture (Y2P) was left to grow for 18 hours before plating to potentially represent the stationary phase of growth, with regards to the finds from the OD measurements visualized in Figure 7. Another one (Y3P2) was plated after almost 9 hours, supposedly in the exponential growth phase. Inoculation of the flasks had been performed with 100–600 µL of bacterial culture, adapted to ensure sufficient growth after a longer storage time at -20°C or despite shorter growth times.

Table 3: CFU counts of plating experiments, including calculated average cell number/µL and standard deviation.

Sample	Growth	Dilution	Plate 1	Plate 2	Plate 3	Average cells/µL	Average cells/µL	Std dev. all plates
Y1P	43.5 h	1:100,000	131	87	286	336,000.00	425,333.33	133,303.33
		1:500,000	50	40	62	506,666.67		
		1:1,000,000	19	23	23	433,333.33		
Y2P	18 h	1:100,000	465	690	434	1,059,333.33	1,208,666.67	378,189.84
		1:500,000	86	170	142	1,326,666.67		
		1:1,000,000	45	95	46	1,240,000.00		
Y3P	24 h	1:100,000	360	340	453	768,666.67	736,222.22	137,286.22
		1:500,000	87	67	90	813,333.33		
		1:1,000,000	40	30	24	626,666.67		
Y3P2	8.83 h	1:100	435	439	479	902.00	3,063.33	831.42
		1:1,000	128	174	87	2,593.33		
		1:10,000	22	14	17	3,533.33		
Y3P3	41 h	1:100,000	71	116	111	198,666.67	269,555.56	99,571.43
		1:500,000	21	37	15	243,333.33		
		1:1,000,000	19	14	22	366,666.67		

The 1:100 dilution of Y3P2 was identified as an outlier and excluded from calculations for the average cell number and standard deviation. The corresponding values are thus displayed in gray. The data from Table 3 is visualized in Figure 8. The separate CFU experiments are listed on the x-axis in order of increasing growth time. However, it should be noted that the x-axis is not scaled to growth time. The calculated average for each experiment is plotted as black asterisk, the median is marked by a red line. The whiskers span to the highest and lowest CFU count. They should not be confused with error bars, as they do not represent the standard deviation.

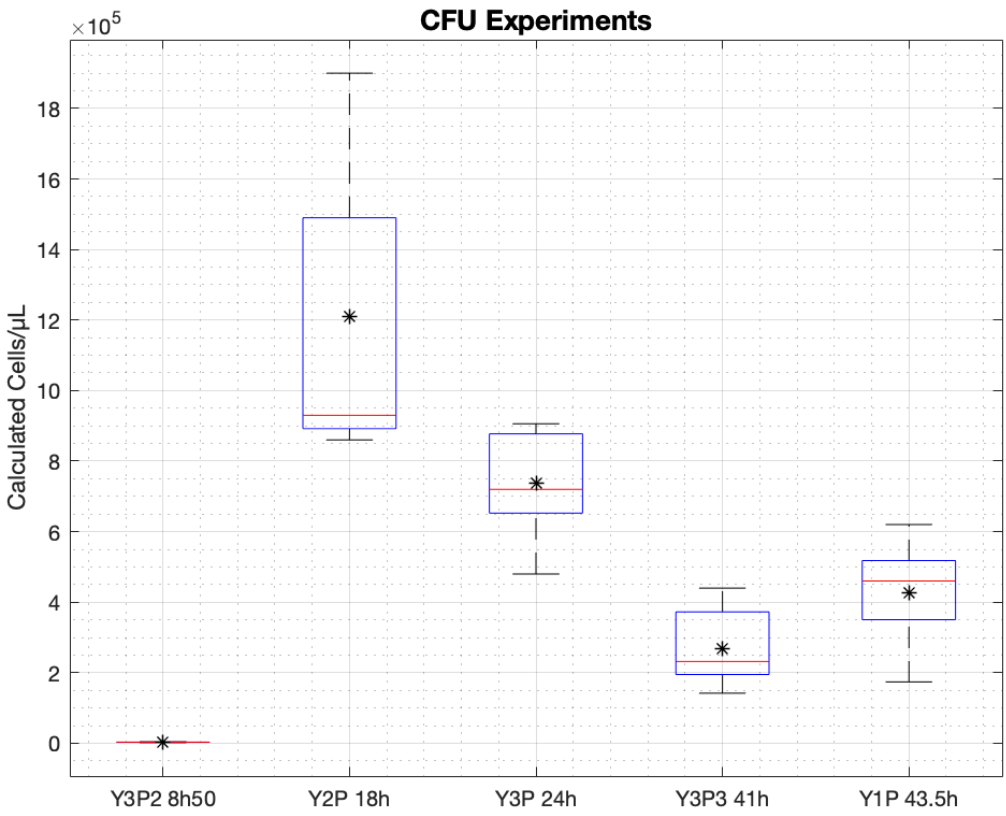

Figure 8: Boxplots of CFU experiments, listed in order of growth time. Whiskers extend to maximum and minimum values. Mean (asterisk) and median (red line) included.

3.2 Creation of a Quantification Standard

The first standard series for qPCR quantification was created using a PCR product spanning the qPCR primer region. Figure 9 shows the agarose gel that was used to check for successful PCR amplification.

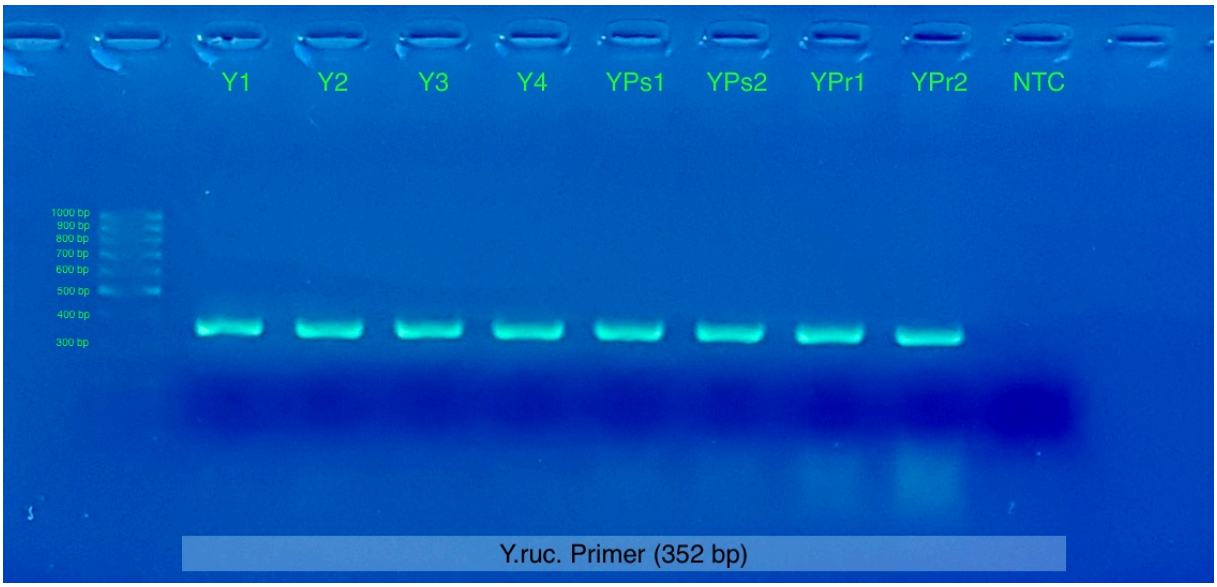

Figure 9: Agarose gel of PCR products with *Yersinia ruckeri*-specific PCR primers. Second well: 100 bp DNA ladder, labelled from 1000 bp to 300 bp.

The PCR products were extracted from an agarose gel using the QIAEX® II Gel Extraction Kit. Five PCR products were extracted that way. The resulting DNA extracts were measured thrice on a NanoDrop™ One to assess DNA concentration and purity, the resulting average values and standard deviations are displayed in Table 4. The copies/µL of DNA extract were calculated using the measured concentration and the molecular weight of the target gene.

Table 4: Concentration and purity measurements of PCR products extracted from an agarose gel. Y1P and Y4P (bold) were used to test their performance as standard series. Y1P (highlighted) was chosen to be used as standard.

	Nucleic Acid (ng/uL)		A260/A280 purity		A260/A230 purity		copies/µL
	Mean	Std	Mean	Std	Mean	Std	
Y1P	**18.034**	**1.655**	**1.739**	**0.024**	**0.712**	**0.020**	**4.81E+10**
Y3P	10.042	0.389	1.831	0.103	0.640	0.025	2.68E+10
Y4P	**11.867**	**0.318**	**1.835**	**0.038**	**0.815**	**0.010**	**3.17E+10**
Ps1	12.663	1.977	1.753	0.061	0.715	0.002	3.38E+10
Pr1	16.134	1.322	1.762	0.051	0.898	0.054	4.31E+10

The bold marked extracts Y1P and Y4P were chosen for the creation of a standard dilution series. Y1P was selected due to its concentration being the highest. Y4P was chosen for highest A260/280 ratio (1.835). The A260/280 ratio is used to assess protein impurity in DNA samples. A value of 1.8 is the reference value for pure DNA (Matlock, 2015). The two standard dilution series were used for a qPCR and the Y1P series was chosen due to better performance (Y1P – E: 92.0%, R^2: 0.999; Y4P – E: 80.1%, R^2: 0.997). The performance of the Y1P standard over several qPCRs can be seen in Table 5 and Figure 10. The wrong molecular weight of the target gene was used initially, so the separate dilution steps contain only 96.67% of the target starting quantity. The lowest starting quantity that was reliably detected were 100 copies. The NTC was positive twice, which is further analyzed in 3.10 Specificity Testing.

Table 5: Overview of Y1P standard series performance. Samples that could not be detected and positive NTCs are marked in red. The average Cq values with standard deviation and coefficient of variation (CV%) of all runs were calculated.

Cq values Y1P Std									
Starting Quantity (SQ)	Corrected SQ	21.02.22	08.03.22	14.03.22	18.03.22	Averages	Std dev.	CV% (inter)	Detected
100,000,000.00	96,674,683.29	12.67	13.41	14.03	14.15	13.56	0.68	5.006%	4/4
10,000,000.00	9,667,468.33	16.10	16.85	17.51	17.57	17.01	0.69	4.041%	4/4
1,000,000.00	966,746.83	19.92	20.55	21.64	21.91	21.00	0.93	4.439%	4/4
100,000.00	96,674.68	23.46	24.57	25.75	26.22	25.00	1.24	4.960%	4/4
10,000.00	9,667.47	26.92	28.09	29.04	29.25	28.33	1.06	3.755%	4/4
1,000.00	966.75	30.68	31.99	32.89	32.46	32.00	0.96	2.989%	4/4
100.00	96.67	33.56	36.27	N/A	35.64	35.16	1.42	4.035%	3/3
50.00	48.36	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/1
25.00	24.18	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/1
10.00	9.67	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/2
NTC	NTC	36.02	N/A	37.29	N/A	36.66	0.90	2.450%	2/4
Efficiency		92.00%	83.29%	83.30%	88.64%	88.02%			
R^2		0.999	0.999	0.999	0.996	0.9989			
Threshold		153.18	178.94	281.05	213.24	-			
Slope		-3.529	-3.8002	-3.801	-3.628	-3.6468			

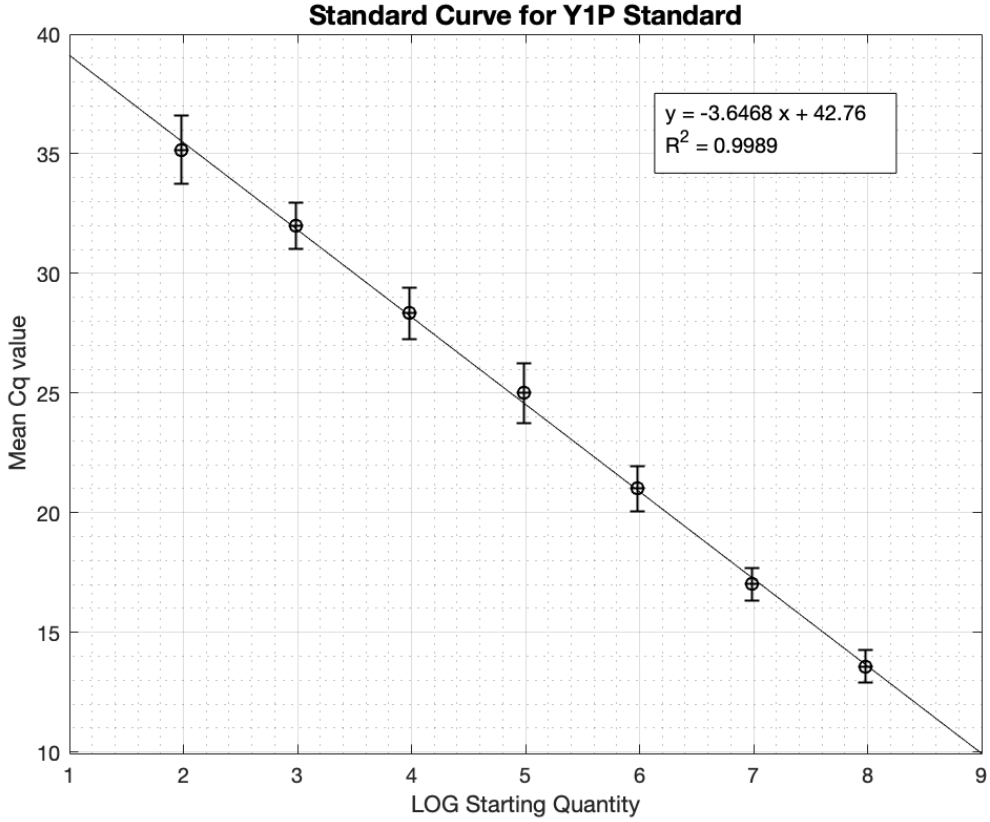

Figure 10: qPCR standard curve for Y1P standard. Plotted from average values of several qPCR runs. Regression line equation used for quantification of samples with unknown starting quantities.

3.3 First Quantification Experiment

In a first quantification experiment, four sets of samples were quantified in a qPCR with the Y1P Standard described above. Two sample sets, Y1P and Y2P, consisted of DNA extracted from bacterial culture. The other two sample sets were obtained through a

filtration experiment in which bacterial culture was diluted in 1 L of distilled water and then filtered through a filtration setup as described in 2.9 Filtration Experiments. The DNA that was retained in the filters was then extracted. The experiment was performed once with cellulose acetate filters (CA) and once with EO-treated glass fiber filters (EO). The number of cells given into qPCR after extraction was expected to span a range from 10 million cells to 10 cells, in decimal steps. However, the quantification output showed results that were higher than the expected starting quantity with factors of detection spanning from 12.15 to 174.61 times higher. The calculated detection factors are displayed in Table 6. The numbers on top stand for the expected starting amount. E.g., the DNA extract from the cellulose acetate filtration with an expected starting quantity of 1 million cells in the qPCR was quantified 23.58 times higher, at 23.58 million cells.

For both filtrations, an NTC was filtered as the last sample that contained no bacterial culture, to check for potential contamination due to insufficient cleaning of the filtration setup between filtration samples. For CA and EO, these NTCs were detected at a Cq of 37.62 and 37.15 respectively, corresponding to a starting quantity of 35.9 and 47.8 cells based on Y1P quantification. The qPCR NTC that can be used to assess pipetting cleanliness or contamination of mastermix components remained empty.

Table 6: Overview of detection factors by which qPCR quantification with the Y1P standard was higher than the expected values. Expected starting quantities were obtained from CFU counts.

	Expected Starting Quantity (Cells)						
	10,000,000	1,000,000	100,000	10,000	1,000	100	10
CA	N/A	23.58	30.87	28.85	56.34	37.89	92.26
EO	N/A	12.15	14.73	19.23	38.21	47.37	38.53
Y1P	N/A	60.84	99.51	159.37	67.47	67.59	174.61
Y2P	18.30	28.09	34.48	13.52	85.23	84.46	N/A

The quantification output summarized in Table 6 raised questions concerning both the accuracy of the expected number of cells added to the qPCR and the accuracy of the created Y1P standard series. For the latter, a new standard was created based on gBlocks Gene Fragments ordered from IDT. As these fragments undergo a thorough quality control, concentration specifications from the manufacturer were expected to be potentially more reliable than the self-made quantification standard based on the NanoDrop™ measurements (Table 4, p.27).

3.4 Creation of a gBlocks Standard Series

An overview of several qPCR runs with the gBlocks standard series can be found in Table 7, which also features the calculated average Cq value for the different starting quantities including their respective standard deviation and coefficient of variation (CV%) between runs. One NTC was positive, which is further analyzed in 3.10 Specificity Testing. The lowest starting quantity that was detected in all runs was 10 cells. 5 cells could be quantified in five out of six runs. The average Cq values were plotted against the LOG starting quantity in an average standard curve (Figure 11), with an efficiency of 91.6% and a R^2 value of 0.9966.

Table 7: Overview of gBlocks standard series performance. Samples that could not be detected and positive NTCs are marked in red. The average Cq values with standard deviation and coefficient of variation (CV%) of all runs were calculated.

Cq values gBlock Std										
Starting Quantity (SQ)	18.03.22	28.03.22	29.03.22	04.04.22	07.04.22	11.04.22	Averages	Std dev.	CV% (inter)	Detected
100,000,000.00	9.56	N/A	N/A	8.29	7.36	6.88	8.02	1.18	14.71%	4/4
10,000,000.00	13.58	N/A	N/A	13.55	12.64	12.48	13.06	0.58	4.47%	4/4
1,000,000.00	17.32	N/A	N/A	17.18	16.31	16.31	16.78	0.55	3.25%	4/4
100,000.00	20.51	20.58	20.24	20.67	19.7	19.65	20.23	0.45	2.22%	6/6
10,000.00	24.09	24.16	23.56	23.8	23.05	23.12	23.63	0.47	2.01%	6/6
1,000.00	27.06	27.52	27.33	27.54	26.9	26.6	27.16	0.37	1.37%	6/6
100.00	30.47	30.24	30.59	30.41	30.06	29.64	30.24	0.35	1.14%	6/6
50.00	31.86	31.82	N/A	31.47	30.35	30.27	31.15	0.79	2.52%	5/5
25.00	32.14	32.15	N/A	32.27	31.65	32.07	32.06	0.24	0.74%	5/5
10.00	33.27	34.27	N/A	33.37	34.1	32.59	33.52	0.68	2.03%	5/5
5.00	37.38	N/A	35.05	36.26	34.03	34.29	35.40	1.40	3.97%	5/6
NTC	N/A	N/A	37.17	N/A	N/A	N/A	N/A	N/A	N/A	1/6
Efficiency	93.40%	100.80%	94.77%	92.00%	91.10%	91.40%	91.60%			
R^2	0.992	0.996	1	0.994	0.995	0.994	0.9966			
Threshold	225.26	197.03	134.99	176.63	127.48	125.06	-			
Slope	-3.49	-3.302	-3.454	-3.53	-3.557	-3.548	-3.5412			

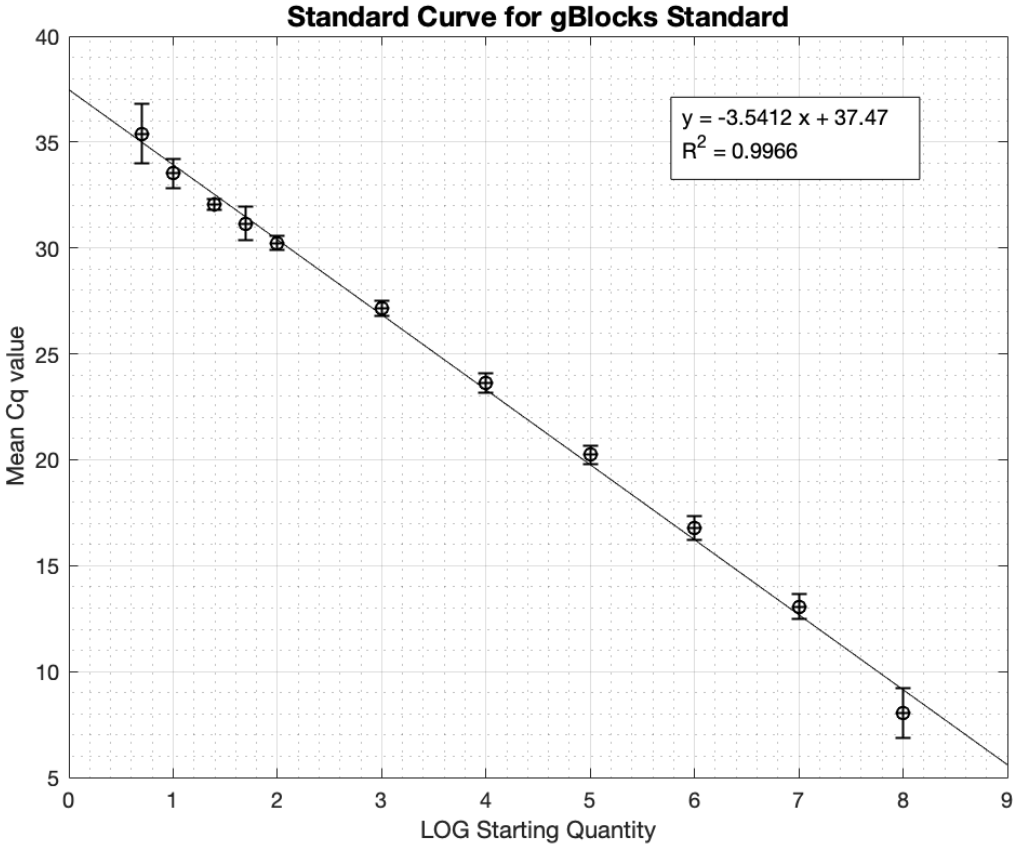

Figure 11: qPCR standard curve for gBlocks standard. Plotted from average values of several qPCR runs. Regression line equation used for quantification of samples with unknown starting quantities.

3.5 Repetition of Quantification Experiment

In a second attempt, the quantification in 3.3 First Quantification Experiment was repeated with both the Y1P standard used before and the newly made gBlocks standard. A fifth set of samples was added, extracted from culture after nearly 9 hours of growth in what was assumed to be the exponential phase. This was done to check whether longer growth negatively influenced the detection factor by which quantification disagreed with an expected value. Results can be found in Table 8. Results are compared between quantification based on gBlocks standard and quantification based on Y1P standard.

Table 8: Overview of detection factors by which quantification varied from expected values in a second quantification attempt. Comparison of quantification by Y1P standard and gBlocks standard. Samples that were not detected are marked in red.

		Expcted Starting Quantity (Cells)						
Sample	SQ based on	10,000,000	1,000,000	100,000	10,000	1,000	100	10
CA Filtrates	gBlocks Std	N/A	2,779,857.19	314,313.56	21,055.70	3,575.15	224.30	35.72
	Factor	N/A	2.78	3.14	2.11	3.58	2.24	3.57
	Y1P Std	N/A	59,251,412.85	7,327,781.90	545,325.33	98,613.46	6,890.78	1,175.15
	Factor	N/A	59.25	73.28	54.53	98.61	68.91	117.51
EO Filtrates	gBlocks Std	N/A	777,289.37	88,428.16	8,041.03	518.21	120.17	13.95
	Factor	N/A	0.78	0.88	0.80	0.52	1.20	1.39
	Y1P Std	N/A	17,769,115.07	2,212,948.25	220,267.47	16,169.11	3,867.81	485.46
	Factor	N/A	17.77	22.13	22.03	16.17	38.68	48.55
Y1P	gBlocks Std	N/A	4,132,814.07	436,950.56	63,076.20	N/A	N/A	N/A
	Factor	N/A	4.13	4.37	6.31	N/A	N/A	N/A
	Y1P Std	N/A	87,540,613.13	10,156,834.09	1,552,609.17	N/A	N/A	N/A
	Factor	N/A	87.54	101.57	155.26	N/A	N/A	N/A
Y2P	gBlocks Std	10,353,705.07	1,666,010.93	199,448.14	N/A	N/A	N/A	N/A
	Factor	1.04	1.67	1.99	N/A	N/A	N/A	N/A
	Y1P Std	211,860,453.11	36,458,563.73	4,691,800.90	N/A	N/A	N/A	N/A
	Factor	21.19	36.46	46.92	N/A	N/A	N/A	N/A
Y3P2	gBlocks Std	N/A	N/A	573,964.91	55,348.33	15,234.39	1,772.32	N/A
	Factor	N/A	N/A	5.74	5.53	15.23	17.72	N/A
	Y1P Std	N/A	N/A	13,082,948.15	1,363,259.41	393,147.24	49,894.98	N/A
	Factor	N/A	N/A	130.83	136.33	393.15	498.95	N/A

Six extracts, three of Y1P and three of Y2P, which had been prepared in one setup, could not be detected. Y1P detection factors spanned a range from 16.17 to 498.95, while gBlocks detection factors spanned a range of 0.52 to 17.72. For each sample that could be detected, the detection factor for gBlocks quantification was lower than for Y1P quantification. Y1P detection factors were bigger than in the previous measurement (Table 6, p.29). Y3P2, which was sampled after the shortest growth time, showed relatively high detection factors, including the highest factor of the experiment. The EO filtrates were the first samples with a detection factor smaller than 1, meaning that less cells were detected than initially added in the experiment. This result was not unexpected, as a loss of cells through filtration and subsequent extraction seemed very likely. Different from the first quantification, the filtration NTCs for which water was filtered without the addition of bacterial culture, were not detected in this qPCR run.

3.5.1 Comparison of Cellulose Acetate and EO-treated Filters

Figure 12 shows more clearly what can already be seen from the data in Table 8. For the same expected starting quantities, more DNA was detected after filtration and extraction from cellulose acetate filters (red curves) than from EO-treated glass fiber filters (blue curves). Figure 12B and C present the separate curves for each filter, with the curves corresponding to, in order: 1,000,000 cells; 100,000 cells; 10,000 cells; 1,000 cells; 100 cells; 10 cells. However, combined in Figure 12A, the curves of the

EO-treated filters are shifted to the right compared to the cellulose acetate filters, indicating a higher Cq value and thus lower starting quantities. RFU on the y-axis stands for relative fluorescence units.

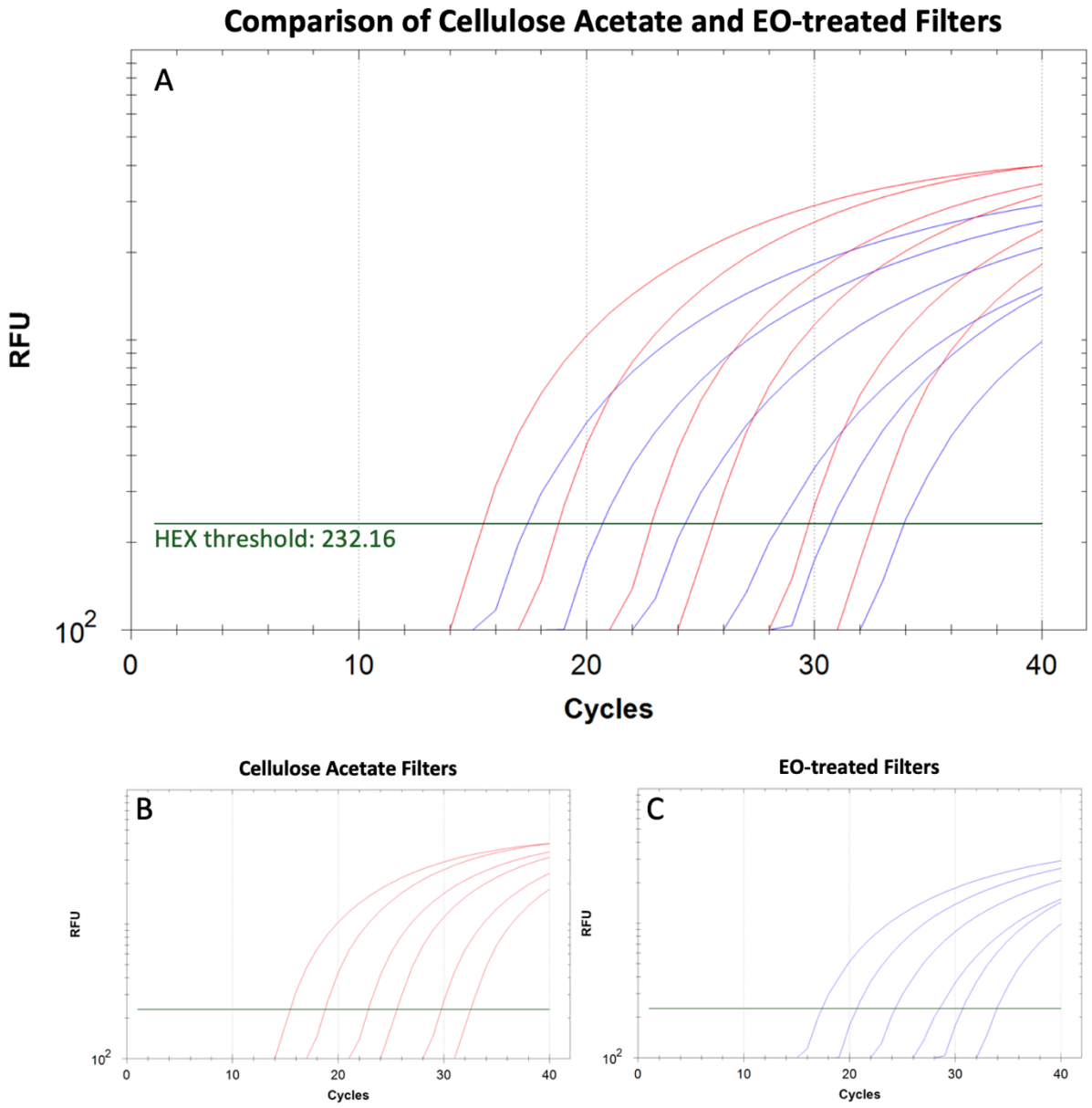

Figure 12: Comparison of qPCR curves of samples filtered with cellulose acetate (CA; red) and EO-treated (EO; blue) filters. Concentration range in decimal steps from 1 million to 10 cells expected SQ. HEX threshold at 232.16 RFU (auto-calculated) for all curves. **A)** Cellulose acetate and EO-treated filter curves. EO-treated curves are shifted to the right compared to CA samples. **B)** Cellulose acetate filter curves. **C)** EO-treated filter curves.

3.6 Creation of a CFU Standard

The gBlocks standard was expected to lead to reliable quantification results due to the manufacturer's specifications and quality control. However, while the factor of disagreement between expected and measured starting quantities was reduced by gBlocks quantification, a detection factor of 0.52 to 17.72 remained (Table 8, p.32). The detection factors were highest for the two sample sets Y1P and Y3P2 which were obtained directly from bacterial culture. Starting quantity estimates had been obtained by plating experiments and counting of colony forming units (CFU counts, Table 3, p.25). Based on the remaining disagreement factor, it appeared that the CFU counts underestimated the amount of DNA in the bacterial culture that could be found through gBlocks quantification. Literature research revealed a paper by Bastardo et al. (2012) on qPCR quantification of *Yersinia ruckeri*. Their approach to quantification of unknown samples was based on a standard curve in which Cq values were plotted against CFU per milliliter. A similar strategy had been applied in this thesis to obtain the starting quantity estimates. However, in this thesis, the expected starting quantities were then measured against a standard for detection of DNA copies, whereas Bastardo et al. measured their other samples against the standard curve created by CFU counts. To test whether this approach would lead to results that agreed with the expected starting quantities, a third standard was created based on CFU counts of the culture Y2P.

The CFU standard was measured in three separate qPCRs and one qPCR in which each sample was measured in triplicate (Triplicate run, 04.04.2022). Results can be seen in Table 9. All samples were detected in all runs, with the lowest starting quantity corresponding to 1 CFU. NTCs remained empty for all measurements. qPCR efficiency varied between 104.9% and 107.1%, the coefficient of determination (R^2) had a range from 0.984 to 0.998. The average Cq values, standard deviations, and coefficients of variation (CV%) were calculated both for the triplicate run (intra-assay evaluation) and for measurements of different qPCR runs (inter-assay evaluation).

Standard deviation was found to be between 0.04 to 0.49 for the triplicate run Cq values, compared to 0.21 to 1.21 between runs. The variation coefficients for the intra-assay evaluation ranged from 0.15% to 1.91% of their respective Cq value. For inter-assay assessment, CV% ranged from 0.66% to 4.27% of their respective Cq value. Overall, efficiency was found to be at 105.5% with a determination coefficient of

0.9969. The standard curve with the average Cq values and standard deviations from all runs can be seen in Figure 13.

Table 9: Overview of CFU standard series performance. The average Cq values with standard deviation and coefficient of variation (CV%) of all runs were calculated. For the triplicate run 04.04.2022, intra-run average Cq values with standard deviation and coefficient of variation (CV%) were calculated.

Cq values CFU Standard										
	31.03.22	Triplicate Run: 04.04.2022			07.04.22	11.04.22	All			
Starting Quantity (SQ)		Average	Std dev.	CV% (intra)			Cq Averages	Std dev.	CV% (inter)	Detected
1,000,000.00	15.85	15.62	0.038	0.243%	15.29	15.75	15.62	0.244	1.562%	6/6
100,000.00	18.12	17.65	0.035	0.199%	17.53	17.95	17.76	0.276	1.555%	6/6
10,000.00	21.33	20.92	0.183	0.877%	20.77	21.11	20.99	0.247	1.175%	6/6
1,000.00	24.82	24.34	0.104	0.429%	24.15	24.55	24.42	0.292	1.194%	6/6
100.00	30.20	27.96	0.494	1.765%	27.72	27.78	28.27	1.206	4.268%	6/6
10.00	30.86	30.83	0.107	0.347%	30.51	31.05	30.82	0.224	0.727%	6/6
5.00	32.12	32.14	0.049	0.154%	31.73	32.18	32.07	0.212	0.662%	6/6
1.00	34.05	33.76	0.644	1.909%	34.27	34.52	34.23	0.333	0.972%	6/6
NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0/4
Efficiency	107.10%	106.94%			104.90%	105.50%	105.54%			
R^2	0.984	0.9966			0.998	0.998	0.9969			
Threshold	113.59	176.63			147.39	163.9	-			
Slope	-3.164	-3.1662			-3.21	-3.196	-3.196			

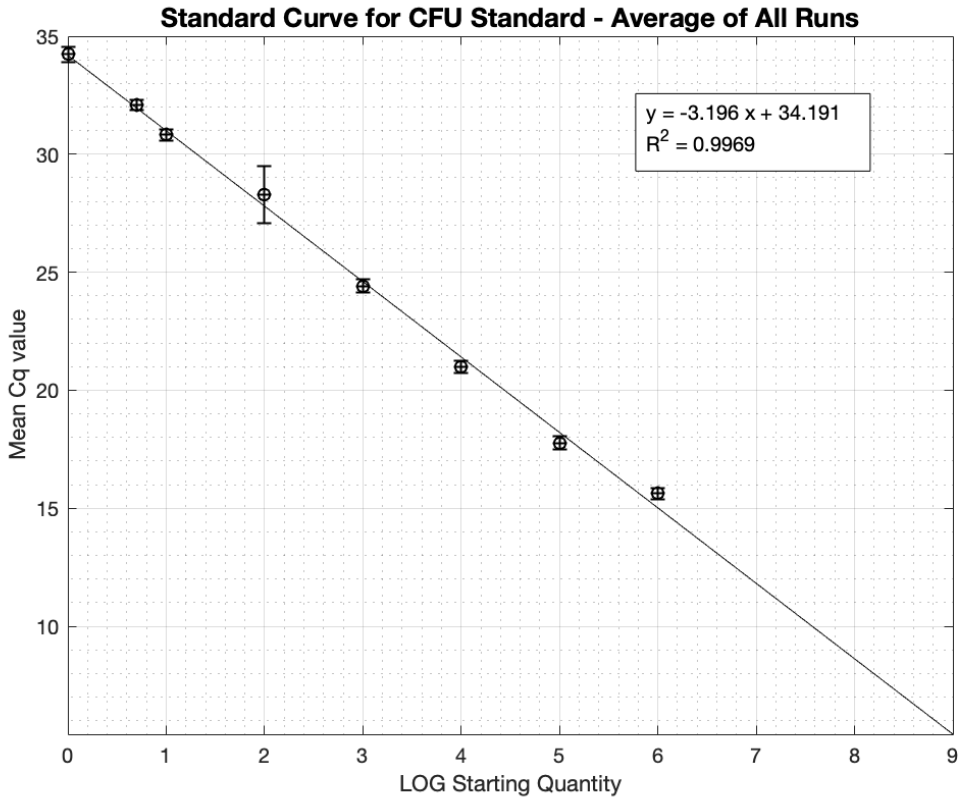

Figure 13: qPCR standard curve for CFU standard. Plotted from average values of several qPCR runs. Regression line equation used for quantification of samples with unknown starting quantities.

3.7 Application of CFU Standard for Quantification Calculations

Table 8 (p.32) showed the quantification results of a second quantification attempt in which a first standard series was compared to the gBlocks standard. The measurements made for the gBlocks standard were now compared to the CFU standard. The Cq values that were recorded in Table 8, quantified by the gBlocks standard, were entered into the regression line equation of the CFU standard series. Same as before, a detection factor was calculated of which starting quantity was calculated compared to an expected value. Table 10 shows both the results that were recorded before in Table 8 and the newly calculated quantification based on the CFU standard equation with their respective detection factors.

Table 10: Overview of factors by which quantification varied from expected values. Comparison between quantification by gBlocks standard (Table 8) and calculated quantification based on CFU standard equation.

Sample		SQ (measured)						
		10,000,000	1,000,000	100,000	10,000	1,000	100	10
CA Filtrates	gBlocks Std	N/A	2,779,857.19	314,313.56	21,055.70	3,575.15	224.30	35.72
	Factor	N/A	2.78	3.14	2.11	3.58	2.24	3.57
	CFU (calc)	N/A	828,062.53	76,332.26	3,969.35	570.81	27.63	3.70
	Factor	N/A	0.83	0.76	0.40	0.57	0.28	0.37
EO Filtrates	gBlocks Std	N/A	777,289.37	88,428.16	8,041.03	518.21	120.17	13.95
	Factor	N/A	0.78	0.88	0.80	0.52	1.20	1.39
	CFU (calc)	N/A	205,480.11	19,069.18	1,385.13	69.04	13.96	1.32
	Factor	N/A	0.21	0.19	0.14	0.07	0.14	0.13
Y1P	gBlocks Std	N/A	4,132,814.07	436,950.56	63,076.20	N/A	N/A	N/A
	Factor	N/A	4.13	4.37	6.31	N/A	N/A	N/A
	CFU (calc)	N/A	1,277,681.75	109,441.48	13,178.31	N/A	N/A	N/A
	Factor	N/A	1.28	1.09	1.32	N/A	N/A	N/A
Y2P	gBlocks Std	10,353,705.07	1,666,010.93	199,448.14	N/A	N/A	N/A	N/A
	Factor	1.04	1.67	1.99	N/A	N/A	N/A	N/A
	CFU (calc)	3,488,527.64	473,027.40	46,415.98	N/A	N/A	N/A	N/A
	Factor	0.35	0.47	0.46	N/A	N/A	N/A	N/A
Y3P2	gBlocks Std	N/A	N/A	573,964.91	55,348.33	15,234.39	1,772.32	N/A
	Factor	N/A	N/A	5.74	5.53	15.23	17.72	N/A
	CFU (calc)	N/A	N/A	147,480.04	11,423.01	2,786.16	264.97	N/A
	Factor	N/A	N/A	1.47	1.14	2.79	2.65	N/A

The CFU detection factors from Table 10 were converted to percentages and documented in Table 11. It is important to know that the samples of the CA filtrates, EO filtrates and Y2P were all taken from the same original culture that was used to create the CFU standard series. To remember the logical workflow: Y2P culture was plated and a CFU count was performed. A CFU standard series was created with a dilution series from DNA corresponding to 10 million CFU. Three sample sets were measured with the CFU standard: DNA extracts directly from the original culture, DNA extracts after filtration of bacterial culture with cellulose acetate filters and DNA extracts after filtration of bacterial culture with EO-treated glass fiber filters. Thus, the percentages

in Table 11 can be seen as a theoretical recovery rate of how many cells could be detected after the different experimental treatments.

Table 11: Potential recovery rate from calculated quantification by CFU standard. Detection factors from Table 8 converted in percentages. Represents percentage of CFU that were detected after extraction.

Sample	Potential Recovery Rate Based on CFU Standard						
	10,000,000	1,000,000	100,000	10,000	1,000	100	10
CA Filtrates	N/A	82.81%	76.33%	39.69%	57.08%	27.63%	37.04%
EO Filtrates	N/A	20.55%	19.07%	13.85%	6.90%	13.96%	13.24%
Y1P	N/A	127.77%	109.44%	131.78%	N/A	N/A	N/A
Y2P	34.89%	47.30%	46.42%	N/A	N/A	N/A	N/A
Y3P2	N/A	N/A	147.48%	114.23%	278.62%	264.97%	N/A

CA filtrate samples were found to have a recovery rate between 27.63% and 82.81%. EO filtrates were lower, at 6.90% to 20.55%. Y2P extracts had higher recovery rates than the EO filtrates, but lower rates than the CA filtrates, at 34.89% to 47.30%. Y1P and Y3P2, which originated from different cultures than the other three sample sets and the CFU standard, were the only samples that had a recovery rate over 100% (corresponding to a detection factor bigger than 1). In fact, all Y1P and Y3P2 samples had a recovery rate over 100%, ranging from 109.44% to 131.78% for Y1P and from 114.23% to 278.62% for Y3P2 which was sampled in its exponential phase.

3.8 Comparison of Extraction with Chelex and with Extraction Kit

In the scope of a spiking experiment which is described further in 3.9 Spiking of Fish Tissue with Bacterial Culture, a comparison was made for DNA extractions using the peqGOLD Tissue DNA Mini Kit from PEQLAB and the Chelex protocol applied to most samples in this thesis. The samples consisted of different starting quantities of bacterial culture, half of which also contained 20 mg of fish tissue. The results are summarized in Table 12. Expected starting quantities are shown only for relative orientation of how much bacterial culture was added.

Table 12: Measured starting quantities for Chelex- and kit-extracted samples with and without addition of fish tissue.

Expected SQ (CFU)	Measured SQ (quantified by gBlocks)					
	without fish			with fish		
	Chelex	Kit	Kit/Chelex	Chelex	Kit	Kit/Chelex
5,000	15,108.39	228.33	1.51%	6,129.76	518.93	8.47%
500	2,200.64	31.55	1.43%	946.86	163.25	17.24%
50	273.75	1.88	0.69%	109.24	15.57	14.25%
25	117.00	3.52	3.01%	63.60	40.98	64.43%

For all samples, regardless of whether they did or did not contain fish tissue, more DNA was detected after Chelex extraction than after extraction with the extraction kit. To compare quantification performance of the two methods, a ratio was calculated for each sample between detection of kit-extracted and Chelex-extracted DNA. As Chelex quantification showed the higher results, this was assumed to be the maximum amount of extractable DNA between the methods, and the ratio determined which percentage of DNA was extracted with aid of the kit. Among the samples not containing fish, qPCR after kit-extraction only detected 0.69% to 3.01% of DNA extracted with the Chelex method. The gap between the extraction methods was closer for the samples containing fish, where it was possible to extract and detect between 8.47% and 64.43% of Chelex-extracted DNA with the extraction kit. The highest percentage (64.43%) was detected for the lowest starting quantity added. While 40.98 cells were detected in the sample with expected SQ of 25, less than half of that amount (15.57 cells) were detected in the sample that was supposed to contain twice as much cells. It is possible that the two samples were switched out by accident during the experiment. If they were switched, percentages of 37.51% (= 40.98/109.24) and 24.48% (= 15.57/63.60) would be reached.

Same as for the samples in 3.7 Application of CFU Standard for Quantification Calculations, the Cq values measured for each sample were used for quantification calculations based on the CFU standard curve equation. Again, the bacteria used in this experiment were from the same aliquots of bacterial culture that had been used to establish the CFU standard (Y2P). A recovery rate was calculated of how much DNA was detected as quantified by the CFU standard, compared with the expected starting quantities that had been based on the same CFU count. Results are summarized in Table 13 and visualized in Figure 14.

Table 13: Calculated recovery rates of Chelex- and kit-extracted samples with and without fish tissue. Based on quantification by CFU standard.

CFU Standard		5,000		500		50		25		Mean Recovery
		SQ (CFU)	Recovery	SQ (CFU)	Recovery	SQ (CFU)	Recovery	SQ (CFU)	Recovery	
Chelex	without fish	3,971.87	79.44%	471.06	94.21%	45.50	91.01%	17.25	68.99%	83.41%
	with fish	1,408.64	28.17%	177.94	35.59%	16.38	32.77%	8.83	35.32%	32.96%
Kit	without fish	36.30	0.73%	3.99	0.80%	0.19	0.38%	0.38	1.50%	0.85%
	with fish	92.94	1.86%	26.27	5.25%	1.88	3.77%	5.65	22.61%	8.37% | 3.63%

A mean recovery rate was calculated for each sample set (Table 13), which was at 83.41% for Chelex extraction without fish tissue, 32.96% for Chelex extraction with fish

tissue and 0.85% for kit extraction without fish tissue. For kit extraction with fish tissue, the average recovery was calculated at 8.37% for all samples and at 3.63% if the sample with 22.61% was treated as an outlier and thus excluded.

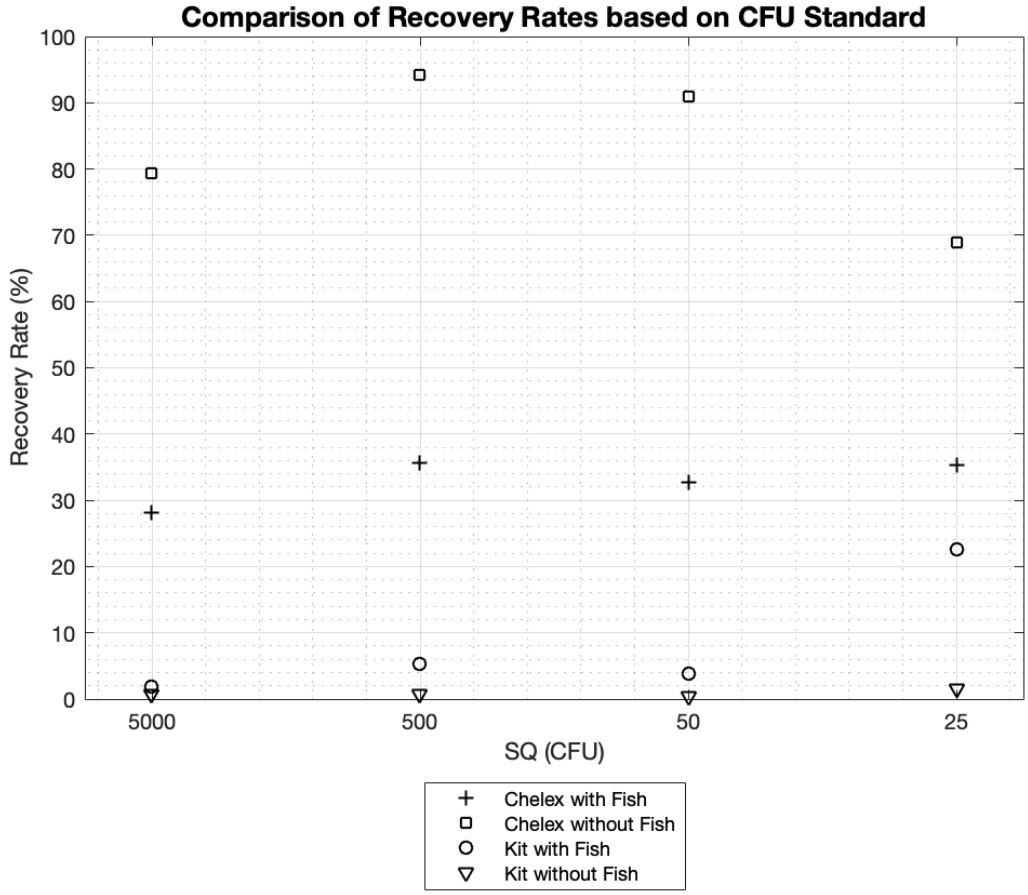

Figure 14: Comparison of CFU-based recovery rates of Chelex- and kit-extracted samples with and without fish tissue as a matrix. The percentages indicate the fraction of DNA that was detected after qPCR with quantification based on the CFU standard, compared to the expected starting quantities based on CFU counts.

3.9 Spiking of Fish Tissue with Bacterial Culture

Initially, spiking of fish tissue with bacterial culture was performed for the 16 samples analyzed in 3.8 Comparison of Extraction with Chelex and with Extraction Kit. From the results displayed in Table 12 (p.37), it was seen that less cells were detected from the Chelex-extracted samples with fish tissue compared to their respective pendants without fish tissue. For the kit extraction, the opposite was true with more cells being detected after extraction with fish tissues compared to without. As the recovery rate for kit extraction was rather low, with average recovery of less than 10% (Table 13), the focus for evaluation of the spiking experiment was set on Chelex extraction, with the

aim to check whether the presence of fish tissue influenced the extraction and detection of bacterial cells. For statistical analysis, a sample size of n=10 was needed, meaning ten samples with fish tissue that were compared to ten samples without fish tissue. Thus, in addition to the four sample sets already displayed in Table 12, another six sets of samples were prepared. Quantification was performed using the gBlocks standard. Table 14 shows the detected cells for all samples as well as their difference, which refers to how many more cells were detected when no fish tissue was present. The difference is visualized in Figure 15, in which a positive difference stands for the extract without fish tissue having a higher detected starting quantity compared to its counterpart with fish tissue, while a negative difference would indicate the opposite. Note that the CFU values on the x-axis are only an orientation for how much bacterial culture was added for each sample, and not an accurate estimate for how many cells were present. For better readability, the bar plots are not spaced according to their respective expected starting quantity given on the x-axis.

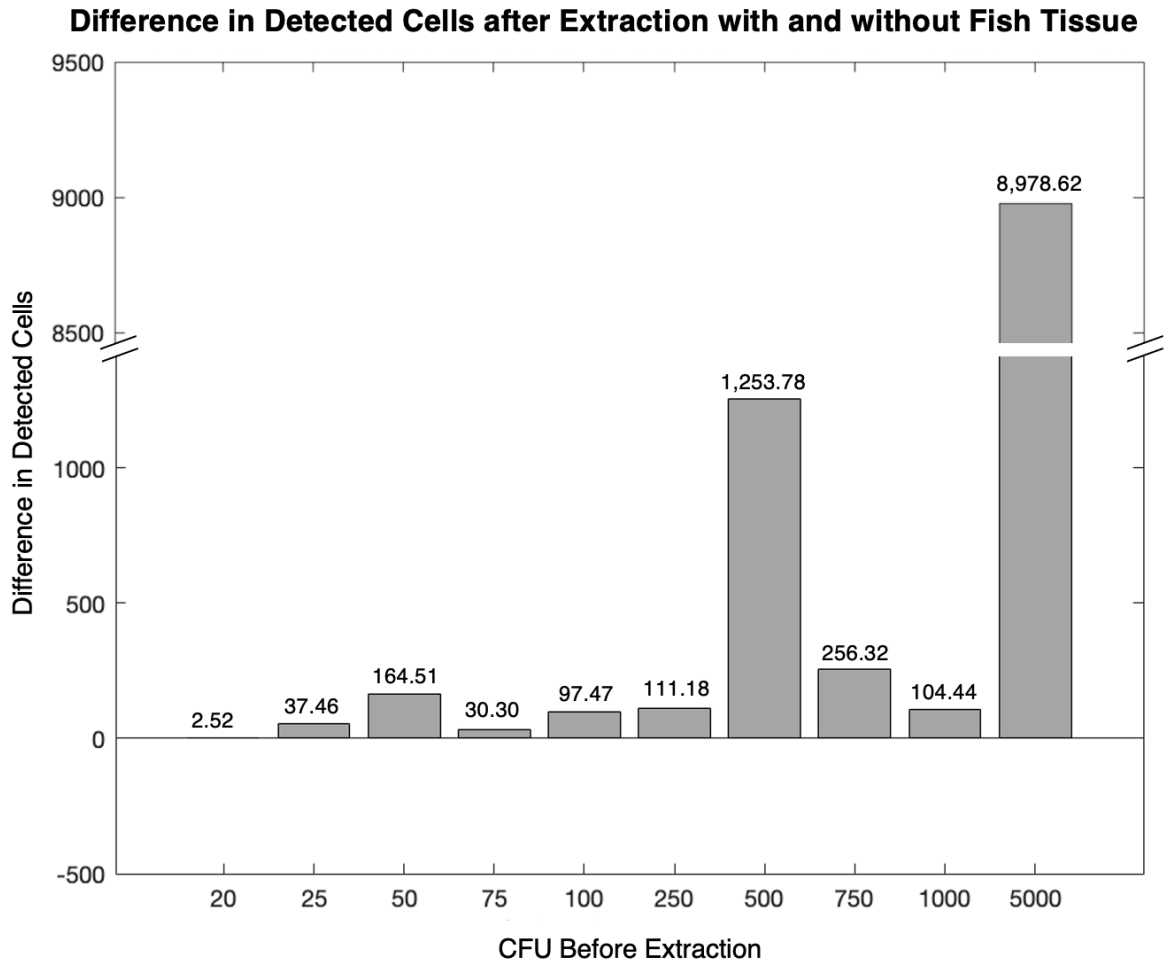

Figure 15: Difference in detected cells after extraction with and without fish tissue. A positive difference refers to more DNA being detected after extraction without fish tissue compared to their counterparts with fish tissue. A negative difference indicates the opposite.

To check for statistical significance, a Wilcoxon signed-rank test was performed. All sample sets were assigned a positive sign, as in each one, a positive difference was detected between samples without fish tissue and samples with fish tissue (e.g., 15,108.39 - 6,129.76 = + 8,978.62). Sample sets were ranked based on the magnitude of their difference (Table 14).

Table 14: Comparison of detected cells from samples with and without fish tissue. Fish recovery (%): percentage of DNA detected in samples containing fish tissue compared to total amount of detected DNA in samples without fish tissue. Positive signs were assigned for samples in which more DNA was detected without fish matrix compared to samples with fish matrix.

Chelex								
CFU	without fish	with fish	difference	fish recovery (%)	sign	rank	positive	negative
5000	15,108.39	6,129.76	8,978.62	40.57%	+	10	10	
500	2,200.64	946.86	1,253.78	43.03%	+	9	9	
50	273.75	109.24	164.51	39.91%	+	6	6	
25	117.00	63.60	53.40	54.36%	+	3	3	
1000	2,287.60	2,183.16	104.44	95.43%	+	7	7	
750	2,148.83	1,892.51	256.32	88.07%	+	8	8	
250	928.19	817.01	111.18	88.02%	+	5	5	
100	428.32	330.85	97.47	77.24%	+	4	4	
75	282.67	252.38	30.30	89.28%	+	2	2	
20	73.79	71.27	2.52	96.59%	+	1	1	
						Total	55	0

The test statistic was W=0, since no sample set had been assigned a negative sign. From a table of critical values, it was determined that the difference between samples with and without fish tissue was statistically significant at a level of $\alpha = 0.0025$, one-sided ($\alpha = 0.0050$ two-sided).

Similar as before for the comparison of kit extracted samples with Chelex extracted samples, a recovery rate was calculated. In this case, it was calculated how many cells were detected after extraction with fish tissue relative to the number of cells that were detected after extraction without fish tissue. The calculated percentages can be found in Table 14 above and are visualized in Figure 16 below. The bar plots are colored according to these percentages, which is explained looking at the following example. For the 5000 CFU samples, ~15,000 cells were detected without fish tissue, so the dark grey bar reaches up to that value on the y-axis. With fish tissue, ~6,000 cells were detected, corresponding to 40.57% of the cells detected without fish tissue. The light grey portion of the bar represents those 40.57% and reaches to ~6,000 on the y-axis.

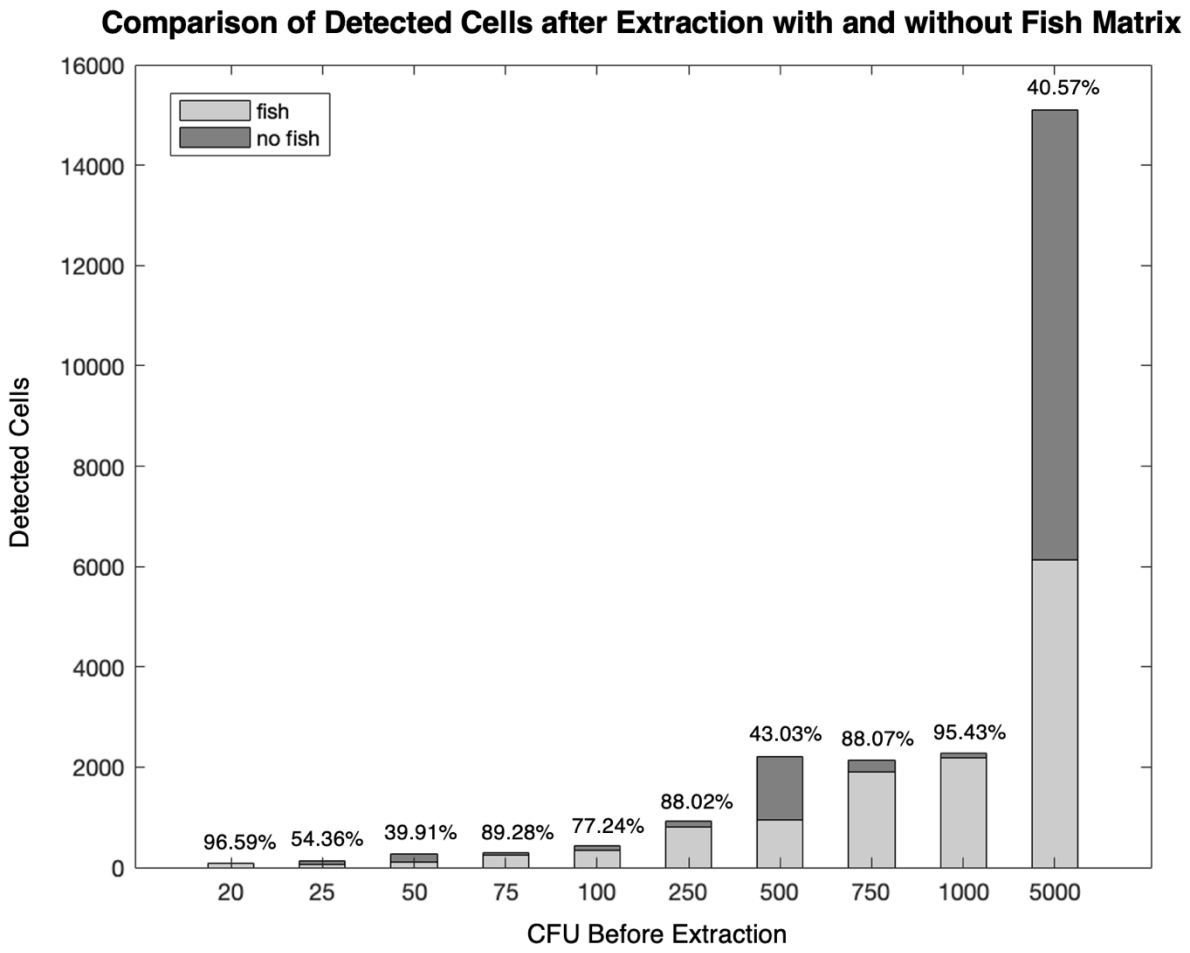

Figure 16: Comparison of detected cells after extraction with and without fish matrix. Light grey parts represent the percentage of cells that were detected after extraction with fish tissue from the total amount of cells that were detectable without fish tissue. The samples with 25, 50, 500 and 5000 CFU before extraction were prepared first. The other samples were prepared on a second day.

Compared to the samples without fish tissue, between 39.91% and 96.56% of cells were also detected after extraction with fish tissue. A difference can be seen between the samples prepared on different days. While for the first four sample sets, relative recovery rates for samples with fish tissue was between 39.91% and 54.36%, the samples that were prepared later had higher relative recovery rates, at 77.24% to 96.59%.

3.10 Specificity Testing

Throughout the different experiments, several negative controls and no template controls were measured via qPCR. A list of the different samples can be found in Table 15, which also indicates whether these samples produced a signal. For samples that were detected, their Cq value is given.

Table 15: Record of detection of negative controls and no template controls (NTCs). Annotations include information on whether the qPCR was performed with qPCR 8-tube strips or 96-well plates.

	Detection										
Sample	21.02.22 strips	24.02.22 strips	08.03.22 plate	14.03.22 strips	18.03.22 plate	28.03.22 plate	29.03.22 strips	31.03.22 strips	04.04.22 plate	07.04.22 plate	11.04.22 plate
NTC (H2O)	36.02	N/A	N/A	37.29	N/A	N/A	37.17	N/A	N/A	N/A	N/A
Aeromonas salmonicida 1:20		36.23	N/A		N/A					N/A	N/A
Aeromonas salmonicida 500			N/A								
Vagococcus salmoninarum 1:20		N/A									
Salmon 1:20		N/A	N/A		N/A					N/A	
Sea Trout 1:20		35.68									
Rainbow Trout 1:20		N/A							N/A		
Brown Trout 1:20		N/A									
Amberjack 1:50					N/A						
Salmon Eggs			N/A								
Salmon Hatchlings			N/A		N/A						
Chelex					N/A						

In all cases where a negative control or NTC was detected, the qPCR had been performed using the 8-well Real-Time lowprofile PCR tubes with attached lids from Brand. Cq values were between 35.68 and 37.29. According to Table 16, in which the theoretical Cq values for starting quantities of one to five cells were calculated, the signal from the controls corresponded to one to three cells.

Table 16: Theoretical Cq values for starting quantities of one to five cells, calculated from gBlocks standard curve equation.

SQ	Theoretical Cq (gBlock)
5	34.99
4	35.34
3	35.78
2	36.40
1	37.47

For all measurements performed in Bio-Rad Hard Shell 96 microplates and sealed with Microseal® B Adhesive Sealer, negative controls and NTCs remained empty, including controls that had been detected before with strips.

4. Discussion

4.1 qPCR Performance

The efficiency value of a qPCR gives an insight on how many times one DNA copy is amplified in a qPCR cycle. An efficiency of 100% indicates that the amount of DNA was exactly doubled in each cycle (Kralik and Ricchi, 2017). However, the apparent efficiency can be influenced by the presence of inhibitors, inaccurate pipetting and DNA template concentration (Johnson et al., 2013). In practice, qPCR efficiencies are likely to be at 90–105% (Johnson et al., 2013).The Bio-Rad qPCR assay guide notes that efficiency should be between 90–110% for assay validation (Bio-Rad Laboratories, 2019). A stable qPCR assay should span at least six logarithmic steps with a linearity of R^2 >0.98 for three technical replicates (Johnson et al., 2013). Furthermore, it is recommended to perform experiments with technical replicates, in which the standard deviations for each set of technical replicates should be <0.2 (Bio-Rad Laboratories, 2019).

All standard series tested in this work fulfill the stability criteria concerning linearity and a range of at least six logarithmic steps. However, the calculated qPCR efficiencies varied for the different standard series. For the first, Y1P standard, efficiencies were between 83% and 92%. The standard curve plotted from the average Cq values resulted in an overall efficiency of 88%, with a linearity coefficient of 0.9989. The low efficiency indicates that the assay conditions were not optimal for this standard. For the gBlocks standard, efficiency was between 91% and 101%, with 91.6% calculated from the averaged standard curve at a linearity coefficient at 0.9966. For the CFU standard, efficiency was between 104% and 108%, with 105.5% calculated from the averaged standard curve at a linearity coefficient at 0.9969. The CFU standard was also tested once with three technical replicates, with intra-run standard deviations being <0.2 for most samples except for the 100 CFU and 1 CFU samples.

4.2 Assay Specificity

As depicted in Table 15 (p.43), the DNA of different fish and bacteria were used as negative controls in different qPCR runs. Also tested were molecular grade water and Chelex solution as NTCs. Looking at the experimental setup, it was noted that positive

NTCs or negative controls only appeared when the 8-well qPCR strips with attached lids were used. For the Bio-Rad Hard Shell 96 microplates that were sealed with Microseal® B Adhesive Sealer, NTCs and negative controls remained empty. A probable source of contamination was found in the opening and closing of the lids on the 8-well strips. As the strips themselves were made from rather flexible material, it was not so easy to tightly close the lids, and even more difficult to re-open them afterwards. In addition, due to delivery shortages, low quality gloves were used in the laboratory that were rather slippery and not very tight around the fingers, which would sometimes lead to gloves getting stuck in a test tube, and generally increase the risk of contact with contaminants. It was assumed that during the struggle of opening and closing of the lids, combined with the sub-optimal gloves, small amounts of DNA came in contact with the gloves and were potentially carried over to the qPCR tubes. As shown in Table 16 (p.43), the measured Cq values for the positive NTCs and negative controls would correspond to 1–3 DNA copies as SQ. According to the MIQE Guidelines for qPCR experiments, the lowest concentration that can be stochastically reliably detected with a 95% confidence interval are three copies (Bustin et al., 2009). Under these circumstances, it can be assumed that the positive negative controls and NTCs in the detected range can be neglected. The positive signals can be interpreted as an admonition for clean handling of samples and/or the use of materials with reduced risk of contamination. This is supported by the evidence that measurements for the controls in question were repeated several times without a signal, with exception of a rainbow trout control that was measured only once. While it is also possible that the rainbow trout was infected with *Yersinia ruckeri* and produced a true positive result, the sample should be measured repeatedly for better reliability.

4.3 Quantification and Factors

While the detection of *Yersinia ruckeri* DNA worked in all but six samples, the quantification presented a challenge. Samples quantified with the first and self-made standard series Y1P were measured to be 12.15 to 174.61 times higher than expected (Table 6, p.29), which raised doubts about the accuracy of both the standard series and the expectation values. When comparing the average Cq values of the Y1P standard series to those of the gBlocks standard, one may notice that the Y1P Cq values are almost one logarithmic step ahead of the gBlocks Cq values (Table 17). For instance, the 100 million expected SQ sample of Y1P has a Cq of 13.56, a value similar

to the gBlocks Cq of 10 million expected SQ (13.06). This suggests that the Y1P standard might have had ~10x less DNA than was assumed. If this was the case, a sample containing 1 million cells (gBlock reference Cq for 1 million copies: ~16.78) would be quantified as 10 million cells by the Y1P standard (Y1P quantification at Cq of ~17.01: 10 million copies). Based on this assumption, the factors of disagreement that were measured in the first quantification experiment could be reduced approximately 10-fold. However, detection factors of 1.2 to 17.5 would remain. These values are in fact very close to the actual detection factors that were measured in the second quantification experiment with the gBlock as standard (gBlock detection factor range: 0.52 to 17.72; Table 8; p.32).

Table 17: Comparison of average Cq values for the different standard series.

	Averge Cq Values		
Expected SQ (CFU)	Y1P Std.	gBlocks Std.	CFU Std.
100,000,000	13.56	8.02	N/A
10,000,000	17.01	13.06	N/A
1,000,000	21.00	16.78	15.62
100,000	25.00	20.23	17.76
10,000	28.33	23.63	20.99
1,000	32.00	27.16	24.42
100	35.16	30.24	28.27
50	N/A	31.15	N/A
25	N/A	32.06	N/A
10	N/A	33.52	30.82
5	N/A	35.40	32.07
1	N/A	N/A	34.23
Efficiency	88.02%	91.60%	105.54%
R^2	0.9989	0.9966	0.9969

As for the explanation of why the standard series might have been 10-fold lower than supposed - despite multiple revisions, no error factor of 10 could be found in the calculations for the creation of the Y1P standard. It was considered whether the Nanodrop™ concentration measurements had been faulty. However, the measured concentrations (Table 4, p.27) were within the detection limits specified by the manufacturer. Furthermore, measurements had been performed in triplicates and the calculated standard deviations were all within the ± 2.0 ng/µL that are given as typical reproducibility variations for concentrations in the measured range (ThermoFisher Scientific Inc, 2016). A study by Masago et al. (2021) did find that DNA concentration measurements with a NanoDrop™ Lite were higher than with a Qubit 4™ fluorometer

for their analyzed samples. However, their research does not specify a certain factor. The 10-fold difference recorded here is more likely explained by improper setup or handling of the Nanodrop™ One, for example by improper cleaning of the measurement pedestal, by hidden calculation errors or by other, unknown reasons.

While the gBlocks standard was assumed to accurately quantify the number of copies present in a sample, a detection factor of up to 17.72 remained (Table 8, p.32). This was attributed to inaccurate expected SQ values, as CFU counts underestimate the amounts of detectable DNA (Sutton, 2011). CFU counts are live cell counts and do not account for the presence of dead cells or free DNA in a sample. While the latter have no colony forming ability, they are detectable by qPCR after DNA extraction. Second, colonies on the agar plates could stem from a cluster of cells (Sutton, 2011). For example, five cells in a cluster would be visible as only one colony, but up to five copies could be detected through qPCR after extraction. *Yersinia ruckeri* has been found to have the ability to form biofilms, which consisted of dispersed and interconnected microcolonies (Kumar et al., 2015; Wrobel et al., 2020). Most detection factors for the gBlocks standard were in the range from 1–7, which seems like a range that could indeed be explained by these microcolonies, dead cells and free DNA.

A CFU standard series was created to increase the compatibility of the measured samples with the standard series. This reduced the detection factors of the samples from the same CFU origin below one. Converted to percentages, the resulting values could now be assumed to represent a recovery rate, providing insight on how much quantifiable DNA might be lost in the extraction process (Table 11, p.37). Comparing the average Cq values of the CFU standard to those of the gBlocks standard, the Cq for a SQ corresponding to 1 CFU was at 34.23 for the CFU standard, which would correspond to 5-10 copies as quantified by the gBlocks standard (Table 17, p.46). The CFU standard Cq for 5 CFU is close to the Cq value that corresponded to 25 copies in the gBlocks standard, supporting the assumptions made above on detectable DNA compared to DNA that was accounted for in CFU counts.

It should be noted that the comparisons between Cq values of different standard series are only approximations. Different standard series have different efficiencies and different threshold values between different runs and cannot be used interchangeably.

For accurate and reliable quantification, a standard series must be included in each qPCR run. All qPCR runs in this work were performed in agreement with the previous statement, a standard was always included. Calculations comparing the different standards were merely performed to better understand the collected data and identify possible error factors and sources.

While the gBlocks standard was assumed to be most reliable for the exact detection of DNA copies, a CFU led to more coherent results in the laboratory environment when comparing qPCR quantification to expected values that were based on CFU counts. A CFU standard should originate from the same aliquots as the samples to be quantified for better comparability. Furthermore, the general detection of *Yersinia ruckeri*, which is the key criterium for the PHOTO-SENS project, was possible for almost all samples except for six samples that were extracted together but could not get detected, suggesting errors in the extraction. Quantification, while not accurate down to exact numbers, could provide information on the order of magnitude of bacterial DNA.

4.4 Comparison to Other Research

The limit of detection (LOD), which is the concentration at which a sample can be detected with 95% probability, was found to be in this study at 10 copies, based on gBlocks quantification, and 1 CFU equivalent. Bastardo et al. (2012) recorded a similar value of 1.7 CFU for their qPCR assay targeting the recA gene of *Yersinia ruckeri*. Other than from purified DNA, they also tested the detection from seeded organs and blood, with detection limits of 3.4 CFU/g and 0.34 CFU/100 µL, respectively. Coefficients of variation for five replicates of purified DNA ranged from 0.42–2.94 CV% within runs, and from 0.88–6.34 CV% between three runs. Unfortunately, it is not specified whether these variations refer to the quantification results or the measured Cq values. In this present thesis, the Cq-related intra-run CV% was at 0.15–1.91% for one triplicate measurement of the CFU standard. Inter-run CV% were found at 0.73–4.27% for four runs of the CFU standard, and at 0.74–14.71% for six runs of the gBlocks standard, which, however, did not always include all samples. The study of Bastardo et al. (2012) deserves highlighting due to the versatility of their study, which included optimizing of the qPCR conditions for best efficiency and linearity of the standard curve and performing experiments challenging rainbow trout fish with two strains of *Yersinia ruckeri*.

Ghosh et al. (2018) performed a qPCR for quantification of *Yersinia ruckeri* from fish feces as non-invasive method and chose the 16S rRNA gene as target. *Yersinia ruckeri* is estimated to contain the 16S rRNA gene as approximately 7 copies/cell. Ghosh et al. (2018) determined the limit of detection for their assay at four copies, corresponding to 0.5 cells. As limit of quantification, they defined the minimum cell concentration of *Yersinia ruckeri* at which the CV% across all replicates was less than 35%. The lowest concentration fulfilling this requirement was 10 copies, which were on average detected as 7.37 copies, equivalent to approximately one cell. The advantage of choosing a multicopy target gene lies in an increased sensitivity, as each cell contains the target gene multiple times. This factor must however be considered when evaluating quantification results. Ghosh et al. (2018) did mention that the qPCR detection includes dead cells, which was assumed to be a major reason that quantification results in this thesis varied from expected values by certain detection factors. They suggest reverse transcriptase qPCR or viable colony counts as supplementary assays for confirmation of viability.

4.5 DNA Extractions

4.5.1 Kit Extraction

While all kit extracted samples could be detected and quantified by qPCR with the gBlocks standard series, recovery rates were low in comparison to Chelex extractions (Table 12, p.37). Only 0.69% to 3.01% of DNA extracted with Chelex was also detected after kit extraction. It has already been found in other studies that DNA extraction methods based on boiling, specifically boiling in a Chelex solution typically yield higher amounts of genomic DNA compared to commercially available extraction kits (Dimitrakopoulou et al., 2020; Simon et al., 2020). Chelex protocols have become a popular choice for fast DNA extractions, particularly when low amounts of DNA are present (Singh et al., 2018). However, major drawbacks in Chelex extraction methods are the presence of impurities after extraction and the stability of the extracted DNA (Simon et al., 2020; Singh et al., 2018). Because of these concerns, this work included the comparison of kit- and Chelex-extracted DNA.

The main reason for the difference in detection levels between extraction methods in this thesis can most likely be found in the nature of the extraction kit. The peqGOLD Tissue DNA Mini Kit is meant for the extraction of up to 30 µg of DNA from eukaryotic

cells or tissues, specifically from human or animal cells (Peqlab, n.d.). As the extraction kit is not intended to be used for bacterial cells, its deficit in this area is not surprising. The different compositions of the cell envelope of *Yersinia ruckeri* compared to human or animal eukaryotic cells may pose a major hindrance in the first step of the extraction, cell lysis. As a Gram-negative bacterium, *Yersinia ruckeri* possesses an outer glycolipid bilayer membrane, a peptidoglycan cell wall, and an inner phospholipid bilayer membrane (Silhavy et al., 2010). In comparison, animal and human cells are only surrounded by a phospholipid bilayer membrane. The more complex and multilayered composition of Gram-negative bacteria might greatly decrease the success of cell lysis and thus result in lower amounts of DNA extracted. The assumption is supported by the fact that the extraction kit is specifically targeted at animal or human eukaryotic cells. Plant cells, while also eukaryotic, are excluded, possibly due to the different composition of their cell walls with the main components being cellulose, hemicelluloses and lignin (Keegstra, 2010).

When fish tissue was added as a matrix for the experiment, the relative recovery rate for the kit extracts was higher than without fish tissue. 8.47% to 64.43% of the number of cells detected after Chelex extractions could also be found after kit extractions. While it can be expected that the kit works better for fish tissues than for bacteria, the fish (salmon) DNA extracted this way should not be detected with the *Yersinia ruckeri*-specific qPCR primer and probe set. It seems that the presence of fish tissue increased the success rate for the extractions of bacterial DNA. It is possible that kit-extracted samples with and without fish were accidently switched out in one step of the experiment. If that were the case, findings would be consistent with the Chelex samples, in which less DNA was detected after extraction with fish tissue than without. However, it seems unlikely that a confusion like that, concerning eight samples in total, would remain unnoticed. In the end, while the reason for higher detection of DNA from kit-extracted samples containing fish compared to kit-extracted samples not containing fish remains unknown, a further investigation of the matter seems unreasonable, as the application of extraction kits for bacterial cell extractions poses a better alternative.

4.5.2 Chelex Extraction

A Chelex DNA extraction protocol was first developed by Walsh et al. in 1991 and has since been adapted and optimized for several uses (Simon et al., 2020; Singh et al., 2018; Walsh et al., 2013). Chelex protocols have certain disadvantages concerning

the purity and stability of extracted DNA. However, these downsides are often outweighed by the simplicity, cost-efficiency, and rapidness of Chelex extraction methods (Singh et al., 2018). Compared to extraction kits, Chelex protocols generally require less steps and less chemicals (Walsh et al., 2013).

In the scope of this thesis, the target DNA was successfully detected through qPCR from almost all Chelex extracted samples. A Chelex NTC remained empty (Table 15, p.43), supporting the assumption that Chelex itself does not interfere with qPCR detection. It was expected that recovery rates of Chelex extracts would be lower than 100%, as it is unlikely that all cells in a sample are successfully lysed, and that no DNA is lost during transfer of supernatant to new tubes. However, in initial quantification experiments, more DNA was detected than was expected to even be present in the samples. These issues were likely related to disagreements between the different standard series that were applied, as was mentioned in 4.3 Quantification and Factors. Consequently, it is difficult to estimate recovery rates for the Chelex extraction method. The best approximation was made in Table 11 (p.37), in which calculated quantification by the CFU standard was compared to expected values. The expected starting quantities for the CA Filtrates, EO Filtrates and Y2P samples were all calculated from the same CFU count that was used to create the CFU standard series. This allows for a somewhat reliable quantification result under the assumption that intra-aliquot variation or inter-aliquot variation between aliquots taken from the same flask at the same time point are rather low. Aliquots were stored at -20°C for two to three weeks and thawed two or three times between the CFU count and extraction, which can potentially cause a certain loss of extractable DNA that is not accounted for in this analysis. Lastly, the quantification in this case was performed by calculation using the CFU standard curve created from average measurements over several runs (Table 9, p.35; Figure 13, p.34).

The recovery rates calculated in Table 11 (p.37) were obtained by dividing the starting quantity calculated from measured Cq values by the expected starting quantity. Recovery rates for the Y2P samples were calculated at 34.89% to 47.30% for expected SQs from 10 million to 100,000. For the EO Filtrates, recovery rates were lower, between 6.90% to 20.55% for a sample range from 1 million to 10 cells that were expected. Recovery rates were relatively stable at 13-14% for the samples of 10,000 cells

to 10 cells, with the lowest recovery rate of 6.90% at 1,000 cells (expected SQ). The highest overall recovery rates were calculated for the CA Filtrates, with the lowest rate being 27.63% at 100 cells expected SQ and the highest rate reaching 82.81% at 1 million cells expected SQ. The measurements at 1 million cells and 100,000 cells expected SQ were with 82.81% and 76.33% much higher than for their equivalent samples extracted directly from Y2P aliquots (47.30% and 46.42% respectively).

4.6 Spiking of Fish Tissues with Bacterial Culture

For the spiking of fish tissues with bacterial culture, a significant difference was found between samples containing fish tissues and samples not containing fish tissues. In all Chelex extracted samples, more DNA was detected in samples without the fish matrix. From Table 14 (p.41), the relative recovery rates of samples containing fish tissue compared to their no-fish equivalents becomes apparent. Within the first four sample sets, which were all prepared on the same day, fish-containing samples had relative recovery rates of ~40-55% compared to their counterparts. For the other six sample sets that were prepared on a different day, relative recovery was at 77-97%. This observation was independent from the starting quantities of *Yersinia ruckeri* cells, as the sample sets of the second experiment were interjected between the initial measurements, and no trends regarding the SQ could be seen. Instead, the noticeable difference suggests that sample handling might have differed between the two days. The lipids and various other components from the fish tissue samples caused a much dirtier extraction and made it difficult to transfer only the middle layer after centrifugation to a new tube supposedly containing the DNA extract, without carrying over parts of the precipitated debris or the upper lipid layer. It seems that on the first try, the focus during supernatant transfer had been set on not transferring impurities, while during the second attempt, more attention was paid to the complete transfer of the DNA extract layer, resulting in the relative recovery rates of up to 97% compared to samples from pure bacterial culture. When comparing detection of *Yersinia ruckeri* from seeded organs and blood, Bastardo et al. (2012) found that the presence of fish tissue did not affect qPCR detection, as the detection limits were equivalent for both sample types. However, the detection limit for both was twice as high as the measured detection limit for samples from pure bacterial culture (3.4 CFU/g and 0.34 CFU/mL compared to 1.7 CFU/mL, respectively). This indicates that a certain decrease in sensitivity compared to pure culture samples may be typical. It should be noted that the organ and blood

samples in the study of Bastardo et al. (2012) stemmed from experimentally infected fish, whereas the samples in this work were only combined with fish tissue, without longer incubation.

4.7 Filtration Experiments

As the PHOTO-SENS biosensing platform is meant to detect the environmental DNA (eDNA) of *Yersinia ruckeri* from water samples, filtration experiments were performed to mimic the detection of pathogenic cells highly diluted by water. Filtration through a membrane is one of the primary methods to collect and concentrate eDNA for extraction (Bessey et al., 2021). In this work, cellulose acetate filters and EO-treated glass fiber filters were compared. More DNA was detected after extraction from CA filters than from EO filters. Pore sizes for both filters were similar, but they differed in rigidity and characteristics of the material. CA filters were more rigid and plastic-like, while EO filters resembled paper. However, as both filter types are designed to be used in pressure filtration, it would be surprising if tears for example had caused a loss of retention in the EO filters. While EO filters were a bit smaller than CA filtrates, both filter types completely covered the filtration sieve, so bypassing of liquid should not have occurred. One possible explanation could be in the method and handling of DNA extractions from the filter paper, for example because the CA filter cuts kept their form while the cut up EO filters slumped together much more, potentially enclosing more of the supernatant after extraction. Other explanations include the possibility that Chelex extractions may work more effectively with the CA filters, or that the DNA might be more easily extractable from the plastic-like surface of the CA filters, whereas the softer and paper-like EO filters may absorb more of the DNA. Note that these explanations are purely hypothetical and were not tested further.

The filtration experiment was first performed with the CA filters and repeated with the EO filters on the next day, which is also a factor of variation. However, if any difference in filtration efficiency had been expected, the author would have assumed to see a “training effect” with increased detection from the second experiment compared to the initial one. For a more reliable and detailed comparison of the two filters and identification of error sources, the filtration experiments would have to be repeated several times in different order and with slight adaptations. For the evaluation in the scope of this work, it shall suffice to say that after extraction from both filters, all samples could

be detected and quantified via qPCR. The lowest SQ as quantified with a gBlocks standard were 13 copies. Higher starting quantities were measured for the CA filters compared to the EO filters.

4.8 Outlook

To further analyze the efficiency of Chelex DNA extractions, known amounts of gBlock standard could be given into the qPCR immediately and after extraction with the Chelex protocol. The comparison could give an insight on how much DNA is lost simply due to handling of the samples during Chelex extraction. It does not, however, provide an insight on the success rate for cell lysis, which is another possible source for DNA loss.

As also noted by other researchers, qPCR detection does not provide information on viability of the detected DNA. The presence of dead cells is directly linked to the presence of living cells and thus not completely irrelevant. However, the contribution of dead cells to quantification might be problematic when assessing treatment measures, potentially resulting in treatments that are disproportionate to the number of living cells. Ghosh et al. (2018) suggest the performance of viable cell counts or reverse transcriptase qPCR to confirm the load of live bacteria. While this may be applicable in the laboratory, it is impractical considering the supposed fast and effortless application of the PHOTO-SENS biosensing platform. It could thus be further investigated whether a distinction between living and dead cells may be relevant for the PHOTO-SENS project, and how such differentiation could be performed, including the potential establishment of orientation values for typical percentages of live and dead cells.

Due to complications with the standard series, it was not possible to conduct experiments testing the simultaneous detection of several pathogens. The main point of interest for the PHOTO-SENS project would be whether the presence of other bacterial DNA influences the detection and quantification of *Yersinia ruckeri*. Similar to the fish tissue experiments, bacterial culture could be used as a matrix. Furthermore, a multiplex qPCR could be tested, utilizing a mix of primers and probes specific to different pathogenic bacteria on a mix of the respective bacterial cultures. Multiplex detection should be possible as long as the probes are labelled with different fluorescent dyes that can be measured simultaneously without wavelength overlaps. One multiplex

qPCR attempt screening for three rainbow trout pathogens (*Flavobacterium psychophrilum, Lactococcus garvieae,* and *Yersinia ruckeri*) has been performed by Chapela et al. (2018) and could successfully detect all three pathogens. However, the study was focused on detection and did not analyze quantification.

5. Summary

Aquaculture is a relevant sector for the satisfaction of increasing demands for fish as a sustainable source of human nutrition as well as for other purposes. The safety and reliability of production, however, is at times threatened by outbreaks of different kinds of fish pathogens. One of such pathogens is *Yersinia ruckeri*, a Gram-negative bacterium that is the causative agent of enteric redmouth (ERM) disease in different fish species. While infections can pass unnoticed due to an asymptomatic carrier state, ongoing and recurrent outbreaks often lead to high cumulative losses. Thus, early recognition methods are needed to prevent and counteract ERM outbreaks.

The PHOTO-SENS project aims at the creation of a photonic biosensing platform for the fast, simple, and affordable detection of multiple fish pathogens. The method is based on short DNA sequences called probes that can complementarily bind the target pathogen DNA, resulting in a detectable signal. In the scope of this thesis, a probe and set of primers were designed to test the detection and quantification of *Yersinia ruckeri.* A valuable tool was qPCR, which allows the quantification of unknown samples by a standard curve.

Three different standard series were prepared following different strategies for accurate quantification. A gBlocks standard was assumed to provide accurate insight on the number of cells present in a sample, while the CFU standard constituted a method to relate detected quantities to expected values as determined by counting of bacterial colonies on agar plates. With a 95% probability, 10 cells or 1 CFU could be reliably detected in several qPCR runs. Difficulties in quantification were attributed to a faulty first standard series as well as a disagreement between the amount of detectable DNA in a sample compared to the number of colonies counted in a CFU count. It was assumed that the CFU counts underestimate the amount of DNA due to the presence of dead cells and potentially the formation of cell clusters.

In addition to samples from bacterial culture, filtrations were performed with two different filters to mimic eDNA detection. More DNA was detected after filtration with cellulose acetate filters than with EO-treated glass fiber filters, with potential recovery rates of 28-83% based on CFU. Furthermore, it was found that the presence of a fish matrix

in samples resulted in losses of detectable DNA, likely attributed to handling of the samples. In a comparison of Chelex DNA extractions with extractions using the peqGOLD Tissue DNA Mini Kit, it was found that Chelex was much more efficient. This was explained by the fact that the extraction kit was meant for the extraction of human or animal eukaryotic cells and not appropriate for application with bacterial cells.

Many of the experiments can still be refined in future works, to optimize qPCR conditions or establish estimates of DNA loss through Chelex extractions. A main challenge could be the differentiation between living and dead cells for detection, as it is much more relevant to quantify the living cells. Furthermore, quantification in multiplex assays has yet to be tested. Despite these potential research topics for a deep-level understanding of the matter, it can be said that the probe tested here was successfully applied for the detection and quantification and can thus be included in the PHOTOSENS project as a biomarker for *Yersinia ruckeri.*

6. References

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7. Statutory Declaration

I hereby declare that I wrote the present dissertation with the topic: “Quantitative real-time PCR Detection of Yersinia ruckeri for Application in 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.

8. Acknowledgments

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9. Annex

9.1 Supplementary Data

Supplementary Table 1: List of fish pathogenic bacteria used for primer design, and their main host fish. Adapted from Sudheesh et al., 2012, Table 2, with the addition of *Vagococcus salmoninarum* from Mishra et al., 2018.

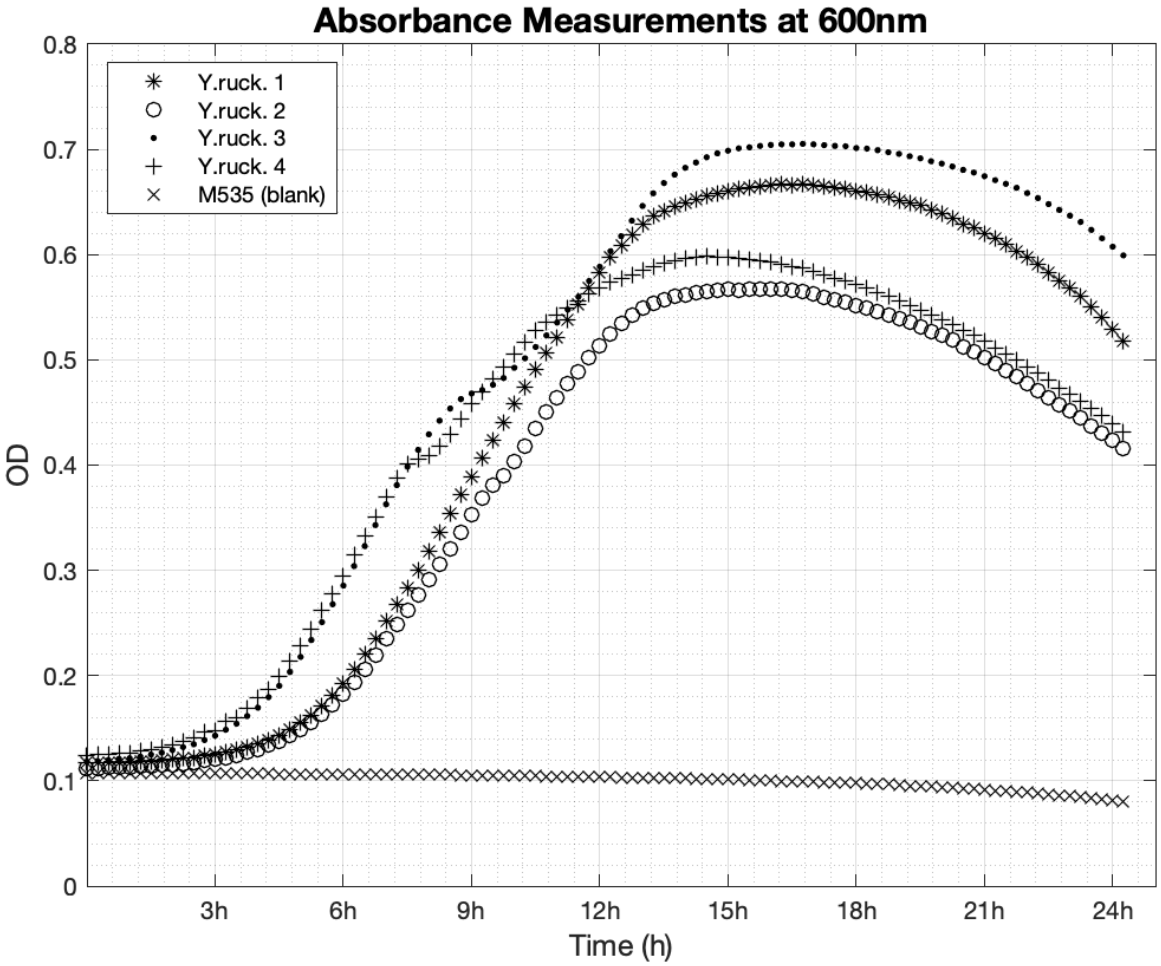
Causative Agent/Species	Disease	Main Host Fish
Aeromonas salmonicida	Furunculosis	Salmon, trout, goldfish, koi
Flavobacterium branchiophila	Bacterial gill disease	Broad range of cultured cold water and warm water salmonid and nonsalmonid fishes
Flavobacterium columnare	Columnaris disease	Cyprinids, salmonids, silurids, eel, sturgeon
Pasteurella skyensis	Pasteurellosis	Salmonids, turbot
Piscirickettsia salmonis	Piscirickettsiosis	Salmonids
Renibacterium salmoninarum	Bacterial kidney disease	Salmonids
Streptococcus phocae	Streptococcosis	Atlantic salmon
Vagococcus salmoninarum	Vagococcosis	Rainbow trout, atlantic salmon, brown trout
Vibrio anguillarum	Vibriosis	Salmonids, turbot, sea bass, striped bass, eel, ayu, cod, red sea bream
Yersinia ruckeri	Enteric redmouth	Salmonids, eel, minnows, sturgeon, crustaceans

Supplementary Table 2: NCBI accession numbers of the gene sequences of ten bacterial reference strains that were used for primer design. Adapted from Sudheesh et al., 2012, Table 2, with the addition of *Vagococcus salmoninarum* from Mishra et al., 2018.

Bacterium	NCBI Accession
Aeromonas salmonicida	MW441232.1 LC499765.1 LC312129.1 LT628040.1 LT628039.1 HG938306.1 AB027546.1 AB027545.1 HG941669.1 HE979858.1 AB027006.1 AB027005.1
Flavobacterium branchiophila	MZ269383.1 MZ269382.1 MZ269381.1 NR_104713.1 AB871648.1 JX657048.1 AB680752.1
Flavobacterium columnare	AB016515.1 AB015481.1 AB010951.1 AB015480.1 AB010952.1 AJ831826.1 AJ831830.1 AJ831829.1 AJ831828.1 AJ831827.1 AJ831825.1 AJ831824.1
Renibacterium salmoninarum	X51601.1 MT023376.1 NR_074198.1 NR_118874.1 NR_041773.1 AY764166.1 AY764165.1 AY764164.1 AY764163.1
Streptococcus phocae	OK033871.1 NR_114556.1 NR_042227.1 FR846243.1 HM032023.1 EF599165.1 AJ621053.1 AF235052.1
Vagococcus salmoninarum	OK376503.1 MW622073.1 MW622072.1 MW622071.1 MW622070.1 MW151830.1 MT998233.1 MK226201.1 NR_044939.1 AM490375.1 AM490374.1 Y18097.1 X54272.1

Pasteurella skyensis	NZ_FOBN01000052.1 NR_025359.1 FOBN01000052.1 KJ396678.1 KJ396677.1 KJ396676.1 KJ396675.1 AJ243202.1
Piscirickettsia salmonis	MW433822.1 MN023100.1 MN023099.1 MN023098.1 MN023097.1 MN023096.1 MN023095.1 MN023094.1 MN023093.1 MN023092.1 MN023091.1 MN023090.1 NR_025980.1 U36941.1

Vibrio anguillarum	MZ007848.1 MZ007847.1 MN900590.1 MN900589.1 LC192806.1 LC365684.1 LC365683.1 LC365681.1 KX238882.1 X16895.1 LN555639.1 KF978124.1 KF150788.1 KF150787.1 KF150786.1
Yersinia ruckeri	MN505173.1 MN505172.1 MN505171.1 LT899970.1 AB681666.1 FN668391.1 FN668390.1 FN668389.1 FN668388.1 FN668387.1 FN668386.1 FN668385.1 FN668384.1 FN668383.1 FN668382.1 FN668381.1



Supplementary Figure 1: Average optical density (OD) measurements of four *Yersinia ruckeri* samples and M535 as a blank. A slight decrease of OD was detected for the medium. However, the difference was rather small and accounted for in the other samples through blanking (subtracting the OD of medium from the measurements of bacterial culture).

9.2 Laboratory Protocols (Short Version)

9.2.1 Chelex Extraction

- 98 µL of Chelex 5% solution (0.5g Chelex 100 Resin in 10 mL H_2O or TE Buffer)
- 2 µL of Proteinase K

1) 56°C and 1,300 rpm shaking for 15 minutes
2) Briefly vortex (e.g., 2,400 rpm for 10 seconds)
3) Repeat Step 1 and 2
4) 99°C and 1,300 rpm shaking for 20 minutes
5) Centrifuge at 15,000 rpm for 5 minutes
6) Transfer supernatant (DNA extract) to new tube

9.2.2 PCR Mastermix Composition

- 2.5 µL OneTaq Standard Reaction Buffer B9022S (clear, 5x concentrated)
- 0.25 µL dNTPs (10 mM)
- 0.25 µL forward primer (10 µM)
- 0.25 µL reverse primer (10 µM)
- 0.0625 µL OneTaq DNA Polymerase M0480G
- 8.2 µL molecular grade water

Add 1 µL of template to 9 µL of mastermix.

9.2.3 PCR Program

1) Initial denaturation: 95°C, 5 minutes
2) Denaturation: 95°C, 40 seconds
3) Annealing: 55°C, 2 minutes
4) Elongation: 72°C, 1 minute
5) Repeat steps 2-4 29x (30 cycles)
6) Final Elongation: 72°C, 7 minutes

9.2.4 qPCR Mastermix Composition

- 5 µL Sso Advanced Universal Supermix
- 1 µL Primer Probe Mix from IDT
- 3 µL molecular grade water

Add 1 µL of template to 9 µL of mastermix.

9.2.5 qPCR Program

1) Initial denaturation: 95°C, 3 minutes
2) Denaturation: 95°C, 10 seconds
3) Annealing: 60°C, 10 seconds
4) Repeat steps 2 and 3 39x (40 cycles)

9.3 Lab Equipment

9.3.1 Devices

- qPCR: Bio-Rad CFX96 Touch™ Real-Time PCR Detection System

- PCR: SensoQuest Labcycler Gradient (Thermoblock 96 wells)
- Centrifuge: Select BioProducts SelectSpin™ 21 Ambient Microcentrifuge
- Photometer: Tecan Sunrise absorbance microplate reader
- Thermomixer: Eppendorf Thermomixer compact
- Incubator: VWR® Incubating Orbital Shaker, Model 3500l
- Nanodrop: Thermo Scientific™ NanoDrop™ One

- Other devices: Vortex, UV table, Incubator Oven

9.3.2 Software

- CFX Maestro™ Software Version 2.2
- Unipro UGENE Version 38.1 (64-bit) – March 2021
- MathWorks MATLAB R2020b Update 5 (64-bit) – Feb 2021
- Microsoft® Excel for Mac Version 16.56 – 2021
- XFluor4 Version 4.51