Norwegian University of Life Sciences

Master's Thesis 2018 60 ECTS

Faculty for Chemistry, Biotechnology and Food Science (KBM)
Knut Rudi

The Host DNA Challenge in the Analysis of Microbiota from Atlantic Salmon (*Salmo salar)*

Daniel Lycke Kristiansen
Microbiology
Faculty for Chemistry, Biotechnology and Food Science (KBM)

Acknowledgements:

This thesis was performed at the Norwegian University of Life Sciences (NMBU), Faculty of Chemistry, Biotechnology and Food Sciences (KBM), under the supervision of Professor Knut Rudi.

First and foremost, I would like to thank my supervisor Knut Rudi for having me on this project. During times of struggle and confusion, he has been there as a guiding hand, answering all my questions and helping solve the challenges which have arisen, all while keeping my spirits up with his cheerful self. For that, I am very grateful.

Secondly, I would like to thank Jane Ludvigsen and Inga Leena Angell for always being open to help, no matter the questions, and for all the help in the laboratory. I would not have made it without you.

Further, a massive thanks to the other Master students, Morten Nilsen, Inger Andrea Goa, Katrine Amlie, Amanda Trueman Morken and Siril Malene Isaksen for being such helpful, amazing people, you have all made working in the lab a blast. Furthermore, the employees at the MiDiv lab also deserve a big thank you, as they have shared their knowledge and experience willingly, and have also largely contributed to the amazing atmosphere at the MiDiv lab.

A thank you to those I've met and become close with during my time at NMBU, you've made these years the best of my life.

Also, a big thank you to my late grandparents for always being there for me throughout my life, I miss you dearly, and I wish I could have shared this moment with you.

Lastly, a special thanks is directed to my friends, girlfriend and family for always supporting me and believing in me. And to my parents, Eva and Jan-Erik, thank you so much for everything. You are all the reason I have come this far.

Ås, 2018

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Daniel Lycke Kristiansen

Abbreviations

DNA – Deoxyribonucleic acid

dsDNA – double-stranded deoxyribonucleic acid

ssDNA – single-stranded deoxyribonucleic acid

PCR – Polymerase Chain Reaction

NGS – Next-generation sequencing

SBS – Sequencing by synthesis

rRNA – Ribosomal ribonucleic acid

CFU – Colony forming units

PMA- Propidium Monoazide

EMA – Ethidium Monoazide

GI – Gastro-intestinal

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Abstract

DNA contamination is a challenge in the modern age of new generation sequencing. Contaminants lead to misassembly, wrongful associations and a decrease in cost efficiency of sequencing. The same is true for host DNA contamination. Outnumbering the total DNA of target microorganisms in samples from blood or faeces, eukaryotic host DNA not only increase the general costs of sequencing many samples but also reduce general sequencing yield and depth. Most protocols and techniques in place today for dealing with contaminations is based on the level of methylation present in humans but lack the broad spectrum required for general use regardless of the target host organism. The use of propidium monoazide (PMA) has been proposed as a solution. Coupled with an appropriate lysis protocol, the PMA method may even be used for selective microbial assays, as well as for removal of DNA contaminations. Atlantic Salmon (*Salmo salar*) was used as a model organism representing the eukaryotic host. The effect of PMA was evaluated using in vitro samples of live and dead *E.coli* DH5α and pure salmon DNA from salmon sperm in conjunction with qPCR, and the effect of PMA treated samples were compared against control samples. In addition, a detergent varying in concentration was used to selectively lyse eukaryotic cells without harming *E.coli* or *E.faecalis* as representatives of gram-negative and gram-positive bacteria. Lastly, pilots were treated using a finalized PMA method on real salmon intestine samples inoculated with *E.coli*.

The use of PMA for removal of contaminating DNA in vitro samples was deemed promising, as living cells treated with the method showed no inactivation while specifically killed bacteria and free eukaryotic DNA was inactivated. However, results were difficult to replicate due to difficulties regarding the viability of the *E.coli* cultures. Using the Triton x-100 detergent on bacteria, *E.coli* showed a higher tolerance in comparison to the *E.faecalis*. Furthermore, procured salmon samples showed a low abundance of eukaryotic DNA present, as well as inconclusive PMA results due to varying inactivation between live samples of *E.coli* in the same assays and between assays. Sequencing of bacterial diversity observed the same species of which have been earlier described as the most dominant in Atlantic Salmon, while a selective inactivation of *E.coli* by PMA and not the microorganisms naturally present was observed. Use of the PMA method for general inactivation of a wide array of samples is deemed promising, albeit further evaluations of a selective lysis protocol targeting eukaryotic cells, as well as an evaluation of the PMA effect on real-life samples is required.

Sammendrag

DNA kontaminasjon er en utfordring for dagens sekvensering. Kontaminanter kan føre til feil ved DNA-sammensettinger, feilaktig assosiasjoner og mindre kostnadseffektiv sekvensering. Kontaminasjon av DNA fra verter viser også de samme problemene. Eukaryot DNA er i overtall i forhold til mikroorganismer fra blod- og faeces-prøver, og fører dermed til høyere kostnader for sekvensering og lavere sekvenseringsmengde og dybde. Det finnes protokoller i dag som har som hensikt å løse problemene med verts-DNA, men de fleste er basert på metyleringsnivå for mennesker, og kan dermed ikke brukes uavhengig av verts-organisme.

Bruk av propidium monoazid (PMA) er blitt sett på som en mulig løsning. Sammen med en passende lysis protokoll kan PMA ikke bare brukes for selektive undersøkelser for mikroorganismer, men også for generell fjerning av DNA. Atlanterhavslaks (*Salmo salar*) ble brukt som modellorganisme for å representere en eukaryot vert. Effekten av PMA ble så undersøkt ved bruk av enkle prøver med levende og døde *E.coli* DH5α og rent DNA fra laksesperm. Disse prøvene ble undersøkt med qPCR og sammenlignet med ubehandlede kontrollprøver. Triton-detergent ble også testet med varierende konsentrasjoner på *E.coli* og *E.faecalis* som representanter for henholdsvis gram-negative og gram-positive bakterier for å finne en optimal konsentrasjon som selektivt lyserer eukaryote celler. Til slutt ble PMA-behandling testet på pilotprøver med tarmskvis fra atlanterhavslaks inokulert med *E.coli*, prøver som senere ble sekvensert.

PMA for fjerning av kontaminerende DNA viste gode indikasjoner, da levende celler behandlet med metoden ikke viste tegn til inaktivering i motsetning til døde bakterier og rent DNA fra laks. Resultatene var derimot vanskelige å replikere grunnet problemer med *E.coli*-kulturer som viste seg å ikke være levedyktige. Forsøk med Triton-detergent på bakterier viste en høyere toleranse for *E.coli* i forhold til *E.faecalis*. Videre viste forsøk med prøver fra laksetarm lave nivåer av 18S DNA samtidig som at PMA-behandling på disse prøvene viste upålitelige resultater på grunn av mangelfull levedyktighet for *E.coli*. Videre sekvensering av prøver fra laksetarm viste samsvar mellom bakterier som tidligere er funnet i laksetarm og de aktuelle bakteriene som ble observert. I tillegg ble det gjennom sekvenseringen observert en selektiv inaktivering av *E.coli* etter behandling med PMA. Siden de tidlige simulerte og enkle prøvene viste selektiv inaktivering av forventet fritt DNA og sekvensering av prøvene bekreftet dette, ser bruken av PMA-metoden for generell inaktivering av kontaminerende DNA lovende ut. Videre evalueringer er derimot nødvendig for å utvikle en brukbar protokoll for selektiv lysis, samtidig at det er nødvendig å stadfeste effekten av PMA på ekte prøver.

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1. Introduction

1.1 Atlantic salmon

Atlantic salmon is part of the larger group of Salmonids, containing 11 genera including salmon, trout, charr, freshwater whitefishes, ciscos and graylings (Davidson et al., 2010). The Salmonids are of social and economic importance today due to their prominent position within aquaculture, wild fisheries and recreational sports fishing, in addition to serving as a key indicator species for coastal and river health in their own ecosystems (Lien et al., 2016). Atlantic Salmon (*Salmo salar)* and the relative Brown Trout (*Salmo trutta)* are both found in the Atlantic sea and the connected rivers (Klemetsen et al., 2003), and the pair are similar enough to have been found to successfully mate and produce hybrids (Jansson, Holmgren, Wedin, & Andersson, 1991).

Atlantic Salmon and the other species of salmonids are studied not only due to their social and economic impact but also due to their genetics. The common ancestor of the salmonids experienced a whole genome duplication event about 80 million years ago, making the salmonids a well-suited model organism to study genome evolution and gene functionalisation in higher organisms like eukaryotes (Davidson et al., 2010)

1.1.1 Lifecycle of Atlantic salmon

Atlantic salmon are known as the prime example of an anadromous fish species, migrating from seawater to freshwater to spawn. The adult salmon return to their natal river or lake system during fall to spawn (Hansen & Quinn, 1998), and in most cases die by stress, disease, or due to programmed degeneration of their bodies (Patnaik, Mahapatro, & Jena, 1994). Only some females survive and return to the ocean (Baglinière, Maisse, & Nihouarn, 1990). The fertilized eggs are incubated during the winter hidden under gravel in what is known as a redd, hatching as alevin when the time is right. In this early life stage, salmon mortality is high, and spawning time is therefore adapted in each river as a means of securing optimal conditions for the alevin spawn (Solberg, Fjelldal, Nilsen, & Glover, 2014; J. H. Webb & McLay, 1996).

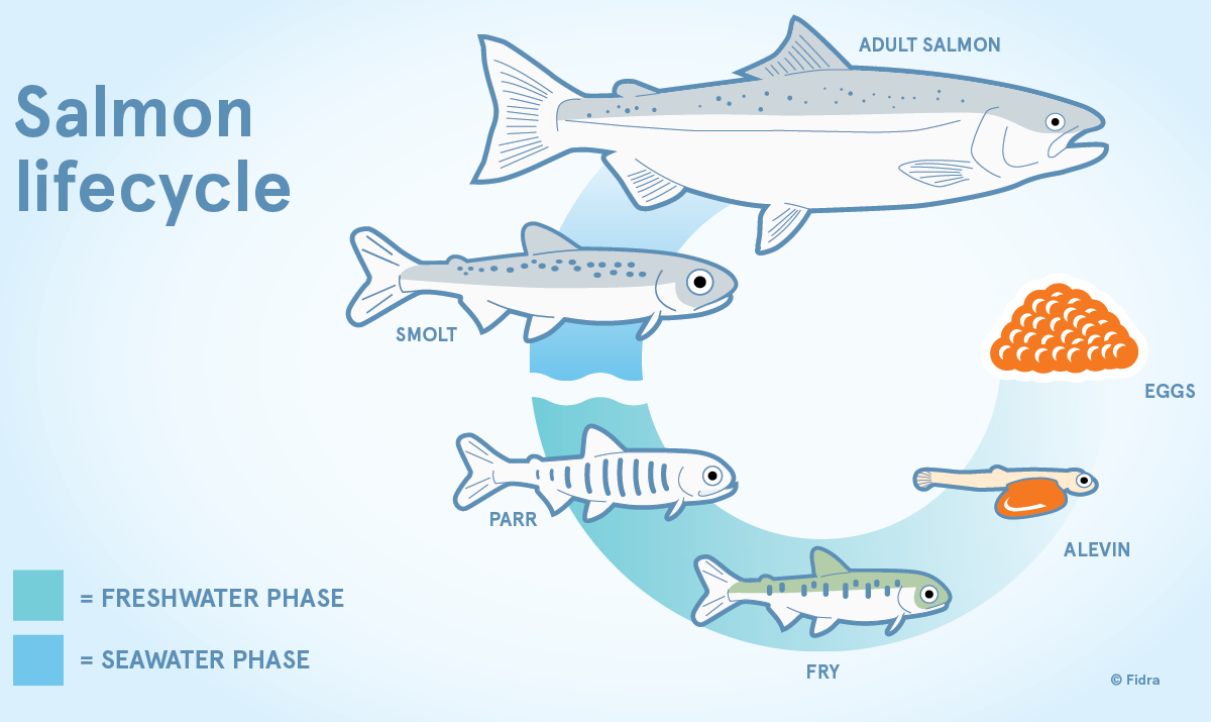

Figure 1.1: Atlantic salmon life cycle: Illustration of the general life cycle of Atlantic salmon (*Salmo salar)* from eggs to the later spawning of adult salmon from where it begins again. The illustration shows the alevin after hatching from the eggs, their transformation to fry after using up the yolk sac, the emerging of parr after independence from their redds and the transformation of parr to smolt prior to venturing out into the ocean and becoming adult salmon. The illustration is divided into two parts for the freshwater phase and the seawater phase, as shown by the colours on the left. Required from https://www.bestfishes.org.uk/did-you-know/scottish-salmon-life-cycle/ 04.08.18, 10:49.

Salmon alevin are characterised by being dependent on their yolk sac as a primary source of nutrition and stay inside their redds before emerging as fry after becoming independent of their yolk sac (Allan & Ritter, 1977). After becoming independent of their redds, the salmon spawn goes on to live like parr for 2-4 years, before undergoing smoltification prior to migrating to the ocean (Hansen & Quinn, 1998). This process involves morphological, biochemical, physiological and behavioural changes in order to prepare the salmon offspring for their migration and survival in the saline ocean (Thorstad et al., 2012). These changes include a change in colouration from darkly pigmented melanin bars known as parr marks to a silvery colouration, a sleeker and more streamlined body, a change from hyper-osmoregulators (discharge of water, uptake of saline) to hypo-osmoregulators (discharge of saline, uptake of water), as well as gaining a preference for saline water (Folmar & Dickhoff, 1980). The scale and onset of these changes as well as differences in both migratory mortality and size are subject to ecological, geographical and genetical differences between individual lake and river systems and their residents (Fleming, 1996).

Following the smoltification process, the salmon offspring are known as smolts for the freshwater phase of their journey, gaining the name of post-smolts from their entry into the marine environment until after their first winter at sea (Thorstad et al., 2012). After maturing, the adult salmon again return to their natal river to begin the cycle anew (Hansen & Quinn, 1998).

1.1.2 Teleost anatomy and digestive system

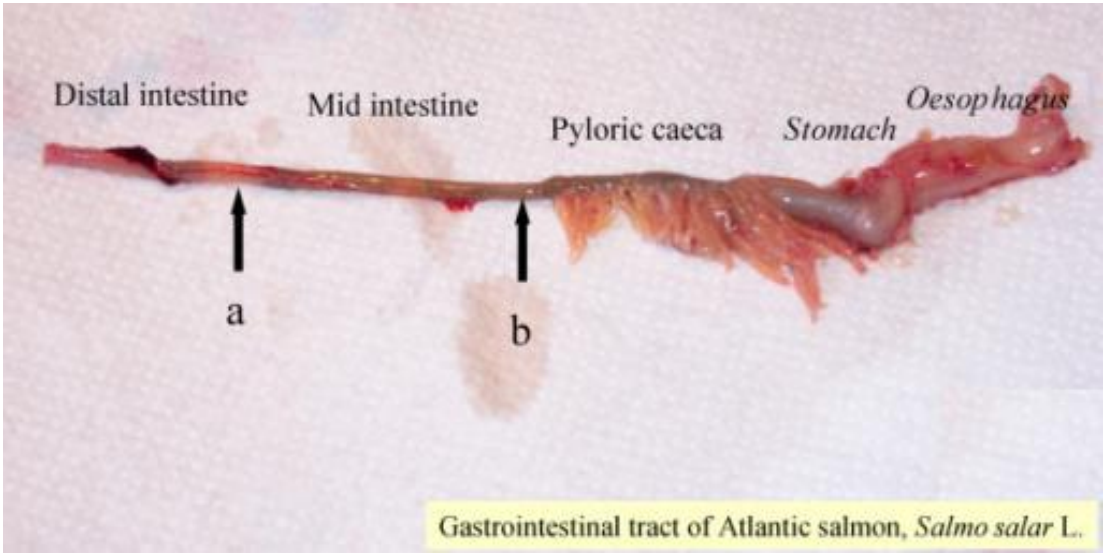

Figure 1.2: Illustrative Photograph of the GI tract of Atlantic Salmon. The illustration shows the different parts of the Gastrointestinal tract of a young individual of the Atlantic Salmon species. Samples were procured from the distal part of the intestine, outlined by the letter a. Illustration acquired from https://openi.nlm.nih.gov/detailedresult.php?img=PMC2667469_1472-6793-9-3-1&req=4,12.06.18, 19:34

Teleost anatomy of the gastrointestinal tract, specifically that of the Atlantic Salmon, is as shown in figure 1.2. Most teleosts are predatory fishes feeding on small invertebrates or smaller fishes, and most of these predatory fishes have what is called throat teeth. After eating, the often still living food enters the oesophagus and is transported to the stomach, both trapped and oriented by throat teeth. The oesophagus is often short, but very distensible once food of a certain size enters. The oesophagus transfers the food into the stomach, where the food is broken down and leaves the stomach as a liquid. After leaving the stomach, food meets a muscular valve, where some teleosts like the Atlantic Salmon have a distinct structure called the pyloric caeca (blind sacs). These blind sacs can have either a digestive function, an absorptive function or both (Parenti & Weitzman, 2018). Additionally, they can also vary in both size and numbers amongst species, from in the thousands to only one, or even being totally absent (European Association of Fish Pathologists, 2018) depending on the diet (Dos Santos, Arantes, Santiago, & Dos Santos, 2015).

Further, the intestine of the teleost emerges, a tubular organ varying in size also depending on the diet. Carnivorous teleosts have shorter intestines than herbivorous teleosts, owing to the difficulties of absorbing nutrients from a herbivorous diet (Dos Santos et al., 2015). The intestinal mucosa of the teleosts is also ripe with mucosa-producing goblet-cells, helping with the passing of food by releasing lubricative mucosubstances, which also increase absorption of nutrients (Dos Santos et al., 2015; Løkka, Austbø, Falk, Bjerkås, & Koppang, 2013). For some teleosts, no real difference from the proximal to the distal parts of the intestine can be seen (Løkka et al., 2013), however it has been shown that zebrafish intestine can be distinctly divided into topographical regions with specific functions (Wallace, Akhter, Smith, Lorent, & Pack, 2005). This has also been shown to be true for many other teleosts (Egerton et al., 2018). Such a division can be seen in most parts for the Atlantic Salmon, as it shows a higher absorptive function of nutrients in the mid-parts of the intestine, and less absorption near the distal parts before ending with the anus and defecation (Løkka et al., 2013)

1.2 Gut microbiota

1.2.1 Gut flora and health impacts

Mucosal bacteria are important for many organisms. They are found in the periphery of the mucosa, helping in the uptake of nutrients and the production of vitamins and short chain fatty acids from often non-digestible complex carbohydrates (LeBlanc et al., 2017). These short chain fatty acids are also important factors contributing to the growth, sustainability and differentiation of epithelial tissue for a wide array of organisms (Guarner & Malagelada, 2003; Løkka et al., 2013).

Mucosal bacteria are part of an important defence mechanism against foreign potentially pathogenic bacteria (Becattini et al., 2017). Many mucosal bacteria produce organic acids, creating a hostile microenvironment near the mucosa (Zhang et al., 2015). Moreover, these bacteria outcompete pathogens due to a better nutrient adaption and produce bacteriocins limiting the number of foreign bacteria able to survive and thrive (Abt & Pamer, 2014). Interestingly, even the microviome of the gut help protect against the colonisation of foreign gut bacteria, as it has been shown that a bacteriophage colonising *E. faecalis* actually provide the bacteria with a competitive edge compared to non-viral *E.faecalis* (Duerkop, Clements, Rollins, Rodrigues, & Hooper, 2012)

1.2.2 Salmon gut flora

Many symbiotic gut microbes in the gut flora of fishes exhibit a positive activity for the host, playing a role in nutrition, immunity, defence and epithelial development (Gómez & Balcázar, 2008; Nayak, 2010). The colonisation of the salmon larvae originate from the eggs, immediate environment and first feed, and include a diverse microbiome of protists, fungi, yeasts, viruses, Bacteria and Archaea (Egerton et al., 2018). For bacteria, there are two types found commonly in the GI-tract, the adherent and the transient. The adherent bacteria are able to adhere to the mucus layer and survive in the presence of bile salt and low pH, while the transient bacteria are unable to adhere to the mucus layer and are therefore just passing through (Nayak, 2010).

Water salinity has been found to be a driving force towards specific gut floras. Freshwater fishes tend to be dominated by bacterial species such as Aeromonadales and Enterobacteriales, while saltwater fishes are most often dominated by Vibrionales (Sullam et al., 2012). A distinct difference has been observed between wild and farmed salmon, even if attempts are made to simulate the wild salmon breeding for farmed salmon. A differing microbiota was also observed between farmed salmon, varying based on geographical location and feed, and mostly constituted *Acinetobacter junii*. For the wild salmon, *Mycoplasma* was found to clearly dominate the microbiota, constituting 96% of the microbiota of the wild salmon (Holben, Williams, Saarinen, & Apajalahti, 2002). Even though the wild salmon was found thoroughly dominated by *Mycoplasma*, presence of other bacterial groups such as *Aeromonas, Pseudomonas* and *Psychrobacter* are also often found in salmon (Egerton et al., 2018).

Salmon shows a tolerance towards their own microbiota, reacting towards foreign microorganisms while sparing the native microbiota (Gómez & Balcázar, 2008). Protection of the fish intestines is mediated by a complex and vast system credited the innate immune system and the commensal gut flora, from acids and enzymes providing a hostile environment to antibacterial peptides (piscidins) isolated only from fish, proteases and specific proteins outright killing or immobilizing the potential pathogens (Gómez & Balcázar, 2008; Silphaduang & Noga, 2001).

1.3 Conventional Nucleic Acids methods

1.3.1 DNA extraction and isolation

1.3.1.1 Cellular lysis

DNA extraction is very important due to the direct effect on later analysis, meaning an unsuccessful extraction will lead to results not being representative for a given sample. In order to first obtain cellular DNA, a disruption of cellular structures and membranes is required (Moré, Herrick, Silva, Chiorse, & Madsen, 1994). This can be accomplished either using chemical, enzymatic or mechanical means, such as the use of chaotropic salts, detergents or alkaline denaturation for chemical lysis (Pethica, 1958) or the use of enzymes to break down the cell wall for enzymatic lysis. Due to the differences between gram positive and gram negative bacteria, as well as the differing composition between eukaryotic and prokaryotic cells, different preparations are needed for an optimal enzymatic lysis (Salazar & Asenjo, 2007).

Mechanical lysis on the other hand is based on the mechanical destruction of the cell walls by means of force. One such method is the bead-beating technique. Glass spheres are added to the sample liquid and the mixture is shaken violently at high speed for some time. Studies have reported that a mixture of differently sized beads performs better than beads of similar size (Bakken & Frostegård, 2006). Other mechanical lysis techniques include grinding the cells either dry or with liquid nitrogen, as well as freeze-thawing of the cells (Bakken & Frostegård, 2006).

1.3.1.2 Purification of DNA

Several methods are used for the purification of DNA following lysis. They include column-, solution- and bead-based techniques (Tan & Yiap, 2009). Solution-based techniques include methods like CTAB (Cetyltrimethylammonium bromide) precipitation (Porebski, Bailey, & Baum, 1997), phenol-chloroform extraction (Green & Sambrook, 2017) and alkaline lysis coupled with centrifugation (Tan & Yiap, 2009).

The column techniques are based on the use of columns of matrixes made with materials like silica or glass powder, coupled with centrifugation for binding and elution of DNA (Esser, Marx, & Lisowsky, 2005). Matrices made with nitrocellulose or polyamide are also used, but bind DNA with less specificity (Arnold, Meyering, & Chesterson, 2005; Tan & Yiap, 2009).

The basis behind the silica and paramagnetic beads methods are the same as with the columns. Both techniques rely on the ability of the material, e.g. silica matrices or carboxyl-coated magnetic particles (Hawkins, O'Connor-Morin, Roy, & Santillan, 1994) to selectively bind the DNA molecules in the presence of a chaotropic reagent. The chaotropic agent acts in the solution as a hydrogen bond breaker, breaking the hydrogen bonds of the negatively charged oxygen ions of the silica material, thus facilitating binding of the negatively charged phosphate backbone of DNA to the silica matrices or the beads supported by a salt cation bridge (Breadmore et al., 2003).

Earlier methods have used salts like guanidium thiocyanate as the chaotropic reagent (Boom et al., 1990), while other methods have used conditions like high concentrations of polyethylene glycol (PEG) and NaCl to facilitate binding of a DNA precipitate (Hawkins et al., 1994). Rudi, Kroken, & Jakobsen (1997) showed that adsorption to the beads could be facilitated using direct cell lysis with a detergent without any precipitating steps. The beads or matrices can further be washed using chaotropic-acting alcohol solutions, retaining binding of the DNA while removing impurities and salts (Engelstein et al., 1998). Utilising magnetic beads, the DNA bound to the beads can be isolated from the resulting impure solution following an alcohol wash.

Lastly, DNA can be eluted from the materials using low-ionic solutions such as distilled water, reintroducing the hydrogen bonds and thus removing the cation bridge supporting the bond (Esser et al., 2005). This results in the release of DNA from the material, and the DNA elute can be directly used in nucleic acid analyses. Nowadays, many commercial kits are available, simplifying the method drastically while also providing specific binding of a specific target. This is done using material coated with antibodies or functional groups much like carboxyl coated beads (Dhaliwal, 2013), binding specifically to e.g. DNA of a certain methylation level (Feehery et al., 2013).

1.3.2 PCR

PCR (Polymerase chain reaction) is a method for amplification of target DNA sequences first properly developed and patented by Kary Mullis and his team in the 1980s (Mullis et al., 1986). It is based on the ability of DNA polymerase to synthesize new strands of DNA based on a given sequence. The core components required is a template of a target DNA sequence, DNA polymerase, primers complementary to the target sequence, nucleotides (dNTP's) and heat (Garibyan & Avashia, 2013).

The strands of the added template DNA are initially separated through heat denaturation, making way for the primers to attach to their complementary sequence during the annealing process. For primer annealing to occur, the mixture is cooled to the optimal annealing temperature for the specific primers used. Once the primers are in place, DNA polymerase can attach complementary nucleotides from the free 3' OH-group on the primer and further 5' to 3' onto the target sequence during the extension process. This results in one new copy of the forward strand, and one new copy of the reverse strand. This process can be repeated multiple times as cycles, each time in theory doubling the amount of target DNA present (Schochetman, Chin-Yih, & Jones, 1988).

Because of potential polymerase inhibitors present in the sample, reagent limitations and self-annealing of the resulting DNA strands, the PCR is limited to roughly 40 cycles before effectivity decrease and the PCR enter a plateau phase (Kainz, 2000). Prior to this maximum cycle limit, the method is relatively accurate (Mullis et al., 1986), though it is prone to amplify even small amounts of contaminants present in the samples, possibly resulting in misleading information (Schochetman et al., 1988). PCR in the most basic qualitative form is innately an end point-analysis, requiring a second verification such as agarose gel electrophoresis to visualize the target fragments, though the PCR is unable to accurately quantify using the endpoint due to the plateau phase since most samples at this point cannot be distinguished from each other (Peirson & Butler, 2007).

Quantitative real-time PCR is one solution to the end-point problems of qualitative PCR. The principle of qPCR is the same as PCR, except a fluorophore reagent is used to visualize the DNA in real time together with a light source, optics and a detector system (Peirson & Butler, 2007). The result is an amplification plot depicting the growth curve of fluorescent light. The amount of fluorescence is proportional to the DNA concentration present in each sample, but

a threshold value and a threshold cycle (called C_t) is also needed to understand the amplification curve. The threshold value is a given level of fluorescence during the exponential growth of fluorescence where all similar plots are analysed, and the C_t-value the specific cycle where the threshold value was exceeded (Peirson & Butler, 2007). An earlier C_t-value in relation to the PCR efficiency therefore means more target DNA from the start point of the analysis. By using the PCR efficiency together with the C_t-value, it is possible to quantify the DNA present in the sample from the start.

1.3.3 Sequencing

Today, sequencing is the bread and butter of molecular biology studies. From the fields of structural genomics, transcriptomics and functional genomics to metagenomic studies, most approaches converge towards at least some form of sequencing. It all started with the theory that phylogenetic analysis of organisms could be performed using ribosomal RNA sequence characterisation (Woese & Fox, 1977). Further progress discovered that using the conservative regions of the 16S rRNA gene as a baseline, it was possible to map the phylogeny of bacteria using the 9 hypervariable regions of the same gene. Having a 70% or greater similarity of the 16S rRNA gene, it is estimated that the general sequence similarity constitutes more than 97% sequence similarity (Stackebrandt & Goebel, 1994).

During this time, the invention of Sanger sequencing by Sanger, Nicklen, & Coulson in 1977 revolutionized the study and classification of microorganisms (Escobar-Zepeda, Vera-Ponce de León, & Sanchez-Flores, 2015). Sanger sequencing is based on the use of a mix of a chain-terminating 2’3’-dideoxynucleotide (ddNTP) together with the 3 analogous dNTPs to stop DNA extension once the ddNTP is integrated into the growing DNA strand (Sanger et al., 1977). In conjunction with restriction enzymes and agarose gels, 96 sequences with an average length of 650 base pairs could be read at once, thereby providing enough length for use in 16S phylogeny (Escobar-Zepeda et al., 2015).

With the later invention of PCR, phylogenetical studies started the shift from DNA reassociation to the study of 16S rRNA (Stackebrandt & Goebel, 1994), only becoming more and more widespread over the years. The widespread use of 16S rRNA classification has even resulted in the reclassification and renaming of whole bacterial genera and species (Woo, Lau, Teng, Tse, & Yuen, 2008).

However, with the increased use of 16S rRNA taxonomy and analysis of metagenomics, sequencing technology present at the time proved to be a major bottleneck (Scholz, Lo, & Chain, 2012). From this need, next-generation sequencing (NGS) arose with pyrosequencing (Margulies et al., 2005). Pyrosequencing is based on one-by-one synthesis of DNA succeeded by the release of pyrophosphate, which in turn is transformed into a luminous signal. After identification of each well containing template DNA, a flow of a specific dNTP is added, and the resulting intensity of illumination for any given well represents the number of nucleotides which were incorporated (Margulies et al., 2005). Using this method, an output of 1 gigabases could be produced for each run (Escobar-Zepeda et al., 2015), a new world compared to Sanger sequencing, though at the cost of shorter read lengths (Margulies et al., 2005). Further progress yielded the analogous Ion Torrent platform in 2010 (Rusk, 2010). The system detects the change in hydrogen potential each time a nucleotide is incorporated, mimicking the basis of the 454 pyrosequencing (Escobar-Zepeda et al., 2015), while cutting cost further (Whiteley et al., 2012).

As a contender to 454 pyrosequencing and Ion Torrent, Illumina sequencing further sheared costs of sequencing. Illumina is a sequencing by synthesis (SBS) technology. Each DNA fragment present in the sample is amplified using PCR together with specific Illumina primers, resulting in the incorporation of adapters to the end of the DNA fragments. These adapters are complementary to oligonucleotides on the Illumina sequencing chip, while also containing an index barcode representing a given sample (Illumina Inc, 2010).

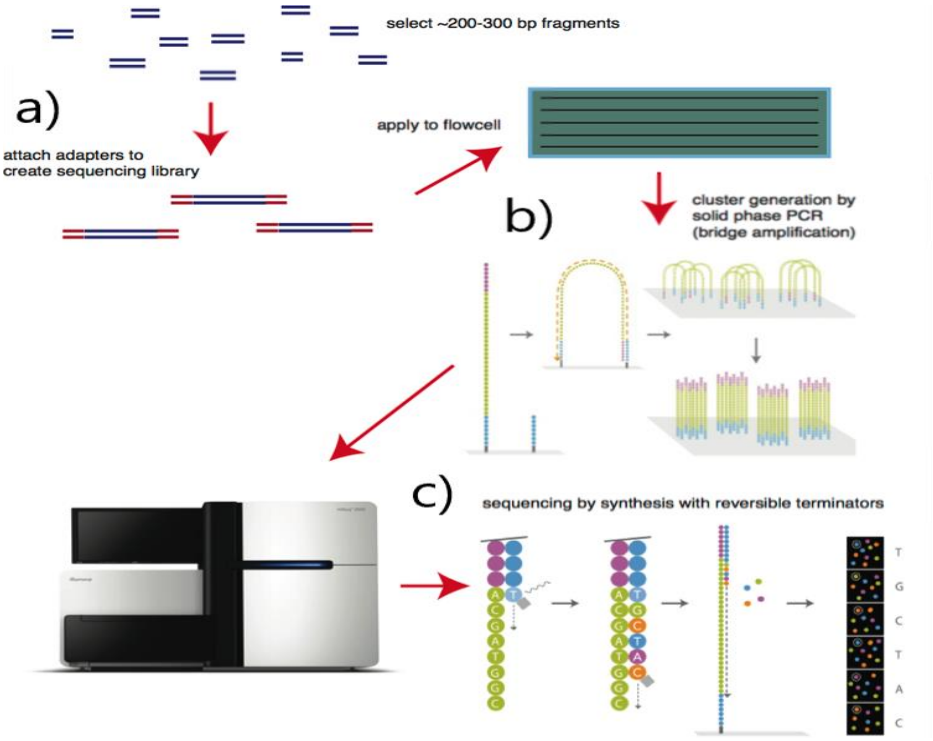

Figure 1.3 Illumina sequencing. Figure illustrates what happens during an Illumina sequencing, from adapter ligation and application shown by a), cluster generation by b) and SBS by c). Acquired from https://bitesizebio.com/13546/sequencing-by-synthesis-explaining-the-illumina-sequencing-technology/ and further modified 02.08.2018 16:22

dsDNA is denatured to ssDNA so that the adapter sequence of the ssDNA can bind to the complementary oligonucleotide, facilitating the synthesis of a new strand attached to the oligonucleotide. This process can be seen by b) of figure 1.3. The template strand is denatured and washed away, prompting the binding of the other end of the attached DNA to another oligonucleotide, resulting in the synthesis of a new attached DNA strand. This is performed several times until a cluster of DNA originating from a single strand of DNA has been created (Bentley et al., 2008). Utilizing a set of four reversible terminators, 3'-O-azidomethyl 2'-deoxynucleoside triphosphates (A, C, G and T) labelled with a different removable fluorophore, each competitive incorporation of a new complementary base releases the fluorophore which is excited by a laser (Bentley et al., 2008). Thereby, each cluster emits a single colour, with a decrease in uniformity of the colour being representative of the quality of the given base incorporation. Due to the unnatural nucleotides used, the sequencing is terminated until a free 3'- OH group can be regenerated, after which the nucleotides are added again for another incorporation.

Due to the low cost, high data output and ability to sequence both ends of a given DNA strand (Lahens et al., 2017), Illumina has become one of the most important sequencing technologies today. It is however limited by read length, affecting the sequencing of e.g. repetitive regions

and discovery of large segmental duplications (Pollard, Gurdasani, Mentzer, Porter, & Sandhu, 2018). Most 2nd generation NGS like Illumina and Ion Torrent are also limited by error rates between 0.8% and 1.7%, making sequencing and assembly of novel genomes a difficult task (Hebert et al., 2018). Several other methods have been developed, such as the Oxford Nanopore and PacBio. These novel technologies are hoping to change the playing field from short-read to long-read sequencing, while at the same time improving cost efficiency (Hebert et al., 2018)

1.4 Microbial growth, biology and analysis

Microorganisms like bacteria are diverse organisms able to occupy most niches that can be theorised. This is due to a fierce competition over resources, driving an extensive evolution in order to fit a niche, dominate or become extinct (Bauer, Kainz, Carmona-Gutierrez, & Madeo, 2018). Bacteria increase their numbers in a given environment by binary fission, wherein one bacteria split into two distinctly similar cells which often continue to divide (Kelly & Rahn, 1932). Therefore, for a given population of bacteria growing under favourable conditions, it is said that bacteria exhibit exponential growth, doubling at regular intervals linked to the growth time of the specific species (Monod, 1949). However, most bacteria living in a given environment never exhibit such exponential growth but are sustained and inhibited by a complex system of co-dependency, competition, abiotic factors and metabolic nutrient availability (Bauer et al., 2018).

1.4.1 Bacterial growth rate

When a fresh medium is inoculated as a closed system with bacteria where no change is made to the medium over time, prokaryotic population growth rate is generally divided into four phases. The first phase is called the lag phase, in which the inoculum needs time to recover from the physical damage related to the transfer of cells, adapt and express the required enzymes and co-enzymes for proper utilisation and survival in a new environment (Rolfe et al., 2012).

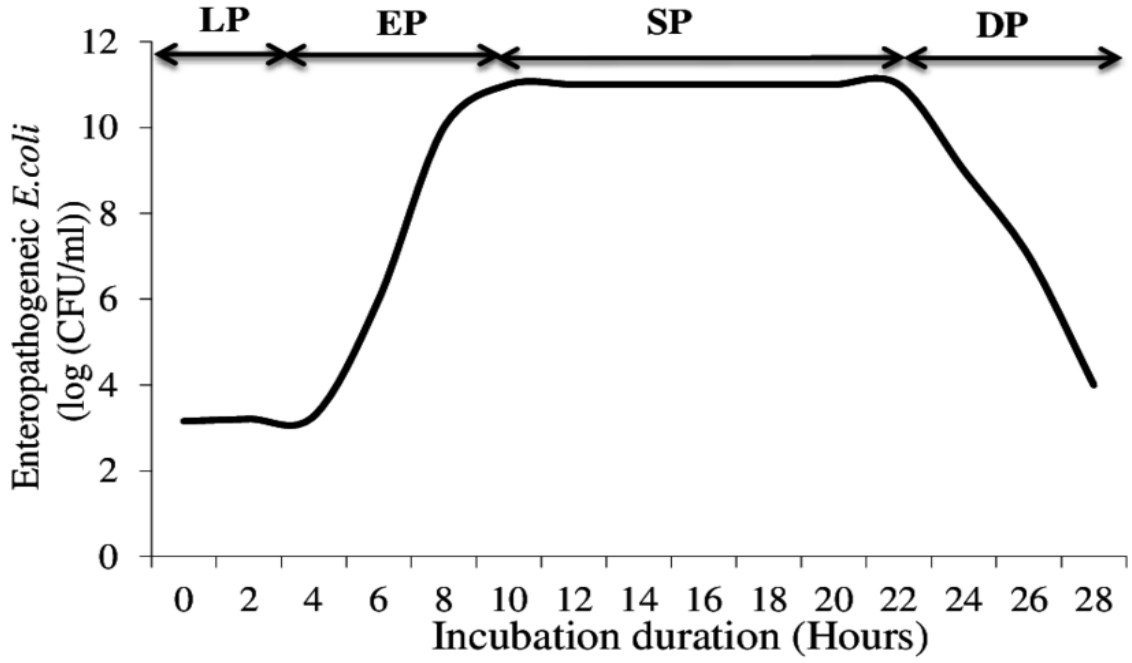

Figure 1.4: Bacterial growth in a closed medium: The illustration shows the growth curve of an Enteropathogenic *E.coli* as a closed system where no changes are made after inoculation. The four phases are outlined as lag phase (LP), exponential phase (EP), stationary phase (SP) and death phase (DP). The log (CFI/ml) is shown on the y-axis, while the incubation time in hours is shown on the x-axis. The figure is retrieved from (Arfao et al., 2016) Figure 1.

However, after the bacteria have passed the initial hurdle of a new environment and all prerequisites are in place, the bacterial population starts to double at regular intervals in what can be seen in figure 1.4 as the exponential phase (EP). In this phase, the generation time of a given bacteria under optimal conditions can be measured by each doubling interval, while the optimal growth requirements of a given bacterial species can be seen by observing the change in generation time (Monod, 1949).

Once the population becomes dense, waste products are accumulated and the abundance of nutrition decreases, population growth comes to a halt. The population enters the stationary

phase, in which the population is sustained by an equal growth and death rate. If no change is made to the culture, the population will enter the death phase with a rapid death rate. Most bacteria present at this point are either dead or non-viable, though some will still be viable (Finkel, 2006).

1.4.2 Microscopy techniques

The microscope as we know it has a long history. From the first microscope created in 1590 to the observations of Robert Hooke in 1667 and the progress of Anton van Leeuwenhoek as the first to successfully observe bacteria, the microscope has changed significantly. A major progenitor in the pursuit of knowledge regarding microorganisms, the microscope has gone from a single lens to becoming a complex system of parts, increasing the resolving power (resolution) and magnification to study the most minute details of the micro world. Many types of microscopes have been devised, from the stereoscope used for dissecting and observation of small organisms, compound microscopes able to magnify around 1000 times using visual light (however with a low resolution), confocal fluorescence microscopes using fluorescent light to the transmission electron microscope (TEM) and scanning electron microscopes (SEM) using electrons rather than light sources in order to provide an image, able to increase magnification greatly while still providing clear images (Van Meerbeek et al., 1993). Also present are the phase contrast and dark field microscopes, which are able to scatter light in different ways so as to visualize not only live cells but also the internal cell parts (Burch & Stock, 1942)

Confocal microscopes using not visual light, but lasers or UV lamps to excite fluorescent reagents can be used in specialised assays such as live/dead staining or selective cell structure assays. While a normal compound microscope visualize the same amount of light reflected from an object, confocal microscopes are able to selectively remove out-of-focus fluorescence by using a beam splitter in conjunction with pinholes, resulting in the isolated visualisation of in-focus fluorescence, which in turn drastically improve image quality (R. H. Webb, 1996; Wolenski & Julich, 2014)

1.4.3 *Escherichia coli*

Escherichia coli is a species of bacteria first discovered by Theodor Escherich in 1884 (Blount, 2015). It is a facultative aerobic gram-negative bacillus, able to exhibit either flagella for self-motility or pili for attachment to surfaces (Blount, 2015). *E.coli* is mostly a commensal bacterium, typically occupying the lower intestines of many animals, but it is also a widely spread organism able to adapt and survive under most conditions (Hufnagel, DePas, & Chapman, 2015). However, *E.coli* can also exhibit many pathogenic strains such as Shiga-toxin producing *E.coli*, EHEC, ETEC and EPEC (Palaniappan et al., 2006).

E.coli is one of the world's most understood and researched organism and is frequently used as a model organism in the lab, such as *E.coli DH5α,* a non-pathogenic lab strain first described by Hanahan, Jessee, & Bloom, 1991, able to accept plasmids for transformation extremely well (Taylor, Walker, & McInnes, 1993). *E.coli* is part of the large family Enterobacteriaceae (Miles, 1985), and is a diverse bacteria known to harbour many strains with different adaptations (Kaas, Friis, Ussery, & Aarestrup, 2012).

1.4.4 *Enterococcus faecalis*

Enterococcus faecalis is a gram-positive member of the *Enterococcus* genus. It is a commensal bacteria in a diverse range of organisms (Van Tyne, Martin, & Gilmore, 2013), and proliferate in the anaerobic and nutrient-rich environment of the intestines (Van Tyne et al., 2013). *E.faecalis* is, for the most part, a sturdy and survival-focused bacteria, as studies have shown how starvation induces drug multiresistance and increases general resistance towards outer forces (Giard et al., 1996; Portenier, Waltimo, Ørstavik, & Haapasalo, 2005). As well, due to an increased antibiotic use, the core presence of *E.faecalis* in the gut microbiota coupled with it being an opportunistic pathogenic bacteria, it has established itself as a large clinical problem (Castillo-Rojas et al., 2013; Gilmore, Lebreton, & van Schaik, 2013; Paulsen et al., 2003; Van Tyne et al., 2013).

1.5 Eukaryotic DNA contamination

DNA contamination is a widespread problem in many fields. In viral association studies of diseases originating from retroviruses, several associations have been shown to originate not from retroviruses, but from mouse DNA contamination (Robinson et al., 2010). Other examples include the presence of human contaminant DNA from ancient animal bones (Malmström, Storå, Dalén, Holmlund, & Götherström, 2005) to repetitive elements originating from humans being observed during routine checking of the NCBI gene databases in species ranging from bacteria to fish (Longo, O'Neill, & O'Neill, 2011), as well as fungus DNA from the *Aspergillus* genus contaminating clinical blood collection tubes (Harrison et al., 2010).

Another difficult problem is the isolation of high-quality nucleic DNA without the presence of mitochondrial or chloroplast DNA for shotgun sequencing. This is especially difficult in plants, due to the high number of mitochondria and chloroplasts present in cells (Lutz, Wang, Zdepski, & Michael, 2011). Sadly, no general protocol excelling for all types of plant species has been found, and there are even large differences in between same species based on tissue type, age, storage and molecular content (Varma, Padh, & Shrivastava, 2007).

Such contaminations can have a wide array of results as can be seen by these examples, resulting in wrongful associations, downright errors in important gene databanks as well as perhaps life-threatening clinical diagnostic errors stemming from contaminating DNA. Contaminating DNA can also result in the direct misassembly of sequencing data, grouping of unrelated sequences, and of course the waste of time, effort and money on unsuccessful analyses (Schmieder & Edwards, 2011)

1.5.1 Host DNA contamination

Similar to DNA contamination, the DNA present in samples taken from a host in order to study the microorganisms present may also prove problematic, especially using whole genome sequencing. Whole genome sequencing has become an important tool for clinical pathogenic analysis, providing information regarding origin, strain, mutations and characteristics of the infecting pathogen (Chin et al., 2010; Gardy et al., 2011). With progress in NGS technology leading to further cost reductions for sequencing, widespread use of sequencing for public health applications and diagnostics is nearer than ever, especially since

the method could be much more informative than clinical microbiology (Chin et al., 2010; Oyola et al., 2013). The problem with such clinical samples is the presence of high amounts of host DNA contamination in comparison to the low abundance of pathogenic and parasitic DNA, reducing the sequencing coverage and drastically increasing the cost per sequenced genome of the microorganisms present (Auburn et al., 2011).

Multiplexing samples is a method used to reduce the cost of sequencing for a set of samples, as many samples from many experiments can be sequenced together, thus reducing overall cost. If one lane of an Illumina High-seq chip is able to run 50 multiplexed samples of e.g. the malaria parasite *Plasmodium falciparum* if the samples were pure, human DNA contamination will reduce the number of samples to the range of a single sample or only a few, while systems like the Illumina MiSeq or the Ion torrent will be much less likely to produce enough coverage for the parasite genome in comparison to the human contaminant (Oyola et al., 2013)

Several methods have been attempted to reduce the amount of human contaminant present in such samples. Oyola et al., 2013 reported an enzyme-based DNA degradation method which selectively digests human DNA present, taking advantage of the difference between highly methylated human DNA in comparison to the mostly unmethylated parasite genome of *P.falciparum*. This method yielded a 4- to 7-fold enrichment of the parasitic DNA and showed coverage of at least 20 times of the parasite DNA increased from 0% of the samples to over 95% (Oyola et al., 2013). However, the method requires an incubation time of 16-hours, being unfavourable in locations where storage and processing could prove difficult (Feehery et al., 2013).

Methylated beads have also been used for the same purpose, with magnetic beads targeting large pieces of methyl-CpG DNA stemming from humans, binding and separating the DNA of humans from the DNA of other low-methylated organisms (Feehery et al., 2013), resulting in an 8-fold enrichment of *P.falciparum* from in vitro samples of 90% human DNA and 10% parasitic DNA. This method also managed to enrich the microbiome of Black Molly fish (*Poecilia* cf. *sphenops*) with an even enrichment of the microbiome, accurately depicting microbial species present in natural levels. Since the method is based on the use of beads binding to the methylated regions and does not require extensive work or time, it has been deemed a reliable and quick enrichment protocol for higher vertebrate DNA (Feehery et al., 2013).

1.5.2 PMA & EMA – Propidium monoazide and Ethidium monoazide.

Ethidium monoazide (EMA) has been used as a reagent for what is known as viability PCR (vPCR). EMA is a DNA intercalating reagent (Waring, 1965) able to produce a highly reactive nitrene when photoactivated, which in turn result in irreversible covalent binding to DNA (Nocker & Camper, 2009). It was proposed by Nogva, Drømtorp, Nissen, & Rudi (2003) as a means of removing membrane-compromised cells from the PCR analysis due to the reagent selectively entering dead and membrane-compromised cells. After entering the compromised cells, the EMA would bind to the DNA and upon photoactivation would irreversibly bind to the DNA and inhibit PCR. In addition, unbound EMA would during light treatment simultaneously react with water and also be inhibited (Nocker & Camper, 2009), thereby no longer being able to deactivate the DNA present in the live cells, leading to a viability-selecting PCR. Using this method, Rudi, Moen, Drømtorp, & Holck, 2005 were able to detect a decrease of 4 $\log_{10}$ for killed bacteria treated with EMA compared to the untreated samples.

However, it was shown that EMA did penetrate not only compromised cells, but also uncompromised live cells (Nocker, Cheung, & Camper, 2006). The potency of EMA was observed to be as much as 60% removal of viable *E.coli* 0157:H7 DNA (Nocker & Camper, 2006). Therefore, the eyes were set upon the reagent Propidium monoazide (PMA) as a potential solution due to successful selective staining of non-viable cells (Nocker, Cheung et al., 2006).

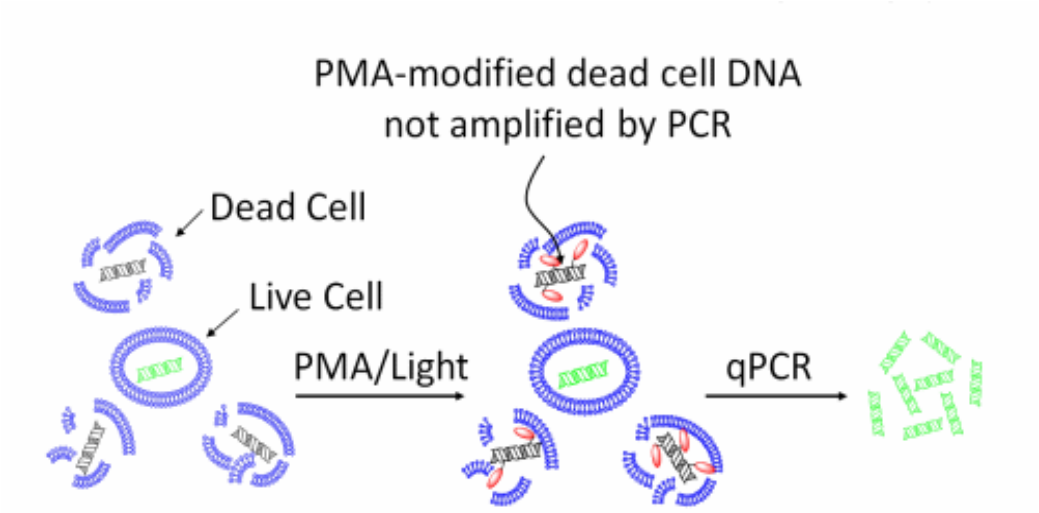

Figure 1.5: Working mechanism of PMA: The working mechanism of PMA is illustrated for live impermeable cells compared to dead permeable cells. As the permeable cells are invaded by PMA, the reagent binds to DNA and inactivates it in conjunction with light. The live cell is impenetrable to PMA and is therefore selectively amplified using qPCR. Illustration required from: https://biotium.com/product/pmatm-dye-propidium-monoazide/, 02.08.2018, 10:10

Just like EMA, PMA is also an intercalating reagent that can bind and inhibit DNA in the same manner (Waring, 1965). As was theorised with EMA, PMA is barred from entering viable impenetrable cells but can enter dead compromised cells and inactivate the DNA within. Thereby, DNA from live cells can be selectively amplified using PCR or qPCR. PMA is in theory usable for all types of organisms enclosed by a lipid membrane barrier, from viruses and bacteria to bacterial spores, protozoa and fungi. However, not all non-viable cells are permeable to PMA, as antibiotics or UV light may inactivate a cell without a rapid increase in permeability (Cangelosi & Meschke, 2014).

1.6 Aim of the thesis

Host eukaryotic DNA contamination is a large problem and bottleneck in the reduction of costs associated with NGS. Eukaryotic DNA is often a large constituent of many faeces-, blood- or tissue samples, and as so interferes with NGS in a way which effectively limits the level of multiplexing available, a possible source of cost reduction. Not only that, but the presence of host eukaryotic DNA also interfere with the available sequencing depth, possibly resulting in the lack of confidence for a given rare sequence present due to the overrepresentation of host DNA in the sample (Illumina Inc, 2018). This is especially true for assays targeting mucosal bacteria, which are most often outnumbered due to epithelial

shedding and the subsequent large amounts of 18S DNA. It is therefore important to remove or limit the magnitude of host DNA presence in microbial samples.

Today, there are several techniques able to selectively remove eukaryotic DNA from samples, but these are based on levels of CpG-methylation based on the human genome (Feehery et al., 2013; Oyola et al., 2013). Thereby, the use of such methods would prove disadvantageous on organisms where the level of methylation is unknown, such as the Atlantic Salmon, or for less methylated organisms. Since most prokaryotic cells are enclosed in a strong cellular membrane or wall while eukaryotic cells are mostly enclosed in a weak cellular membrane, the use of an inactivation protocol resulting in the inactivation of all DNA not protected by the prokaryotic defensive structures could be a possible method.

The use of PMA has been proposed as a possible solution for the full-scale inactivation of free and accessible DNA present in samples. Since PMA is unable to access enclosed cells, and therefore unable to inactivate DNA of viable living cells, the performance of PMA could be perfected using a selective lysis protocol targeting eukaryotic cells present in samples. Furthermore, the PMA method could function as an enrichment step in selective microbial assays by using selective lysis protocols for the enrichment of target bacteria. Thereby, the aim of this project constitutes:

- The evaluation of the use of PMA for the inactivation of host DNA contamination.
- The development of a selective lysis protocol in which viable prokaryotic cells survive.
- The establishment of a working PMA method for use in bacterial assays.

The invention of a ready to use, easy and general method of selective inactivation of target DNA is bound to drastically improve cost efficiency, accuracy and quality of life for microbial assays. Such a method could also provide better assays for studying the important roles of not only pathological organisms, but also important commensal bacteria colonising humans and animals alike.

2. Materials and methods

To solidify a working method to selectively inactivate eukaryotic DNA from mixed bacterial and eukaryotic samples, several experiments were performed to investigate different aspects of the method, and the resulting workflow can be seen by figure 2.1.

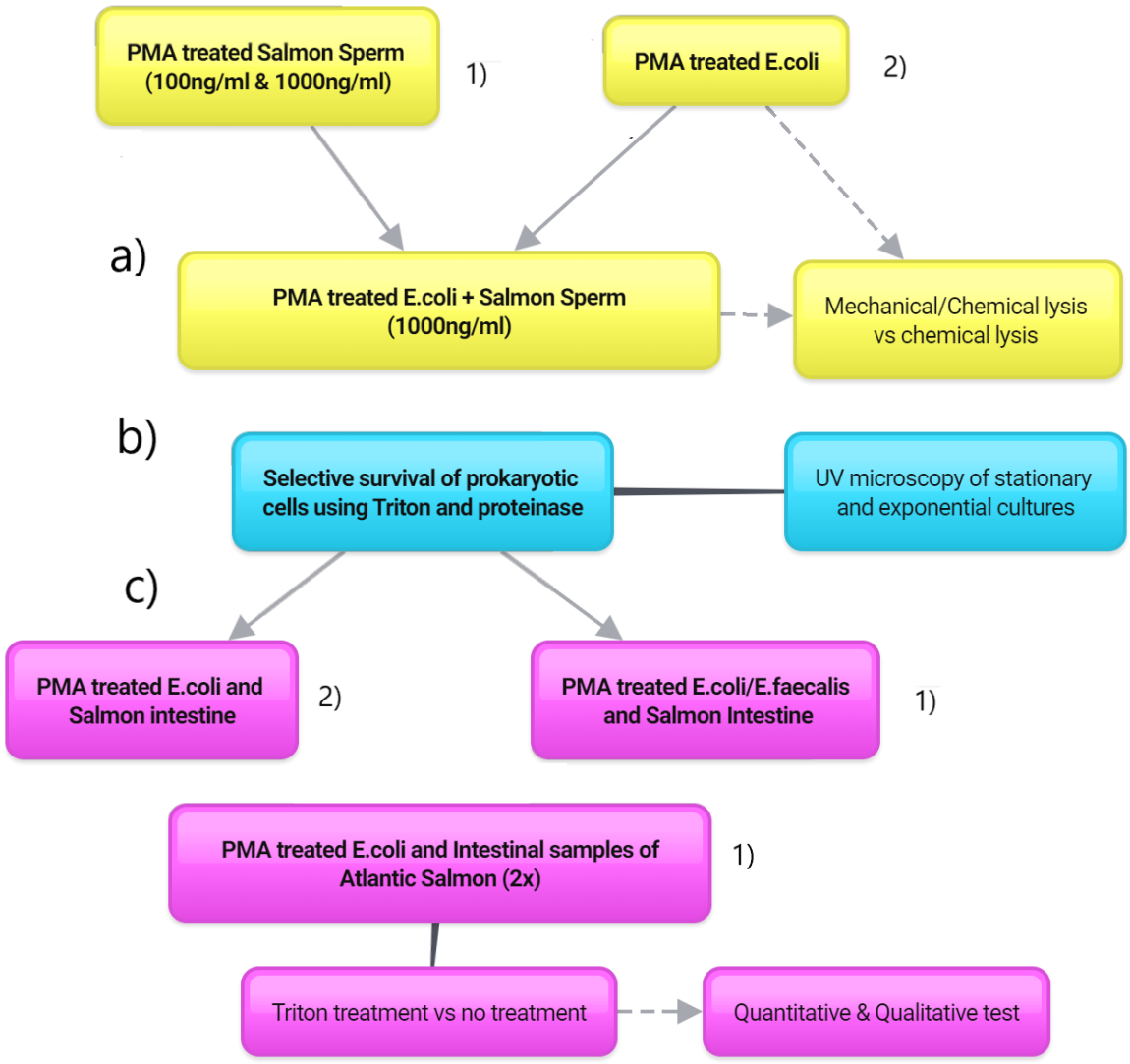

Figure 2.1: Flowchart. The flowchart shows the workflow of the project with a general description of the samples and methods used. Numbers 1) and 2) represents the type of DNA extraction used for the respective experiment, with 1) representing robotic extraction and 2) representing manual extraction. Furthermore, a) represents initial testing of the PMA method with salmon sperm and grown *E.coli*, as well as the testing of chemical and mechanical lysis in regard to the method. b) represents the testing of Triton x-100 and proteinase K for use in the method, and c) represents the testing for the finished method following progress in a) and b) with procured salmon intestinal samples. In a), only Qubit, 16S qPCR and 18S rRNA qPCR were used as quantitative methods, while in c), the quantitative and qualitative tests used also included UV-microscopy, Illumina sequencing and an incubation control.

2.1 PMA treatment and sample preparation

2.1.1 PMA treatment

For all PMA treatments, 2.56 µL of a 2mM PMA (Biotium, USA) solution was added to half of the samples already containing 100 µL, resulting in a final concentration of 50 µM. The other half of the samples acted as controls for the treatment. The treatment was conducted using 96-well PCR plates. The samples were incubated in the dark for 5 minutes, mixing occasionally, before being exposed to light in a lightbox (Geníul, Spain) for 30 minutes to inactivate the PMA. The treated and untreated samples were stored in a -20ºC freezer awaiting DNA extraction.

2.1.2 Live culture preparation

Live cultures of *E.coli* DH5α (Thermo Fischer Scientific, USA) and *E.faecalis* (procured from a course lab at NMBU) were prepared from -80ºC frozen glycerol stocks grown to an exponential phase before freezing. Cultures were started using 10 µl of frozen culture in 5ml of prepared and autoclaved Brain Heart Infusion broth (Sigma Aldrich, USA) and incubated at 37 ºC overnight with moderate shaking to avoid sedimentation. From these start cultures, cultures for both bacteria was prepared using 50 µl of the last prepared overnight culture in 5 ml of BHI broth.

2.1.3 Dead culture preparation

Dead cultures used in experiments was freshly prepared using the live culture from the respective day. Preparing the dead cultures, 100% Isopropanol (Sigma Aldrich, USA) was diluted using a mixture of 1:2 bacteria and MilliQ water to a concentration of 70%. The mixture was incubated at room temperature for 5 minutes before centrifuging at 13000rpm for 5 minutes. Removing the resulting supernatant, the cells were resuspended using 125 µl of 0,85% NaCl and further treated like the live cells.

2.1.4 Atlantic Salmon intestinal sample procurement

Atlantic salmon fishes of roughly 500g were procured from the Fish Farming Laboratory of NMBU. The specimens were dissected shortly after being euthanized. The distal part of the salmon intestine as shown in Figure 1.1 (a) was scraped and emptied into 1ml of PBS buffer and vortexed to homogenize the sample.

2.1.5 MacFarland turbidimeter

A McFarland turbidimeter was used to measure the density of bacterial cultures as an evaluation of their growth. The MacFarland scale measures the number of CFU from a cell culture at roughly $1*10^8$ CFU/mL from the start of the scale. At 1 MacFarland, this represents $3.0 * 10^8$ CFU/mL. For every further MacFarland value, the density increases by $3.0 *10^8$, yielding roughly $6.0 *10^8$ CFU/mL for a MacFarland value of 2.

2.2 Nucleic Acids Extraction

2.2.1 Mechanical cell lysis

Mechanical lysis was performed using a FastPrep96 machine (MP Biomedicals, USA) prior to DNA extraction. A mixture of 100 µl sample and 200 µl STAR buffer (Roche, USA) was processed together with 3 different sizes of acid washed beads (20 µg of ≤106 µm beads, 20 µg of 425-600 µm beads, and two 2.5-3.5mm beads (Sigma Aldrich, USA)). The samples were processed twice at 1800 rpm for 40 seconds and cooled on ice between runs. Samples were centrifuged at 13000 rpm for 5 minutes to aggregate beads and cell fragments in a pellet, before 100 µl of the supernatant was transferred to new Eppendorf-tubes and stored at -20ºC prior to extraction.

2.2.2 DNA extraction

Manual DNA extraction was performed using paramagnetic beads from the Mag Midi DNA extraction kit (LGC Genomics, UK) to bind the negatively charged DNA. A volume of 50 µL of Lysis buffer and 5 µL of Proteinase K was added to 50 µL of each sample to lyse the

bacterial cell walls and degrade proteins, thereby releasing free DNA from the cells, before being thoroughly mixed and incubated at 55ºC for 10 minutes. After cooling to room temperature, 16 µL of the fully suspended mag particle suspension was added to each sample together with 50 µL of 96% ethanol and further mixed. The samples were incubated for 2 minutes at room temperature to allow bead-binding, before bringing the samples in contact with the magnet. The supernatant was removed after pellet-formation before removing the magnet and mixing in 170 µL of Wash Buffer until the pellet was fully resuspended. The samples were incubated at room temperature for 10 minutes, shaking periodically, before bringing the samples in contact with the magnet again, removing the supernatant after pellet formation. This was repeated twice using 175 µL Wash Buffer containing acetone to remove salts and impurities. After removing all the supernatant, the pellets were air-dried at 55ºC for 6 minutes, allowing evaporation of the ethanol to occur. To elute the sample DNA from the beads, 50 µL of Elution buffer was added, resuspending the pellet and incubating at 55ºC for 10 minutes at 800 rpm. The samples were placed on the magnet, and the following elute after pellet formation was kept and stored at -20ºC before further analysis.

Automatic DNA extraction was also performed using 96 well KingFisher plates (Thermo Fisher Scientific, USA) with a KingFisher Flex robot (Thermo Fisher Scientific, USA), performing the same steps as the manual extraction.

2.3 Quantitative and qualitative nucleic acid measurements

2.3.1 Qubit

Qubit fluorometer (Life Technologies, USA) was used to measure the amount of DNA present in samples using the dsDNA High Sensitivity Assay Kit (Life Technologies, USA). According to protocol, a working solution was prepared using Quant-iT reagent diluted 1:200 in a Quant-iT buffer. A mixture of 2 µL of each sample to be measured was further mixed with 198 µL of prepared working solution, vortexed and incubated at room temperature for 5 minutes before measuring. Calibration of the instrument was done using standards provided by the manufacturer.

2.3.2 PCR

Different sets of primers were used for quantitative and qualitative PCR runs and can be seen in table 1.

Table 2.1: Primers used for PCR. *Table shows the different primers used for all experiments, their sequence, specific annealing temperature as described by their respective references, experimental temperature used, normal usage of the primers, as well as important references.*

Primer	Primer sequence	Annealing temp (C)	Protocol temp (C)	Usage	References
Forward 3NDF 18S	GGC AAG TCT GGT GCC AG	57	59	Eukaryote	(Cavalier-Smith, Lewis, Chao, Oates, & Bass, 2009)
Reverse V4 Euk R2 18S	ACGGTATCT(AG)ATC(AG)TCT TCG	60	59	Eukaryote	(Bråte et al., 2010)
PRK341F forward	CCTAC GGGRB GCASC AG	61.0	55	Prokaryote	(Yu, Lee, Kim, & Hwang, 2005)
PRK806R reverse	GGACT ACYVG GGTAT CTAAT	59.4	55	Prokaryote	(Yu et al., 2005)
EC23S857 forward	GGTAGAGCAC TGTTTtGGCA	60	60	*E.coli* specific	(Chern, Siefring, Paar, Doolittle, & Haugland, 2011)
EC23S857 reverse	TGTCTCCCGTG ATAACtTTCTC	60	60	*E.coli* specific	(Chern et al., 2011)

2.3.2.1 Quantitative PCR

Quantitative PCR was performed for both 18S rRNA fragments and 16s rRNA fragments individually using LightCycler 480 II in 96 well Light Cycler qPCR plates (Roche, Germany). A mixture was prepared for each reaction using a concentration of 1x HotFirePol EvaGreen qPCR supermix (Solis BioDyne, Estonia), 0.2 µM forward and reverse primer respective to the target sequence as shown by table 1, as well as 13.2 µL nuclease-free H_2O (VWR International, USA). A 2µL volume of Template DNA was added for each reaction, resulting

in a total volume of 20 µL. Initial denaturation took place at 95ºC for 15 minutes, following 45 cycles of denaturation at 95ºC for 30 seconds, 30 seconds annealing at specific temperatures for 18S and PRK-primers as shown in table 1, followed by elongation at 72ºC for 45 seconds, at which point fluorescence was measured for each cycle. *E.coli* gDNA or 1000ng/ml working solution of salmon sperm DNA was used as positive controls, respectively, while nuclease-free H_2O (VWR International, USA) was used as negative control. Data following the qPCR runs using the LightCycler 480 was further processed using LinRegPCR for baseline regression (Ruijter et al., 2009).

An *E.coli* specific qPCR was also performed using a Biorad CFX 96 Touch Real-Time PCR system (Biorad, USA), together with specific *E.coli* primers as shown in table 1. All other reagents were like the qPCR runs on the Light Cycler 480 II. Initial denaturation took place at 95ºC for 15 minutes, followed by 40 cycles of denaturation at 95ºC for 30 seconds. A combined annealing and elongation step at 60ºC for 1 minute was used, at which point the fluorescence was measured. Data from the qPCR run was analysed using Bio-Rad CFX Maestro 1.1 (Bio-Rad, USA).

2.3.2.2 Qualitative PCR

Qualitative PCR was performed on the 2720 Thermal Cycler 17 (Applied Biosystems, USA). For each reaction, a final concentration of 1x 5xHotFirePol Ready to Load (Solis BioDyne, Estonia), 0.2 µM Forward and reverse PRK primers, 17 µL nuclease-free H_2O (VWR International, USA) and 2 µL sample DNA was used for a total volume of 25 µL. For the initial denaturation, the samples were held at 95ºC for 15 minutes, before undergoing 35 cycles of denaturation at 95ºC for 30 seconds, 30 seconds of annealing at 55ºC, and lastly extension at 72ºC for 45 seconds. After all cycles had been completed, a final elongation at 72ºC for 7 minutes was completed, before the samples were held at 10ºC indefinite. *E.coli* gDNA was used as a positive control, while nuclease-free H_2O was used as a negative control.

2.3.3 Gel electrophoresis

Gel electrophoresis was used as a qualitative control method to check samples from qPCR and PCR for contamination, proper extraction or for impurities. For each run, an agarose gel of 1% was prepared using 1x TAE buffer (Thermo Fisher Scientific, USA), 1% agarose powder (Sigma Aldrich, Germany) together with 4 µl of PeqGreen (Peqlab, UK) per 100ml prepared gel solution. Each run of electrophoresis was run at 85V for 30 minutes, using a 100bp DNA ladder (Solis BioDyne, Estonia) for DNA fragment size control. The gel was photographed using UV light from The Molecular Imager Gel Doc XR Imaging system (Bio-Rad, USA) together with Quantity One 1-D analysis software v.4.6.7 (Bio-Rad, USA).

2.3.4 UV Microscopy

UV light microscopy was used both as a qualitative control method to check for culture viability, but also as a quantitative method. A Leica DM RXE Microscopy (Leica Camera, Germany) coupled with a Leica DFC425 C (Leica Camera, Germany) camera was used in conjunction with a 100W UV lamp for all microscopy needs. A 1 µl combined solution of Live/Dead BacLight Bacterial Viability kit (Thermo Fisher Scientific, USA) was added to 1ml of each sample to be visualized. The samples were vortexed to ensure a proper spread of the BacLight reagent, before being incubated in the dark for 15 minutes. About 20 µl of each sample was added to a clean glass slide, visualized and photographed using optimal camera settings.

2.5 Selective survival of prokaryotes following a Triton x-100 and proteinase K treatment

2.5.1 Triton survival of stationary phase cultures

Survival of stationary phase bacteria and the coincident lysis of eukaryotic cells was required for the successful usage of the PMA method. Triton x-100 (Sigma Aldrich, Germany) was a possible detergent for this use. The amount needed to sustain the bacterial populations while lysing the weaker eukaryotic cells was unknown, and therefore an experiment was set up to determine the optimal concentration of Triton x-100 to avoid killing the bacterial populations. *E.coli* and *E.faecalis* were used as representatives of both Gram-negative and Gram-positive

bacterial populations in the salmon gut. Cultures were grown overnight in 5 ml BHI-broth (Sigma Aldrich, USA) and checked on a McFarland turbidimeter for circa number of bacteria before being diluted. A value of 4.30 McFarland was used for all dilutions after correcting for the turbidity of the culture medium itself in order to reach a concentration of roughly 10^9 bacterial cells. A volume of 500 µL of the cultures was transferred to new Eppendorf tubes and centrifuged at 10 000 rpm for 10 minutes. The subsequent supernatant was removed, and all tubes were added a treatment solution of either Triton x-100 or 0,85% NaCl/ MilliQ water as controls.

For the samples where Triton was added, a chain of dilution of Triton x-100 was prepared to the concentrations of 1%, 0.5%, 0.25%, 0.1%, 0.050% and 0.025%. Each solution was thoroughly vortexed before transferring to the next. The tubes were incubated at room temperature at 800 rpm for 1 hour to simulate proteinase treatment without killing the bacteria. The treated cultures were transferred to new Eppendorf tubes and added 1:200 Bac Light mix. The samples were placed in the dark for 15 minutes before pictures were taken for counting using UV microscopy. The sample series were analysed using the 0,85% NaCl/MilliQ controls as a basis for the analysis.

2.5.2 Selective survival of exponential phase cultures

A triton-addition experiment was performed to test the effect of Triton x-100 on cells in the exponential phase. From overnight cultures of both *E.faecalis* and *E.coli* grown in 5 ml of BHI-broth at 130 rpm shaking and 37ºC, 50 µL was transferred to a new 5 ml BHI-broth and incubated at the same conditions. The cell cultures were monitored and checked every 30 minutes with a McFarland-apparatus. After reaching 1 McFarland, a clear indication that the cultures were starting to grow and had reached the exponential phase, the cultures were stopped by placement on ice, and the samples were treated and analysed similarly to the stationary phase experiment.

The cultures were also checked for growth rate using the same conditions.

2.5.3 Selective survival of prokaryotes added Triton and proteinase K

Prokaryotic cells growing in the exponential and stationary phase were individually tested with a mixture of 0.025% Triton x-100 and varying concentrations of Proteinase K for post-treatment viability. A 1:2 dilution series of 50 µM Proteinase K concentration was set up, ending at a final concentration of 3.125µM. The samples were treated similarly to the prior triton experiments, visualized using UV microscopy and counted live versus dead cells for each concentration. Control series used as a basis for the analysis were similar to the stationary phase experiment but included a control of pure 0.025% concentration of Triton x-100.

2.6 Salmon intestine incubation

An incubation experiment was set up in order to test if *E.coli* would be inhibited being submerged in salmon intestine, and thereby not being viable for treatment. For this, 100 µl of salmon intestine was inoculated with 100 µl *E.coli* 10^9 culture, together with 800 µl of BHI-broth up to a total volume of 1ml. A control of 100 µl *E.coli* together with 900µl BHI medium acted as a control, and all mixed cultures were incubated at 4ºC overnight. A series of dilutions of each mixed incubated culture was performed, following a 100 µl spread inoculation on BHI-agar. The inoculated concentration for each culture was 10^5, 10^4, 10^3, 10^2 and 10^1. The agar plates were incubated overnight at 37ºC, before being counted the next day.

2.7 Illumina Library preparation and sequencing

An Illumina sequencing library was prepared in order to sequence using the Illumina MiSeq machine (Illumina, USA).

A first step qualitative PCR targeting the 16s rRNA gene using PRK primers was performed using 28 cycles and 2µl of template DNA with the same reagents, controls, settings and machine as described for 2.3.2.2 Qualitative PCR. The samples were checked for the presence of amplicons of the right size on a 1% agarose gel. The samples were further purified using 0.8x volume of 0.1% Sera-Mag beads (Thermo Fisher Scientific, USA) to the volume of DNA

sample. Several washing steps were performed using 80% ethanol whilst keeping the samples on magnets before eluting in an equal volume of nuclease-free H_2O.

To be able to sequence several samples in one run, Illumina adapters were attached to the 16S rRNA fragments by performing an indexing PCR. Combining 8 different forwards and 3 different reverse primers, all 18 samples and controls were barcoded. A concentration of 1x 5x FIREpol Master Mix Ready to Load (Solis-BioDyne, Germany), 0.2µM forward and reverse primer (see Appendix X), nuclease-free H_2O and 2 µl of sample DNA was used for each reaction. Amplification was performed using an initial denaturation at 95ºC for 5 minutes, followed by 10 cycles of 30 seconds denaturation at 95ºC, 1-minute annealing at 55ºC, and lastly 45 seconds elongation at 72ºC. A final hold at 72ºC for 7 minutes concluded the index PCR.

The PCR product quality was checked on a 1% agarose gel before quantifying using Qubit. Quantified samples were pooled together with a maximum volume of 15µl per sample, purified using the earlier described 0.8x Sera Mag 0.1% beads and checked for quality on a 1% agarose gel. The pooled sample was quantified using droplet digital PCR (BioRad, USA) and 6 pM of the pooled sample was loaded to the Illumina MiSeq following Illumina's instructions. A PhiX control was added to the pooled sample to a concentration of 15% prior to loading.

Illumina data was stored as a FASTQ file following the Illumina MiSeq run. The sequencing data was processed from this file using the QIIME pipeline.

2.8 Statistical analysis

Paired two-tailed t-tests were performed to evaluate if the change in qPCR C_t-values for the PMA treated and untreated samples were due to chance or due to an actual effect. This was also done to check for other differences between sample pairs and treatments. The paired two-tailed t-tests were performed using the Excel command $= TTEST(data1; data2;2;1)$, providing a p-value representing the statistical significance regarding the differences in the data evaluated. Lower p-values represented a larger significance of the differences. The calculations were done using Excel (version 16.0.10228.20134) from the Office 365 package.

3. Results

3.1 Initial study of PMA effect on eukaryotic DNA

3.1.1 Simulation of the expected salmon gut using *E.coli*

Initial experiments trying to simulate the expected salmon gut was performed using 100ng of Salmon DNA. Such an amount was too small in comparison to the *E.coli* and it was increased to a concentration of 1000ng for the PMA method. For these experiments, a clear effect of PMA was seen for the bacterial samples. Several other experiments were also performed, all using automatic extraction of the samples. These early automatic extraction experiments showed large scale inactivation of PMA treated live *E.coli* samples, as well as low general DNA yield.

An experiment testing the effect of treating salmon DNA from salmon sperm (10mg/ml) (Thermo Fisher Scientific, USA) and live *E.coli* with PMA was performed to control for the experiments prior. A salmon DNA concentration of 1000ng was used. The 1/10 samples had a total *E.coli* concentration of 5.5×10^8 cells/ml, while the 1/100 samples had a concentration of 5.5×10^7 cells/ml.

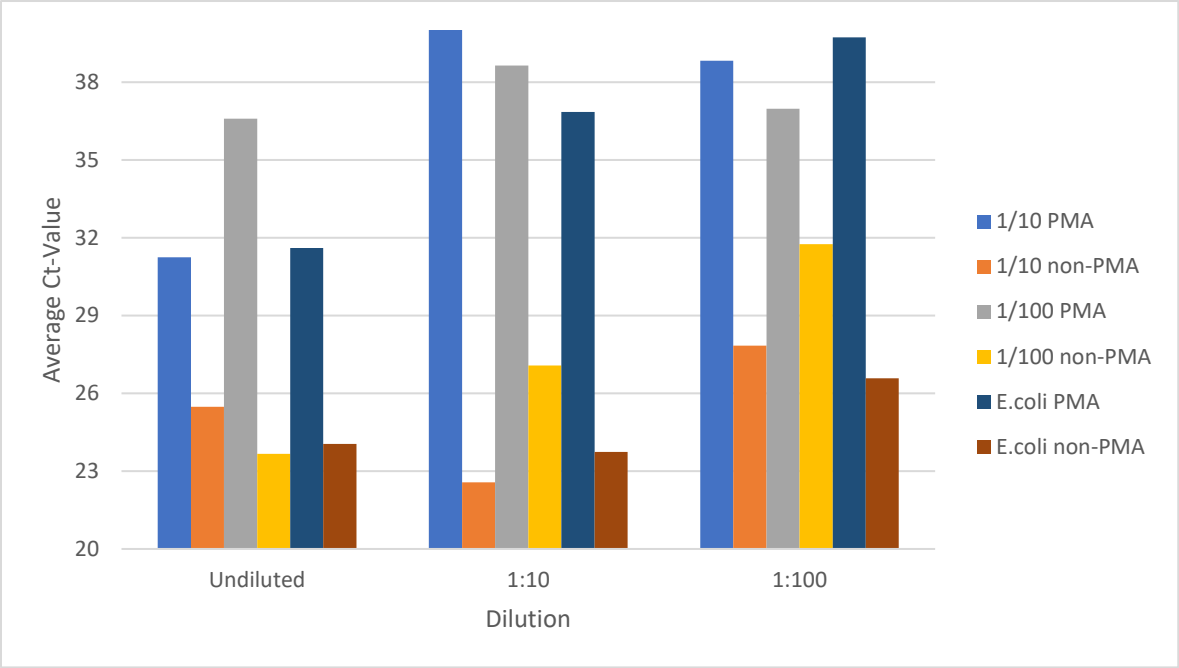

Figure 3.1: 16S qPCR of initial in vitro salmon sperm and *E.coli* samples following treatment of PMA. The bar chart illustrates the average *C_t-value*s following a 16S qPCR for varying samples treated with PMA or not. The average C_t-values were calculated using the triplicates for each sample type after removing clear outliers. The chart is divided into samples which were undiluted, diluted 1:10 and diluted 1:100 from the original concentration after addition of salmon sperm and/or *E.coli*. The different samples, comparisons and their colouring are shown on the right. Equal samples which were either PMA treated or left untreated were compared using their C_t-values. A comparison was also made regarding differences in C_t-values based on dilution.

A clear trend towards a high C_t-value for the PMA-treated samples can be seen by figure 3.1, as well as a low C_t-value for the untreated samples ($p = 9{,}21*10^{-8}$), showing a low DNA content for the PMA-treated and a high DNA content for untreated. Undiluted PMA-treated and untreated samples show a p-value of $8.78*10^{-5}$.

The C_t-values observed between the dilutions were statistically significant, with undiluted against 1:10 (p=0.0363), 1:10 against 1:100 (p=0.0042) and undiluted against 1:100 (p=0.0012).

The similar samples like 1/10 non-PMA and *E.coli* non-PMA differ only slightly from each other for all dilutions, while the 1/100 show a slightly higher C_t-value on average, except for the untreated and undiluted sample. The PMA samples all show a C_t-value of 31 or higher, while the negative controls showed a value of roughly 40. The mean differences between the PMA treated and untreated samples was 9.38 cycles, while the standard deviation between the samples was 6 cycles.

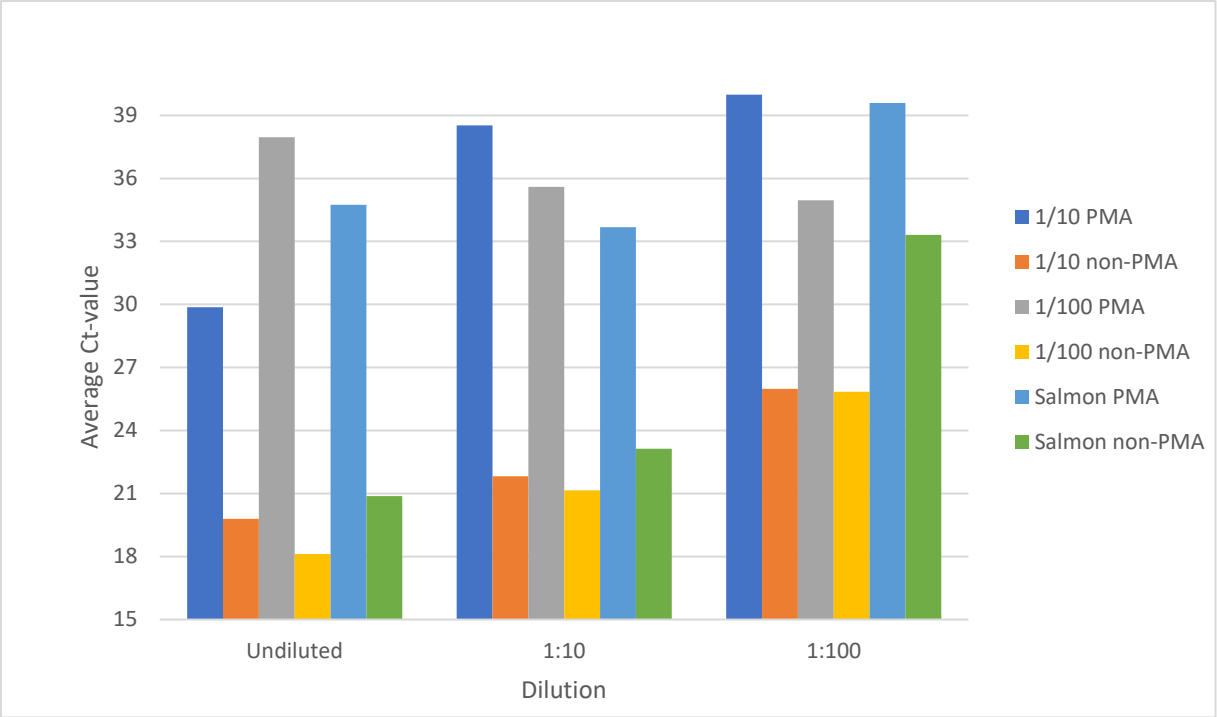

Figure 3.2: 18S rRNA qPCR of initial in vitro salmon sperm and *E.coli* samples following treatment of PMA. The bar chart illustrates the average 18S rRNA C_t-values of PMA-treated or untreated samples. The average C_t-values were calculated using the triplicates for each sample type. For outlier replicates or failed replicates, average C_t-values was calculated using the remaining replicates. The chart is divided into 3 parts for each dilution, and further into different sample types as shown on the right with varying colours. A comparison between PMA treated samples and untreated samples was made, as well as a minor comparison in regard to the dilutions.

A t-test examining the differences between PMA and untreated samples for the 18S samples yielded a p-value of $7.35*10^{-13}$, showing there is a clear trend towards a higher C_t-value for the PMA-treated samples as opposed to the untreated counterparts. The undiluted samples as seen in figure 3.2 yielded C_t-values in the range of 18 to 21, while their counterparts yielded C_t-values in the range of 30 to 37. Further dilutions seem to display the same trend as 16S but show higher C_t-values on average for all treatment types and samples. A mean increase of 11.51 cycles was observed for the PMA treated 18S samples in comparison to the untreated, as well as a standard deviation of 5.21 cycles.

3.1.2 Pure *E.coli* control

Due to the loss of *E.coli* cells either before or during the DNA extraction, a control experiment was set up including dead *E.coli* cells to see if loss of E.coli DNA was a result of dead cultures. The *E.coli* control included dead *E.coli* cells instead of salmon DNA, but samples were treated equally to the prior experiments with salmon sperm.

In this experiment, mechanical and chemical lysis was also tested against only chemical lysis. For mechanical lysis, the samples were processed in a Fastprep 96 machine prior to extraction. The samples were analysed using Qubit and qPCR, and the live and dead *E.coli* cultures were quantified using a McFarland densitometer.

The McFarland density was measured for the *E.coli* after growth at 3.58, including the medium in which it was grown at 0.37, yielding a McFarland density of 3.21. The density of the same culture of *E.coli* after killing was measured at 4.40. The 0.85% NaCl solution used to resuspend the culture after killing and centrifuging was measured to 0.12, yielding a total McFarland density of 4.28. The killed *E.coli* culture thereby increased the turbidity of the fluid, resulting in a higher McFarland value.

Automatic extraction of the samples yielded high C_t-values, however, all samples showed similarities between the mechanical and chemical lysis. Due to the C_t-values approaching the negative control, a second experiment was set up, testing the manual extraction of *E.coli* cells instead.

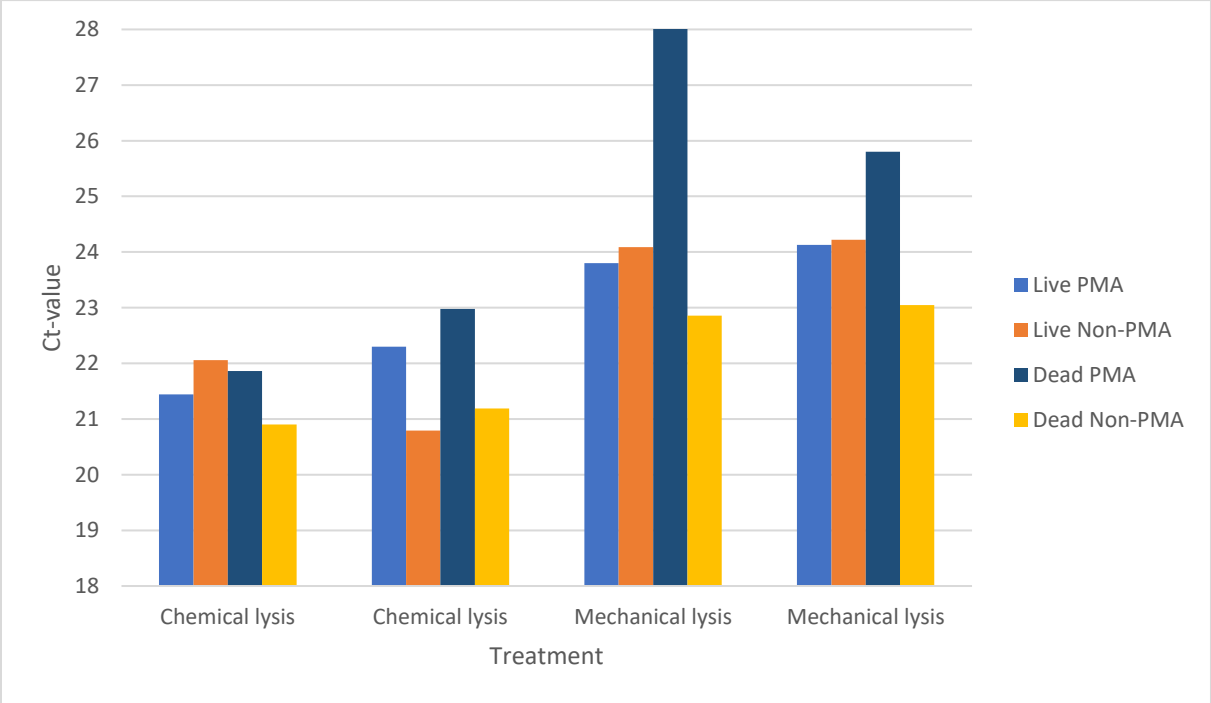

Figure 3.3: qPCR of manually extracted *E.coli* with varying lysis methods. The figure shows a bar chart illustrating the C_t-values from qPCR of manually extracted *E.coli*. The varying samples of *E.coli* were treated using either chemical lysis or mechanical lysis in conjunction with the chemical lysis. Additionally, the samples were either treated with PMA or not. The duplicates are depicted individually to show the differences equal samples show. The exact C_t-values for the samples are shown together with an explanation for the varying samples on the bottom of the bar chart together with a colour code. Comparisons were made between PMA treated and untreated samples of both lysis methods, as well as between the lysis methods.

As seen by figure 3.3, C_t-values seem generally similar following qPCR of the manually extracted E.coli. PMA-treated and untreated yielded very similar values, with an observed mean difference of 1.67 and a standard deviation of 2.56 (0.86 and 1.24 excluding the high-Ct dead replicate). The exception is the first mechanically lysed duplicate of dead E.coli cells, with a difference of roughly 7 cycles. PMA treatment on these samples yielded no statistically significant differences with a p-value of 0.108 (p=0.245 excluding the dead PMA samples).

Mechanical and chemical lysis differ somewhat with a mean difference of 3.08 and a standard deviation of 2.20 (2.33 and 0.60 if the dead outlier is excluded). Mechanical lysis generally yielded a higher C_t-value on average, as seen by a p-value of 0.0055. Calculating the p-value without the mechanically lysed dead *E.coli* yields a p-value of $4.92*10^{-5}$. The differences between the PMA treatment of either live or dead *E.coli* were not statistically significant.

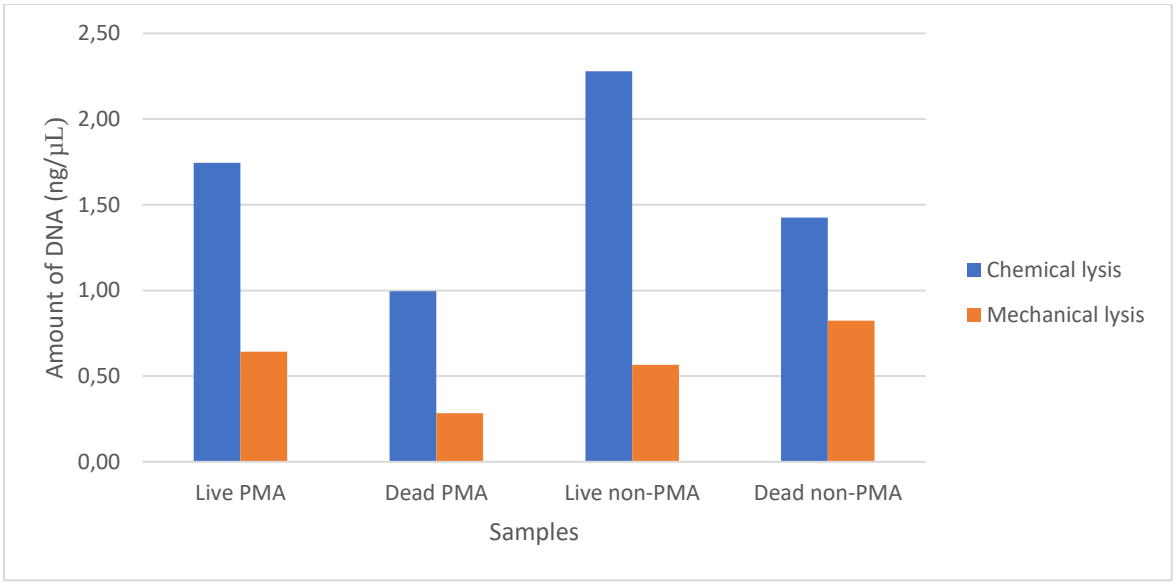

Figure 3.4: Bar chart of DNA concentrations following Qubit analysis of manually extracted samples. The bar chart represents the DNA concentration for live and dead *E.coli*. The amount of DNA content present for either chemical or mechanical lysis is shown for each sample on the y-axis (ng/µL), and a higher bar represents a higher DNA content. The lysis used is given different colours as seen on the right, and the specific treatment is given on the x-axis. A comparison between PMA treated lysed samples was made against their untreated counterparts, as well as the difference inbetween the lysis methods.

Qubit analysis of the samples showed that the largest amount of DNA content was provided by chemical lysis, while mechanical lysis provided a DNA content of roughly 1ng/µL less than the DNA content of chemical lysis, which can be seen by figure 3.4. Positive control yielded a higher DNA content than the samples, while negative control was undetected.

3.1.3 *E.coli* and salmon DNA control

A similar experiment was set up to test the same conditions as *E.coli* on salmon sperm. The experiment was set up including live and dead *E.coli* cells, salmon DNA from salmon sperm (1000ng), and combined samples of live *E.coli* and salmon.

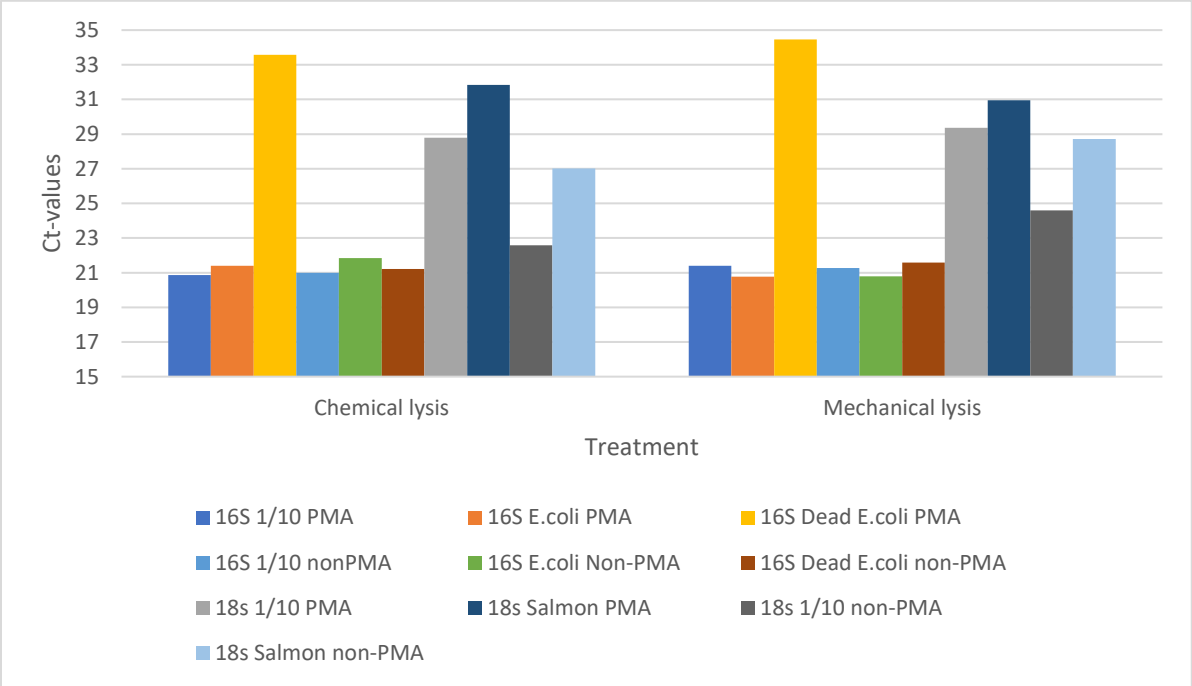

Figure 3.5: Manually extracted salmon sperm control qPCR bar chart: The bar chart illustrates the differences in C_t-values between chemically lysed samples and mechanically lysed samples prior to manual DNA extraction. The bar chart is based on average values calculated from the duplicates for each sample type. Samples are in addition either treated with PMA or not. The explanation for each bar is given underneath the bars, read from left to right. The graph is a combination of 18S and 16S, with the first 6 bars being viable 16S samples and the remaining bars being viable 18S rRNA samples. 16S and 18S samples were compared in regard to their individual inactivation by PMA versus no treatments for both lysis methods.

16S rRNA generally yielded no significant differences in C_t-values, both between the PMA-treated and untreated as well as between chemical and mechanical lysis. Most 16S rRNA samples yielded C_t-values between 20 and 22, with the only exception being PMA-treated dead *E.coli* cells which yielded C_t-values of roughly 34 for both lysis treatments. Looking at figure 3.5, 18S rRNA showed higher Ct-values than the 16S rRNA, yielding C_t-values in the range of 28 to 31 for the PMA-treated samples. Untreated samples yielded lower C_t-values, with the 1/10 yielding the lowest C_t-values at roughly 22 and 24 for chemical and mechanical lysis respectively while pure salmon yielded roughly 27 and 28 for the same treatments. Statistics indicate there is a significant difference between the PMA-treated and untreated 18S samples with a p-value of 0.0121.

Following a second qPCR, the samples containing PMA were completely lost resulting in null-values and C_t-values higher than 35. The untreated samples showed the same trend as the experiment prior.

UV microscopy was performed to check the culture viability of the two distinct qPCR cultures following diverging results. The UV microscopy was performed using Bac Light Viability staining, and the two cultures were photographed.

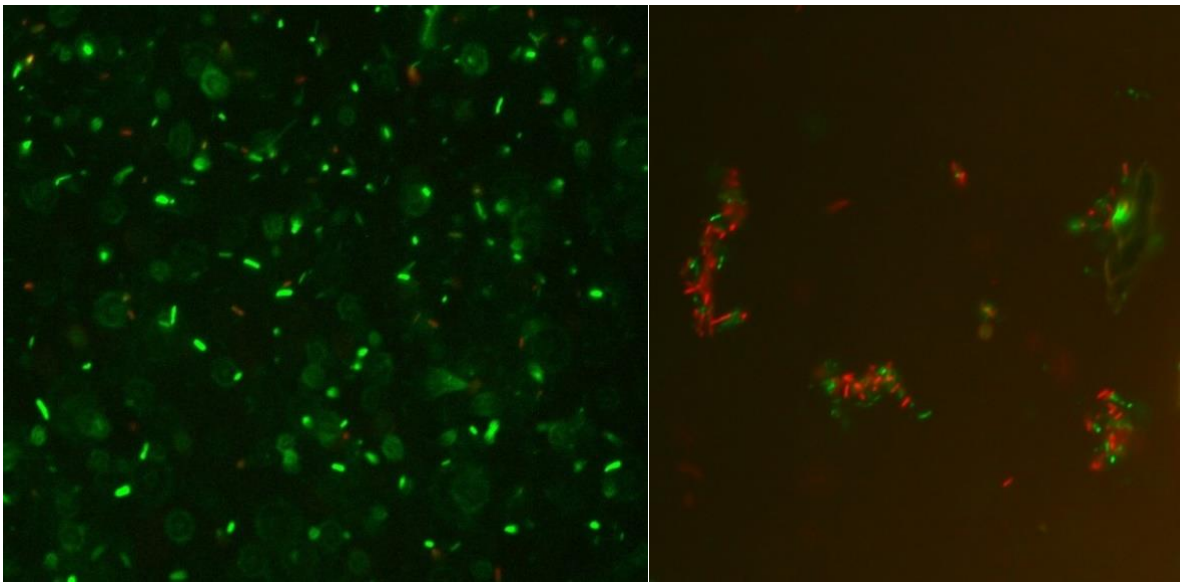

Figure 3.6: Medium comparisons of qPCR analysed cultures. Two distinct live E.coli cultures were treated with BacLight bacterial assay and photographed to understand the differing results after being subjected to a PMA treatment. The left culture did not differ between PMA-treated or not, while the right culture differed greatly.

There is a distinct difference between the two cultures as seen in figure 3.6. The first culture is distinctly green coloured with few red cells, while the second culture is distinctly red with few green cells. The right culture shows more aggregated cells clumped together, while the left culture shows dispersed green cells.

3.2 Salmon intestinal pilot

3.2.1 Pilot optimisations

Following the initial testing of the PMA method, improvements to the method were required. There were uncertainties regarding the viability of the cultures, as well as a need for a viable lysis protocol for eukaryotic cells. A series of optimisation experiments were performed to evaluate the growth and use of the bacteria, as well as the survival of these bacteria under lysis conditions.

3.2.1.1 Bacterial growth rates

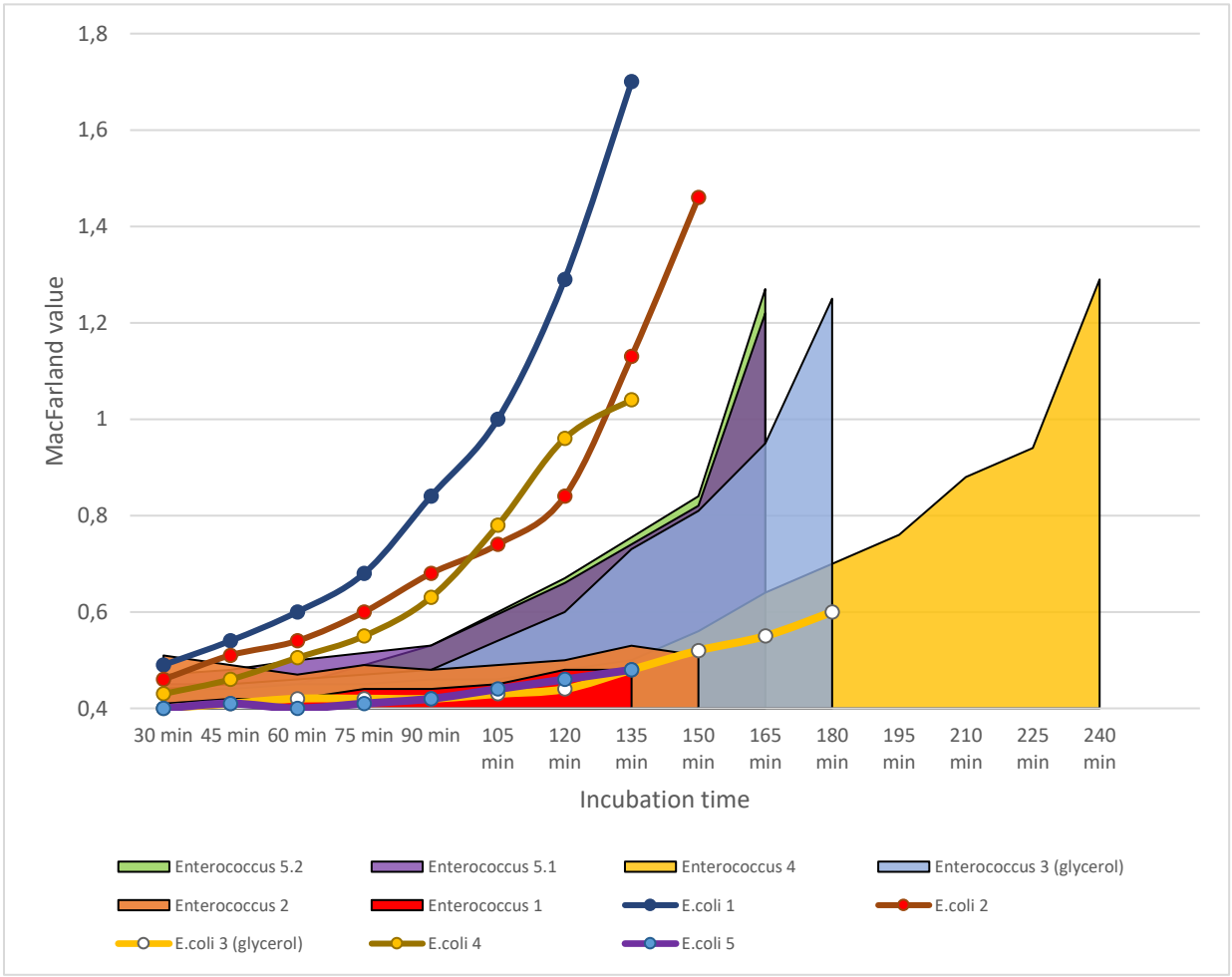

Figure 3.7: Growth rates of *E.faecalis* and *E.coli*. The figure depict the growth rate of *E.faecalis* and *E.coli* over time following varying inoculation from either stationary or stopped exponential cultures. The cultures inoculated with exponential cultures were *E.coli* 3 and *E.faecalis* 5.1 and 5.2. The McFarland value of each culture at a certain time is given on the y-axis, while the x-axis show the time the cultures were analysed with the McFarland apparatus.

Looking at the growth curves for *E.coli* and *E.faecalis* given in figure 3.7, it is possible to see that *E.coli* 1, 2 and 4 grew optimally from the start, showing a clear increase for each sampling time. *E.coli* 3 and 5 grew exceptionally slow and did not even begin to grow properly past 3 hours.

E. faecalis had trouble growing from the stationary cultures, showing an incredibly long lag phase for cultures 1 and 2 while still managing to grow overnight to a McFarland of 3.4 and 6.58 respectively. The growth of *E.faecalis* taken from a 1 McFarland culture stored at -80ºC with glycerol resulted in a much shorter lag-phase, as seen by culture 3. Following this culture overnight resulted in a McFarland of 6.58 in stationary phase. Furthermore, the resulting culture from this stationary phase yielded a long lag phase as seen by culture 4. This is fairly similar to the 1st and 2nd culture of *E.faecalis*. The 5.1 and 5.2 cultures were grown in parallel from a 1 McFarland cultures stored at 4ºC overnight and showed just like culture 3 a quick growth rate.

3.2.1.2 Optimisation of Triton concentration

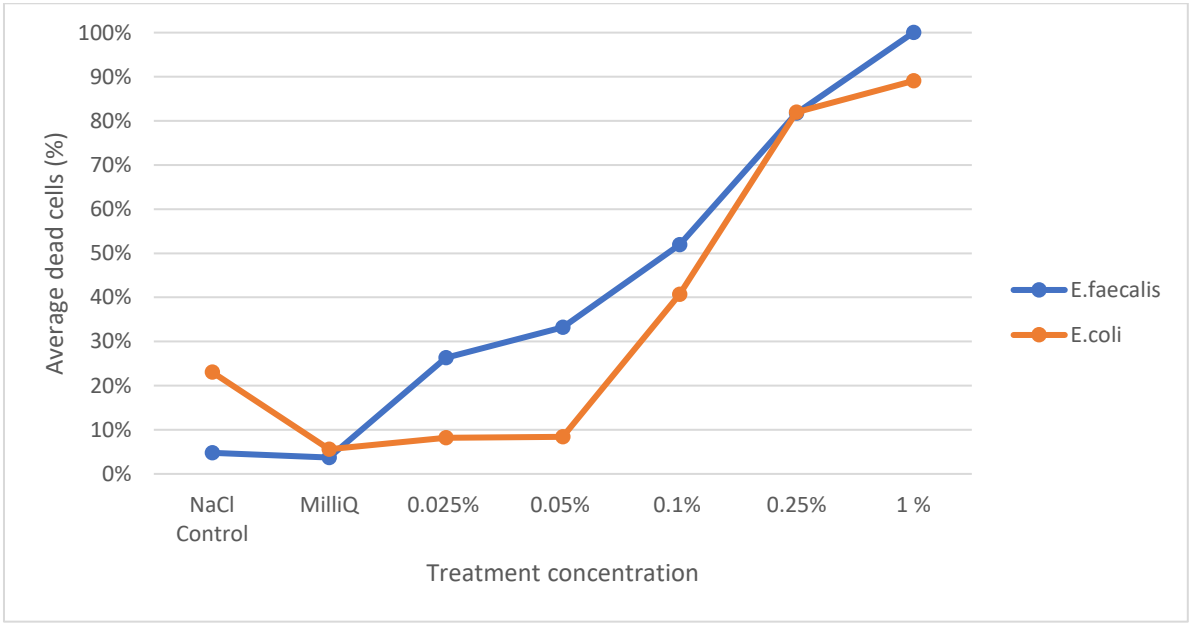

Figure 3.8: Percentage of dead cells following Triton x-100 treatment of stationary cultures. The graph is an illustration of the number of live *E.coli* and *E.faecalis* cells versus dead cells counted for each Triton x-100 treatment. Pictures were taken from around the cultures using UV microscopy, and the cells were later counted from the pictures. The dead cells were then divided by the number of cells present for each picture, and further calculated to an average percentage for each treatment based on a number of replicates, thereby showing average dead cells for each treatment in percentage on the y-axis. The x-axis shows the concentration of added Triton x-100 during the treatment. Controls of MilliQ water and a 0.85% NaCl solution is also shown. An explanation of which line representing which bacteria is given on the right.

The number of dead cells for the controls were negligible for *E.faecalis*, while the MilliQ control of *E.coli* showed a much higher dead cell count at 23% which can be seen in figure 3.8. The MilliQ control of *E.coli* varied much, having a dead cell content of 40% for one of the controls and 6% for the other.

Increasing the concentration of Triton resulted in a much higher dead cell content as soon as 0.025%, having a content of 26% dead cells. Increasing further resulted in a steadily growing percentage of dead cells, up to 100% dead cells at 1% Triton concentration. The *E.coli* did not see much change until a triton concentration of 0.1%, jumping from 8% to 41% dead cells, and further increasing to 82% dead cells for the 0.25% Triton concentration.

For the exponential phase, *E.faecalis* showed even less resistance, resulting in a 100% death count for almost all treatment concentrations. *E.coli* was able to survive concentrations of up to 0.1% Triton with a 100% survival rate, and perhaps higher since no higher concentrations were tested.

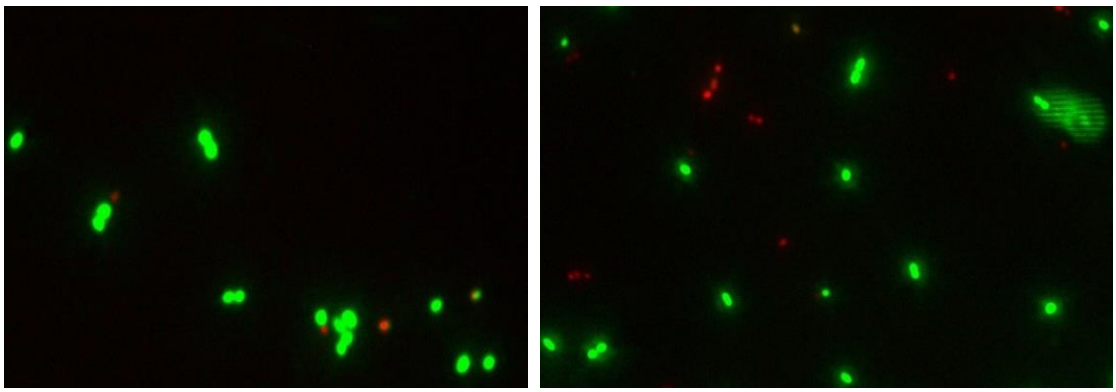

Figure 3.9: Live/dead fluorescence microscopy of E.faecalis after proteinase K treatment. The pictures represent *E.faecalis* treated with 50 µg/ml proteinase K and 6.25µg/ml respectively before live/dead staining. The green cells represent live cells, and the red cells represent dead cells.

Furthermore, an optimal concentration of *Proteinase K* together with 0.025% Triton x-100 was tested, but quantification was difficult to establish. It is possible to see by figure 3.9 that the 50 µg/ml concentration of proteinase K resulted in almost only living cells, while the 6.25 µg/ml concentration resulted in a mix of live and dead cells, with live cells slightly dominating. For the highest concentration, a veil of red was observed, including what could be best described as cellular residual floating around. This was not observed for the lowest concentration. The same was true for *E.coli*, but these cells were not as readily affected. The lowest concentration of proteinase K yielded what was thought to be the most live culture following treatment for *E.faecalis*.

3.2.2 Pilot study of PMA effect using *E.coli*

To reduce the density of the sample, the intestinal sample was diluted using an equal volume of Milli Q water prior to treatment. The samples included in the pilot were live and dead *E.coli*, salmon intestinal samples of either live or dead *E.coli* and a pure salmon intestine sample. These were either treated with PMA or left untreated as controls.

An *E.coli* overnight culture was spun down in a microcentrifuge and added to a final concentration of 10^7 *E.coli* cells/ml together with 0.025% Triton x-100 and 6.25µg/ml concentration of Proteinase K to a total volume of 100 µL. For samples only including either *E.coli* or salmon intestine, a larger volume of Milli-Q water was added to compensate. Due to the dense nature of the intestinal samples, all pipette tips used were cut near the end as to not introduce differences. During PMA treatment, the samples were mixed several times to avoid intestinal sedimentation. The samples were mechanically lysed and manually extracted before analysing with qPCR.

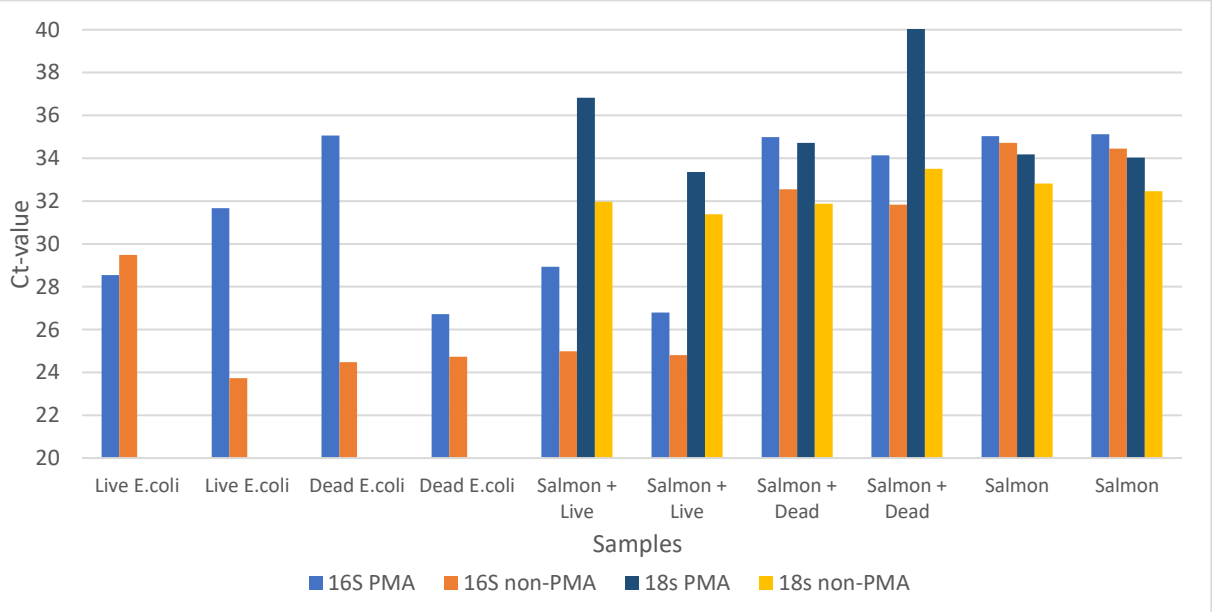

Figure 3.10: 16S qPCR following Pilot study of the PMA effect on real Atlantic salmon using *E.coli.* The bar chart depicts the effect of PMA treatment in C_t-values on pilot samples taken from a euthanized Atlantic Salmon. Due to the differing results in between sample types, the samples were shown individually. The bar chart shows both 16S and 18S rRNA qPCR values. For samples containing only bacteria, 18S rRNA values were omitted since the samples did not contain any eukaryotic DNA. These were similar to the negative control. Due to uneven replicates, each sample is shown as an individual bar. An explanation for the samples is given underneath the chart. 16S and 18S inactivation of PMA samples was compared against their non-treated counterparts, as well as inactivation in comparison to each other. A comparison of dead *E.coli* cell inactivation and live *E.coli* cell inactivation was also made.

The 16S Pilot qPCR yielded generally lower C_t-values for the PMA-treated samples than the untreated (p=0.0214) which can be seen in figure 3.10. However, one of the live *E.coli* samples yielded a higher C_t-value for the untreated sample. The samples containing only salmon intestine showed a high C_t-value for both treated and untreated. Several samples approach the negative control, such as one replicate of the dead *E.coli* and most of the intestinal salmon samples. Samples containing dead *E.coli* seem in most part to differ more than the live E.coli, though some of the PMA-treated live and dead E.coli differed enormously from their counterparts, with a difference of almost 8 cycles for the live and 10 cycles for the dead sample. The p-value testing the effect of PMA for only *E.coli* is 0.162, due to the large differences in between both the live and dead *E.coli.*

For the 18S qPCR, samples treated with PMA generally yielded a higher C_t-value than their counterparts (p=0.0152). They did although yield a relatively high C_t-value, with a low of 31 and a high of 40. Even though the PMA against untreated yielded a low p-value, the results are still slightly uncertain due to the high C_t-value. Negative control yielded a C_t-value of 38.

A second experiment was set up including *E. faecalis* while adding another replicate for each treatment, resulting in 3 replicates per treatment. The salmon intestine from the first pilot was diluted 1:2 to check if the density of the salmon intestine represented a bias towards the treatment, while also being required to dilute due to lack of salmon intestine. Samples were processed using automatic DNA extraction.

16S qPCR showed a general inactivation of PMA-treated samples. For pure E.coli, live E.coli showed a slightly larger inactivation, while the pure *E. faecalis* showed a larger inactivation for the dead cells. For the samples spiked with either *E.coli* or *E.faecalis*, the dead *E.coli* cells showed the largest inactivation differing from 30 to 26 by 4 cycles. The live cells however differed only by 2 cycles. The spiked *E.faecalis* showed an inactivation for both live and dead spiked cells by 3 cycles, while the pure salmon samples showed the largest inactivation with a difference of roughly 5 cycles. 18S rRNA, however, yielded extremely high C_t-values close to the negative control and were deemed almost void of 18S DNA, both for PMA treated and untreated samples.

3.3 PMA inactivation for Atlantic Salmon sample DNA

3.3.1 Use of a finalized PMA method on two Atlantic salmon samples (F1&F2)

A final experiment was set up to test the practical use of the combined PMA and Triton treatment on spiked salmon intestinal samples. Two Atlantic salmon of roughly 500g were procured from the Fish Farming Laboratory of NMBU. After procurement, the samples were stored 4 and 5 days at 4 ºC respectively before further treatment. Unlike the pilot experiment, the intestinal scrape was processed undiluted to check if dilution was the cause of low DNA content for the 18S. The samples included triplicates of PMA-treated and untreated, as well as variations for each sample type between Triton-treated and untreated.

Triton and PMA treatment was performed similarly to the previous pilot. The samples were processed using automatic DNA extraction, before being analysed using Qubit and qPCR. The samples were also visualized using live/dead BacLight staining in conjunction with UV microscopy both before triton-treatment and after as a control for bacterial viability.

Following UV microscopy of the samples before the treatment, a red veil was observed in the background, most likely representing free DNA from the salmon epithelia or from dead bacteria. Microscopy before the treatment also showed many live and green cells, both for the salmon intestinal samples and the pure *E.coli*. The green bacteria from the pure *E.coli* were morphologically like *E.coli*, while the bacteria from the pure salmon samples were mostly round or elongated and truncated in the middle. The mostly round bacteria showed self-motility.

After triton-treatment, the samples still showed many live cells while retaining the red veil in the background. The number of live cells present seemed to decline slightly.

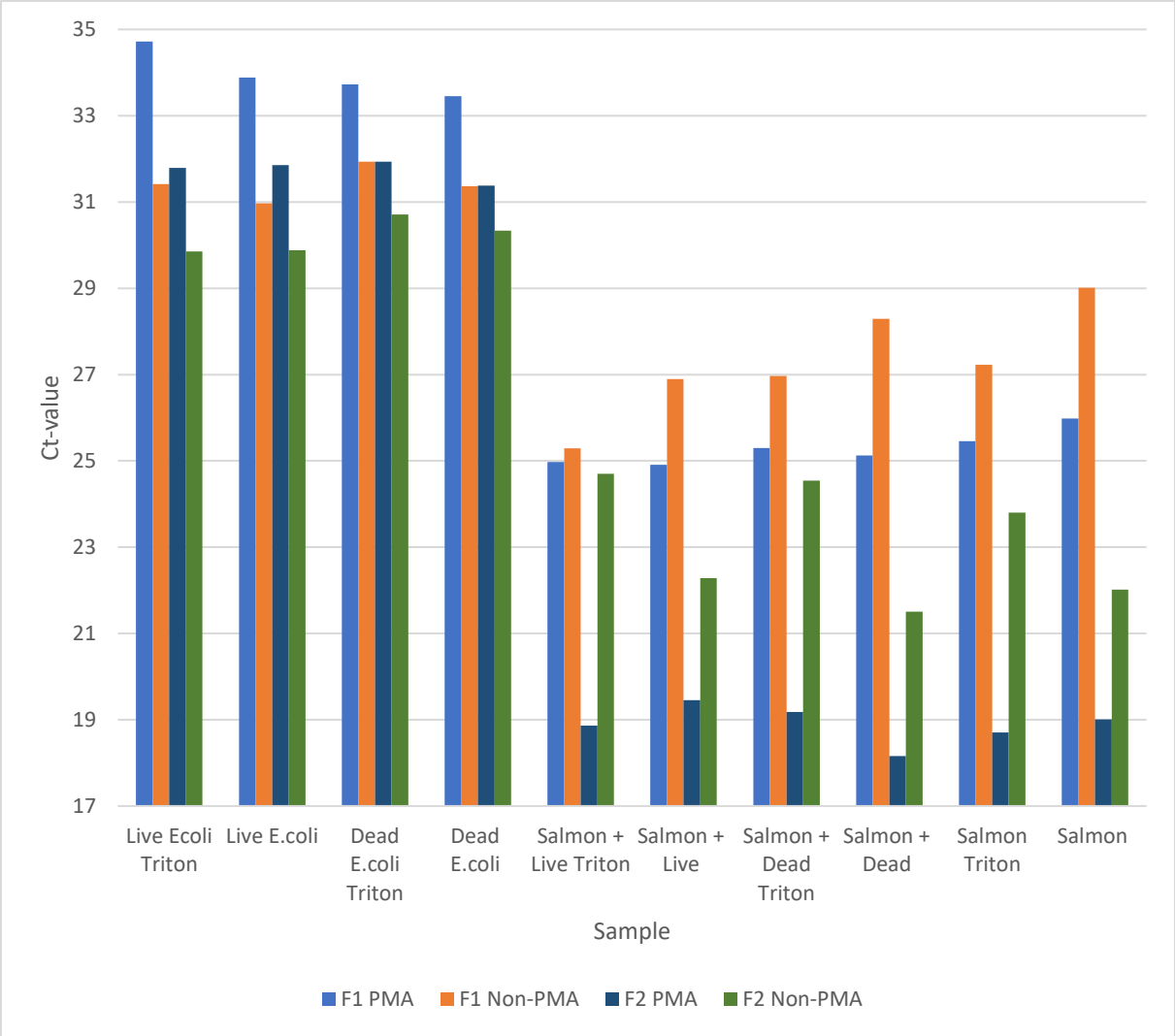

Figure 3.11. Bar chart of 16S qPCR for the F1 and F2 pilot fishes. The bar chart represents the average 16S qPCR C_t-values for the fishes included in the experiment, calculated from the triplicates for each sample. The bar chart further depicts the effect of PMA treatment on the samples, as well as the effect of Triton treatment or no treatment. F1 and F2 represent the fishes used in the study, with F1 representing the first treated fish, and F2 representing the latter. The type of fish and PMA treatment is explained underneath, while the type of sample and Triton treatment is shown on the x-axis. PMA treated living *E.coli* and dead *E.coli* was compared against the respective counterpart, as well as between pure bacterial samples and samples including salmon intestine. The treated salmon samples were also compared against their non-treated samples, as well as the differences between samples added *E.coli* or not. In addition, the differences between the analysed fishes were compared, both for the inactivation and the innate differences.

As figure 3.11 shows, a large difference can be seen between the samples containing only *E.coli* and the samples containing salmon intestinal material in addition ($p=3.99*10^{-22}$), in which pure *E.coli* yielded very high C_t-values at an average of 31.87 in comparison to the salmon intestine, which yielded 23.65 cycles. The F2 fish yielded generally lower C_t-values, differing with 4 cycles between 28.8 for the F1 and 25 for the F2 fish.

For samples containing only *E.coli,* PMA-treated yielded a lower DNA-content than their untreated parallels ($p=1.25*10^{-10}$).

However, every sample containing salmon intestine yielded a higher DNA content for the PMA-treated samples at C_t-values as low as 18 for the treated and 21 for the untreated, contradicting the trend from the pure *E.coli* ($p=5.23*10^{-12}$). No real trend of difference could be seen between Triton-treated and the untreated samples. A higher DNA content for the Triton-treated F1 fish could be seen, while the F2 fish had a higher DNA content for the untreated.

For the 18S rRNA qPCR however, little to no DNA was present. There seem to be small differences between the F1 and F2 fish, but these differences are very small, differing at most by 4 cycles though this is in the upper part of the C_t-values. This is further indicated by a p-value of 0.724. Almost all samples yielded C_t-values of no less than 31, except for the Triton-treated non-PMA F2 fish, which yielded a C_t-value of 26. Some replicates even resulted in a C_t-value of 40 while the negative control had a Ct-value of 38. Even though the samples showed high C_t-values, the mean PCR efficiency was 1.798. A trend towards a higher C_t-value for the untreated samples can be established from the F1 samples with a p-value of 0.0044, though the F2 show no such differences with a p-value of 0.85.

A control sample of salmon sperm 1000ng concentration was added prior to the automatic extraction, resulting in a C_t-value of 14.45. This is quite similar to the positive control of 1000ng salmon sperm DNA which was added as a qPCR control, yielding a C_t-value of 14.15. A negative control was also added prior to the DNA extraction, yielding a C_t-value of 37.2, quite similar to the negative control.

The *E.coli* cells were also incubated overnight in the salmon intestinal scrape from the two fishes along with a control. No clear inhibition could be seen, with the F1 and F2 fishes resulting in roughly equal amounts of colonies on the agar plates for most of the dilutions in comparison to the *E.coli* control.

3.3.2 E.coli specific qPCR

Due to the long storage at 4ºC, an *E.coli* specific qPCR was also performed as described to remove the possible background bias introduced by growth during this time.

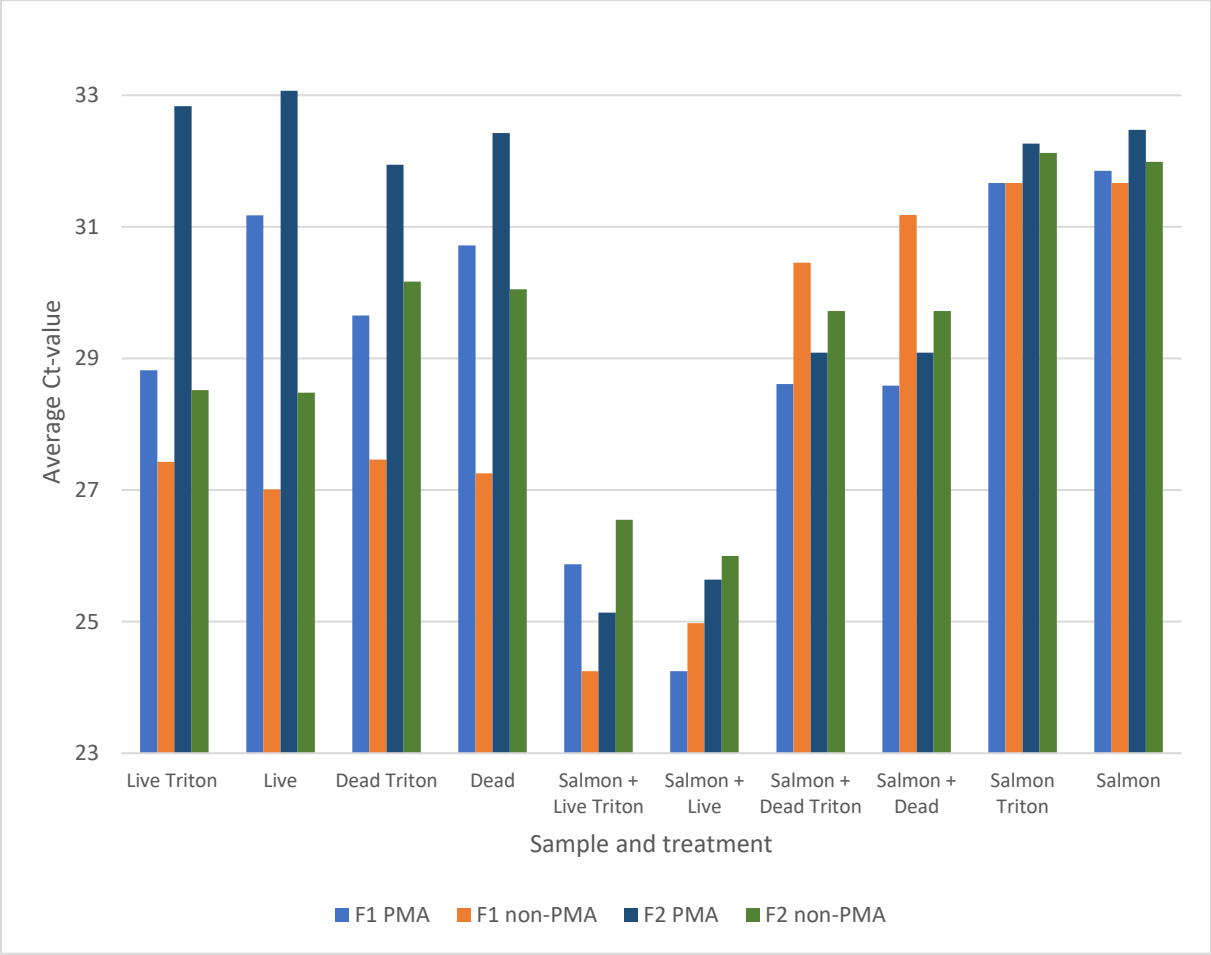

Figure 3.12: *E.coli* specific qPCR bar chart of Salmon Pilot F1&F2. The bar chart depicts the effect of PMA and Triton treatment on samples of pure *E.coli* and spiked salmon intestine spiked with *E.coli*. The *E.coli* used in the study was either live or dead. All sample types and treatments can be seen on the x-axis, while the y-axis shows the average C_t-values of each sample type calculated from all viable triplicates. The explanation for the fishes used and if the samples were PMA-treated or not is given on the right. PMA treated living *E.coli* and dead *E.coli* was compared against the respective counterpart, as well as between pure bacterial samples and samples including salmon intestine. The treated salmon samples were also compared against their non-treated samples, as well as the differences between samples added *E.coli* or not, such as the pure salmon samples. In addition, the differences found between the analysed fishes were compared, both for the inactivation and the innate differences. Effect of Triton was also evaluated using the difference in C_t-values.

Contrary to the prior general 16S qPCR, the *E.coli* specific qPCR which can be seen in figure 3.12 yielded lower C_t-values for the pure *E.coli* at a value of 28.82 for the F1 triton and PMA-treated live *E.coli*, while the F2 fish yielded higher C_t-values than the F1 at roughly 33 cycles for the same treatment. The two fish samples showed a clear difference between each other

for the *E.*coli samples ($p=1.324*10^{-8}$), but not as much for the salmon intestine samples ($p=0.035$). The F1 and F2 fishes showed a general inactivation of prokaryotic DNA following the PMA treatment ($p=7.86*10^{-10}$). The spiked live and dead *E.coli* showed a lesser difference, though against less DNA for the untreated samples ($p=0.013$). Triton-treated live and dead *E.coli* yielded generally lower C_t-values than their untreated counterpart ($p=0.0768$), though the salmon intestine showed absolutely no such trend ($p=0.99$).

The salmon intestine spiked with live *E.coli* yielded C_t-values of roughly 24 to 26, much lower than the pure bacterial samples ($p=8.62*10^{-6}$). Contrary to these live spiked samples, the salmon intestine spiked with dead *E.coli* showed a higher Ct-value at roughly 28 to 30 ($p=3.86*10^{-13}$). The pure salmon intestine yielded steadily C_t-values of 32. The spiked dead samples showed the same trend as the general 16S qPCR with a decrease in C_t-value for the PMA-treated samples ($p=0.00175$). The PMA-treated and untreated spiked samples, however, did not differ by more than at most 2 cycles. No real difference was observed for any of the other Triton-treated and untreated samples.

3.3.3 Illumina sequencing of pilot samples

Some samples from the F1 and F2 fishes were selected to be sequenced using the Illumina MiSeq Machine (Illumina, USA). The exact samples chosen were based on likeness to the average of each treatment triplicate containing salmon intestine and live *E.coli*, as well as pure salmon intestine.

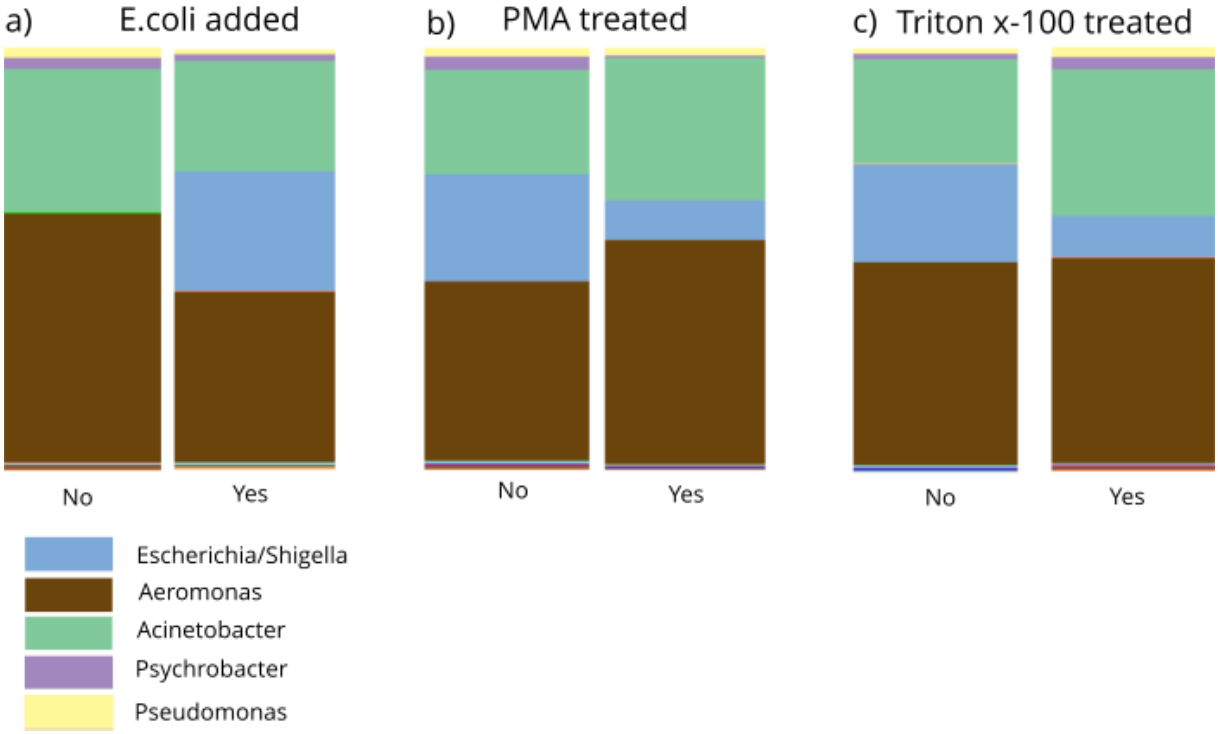

Figure 3.13: Relative diversity of salmon intestinal samples based on metadata. The bar chart showcases the relative core species abundance of the salmon intestine and the varying treatments. The dominant bacterial species are displayed in the bottom part with their respective colours. The bar charts are divided into three parts, where a) shows relative species abundance for samples added *E.coli* or not, b) shows relative species abundance based on if samples were PMA-treated or not, and c) shows relative species abundance based on if samples were Triton x-100 treated or not.

Illumina sequencing data for the two fishes was analysed based on their metadata. As seen for a) of figure 3.13, samples added *E.coli* show a clear blue part of the bar, a colour representing the *Escherichia/Shigella* species. However, looking at the part of a) where no *E.coli* was added, it is impossible to spot even a slight blue colour. Furthermore, *Aeromonas*, *Acinetobacter*, *Psychrobacter* and *Pseudomonas* decrease in relation with the addition of *E.coli*.

Looking further at b), PMA-treated and untreated samples were compared against each other. For untreated samples, *E.coli* constitute a major part of the relative abundance, but treatment

with PMA seems to decrease the relative abundance of *E.coli* quite a bit. For the PMA-treated samples, a decrease is also seen for *Psychrobacter* and *Pseudomonas*, while *Acinetobacter* and *Aeromonas* increase in relative abundance compared to the other bacteria. This is also true for the Triton-treated which can be seen by c), except for *Psychrobacter* and *Pseudomonas* which increase in relative abundance with Triton treatment. No other major bacterial species can be seen for the salmon samples.

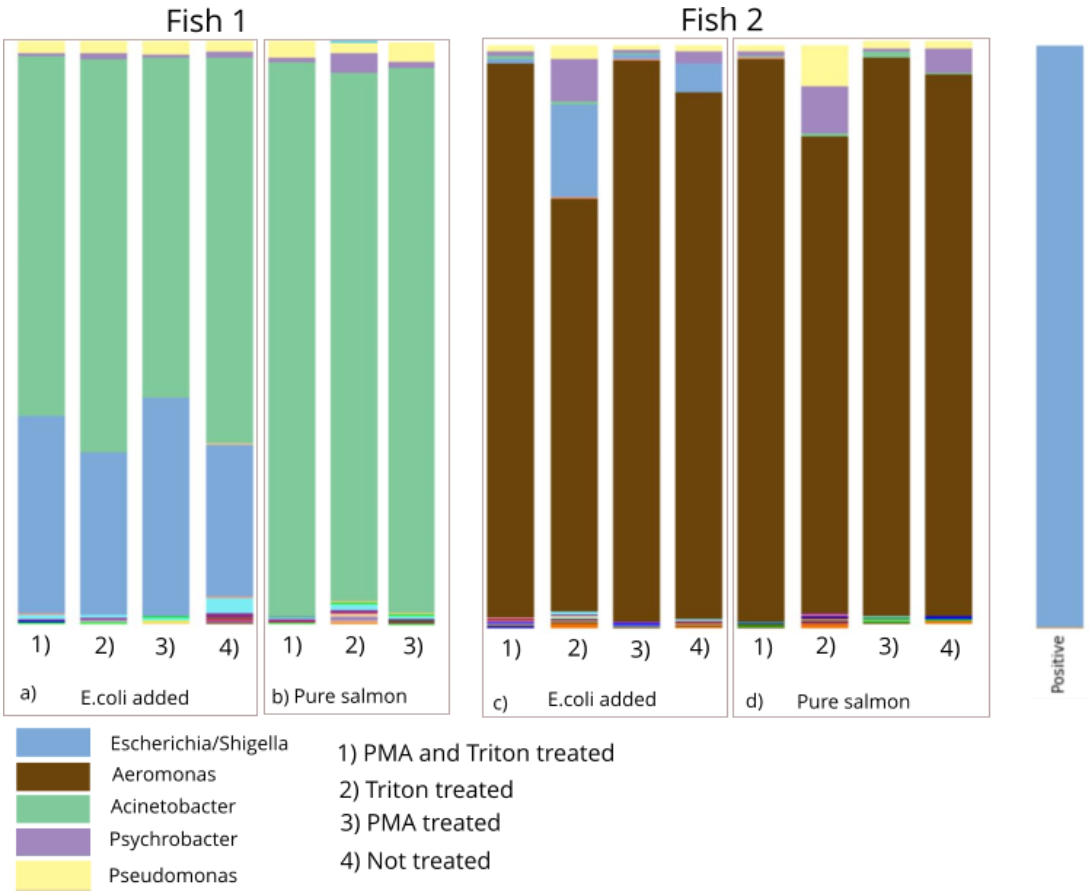

Figure 3.14: Relative microbial diversity for salmon intestinal samples. The bar chart shows relative species abundance for each sample sequenced for the two fishes. The chart is divided into a), b), c) and d), with each division showing whether the bar is pure salmon intestinal scrape or includes *E.coli* for both fishes. The chart is further divided into 1), 2), 3) and 4), with each number corresponding to a varying treatment of either PMA, Triton, both or nothing, as shown below the chart. The dominant bacterial species are displayed in the bottom left part with their respective colours. Pure *E.coli* DNA was included as a positive control, which can be seen furthest to the right. Negative control yielded no sequences. The untreated pure F1 salmon sample did not yield enough sequences to make it through the analysis pipeline and is therefore not shown.

Following the Illumina data analysis of the individual samples, there is a clear difference between the two salmon fishes. As seen in figure 3.14, the F1 fish is clearly dominated by *Acinetobacter*, with *E.coli* being the other major constituent for the samples where it was added, given by a). Furthermore, *Pseudomonas* and *Psychrobacter* show a low relative abundance of all the F1 samples. The combined PMA and Triton treatment and purely PMA

treatment shows the largest relative abundance for *E.coli,* while the purely Triton-treated and the untreated sample show an equal abundance of *E.coli*. For samples where *E.coli* was not added, purely Triton-treatment seem to show the most diverse abundance and therefore the lowest relative abundance for *Acinetobacter*.

For the F2 fish, it can be seen by the pure salmon of d) that *Aeromonas* is the dominant bacterial species, with *Psychrobacter* and *Pseudomonas* being the two other constituents. The *Acinetobacter* dominating the F1 fish is barely visible. For the combined treatment of Triton and PMA for the samples containing *E.coli,* there is barely any *E.coli* present. This is also true for the PMA-treated sample. Triton-treated and untreated samples retain the added *E.coli*, but to a lesser extent for the untreated. The Triton-treated also display the most diverse abundance, with *Psychrobacter* and *Pseudomonas* constituting a much larger percentage of the samples than for any other.

4. Discussion

4.1 PMA effect

PMA treatment of in vitro samples indicates a promising use for future selective inactivation of free DNA. However, the assays were based on *E.coli* cultures which were viable, and not readily inactivated by PMA due to culture weakness and extensive death. It has been shown that such cell death can be avoided, in which the treatment should work as intended.

4.1.1 PMA effect on in vitro combined samples of *E.coli* and Salmon DNA

A significant initial inactivation was observed for PMA treated 16S live, dead and 18S DNA samples, with 18S samples showing the largest inactivation. Additionally, no differences were observed between pure *E.coli* samples and mixed salmon sperm and *E.coli* samples regarding PMA inactivation. The initial inactivation was avoided for live *E.coli* following further work with the simulated samples. During chemical and mechanical lysis assays, inactivation was seen selectively for PMA treated 18S salmon DNA and 16S DNA from dead *E.coli*, while DNA was retained for live *E.coli*. This indicates PMA does not inactivate viable, impenetrable cells, which corresponds with earlier findings (Nocker & Camper, 2006).

Extensive inactivation of 16S DNA was later observed for a replicate experiment of the lysis assays. UV-microscopy of the replicate samples showed a large percentage of dead cells present for the inactivated samples, while unaffected samples prior showed no dead cells. As such, PMA treatment shows promise as viable bacterial cells were unaffected by treatment under simple conditions, though cell viability seems to affect the treatment assay greatly.

4.1.2 PMA effect on real Salmon Pilot samples

Following promising results for the selective inactivation of free DNA by PMA treatment of in vitro samples, treatment was evaluated using procured intestinal samples from three Atlantic salmon individuals. Inactivation of both 16S and 18S samples was observed, but inactivation also varied between the different salmon individuals.

Salmon 18S samples generally yielded low DNA content. A 1000ng control added during one of the assays before the DNA extraction showed that the DNA extraction worked as expected. Either low amounts of DNA are natural for the respective salmon samples, or method bias is introduced during treatment, possibly from mechanical lysis (see 4.2 Technical challenges).

Sequencing of the 16S gene showed a selective inactivation of *E. coli* cells in one of the two salmon samples, wherein the native microbiota was left unaffected. This inactivation may indicate an unviable *E. coli* culture, or antimicrobial contents of the salmon intestine, such as piscidins, which may have killed the *E.coli* cells.

Triton x-100 was added to selectively lyse the eukaryotic salmon cells present while retaining the bacterial cells. Addition of Triton x-100 showed no significant effect on PMA treated live or dead *E.coli* compared to untreated samples, indicating no additional inactivation of 16S DNA. Since 18S DNA content was low, no conclusions could be drawn regarding cell lysis, digestion and additional inactivation of 18S DNA by Triton treatment.

4.2 Technical challenges, optimisations and progress

4.2.1 Optical density for normalising of dead cells

Normalising of *E.coli* sample concentrations was desirable due to the expected loss of DNA following isopropanol treatment. Isopropanol is known to denature proteins in high concentrations, resulting in an increased turbidity of the solution (Bobaly, Beck, Veuthey, Guillarme, & Fekete, 2016; Suzuki, Miyosawa, & Suzuki, 1963). As isopropanol treatment is bound to result in cell contents leaking into the culture solution, the subsequent denaturation of proteins is likely. The increased turbidity observed for the treated cells proved that normalisation using turbidity measurements was impractical. A consideration of sample preparation bias would be required between live and dead cells.

4.2.2 Technical challenges regarding cell lysis

Cell lysis was evaluated using chemical and mechanical lysis. The use of mechanical lysis yielded less DNA for *E.coli* but replicates observed equal levels of 16S and 18S DNA for both chemical and mechanical lysis. The differences observed for the mechanical lysis may be due to human error, such as inexperienced treatment of the samples. Earlier studies have shown that lysis by bead beating provide a higher diversity of microorganisms (Bakken & Frostegård, 2006; Smith, Li, Andersen, Slotved, & Krogfelt, 2011) and therefore, bead beating was thought to provide both a chemical-independent lysing step, as well as providing higher diversity and DNA yield for later treatment. This is required due to the varying complexity and chemical composition in a wide array of samples susceptible to host DNA contamination, such as blood, tissue and faecal samples.

4.2.3 Technical challenges regarding extraction of DNA

Initial experiments showed PMA samples were thoroughly lost after treatment. This contradicted the theory that the PMA reagent would not penetrate and inactivate DNA from live cells (Nocker et al., 2006), and it was thought that the use of automatic extraction reduced overall yield of DNA following PMA treatment and was responsible for the low C_t-values for both 16S and 18S. Other types of automatic extraction using magnetic beads are known to

slightly reduce DNA yield of extraction in comparison to more conventional, manual methods (Smit et al., 2000), but not to this degree. After observing the same large-scale inactivation for manually extracted cells, the probability of automatic extraction being the causative factor was significantly reduced. This was further indicated following qPCR of the last salmon intestinal samples which yielded low C_t-values of *E.coli* submerged in the salmon intestine. Sequencing of these samples showed that only *E.coli* to a large extent was removed by PMA treatment, pointing at cell viability as the likely cause. Automated extraction was therefore deemed non-causative for the extensive disappearance of PMA treated DNA.

4.2.4 Selective survival of bacterial cultures

Selective survival experiments indicated a lower resistance towards the Triton x-100 treatment for exponential cultures compared to stationary cultures of both bacteria. The gram-positive *E.faecalis* indicated a lower resistance towards the treatment compared to the gram-negative *E.coli,* as seen by a generally higher death rate for low concentrations of Triton x-100 compared to *E.coli*. *E.coli* survival during stress has been shown to be dependent on many factors, such as growth phase, growth medium, pH, temperature, and preadaptation of strains, but in general, stationary phase cultures are more resistant to stress than exponential phase cultures (Lindqvist & Barmark, 2014). To include *E.faecalis* in the salmon sample PMA assays, a concentration of 0.0025% of Triton x-100 was required to retain the cells, still yielding a death rate of 26%. A compromise was made between retaining these cells and including a high enough concentration of Triton x-100 to lyse eukaryotic cells. Since the gram-positive *E.faecalis* showed such sensitivity to low concentrations of Triton x-100, increasing Triton concentrations together with the PMA treatment could lead to selective gram-negative bacterial assays in the future.

E.coli cultures showed a higher percentage of dead control cells compared to the *E.faecalis,* indicating something resulted in the death of a relatively high amount of *E.coli* cells. It has been shown that *E.coli* persists to a large degree at 0-2% salinity (Stahl, Frost, Heard, & Hill, 2016), additionally surviving high concentrations of Triton x-100 for the assays included in this thesis. Therefore, the 0.85% NaCl and MilliQ water controls should have minimal implications. In the stationary phase of bacterial growth, cultures are sustained by equal growth and death rates (ref stationary phase). Since few dead cells were observed from the exponential phase of *E.coli,* this equilibrium of dead and living cells could have resulted in the relatively high amounts of dead *E.coli* cells observed in the controls.

Concentrations ranging from 6.25µg/ml – 50µg/ml of Proteinase K were also evaluated for use with the Triton x-100 selective lysis protocol. Quantification was difficult to establish due to extensive cell digestion for high concentrations of Proteinase K, resulting in only viable cells being visualised during the UV microscopy. Due to the sensitivity of *E.faecalis* to Triton x-100 concentrations, the lowest evaluated concentration of 6.25 µg/ml for Proteinase K was used as to not further remove potentially viable *E.faecalis* cells.

4.2.5 Bacteria culturing challenges

Culturing of *E.coli* and *E.faecalis* presented some challenges, indicating a potential impact on their viability during PMA- and Triton-treatment. Stationary cultures of *E.faecalis* always exhibited a prolonged lag-phase, while the contrary for exponential cultures was observed for *E.coli*.

Cell to cell communication during nutrient scarcity is known to influence a decrease in growth of cell-dense cultures of *E.coli* (Carbonell, Corchero, Cubarsí, Vila, & Villaverde, 2002), while *E.coli* generation time has been shown to vary by as much as 0.22±0.02 hours (Plank & Harvey, 1979). Generation time is therefore not static and is subject to differences for every *E.coli* cell present. The prolonged lag-phase of *E.coli* cultures may be down to these intraspecies differences, as well as glycol storage leading to reduced fitness of *E.coli* cells. Additionally, such cell-to-cell communication may limit the amount of nutrients used by the stationary cultures, preventing them from reaching an early death phase.

E.faecalis cells exhibiting a long lag phase after reaching the stationary phase may be due to an induced starvation-phase affecting initial growth rate, as has been reported earlier (Giard et al., 1996; Portenier et al., 2005). As a reaction to low nutrient availability and subsequent starvation of *E.faecalis* cells, a deregulation of growth factors is induced, leading cells to focus on own survival. This leads to a low population growth, and a high resistance to outer forces could be favourable to sustain the population. This induced slow-growth starvation phase is most probable as to what caused the initial problems of growing and using the *E.faecalis* cells. Cultures stopped during exponential growth, either frozen with glycol for a prolonged time or stored in a refrigerator overnight, did not exhibit an equally long lag phase.

4.3.6 *E.coli* viability challenges during thesis

E.coli samples showed inactivated DNA for dead samples, but often showed significant inactivation for live cells also. A possible explanation is a large percentage of unviable cells present in the expected live samples. Cells may have quickly reached the stationary phase and died during the overnight incubation. Otherwise, the culture medium of BHI-broth could have been unsuited for use with the *E.coli* and *E.faecalis* and resulted in weak and unviable cells readily penetrated by PMA. Another explanation may be that the cell cultures reached a death phase resulting in mass inactivation of already free DNA due to viral infections.

Seeing cultures of *E.coli* often quickly reached a high cell density, there is a possibility that cells also quickly reached a death phase. This could explain the inconclusive results initially, as a potential viral infection would most likely be spread during inoculation of fresh cultures later. Furthermore, if the bacteria were unsuited for growth in the BHI-broth, *E.coli* most likely would exhibit a much longer lag-phase or growth would be otherwise inhibited during the exponential phase. Furthermore, BHI-broth is a medium often used in the lab, showing good growth for many other students for the respective bacteria included in the assay. Therefore, quickly reaching the stationary phase overnight before transgressing into the death-phase either before or during cell treatment is plausible.

4.3 Gut microbiota of Atlantic Salmon

Sequencing by Illumina was performed as a control to check if observed species correlated with prior studies, in addition to acting as a control for the effect of PMA and Triton x-100 treatment on the bacterial species present.

Acinetobacter was found to significantly dominate the gut flora of one of the fishes, while the other fish was largely dominated by *Aeromonas*. Even though *Acinetobacter* is commonly found on human skin and mucous membranes and can be found on up to 44% of non-hospitalised, healthy human individuals (Wisplinghoff & Seifert, 2010), it has also been found to be a major constituent of some farmed salmon, more specifically *Acinetobacter junii* (Holben et al., 2002). It was feared that the *Acinetobacter* and *Aeromonas* observed was due to contamination, however, many other bacteria reportedly native to the salmon GI-tract was also found by sequencing, such as *Psychrobacter* of the Moraxella family and *Pseudomonas* (Egerton et al., 2018).

The relative composition of the microbiota and the significant domination of *Aeromonas* and *Acinetobacter* for the two fishes respectively is although questionable. Only 1% of the salmon gut microbiota can be cultured, including the *Aeromonas, Acinetobacter*, *Pseudomonas, Enterobacteriaceae, Staphylococcus, Vibrio* and *Bacillus* (Dehler, Secombes, & Martin, 2017). Since the samples were stored for some days in the refrigerator, and these specific bacteria were found in high relative amounts to the other bacteria, there is a possibility that they were cultured in the intestinal scrape over this time. *Psychrobacter* is, in addition, a psychrophilic bacteria readily growing under cold conditions (Kim et al., 2012).

Interestingly, no native Vibrionaceae or Enterobacteriaceae were found during this study, of which have been described as belonging to the normobiota of Atlantic Salmon (Egerton et al., 2018; Sullam et al., 2012). The lack of Vibrionaceae may be due to the salmon samples originating from freshwater tanks, as Vibrionaceae are most often found in the gut microbiota of farmed saltwater adult salmon and other saltwater fishes (Egerton et al., 2018; Sullam et al., 2012).

4.4 Shortcomings of the study

Due to the indicated extensive cell death of *E.coli* cultures, not much can be said for the use of the PMA method on real salmon intestine samples. Additionally, the minuscule concentrations of Triton x-100 required before observing a high death-rate for *E.faecalis* provided uncertainty on the use of Triton as a viable detergent for general microbial assays of the salmon intestine.

As for the inconsistencies showed by the last salmon intestine assay, which yielded a higher DNA-content for PMA treated cells, no clear conclusions can be drawn as to what has happened other than human error during sample preparation. An increase and higher DNA content of PMA treated samples compared to untreated samples completely contradicts the theory. This has even been shown to not be the case earlier on during the thesis, as viable cells were unaffected by PMA while free DNA from dead cells was inactivated.

To further investigate the effect of PMA on free DNA in the salmon intestine, samples could have included a salmon intestine spiked with pure salmon DNA. This could have tested if low 18S DNA content was due to the nature of the samples or due to the nature of the method.

5. Conclusion and further work

Even though there are some shortcomings to the thesis, especially regarding the salmon intestinal samples, the general trend points towards an inactivation only if free DNA is present. However, the present study failed to show conclusive proof that PMA would work on real-life samples, although several steps have been made, such as evaluating potential pitfalls. Initial in vitro samples provided positive indications for PMA-treatment as a working method, in fact selectively inactivating DNA originating from dead cells while retaining viable cell DNA. It seems plausible that inactivation of live *E.coli* cells initially is due to using unviable and weak cultures. These weak and unviable cultures are readily invaded and inactivated by PMA, which otherwise does not penetrate viable cells. This is further proven by mechanical and chemical lysis evaluations showing no inactivation of live *E.coli* DNA.

If precautions are made to make sure cultures are viable, future assays using the PMA method could work for inactivation of host DNA contamination which is free and readily available in the samples. Furthermore, the selective inactivation of mostly *E.coli* DNA for salmon samples as seen by the sequencing results continues to send positive sentiments regarding the potential use of the method.

More work should be put down towards finding a selective lysing protocol targeting eukaryotic cells, as the present study could not conclude with confidence that the use of Triton x-100 is a viable method. Such future selective lysing protocols could even potentially include selective antibiotic treatments for use in selective bacterial assays. Moreover, further evaluation is required for the PMA method to become a ready-to-use method in the general lab environment. However, PMA shows promise as a general inactivation method which may be tailored to suit specific assay needs while also providing a general inactivation suitable for a wide array of host organisms.

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Supplementary figures and tables

Appendix A: Primer sequences

PRK Illumina primer sequences for Index PCR:

PRKi forward (5'-3'):

1. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagtcaaCCTACGGGRBGCASCAG

2. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctagttccCCTACGGGRBGCASCAG

3. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctatgtcaCCTACGGGRBGCASCAG

4. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctccgtccCCTACGGGRBGCASCAG

5. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtagagCCTACGGGRBGCASCAG

6. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtccgcCCTACGGGRBGCASCAG

7. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtgaaaCCTACGGGRBGCASCAG

8. aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctgtggccCCTACGGGRBGCASCAG

PRKi Reverse (5'-3'):

31.caagcagaagacggcatacgagatATCGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT

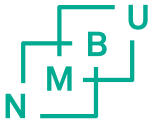
32.caagcagaagacggcatacgagatTGAGTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT

33.caagcagaagacggcatacgagatCGCCTGgtgactggagttcagacgtgtgctcttccgatctGGACTACYVGGGTATCTAAT

Appendix B: Species sequenced

Table B.1. Bacterial composition at family level in percentage. The supplementary table shows the abundance in percent for bacterial families sequenced from two Atlantic salmon indiviuals, further processed using the QIIME pipeline

Legend	Taxonomy	Total	F1.10B	F1.5C	F1.5D	F1.6C	F1.6D	F1.9C	F1.9E	F2.10B	F2.10F	F2.5B	F2.5F	F2.6B	F2.6F	F2.9A	F2.9E	Positive
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
	D_0__Archaea;D_1__Euryarchaeota;D_2__Methanobacteria;D_3__Methanobacteriales;D_4__Methanobacteriaceae;D_5__Methanobrevibacter	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Bifidobacterium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Arthrobacter	0.2%	0.0%	0.0%	0.1%	0.0%	0.1%	0.0%	0.3%	0.0%	0.4%	0.1%	0.7%	0.0%	0.2%	0.0%	0.9%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Rarobacteraceae;D_5__Rarobacter	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteria;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Collinsella	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteria;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Enterorhabdus	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteria;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Senegalimassilia	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Actinobacteria;D_2__Coriobacteria;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__uncultured	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidaceae;D_5__Bacteroides	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.1%	0.0%
	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidales S24-7 group;D_5__uncultured bacterium	0.1%	0.1%	0.0%	0.0%	0.0%	0.1%	0.1%	0.1%	0.2%	0.1%	0.1%	0.2%	0.2%	0.2%	0.2%	0.3%	0.0%
	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Parabacteroides	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Alistipes	0.1%	0.1%	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.1%	0.1%	0.1%	0.0%	0.1%	0.0%	0.0%
	D_0__Bacteria;D_1__Cyanobacteria;D_2__Chloroplast;D_3__Bromus tectorum;D_4__Bromus tectorum;D_5__Bromus tectorum	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Cyanobacteria;D_2__Chloroplast;D_3__Phaseolus acutifolius (tepary bean);D_4__Phaseolus acutifolius (tepary bean);D_5__Phaseolus acutifolius (tepary bean)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Deferribacteres;D_2__Deferribacteres;D_3__Deferribacterales;D_4__Deferribacteraceae;D_5__Mucispirillum	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Bacillus	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Family XI;D_5__Exiguobacterium	0.1%	0.1%	0.0%	0.2%	0.1%	0.3%	0.0%	1.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Paenibacillaceae;D_5__Brevibacillus	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.1%	0.2%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Lactococcus	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.1%	0.1%	0.0%	0.1%	0.1%	0.1%	0.0%	0.0%	0.1%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__Christensenellaceae R-7 group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Candidatus Arthromitus	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Clostridium sensu stricto 1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XI;D_5__Tepidimicrobium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Anaerostipes	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia	0.1%	0.1%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Coprococcus 2	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Fusicatenibacter	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NC2004 group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.2%	0.1%	0.1%	0.1%	0.0%	0.2%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Marvinbryantia	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Tyzzerella 4	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] hallii group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] rectale group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ventriosum group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Ruminococcus] torques group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.1%	0.1%	0.0%	0.1%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Intestinibacter	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Anaerotruncus	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Faecalibacterium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 9	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-002	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-003	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-004	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-013	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 2	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Subdoligranulum	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__[Eubacterium] coprostanoligenes group	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__uncultured	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Catenibacterium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelatoclostridium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelotrichaceae UCG-003	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Holdemanella	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Dialister	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Bradyrhizobiaceae;D_5__Bradyrhizobium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rickettsiales;D_4__Mitochondria;D_5__Oryza meyeriana	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Sphingomonadales;D_4__Sphingomonadaceae;D_5__Sphingomonas	0.2%	0.3%	0.2%	0.2%	0.1%	0.9%	0.1%	0.5%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.0%	0.1%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Burkholderiaceae;D_5__Ralstonia	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Delftia	0.5%	0.7%	0.8%	0.5%	0.5%	2.5%	0.4%	1.1%	0.1%	0.1%	0.1%	0.6%	0.1%	0.1%	0.0%	0.2%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Betaproteobacteria;D_3__Burkholderiales;D_4__Comamonadaceae;D_5__Variovorax	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Aeromonadales;D_4__Aeromonadaceae;D_5__Aeromonas	45.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.1%	0.1%	95.9%	92.8%	95.1%	70.8%	96.3%	90.4%	96.7%	81.6%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Moritellaceae;D_5__Moritella	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Alteromonadales;D_4__Pseudoalteromonadaceae;D_5__Pseudoalteromonas	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Citrobacter	0.1%	0.1%	0.1%	0.1%	0.0%	0.1%	0.1%	0.1%	0.1%	0.0%	0.1%	0.2%	0.1%	0.1%	0.1%	0.1%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella	18.5%	0.0%	34.0%	27.7%	37.6%	26.1%	0.0%	0.0%	0.0%	0.0%	0.9%	16.0%	1.0%	4.8%	0.0%	0.0%	99.7%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Halomonadaceae;D_5__Halomonas	0.1%	0.2%	0.0%	0.1%	0.1%	0.3%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Acinetobacter	33.8%	93.6%	61.5%	67.4%	58.3%	66.0%	94.8%	90.6%	1.0%	0.1%	0.3%	0.4%	0.2%	0.1%	0.4%	0.5%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Moraxellaceae;D_5__Psychrobacter	2.1%	1.0%	0.7%	1.1%	0.4%	1.2%	0.8%	3.6%	0.5%	4.3%	0.8%	7.4%	0.4%	2.1%	0.6%	8.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pseudomonadales;D_4__Pseudomonadaceae;D_5__Pseudomonas	2.0%	3.4%	1.9%	1.9%	2.3%	1.7%	3.0%	1.9%	1.2%	1.3%	1.1%	2.2%	0.8%	0.9%	1.1%	7.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Aliivibrio	0.0%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Vibrionales;D_4__Vibrionaceae;D_5__Photobacterium	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Xanthomonadales;D_4__Xanthomonadaceae;D_5__Stenotrophomonas	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.0%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	D_0__Bacteria;D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Verrucomicrobiales;D_4__Verrucomicrobiaceae;D_5__Akkermansia	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway