Master's thesis

NTNU
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Effect of Season, Fjord Locality and Food Safety Treatment on Microbial Composition of Norwegian Blue Mussels (*Mytilus Edulis*)

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Anita Nordeng Jakobsen
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Preface

This master's thesis was completed as part of the course *TBT4900 - Biotechnology, Master's Thesis* in the five-year Master's Degree Programme in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU) in Trondheim. The research focused on investigating the effect of different parameters in mussel cultivation on the microbial composition of blue mussels (*Mytilus edulis*) cultivated in Åfjord, a fjord located in the Trøndelag region of Norway. The report is titled *"Effect of Season, Fjord Locality and Food Safety Treatment on Microbial Composition of Norwegian Blue Mussels (Mytilus Edulis)"* and aims to fill knowledge gaps in unexplored sides of blue mussel aquaculture.

Abstract

Blue mussels (*Mytilus edulis*) cultivated in Åfjord in the Trøndelag region of Norway were sampled from the autumn of 2022 to the early summer of 2023. The mussels were used to explore different aspects of mussel cultivation and the associated effects on the microbial composition of the blue mussels. Several localities within Åfjord were used to compare the effects of location on microbial contents, alongside the seasonal variations. Additionally, the food safety measures of depuration and heat treatment of mussels were evaluated.

The microbial characteristics of blue mussels from Åfjord were investigated and results revealed that blue mussels from Åfjord contain a low amount of bacteria in general. Seasonal variations of *Aeromonas* spp. were shown in the inner and middle parts of Åfjord, but no further bacterial parameter varied by season nor location. The process of depuration was shown to be able to significantly ($p<0.05$) reduce the levels of *Aeromonas* spp. and *E. coli* in blue mussels. With *E. coli*'s role as an indicator organism, there was presumably a reduction of other enteric contaminants like norovirus as well. This underlined the ability to use depuration as a food safety measure. Heat treatment similar to cooking conditions was tested and shown to not be an adequate food safety measure by itself, with conditions being far from the industrial requirements of sufficient heat treatment of mussels in Norway.

Still, further research should be conducted to directly investigate the reduction of viral content by depuration, going beyond the use of *E. coli* as an indicator. Additionally, the use of DNA sequencing to characterise blue mussels' microbiota in a broader scope should be explored further to gain new insight into ensuring the quality and safety of blue mussels.

Sammendrag

Blåskjell (*Mytilus edulis*) dyrket i Åfjord i Trøndelag i Norge, ble undersøkt fra høsten 2022 til forsommeren 2023. Blåskjellene ble brukt til å utforske ulike sider ved skjelldyrking og tilknyttede effekter på den mikrobielle sammensetningen av blåskjellene. Flere ulike lokaliteter i Åfjord ble brukt for å sammenligne lokale effekter på mikrobielt innhold, i tillegg til sesongvariasjoner. Det ble også gjort undersøkelser av to matsikkerhetstiltakene til muslinger: revanning og varmebehandling.

På denne måten ble blåskjell i Åfjord sine mikrobielle kjennetegn undersøkt, og resultatene avslørte at blåskjell fra Åfjord generelt inneholder et lavt antall bakterier. Sesongvariasjoner av *Aeromonas* spp. ble påvist i indre og midtre del av Åfjord, men ingen andre bakterielle parametere varierte som følge av sesong eller lokasjon. Revanning viste seg å kunne redusere nivåene av *Aeromonas* spp. og *E. coli* signifikant ($p<0.05$) i blåskjell. Siden *E. coli* brukes som indikatororganisme, antas det også å være en reduksjon av andre fekale forurensninger, som norovirus. Dette understreket muligheten for å bruke rensing som et mattrygghetstiltak. Varmebehandling tilsvarende tilberedning av blåskjell som mat ble testet og viste seg å ikke være tilstrekkelig som et enkeltstående mattrygghetstiltak, med forhold langt unna de industrielle kravene for tilstrekkelig varmebehandling av muslinger i Norge.

Likevel bør det utføres videre forskning for å direkte undersøke reduksjonen av viralt innhold ved revanning, som burde gå utenfor bruken av *E. coli* som indikator. Videre bør bruken av DNA-sekvensering for å karakterisere det større mikrobielle samfunnet i blåskjell undersøkes nærmere for å oppnå ny innsikt i sikringen av kvalitet og mattrygghet i blåskjell.

Acknowledgements

This master's thesis would not be possible without the aid and guidance of my supervisors, Anita Nordeng Jakobsen and Sunniva Hoel. Ever since the initial conversations about pursuing this project, they have shown unwavering support and dedication to developing a worthy master's thesis. Only with their invaluable feedback and experience could this project be finalised, for which I am sincerely grateful. I would also like to thank my lab partner, Marte Holm, and other associates in the laboratories at NTNU, for all the hours we have shared in the laboratory. Long days of lab work were not as daunting in your company. Additionally, I am grateful for the year-long collaboration with our blue mussel supplier in Åfjord. The generosity and cooperation in the deliverance of blue mussel samples for every thinkable analysis, as well as the hospitality shown to me during my visit to the facilities, were signs of the trust and interest that was given to this project and NTNU.

Throughout this project, I have been given the opportunity to plan and execute laboratory and scientific work independently, while also being allowed to work in conjunction with others. Both aspects have allowed me to develop my own skills in teamwork and work management, which I will carry with me going forward. The added chance to get in contact with and meet a wide variety of people in different occupations is something I appreciate and will remember fondly. Through this work, I have also been allowed to develop my skills in conveying data and science, giving me valuable experience in scientific work.

Lastly, I would like to extend a thank you to my friends and fellow students, who have shown support and cheered me on through a long, and sometimes arduous, journey to finish this master's thesis.

Trondheim, June 12, 2023

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1 | Introduction

The history of mankind is also the history of food, yet an oft-forgotten aspect taken for granted is food safety. Dating back to ancient civilisations, a constant challenge for mankind has been to produce sufficient quantities of food and ensure its safety and suitability for human consumption. As society has developed and industrialised, the challenge of providing safe and nutritious food has only grown. Today, the food industry is faced with meeting the nutritional needs of a growing population while ensuring that the food we consume is safe and free of pathogens and harmful contaminants.

At first, food safety measures were primitive in nature. However, with advancements in technology and science, the means have become understood, not just the ends. From discovering microorganisms to developing modern technologies for food preservation and testing, scientific breakthroughs have been instrumental in improving the safety and quality of our food supply. Despite these advances, the challenge of ensuring food safety remains as crucial as ever. In recent years, foodborne illness outbreaks have continued, and new contaminants and pathogens have emerged. The rise of the global food trade has thus produced new challenges for ensuring the safety of imported foods.

Additionally, a new consideration of food safety has emerged due to how the general public views food. Where before food was simply the only solution to resolving hunger, it is now viewed as a key piece of a healthy lifestyle. The perception of the utility of food has shifted from being an energy source to being a source of targeted macronutrients and micronutrients. This way of thinking is the cause of the concept of having a specific diet for health reasons. This has led many to focus on seafood as a valuable source of infrequent micronutrients and desirable macronutrients, such as unsaturated fatty acids. Although historically associated with fish dishes, new ways to prepare and consume seafood have emerged in recent years, leading to increased interest and curiosity in these food products. The most prevalent example of this development is the popularity of sushi, but other seafood dishes with light

preparation, like poke, have also sparked interest. In any case, eating fresh seafood has become a mainstay in many households.

Institutions like the Norwegian Food Safety Authority administer legislation for the safety of the new food products that are introduced into the market. In fact, the idea of prohibited food dates back to the Viking age in Norway, with laws that banned eating animals that died from natural causes in the wild[1]. Today state institutions like the Norwegian Food Safety Authority are primarily involved in preventing unsafe food items from ever reaching the consumer, as opposed to placing bans on the act of eating specific food products. In this way, the producers are responsible for maintaining food safety regulations in all stages of production. This entails that all food products available to the customer have met safety standards and are suited for human consumption. The Norwegian Food Safety Authority regulates the bivalve mollusc industry in Norway in this fashion. The authority requires molluscs to contain a limited level of *E. coli* before being commercially available.

Blue mussels (M. edulis) represent the overwhelming majority ($> 90\%$) of combined sales of molluscs, crustaceans and echinoderms in Norway. Most years, the annual gross sale of blue mussels averages over 2 000 metric tons, with a value of over 20 million NOK[2]. Whilst small compared to the Norwegian fish industry, the blue mussels industry is important in a growing seafood-dependent market. With the mussels known capability to cause foodborne illness outbreaks[3], it is vital to continue research to ensure food safety in blue mussels.

1.1 Project objectives

This study aims to investigate the microbial composition in blue mussels (*M. edulis*) and their effect on food safety, as well as enhance our understanding of the microbiota present in blue mussels. To achieve these goals, the microbial composition of blue mussels from different seasons, as well as blue mussels with various treatment methods, will be analysed using traditional selective growth media. Additionally, a more comprehensive understanding of the microbiota of blue mussels was attempted to be mapped by using the modern DNA sequencing method 16S rRNA amplicon

sequencing but was unable to be finalised within the deadline of the project.

The project's findings will be evaluated to determine the effects of season, cultivation locality and food safety treatment on the microbial composition of blue mussels from Åfjord. The results will also be used to determine the quality of the blue mussels in terms of microbial contamination. Ultimately, this study will provide valuable insights into the microbiota of blue mussels. The findings can be employed to improve food safety in blue mussel production in Norway and the shellfish industry at large.

A part of the present project was undertaken as a prestudy over the course of the autumn of 2022, titled "*Interplay of Microbial Diversity and Food Safety in Blue Mussel Aquaculture*". The prestudy's methods, principles and findings will be used in the present project, both as citations and elaborated on for the purposes of the larger scope of the present project.

2 | Background

2.1 *Mytilus edulis* - The Blue Mussel

The blue mussel, scientifically known as *Mytilus edulis*, is a bivalve mollusc belonging to the Mytilidae family[4]. During their early development, blue mussels undergo a pelagic larvae phase lasting approximately three to four weeks[5]. Subsequently, they settle on the seafloor, settling onto thin algae, primarily inhabiting the tidal zone and descending to depths of up to ten meters[6]. Over time, these mussels develop their characteristic shells as they thrive in the demersal zone, employing a filtration mechanism to extract nutrients from the surrounding seawater[7]. The shells of blue mussels can reach an impressive size of almost 80 mm[6]. The species exhibits a wide distribution across the oceans of the world, with a preference for colder waters.

2.1.1 Role as a food resource

Blue mussels hold notable value as a consumable resource and have historically, as well as contemporarily, found importance in numerous societies[8][9]. In Norway, for instance, the annual sale in 2021 reached 2,163 tonnes of blue mussels[2]. While in Europe at large, the average annual production of marine bivalves between 2010 and 2014 averaged 560 000 tonnes[9]. Several species of marine bivalves are included in the European statistic, but blue mussels represent an important portion of the industry, particularly in specific countries like Denmark and France[9].

From a nutritional perspective, blue mussels represent a prime source of protein and fatty acids[10]. When compared to conventional terrestrial livestock, molluscs offer protein with high digestibility and considerable levels of essential amino acids[11]. Notably, molluscs possess a more favourable fat composition, exhibiting lower overall fat content but with especially high concentrations of omega-3 fatty acids[11], which are renowned for their anti-inflammatory effects and various health benefits[12]. Furthermore, molluscs encompass essential minerals and vitamins, such as selenium, iron, and vitamin B12. Given their low carbohydrate content, blue mussels

position themselves as a nutrient-rich, low-calorie food.

2.1.2 Ecological characteristics

From an ecological perspective, blue mussels are beneficial agents in their local environment, as they demonstrate the potential to enhance coastal water quality due to their unique capacity to filter and remove nitrogen [13]. Additionally, blue mussels can act as sentinel organisms for pollution of seawater [8]. These remarkable abilities render them effective tools both in wild populations and for cultivation within constructed coastal frameworks. Given the inherent advantages of blue mussels in terms of sustenance and their positive ecological impact, the cultivation of blue mussels emerges as an advantageous pursuit in several contexts.

2.1.3 Mussel cultivation

During the 1960s, studies aimed at commercialising blue mussel production in Norway were conducted [6] [14]. These pioneering efforts revealed the potential for establishing a thriving mussel industry across numerous coastal areas in Norway. While blue mussels typically grow naturally on the seafloor, they can be cultivated for commercial purposes using suspended ropes, or similar alternatives, anchored to floating structures to facilitate anthropogenic production [5].

The adolescent stage of blue mussels as pelagic larvae enables their cultivation in specialised structures. In blue mussel production facilities, ropes are strategically deployed in the ocean during the spring season to allow the larvae to attach and grow on the ropes [5]. Over the course of the following year, these larvae transform into fully formed mussels with shells. However, if left unattended, mussels tend to grow atop one another, leading to competition and subsequent mortality. To address this challenge, after the initial year of development, the mussels are transferred to specially designed stockings [5]. These stockings are woven with cotton mesh and a growth band, and filled with young mussels [15]. The cotton mesh will rot over the course of a couple of weeks, in which time mussels will have attached themselves to the growth band [15]. The mussels are subsequently left to grow for an additional two years before reaching the optimal stage for harvesting [15].

This cultivation process, involving the use of suspended ropes and stockings, has enabled the successful commercial production of blue mussels in Norway. Through these innovative techniques, the growth and harvesting of mussels have been effectively managed, ensuring high-quality yields for the market.

2.1.4 Regulation of production areas

The Norwegian Food Safety Authority employs a classification system to assess the suitability of coastal waters used for mussel production in Norway, taking into account the presence of enteric bacteria in seawater and local sources of pollution [16]. The indicator organism chosen for testing is *Escherichia coli*, which serves as an indicator for other enteric bacteria and viruses, such as norovirus. The classification process involves conducting twelve tests for *E. coli* at four-week intervals throughout the year, followed by ongoing testing to ensure accurate classification. The Norwegian Food Safety Authority has established four distinct classes of production areas for shellfish: A, B, C, and "Not suited" [16]. While there are distinct criteria for each class, the same testing regime is utilised in all circumstances. Samples are collected 12 times throughout a calendar year, in four-week-long intervals. Following this, a permanent classification is given if continual samples are taken at least six times a year [16].

Class A signifies a low level of *E. coli* contamination, with at least 80% of samples containing less than 230 *E. coli* per 100 grams and no samples exceeding 700 *E. coli* per 100 grams [16]. Mussels harvested from Class A areas can be directly consumed by consumers without further treatment, including the option of consuming them raw without the risk of illness. However, if more than 20% of samples exceed the limit of 230 *E. coli* per 100 grams, the Class A classification is invalidated.

Class B is assigned to areas where at least 90% of samples have less than 4 600 *E. coli* per 100 grams and no values surpassing 46 000 *E. coli* per 100 grams [16]. Mussels originating from Class B areas require treatment by the supplier before sale. Acceptable measures include purification, relaying mussels to a Class A area, or heat treatment. If more than 10% of samples exceed the limit of 4 600 *E. coli* per 100 grams, Class C must be considered instead.

In Class C areas, no sample can exceed 46 000 *E. coli* per 100 grams, and mussels cultivated there must either be moved to a Class A area or cooked prior to being offered to consumers[16]. Harvesting from areas exceeding the limits set by Class C is strictly prohibited by law, as shellfish from these areas are deemed unsuitable for human consumption[16].

2.2 Blue mussels and food safety

Blue mussels possess a remarkable attribute for commercial purposes due to their ability to feed using filtration. However, this characteristic, while advantageous, also presents a notable drawback. On one hand, it makes them highly sought after for commercial production, as they require no extra feed to thrive. On the other hand, this very mechanism renders blue mussels susceptible to the accumulation of pathogens and potentially harmful substances that can pose risks to human health when consumed. Therefore, ensuring the food safety of these mussels is predominantly contingent upon the quality of the water in which they reside.

2.2.1 Algal toxins

Algal toxins are recognised by the Norwegian Food Safety Authority as a major concern related to the risk of severe illness associated with mussel consumption, along with microorganisms[17]. Blue mussels engage in filter feeding, extracting nutrients from naturally occurring plant algae in the ocean. Occasionally, certain toxin-producing algae species, for instance, *Dinophysis* spp. and *Alexandrium* spp.[18], may be present in the waters where mussels are cultivated. These algae are filtered by the mussels, resulting in bioaccumulation of the toxins within the mussels' tissues. Interestingly, the mussels themselves appear to be unaffected by the increased toxin levels they harbour[19].

Globally, various types of algal toxins are acknowledged, but in Norway, the Norwegian Institute of Public Health (NIPH) highlights three distinct types: Diarrhoeic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), and Amnesic Shellfish Poisoning (ASP). Symptoms of DSP contamination include nausea, vomiting,

and stomach pain, while PSP and ASP contamination can lead to more severe symptoms, such as muscle numbness and nerve damage, respectively[19].

To ensure consumer safety, the Norwegian Food Safety Authority has established limits on algal toxin levels in mussels, aiming to prevent the consumption of mussels with high toxin content. The limits are as follows: PSP - 800 μg saxitoxin equivalents diHCl per kilogram, ASP - 20 mg domoic acid per kilogram, the combined quantity of okadaic acid and its derivatives dinophysis toxins - 160 μg okadaic acid equivalents per kilogram, yessotoxin - 3.75 mg yessotoxin equivalents per kilogram, and azaspiracid - 160 μg azaspiracid equivalents per kilogram[20]. Producers of commercially grown mussels bear the responsibility of meeting these requirements. Additionally, the Norwegian Food Safety Authority identifies areas where the collection of wild mussels for private consumption is deemed safe based on laboratory analyses, with the results publicly available[21]. While the Norwegian Institute of Public Health (NIPH) does not maintain records of food poisoning cases specifically attributed to mussels in Norway, incidents of poisoning related to DSP have been reported, with fewer cases associated with PSP and no documented instances of ASP poisoning. Hence, it is crucial to prevent dangerous toxin concentrations in mussels that may reach consumers, as these concentrations are not adequately reduced by consumer-side treatments such as cooking[22].

2.2.2 Bacterial pathogens

Microorganisms are a major concern in ensuring the food safety of mussels[17]. While consumer advice and attention in Norway primarily focus on algal toxins regarding food poisoning from blue mussels, production facilities place considerable focus on monitoring the microbial content of the mussels.

In Norway, cultivation areas are determined by the Norwegian Food Safety Authority based on the occurrence of faecal bacteria, especially *E. coli*, along with information on known contamination sources in or close to the cultivation site[16]. The allowed limits used are identical to those used in the European Union[23]. In these coastal regions, the runoff from agriculture, industrial waste, and particularly sewage pollution raises concerns. Contaminants from land can be rinsed into the waters where

mussels feed, potentially compromising their food safety at the cultivation locality [7]. Given the fact that Norway's population is spread throughout most of the country's coastline, it is evident that anthropogenic pollution near cultivation sites may be especially relevant in the country's many fjords.

Blue mussels cultivated in waters contaminated with sewage may harbour pathogenic bacteria derived from said sewage, which can pose health hazards to human consumers, especially when present in high concentrations [7]. Examples of faecal bacteria originating from sewage include *E. coli*, *Salmonella* spp., and *Shigella* spp. [24] These pathogens can induce gastrointestinal infections and other adverse health effects if consumed by means of contaminated seafood.

In addition to pathogens introduced through human activities, certain pathogenic bacteria species naturally populate seawater and can affect the food safety of blue mussels. For example, *Listeria monocytogenes* and *Clostridium botulinum* are known to be present in marine environments. Genera like *Vibrio* spp. and *Aeromonas* spp. have also been identified in seafood samples in both the US and EU [24]. These bacteria can pose risks to human health if present in mussels at influential levels. A selection of bacteria that can inhabit blue mussels is described in Section 2.3.

While some pathogens may be eliminated through appropriate cooking practices, it is important to note that cooking alone may not be adequate to remove all microorganisms. Residual pathogens can persist in sufficient concentrations to pose a risk for human infection, especially if the cooking process is not performed at the temperatures or durations required to destroy all microorganisms [7]. Therefore, ensuring proper handling, processing, and cooking practices is crucial to minimise the risk of bacterial contamination. The legislative requirements of heat treatment are described in Section 2.4.2.

Furthermore, the potential risk associated with bacterial infection from seafood is intensified by the growing issue of antimicrobial resistance (AMR) in new bacteria. A study from 2022 by Kausrud et al. highlights that there is a concern related to the proliferation of extended-spectrum β-lactamase—producing (ESBL) *E. coli* in blue mussels. This has implications for blue mussels' food safety, as AMR can reduce the effectiveness of antibiotics in treating infections caused by resistant bacteria.

Various studies have demonstrated the presence of AMR in seafood[26], with the use of antimicrobial agents in both wastewater treatment and agriculture as a known cause of AMR in many bacterial species[27][28]. Efforts to address antimicrobial resistance and promote responsible antibiotic use, as well as measures to combat AMR pathogens like that of Kausrud et al., are crucial in ensuring the continued food safety of seafood, including blue mussels.

2.2.3 Viral pathogens

Pathogens associated with foodborne diseases also include viruses. Norovirus infection can cause gastroenteritis, causing symptoms such as nausea, vomiting, and diarrhoea[29]. The virus is present in the faeces of infected individuals, and sewage water serves as a potent source of contamination[30]. Norovirus is highly infectious, requiring only a small number of viral particles to induce illness. Moreover, it has the ability to survive for extended periods in chilled foods[30]. These characteristics make norovirus capable of causing outbreaks of gastroenteritis, particularly in large groups. Given its resilience in cold temperatures and its association with sewage water, norovirus is frequently found in mussels. The NIPH reports that mussel consumption is a primary source of norovirus outbreaks in Norway[30]. In addition to norovirus, the Hepatitis A virus (HAV) is also linked to the consumption of shellfish. Compared to norovirus, HAV-related illnesses tend to be more severe, with some cases even proving fatal[31]. HAV is also associated with sewage pollution of human origin, further raising concerns about the food safety of mussels cultivated in affected waters. It is important to note that the viral contents of mussels are not measured directly, however, *E. coli* is used as an indicator organism for viral contaminants like these from faecal sources. *E. coli* is used in this way because of two distinct reasons: It is easy to detect and measure, and it is invariably found in sewage[32].

2.2.4 Chemical toxicants

The ocean harbours various compounds and elements that are relevant to food safety, including heavy metals and persistent organic pollutants (POP). Mussels have the

disposition to accumulate these substances, particularly lipophilic compounds that accumulate in fatty tissues. Once absorbed, these substances remain stored in the fatty tissue until the mussels digest it, making their excretion complicated. Consequently, mussels are utilised as biomonitoring tools and biomarkers for pollution and climate change[8]. Mussels inhabit a low trophic level in the food chain, and as such they are not subject to biomagnification themselves. Instead, they serve as sources of biomagnification for higher trophic levels in shallow waters. As a result, the overall pollutant levels in mussels are generally lower compared to organisms higher up in the food chain. Nonetheless, mussels do accumulate certain chemicals and compounds that are closely monitored for food safety concerns.

The disturbance of sediment rich in contaminants in mussel growth areas can lead to the re-suspension of these contaminants, facilitating their uptake by mussels through their filtration process. A study from 2020 of Flekkefjord, Norway, reported elevated levels of polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbons (PAH) in blue mussels used for pollution monitoring following an undersea landslide[33]. The recorded levels exceeded the consumption limit set by the European Commission for bivalve molluscs, highlighting the hazards associated with consuming these blue mussels and their aquatic environment[33]. Although the study initially aimed to investigate the effects of sediment restoration activities, such as dredging, on the re-suspension of contaminants, the landslide hindered a conclusive assessment of the impact of such activities. Therefore, caution should be exercised in mussel production in known areas of POP pollution, as re-suspension of contaminants remains a possibility.

While anthropogenic toxicants are not recognised as the primary food safety issue by the Norwegian Food Safety Authority, producers in Norway must adhere to legislation within the European Economic Area (EEA), which defines tolerable concentrations of contaminants in food[34]. In Norway, blue mussels have been found to contain not only PCBs and PAHs[35] but also toxic elements like arsenic, lead and mercury[36]. Although certain chemicals may accumulate in insignificant concentrations for human consumption of mussels, they can still pose a danger to the mussels themselves due to their size and biology, ultimately affecting cultivation.

2.3 Microbiota of blue mussels

A complete understanding of the microbiota present in blue mussels is still narrow. Some studies suggest that the microbiota of blue mussels and other bivalves closely echoes the composition of the surrounding water[37]. Being filter feeders, mussels acquire bacteria from the water they inhabit as part of their sustenance[37]. However, other studies have identified significant variations between the microbiota of mussels and the surrounding water[37]. The prevailing theory suggests that the mussels' microbiota is primarily influenced by the local aquatic environment but is also regulated to some extent by certain biological factors within the mussels themselves. For example, the hemolymph of healthy bivalves is known to host specific bacteria while excluding others[37].

Interestingly, the composition of the microbiota in blue mussels can be influenced by external factors, both by variations in the bateria species' abundance and their behaviour. For instance, certain species of *Vibrio* may dominate in the absence of other bacteria, displaying opportunistic behaviour[37]. However, much of this knowledge is based on traditional testing methods, and a more profound understanding of the microbiota can be gained via approaches like 16S rRNA gene amplicon sequencing. This approach can provide valuable insights into the unknown bacterial contents of mussels, helping to address uncertainties related to their food safety. Nonetheless, it is expected that certain genera and species of bacteria will be present in seafood products. For instance, bacteria capable of producing hydrogen sulphide (H_2S), particularly from the *Shewanella* genus, and the *Pseudomonas* and *Photobacterium* genera are commonly associated with seafood spoilage in Norway[38][39]. Moreover, specific genera like *Listeria*, *Vibrio*, *Aeromonas*, are known to be of concern in terms of food safety[24]. These bacteria can be identified through sequencing techniques as well as traditional tests utilising selective growth media[38]. The ensuing sections will present a selection of bacteria associated with foodborne illnesses.

2.3.1 *Escherichia coli*

Escherichia coli is a facultative anaerobic bacteria that is frequently found in the human intestinal tract[40]. While most strains of *E. coli* are harmless to humans, certain pathogenic strains can cause illness when ingested. Diarrheagenic *E. coli* in particular is recognised as a source of toxicity, leading to diarrheal infections in humans[41]. *E. coli* can be present in mussels and other shellfish, and its occurrence is often linked to contamination from faecal sources, along with other enteric bacteria[40]. Cases of illnesses related to *E. coli* infection from seafood consumption has been reported[40]. However, it is important to note that the presence of non-pathogenic *E. coli* is also a concern because it indicates faecal contamination[40]. Even though the *E. coli* strains themselves may not be harmful, their presence suggests the potential presence of pathogenic contaminants originating from sewage[7]. Therefore, *E. coli* is commonly used as an indicator of sewage pollution and the possible presence of other pathogens derived from sewage[7].

2.3.2 *Aeromonas* spp.

Aeromonas spp. are commonly occurring facultative anaerobic bacteria found in various aquatic environments[42]. Many species within the *Aeromonas* genus are associated with human sickness, particularly mesophilic *Aeromonas* spp[24]. Gastroenteritis is a diagnosis linked to the consumption of seafood contaminated with *Aeromonas* bacteria[42]. The presence of *Aeromonas* extends beyond seafood and can also be found in other food products such as meat and vegetables[24]. Illnesses caused by *Aeromonas* have been reported in different regions ranging from Bangladesh to Finland, involving a wide range of food products[24]. It is important to note that *Aeromonas* species can thrive in cold storage conditions[43], which poses a food safety consideration, especially in foods that are not subjected to strict treatments before human consumption, such as raw seafood.

2.3.3 *Vibrio* spp.

Vibrio spp. are facultative anaerobic motile bacteria that are native to oceanic environments[44]. This genus generally thrives in warm water with appropriate salinity

levels[44], but some species like *V. alginolyticus*, *V. metschnikovii*, *V. anguillarum* can be found in Norwegian ocean water[45]. While most species of *Vibrio* pose no harm to humans, there are certain pathogenic species that are of significant concern for human health. One of the most well-known species, *Vibrio cholerae*, can cause cholera and, in severe cases, result in the death of otherwise healthy individuals[44]. *Vibrio* spp. can find their way into seafood due to their presence in the surrounding environment. *Vibrio cholerae* and *Vibrio parahaemolyticus* have been identified in seafood from various regions, including Germany and Brazil, with the latter species being responsible for a significant proportion of foodborne illnesses in Japan[24].

It is essential to note that *Vibrio* spp. are not typically associated with enteric bacteria or sewage contamination[24]. Therefore, cases of contamination in seafood occur as a result of the bacteria's natural presence in seawater. For mussel producers in Norway, the main concern regarding contamination occurs during the summer months when *Vibrio* spp. thrive in warmer waters with temperatures of 18°C and above[46].

2.3.4 Environmental factors affecting mussels and their microbiota

Norway has a weathered coastline, and environmental factors can affect which bacteria thrive and die in these waters. For instance, *Vibrio* spp., as previously mentioned, is a genus more adapted to warmer sea temperatures. However, with global climate change affecting ocean temperatures worldwide, the issue of *Vibrio* spp. in Norway may become more prevalent. Since 2019, *Vibrio* infections have been mandatory to report in Norway, which may provide new insights into their occurrence[46]. Additionally, the warming of the ocean has led to an increase in *Vibrio*-related infections among both humans and mussels[37]. Notably, there has been a concerning proliferation of *Vibrio cholerae* over the past few decades[47].

Another aspect to consider is the impact of ocean acidification, another product of climate change. Increased carbon dioxide (CO_2) in the atmosphere leads to more dissolved CO_2 in the water, resulting in a lower pH. Research has shown that even a slight change in pH can alter the microbial community in the North Sea, which

could potentially have adverse effects on blue mussels by facilitating the proliferation of pathogenic *Vibrio* spp[48] [37]. Weather conditions and ocean currents also play a role in shaping the mussel microbiota. Severe storms, heavy rainfall, drainage from land, and changes in ocean currents can introduce foreign bacteria into the mussels' habitat, potentially superseding the indigenous bacteria[37]. These changes can have cascading consequences on both the mussels' health and the safety of their consumption by humans. Weather and ocean currents can also affect nutrient concentrations in the water. Increased nutrient levels have been shown to lead to mass mortality in blue mussels due to the increase of heterotrophic bacteria[49]. It is important to note that these changes are often driven by eutrophication, which provides favourable conditions for opportunistic pathogens[37]. Overall the worrying aspect is that these developments have been documented with unknown long-term outcomes and effects due to the accelerating speed of climate change.

The location of mussel production sites is another important consideration. Offshore and deeper waters offer advantages over coastal areas, as they mitigate issues associated with agricultural runoff and faecal contamination[50]. These factors directly affect food safety, particularly concerning pollution from sewage. Consequently, it is reasonable to assume that the risk of antimicrobial resistance (AMR) spread through agricultural runoff is also minimised. Cultivating mussels in deeper water provides the added benefit of a more stable environment, with fewer fluctuations in temperature and other conditions able to stress the mussels. This stability can lead to more favourable growth conditions[50]. With the increasing impact of climate change on oceanic ecosystems, it becomes evident that exploring alternative cultivation locations may be beneficial for the mussel industry.

2.4 Food safety measures for shellfish

Throughout history, the seafood industry has implemented different methods to address the accumulation of pathogenic contamination in shellfish. Since contaminants typically are sensory indiscernible to consumers, ensuring food safety requires measures to be taken before the product reaches the consumers.

2.4.1 Depuration and relaying

One method used to ensure lower levels of enteric bacteria in mussels is placing live mussels in clean seawater after collection, which utilises their natural filtration activity to expel harmful pathogens from their intestines[3]. This approach was initially developed in response to outbreaks of typhoid fever caused by shellfish consumption[7]. In 1911, studies performed in the US indicated the potential for removing all coliforms from contaminated mussels. The process, known as relaying, involves moving the mussels to another location in the ocean with cleaner seawater to facilitate bacterial purging[7]. In Norway, relaying is only permitted to class A waters, as determined by the Norwegian Food Safety Authority[16]. The legislation sets demands for a minimum relaying duration of two months, but sufficient relaying is determined when the mussels meet Class A criteria, and not merely by duration[20].

Another method is depuration, which elaborates on the principle of relaying but instead of finding a suitable location one is constructed. Depuration involves placing the mussels in trays in large seawater tanks within an artificial environment. Seawater is either replaced in batches or continuously renewed through flow. In Norway, there is no specified required duration for depuration. Instead, the depuration is carried out until the harvested mussels meet class A criteria[20]. It is also crucial to ensure the cleanliness of the seawater used in depuration to prevent further contamination or re-contamination. This may involve sterilisation through chemical or physical means, such as using UV light[7]. Bacteria purged during the depuration process are excreted by the mussels as faecal matter. While this method effectively removes bacterial contaminants, it is primarily limited to eliminating intestinal bacteria[3]. Depuration is not as effective in removing viral pathogens or algal toxins[51][22]. It is also of import to remark that depuration is only a viable decontamination measure to a certain extent for bacterial contamination. *E. coli* is commonly used as an indicator organism for faecal contamination, and if the concentration exceeds certain thresholds, depuration may no longer sufficiently remove enough *E. coli* and other pathogens to ensure the mussels are adequately decontaminated for safe human consumption. Both the EU and the US have laws prohibiting the depuration of mussels with excessively high contamination levels[7]. Therefore, mussel producers should

ensure that their production takes place in non-contaminated waters to minimise the need for extensive decontamination processes.

Overall, a combination of practices such as relaying and depuration can be employed to mitigate pathogenic contamination in mussels, but it is essential to understand the limitations of these methods and prioritise production in clean water sources to ensure food safety. This is especially important due to the knowledge gap concerning the depuration's effect on the microbial community in blue mussels as a whole.

2.4.2 Heat treatment

Another commonly adopted food safety measure is heat treatment. Generally, heat treatment involves raising the internal temperature of the mussels, or other food items, to a level that kills or destroys pathogens, ideally without compromising the sensory attributes of the food. Normally the heat treatment is facilitated through cooking, boiling, steaming, or pasteurisation. These methods do not only target pathogenic microorganisms but also help to stem spoilage bacteria, thereby extending the shelf life of the shellfish[24]. For these reasons, heat treatment is commonly employed as a regulatory measure to ensure food safety standards. Regulatory authorities like the Norwegian Food Safety Authority specify requirements for heat treatment methods, including the recommended time and temperature combinations, to ensure pathogen reduction to acceptable concentrations. In Norway, the specified heat treatments for mussels are either boiling or pressure cooking to raise the internal temperature of the mussels to a minimum of 90 °C for a minimum of 90 consecutive seconds, alternatively cooking for 3 to 5 minutes in a closed container holding a temperature of 120 to 160 °C and pressure of 2 to 5 kg/cm^2, followed by shell removal and freezing of the mussel flesh to an internal temperature of -20°C[20]. In the case of the mussel industry, heat treatment is useful as it serves as an effective method of not only killing bacterial pathogens, e.g. *Vibrio* spp. and *E. coli*, but also destroying viral pathogens like norovirus and hepatitis A virus, that may be present in the mussels. This treatment measure thus eliminates the most common potential pathogens associated with mussel consumption[24]. Still, public perception associates blue mussels with fresh quality, and cooked or frozen blue mussels

are far from as sought after as fresh mussels. Consumers typically perform a light heat treatment at home, or in restaurants, by steaming the mussels, often in white wine. This process is focused on the sensory aspects of the mussels more so than food safety, which potentially does not ensure a safe product. This is a particular concern since the mussels generally only are steamed until a large proportion of the shells have opened. The previously mentioned study by Kausrud et al. showed the lacking reduction of *E. coli* content in blue mussels prepared in a similar fashion to in a traditional consumer home, underlining the importance of proper heat treatment or alternative food safety measures. On that note, it is important to recall that heat treatment is just one aspect of a thorough food safety regime. Other critical measures, such as proper cultivation conditions and good hygiene practices in the value chain, also play vital roles in ensuring the continued food safety and quality of mussels.

3 | Materials and method

3.1 Sampling of mussels

The blue mussels (*M. edulis*) used in this study was provided by a Norwegian blue mussel producer. The mussels originated from the producer's cultivation facilities in Åfjord, Norway. Microbial analysis of the mussels was performed in-house at NTNU´s laboratories at Kalvskinnet in Trondheim.

3.1.1 Season, locality and depuration trials

Blue mussels were sampled over the course of half a year, from the autumn of 2022 to the spring of 2023, to evaluate season variations in the mussels' microbial content. The first sampling was performed in November of 2022 as part of the prestudy for the present work, the second in March of 2023, and the third in May of 2023. Each sampling consisted of mussels from three specific localities of the producer´s array of production facilities in Åfjord. A map depicting the localities' sites within Åfjord is illustrated in Figure 3.1.1. The localities are situated as follows in the fjord: Askerholm is closest to the inner end, Oldøya to the outer end, and Rånes/Minde in between. These localities were chosen to compare different segments of the fjord regarding microbial composition. The Norwegian Food Safety Authority classified all three of the localities as class B areas[52] during each sampling event.

Half of the sampled mussels from each locality were depurated to study the effects of depuration on the microbial content of blue mussels, while the other half was untreated. The depuration process was carried out on-site in Åfjord by the producer for a minimum of 12 hours. The live mussels were then shipped chilled to NTNU in Trondheim for microbial examination. A total of eighteen samples with 50g of mussel flash and intravalvular liquid were used for each season: Three localities with two treatment options, each with three parallels. A table showing the breakdown of the sampling regime is shown in Table 3.1.1. The total number of samples for all seasons considered was thus fifty-four.

Figure 3.1.1: Map of the producer's cultivation localities of blue mussels (*M. edulis*) used for seasonal sampling in this study[53]. The three localities used were Askerholm, Rånes/Minde and Oldøya, each representing a distinct part of the fjord. Askerholm is situated in the innermost part of the fjord, Oldøya in the outermost part near the open ocean, and Rånes/Minde between the other two. Samples were collected in November of 2022, March of 2023 and May of 2023, and analysed at NTNU in Trondheim. At the time of sampling, these localities were classified as class B areas by the Norwegian Food Safety Authority[52]. Each sample used for analysis included mussels that were treated with depuration and untreated mussels.

Table 3.1.1: Table of all eighteen blue mussel samples with their corresponding locations of origin, relative location in Åfjord, means of treatment, parallel number and identification code.

Sample number	Origin	Relative location	Treatment	Parallel	ID
1	Askerholm	Inner	No depuration	1	IF1
2				2	IF2
3				3	IF3
4			Depuration	1	IE1
5				2	IE2
6				3	IE3
7	Rånes/Minde	Middle	No depuration	1	MF1
8				2	MF2
9				3	MF3
10			Depuration	1	ME1
11				2	ME2
12				3	ME3
13	Oldøya	Outer	No depuration	1	YF1
14				2	YF2
15				3	YF3
16			Depuration	1	YE1
17				2	YE2
18				3	YE3

3.1.2 Depuration time trial

In March of 2023, two additional separate batches of blue mussels were sampled to be used in a trial to evaluate depuration over time. One batch was depurated before being contaminated with bovine manure to induce *E. coli*-contamination, while the other batch was unaltered after collection. Following this, both batches were depurated for 24 hours, and samples were collected from each batch after 0, 2, 4, 6, 8 and 24 hours of depuration. The samples were then analysed for contents of psychotropic bacteria and *E. coli*.

3.1.3 Heat treatment trial

Alongside the batches for the depuration trial, a separate batch was sampled for a heat treatment trial to evaluate the heat death aspect in the cooking process of mussels. These mussels were collected in March of 2023 and were untreated before the trial. Blue mussels were then steamed with 100 mL water in kettles on a stove pot to simulate how mussels are traditionally prepared in kitchens. Samples were collected at 0, 3, 6 and 10 minutes of steaming. Internal temperatures of the mussel flesh were recorded before the cooked mussels were analysed for contents of psychotropic bacteria and *E. coli*.

3.2 Microbial analysis

In order to enable the mussels for analysis using the selected media, a 1:10 dilution homogenate was prepared with each sample. The homogenate preparation was conducted as described by Grevskott et al., with supplemental peptone water prepared as described in Nordic Committee on Food Analysis method 150[55], with *Bacteriological peptone* (Oxoid LP0037) and *Sodium chloride* (VWR 27810.295). 30 mL of each homogenate solution was stored for DNA sequencing later. A 10-fold dilution series was prepared with each sample using the remaining homogenate, resulting in dilutions ranging from 1:10 to 1:10 000. Afterwards, the homogenate's contents of psychrotrophic bacteria, mesophilic bacteria, H_2S producing bacteria, *Aeromonas* spp., *Vibrio* spp., and *E. coli* were analysed. The microbial parameters' methodolo-

gies described in the prestudy are recited in the following sections for clarity [53].

3.2.1 Psychrotrophic bacteria

Psychrotrophic bacteria count was determined with Long & Hammer (LH) agar. Preparation and incubation were conducted according to Nordic Committee on Food Analysis method 184 [55]. 0.1 mL of each homogenate dilution was inoculated.

3.2.2 Mesophilic bacteria

Mesophilic and H_2S-producing bacteria counts were determined using iron agar - mesophilic count by total colony count, and H_2S-producing count by black colony count. Agar was prepared, and 1 mL of each homogenate dilution was inoculated and incubated, as described in Nordic Committee on Food Analysis method 184 [55].

3.2.3 *Aeromonas* spp.

Yellow colony count on Starch Ampicillin Agar (SAA) was used to determine *Aeromonas* spp. count. Agar was prepared with 31.02 g/L *Phenol Red Agar Base* (HiMedia Laboratories M053-500G), 10.00 g/L *Soluble Starch* (BD 217820) and peptone water, and added 10 g/L *Ampicillin* (Sigma-Aldrich A9393-5G). 0.1 mL from the 10^{-1} and 10^{-2} homogenate dilutions were inoculated and incubated as described in Nordic Committee on Food Analysis method 150 [56].

3.2.4 *Vibrio* spp.

Green-yellow colony count on thiosulfate–citrate–bile salts–sucrose agar (TCBS) was used to determine *Vibrio* spp. count. Agar was prepared with 88 g/L *TCBS Agar* (Millipore 86348-500G). 0.1 mL from the 10^{-1} and 10^{-2} homogenate dilutions were inoculated and incubated as described in Nordic Committee on Food Analysis method 156 [57]

3.2.5 *E. coli*

To study the count of *E. coli* the Most Probable Number (MPN) method for enumeration of *E. coli* as described by Grevskott et al.[54] was used. Double-strength and single-strength solutions of mineral modified glutamate broth (MMGB) were prepared with a kit, *Mineral modified Glutamate Broth (Base)* (Sigma-Aldrich 17171), and *Ammonium chloride* (VWR 21236.291), according to the kit's guide. Inoculation and incubation were conducted as described by Grevskott et al..

TBX agar was prepared using *TBX Agar* (Sigma-Aldrich 92435-100G-F) as described in the manufacturer's manual. Inoculation and incubation were conducted as described by Grevskott et al..*E. coli* per 100g of sample material was estimated using the MPN table described by Lee et al..

3.3 Statistical analysis of mean counts

Using the statistics software IBM SPSS Statistics, log-transformed bacterial counts were used for One-way ANOVA and Post-hoc Tukey multiple comparison tests (95% significance level) to compare groups. In samples were no counts were able to be detected the count was set to zero.

3.4 Microbial community identification

The following paragraphs are recited from the prestudy[53], with alterations to account for changes in this larger project.

3.4.1 DNA extraction

DNA extraction was carried out using the *Genomic Mini AX Food* (A&A Biotechnology 053-60), following the protocol provided in the kit's instruction manual.

3.4.2 Spectrophotometrical determination of DNA concentration

Spectrophotometric analysis was conducted using a Biotek Powerwave XS (MQX200R) spectrophotometer equipped with a Take3 adapter. The DNA concentration in the extracted DNA samples was determined by measuring the absorbance of two parallels, each containing 2 μL of DNA, at three specific wavelengths, 230, 260 and 280 nm. The obtained measurements provided A260/280 and A260/230 ratios as well as the concentration in ng/μL.

3.4.3 Gel electrophoresis

A qualitative assessment of the extracted DNA was performed using gel electrophoresis. A 1.5% agarose gel, supplemented with *GelRec Nucleic Stain (10,000X in Water)* (Merck Life Science SCT123), was prepared. To prepare the gel, 1.5 g of *SeaKem LE Agarose* (Lonza 50004) was dissolved in 75 mL of 1x TAE buffer.

For the electrophoresis run, the first and last wells of the gel were loaded with 5 μL of *GP 1kb Plus Ladder* (Qiagen 239095), while the remaining wells were filled with 5 μL of DNA samples and 1 μL of *GelPilot 5x Loading Dye* (Qiagen 239901). The electrophoresis run was conducted at 90 Volts and 400 Amperes for a duration of 90 minutes.

3.4.4 Sequencing and identification

The extracted DNA samples were shipped to Eurofins Genomics in Konstanz, Germany, for library preparation and 16S rRNA amplicon sequencing. At Eurofins Genomics, Minimum Entropy Decomposition (MED) was used to partition marker gene datasets into Operational Taxonomic Units (OTUs). Then DC-MEGABLAST was performed to assign taxonomic information to each OTU.

3.4.5 Troubleshooting

As part of the prestudy, DNA from blue mussels samples in November was similarly extracted, analysed and sent to Eurofins Genomics for sequencing and identifica-

tion. However, due to unknown reasons, a majority of samples could not be used by Eurofins Genomics, even though the qualitative and quantitative requirements were met. To work around this issue, several troubleshooting steps were undertaken to improve the DNA quality in the hope of a higher success rate of sequencing that Eurofins Genomics could achieve. New DNA samples were extracted and used in multiple polymerase chain reaction (PCR) runs with different annealing temperatures but to no avail. The samples in November were extracted using *DNeasy Powerfood Microbial Kit (100)* (Qiagen 21000-100), and as a final solution the extraction was instead carried out using *Genomic Mini AX Food* (A&A Biotechnology 053-60). However, due to time constraints, the results could not be generated by Eurofins Genomics in time for this study's deadline, but the methods described were still carried out.

4 | Results

Every individual parallel of bacterial count and MPN *E. coli* is supplied in the tables of Appendix A. The corresponding means are presented in figures in the following subsections.

4.1 Impact of season and locality on microbial count

The blue mussels samples in this study were harvested from Åfjord, Norway, in three different months (November, March and May) from the autumn of 2022 to the early summer of 2023. During each sampling event mussels were harvested from three different cultivation localities within Åfjord, enabling comparison of localisation within the fjord as well as season. In this section, only the mean counts from un-depurated blue mussels are presented.

The psychrotrophic bacteria count means ranged from 2.4 ± 2.1 to 4.4 ± 0.29 log CFU/g, the mesophilic mean counts from 2.1 ± 1.8 to 2.9 ± 0.20 log CFU/g, the H_2S producing bacteria mean counts from 0.42 ± 0.72 to 2.0 ± 0.76 log CFU/g, the presumptive *Vibrio* spp. mean counts ranged from 0.65 ± 1.1 to 2.2 ± 0.35 log CFU/g and the mean MPN of *E. coli* ranged from 6.7 ± 12 to 133 ± 174 log CFU/g in the mussel samples. No significant difference from neither sampling locality nor season was shown for any of these five microbial parameters (Figure 4.1.1-4.1.5).

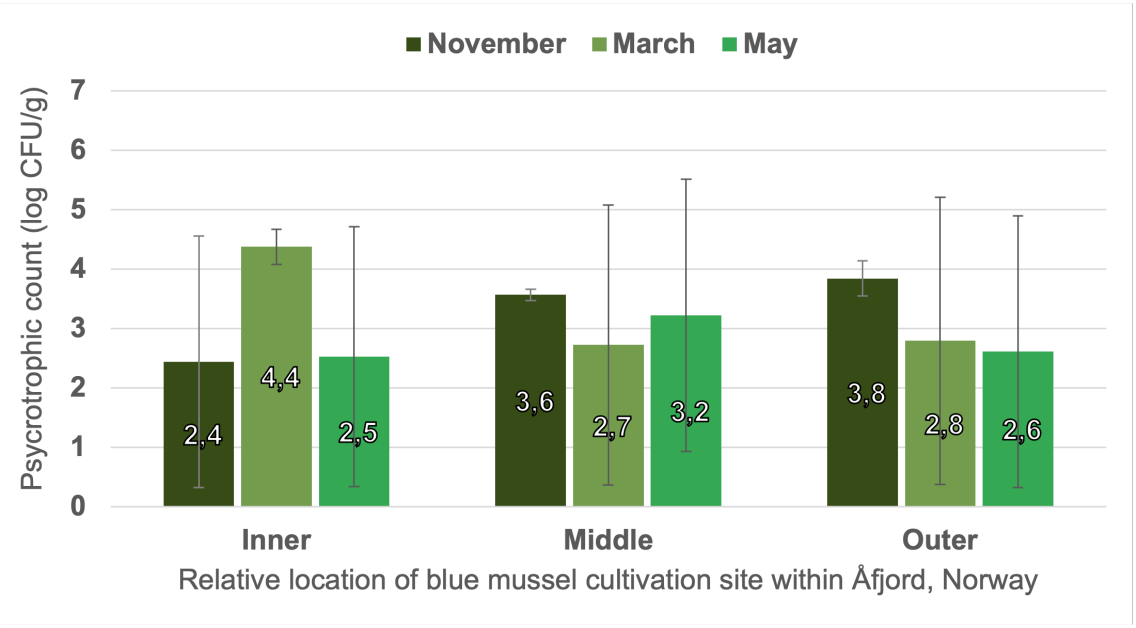


Figure 4.1.1: Bar chart showing mean counts of psychrotrophic bacteria found in blue mussels originating in Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023. The values are given as mean (n=3) counts ± SD. 1 parallel at the inner locality in November, 1 from the middle and outer localities each in March, and 1 from every locality in May did not result in a countable number of colonies

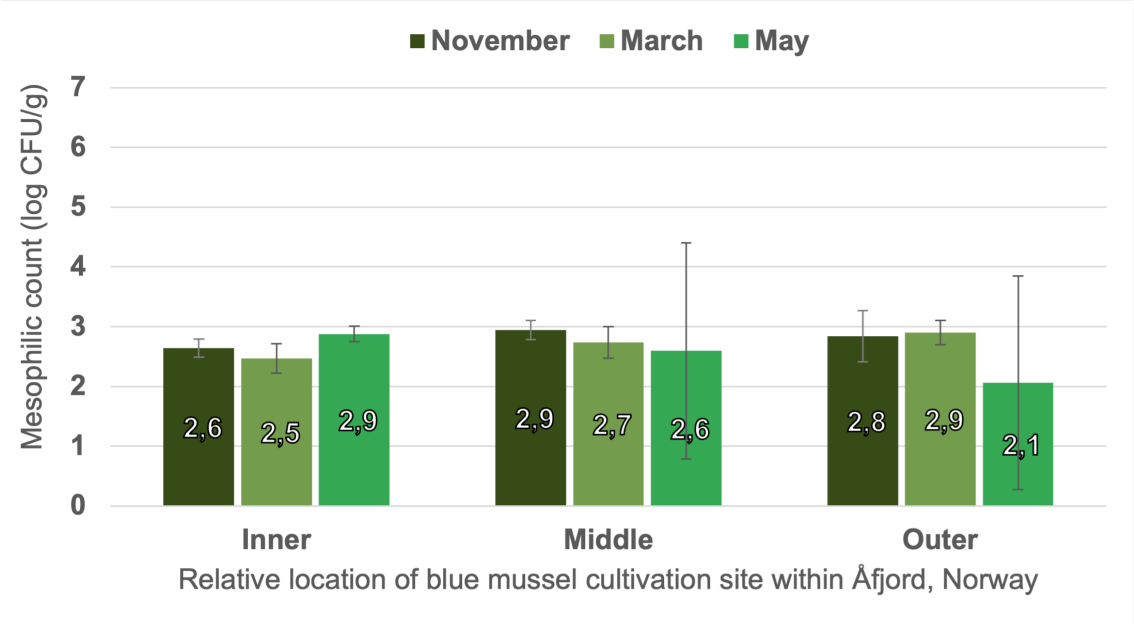


Figure 4.1.2: Bar chart showing mean counts of mesophilic bacteria found in blue mussels originating in Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023. The values are given as mean (n=3) counts ± SD. 2 parallels from the middle and 1 from the outer locality in May did not result in a countable number of colonies.

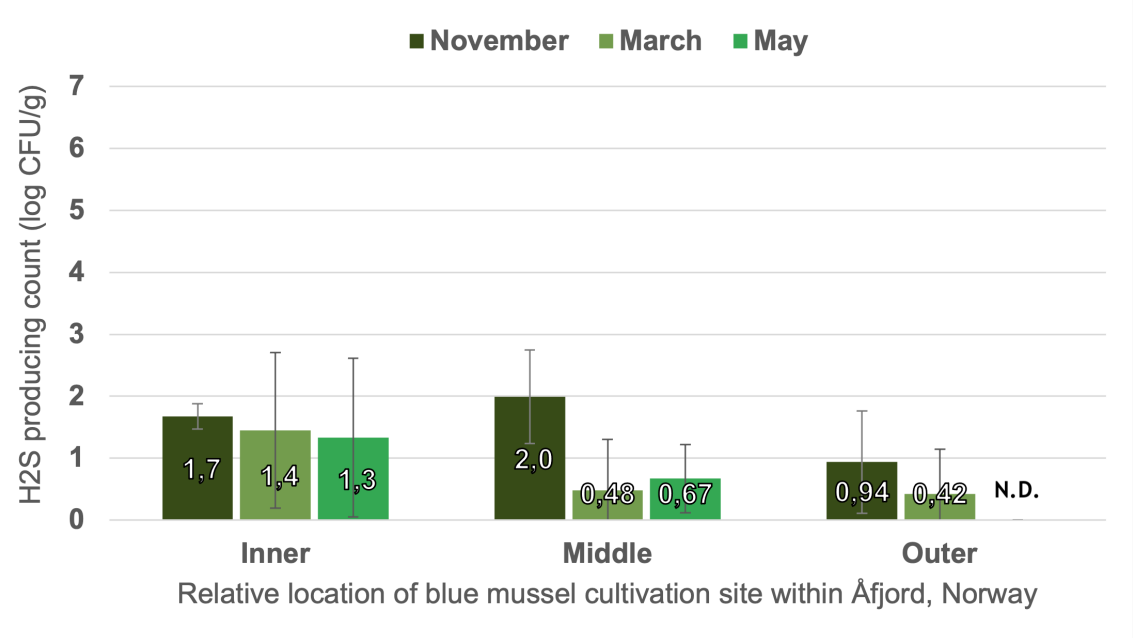


Figure 4.1.3: Bar chart showing mean counts of H_2S producing bacteria found in blue mussels originating in Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023. The values are given as mean (n=3) counts ± SD. 1 parallel from the outer locality in November, 1 from the inner and 2 from the middle and outer localities each in March, 1 from the inner and 2 from the middle locality in May did not result in a countable number of colonies. Means with no detected count in any parallel are denoted (N.D.).

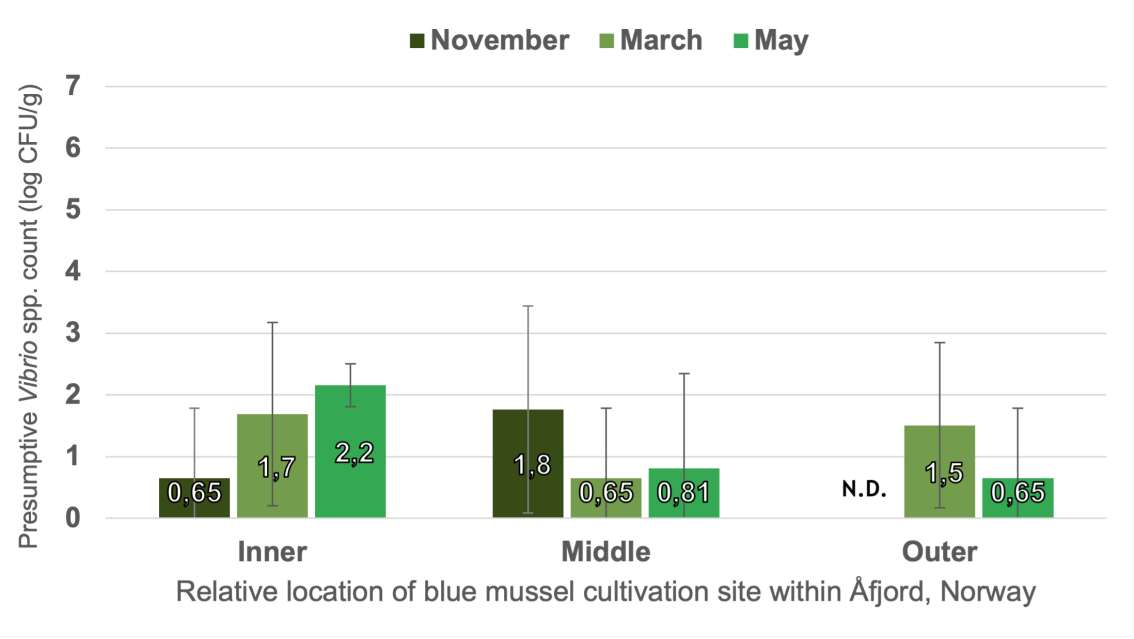


Figure 4.1.4: Bar chart showing mean counts of presumptive *Vibrio* spp. found in blue mussels originating in Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023. The values are given as mean (n=3) counts ± SD. 2 parallels from the inner and 1 from the middle localities in November, 1 from the inner, 2 from the middle and 1 from the outer locality in March, and 2 from the middle and outer localities each in May did not result in a countable number of colonies. Means with no detected count in any parallel are denoted (N.D.).

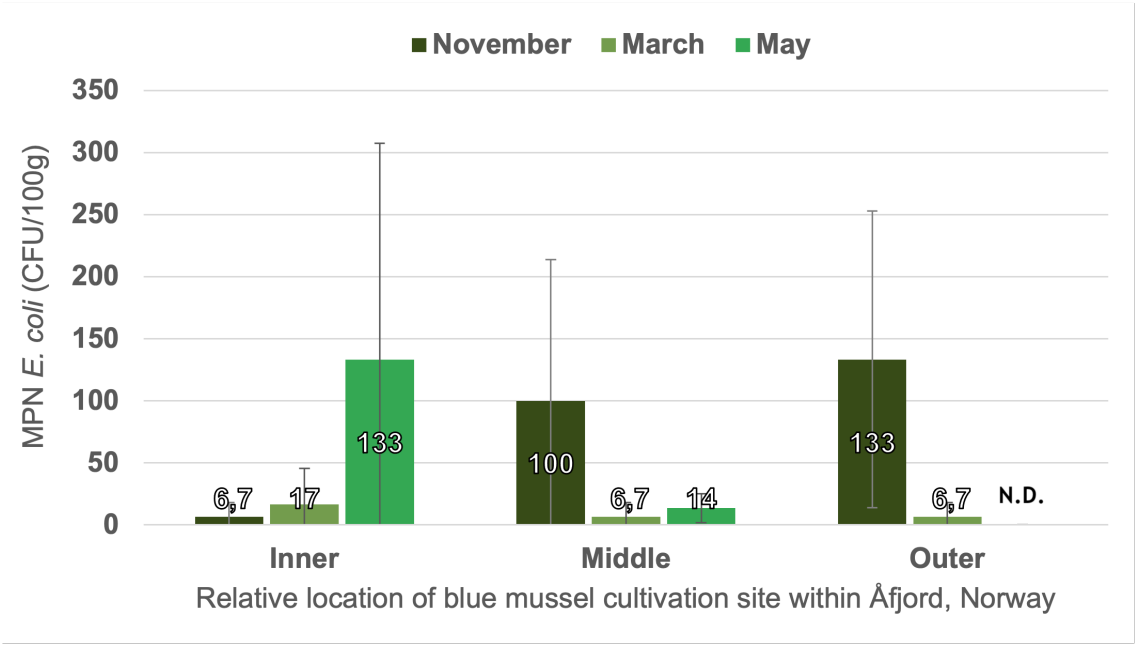


Figure 4.1.5: Bar chart showing mean MPN of *E. coli* found in blue mussels originating in Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023. The values are given as mean (n=3) counts ± SD. 2 parallels from the inner and 1 from the outer locality in November, 2 from the inner, middle and outer localities each in March, and 1 from the inner and 2 from the middle localities in May did not result in detectable levels of *E. coli*. Means with no detected MPN in any parallel are denoted (N.D.).

The mean count of presumptive *Aeromonas* spp. ranged from 1.0 ± 1.8 to 4.1 ± 0.35 log CFU/g in the mussel samples. There was no significant difference between localities, however, within the inner and middle localities significant ($p<0.05$) seasonal differences were noted. At the inner locality, the samples from March had significantly higher *Aeromomonas* spp. contents compared to the samples from November and May. At the middle locality, there was a significant ($p<0.05$) increase of *Aeromonas* spp. content from November to March, while the samples from May were not significantly different from the other two months and acted as a numerical middle point (Figure 4.1.6).

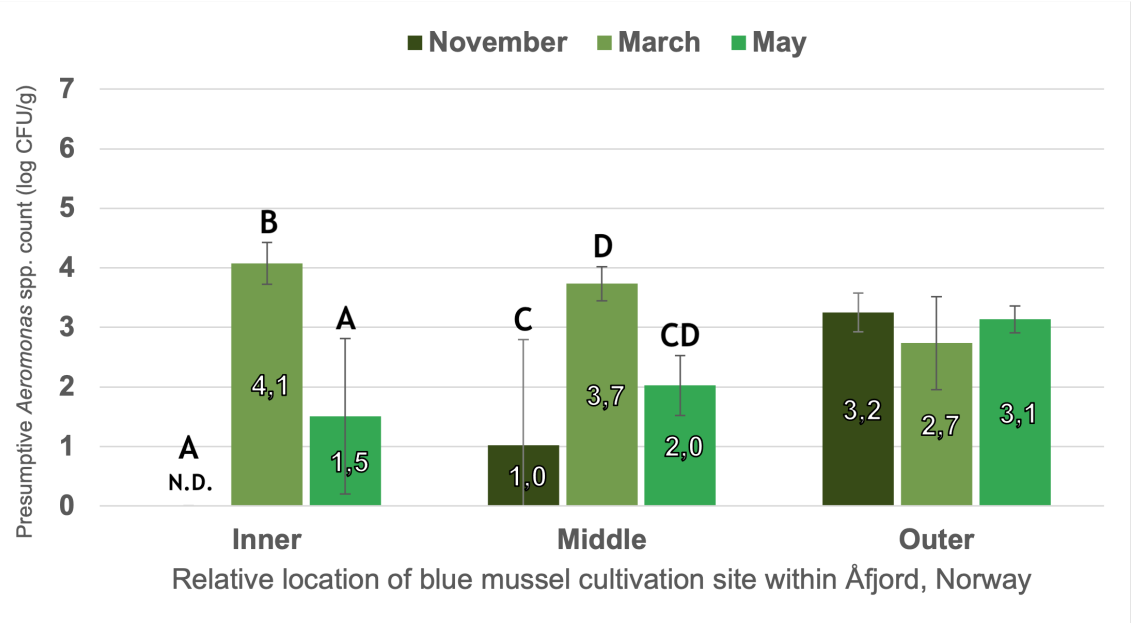


Figure 4.1.6: Bar chart showing mean counts of presumptive *Aeromonas* spp. found in blue mussels originating in Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023. The values are given as mean (n=3) counts ± SD. Significantly ($p<0.05$) different means by season within the bounds of each locality are denoted with respective letters (A, B, C, D). No significant difference was shown between the localities overall. 2 parallels from the middle locality in November, and 1 from the inner locality in May did not result in a countable number of colonies. Means with no detected count in any parallel are denoted (N.D.).

4.2 Impact of depuration on microbial count

For every sample of blue mussels, both in terms of season and locality, samples treated with depuration were also received. By comparing the microbial contents of mussels with and without depuration treatment it is possible to evaluate the effects of depuration.

The psychrotrophic bacteria count means ranged from 3.1 ± 1.7 to 3.3 ± 1.2 log CFU/g, the mesophilic mean counts from 2.5 ± 0.94 to 2.8 ± 0.61 log CFU/g, the H_2S producing bacteria mean counts from 0.78 ± 1.0 to 1.0 ± 0.95 log CFU/g, and the presumptive *Vibrio* spp. mean counts ranged from 0.78 ± 1.2 to 1.1 ± 1.2 log CFU/g in the mussel samples. No significant difference from the depuration process was shown for any of these four microbial parameters (Figure 4.2.1-4.2.4).

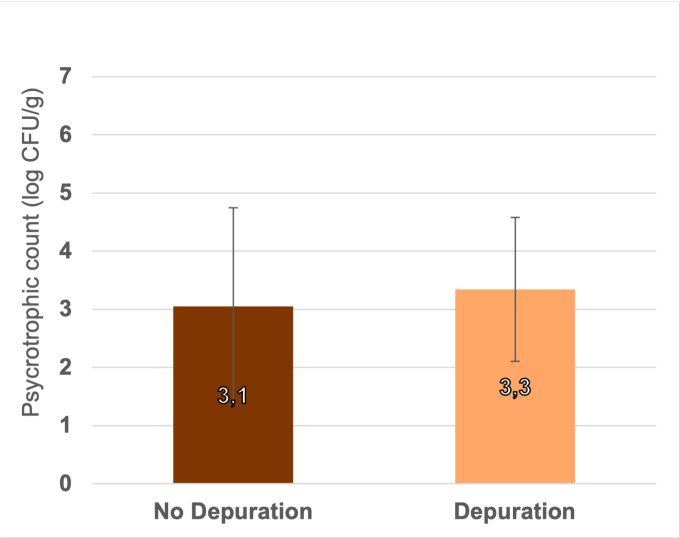


Figure 4.2.1: Bar chart showing mean counts of psychrotrophic bacteria found in depurated and non-depurated blue mussels from Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023, and subsequently half of each batch was treated with depuration. The values are given as mean (n=27) counts ± SD. 6 of the 27 non-depurated samples and 3 of the 27 depurated samples did not result in a countable number of colonies.

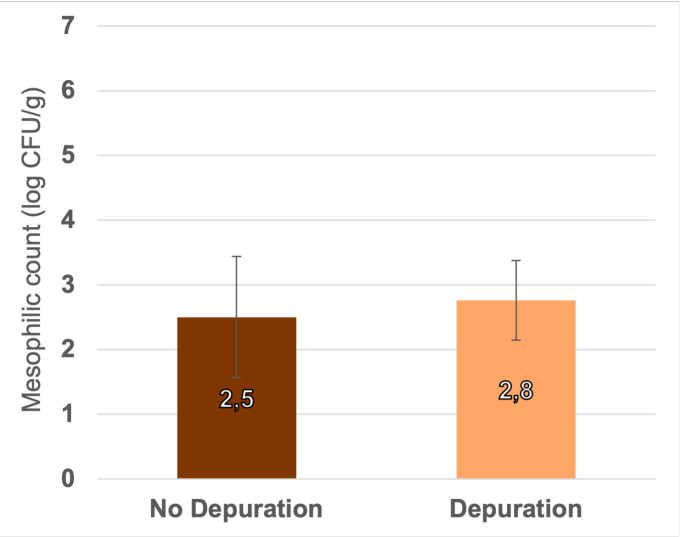


Figure 4.2.2: Bar chart showing mean counts of mesophilic bacteria found in depurated and non-depurated blue mussels from Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023, and subsequently half of each batch was treated with depuration. The values are given as mean (n=27) counts ± SD. 3 of the 27 non-depurated samples and 1 of the 27 depurated samples did not result in a countable number of colonies.

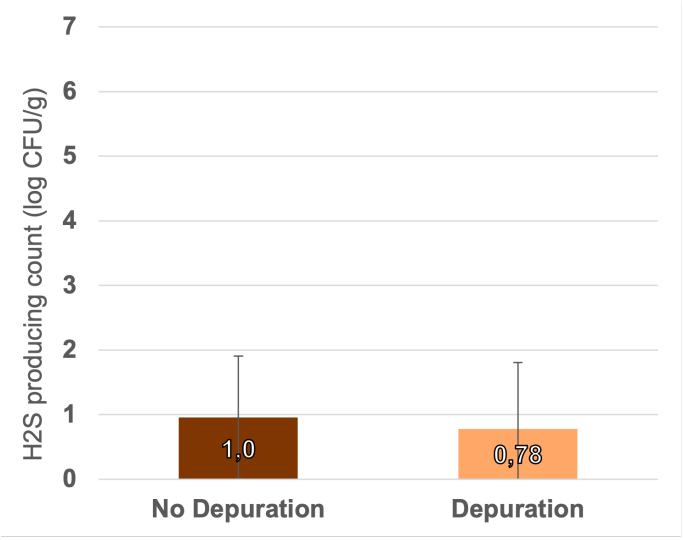


Figure 4.2.3: Bar chart showing mean counts of H_2S producing bacteria found in depurated and non-depurated blue mussels from Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023, and subsequently half of each batch was treated with depuration. The values are given as mean (n=27) counts ± SD. 12 of the 27 non-depurated samples and 16 of the 27 depurated samples did not result in a countable number of colonies.

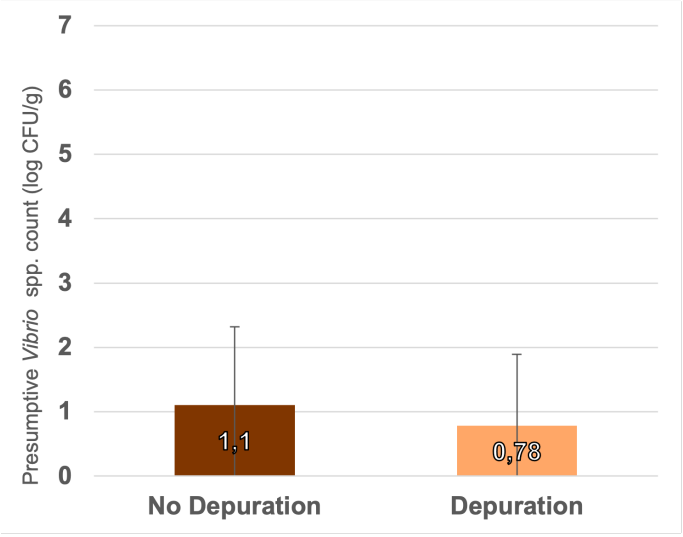


Figure 4.2.4: Bar chart showing mean counts of presumptive *Vibrio* spp. found in depurated and non-depurated blue mussels from Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023, and subsequently half of each batch was treated with depuration. The values are given as mean (n=27) counts ± SD. 6 of the 27 non-depurated samples and 12 of the 27 depurated samples did not result in a countable number of colonies.

The mean count of presumptive *Aeromonas* spp. ranged from 1.5±1.5 to 2.5±1.5 log CFU/g and the mean MPN *E. coli* ranged from 3.3±46 to 46±88 CFU/100g in the mussel samples. There was a significantly ($p<0.05$) lower means in the depurated samples compared to the un-depurated samples for these two microbial parameters

(Figure 4.2.5-4.2.6).

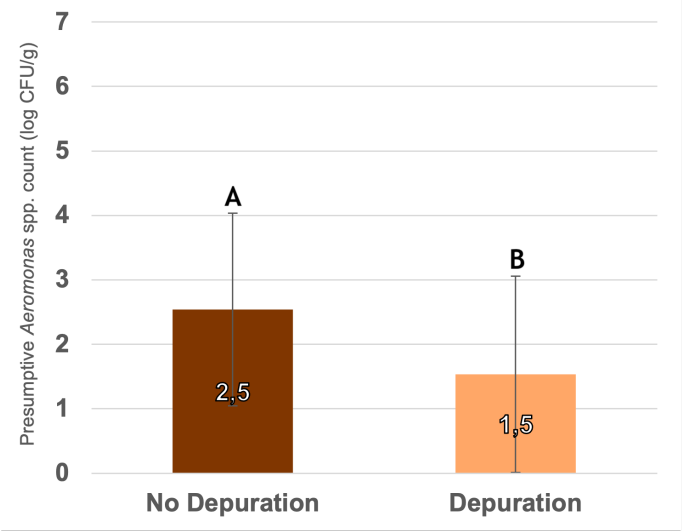


Figure 4.2.5: Bar chart showing mean counts of presumptive *Aeromonas* spp. found in depurated and non-depurated blue mussels from Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023, and subsequently half of each batch was treated with depuration. The values are given as mean (n=27) counts ± SD. 14 of the 27 non-depurated samples and 17 of the 27 depurated samples did not result in a countable number of colonies.

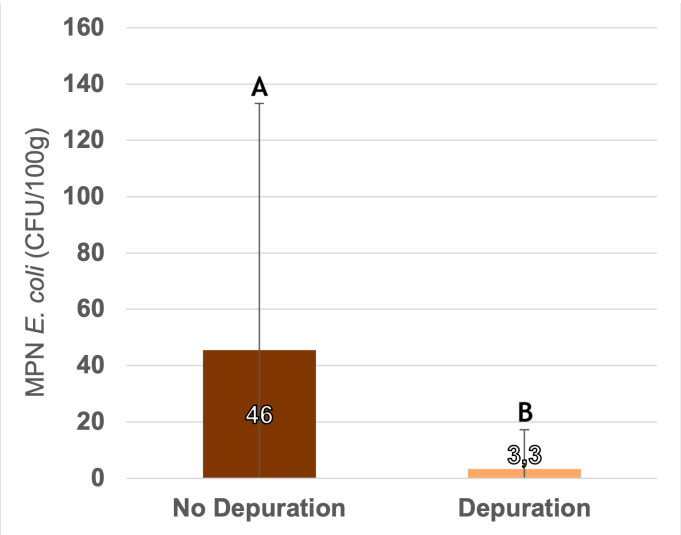


Figure 4.2.6: Bar chart showing mean MPN of *E. coli* found in depurated and non-depurated blue mussels from Åfjord, Norway. The mussels were harvested from 3 separate localities in Åfjord in November of 2022, and March and May of 2023, and subsequently half of each batch was treated with depuration. The values are given as mean (n=27) counts ± SD. 15 of the 27 non-depurated samples and 25 of the 27 depurated samples did not result in detectable levels of *E. coli*.

4.3 Impact of depuration duration on microbial count

Two separate batches of blue mussels were harvested in March of 2023 to be used in an evaluation of the depuration process over time. One batch was unaltered from the ocean, while the other was depurated before being artificially contaminated with bovine manure to induce *E. coli* contamination. Both batches were then depurated and samples were collected every 2 hours for 8 hours, and a final sample was collected after 24 hours. The samples were then analysed for their content of psychrotrophic bacteria and *E. coli*.

The psychrotrophic bacteria count means started at 3.3±1.1 and ended at 4.0±0.20 log CFU/g for the unaltered samples, and started at 4.2±0.17 and ended at 4.1±0.26 log CFU/g for the contaminated samples, in the depuration process (Figure 4.3.1).

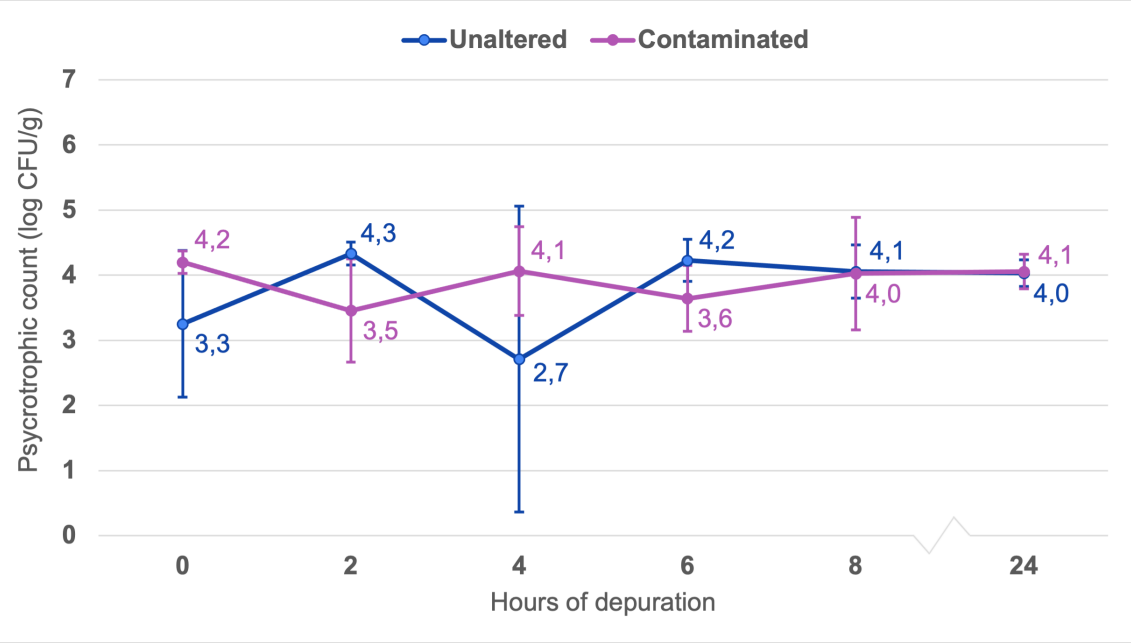
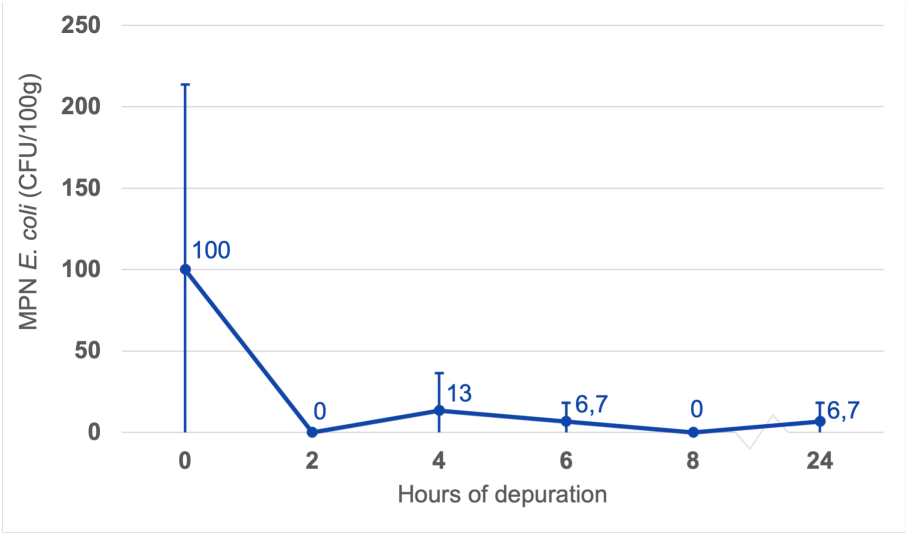
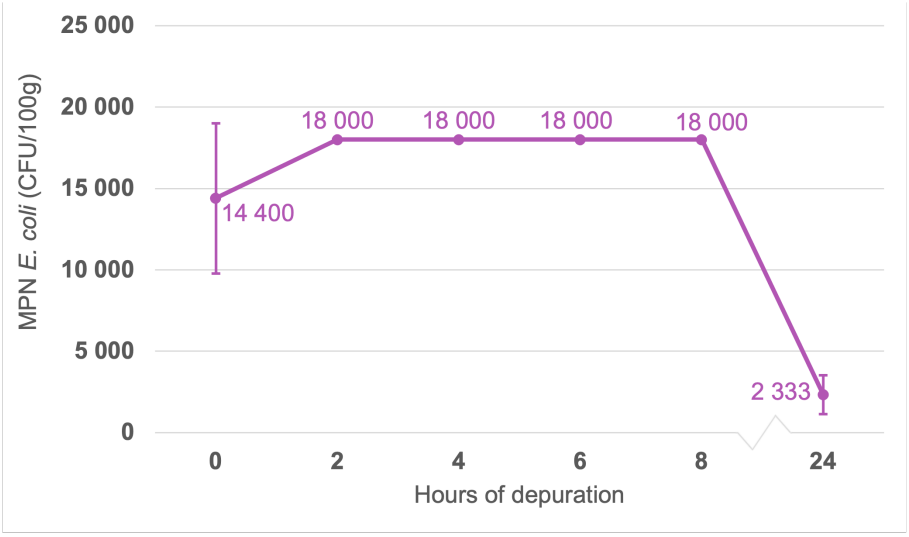


Figure 4.3.1: Scatter plot of mean counts of psychrotrophic bacteria found in blue mussels from Åfjord, Norway, over the course of the depuration process. One batch of mussels was unaltered from the ocean, while the other was artificially contaminated with bovine manure to induce *E. coli* contamination. The values are given as mean (n=3) counts ± SD.

The MPN *E. coli* means started at 100±114 and ended at 6.7±12 CFU/100g for the unaltered samples, and started at 14400 ± 4613 and ended at >= 18000 CFU/100g for the contaminated samples, in the depuration process (Figure 4.3.2).



(a) Scatter plot of mean MPN *E. coli* found in the contaminated mussels. All 3 parallels at the 2 and 8-hour marks, as well as 2 parallels each from the 4, 6 and 24-hour marks, did not result in detectable levels of *E. coli*.



(b) Scatter plot of mean MPN *E. coli* found in the contaminated mussels. The values of 18000 CFU/100g are maximum MPN values in the MPN table[3], and could, in reality, be an underestimate. The real value is therefore unknown, but is at least 18000 CFU/100g.

Figure 4.3.2: Scatter plot of mean MPN of *E. coli* found in blue mussels from Åfjord, Norway, over the course of the depuration process. One batch of mussels was unaltered from the ocean (**a**), while the other was artificially contaminated with bovine manure to induce *E. coli* contamination (**b**). The values are given as mean (n=3) counts ± SD. The values are divided into two separate plots due to their values being several orders of magnitude different.

4.4 Impact of heat treatment on microbial count

A batch of blue mussels was harvested in March of 2023 to be used in an evaluation of steaming as a means of heat treatment for mussel preparation. The mussels were harvested in Åfjord, and no further treatment was employed before this trial. The mussels were steamed in 100 mL of water in a stove pot with a lid, to simulate traditional cooking by consumers. Samples were collected at 3, 6 and 10 minutes of steaming, as well as an uncooked sample. The internal temperature of the mussels was measured immediately following steaming. The samples were then analysed for their content of psychrotrophic bacteria and *E. coli*. The mean internal temperatures of the blue mussels' flesh are shown in Table 4.4.1. Additionally, it should be stated that every single shell treated with steaming in this trial opened on its own, without further human interference.

Table 4.4.1: Table of mean recorded internal temperatures of blue mussels with corresponding minutes of steaming. The mussels were harvested without further treatment, and steamed in 100 mL of water in a stove pot with a lid. The values are given as mean (n=3) temperature ± SD.

Minutes	Temperature [°C]
3	73 ± 4.6
6	76 ± 2.1
10	72 ± 2.1

The psychrotrophic bacteria count started at 4.1 ± 0.58 log CFU/g, and ended at 3.8 ± 0.33, in the steaming process (Figure 4.4.1).

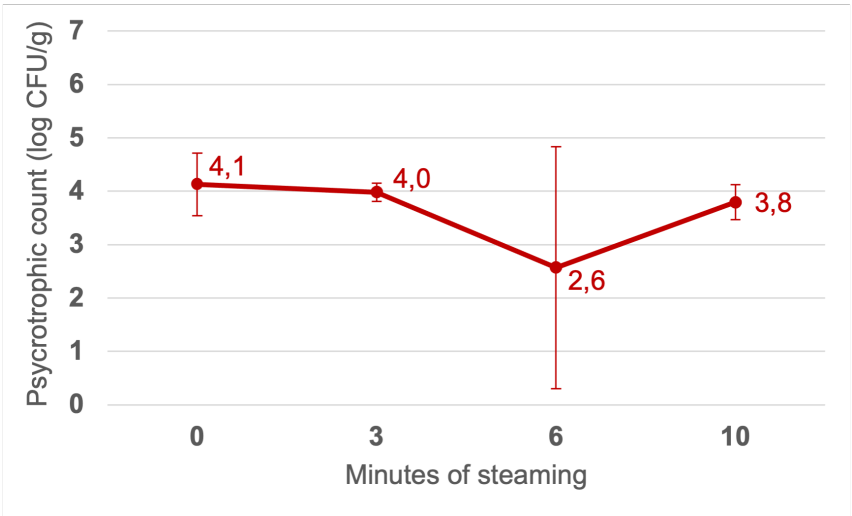


Figure 4.4.1: Scatter plot of mean counts of psychrotrophic bacteria found in blue mussels from Åfjord, Norway, over the course of 10 minutes of steaming. The values are given as mean (n=3) counts ± SD. One parallel at the 6 minute mark did not yield detectable counts of bacteria.

The initial MPN *E. coli* before steaming was 243 ± 236 log CFU/100g. None of the steamed samples resulted in detectable levels of *E. coli*, leaving only the initial value.

4.5 Microbial community identification

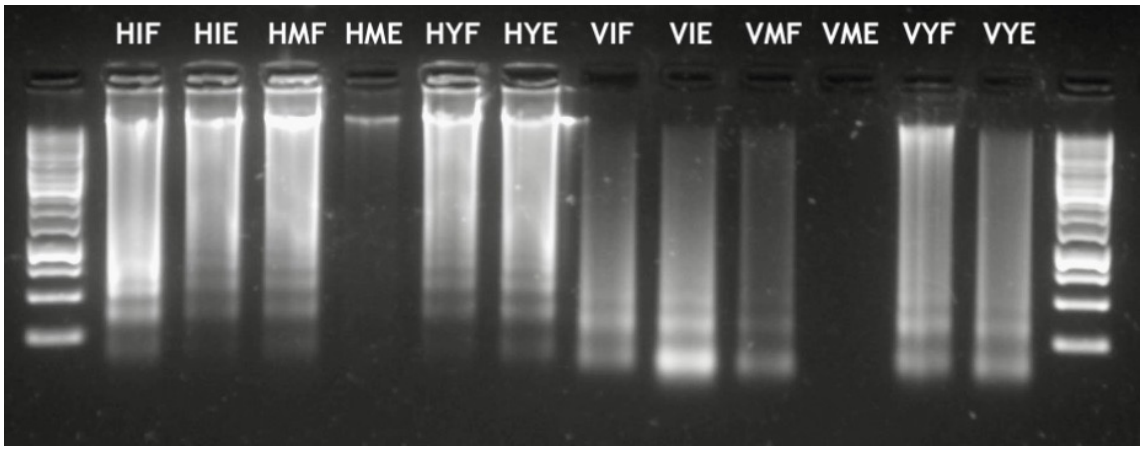
DNA was extracted from blue mussels from Åfjord as part of the trials focused on season, locality and depuration. 18 samples of DNA were extracted and then analysed in-house at NTNU using qualitative and quantitative measures.

The DNA samples had mean DNA concentrations in the range of $48 - 364$ ng/μL, mean A260/280 ratios within the range of $1.772 - 1.927$, and mean A260/230 ratios within the range of $1.362 - 2.195$ (Table 4.5.1).

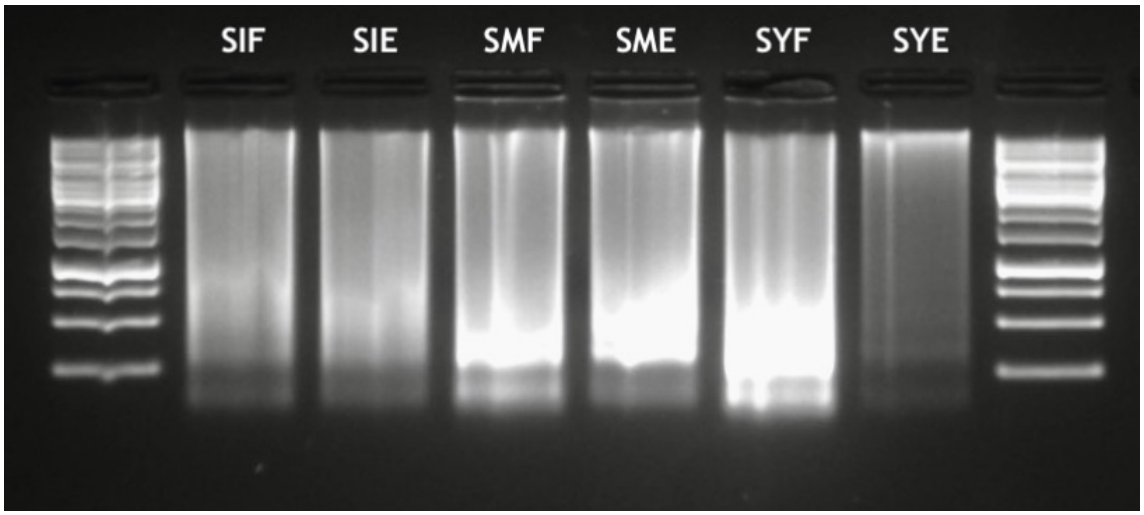
Table 4.5.1: Spectrophotometric measurements of concentration, A260/280, and A260/230 ratio of DNA extracted from blue mussels. Each sample is a combination of parallels (n=3) with a unique combination of sampling month, cultivation locality and depuration treatment.

Month	Locality	Treatment	Sample ID	µg/µL	A260/280	A260/230
November	Inner	No Depuration	HIF	251	1,915	2,188
		Depuration	HIE	235	1,927	2,114
	Middle	No Depuration	HMF	440	1,873	2,195
		Depuration	HME	129	1,877	1,743
	Outer	No Depuration	HYF	272	1,885	2,092
		Depuration	HYE	364	1,820	2,100
March	Inner	No Depuration	VIF	48	1,772	1,362
		Depuration	VIE	65	1,894	1,482
	Middle	No Depuration	VMF	32	1,859	1,615
		Depuration	VME	172	1,838	1,600
	Outer	No Depuration	VYF	97	1,832	2,066
		Depuration	VYE	59	1,823	1,606
May	Inner	No Depuration	SIF	176	1,889	2,138
		Depuration	SIE	238	1,847	1,955
	Middle	No Depuration	SMF	183	1,908	2,104
		Depuration	SME	140	1,839	2,067
	Outer	No Depuration	SYF	172	1,860	1,905
		Depuration	SYE	120	1,787	2,017

The extracted DNA was analysed using gel electrophoresis to determine band length of the DNA fragments. The resulting gels (Figure 4.5.1) were characterised by smearing, indicating fragmentation of the DNA. The VME sample did not yield a band, and the HME sample had a weakly pronounced band.



(a) Gel electrophoresis bands of DNA extracted from 12 blue mussels samples from Åfjord, Norway, sampled in November of 2022 and March of 2023.



(b) Gel electrophoresis bands of DNA extracted from 6 blue mussels samples from Åfjord, Norway, sampled in May of 2023.

Figure 4.5.1: Gel electrophoresis bands of DNA extracted from blue mussels sampled from Åfjord, Norway, in November of 2022 and March of 2023 (**a**), and May of 2023 (**b**). Each band is a combination of parallels (n=3) representing a unique combination of sampling month, cultivation locality and depuration treatment. Sample IDs are denoted above the respective sample band. The corresponding sample to each sample ID is presented in Table 4.5.1

5 | Discussion

In order to assess the food safety and microbiota attributed to blue mussel cultivation in Norway, samples of blue mussels were collected from the cultivation sites of a producer in Åfjord and analysed at NTNU in Trondheim. The microbial contents of the mussels were analysed using six different microbial parameters: psychrotrophic bacteria, mesophilic bacteria, H_2S producing bacteria, *Aeromonas* spp., *Vibrio* spp., and *E. coli*. Samples were chosen to reflect different conditions and were thus collected in three different months (November, March and May) from the autumn of 2022 until the early summer of 2023, as well as from three different localities of blue mussel cultivation in Åfjord, and were or were not treated with depuration for bacterial purging by the mussels. Additionally, extra samples of mussels were harvested separately in late March of 2023 to be used in trials focused on evaluating the depuration process over time, as well as steaming as a heat treatment option in mussel preparation.

A comparison of the mean counts of un-depurated mussels in this study should be drawn to a similar study from 2009 on the effect of depuration conducted by Martínez et al.. Their molluscs were obtained from cultivation areas in Galicia, Spain, with the same classification of *E. coli* concentration limits as class B in Norway, providing grounds for comparison between Åfjord and Galicia. Considering the psychrotrophic counts, there were slightly higher levels in Galicia (3.990 ± 0.725 log CFU/g) compared to Åfjord (3.1 ± 1.7 log CFU/g). The same was also true of mesophilic counts (3.523 ± 0.820 log CFU/g) from Galicia, compared to Åfjord (2.5 ± 0.94 log CFU/g). Taking this into account, Åfjord hosts slightly less of these bacteria overall than Galicia. Nonetheless, the levels are close enough to warrant a comparison of the two areas and studies. On the other hand, the MPN *E. coli* from Galicia and Åfjord tell different stories. Although both areas were classified using the same criteria as Class B cultivation sites, there was a distinctively lower mean MPN value for the un-depurated samples from Åfjord (46 *E. coli*/100g) compared to the un-depurated samples from Galicia (2735 *E. coli*/100g). These results from Åfjord were, surprisingly, well within the limits of the Norwegian Food Safety Authorities'

class A areas (230 *E. coli*/100g). Similarly, a study from 2016 by Grevskott et al. examining bivalve molluscs from various locations along the Norwegian coast found that the majority of samples (87%) met the class A limits, with the remaining samples within class B. With this in mind, the blue mussels from Åfjord used in this study were remarkably uncontaminated by *E. coli*. In fact, as per the definition of class A areas, the mussels could be, if wished, consumed raw. Nonetheless, it is important to note that the sampling locations in this study were categorised as class B areas at the time of sampling and should be considered as such. Studies like that of Grevskott et al. and the present work, simply provide snapshots of microbial content and do not describe long-term trends, as such assertions require comprehensive reviews. The Norwegian Food Safety Authority conducts thorough testing based on continuous sampling to determine classification over time[16], as seasonality is an important parameter for microbial content. Nevertheless, the *E. coli* concentrations observed in this study's samples were well within the food safety standards set by the Norwegian Food Safety Authority.

A common attribute in all of the results was large standard deviations for the mean counts of bacterial parameters, as well as undetectable levels of bacteria. For instance, the psychrotrophic bacteria, mesophilic bacteria, H_2S producing bacteria counts were affected by the quantification limits (25-250) on Long & Hammer and iron agar plates, as described in the Nordic Committee on Food Analysis method 184[55]. thus dragging the average down and increasing the standard deviation of the mean. However, the issue of zero-samples was most apparent in the determination of *Aeromonas* spp., *Vibrio* spp., and *E. coli*, where a large proportion of the samples had undetectable levels. This develops uncertainty in the results and makes concise conclusions ambiguous. Also, There were a plethora of combinations of distinct mussel variations, each characterised by treatment alternative (depuration or no depuration), locality (inner, middle or outer part of Åfjord) and season (November, March or May), but only 3 parallels were used for most results. While this gives a more comprehensive outcome than using a single parallel, the meagre use of 3 parallels has pronounced flaws, which were realised in the results. As stated earlier, the Norwegian Food Safety Authority bases its classification of cultivation areas on 12 samples, one every four weeks, throughout a calendar year. Even after

that, a permanent classification is only given if samples are taken continuously at least six times a year[16]. This clearly underlines the weakness in the number of samples, not just for each sampling season, but also for the number of sampling seasons, that were used in the present study's sampling regime.

5.1 Impact of seasonality on microbial count

Only the presumptive count of *Aeromonas* spp. were significantly ($p<0.05$) distinct throughout the year, at the inner and middle locality, as seen in Figure 4.1.6. At the inner locality, the microbial count in March was significantly ($p<0.05$) higher than the counts in November and May. And at the middle locality, the count was significantly ($p<0.05$) higher in March compared to November, but the mean count in May was not significantly different from the other two. Using the entire year as a reference point it is therefore evident that the *Aeromonas* spp. population decreases significantly over the course of the summer and autumn at these localities. This implies that the *Aeromonas* spp. proliferation reaches its peak in time for, or during, early spring and is in a decline over the coming months approaching autumn. In 2021 Park et al. published a study investigating, among other aspects, the seasonal variation of *Aeromonas hydrophila* in fresh quality fish in South Korea. Their results indicated a higher prevalence of *A. hydrophila* during winter, i.e. January to February, as opposed to during spring and summer, i.e. March to May and June to September respectively. A proposed explanation is the bacteria's inherent ability to resist lower temperatures. Temperatures of 4°C can facilitate *A. hydrophila* proliferation[58], and even a reported case of growth at -0.1°C[59]. With this knowledge, it is unsurprising to find *Aeromonas* spp. in blue mussels from March. However, the significantly ($p<0.05$) higher count in March could be a result of opportunistic behaviour by *Aeromonas* spp. in cold water, when other microbes could be inhibited by the lower temperatures. Still, Park et al. points to other studies showing the prevalence of *A. hydrophila* being noted during the summer, thus complicating an opportunity for a concise conclusion. *Aeromonas* spp. is a known opportunistic pathogen found in several types of food products not limited to seafood but also being registered in processed food and fresh vegetables[58]. Keeping this prolifera-

tion ability in mind', it can be inferred that *Aeromonas* spp. may be introduced to a food product later in the value chain than at its origin. However, given that mussels are scrubbed and packaged on-site in Åfjord, and sold directly after transportation, the number of steps in the value chain is reduced, thus reducing the risk of cross-contamination in the case of blue mussels from Åfjord. This does subsequently suggest that *Aeromonas* spp. contamination is a result of the aquaculture itself in Åfjord.

5.2 Impact of locality on microbial count

No microbial parameter was overall significantly affected by the locality of the mussel cultivation, i.e. the production location within the fjord. However, seasonal variations of *Aeromonas* spp. at two of the localities suggest an important consideration. Though the *Aeromonas* spp. content was not significantly affected by locality overall, as seen in Figure 4.1.6, there was a major difference between the localities: Both the inner and middle locality counts were significantly ($p<0.05$) affected by season. This means that these localities have a fluctuating *Aeromonas* spp. content throughout the year, while the outer locality is more stable. This suggests that the inner and middle locality experience a more diverse set of conditions over the course of a year, compared to the outer locality. There is little research that has investigated the cascading effects locality within a fjord has on mussel cultivation or mussel microbiota, but some aspects could be theorised. Changes in rainfall by season, as well as snow-melting in the spring may have unexplored effects, especially in Norway where seasons and their varying conditions are particularly pronounced. Both runoff from land and overflow from rivers and lakes into the fjord could alter the microbial composition of the fjord. Either by way of diluting the salinity in the fjord or by being a means of carrying bacteria into the fjord from freshwater sources. *A. hydrophila* has for instance been found in freshwater conditions as well[58]. This effect could particularly be true further inside a fjord, where water is less subject to large oceanic currents directly, and a larger portion of the fjord water is surrounded by land, and thus more exposed to pathways for freshwater to enter the fjord. Meanwhile, the stable levels at the outer locality could be a result of a constant external

influx of the genus to Åfjord. *Aeromonas* spp. thrive innately in water, including the ocean, and may thus be transported into Åfjord by ocean currents hitting the inlet of the fjord. The Research Council of Norway has documented that a supply of bacteria pathogenic to humans has been ongoing for a considerable period in Norwegian coastal waters. This is attributed to the migration of bird and whale species between the equator and Svalbard, as well as human activities[60]. This influx could in theory be happening at an overall steady rate throughout the year, establishing a steady state-like condition.

On a similar note regarding the water flow into and inside the fjord, the topography of Åfjord should also be discussed briefly. Åfjord is also a type of sill fjord, a fjord with shallow waters at the inlet with a deeper basin further inside, which results in water being replaced at a slower rate inside the basin compared to traditional fjords. A comparison can therefore be drawn to a well-known and studied sill fjord, Framvaren fjord in southern Norway. Framvaren fjord's characteristic narrow inlet allows oxygen-depleted water to be a persistent characteristic of the fjord. Studies by Behnke et al. and Yao and Millero of the anoxic characteristics of Framvaren fjord point to increased levels of H_2S as a consequence of the lack of oxygen in the water. This feature is caused by the presence of H_2S producing bacteria, which are favoured in environments with low oxygen concentration or anoxic conditions, in the fjord. Although Åfjord also is a type of sill fjord, there was no significant difference in the H_2S producing bacteria content found in the mussels from the different localities. This suggests that Åfjord is not affected by oxygen-depleted water as a consequence of its sill fjord properties. On the flip side, a study of oxygen depletion in Limfjorden in Denmark from 1980, by Jørgensen, points to decreased oxygen levels in seawater over blue mussel beds. The decrease is a product of the mussels' metabolism and oxygen uptake, resulting in less available oxygen for other species. Given the large-scale blue mussel cultivation throughout the entirety of Åfjord, it could be speculated that the fjord is subject to lower oxygen levels than normal. This theory was, however, also debunked due the lack of supporting evidence in the bacterial counts.

Lastly, an aspect of the proliferation of the *Vibrio* genus should be touched upon. In 2016 Froelich and Noble published a study, investigating *Vibrio* in oysters, a similar

bivalve mollusc to blue mussels, and found that comprehensive studies are needed in order to make definitive assertions about the *Vibrio* population of a specific area. They compiled a myriad of studies and were unable to pinpoint a single factor that decisively affects *Vibrio* spp. proliferation in oysters. For instance, certain studies have shown the *Vibrio* population to be negatively correlated with water salinity, while others have shown no correlation. In Åfjord's case, it could be prophesied that the innermost part may contain a heightened population of the *Vibrio* genus due to the estuary conditions in the inner fjord, favouring greater *Vibrio* growth[65]. This could in turn adversely affect the blue mussels' food safety due to the known health hazards of *Vibrio* ingestion. Yet, there were shown no empirical data to support increased *Vibrio* spp. levels in the inner part of the fjord. Still, *Vibrio* spp. content in mussels is a health concern, so the origin of *Vibrio* spp. in mussels is an important factor. Froelich and Noble stated that there is evidence to show that the *Vibrio* spp. concentration inside oysters varies widely between individuals, even in oysters of the same batch, but overall is stable long terms in a single oyster. A theory is that the *Vibrio* spp. take up residence in the mollusc at the larvae stage, and keep residency inside the mollusc throughout its life, thus accounting for the stable presence found in individual molluscs[64]. This could have implications for food safety in mussel cultivation, but in terms of locality, no correlation could be found between *Vibrio* spp. content in the mussels and cultivation location within Åfjord.

5.3 Impact of depuration treatment on microbial count

Two parameters were significantly ($p<0.05$) affected by depuration treatment. Both *Aeromonas* spp. and *E. coli* counts were significantly lower for the depurated samples compared to the un-depurated samples, implying the depuration process can remove these bacteria from the mussels. Martínez et al. demonstrated a significant ($p<0.05$) reduction in *Aeromonas* spp. and *E. coli* levels in blue mussels, as well as psychrotrophic and mesophilic bacteria, using depuration.

The depuration process used by Martínez et al. involved vertical stack purification

systems with filtered UV-disinfected natural seawater. Depuration was conducted for a period of 42 to 48 hours, maintaining a water temperature of 14 to 18 degrees Celsius, dissolved oxygen levels above 5.5 mg/Liter, salinity exceeding 30 PSU, and nephelometric turbidity units below 15. In Norway, the Norwegian Food Safety Authority does not state as rigorous conditions for depuration and only specifies the end goal of mussels fulfilling Class A criteria after depuration[20]. The supplier in the present study has specified that a 12-hour duration depuration is the only condition used for their blue mussels, and thus the samples in this study.

The blue mussels from Åfjord that were depurated yielded a significantly ($p<0.05$) lower MPN value for *E. coli* than the un-depurated samples, as can be seen in Figure 4.2.6. Considering the well-established role of *E. coli* as an indicator organism for evaluating depuration as a decontamination method for bivalves, the successful removal of *E. coli* is unsurprising.

The *Aeromonas* spp. content of the mussels from Åfjord was also significantly ($p<0.05$) reduced by depuration, seen in Figure 4.2.5. There was a comparable level of *Aeromonas* spp. in un-depurated samples from Åfjord (2.5 ± 1.5 log CFU/g) as Martínez et al.'s un-depurated samples (2.853 ± 1.017 log CFU/g) from Galicia. It seems plausible to assert that depuration, therefore, is able to significantly reduce the*Aeromonas* spp. concentration in molluscs, given that both studies were able to replicate this result independently.

The psychotropic mean count from Galicia for un-depurated samples (3.990 ± 0.725 log CFU/g) and for depurated samples (3.643 ± 0.806 log CFU/g), were higher than those found in mussels from Åfjord (3.1 ± 1.7 and 3.3 ± 1.2 log CFU/g, respectively). As was the case for mesophilic counts from Galicia for un-depurated samples ($3.523 \pm$ 0.820 log CFU/g) and depurated samples (3.237 ± 0.985 log CFU/g), compared to Åfjord (2.5 ± 0.94 and $2. \pm 0.61$ log CFU/g, respectively). The results from Åfjord indicated that depuration was not able to significantly ($p<0.05$) lower the levels of neither psychrotrophic nor mesophilic bacteria in the mussels, whereas the depuration in Galicia was able to. However, Martínez et al. states that depuration rarely reduced these counts below between 3 to 4 log CFU/g in their study, and links this trend to the maintenance of the gram-negative bacteria predominant in the

endogenous microbiota[32]. This phenomenon is described further in another study from 1993 by Olafsen et al.. For the present study, the explanation could therefore be that the psychrotrophic and mesophilic concentrations already were at their lower limit levels and that the depuration is not able to reduce their concentrations further. After all, the un-depurated concentrations found in Åfjord are already at a similar level to the depurated samples from Galicia, which were both at the reported lower limit range of 3 to 4 log CFU/g. Interestingly, Martínez et al. found that their described depuration conditions may not always achieve an adequate reduction of *E. coli* levels and a report from 1988 by Richards found that various factors, including water salinity, temperature, mussel species, and geographic harvesting location, can influence the effectiveness of depuration. Given the lack of controlled variables in the depuration process used in Åfjord it is hard to pinpoint how parameters such as temperature and salinity affect the microbial content in the blue mussel. Consequently, it is evident that more research into the depuration parameters and their correlating effects on microbial content in blue mussels is merited.

Regarding the viral contaminants in mussels, their removal through depuration is not as extensively documented as that of bacterial contaminants[67]. Viruses, including norovirus, have been found in mussels and are associated with foodborne disease outbreaks[32]. Enteric viruses, such as norovirus, have been detected in shellfish cultivated along the Norwegian coast, and norovirus-related gastroenteritis outbreaks from oysters contaminated with faecal matter have been reported in Norway[68]. Depuration has shown some potential in reducing viral concentrations[67][32], as evidenced by a scientific report by the European Food Safety Authority that demonstrated effective reduction of viral content in oysters through depuration[69]. However, it's important to note that low levels of enteric viruses may persist in mussel tissues and hemolymph, protecting them from depuration, which primarily purges the mussels' gut and intestines[3]. Therefore, viral contamination remains a concern for mussel food safety. The use of *E. coli* as an indicator for monitoring viral contents in mussels has shown promise but is limited in effectiveness.

5.4 Impact of depuration duration on microbial count

To evaluate the depuration process over time, a batch of mussels contaminated with bovine manure and a batch of unaltered mussels were depurated for 24 hours and sampled every 2 hours for the first 8 hours. The samples were subsequently analysed to find psychrotrophic bacteria and *E. coli* content.

The distinct pattern of psychrotrophic bacteria emerged during the depuration process, as seen in Figure 4.3.1. The unaltered mussels ranged from 2.7±2.3 to 4.3±0.17 log CFU/g, while the contaminated mussels had a similar range of 3.5 ± 0.79 to 4.2 ± 0.17 log CFU/g. The development over the depuration duration did not point to a particular effect of depuration during its run, and the end values after 24 hours were nearly identical to the initial values for both sample types. Given the earlier established result that the psychrotrophic bacteria content in the mussels was not significantly affected by depuration (Figure 4.1.1), it seems evident that the present study shows that depuration does not affect the psychrotrophic bacteria content in blue mussels in a meaningful way.

The *E. coli* content of the batches was vastly different. The unaltered samples had MPN *E. coli* values in the range of 6.7 ± 12 to 100 ± 114 CFU/100g, while the contaminated samples ranged from 2333±1201 to 18000 CFU/100g. This indicates that the bovine manure was a potent source of *E. coli*, as it surpassed the natural levels considerably. Given the fact that all samples from 2 to 8 hours reached the maximum table value, it is difficult to comment on any development during the depuration in this period, as the sample values exceeded the scope of the testing method. All the sample values up until and including 8 hours of depuration place the mussels firmly inside Class C criteria (all sample values have to be less than 46000 CFU/100g) of the Norwegian Food Safety Authority since Class B criteria (90% of samples have to be less than 46000 CFU/100g) are not met. However, the mussel fulfilled Class B criteria after 24 hours of depuration, with a mean count of 2333 ± 1201. The unaltered samples also showed progress over time that would suggest bacterial purging, but importantly the initial value, along with all subsequent values, was within Class A criteria (80% samples have to be less than 230 CFU/100g), suggesting that depu-

ration was not needed for these samples. Nevertheless, the initial value of 100 ± 114 CFU/100g was reduced with depuration, regardless of its necessity. As put forward by Richards, it is known that heavily contaminated bivalves require a longer depuration process compared to lightly contaminated mussels in order to achieve adequate bacterial reduction. As witnessed with this study's contaminated samples even a 24-hour depuration period is not sufficient to reduce *E. coli* levels to Class A criteria and commercial availability, from a Class C start point. Still, given enough time the depuration should prove satisfactory as a *E. coli* reducing means.

5.5 Impact of heat treatment on microbial count

A batch of blue mussels was harvested from Åfjord in March of 2023 and without further treatment steamed with 100 mL of water to simulate a conventional cooking method by a consumer. Three separate steaming sessions were performed for different lengths of time: one for 3 minutes, one for 6 minutes, and one for 10 minutes. The samples were subsequently analysed to find psychrotrophic bacteria and *E. coli* content, and were compared to a raw sample of the same batch.

In the present study, the only samples which displayed *E. coli* content were the three uncooked parallels, with a mean count of 243 ± 236 log CFU/100g. No further count of *E. coli* could be registered. There is a margin of error attributed to these results due to no detection occurring, however, it could imply that all *E. coli* was killed by the steaming process. This is still uncertain and should be explored further, however, the thought can be entertained. On the other hand, the mussels' content of psychrotrophic bacteria after steaming was surprising (Figure 4.4.1), with mean counts ranging from 2.6 ± 2.3 to 4.1 ± 0.58 log CFU/g. Psychrotrophic bacteria are per definition accustomed to colder temperatures of below 7°C[70], and generally not heat resistant, yet in this trial no pronounced decrease in the microbial count was registered over the course of the steaming process. This would suggest that the bacteria were largely unaffected by steaming, which seems implausible as they are heat sensitive. A possible explanation is that the temperatures inside the mussels were not sufficiently high to kill a large fraction of the bacteria. Alternatively, if the bacteria are spore-forming they would be able to survive these conditions.

Regardless, no further knowledge into the spore-forming abilities of the bacteria in the samples it is difficult to pinpoint a certain reason for their survival.

The internal temperatures of the mussel flesh, which ranged from 73 ± 4.6°C, to 76 ± 2.1°C, were similar for all three runs of steaming, regardless of duration. This shows that even with prolonged steaming, the temperature does not increase substantially in the mussels. As a food safety measure, the steaming process should therefore be focused on how keeping the mussel in this temperature range over time affects the bacteria content, i.e. if a longer time span at a given temperature has additional benefits as to momentarily raising the temperature to the same point. Traditionally, mussel preparation is prepared in a similar fashion as this trial, albeit often with white wine instead of water, but instead of steaming the mussel for a predetermined time the cooking time is relegated to being for as long as it takes a majority of the mussels to open up, as they are steamed fresh and alive. A study from 2022 of the heat treatment of blue mussels, by Kausrud et al., concluded that using the proportion of open shells as an indicator of sufficient steaming time carries risks and that a steaming time of over 3.5-4.0 minutes is a better indicator of reducing *E. coli* levels.

Coming back to the heat treatment requirements set by the Norwegian Food Safety Authority, it is evident just how far off conventional consumer cooking is off the conditions defined in the law. The steamed mussels in this study only reached internal temperatures in the middle 70s, far below the 90 °C minimum requirement to entail proper heat treatment[20]. The duality of both having sufficiently high temperatures and duration of heating is of great importance to ensure adequate heat death of pathogens. With these results, and those of Kausrud et al., it can be inferred that the steaming process used in mussel cooking is not satisfactory in ensuring complete food safety. However, the light steaming a consumer does in their home kitchen is not thought to be the only safety measure. The law specifies that the heat treatment regime it describes is an alternative to depuration or relaying. What this means is that any fresh mussels have to be purged of bacteria using other methods before commercialisation. Thus the fresh mussels consumers use in cooking have already been subject to acceptable food safety measures, and the final steaming

by the consumer is not required to enhance safety. In fact, the steaming is legally speaking unnecessary as the mussels will at this point, after depuration or relaying, meet Class A criteria, making them able to be consumed raw without risk. In any event, heat treatment is an adequate measure to reduce the risk associated with mussel consumption. Feldhusen declares that, in food safety terms, there is little risk associated with heat-treated seafood, as the heat-treatment process eliminates pathogenic bacteria.

5.6 Microbial community identification

DNA extraction was performed on the blue mussels used in this study to attain a comprehensive understanding of the mussel's microbiota. The DNA samples were sent to Eurofins Genomics in Germany for sequencing, with the goal of gaining insights into the microbial community of blue mussels from Åfjord, Norway. Unfortunately, as of the time of writing, the final report containing the sequencing and microbial identification results from Eurofins Genomics has not been provided. It should be noted that there have been no reported issues with the process itself, however, the results were not generated in time to meet the deadline of the present project, as it has been undertaken as a master's thesis at NTNU. Consequently, further results will ultimately become available but cannot be discussed at the current moment.

5.7 Choice of methodologies

In this study, a combination of traditional methods using selective agars and modern sequencing methods was employed. A side objective of the study was to combine these methodologies in hopes of generating new knowledge in the usage of these specialisations in conjunction. While the use of selective growth media requires the selection of specific parameters in advance, sequencing methods offer a more experimental approach to identifying microbiota with a broader scope. Because of this, sequencing microbiota has the advantage of being able to discover previously unknown genera in, for instance, seafood [71]. Thus, relying solely on selective growth

media may be limited in the range of results that can be produced. However, sequencing results can be too comprehensive and raise more questions than answers when clear tendencies are not prominent in the samples. Moreover, outsourcing significant portions of the process to external parties can be time-consuming and resource-demanding. On the other hand, selecting parameters in advance requires careful consideration and a deep understanding of the subject matter, front-loading the effort required to undertake a study. Nevertheless, it provides a straightforward yet effective means of quantitatively and qualitatively determining the presence of specific bacterial parameters, such as genera or species. Therefore, the optimal strategy appears to be the combined use of these methods, utilising their respective potentials.

An alternative pathway is to explore the microbiota of the blue mussels by picking individual colonies for further inoculation and identification. This methodology was further explored in Marte Holm's Master's Thesis, titled "Identifisering og karakterisering av mikrobielt samfunn i blåskjell (*Mytilus edulis*) og forekomst av antibiotika resistente *Escherichia coli*", which was carried out in parallel to this Master's Thesis, using the same blue mussel samples.

6 | Conclusion

Legislation for mussel production in Norway plays a crucial role in preserving the food safety of blue mussels for consumers. To follow the regulatory framework set by the Norwegian Food Safety Authority, mussels must be produced in areas of low contamination. The classification system is based on the level of *E. coli* contamination, which serves as an indicator of faecal contamination. Mussels from class B or C areas are too contaminated for commercialisation and are required to undergo relaying or depuration before being sold fresh to consumers[16]. Currently, depuration serves as the chief safety measure to purge blue mussels of pathogens and prevent foodborne diseases associated with blue mussel consumption in Norway. Since most coastal areas in Norway do not meet the determined criteria[52], the depuration process is closely tied to the mussel industry. Alternatively, mussels may be heat treated, but due to consumer demand for fresh mussels, the method is scarcely used, although effective.

The location of mussel cultivation within Åfjord was also explored. Although no significant impact was observed as a result of where in the fjord the cultivation site was placed, the results revealed that blue mussels from Åfjord have particularly low bacteria content throughout the year. Åfjord has a permanent classification of Class B by the Norwegian Food Safety Authority, however, the *E. coli* concentrations found in this study never exceeded Class A levels. A significant ($p<0.05$) difference in *Aeromonas* spp. content of blue mussels throughout the year was revealed. The *Aeromonas* spp. concentration was not at a concerning level, and while no discernible reason was explored for the seasonal variation, it is an important find for the industry. Certain species of*Aeromonas* may induce foodborne illnesses, and season variation may pose a larger threat in other locations. Even with that concern, there was shown a significant ($p<0.05$) ability to reduce *Aeromonas* spp. content of blue mussels using depuration, thereby securing food safety. Additionally, the research demonstrated that depuration also has a significant ($p<0.05$) effect on reducing *E. coli* concentrations in blue mussels. Since the reduction of *E. coli* content through depuration is considered an indication of the removal of other enteric

pathogens, there is now more evidence to support that depuration is a satisfactory food safety measure. Ultimately, the greatest compromise to the food safety of blue mussels arises from viral contaminants, including norovirus. Although this study did not directly analyse viral contamination, it examined *E. coli* as an indicator, following an industrial practice. Given that depuration successfully removes *E. coli*, it seems reasonable to expect similar removal of viruses. However, while depuration has been shown to reduce viral content, complete elimination of viral contaminants has not been demonstrated through this process.

7 | Further Work

The findings and considerations of this study have underlined the existence of various dimensions within blue mussel aquaculture that warrants further research. A clear direction of future study is to focus on the parameters of depuration and corresponding implications on the microbiome of blue mussels. This can show the hitherto unknown effects of the most common practice of ensuring food safety standards in shellfish. By delving into the dynamic interplay between bacterial purging and the composition of the microbiome in blue mussels, there can be gained insights that can be advantageous in optimising their cultivation and processing for commercial purposes.

Moreover, future studies of blue mussels have the potential to shed light on a critical element related to the occurrence of foodborne illnesses, namely the role played by viruses. As *E. coli* is currently used as an indicator organism of other enteric contaminants, there is room to improve the understanding of the direct dynamics of viral contamination in shellfish, as opposed to using a different indicator. For instance, there is a documented increase of norovirus during the winter months [72], a prevalent cause of foodborne illnesses, but little research to show effects on viral concentration by use of depuration. By unravelling the relationship between viral and bacterial contents, a new understanding regarding the emergence and proliferation of these pathogens in mussels can be acquired. Such knowledge can result in more targeted preventive measures to safeguard human health.

The usage of sequencing methods should also be explored further. With the delayed results of microbial community identification in this study, there is still room for further research on this topic, that could yield important discoveries. Additionally, modern methods like these should be explored further in any sense, to gain a better understanding of how they can be used to highlight formerly unexplored areas of research in food.

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A | Appendix

A.1 Bacterial count tables

Table A.1.1: Table of every parallel with corresponding results for trials of season, locality and depuration as described in Section 3.1.1.

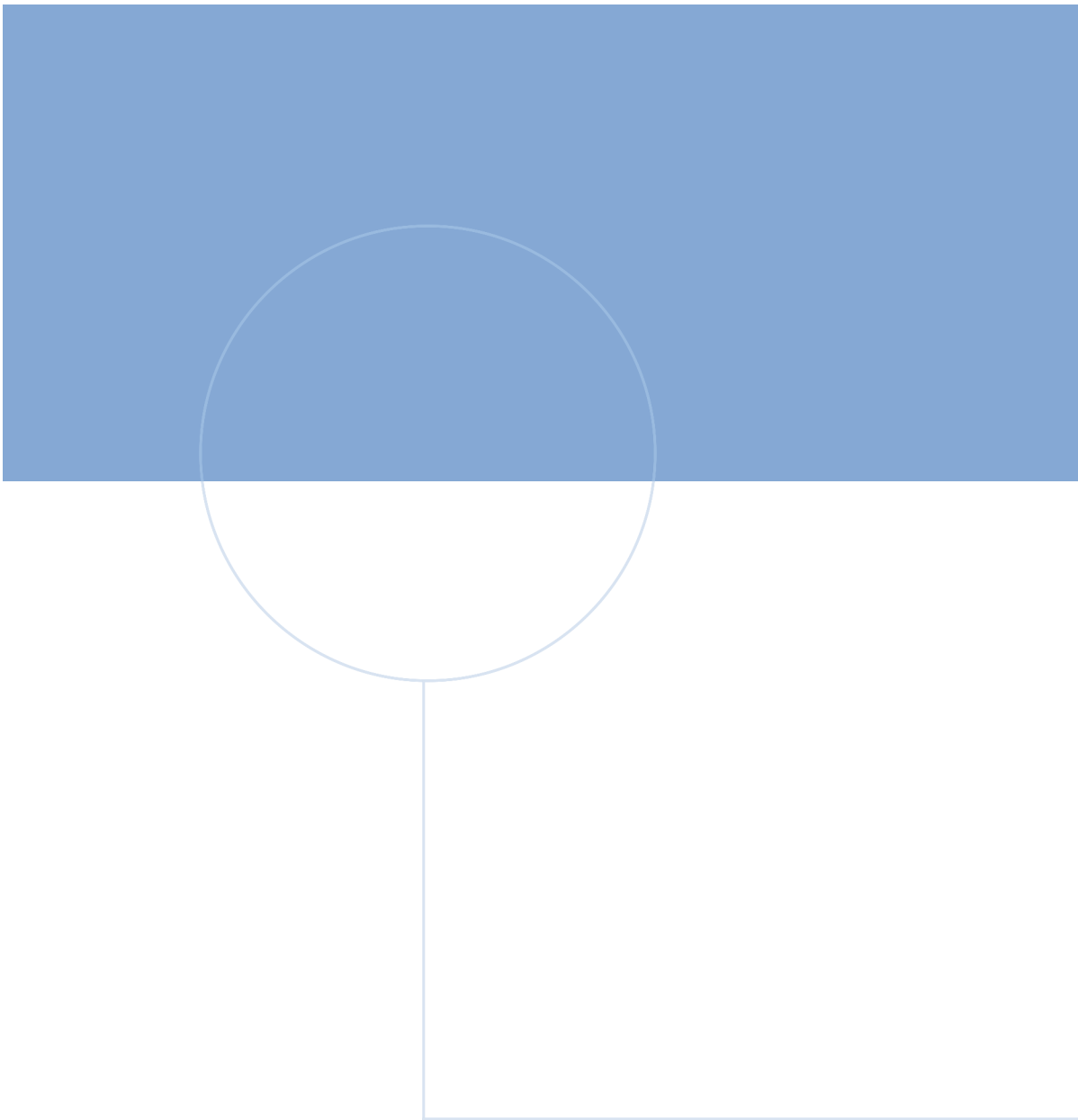
Treatment	Locality	Season	Pyschrotrophic bacteria [log CFU/g]	Mesophilic bacteria [log CFU/g]	H_2S producing bacteria [log CFU/g]	*Aeromonas* spp. [log CFU/g]	*Vibrio* spp. [log CFU/g]	*E. coli* [CFU/100g]
No Depuration	Inner	November	0	2,5	1,6	0	0	0
			3,8	2,8	1,9	0	2,0	20
			3,5	2,7	1,6	0	0	0
		March	4,5	2,7	0	3,7	0	50
			4,6	2,4	2,2	4,1	2,8	0
			4,0	2,3	2,2	4,4	2,3	0
		May	3,8	3,0	2,6	2,3	2,0	330
			0	2,9	0	0	2,6	70
			3,8	2,7	1,4	2,3	2,0	0
	Middle	November	3,6	2,8	1,6	0	0	50
			3,6	3,1	2,9	3,1	3,3	20
			3,5	2,9	1,6	0	2,0	230
		March	0	2,9	0	3,8	2,0	0
			4,2	2,4	1,4	4,0	0	0
			4,0	2,9	0	3,4	0	20
		May	4,4	0	0	2,9	0	20
			0	0	0	3,5	0	0
			3,4	3,1	1,0	3,9	2,7	0
	Outer	November	3,7	2,4	0	3,6	0	170
			3,7	3,1	1,6	3,0	0	230
			4,2	3,0	1,3	3,2	0	0
		March	4,2	3,1	0	2,7	2,0	20
			4,2	2,8	0	3,5	0	0
			0	2,7	1,3	2,0	2,6	0
		May	0	3,2	0	3,4	0	0
			3,6	3,0	0	3,1	2,0	0
			4,2	0	0	2,9	0	0
Depuration	Inner	November	3,8	2,8	1,0	0	2,0	0
			3,4	3,2	2,6	0	2,0	0
			3,8	2,5	1,7	0	2,6	0
		March	4,4	2,6	0	0	0	0
			3,4	3,1	0	0	2,7	0
			3,6	2,7	2,5	3,0	2,0	0
		May	3,6	3,1	1,7	0	0	70
			0	3,4	2,7	2,3	2,3	20
			3,4	0	0	0	0	0
	Middle	November	0	3,0	2,0	0	0	0
			3,3	3,2	2,2	0	0	0
			3,6	3,2	2,3	2,0	2,0	0
		March	3,7	2,5	0	2,3	0	0
			0	2,8	0	4,2	0	0
			4,0	2,7	0	3,9	2,4	0
		May	4,0	3,0	0	0	0	0
			4,2	3,1	0	2,0	0	0
			4,1	3,1	1,4	2,0	0	0
	Outer	November	3,8	2,9	0	3,5	0	0
			3,5	2,7	0	3,7	0	0
			3,8	2,7	0	0	0	0
		March	3,8	2,4	0	3,6	2,3	0
			3,9	2,7	0	2,7	2,6	0
			3,8	3,1	1,3	2,0	0	0
		May	3,4	2,6	0	2,6	0	0
			3,8	2,5	0	0	0	0
			4,3	2,8	0	2,0	0	0

Table A.1.2: Table of every parallel with corresponding results for the depuration duration trial described in Section 3.1.2.

Depuration duration [Hours]	Psychrotrophic bacteria		*E. coli*	
	Unaltered [log CFU/g]	Contaminated [log CFU/g]	Unaltered [CFU/100g]	Contaminated [CFU/100g]
0	4,0	4,1	230	9 200
	2,0	4,4	50	16 000
	3,8	4,2	20	18 000
2	4,5	4,0	0	18 000
	4,3	2,6	0	18 000
	4,2	3,8	0	18 000
4	4,2	4,4	0	18 000
	0	3,3	0	18 000
	4,0	4,5	40	18 000
6	4,6	3,3	0	18 000
	4,0	3,4	20	18 000
	4,1	4,2	0	18 000
8	4,2	3,0	0	18 000
	4,4	4,4	0	18 000
	3,6	4,6	0	18 000
24	4,3	4,3	20	1 100
	4,0	3,8	0	2 400
	3,9	4,1	0	3 500

Table A.1.3: Table of every parallel with corresponding results for the heat treatment trial described in Section 3.1.3.

Steaming duration [Minutes]	Psychrotrophic bacteria [log CFU/g]	*E. coli* [CFU/100g]
0	4,4	20
	3,5	220
	4,5	490
3	3,9	0
	3,9	0
	4,2	0
6	3,4	0
	4,3	0
	0	0
10	3,4	0
	4,0	0
	4,0	0



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