Exploring the Condition of Fish Welfare and Microbial Dynamics in Egget® – a Novel Closed Aquaculture System at Sea

"Eggsploring Egget®"

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Abstract

The Norwegian Atlantic salmon (*Salmo salar*) aquaculture industry faces significant challenges, prompting the need for innovative approaches to enhance sustainability. One such innovation is the prototype Egget®, a novel 1850m^3 floating closed containment aquaculture system developed by Ovum AS for the initial sea phase of salmon production. This study assesses the impact of Egget®'s production routines on fish health and welfare during the first-ever production cycle (October 9th, 2022 - February 22nd, 2023) through three sampling campaigns (November 10th, December 12th, and February 16th). A minor amoebic gill disease (AGD) outbreak occurred on November 29th during the sampling period but was resolved without treatment despite the persistence of the amoeba in the environment. The mortality rate in Egget® was 1.2% over the five months, with a specific growth rate of 1.6% in the four months of sampling. Key health indicators were monitored, including gill and skin mucosal dynamics, as they are vital components of the fish's immune system. Microbiota analyses were conducted on gills, inlet/outlet water, and the system's biofilm. Using PCR sequencing of the 16S rRNA gene, DNA extraction, and bacterial concentration with flow cytometry, this study characterised microbial species and differences in composition from the various sources. The gill mucous barrier showed an acute immune response to the *Paramoeba peruans* amoeba in December, resulting in decreasing mucous cell size. Subsequently, an adaptation to the amoeba resulted in a new homeostatic state of mucous cells with increased size and volumetric density of mucous cells in the gill lamellae. Skin mucous barriers remained consistently stable. Microbiota analyses revealed non-pathogenic bacteria associated with marine aquaculture environments, indicating a healthy ecosystem. No accumulation of bacteria was observed in water samples or biofilm. Furthermore, maintaining stable environmental conditions and minimal fish handling has positively impacted fish health and welfare in this investigation. This first production cycle in this pioneering system highlights the presence of healthy fish exhibiting robust growth and low mortality compared to both standard farming practices and Egget®'s sibling fish raised in open net pen. In addition, an active and responsive mucous barrier in both gills and skin was observed. Notably, there was no observed tendency for microbiota accumulation or alteration in the water samples from the biofilm. These indications suggest that closed systems like Egget® may play a pivotal role in the future of the aquaculture industry, as such systems have the potential to enhance and expedite salmon production while ensuring sustainability, lowering labour, and promoting fish welfare.

Contents

Abbreviations

A – Adenine

A8f – Primer A8 forward

AGD – Amoebic Gill Disease

ANOVA – Analysis of Variance

BD – Becton Dickinson

BLAST – Basic Local Alignment Search Tool

C – Cytosine

CCS – Closed Containment System

CF – Condition Factor

CO_2 – Carbon Dioxide

D – Days

DA – Defence Activity

DNA – Deoxyribonucleic Acid

EB – Ethidium Bromide

FCM – Flow Cytometry

FCR – Feed Conversion Rate

G – Guanine

GRP – Glass Reinforced Polymer

H1542r – Primer H1542 reverse

H_2O_2 – Hydrogen Peroxide

L – Left

Len – Length

MBA – Marine Broth Agar

MCA – Mucous Cell Area

MCD – Mucous Cell Density

MEGA 11 – Molecular Evolutionary Genetics Analysis Version 11

NCBI – National Center for Biotechnology Information

NS – Norwegian Standard

NTU – Nephelometric Turbidity Unit

O – Opposite

O_2 – Oxygen

OTUs – Operational Taxonomic Units

PAA – Peracetic Acid

PAS-AB – Periodic Acid Schiff - Alcian Blue

PCR – Polymerase Chain Reaction

QD – Quantidoc

QQ plot – Quantile-Quantile plot

R – Right

ROV – Remotely Operated Vehicle

rRNA – Ribosomal Ribonucleic Acid

S-CCS – Semi-Closed Containment Systems

SDGs – Sustainable Development Goals

SE – Standard Error

SGR – Specific Growth Rate

T – Thymine

TAE – Tris Acetate EDTA

Tukey's HSD – Tukey's honest significant difference

UiB – University of Bergen

UV – Ultraviolet

W – Weight

1. Introduction

1.1 Background

Norwegian Atlantic salmon (*Salmo salar*) production has plateaued since 2013 due to issues like lice infestations, the impact of treatments on farmed salmon and the surrounding environment, and the escape of farmed salmon that threaten wild populations (Fiskeridirektoratet, 2022; Forseth et al., 2017; Karlsson et al., 2016; Næve et al., 2022). The production of farmed salmon stagnated, but the production cycle during the sea phase has also failed to show improvement, remaining 14 to 20 months, with an average weight range of four to six kilograms (Glover et al., 2009; Norsk laks er viktig for Norge, n.d.; Thorland et al., 2020). The extent to which the growth potential is achieved or compromised depends on various factors, including production practice, diet composition, disease, and consequences of parasites like salmon lice (*Lepeophtheirus salmonis*) (Aas et al., 2019).

Most farmers in Norway employ floating open net pens to exploit the country's favourable natural conditions. However, this method allows the passage of pathogens, such as salmon lice, between wild and farmed salmon. Furthermore, the confinement of stocks, even at low density in a single location, disrupts the host-parasite dynamics (Bergh, 2007; Costello, 2009). Consequently, the fish are subjected to stressful and energy-consuming situations like handling, sorting, transporting, crowding, treatments and delousing, negatively affecting their welfare and growth performance, leading to high mortality (Ashley, 2007; Grønvik et al., 2022).

Salmon lice, a parasitic pathogen, have both farmed and wild salmonids (salmon and trout) as hosts (Costello, 2009). The presence of this pathogen has posed significant challenges to the industry, leading to the exploration of various innovative approaches to mitigate lice infestation. Notably, in addition to the harm caused by the pathogen itself, the handling of fish as delousing and frequent handling procedures induces stress, which affects the immune system, robustness, and welfare of the fish. These practices also influence the mortality rate, fish quality, average slaughtering weight, feed intake, and fish growth (Aas et al., 2019; Iversen et al., 2017; Overton et al., 2019). Thus, the production operations stress the fish, compromising their external immune systems and overall health. Furthermore, these treatments entail economic costs. Disregarding the income loss resulting from impaired growth, the estimated cost for the entire industry was approximately NOK 5 billion (Iversen et al., 2017).

Aquaculture challenges the external environment despite the relatively low risk compared to land-based industries. These challenges include the spread of diseases (Garseth et al., 2013), interactions with wild stocks through escapes, nutrient overloading, and the accumulation of organic materials such

as feed surplus and faeces (Grefsrud et al., 2022a; Naylor et al., 2005). In line with the United Nations' sustainable development goals (SDGs) and their call for action (UN DESA, 2022), the Norwegian salmon farming industry must consistently seek and implement more sustainable and innovative rearing methods.

1.2 Regulation

Regulations from the Norwegian government have prompted efforts to increase production and explore alternative farming methods such as land-based farming, offshore farming, and various closed or semi-closed containment systems (CCSs)/(S-CCSs) due to the impact on the environment and the wild salmon stocks (Grefsrud et al., 2022b; Sommerset et al., 2022). Closed aquaculture systems provide a physical barrier between farmed salmon and the surrounding environment, which have demonstrated potential in mitigating both the environmental impact and the requirement for handling and delousing, even after transferring the fish to open net pens (Øvrebø et al., 2022; Thorarensen & Farrell, 2011). Reducing or avoiding handling and delousing processes can greatly benefit fish welfare and growth by minimising external stress (Ashley, 2007). Additionally, smolt farmed in this system have shown better growth, lower feed conversion rate (FCR) and less stress-related hormones than fish farmed in open net pens (Øvrebø et al., 2022).

1.3 Closed Containment System (CCS)

CCSs and S-CCSs offer several advantages in salmon farming. One key advantage is their ability to pump in water from depths where sea lice do not reside, as sea lice typically inhabit the top layers of the water column (Nilsen et al., 2017). Both systems operate on the same principle, with the main difference in whether the outlet water is treated or cleaned, for simplicity reasons, hereby referred to as CCSs. These systems provide more stable temperatures, positively impacting fish growth, as surface temperatures can be suboptimal during certain periods of the year (Thorarensen & Farrell, 2011). CCSs are more robust and secure than traditional open net pens, significantly reducing escape risk and enhancing environmental safety (Nilsen et al., 2019). Moreover, these systems enable the collection and reuse of organic material, thus reducing the impact on the seabed and the surrounding environment (Nilsen et al., 2019). Prototypes tested indicate that salmon lice entering the system exhibit limited reproductive capabilities and are subsequently washed out. Promising findings have also been observed concerning fish welfare in these systems (Lazado et al., 2022a; Nilsen et al., 2017).

New technologies like this can serve as alternative rearing methods by either housing the fish in an intermediate phase to reduce the time spent in open net pens or keeping the fish until slaughter (Nilsen et al., 2017). Maintaining the fish within a stable and secure system for an extended duration can reduce stress and achieve an increased growth rate, shortened production period, and enhanced feed utilisation (Lazado et al., 2022a; Thorarensen & Farrell, 2011). Several studies have demonstrated positive outcomes in growth and fish welfare within these systems; however, as Lazado et al. (2022a) highlight, these facilities are still in the pilot/prototype phase, and further documentation regarding the different systems is required.

It is essential to note that CCSs are relatively more expensive than open net pens regarding construction and operation costs connected to water pumps and oxygen supply (Calabrese et al., 2017). Today, these systems and open cages are regulated equally, which requires solid documentation of how CCSs compete with open net pens in terms of profitability (Balseiro et al., 2018; Lazado et al., 2022a). Consequently, farming with high densities becomes a potential solution to offset these additional costs and maximise output and return on investment. However, high-density production in CCSs requires careful water quality monitoring (Thorarensen & Farrell, 2011). While water supply from lower depths mitigates the risk of sea lice infestation, it is important to acknowledge that other marine pathogens can still infiltrate the system, and there are likely significant site variations (Grønvik et al., 2022). Water applied from depths exceeding 20 meters can effectively mitigate the presence of numerous marine pathogens. However, bacterial species such as *Tenacibaculum* bacterium and the amoeba *Paramoeba peruans,* responsible for amoebic gill disease (AGD), may persist even in such water sources (Multiple Authors, 2022). Substantial evidence suggests that closed aquaculture systems have the potential to facilitate the accumulation of infectious agents, which can ultimately exacerbate disease outbreaks (Hjeltnes et al., 2019; Riksrevisjonen, 2023; Sommerset et al., 2022). Fish farmers are responsible for ensuring that new technologies are implemented to safeguard fish welfare, as mandated by § 8 of the Norwegian Animal Welfare Act (Dyrevelferdsloven, 2021, § 8). Despite the challenges associated with these systems, several companies are actively pursuing various types of emerging technology. One such technology that shows promise in unlocking growth potential and facilitating more sustainable salmon production is the novel floating CCS developed by Ovum AS, formerly a subsidiary of Hauge Aqua AS.

1.4 Egget®

Innovative solutions are required in Norwegian aquaculture to ensure sustainable growth and minimise the impact on wild salmon stocks and the environment. Egget® (Figure 1), a novel floating closed aquaculture construction, has been developed as a promising solution that aims to solve the major challenges in the salmon production industry. Physical barriers between farmed fish and sea lice prevent lice infestations. Additionally, the solid walls of the "Eggshell" effectively prevent escapes. The system is constructed using a composite sandwich of fibreglass and glass reinforced polymer (GRP), providing a robust container, effectively shielding the fish from the external environment. The interior of Egget® features a smooth surface to inhibit the attachment of organisms. With a height of 21 meters, a diameter of 15 meters, and a volume of 1850 m^3, the system can house up to 100,000 fish at an average weight of 1 kg. Water is supplied from two inlets at a constant depth of 17 meters below the surface, driven by the suction created when outlet water is pumped out at the surface. Subsequently, the incoming water circulates centrifugally with the aid of circulation devices before being discharged through the two outlets. The inlet and outlet areas are double-secured with grating to prevent escapes and large items from entering (Hauge Aqua, n.d.). The construction is certified according to Norwegian Standard (NS 9415:2009). Parameters such as water flow rate, oxygen (O_2) levels, carbon dioxide (CO_2) levels, temperature, turbidity, pH, and salinity are measured by sensors at several depths several times per day, controlled, and automatically registered in a system delivered by Guard Automation AS (Norway). The water undergoes a flow rate of 1-1.5 exchanges per hour, and it is an automatic oxygen supply to the incoming water to ensure a saturation above 95%. Constant light over 21 lux is applied to improve growth with three light sources immersed in the cage and five above the water facing down in the system. A camera delivered by Createview (Molde, Norway) measures biomass, counts lice, and examines external welfare signs daily. A lift-up system uses compressed air to remove dead fish from the bottom and transfers them to an external container daily. The organic material, mainly surplus feed and faeces, accumulates at the bottom and is collected using a continuous suction pump, which transfers it directly to a mechanical filter before being further directed into a collection tank. The inside wall surface is brushed daily by one of the two (Mainstay and Troll Systems) manually controlled remotely operated vehicle (ROV) to reduce biofilm formation.

Anticipated improvements in growth and the development of a more robust fish are expected through the control of critical parameters and the provision of a safe and stable environment for salmon. This environment is characterised by reduced handling, treatments, and a continuous water flow that allows fish to exercise. These could be critical factors in reducing production time during the sea phase and maintaining better fish welfare.

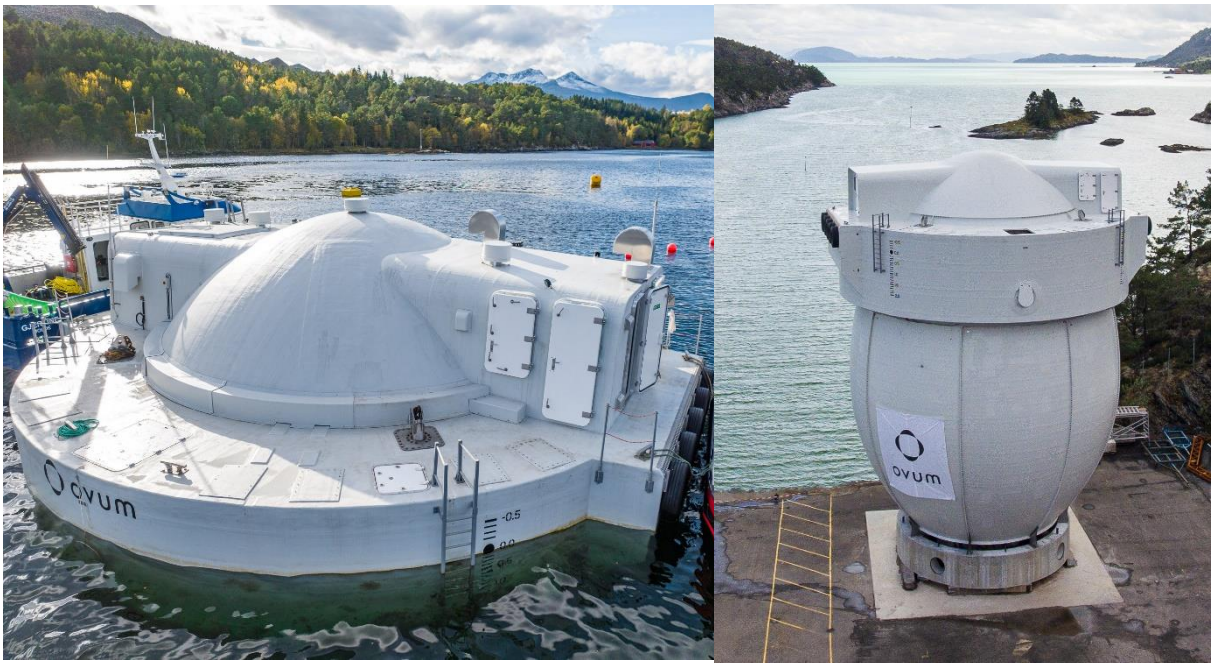

Figure 1: Photo of the floating closed aquaculture systems, Egget®, produced by Ovum AS. Photo: Ovum AS.

Despite the initial capital investment associated with constructing CCSs, a comprehensive master's thesis at the Norwegian School of Economics (Norges Handelshøyskole - NHH) has critically examined the economic viability of adopting such systems (Pedersen & Lyngøy, 2020). The study's findings indicated that, despite the inherent challenges in accurately predicting profitability potential, the prototype Egget® demonstrated significant economic performance compared to conventional open net pens. Specifically, Egget® exhibited a higher estimated real required rate of return of 5.88% compared to the 3.77% of open net pens, resulting in a substantial present value difference of NOK 230 million. Egget® maintained a strong competitive position within the aquaculture industry relative to traditional sea cages (Pedersen & Lyngøy, 2020).

1.5 Water Quality

In total, eleven sensors monitor critical water parameters on the inside and outside of Egget®. Temperature, O_2, CO_2, salinity, pH and turbidity are measured several times daily. These factors have notable impacts on water quality (Thorarensen & Farrell, 2011), which again plays a significant role in the growth of Atlantic salmon. These factors require meticulous monitoring and control to ensure favourable conditions for fish rearing. Within the appropriate ranges, these factors contribute to improved growth rates and the overall welfare of the fish. Oxygen in Egget® is measured in the outlet water, and in cases of 95% or less saturation, oxygen is applied to the inlet water. Maintaining optimal

water quality is paramount when operating a CCS compared to standard open net pens where water flows freely.

In Egget®, the turnover rate of water flow varies from 1-1.5 times per hour and can be adjusted depending on the registered water parameters. Scientific research has demonstrated that in addition to water quality, can high water velocity positively affect fish by promoting robustness, metabolic activity, growth, behaviour, and welfare (Palstra & Planas, 2011). Additionally, these conditions have been found to enhance the texture of fish flesh, reducing stress responses and aggression (Solstorm et al., 2016; Totland et al., 1987). However, it is important to note that excessive water velocity can result in fish exhaustion, leading to decreased appetite, growth, and compromised welfare (Solstorm et al., 2015, 2016).

In addition to abiotic factors, biotic factors also influence water quality. In Egget®, the fish are fed with EWOS Rapid HFT HP 200-1000 pellets from EWOS (Cargill, Norway) 19-20 hours per day with an automated Betten screw feeding system. Feeding strategies, the type and amount of feed, and microorganisms' diversity can impact water quality and subsequently affect fish growth and health. Given that Egget® represents a pilot project, a knowledge gap must be addressed regarding these factors and their impact on the system and the fish.

1.6 Fish Welfare

In addition to continuously monitoring environmental welfare indicators related to water quality surrounding the fish, Egget® employs individual-based welfare indicators to assess fish welfare. Fish welfare refers to the physical and mental well-being of fish, and it can be defined as the perceived quality of life from the fish's perspective (Noble et al., 2018). Egget® utilises a camera system that captures and counts approximately 100-800 fish daily, enabling the estimation of biomass based on length and weight. Furthermore, various health indicators, including lice infestation, skin-, eye-, gill cover- and muzzle- damage, deformities, and external wounds, are recorded under the guidelines outlined in the Fishwell book (Noble et al., 2018; Sommerset et al., 2022). Additionally, group-based indicators such as mortality rate, behaviour, appetite, growth, and disease are registered and monitored daily, as specified in Fishwell. Fish welfare encompasses farmed fish's overall living conditions throughout their life cycle, including stress levels, injuries, diseases, and mortality (Grønvik et al., 2022). Among these indicators, reported fish deaths are widely utilised as the measure of welfare since it is relatively easy to measure; however, it provides limited information regarding the specific welfare concerns and the underlying causes of mortality (Grønvik et al., 2022; Sommerset et al., 2022).

Hence, fish health and welfare are assessed through various methods in this study conducted in Egget®.

Although the salmon louse is a significant challenge, gill diseases emerged as the primary challenge in terms of registered cases, as highlighted by the survey conducted by the Veterinary Institute, and only some of these diseases are mandatory to report (Riksrevisjonen, 2023; Sommerset et al., 2022). Complex gill diseases are considered crucial and an emerging health issue that notably contributes to a decline in the welfare of salmon production (Sommerset et al., 2022). The fish gills play a pivotal role in several critical functions, and their vulnerability to the surrounding environment makes them susceptible to potential damage (Evans et al., 2005; Foyle et al., 2020). The surrounding environment presents challenges in the form of microorganisms and pathogens in aquaculture settings (Akselsen et al., 2019, p. 12).

1.7 Microorganisms

1.7.1 Microbiota

Seawater hosts a diverse range of microorganisms, with estimations suggesting 10^5 –10^6 bacteria per ml and 10^6– 10^7 viruses per ml. These microorganisms play a pivotal role in the carbon cycle by aiding in the decomposition of organic matter and contributing to the circulation of carbon within marine ecosystems (Munn, 2011, pp. 153-157). Aquaculture habitats have a wide range of diverse microorganisms that significantly impact water quality. In farming environments where the density of individuals is high, the presence of beneficial as well as unwanted microorganisms becomes more pronounced. Microorganisms interact with each other and the fish, forming complex ecological relationships. Microbial communities on the fish protect it against pathogens and maintain fish health (Gomez & Primm, 2021), but they can also be the primary source of infections (Sommerset et al., 2023). Psychrophile bacteria, reproducing in low temperatures, thrive predominantly in cold marine environments in surface and deep waters. Psychrophile bacteria encompass a broad spectrum of phylogenetic diversity, becoming numerically dominant when sufficient nutrients are available (Helmke & Weyland, 2004). Considering the characteristics of closed systems like Egget® in Norway, it is reasonable to expect a high concentration of psychrophile bacteria in such cold environments. To better understand Egget®'s effect on the fish and their welfare, it is crucial to investigate the diversity, abundance, and composition of the microorganisms in the water, in the biofilm, as well as on the fish. Since the fish's gills respond to waterborne microbiota and constantly interact with the surrounding

environment, investigating the gill surface microbes provides valuable insights and information about the fish (Merrifield & Rodiles, 2015; Minich et al., 2020).

1.7.2 Biofilm

Biofouling is the process where free-living microorganisms in water accumulate and colonise surfaces in aquatic or high-humidity environments and eventually form biofilm. It involves initial bacterial adhesion, followed by interactions between bacteria, eukaryotes, and substrates (Garibay-Valdez et al., 2022; Iijima et al., 2009). High-density stocks and a continuous nutrient supply can create an environment for pathogens within the biofilm (Cai & Arias, 2017). Closed aquaculture systems like Egget®, with solid walls, offer a large substrate area that may facilitate biofilm production.

Time plays a crucial role in biofouling (Karačić et al., 2022), and to mitigate its natural formation, the interior walls of Egget® are manually brushed daily using an ROV. Regular in-situ net cleaning, typically high-pressure water, is commonly employed to remove biofilm and clean open fish net pens. Biofilm organisms are released into the water as fragments or whole particles during the cleaning process. This has been found to cause discomfort for the salmon, as evidenced by their behaviour and negative impact on their gills (Bloecher et al., 2018; Elsheshtawy et al., 2023). Biofouling has substantial implications for the aquaculture industry, emphasising the need to comprehend its effects and composition in novel systems like Egget®, particularly when evaluating its impact on fish.

1.7.3 Measurement of Microorganisms

To characterise the microbial community in Egget®, sequencing the universal 16S ribosomal ribonucleic acid (rRNA) gene of bacteria present in the water, biofilm, and on the fish's gills provides an overview and insight into the microbial phylogenies communities (Madigan et al., 2018, chap. 12 and 13). Traditional microbiological techniques, such as inoculating and promoting growth on specific growth media, allow for preparing pure colonies that can undergo polymerase chain reaction (PCR) (Polz & Cavanaugh, 1998; Wagner et al., 1994). PCR is a technique that efficiently and precisely amplifies specific deoxyribonucleic acid (DNA) sequences. By comparing the obtained sequences to established databases, information on genetically related bacteria and insight into the present taxonomic groups can be obtained and give an understanding of the diversity of bacteria (Madigan et al., 2018, chap. 12). In microbial community analysis, sequences obtained are commonly subjected to a 97% similarity cut-

off based on the prevailing assumption that this threshold signifies the boundary for identifying the same species (Stackebrandt & Goebel, 1994). This is also applied in this study.

Accurate quantification of DNA and bacterial content in samples from aquaculture are crucial for understanding the microbial community, particularly in the context of new rearing methods such as Egget®. DNA extraction allows for insights into the microbial biomass and genetic potential present in the sample (Madigan et al., 2018, chap. 12). Flow cytometry (FCM) is considered a valuable tool for sensitive and precise quantification of bacterial abundance and providing information on microbial density in aquaculture (Brussaard et al., 2010; Endo et al., 2000). These techniques are instrumental in obtaining knowledge about the microbial diversity and biomass in Egget® and contribute to a comprehensive understanding of its microbial dynamics.

1.7.4 Amoebic Gill Disease (AGD)

Over the past decade, AGD caused by the presence of the amoeba *Paramoeba peruans* has inflicted severe damage and losses in salmon farming. High temperatures and high salinity levels have been identified as associated risk factors (Sommerset et al., 2023). AGD primarily affects the gill lamellae of infected fish, resulting in multifocal hyperplastic lesions and lamellae fusion. Additionally, it leads to an increased number of larger and denser mucous cells in the affected fish, ultimately causing the formation of slimy white mucus spots (Furtado et al., 2022; Lazado et al., 2022b; Young et al., 2007). This increases the gas exchange distance between the water and blood, affecting the fish's respiratory system (Lazado et al., 2022b). Monitoring AGD involves PCR screening, histopathology, and macroscopic examination of the gills (Lazado et al., 2022c; Sommerset et al., 2023). A scoring system has been developed to facilitate assessment to classify the observable changes indicative of AGD from 0= ”clear” to 5= “heavy”, a valuable tool for fish health services. This scoring process must be conducted by trained professionals with the necessary expertise (Sommerset et al., 2023; Taylor et al., 2009). Compared to challenges such as sea lice infestations and escape risk, AGD proves difficult to avoid in CCSs (Sommerset et al., 2023). Moreover, severe outbreaks of AGD have been observed in closed facilities, and the disease seems to develop more rapidly in these systems (Hjeltnes et al., 2019). Experimental treatments using peracetic acid (PAA) on AGD-infected fish have shown changes in behaviour and mortality rates. Downregulation of genes related to immune cells and increased mucous cell size and density have also been observed. Transcriptome profiling of the gills revealed significant changes induced by AGD and PAA treatments, with the effects of PAA being more pronounced within 24 hours post-treatment (Lazado et al., 2022b).

1.8 Gills

The gills are crucial in fish respiration with their highly folded structure. They possess a substantial surface area estimated to be within the range of 0.1–0.4 m^2/kg body weight, accounting for approximately 50% of the fish's total surface area (Akselsen et al., 2019, p. 20; Burton & Burton, 2018, chap. 6; Koppang et al., 2015). Composed of a thin epithelial tissue measuring only a few micrometres in thickness, the gills act as a protective barrier separating the internal environment of the fish from the surrounding environment. These respiratory organs are organised into bilateral series supported by bony structures called gill arches, which provide structural support to the epithelial tissue. Within each gill arch are two rows of epithelial tissue referred to as gill filaments or primary lamellae, referred to as filaments in this thesis. Furthermore, each gill filament exhibits a secondary folding pattern consisting of two rows of folded tissue known as secondary lamellae, referred to as lamellae in this thesis (Figure 2) (Burton & Burton, 2018, chap. 6). Recent histological studies have highlighted distinctive responses in the lamellae and filaments, suggesting separate functional roles within the tissue (Haddeland et al., 2021). Specifically, the mucous cells in the lamellae have demonstrated greater susceptibility to environmental changes, resulting in a higher density than the filaments' relatively unchanged mucous cell density (Dang et al., 2019, 2020).

Fish are aerobic organisms reliant on O_2 for cellular respiration, and the gills serve as specialised respiratory organs facilitating gas exchange between the surrounding water and the bloodstream (Burton & Burton, 2018, chap. 6). A high density of mucous cells in the lamellae potentially increases the respiratory diffusion distance. It challenges efficient gas exchange between the water and the fish's bloodstream, emphasising the gills' critical role in respiration (Dang et al., 2019, 2020).

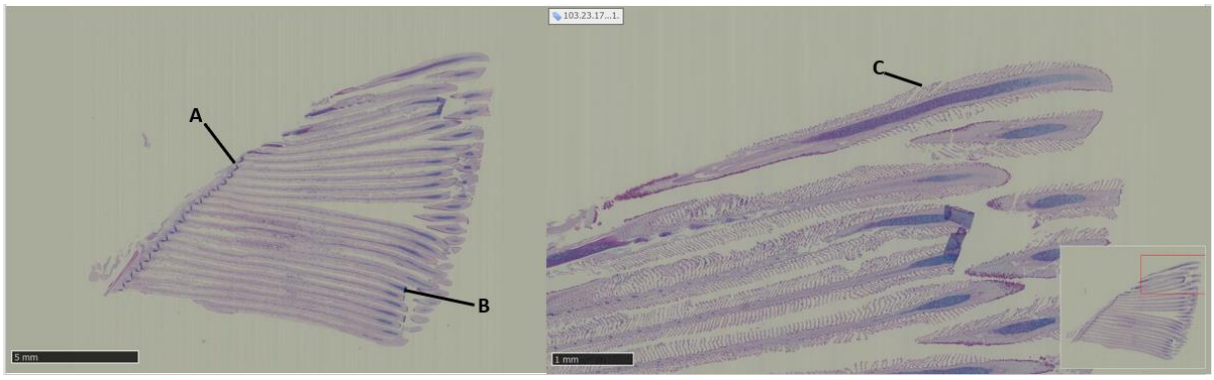

Figure 2: A histological zoom scan of the gill from Atlantic salmon. Letters A-C mark essential tissue of the gill. A= gill arche, B= filament, C= lamellae.

1.9 Skin

In addition to the gills, the skin of fish plays a pivotal role as a protective barrier, regulating the fish's internal environment and maintaining the homeostatic equilibrium of ions, gases, nutrients, and metabolic substrates within the animal (Chuong et al., 2002; Fæste et al., 2020; Glover et al., 2013). Functioning as the primary line of defence, the skin protects against various external threats, including pathogens, parasites, and physical injuries (Dang et al., 2020; Dash et al., 2018; Glover et al., 2013). The fish's skin consists of an epidermis and a dermis, transdermal scales, and mucous cells (Figure 3) (Pittman et al., 2013). The living cell layer secretes a slimy mucus covering the skin's surface, rendering it relatively sensitive to waterborne chemicals and susceptible to physical and biological stressors (Dang et al., 2020). This mucus layer is a chemical barrier containing antimicrobial compounds that effectively prevent infections and preserve skin health (Akselsen et al., 2019, p. 15; Fæste et al., 2020). Despite its protective features, environmental factors such as pollution and fluctuations in water quality pose risks to the skin's integrity, potentially compromising its defensive capabilities (Akselsen et al., 2019, p. 12; Dash et al., 2018).

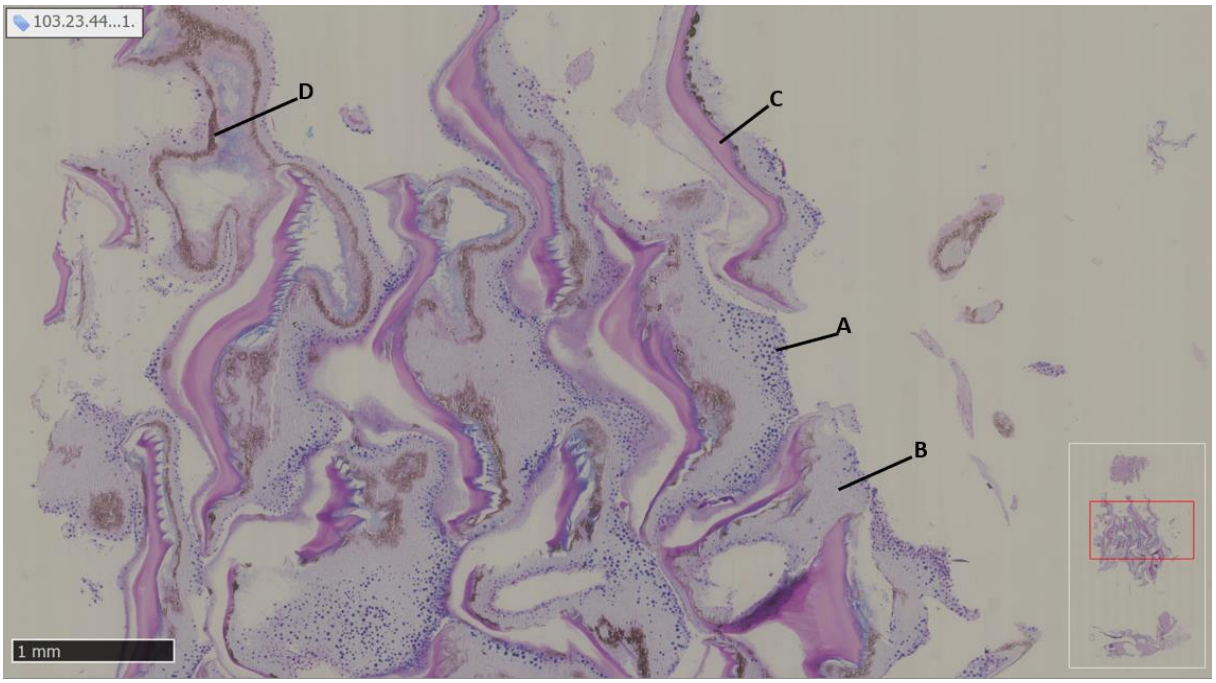

Figure 3: A histological zoom scan of the skin from Atlantic salmon. Letters A-D mark essential tissue in the skin. A= dark blue spots, mucous cells, B= blue tissue, mucosal epithelium, C= pink area, scales, D= brown/dark area, pigments.

1.10 Mucous Cells

With a protective layer of a thin 0.07 mm cell membrane, specialised mucous cells act as the first line of defence, preventing microorganisms from the surrounding seawater from entering the interior of the fish (Akselsen et al., 2019, pp. 8–12; Dang et al., 2020; Maynard et al., 2012). The intestinal barrier is vital for the health of the fish. It blocks harmful substances and pathogens while absorbing nutrients and water (Sundh & Sundell, 2015). These mucous cells are part of the mucosal lining, composed of glycoproteins and water, that covers the skin and other surfaces of the fish, constituting a vital component of the fish's microbiome (Gomez & Primm, 2021; Segner et al., 2012). Among the studied organs, gills, gut, and skin are considered the primary defence barrier against the external environment (Cabillon & Lazado, 2019; Merrifield & Rodiles, 2015). In addition to influencing susceptibility to various diseases, the skin microbiota also likely impacts the fish's response to environmental stresses (Gomez & Primm, 2021).

The mucus layer, which interacts with the environment, continuously undergoes transformation and renewal, enabling rapid responses to external stimuli. In addition to providing a physical barrier, mucous cells play a protective role by nourishing beneficial organisms and eliminating harmful microbes through cleaning, inhibiting growth and the release of antimicrobial agents (Akselsen et al., 2019, pp. 10–16; Bols et al., 2001). Furthermore, the mucous membrane reduces friction and acts as a lubricant between the fish and the water (Akselsen et al., 2019, p. 14). Studies have shown a correlation between environmental conditions, water quality, and the secretion of mucous cells. Recent research indicates a complex communication and interaction system between the components of the fish's normal microbiome and the mucosal surfaces (Merrifield & Rodiles, 2015). Heavy metal poisoning increases mucous cell secretion and density in fish gills, showing a discernible response between the gill lamellae and filaments (Christie & Battle, 1963; Dang et al., 2019; Wu et al., 2007). Notably, the mucous cells in the lamellae demonstrate a more pronounced reaction to the poisoning than the filaments, rendering them a more suitable region to study the environmental impact on fish (Dang et al., 2019).

Research by Dang et al., 2019, on shorthorn sculpins (*Myoxocephalus scorpius*) sampled from different distances from a former mine has revealed that the skin and gill mucosal epithelia respond to environmental pollution and the pressure of parasites. A correlation was observed between the sampling station and the size and density of gill lamellae mucous cells, with the most polluted station exhibiting the largest and densest cells compared to the least polluted station (Dang et al., 2019). Cases of crude oil exposure in Atlantic cod (*Gadus morhua* L.) showed increased mucous-producing epithelial cells and other histopathological changes (Khan & Kiceniuk, 1984). Another study on Atlantic salmon

infected with AGD found that fish treated with PAA and untreated infected fish exhibited significantly larger and denser mucus cells than uninfected fish (Lazado et al., 2022b). These studies highlight the association between the environment, microbial factors, and mucous cells' numerical density and size in the gills and skin. Consequently, alterations in mucous epithelial characteristics can serve as indicators of responses to environmental challenges and early detection of infections (Dang et al., 2019; Lazado et al., 2022b).

Numerical density was deemed inadequate in this study as it does not consider differences in mucous cell size. Instead, in line with Dang et al. (2020), volumetric density was employed as a more precise tool to measure the volume of mucous cells in a given amount of epithelium. This allows for direct comparison across tissues by accounting for the interaction between cell size and number within the reference volume of the mucosa (Dang et al., 2020). In addition, the mucosal mapping method used in this study analyses the surface area horizontally and not only a vertical section of the tissue as done in standard histological quantification of mucous cells (Table 1) (Dang et al., 2020).

Table 1: Presents a depiction of distinctions between the methods, histological quantification of mucous cells and mucosal mapping from Dang et al. 2020.

Table 4
Differences between histological quantification of mucous cells and mucosal mapping.

Comparison	Histological quantification of mucous cells	Mucosal mapping (design-based stereology: 3D from 2D)
Length or area included in the analysis	1–2 mm running length of epithelium	1–2 cm^2 surface area
Unit of measure	relative to existing structures (for example interlammelar units)	universally applicable
Orientation of the section	very important	not important
Standardization	no standardized units	standardized reporting
	not directly comparable across treatment and organs	comparable across treatment and organs
Qualitative or quantitative	qualitative and quantitative	quantitative
Method	Manual	semi-automated
Bias	unbiased only if random selection rules followed (see Methods in and [28])	unbiased

The staining technique used in this study to examine the mucous cells resulted in these cells appearing in a blue/purple colour. A combination of periodic acid Schiff and alcian blue (PAS-AB) staining was employed, explicitly targeting the mucosa's glycoproteins (Figures 2 and 3). This approach differs from haematoxylin and eosin staining, which may introduce artefacts and incorrectly stain glycoproteins, making it unsuitable for the intended purposes of this study.

1.11 Aims of the Study

This study aims to investigate the welfare of young salmon (*Salmo salar*) within the new floating closed containment system, Egget®. Particular attention is given to the sensitive mucous cells on the fish's gills and skin, constantly exposed to the surrounding environment. In addition, microbial colonisation on the fish's gills, in the water, and the biofilm on the surface inside Egget® is investigated in the first production cycle to gain insight into the bacterial abundance and diversity (community composition) present in the system that can affect the fish.

Hypothesis ($H0_1$): The new floating closed containment system, Egget®, does not affect the welfare of fish farmed in the system.

Hypothesis (HA_1): The new floating closed containment system, Egget®, positively affects the welfare of fish farmed in the system.

Hypothesis ($H0_2$): The routines for fish rearing in Egget® do not improve resilience to ambient (pathogenic) organisms.

Hypothesis (HA_2): The routines for fish rearing in Egget® improve resilience to ambient (pathogenic) organisms.

2. Materials and Methods

2.1 Study Area and Facility

Samples are collected from the newly installed (October 2022) floating CCS, Egget® (Figure 4). The facility is located in Romsdalsfjorden at site 13852 Gjermundnes (62° 37.650'N 7° 11.569'E) in Møre and Romsdal in western Norway (Figure 5). 49,895, 150-gram Atlantic salmon smolt (*Salmo salar*) from Eik Settefisk (Vestrefjord) were delivered according to a standard protocol to Egget® October 9th, 2022, and cultivated to February 22nd, 2023. Fish, water, and biofilm samples were collected to investigate fish health and microbial soundings in the system from November 2022 – February 2023. A total of three sampling campaigns were done at Egget®: one month (November 10th, 2022), two months (December 12th, 2022) and four months (February 16th, 2023) after the fish entered. The seawater temperature declined by 5°C during the sampling period. Temperature, O_2, CO_2, salinity, pH, and nephelometric turbidity units (NTUs) were automatically measured (Table 2) with sensors and systems from Guard Automation. Some inconsistencies were noted in the measurement locations and the recording of all parameters. Additionally, significant noise was observed in the turbidity measurements, indicating possible errors. It is worth noting that this is the first prototype of Egget®, and continuous efforts are being made to improve various aspects.

*Table 2: Overview of temperature, oxygen (O_2), carbon dioxide (CO_2), salinity, pH, and turbidity measured with Guard system at different depths inside and outside the closed aquaculture system, Egget® at three sampling dates. *-marked values were the average of the sampling that day, and values without were single values when sampling.*

Sample	Temperature (°C)	O_2 saturation (%)	O_2 (mg/l)	CO_2 (mg/l)	Salinity (‰)	pH	Turbidity (NTU)
November Surface Outlet water	-	95.4–96.6	8.6	-	-	-	-
10 m depth	11.3	104.6	9.4	0	29.9	8.01	0
20 m depth	11.3	91.1	8.1	-	32.2		8.9
December surface outlet water	7.9	94.5–96.0	9.3–9.1	1*	33*	7.9	-
10 m depth	7.8	94.2	9.1	-	-	-	0
20 m depth	7.9	91.3	8.8		33.1		1.8*

February surface outlet water	5.8	92.9–93.6	9.3–9.4	0	33.5	7.9	87
10 m depth	5.7	94.5	9.6	-	-	-	0.2
20 m depth	5.9	94.9	9.5	-	33.9	-	0.8

The roe of the fish introduced to the system was produced by Benchmark Genetics Salten AS from the strain SalmonBreed from Sørfjorden Stamfisk, fed with pellets from EWOS (Cargill, Norway). Before exposure to seawater, the fish were reared in a flow-through system with a 24-hour photo regime and were introduced to seawater before being transferred to Egget®. The accumulated mortality before the fish were transferred to Egget® was 7%, with no disease outbreaks or treatments. Due to delayed sea release, the fish were initially smoltified with a photo regime and Flex feed (EWOS, Cargill, Norway) and subsequently fed with Supersmolt (Polarfeed) before transfer to Egget®. While farmed in Egget®, the fish was fed with Rapid HFT HP 200–1000 pellets from EWOS (Cargill, Norway) according to fish size, 19–20 hours daily. Water turnover was 1–1.5 per hour. The sludge was continuously removed using a continuous suction pump, while dead fish were removed daily using compressed air. The walls inside Egget® were manually brushed daily with ROV (from Mainstay or Troll Systems).

Figure 4: Photo of the new floating closed aquaculture system, Egget®, from the main entrance. Photo: Andrea C. Opshaug

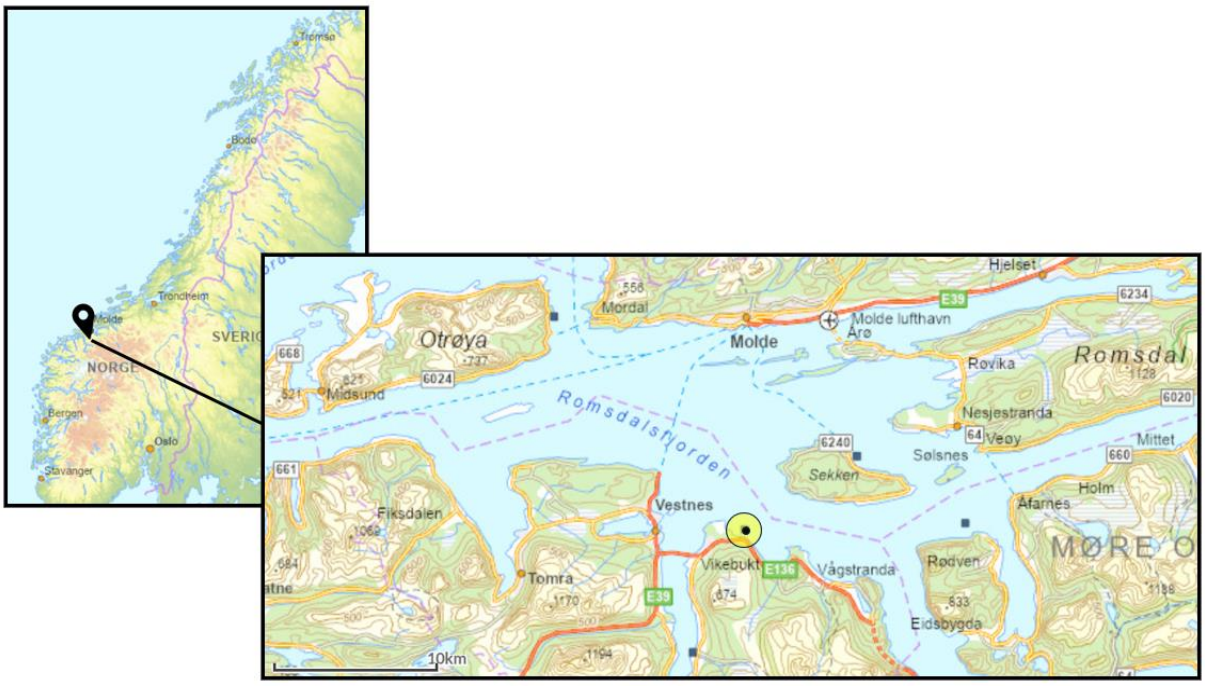

Figure 5: The top map shows where in Norway the facility Egget® is located, while the lower is zoomed in on the position of the sampling site Gjermundnes in Romsdalsfjorden, Møre and Romsdal, Norway (Kartverket, n.d.).

2.2 Sample Collection

In each sampling campaign, ten fish were sampled (N=30, n=10), and gills, skin and gut from each specimen were collected (Figure 6). Due to time limitations, only gills and skin samples were analysed, 60 samples in total. The average weight of the fish started at 235g ± 27g and increased almost five times during the sampling period. In addition, inlet- and outlet water samples and biofilm from three locations inside Egget® were collected and analysed. Furthermore, an outbreak of AGD occurred during the sampling period. An average AGD score above one was recorded on November 29th, 2022 (Figure 6) through gill checks and PCR testing (n=10) conducted by the director of fish health and welfare, Siri Vike, at Ovum AS.

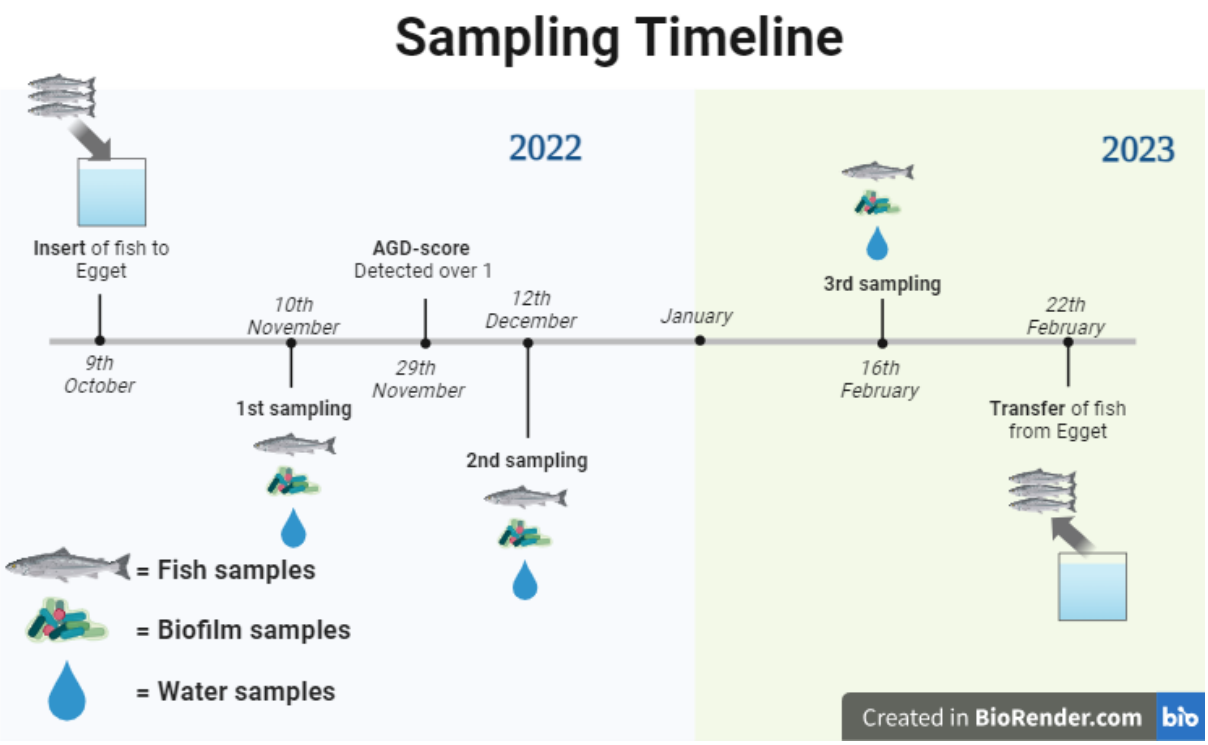

Figure 6: Timeline for sampling at Egget® between October 2022 and February 2023 (Biorender).

2.3 Fish Samples and Mucous Mapping

Ten salmon smolts were randomly selected each sampling day (N=30, n=10) by throwing pellets in Egget® to attract fish before raising a net and collecting a few fish each time (Figure 7). The fish was humanely euthanised with a double lethal dose of the anaesthetic Benxoak 200 mg/ml. One to five fish were transferred simultaneously from the net to the euthanising bath to maintain fresh tissue. Total length, fork length, and weight were measured by the standard sampling protocol of Quantidoc (QD) for Veribarr™ (Appendix A), and an external photo of the fish was taken. Due to certain challenges encountered during the initial sampling, the weight measurements obtained for the fish on the first sampling day exhibit varying levels of reliability. Furthermore, the conditions of the gills were assessed and registered with an AGD score ranging from 0 to 5 in relation to severity by qualified personnel: Siri Vike, Ovum AS or Oda Mittet, a veterinarian, Åkerblå. The mucous membranes were sampled from the left side, and to minimise disturbance to this side, the fish were primarily handled on the right side. The specific growth rate (SGR) was calculated based on the individual's current and previous average weight of the stock and the corresponding period in days (D). Overall SGR in % were also calculated based on the average weight from the first to the last sampling. Additionally, the condition factor (CF)

was calculated with the Fulton formula based on the weight (W) in g and length (Len) in cm (Tvenning, 1991, p. 25).

Specific Growth Rate formula:

$$SGR = \sqrt[D]{\frac{W_{current}}{W_{previous}}}$$

Specific Growth Rate in% formula:

$$SGR\ \% = (\ln(W_{current}) - \ln(W_{previous})) * 100/\mathrm{D}$$

The Fulton formula:

$$CF = \frac{100 * W}{Len^3}$$

Tissues were sampled within 30–40 minutes of lethal anaesthesia in several rounds. The sampling process took longer than desired due to the concurrent need for additional tests. Tissue samples followed standard QD protocol (Appendix A) (Figure 7). Gill tissue were dissected by cutting a part of the second gill arch from the fish's left side. The gill pieces were then carefully and, by only touching the cartilage with tweezers, transferred to a labelled histocassette. Skin samples were collected from the lateral dorsal area, while gut samples were taken from the foregut, following the QD protocol. Three separate labelled histocassettes containing one fish's skin, gills, and foregut were stored in a labelled BiopSafe and filled with 10% buffered formalin.

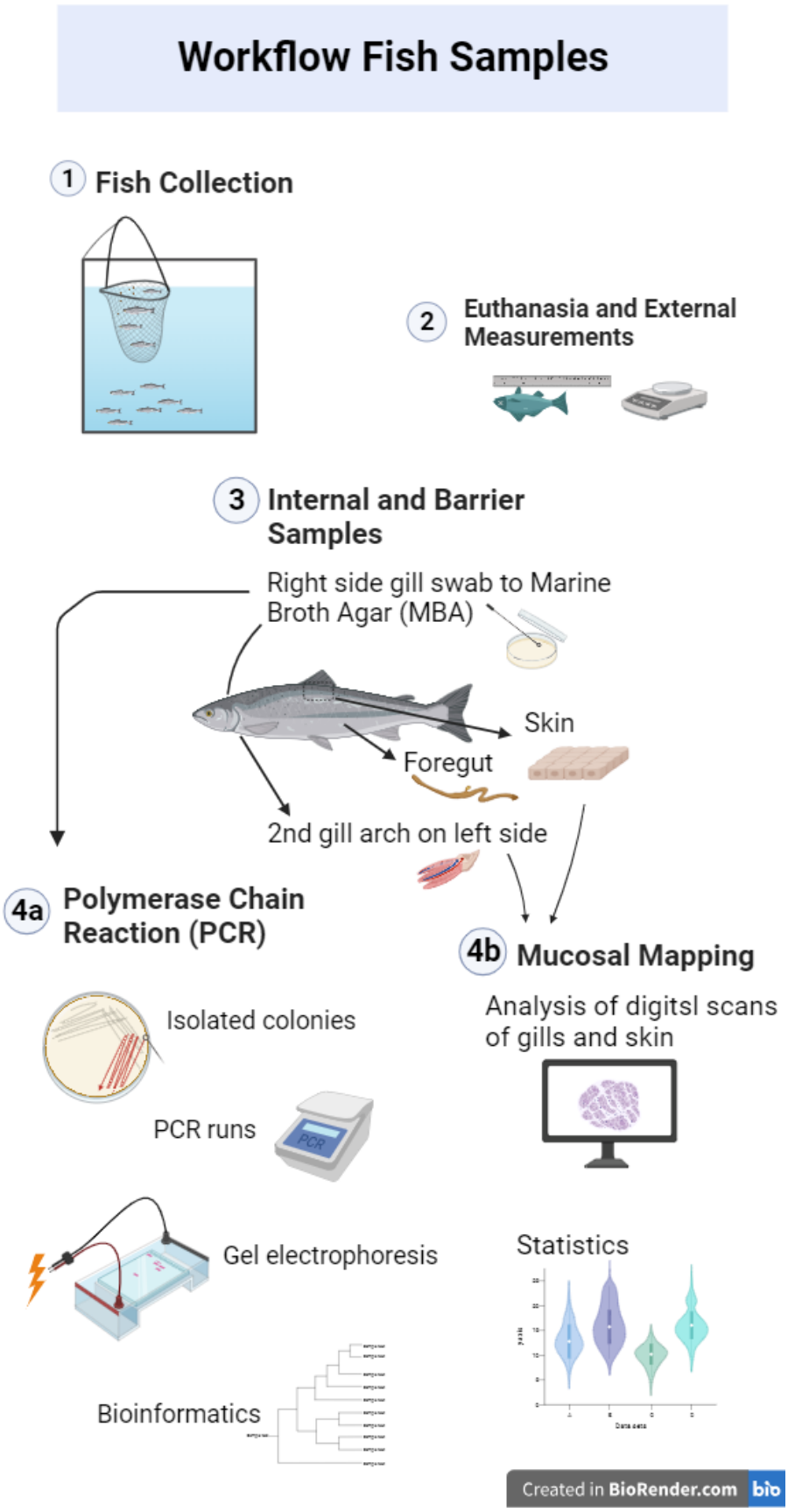

Figure 7: Workflow illustration of fish samples (N=30, n=10) collected from Atlantic salmon farmed in the closed aquaculture system, Egget® (Biorender).

30 BiopSafes containing the three different types of tissue, gill, skin, and gut for each fish, 60 samples, were prepared by Quantidoc for analysis. The standard procedure includes dehydration, decalcifying tissue embedding in paraffin, sliced at 2–3 µm thickness, tangentially stained with PAS-AB, and scanned with a high-resolution digital scanner. The scans were analysed in collaboration with Veribarr. To secure equal evaluation of the scan, all were analysed two times, with evaluation and calibration from Dr. Grigory Merkin, QD.

Mucosal analysis was conducted following the methods described by Pittman and colleagues (Pittman et al., 2011, 2013). The areas of the epithelium and the area of mucus cells were marked on randomised frames on the scanned tissues (Figures 8 and 9) and registered following the established protocol. The Mucomaster™ software of QD was employed to analyse and estimate the mean mucous cell area (MCA) near the equator and mean volumetric mucous cell density (MCD) in gill lamellae and skin. The defence activity (DA) in the mucosal epithelium was calculated using MCA and MCD as inputs, with the formula:

$$Defence\ Activity = 1000 * \frac{1}{MCA/MCD}$$

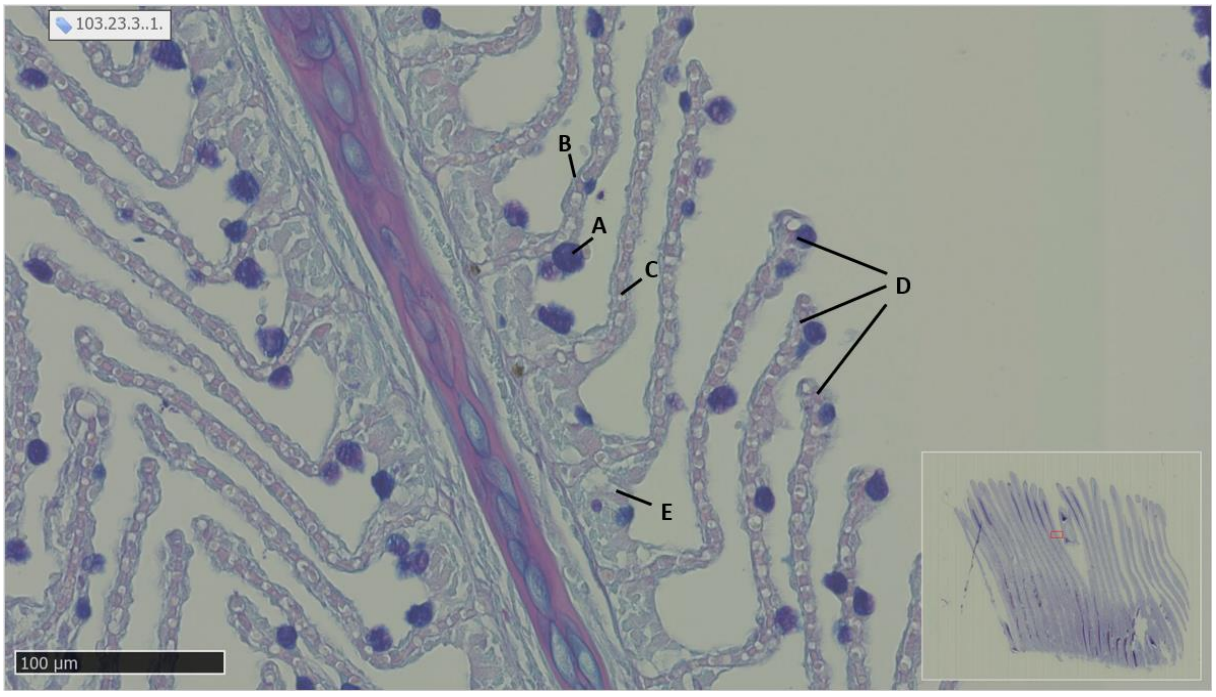

Figure 8: A histological zoom scan of the gill from Atlantic salmon in size 100µm. Letters A–F mark essential tissue of the gill. A= mucous cell, B= epithelium in gill lamellae, C= pillar cell, D= gill lamellae, E = gill filament.

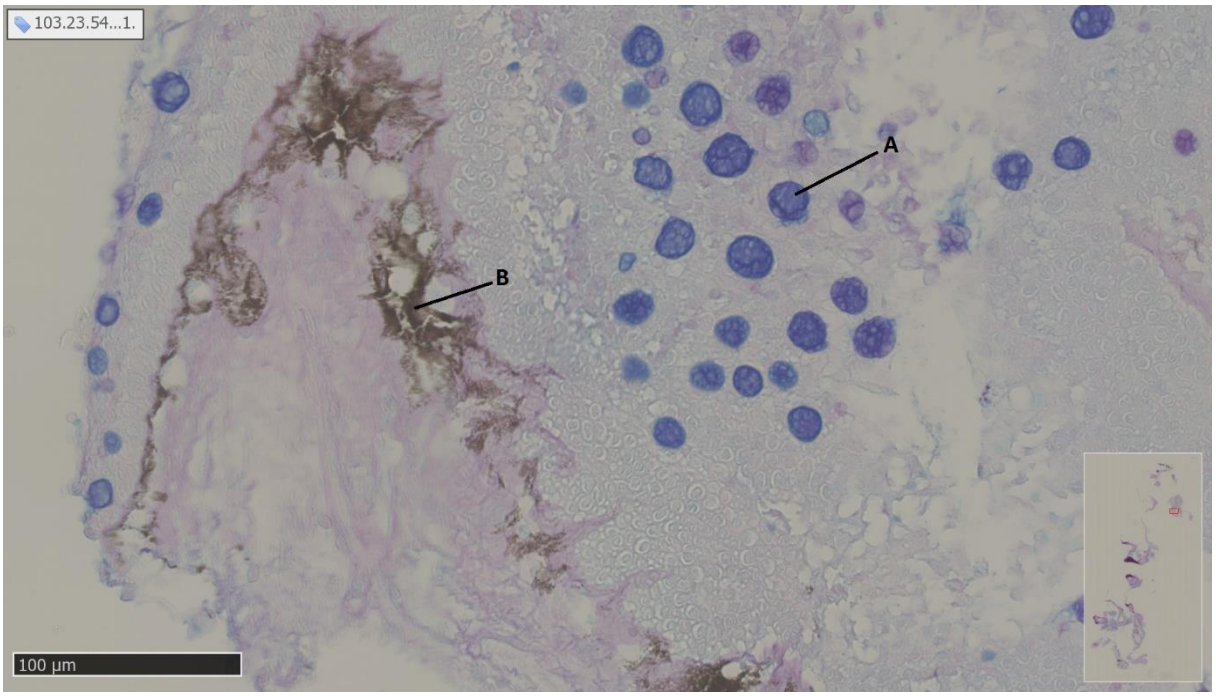

Figure 9: A histological zoom scan of the skin from Atlantic salmon in size 100µm. Letters mark tissue showed on the scan, A= mucous cell and B= pigment cells.

2.4 Polymerase Chain Reaction (PCR) on Isolated Colonies

Concurrent with the mucosal mapping of the fish, the right gill was swabbed with a sterile cotton swab. The swab was then directly inoculated onto a labelled petri dish containing the growth medium marine broth agar (MBA) to facilitate the colony-forming units (Figure 7). Additionally, water from the inlet and outlet was inoculated on MBA plates and treated with the same procedure as the gill samples on MBA. This chapter describes the procedure with gill samples but applies to all the MBA plates. The MBA plates were incubated at 4°C for 6–11 days before further analyses. An overview of the incubation period is provided in Appendix B. Colonies with different morphology regarding structure, shape, and pigmentation were re-streaked on a new sterile MBA plate in a specific pattern to obtain pure cultures (Figure 7). The re-streaking process was performed multiple times, with checks approximately once a week, documenting growth and changes in morphology for each dish.

PCR was used to identify the 16S rRNA gene from bacterial DNA of the microbial community on the fish gill, in the surrounding water and from the biofilm on the walls. Specific primers A8 forward (A8f) and H1542 reverse (H1542r) (Edwards et al., 1989), targeting bacteria, were used in the PCR. First, one isolated colony unit was selected from growth on incubated MBA agar plates and diluted in distilled water in an Eppendorf tube using a sterile toothpick. A positive control using *E. coli* and a negative control with water were also included. Next, a heating-cooling presider was performed to open the

cell membrane. The samples were heated to 80°C for 2 minutes, followed by 2 minutes on ice, two consecutive times. A HotStar master mix was made with the A8f and H1542r primer (Edwards et al., 1989) to amplify nearly the whole length of the 16s rRNA molecule. The master mix, to which the DNA target was added, was analysed using the PCR program at 95°C for denaturation (5 min). Followed by 30 cycles of 30 seconds of denaturation (95°C), 55°C annealing for 30 seconds, and extension at 72°C for 1 min, with a final elongation step at 72°C for 7 min.

2.4.1 Agarose Gel Electrophoresis

An agarose gel electrophoresis was employed to ascertain the presence and evaluate the quality of the PCR product (Figure 7). The DNA migrated through tiny pores in the gel matrix, driven by an applied electrical charge. GelRed, a fluorescent dye with a high affinity for DNA, was used to visualise the fragments. A 1% agarose gel was made with tris acetate EDTA (TAE) buffer and GelRed. The first and last well in the gel contained 5 µl GenRuler 1kb DNA Ladder (Thermo scientific™) used as a ladder to estimate the size of the DNA fragments in the range 100 – 5000 bp; an image of the ladder is found in Appendix C. The gel was prepared with 16 x 2 wells divided into two rows, resulting in four GenRuler ladders on a single gel. The remaining wells were filled with 4 µl DNA product mixed with 1 µl loading dye (6x Loading dye) and subjected to electrophoresis at 200 volts for 20–30 minutes, depending on the migration speed, manually rated. Subsequently, the DNA bands were visualised using ultraviolet (UV) light and the ImageLab software to determine their quality and suitability for subsequent sequencing.

2.4.2 Purification of PCR-Products, ExoSol and BigDye

PCR products were evaluated based on the fluorescence of UV light to determine whether further analysis should be carried out. Subsequently, a second analysis involving purification and removal of unnecessary components was performed on selected samples before sequencing. Most samples were purified and subsequently subjected to sequencing among the initial colonies selected. For the purification process, 5 µl of PCR product and 2 µl of ExoSol were vortexed, centrifuged, and placed in the PCR machine under the following thermocycler conditions: 37°C for 15 minutes and 80°C for 15 minutes. Next, a one-direction BigDye sequencing reaction was conducted. The master mix contained 1 µl BigDye, 1 µl sequence buffer, 3.2 µl primer A8f (1 µ Molar), and 2.8 µl PRC water was multiplied by the number of samples. 8 µl of the BigDye master mix and 2 µl cleaned PCR product were centrifuged, and the following PCR program was performed: 96°C for denaturation (5 min), followed

by 25 cycles of 10 seconds denaturation (96°C), 55°C annealing for 5 seconds, and extension at 60°C for 4 min. 10 µl nuclease-free water was added at the end of the program, adding up to a total volume of 20 µl in each PCR tube. Samples were then delivered to the Sequencing Facility at the University of Bergen (UiB) for sequencing.

2.4.3 Bioinformatics

DNA sequences were trimmed and analysed using the Chromas software. This program visually represents the nitrogen bases in the sequence as coloured peaks corresponding to adenine (A), thymine (T), cytosine (C), and guanine (G) (Figure 10). Any noise or disrupted peaks at the beginning and end of the sequences were removed, illustrated with the dashed line in Figure 10. Nucleotides in the sequence not accurately registered during sequencing were labelled as "N". These instances were manually inspected, and when a definitive nucleotide base was observed with a coloured peak, manual editing was performed to correct the sequence. A lowercase letter was used for the substituted letter to make the differences (Figure 10).

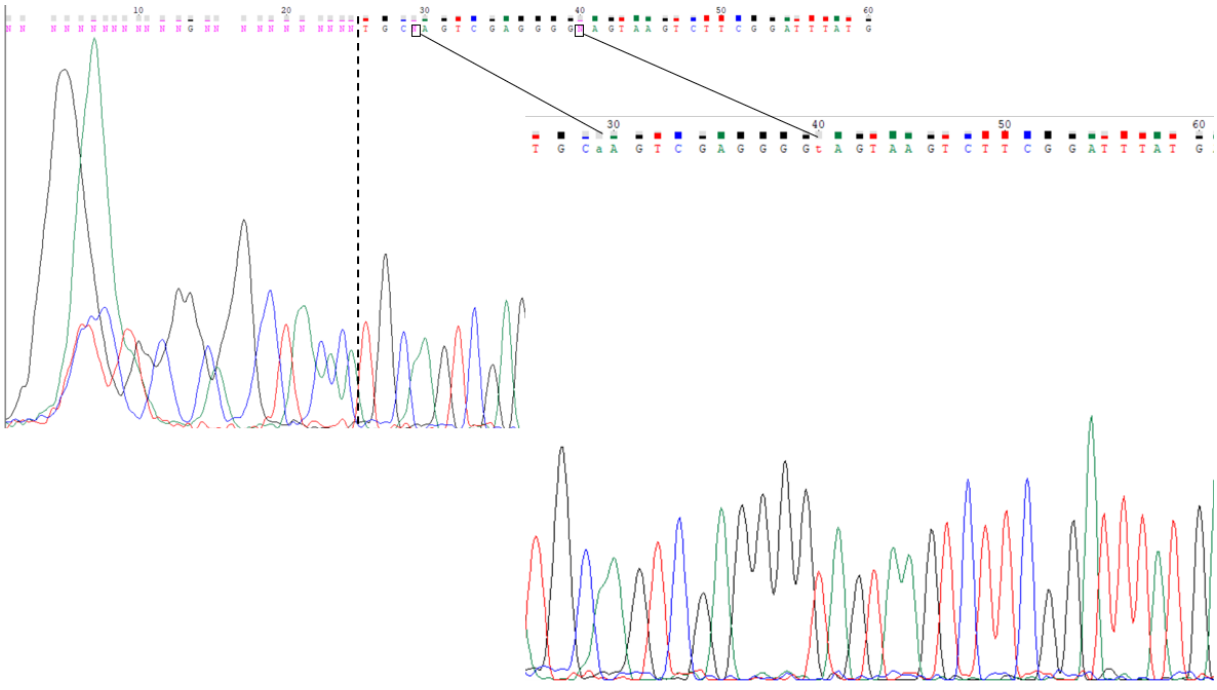

Figure 10: Example of trimming and cleaning of one DNA-sequence in the Chromas software. Source: images from work with sequences in Chromas.

2.4.4 Alignments, BLAST and Phylogenetic Trees

After cleaning, the DNA sequences were further analysed and aligned using Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) software (Tamura et al., 2021). The trimmed sequences were imported to a Plain Text Editor, "Notepad", and aligned utilising the ClustalW option to ensure accurate alignment. Non-conserved regions outside the aligned samples were removed to focus solely on the conserved sequences. Each sequence was also analysed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to compare the DNA sequences with known sequences in the GenBank database. This facilitated the similarity assessment between our collected sequences and established bacterial sequences. The results of these comparisons were then utilised as references for the inclusion of the corresponding bacterial species in the phylogenetic tree analysis.

Phylogenetic trees were constructed using MEGA11, based on the bacteria sequences and followed the protocol for constructing a Phylogenetic tree, see Appendix D. The "Neighbor-joining" method was used, and the setting "Bootstrap method" was set to 1000 iterations to provide robust statistical support. The trees were further built and edited with outgroups, colours, and aesthetics. Different geometric shapes and colours were used to represent fish samples, water samples, and the reference bacteria. These visual elements were utilised to present best the kinship between the bacterial species in the constructed trees.

2.5 Harvesting of Microbial Biomass – Water and Biofilm

To perform DNA extraction, samples were collected from inlet and outlet water on all three sampling days, close to the inlet/outlet area on the right side of the facility's main entrance. A total of 2.1 litres of water was collected using a washed water collector (7670.17, 1L from Frederiksen). The collector was washed with freshwater before and between the sampling days and with seawater from the site before sampling. Inlet water was collected from the outer inlet area of Egget® using the cylinder-shaped collector, which was lowered and sealed at a depth of 17 meters, corresponding to the inlet water level. Outlet water was collected with the same method less than a meter from the outlet area inside Egget®.

Microbial biomass from the biofilm was collected using sterile swabs with a 4 cm sponge at the end, connected to a telescopic rod, and swabbed directly at visible fouling on the wall. Approximately 0–0.5 meters below the surface, the sponges were swabbed 3–4 times up and down the wall. Biofilm harvesting was performed at three different locations inside Egget®: 1st Right (R), from the main

entrance around the outlet water area, 2nd Opposite (O) from the main entrance, and 3rd Left (L), from the main entrance (Figure 11).

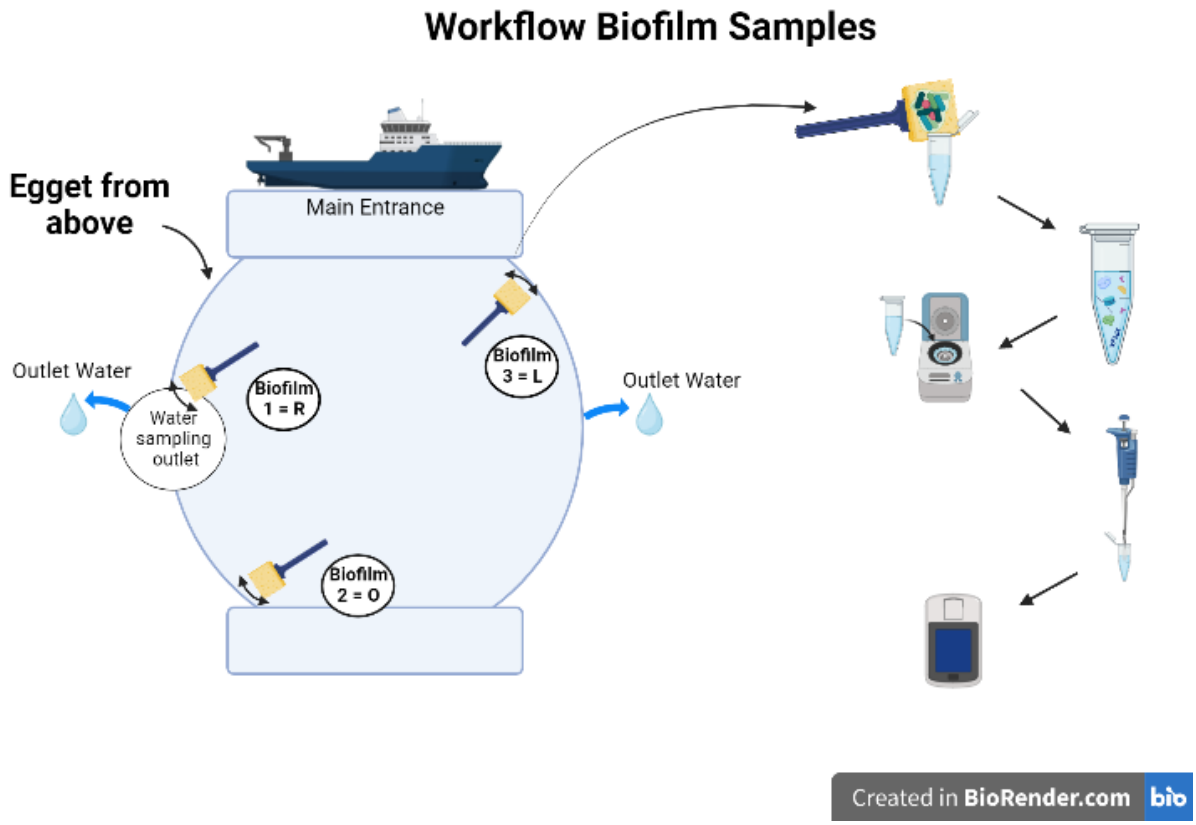

Figure 11: Illustration of the closed aquacultur system, Egget®, seen from above. The three collection points for the biofilm are marked from 1–3 (Biorender).

2.6 Isolation of Microorganisms from Water

Concurrent with the microbiology investigation on the fish's gill, a cotton swab was soaked in collected inlet/outlet water and spread on a petri dish containing MBA growth medium. This was done in triplicates: three petri dishes for the inlet and three for the outlet, a total of 6 dishes each sampling day, a total of 18 dishes. Further, these samples were treated with the same procedure as the microbiology samples from the gills, as described in Chapter 2.4.

2.7 Nucleic Acid Extraction of DNA from Water

Before DNA extraction, 1 litre of collected seawater at the site was filtrated manually through a 0.22 µm Sterivex filter (SVGPL10RC) using a handheld 60 ml syringe following the protocol (Appendix E) (Figure 12). After one litre of seawater had been pushed through the filter, 2 ml of RNA-later (AM7021) was immediately added to preserve the filters for transportation before storage at 4°C. On each sampling day four filters were collected, two for inlet and two for outlet water, in total, 12 samples.

Nucleic acid extraction of DNA was done following the AllPrep DNA/RNA Kit (Qiagen) protocol (Figure 12), slightly modified from Müller 2014. First, Sterivex filters were drained from RNA-later, rinsed with nuclease-free water and dried with air before 1 ml RLTplus buffer was added and incubated for 2 x 2 minutes (turned 180° after 2 minutes) while vortexed at level 5, out of 10 levels. Next, lysate was transferred and centrifuged with Sigma 1–14K for 30 seconds at 10 000 rpm in the Allprep DNA spin column before first, 500 µl, AW1 (AllPrep Wash Buffer 1) was added and centrifuged at the same speed, intensity and seconds, 500 µl AW2 (AllPrep Wash Buffer 2) was added and centrifuged for 2 minutes at full speed (14 800 rpm). AW1 and AW2 were washing solutions and used to remove any remaining impurities and contaminants in the samples. Further, 50 µl of Ethidium Bromide (EB) buffer (Elution buffer), at 70°C, was added to the centre of the column with a new tube and incubated for 5 minutes at room temperature to elute/release the DNA. Finally, the column was one last time centrifuged and the previous step with EB buffer was repeated for a second elution. Samples were further incubated in a refrigerator at 4°C and measured with a Qubit-fluorometer the following day, further described in Chapter 2.9.

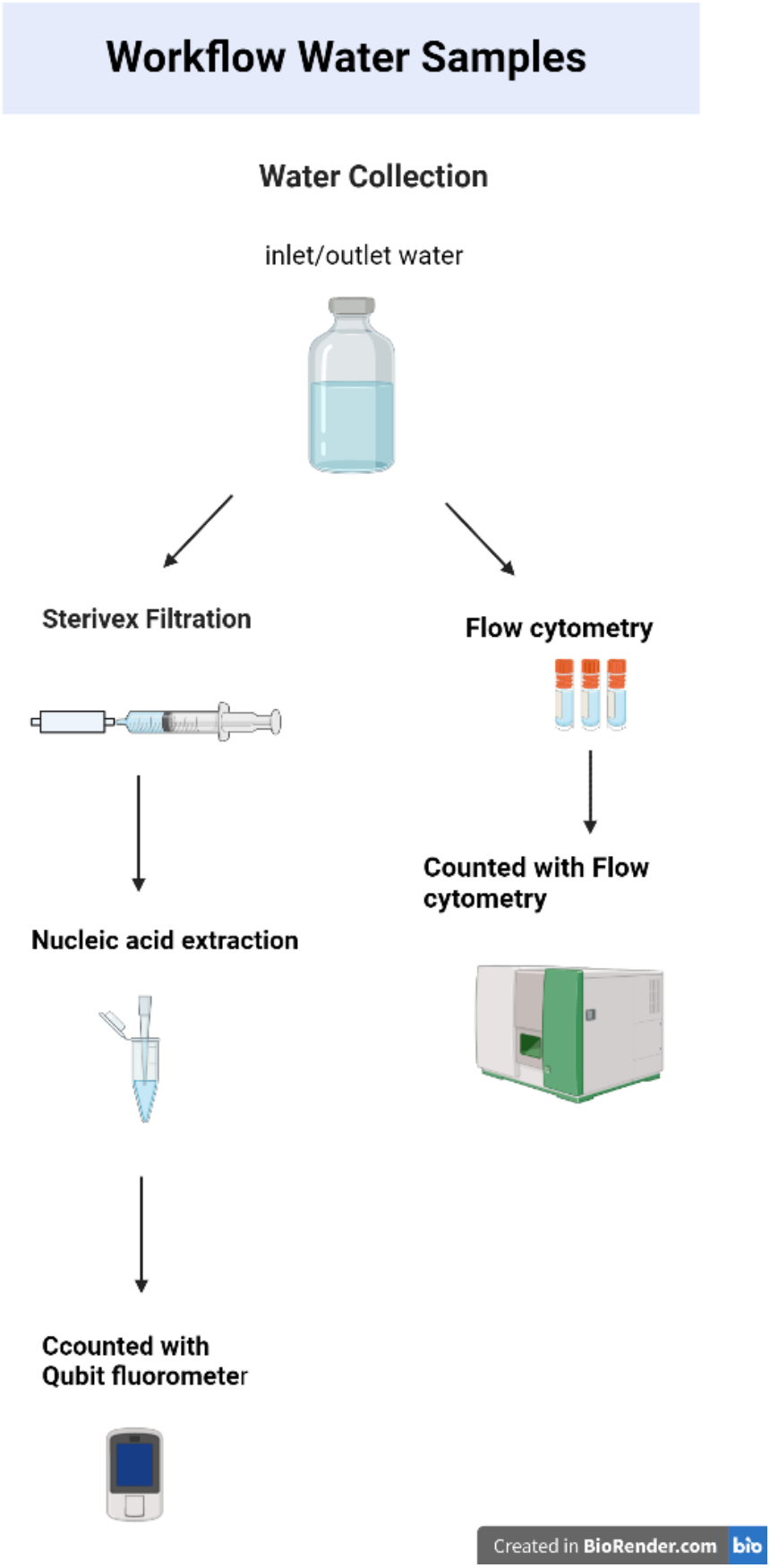

Figure 12: Flow chart over water samples collected at Egget® from inlet and outlet water (Biorender).

2.8 Nucleic Acid Extraction of DNA from Biofilm

Before using the DNeasy soil kit to isolate DNA, the biofilm samples were transferred directly from the sponge to an Eppendorf tube filled with RNA-later (AM7021) at the site (Figure 11). Three tubes were collected each sampling day to measure the DNA from the microbial biofilm community on the wall at Egget®, nine samples in total.

To remove RNA-later, samples were centrifuged at 14 800 rpm with centrifuges Sigma 1–14K for 25 minutes to ensure the pellet had settled at the bottom. RNA-later was then carefully removed with a syringe. The procedure was done following the Quick-Start Protocol, May 2019. First, 800 µl of CD1 solution was added to the tubes, whirled, and transferred to the PowerBead Pro Tube before being vortexed for 10 minutes at full speed. Then, several steps with adding, homogenisation and centrifuge were performed by adding 200 µl of CD2 solution, 600 µl of CD3 solution, 500 µl of EA-solution and 500 µl of CD5 solution. Further, 50 µl of CD6 at 60°C was added to the centre of the column with a new tube and incubated for 20–25 minutes at room temperature to elute /release the DNA. After one more centrifugation, the last step with CD6 was repeated for a second elevation. Finally, all samples were incubated in a refrigerator at 4°C and measured with a Qubit-fluorometer the following day, further described in Chapter 2.9.

2.9 DNA Measures of Water and Biofilm Samples

Quant-iT™ double-stranded DNA high sensitivity assay kit was used to measure the DNA concentration in both samples from Sterivex, AllPrep DNA/RNA kit, Chapter 2.7 (Figure 12) and biofilm Eppendorf tubes, DNeasy soil kit, Chapter 2.8 (Figure 11), in total 21 samples. The kit protocol was followed, and a working solution containing Quant-iT™ reagent diluted with Quant-iT™ buffer was made based on the number of samples. Qubit assay tube was filled with 197 µl of working solution and 3 µl of DNA extraction (samples from Chapters 2.7 and 2.8), while two standards were filled with 190 µl working solution and 10 µl Quant-iT™ standard. Each sample was vortexed for 3–4 seconds and then incubated at room temperature for 2 minutes before being analysed using a Qubit 2.0 fluorometer. The analysis began with creating a standard range from 0 ng/µl to 10 ng/µl. The Quant-iT™ Assay results for water and biofilm samples were presented in separate bar charts created in Excel.

2.10 Flow Cytometry (FCM) Analyses

The number of microbial cells in the inlet and outlet water in Egget® was estimated using FCM (Figure 12). 1.8 or 1 ml of collected water was transferred with a sterile syringe to a cryo-tube, prefilled with glutaraldehyde to a final concentration of 2.5%. This was done in triplicate: three tubes for inlet and outlet water, a total of six tubes each sampling day, 18 samples in total. FCM tubes were transported in a freezer bag and stored in a – 80°C freezer at UiB until further analyses.

FCM detects and characterises microbial cells based on their light scatter and fluorescence features. A Becton Dickinson (BD) FACS Calibur, 1998, was used with an argon laser (488 nm, blue) to analyse the cells. The 18 samples (inlet water N=9, outlet water N=9) were moved from the -80°C freezer to a 4°C refrigerator two hours before analyses. Together with Senior Engineer Elzbieta Anna Petelenz, the protocol by Brussaard, 2010 for flow cytometry was performed (Brussaard et al., 2010). Using SYBER Green, each sample was counted for 60 ± 2 seconds at a flow rate of 55.4 µl/min. The data were visualised using CellQuest Pro Software and the mean of the samples was presented in an Excel bar chart.

2.11 Library Construction, Quality Control, and Sequencing

To enhance the understanding of the microbial community, the water and biofilm samples, which had been quantified using Qubit, were sent to Novogene for metabarcoding and DNA sequence analyses (Figure 13). These analyses used specific primers containing barcodes to amplify targeted DNA regions. Subsequently, the resulting PCR products were selected based on their size using gel electrophoresis. Equimolar amounts of PCR products from each sample were pooled together and underwent library preparation, which involved the addition of adapters for sequencing on an Illumina platform. The resulting library was subjected to thorough quantity and quality assessment using Qubit, real-time PCR, and bioanalysis. The quantified libraries were subsequently combined and sequenced on Illumina platforms, considering the required library concentration and desired amount of data.

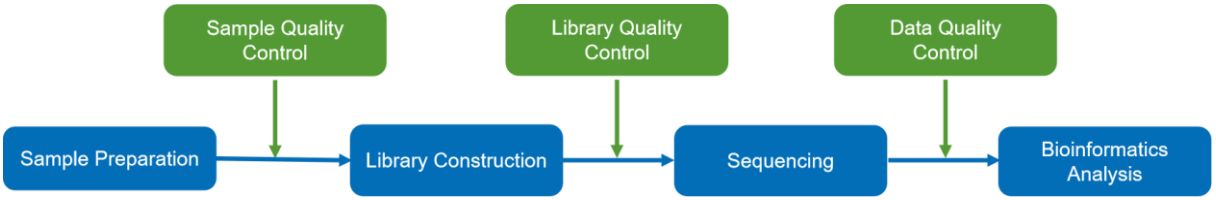

Figure 13: Workflow for sample analyses conducted by Novogene.

2.12 Statistical Analyses

Statistical analyses were conducted using RStudio (RStudio Team, 2023) and R (R Core Team, 2023), with the packages “car” (Fox & Weisberg, 2019), “emmeans” (Lenth, 2023) and “ggplot2” (Wickham, 2016). A preliminary check was performed before running the Analysis of Variance (ANOVA) model. Firstly, the assumptions necessary for the validity of the ANOVA were carefully evaluated. This involved examining the data distribution's normality using a Quantile-Quantile plot (QQ plot), testing the homogeneity of variances with Levene's test, and assessing the independence of samples. Subsequently, an ANOVA test was applied. A P-value ≤ 0.05 was considered statistically significant, and to further elucidate the nature and magnitude of these differences, a post hoc Tukey's honest significant difference (HSD) test was employed. The post hoc analysis aimed to identify groups that exhibited statistically significant discrepancies, providing a more precise understanding of the observed variations in the ANOVA (Zar, 2010, pp. 189–224). The statistical analysis was done on gill lamellae MCA, MCD and DA against the sampling months, November, December, and February. Due to minor differences in the skin mucous samples and a low probability of significant variance, these samples were not statistically analysed.

3. Results

Analysis was conducted on 60 mucous samples obtained from the gills and the skin of farmed salmon harvested from the newly implemented floating closed containment system, Egget®. The microbial communities in the gills, inlet and outlet water, and the biofilm on the system's walls were examined.

3.1 Fish Samples

Thirty Atlantic salmon (20 males, nine females and one unidentified), with average weight; 1st sampling: 235g ± SE 27g, 2nd sampling: 602g ± SE 30g, 3rd sampling: 1157g ± SE 69g, were analysed in this study. The seawater temperature inside Egget® declined during the sampling period, initially from 1st sampling at 11.3°C, 2nd sampling at 7.9°C and 3rd sampling at 5.9°C. During the production period from October 9th, 2022, to February 22nd, 2023, 588 fish mortalities were recorded, resulting in a mortality rate of approximately 1.2% (Table 3). Throughout this period, fewer than 20 sea lice, no wounds, and very few other welfare-related issues, such as scratches and fin damage, were observed on the fish while investigating approximately 100–800 fish daily using a camera in the cage.

Table 3: Cumulative mortality of Atlantic salmon produced in the closed aquaculture system, Egget®, in Norway over 21 weeks in 2022/2023

Year/Week number	Cumulative Mortality in Egget® (%)
2022/40	0,00
2022/41	0,03
2022/42	0,06
2022/43	0,10
2022/44	0,17
2022/45	0,24
2022/46	0,32
2022/47	0,40
2022/48	0,46
2022/49	0,53
2022/50	0,57
2022/51	0,62
2022/52	0,71
2023/01	0,78
2023/02	0,86
2023/03	0,91
2023/04	0,97
2023/05	1,05
2023/06	1,10
2023/07	1,15
2023/08	1,18

The length and weight measurements of the fish sampled showed a linear and proportional relationship between the two variables (Figure 14). The fish's specific growth rate (SGR) ranged from -0.8 to 3.9 g/day (Figure 15). In November, three instances exhibited a negative SGR, while the remaining instances showed a positive SGR ranging between 0.9 and 2.6 g/day. In December, the range was between 2.1 and 3.9 g/day, and in February, the range was between 0.7 and 1.6 g/day. The overall SGR in % from November to February was 1.6. The condition factor (CF) ranged from 0.3 to 1.3 and increased overall over the sampling period (Figure 16). November displayed a larger variation in CF, with values ranging from 0.3 to 1.1. In December, the CF values were more concentrated around 1.1, while in February, values clustered around 1.2.

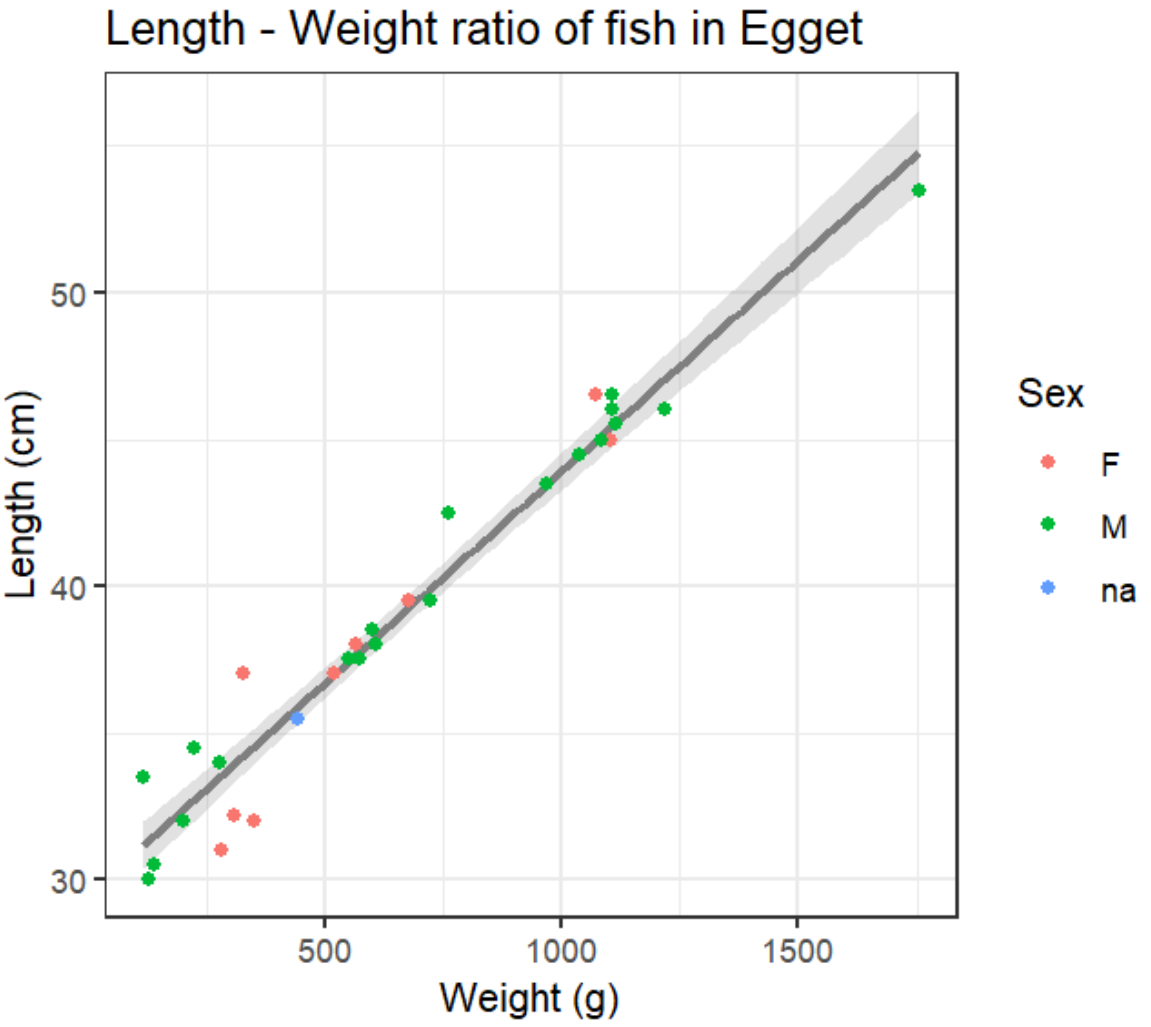

Figure 14: Ratio between length and weight of Atlantic salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. The different colours indicate sex differences.

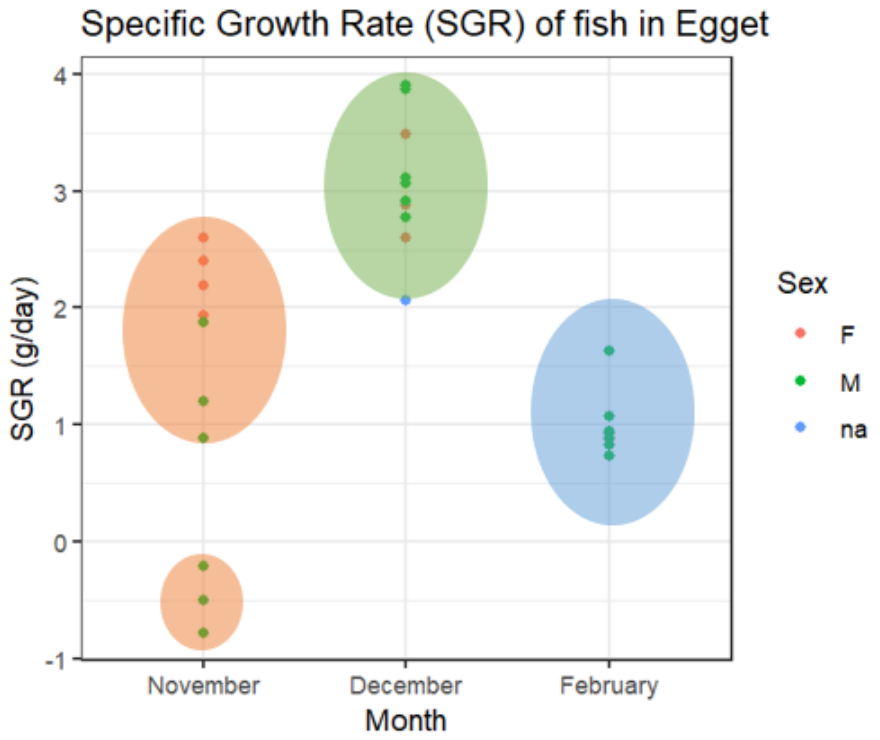

Figure 15: Specific growth rate (SGR) of Atlantic salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. The different colours indicate sex differences.

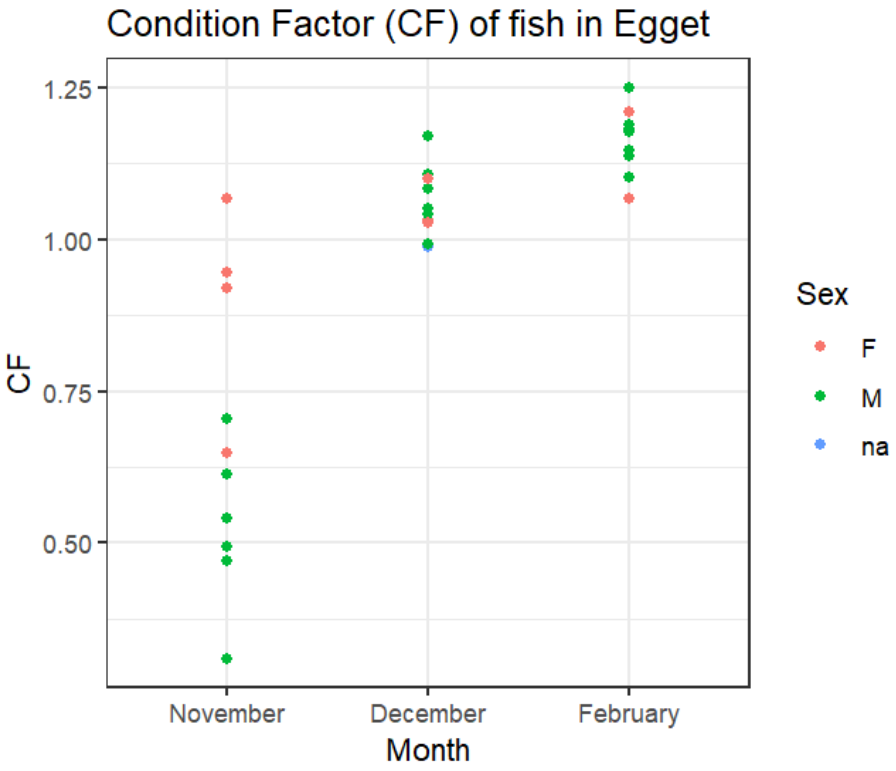

Figure 16: Condition factor of Atlantic salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. The different colours indicate sex differences.

3.2 Gill Lamellae

The mean mucous cell area (MCA) in the gill lamellae of the sampled fish ranged from 64–79 µm^2, and the median from 63–77 µm^2. The mean MCA decreased from November (74 µm^2) to December (64 µm^2) and increased to February (79 µm^2) (Figure 17). The mean volumetric mucous cell density (MCD) ranged between 6%–14%, and the median ranged from 5%–14%, gradually increasing from November to February (Figure 18). The recorded mean values for defence activity (DA) in the gill lamellae mucosal epithelium varied from 0.8–1.8, with the median from 0.7–1.7 (Figure 19), exhibiting the same trend as in the MCD. Significant changes in MCA were observed between the 2nd and 3rd samplings. However, no significant alterations in MCA were noted between the remaining samplings (Figure 17). Volumetric MCD and DA gradually increased, with significant changes observed between all sampling months (Figures 18 and 19). The results showed changes in mucus dynamics in gill lamellae, with more but smaller cells in December than in November and an increase in both size and density of cells in February (Figure 20).

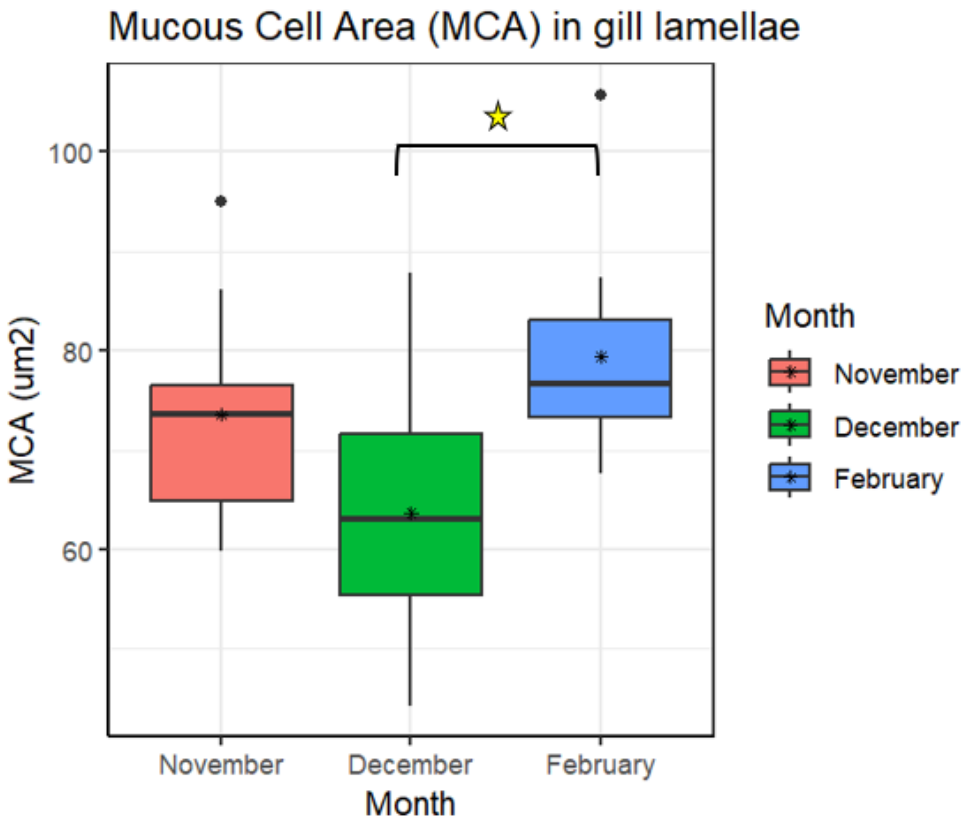

Figure 17: Mucous cell area (MCA) in square micrometres (µm²) in gill lamellae of Atlantic Salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. Within each box, the black line represents the median MCA, and the black star indicates the mean MCA value. Significant differences are determined using the statistical analysis ANOVA and are shown as yellow stars in the plot. The plot was generated using RStudio.

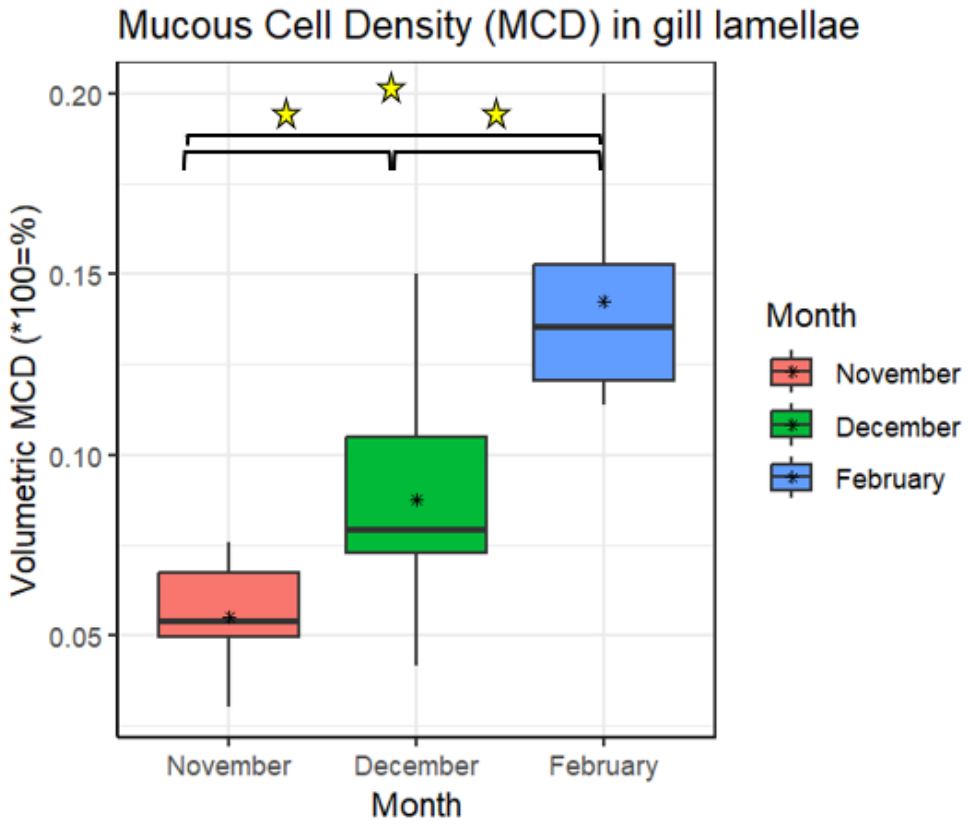

Figure 18: Volumetric mucous cell density (MCD) in gill lamellae of Atlantic Salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. Within each box, the black line represents the median MCD, and the black star indicates the mean MCD value. Significant differences are determined using the statistical analysis ANOVA and are shown as yellow stars in the plot. The plot was generated using RStudio.

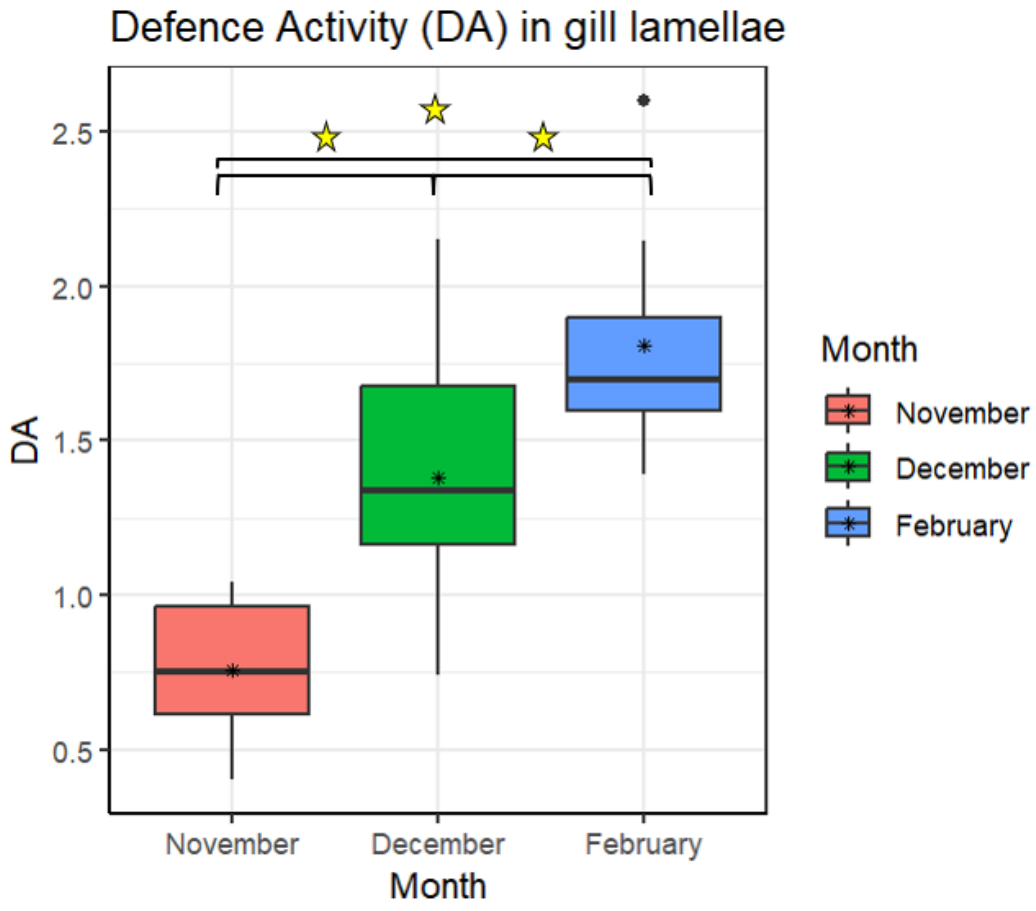

Figure 19: Defence activity (DA) in the mucosal epithelium in lamellae gill of Atlantic Salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. Within each box, the black line represents the median MCD, and the black star indicates the mean MCD value. Significant differences are determined using the statistical analysis ANOVA and are shown as yellow stars in the plot. The plot was generated using RStudio.

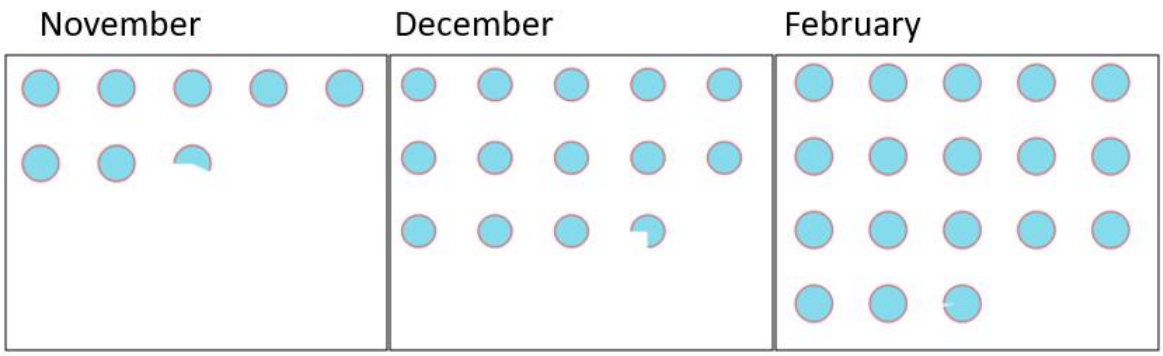

Figure 20: Standardised representations of the variations in the average dynamics of mucous cells found in the gill lamellae of Atlantic salmon (N=30, n=10) farmed in the closed aquaculture system, Egget®. Data were collected during three different months in the 2022/2023 period and are visualised and represented using a dice diagram generated using Quantidoc's Dice App v2, highlighting the differences between the various samplings.

3.3 Skin

The mean MCA in the skin of the sampled fish exhibited an unaltered value between 137–153 µm² throughout the study period and with a median between 132–147 µm² (Figure 21). Mean MCD was maintained at 9–11% and the median at 8–10% (Figure 22). The DA also remained stable, with mean and median values within 0.6–0.7 (Figure 23). None of the parameter's MCA, MCD, or DA demonstrated statistically significant changes across the sampling days (Figure 24). The mucous skin results from this study, alongside the database of Quantidoc, showed a clustering of the skin samples (the coloured dots) within the average range of mucous cell size and defence activity indicated by the green circle (Figure 25).

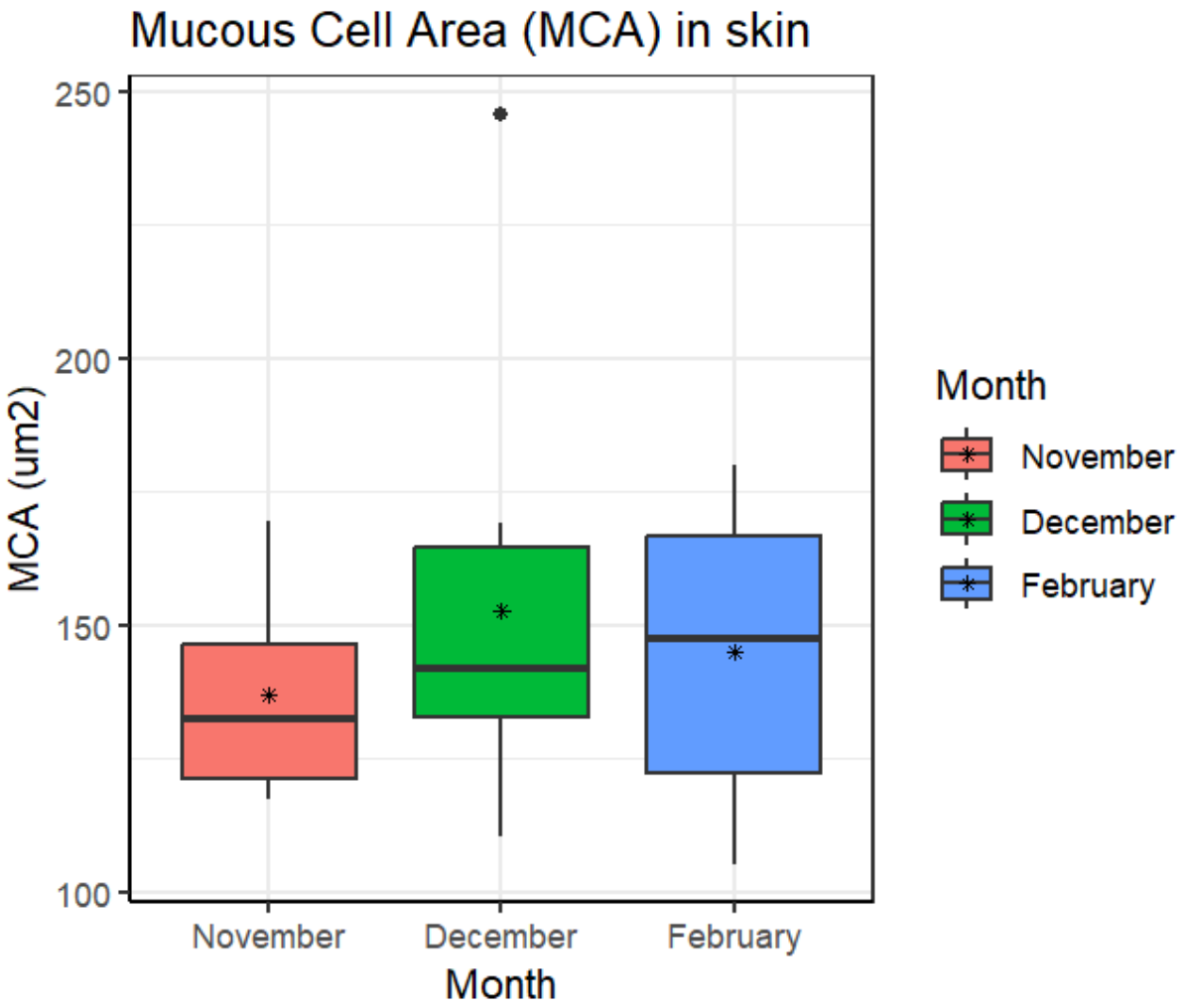

Figure 21: Mucous cell area (MCA) in square micrometres (µm²) in the skin of Atlantic Salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. Within each box, the black line represents the median MCA, and the black star indicates the mean MCA value. The plot was generated using RStudio.

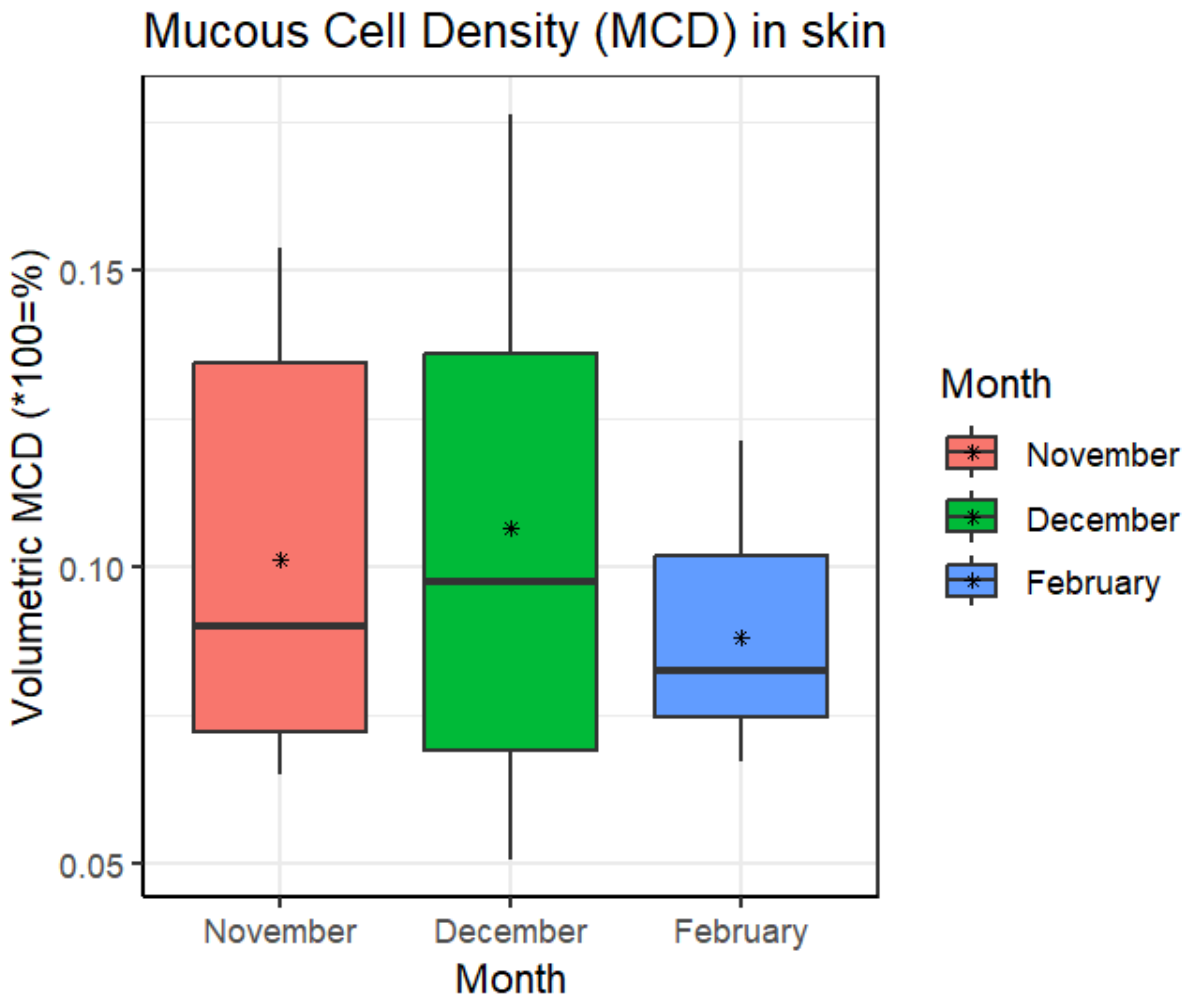

Figure 22: Volumetric mucous cell density (MCD) in the skin of Atlantic Salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. Within each box, the black line represents the median MCD, and the black star indicates the mean MCD value. The plot was generated using RStudio.

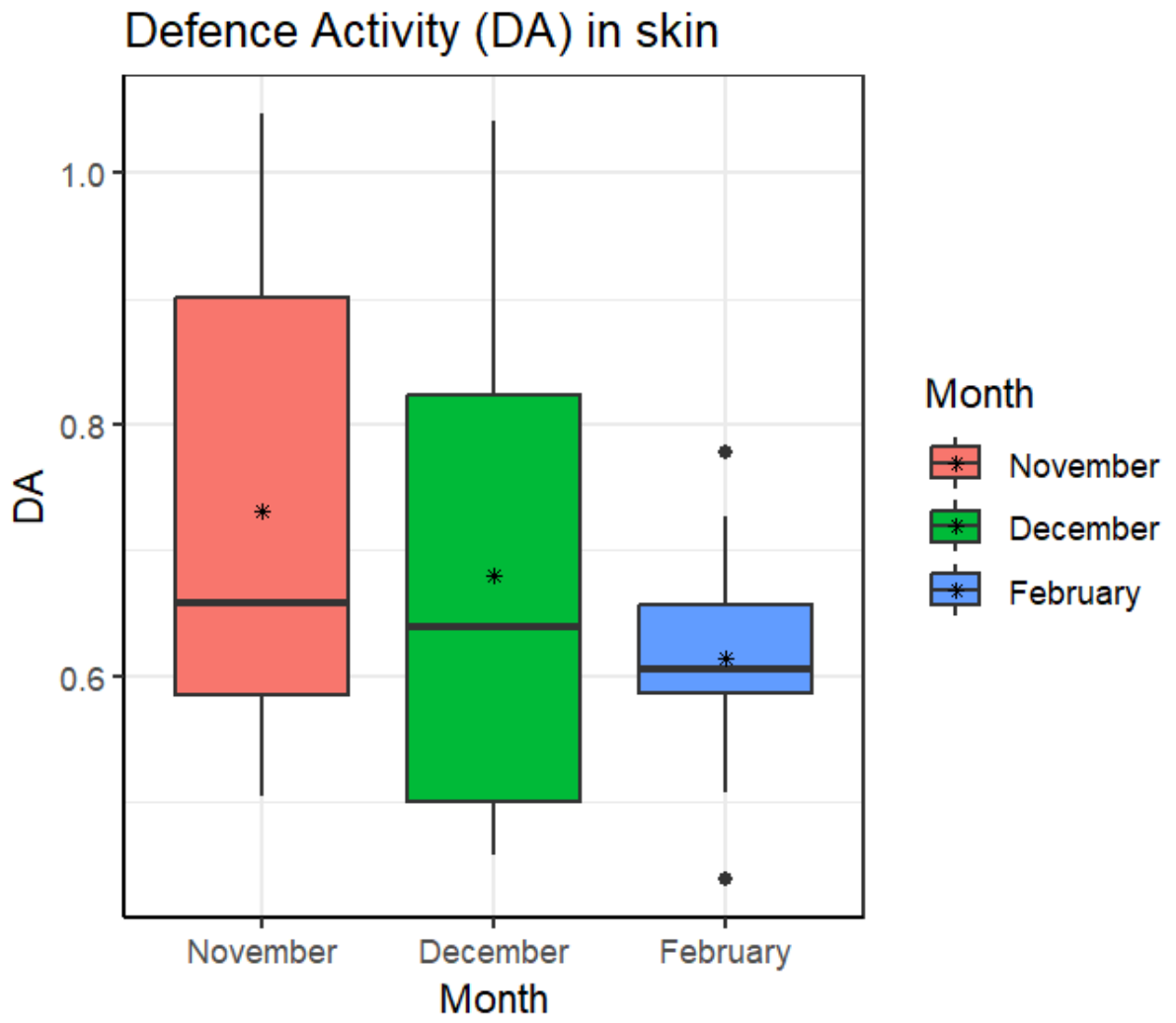

Figure 23: Defence activity (DA) in the mucosal epithelium in the skin of Atlantic Salmon (N=30, n=10). Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. Within each box, the black line represents the median MCD, and the black star indicates the mean MCD value. The plot was generated using RStudio.

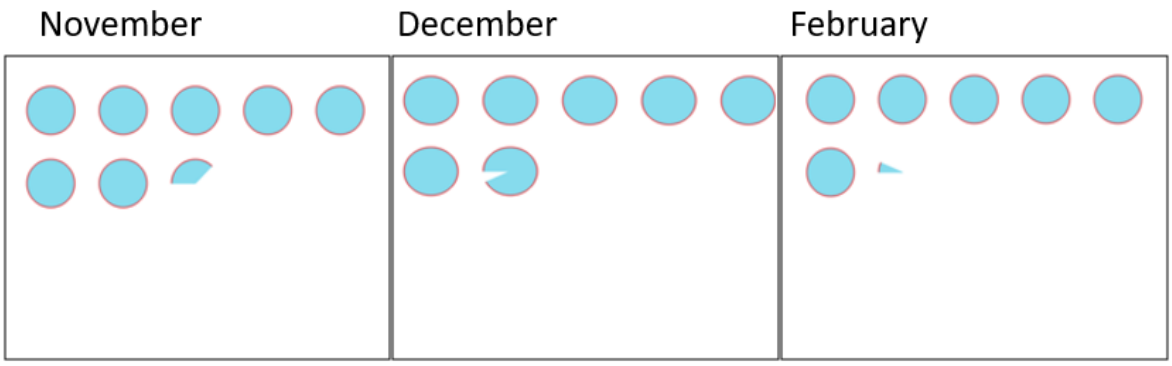

Figure 24: Standardised representations of the variations in the average dynamics of mucous cells found in the skin of Atlantic salmon (N=30, n=10) farmed in the closed aquaculture system, Egget®. Data were collected during three different months in the 2022/2023 period and are visualised and represented using a dice diagram generated using Quantidoc's Dice App v2, highlighting the differences between the various samplings.

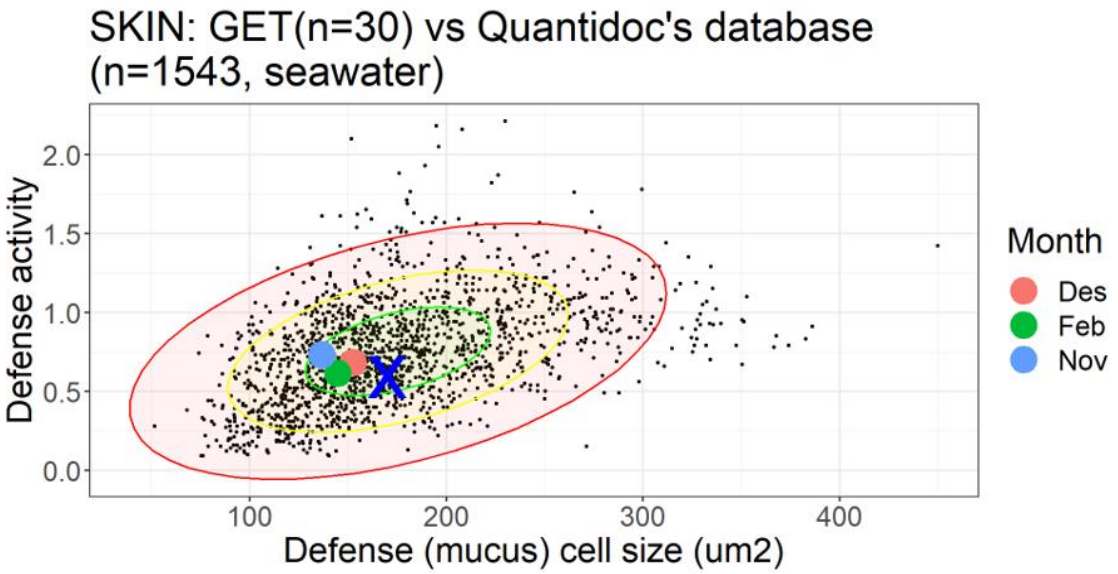

Figure 25: Comparison of mean mucosal parameters (defence activity and cell size) in the skin of Atlantic salmon farmed at the closed aquaculture system, Egget®, relative to Quantidoc's database for farmed and wild salmon from various aquaculture systems in seawater (n=1543). In the figure, the blue 'X' represents the mean values of wild salmon, while the black dots represent individual values from the database. The pink, green, and blue-coloured dots depict the mean values of samples collected in Egget® (N=30, n=10), collected during three different months in the 2022/2023 period. The green, yellow, and red circles represent the distance from the average standard.

3.4 Polymerase Chain Reactions, Agarose

Utilising the A8f and H1542r primers, sequencing efforts encompassed a substantial portion of the 16S rRNA gene, specifically focusing on a 1500 bp segment. Post-PCR reaction products were assessed through 1% agarose gel electrophoresis, confirming successful amplification and determining suitability for subsequent sequencing. In this study, 108 of 123 products exhibited distinct, well-defined bands at the anticipated fragment size (~1500bp) during electrophoresis, corroborating positive and negative control outcomes (Figure 26).

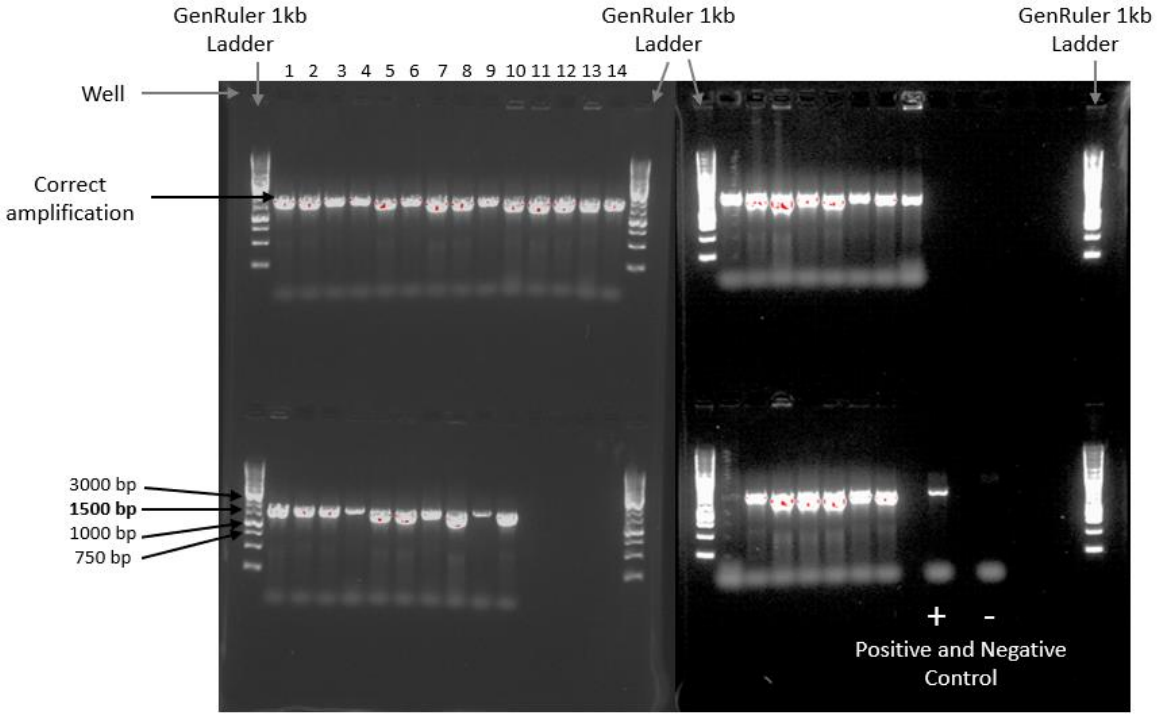

Figure 26: PCR products obtained from isolated colonies originating from Atlantic salmon gills and water samples collected at the inlet and outlet of the closed aquaculture system, Egget®. Electrophoresis was conducted on a 1% agarose gel containing GelRed in TAE buffer, with an applied voltage of 200V for 20–30 minutes.

3.4.1 Phylogenetic Tree

A phylogenetic tree was made from 82 fish- and 19 water samples (inlet and outlet water) from the 1st, 2nd and 3rd sampling with 25 reference bacteria (Figure 27). The phylogenetic tree showed a distribution of all the samples collected clustering together, with the most dominant genus being *Pseudoalteromonas sp.*, *Psychrobacter sp.*, *Colwellia sp., Flavobacterium sp.* and *Polaribacter sp*.

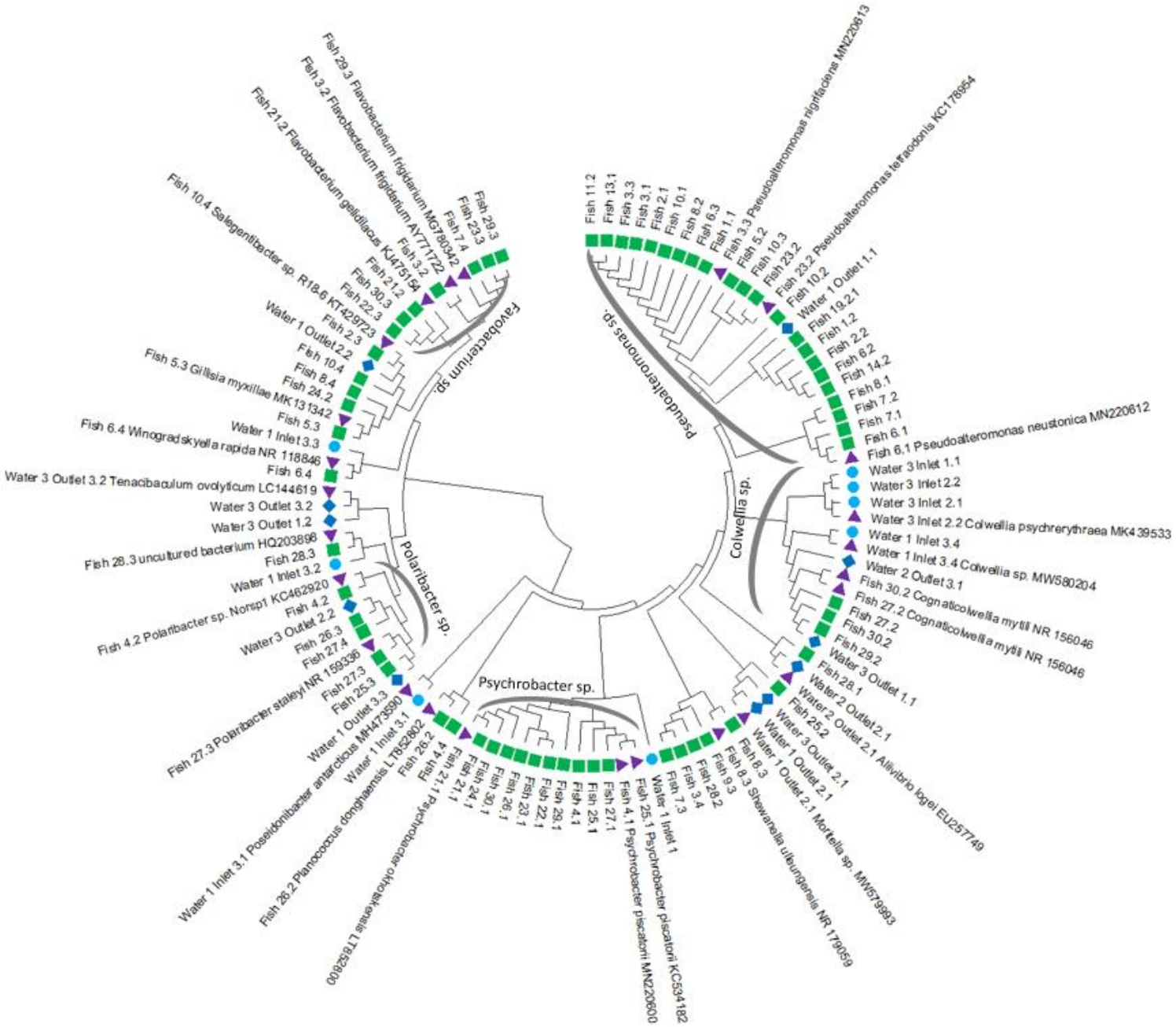

Figure 27: Phylogenetic tree constructed using the neighbour-joining method in MEGA11. Green squares represent bacterial sequences isolated from Atlantic salmon gills (N=30), while inlet water samples (N=8, n=3) are depicted as light blue circles, and outlet water samples (N=10, n=3) as dark blue diamonds. All samples were collected from the closed aquaculture system, Egget®. Purple triangles indicate reference bacteria showing the closest matches in the sequence database from the National Center for Biotechnology Information, BLAST function.

3.5 Bacteria Isolated from Gills

The bacterial sequences obtained from the gills of the salmon (N=30) are summarised in Table 4; a complete overview is found in Appendix F. Table 4 presents the similarity percentages and the distribution of the closest associated bacteria identified through BLAST analysis. It was observed that most bacterial strains exhibited a similarity level above 97% with previously sequenced bacteria.

Table 4: An overview of fish and colony numbers, along with the corresponding bacterial species and sequence similarity percentages, derived from the gills of Atlantic salmon (N=30, n=10) farmed at the closed aquaculture system, Egget®. Bacterial samples were obtained by swabbing the fish's gills and subsequently cultured on marine broth agar before undergoing PCR sequencing.

Fish and Colony number	**Bacterial Species**	**Sequence Similarity %**
28.1	*Alteromonas naphthalenivorans*	99.39
27.2, 29.2, 30.2	*Cognaticolwellia mytili*	≥97
27.4	*Flavobacteriaceae bacterium*	99.70
3.2, 7.4, 23.3, 29.3, 21.2, 22.3, 30.3	*Flavobacterium frigidarium/gelidilacus*	≥99
8.4, 5.3, 9.4, 24.2	*Gillisia mitskevichiae/myxillae*	≥97
25.2	*Moritella marina*	99.50
26.2, 4.4	*Planococcus donghaensis/halocryophilus*	≥99
22.2	*Planococcus kocurii*	95.17
25.3, 26.3, 4.2, 27.3	*Polaribacter sp./irgensii/staleyi*	≥98
19.2.1, 5.2, 10.3, 2.1, 5.1, 3.1, 6.1, 7.1, 1.1, 3.3, 6.3, 7.2, 8.1, 1.2, 9.2, 11.2, 13.1, 6.2, 8.2, 10.2, 10.1, 14.2, 2.2	*Pseudoalteromonas sp./arctica/atlantica/distincta/elyakovii/tetraodonis/neustonica/prydzensis/nigrifaciens/mariniglutinosa/translucida*	≥99
23.2	*Pseudoalteromonas tetraodonis*	92.39
4.1, 24.1, 26.1, 30.1, 29.1, 22.1, 23.1, 21.1, 25.1, 27.1	*Psychrobacter (sp.) piscatorii/proteolyticus/aquimaris/cryohalolentis/ glaciei/ glacincola/ nivimaris/ okhotskensis/ piscatorii*	≥99
2.3, 10.4	*Salegentibacter sp./ Mesonia sp.*	≥99
28.2, 3.4, 7.3, 9.3, 8.3	*Shewanella sp. frigidimarina/ livingstonensis/ vesiculosa/ ulleungensis*	≥99
28.3	*Uncultured bacterium*	99.19
6.4	*Winogradskyella rapida/pacifica/thalassocola*	97.88

3.6 AGD Effect on Gill Lamellae

The same veterinarian from Åkerblå examined the fish during the 2nd and 3rd sampling events after the AGD outbreak was detected. The results of the AGD scores plotted against the MCA, MCD, and DA showed a slight increase in MCA between the 2nd and 3rd samplings, accompanied by lower AGD scores (Figure 28). The same trend but more pronounced was observed in MCD and DA between the two last sampling months (Figures 29 and 30).

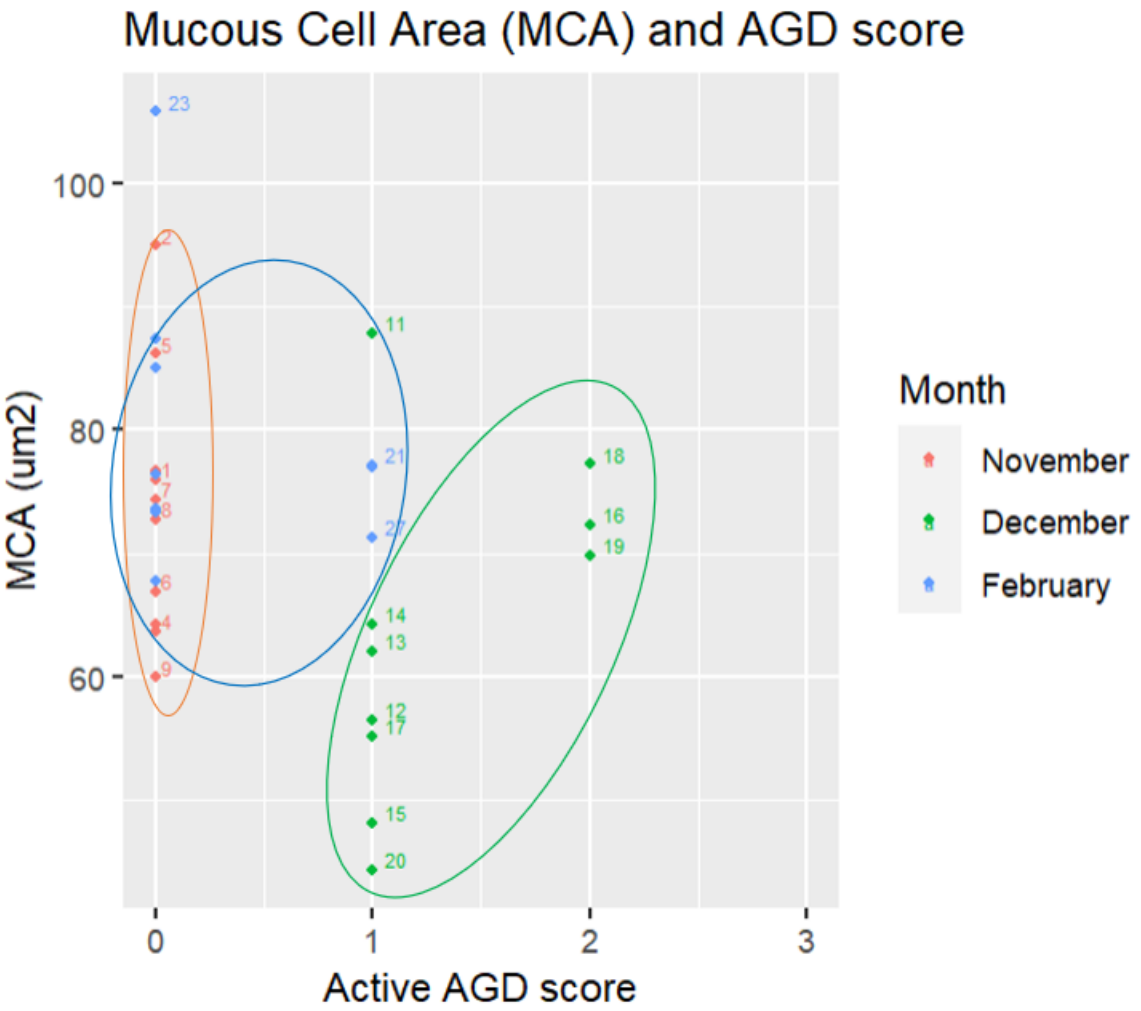

Figure 28: Mucous cell area (MCA) in square micrometres (µm²) in gill lamellae of Atlantic Salmon (N=30, n=10) and corresponding AGD score ranging from 0-5 in severity. Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. The plot was generated using RStudio.

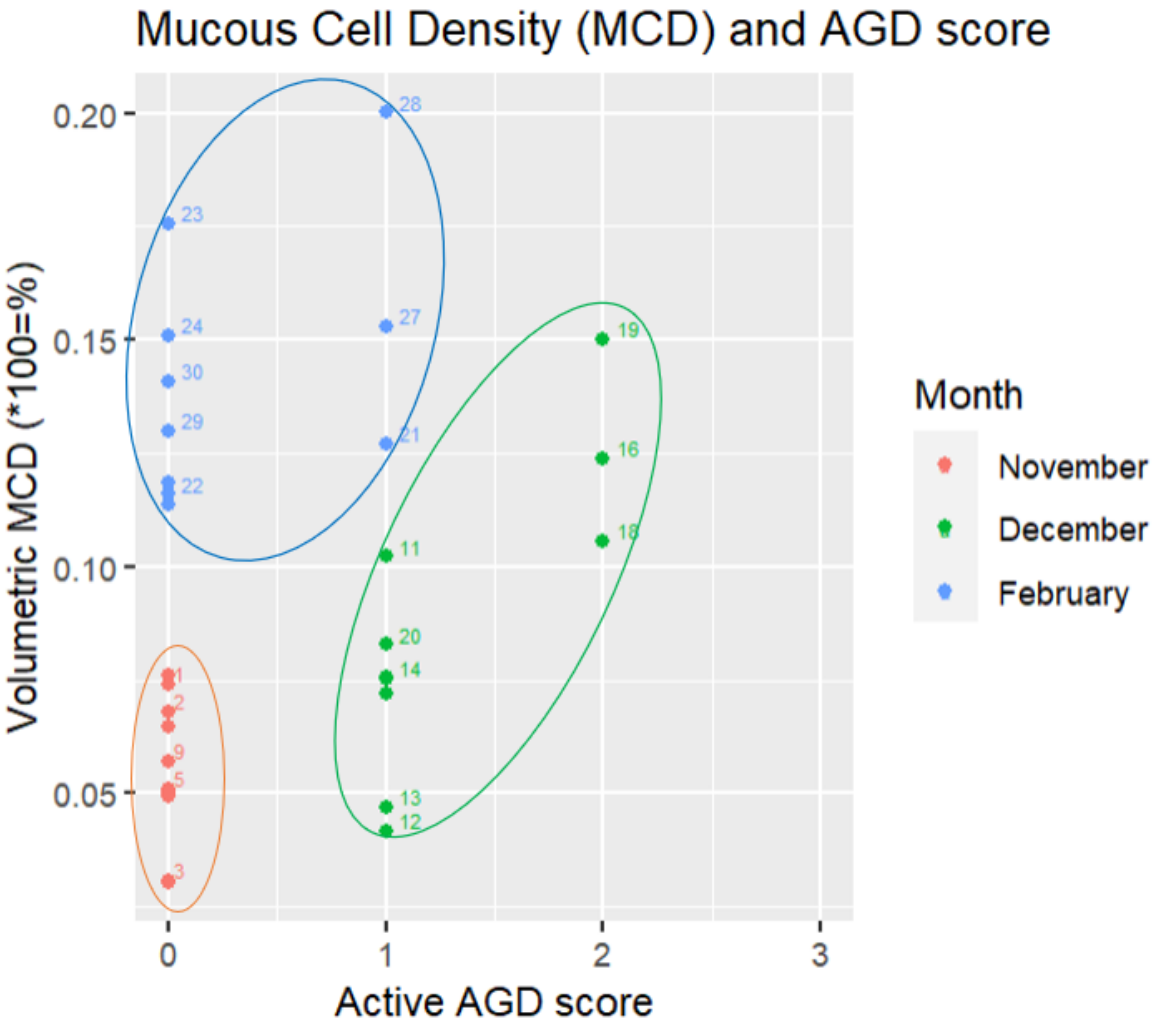

Figure 29: Mucous cell density (MCD) in gill lamellae of Atlantic Salmon (N=30, n=10) and corresponding AGD score ranging from 0-5 in severity. Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. The plot was generated using RStudio.

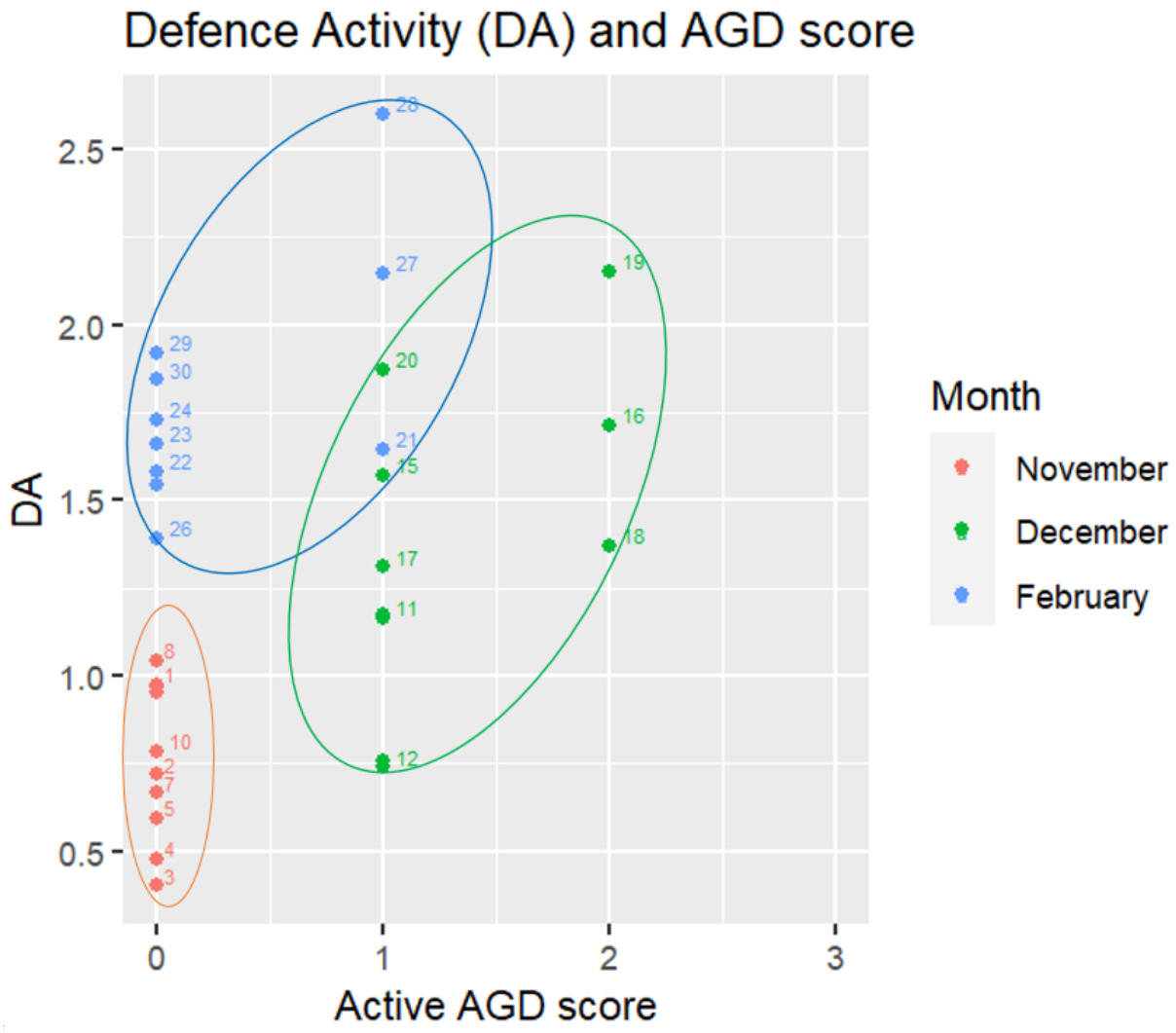

Figure 30: Defence activity (DA) in mucosal epithelium in gill lamellae of Atlantic Salmon (N=30, n=10) and corresponding AGD score ranging from 0-5 in severity. Data were collected during three different months in the 2022/2023 period at the closed aquaculture system, Egget®. The plot was generated using RStudio.

3.7 DNA Extraction from Water

Mean DNA concentrations from inlet- and outlet water from November were measured to 6.15 and 4.87 ng/sample. For December, inlet- and outlet mean concentrations were 1.94 and 3.79 ng/ sample. For February, the inlet- and outlet mean DNA concentrations were 3.47 and 4.15 ng/ sample. The discrepancies between the inlet and outlet water samples collected on the same day were smaller than those observed between different sampling days (Figure 31). An overview of mean concentration ± standard error (SE) is provided in Appendix G.

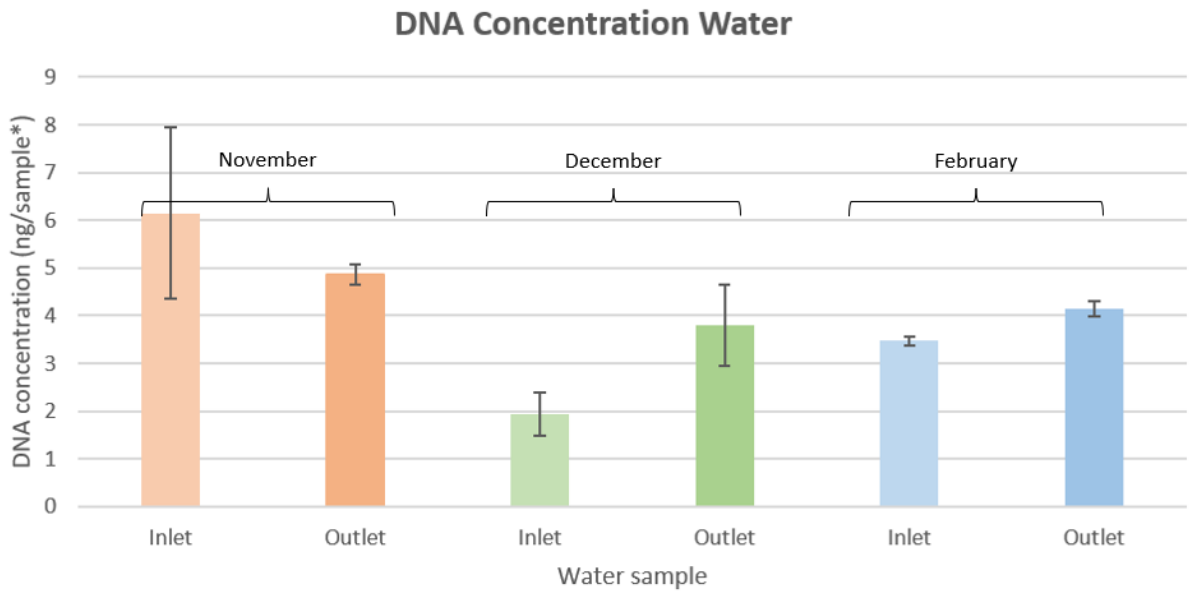

*Figure 31: Mean DNA concentration measured with Quant-iT™ DNA Assay Kit from inlet and outlet water (N=12, n=2), collected at the closed aquaculture system, Egget® at three sampling dates in 2022/2023. *Each sample contained filters after filtration of 2 litres of seawater through a pore size of 0.22 µm.*

3.8 DNA Extraction from Biofilm

The DNA concentrations in the biofilm obtained from the sampling locations, right (R), from the main entrance, exhibited values of 6.69, 2.74, and 0.06 µg/sample for November, December, and February, respectively. Similarly, the corresponding concentrations at the sampling location, opposite (O) from the main entrance, were 0.41, 10.73, and 43.50 µg/sample. For the third sampling location, and left (L), from the main entrance, the concentrations measured were 42.50, 1.53, and 12.31 µg/sample. The results indicated large variations in DNA concentration, and no discernible systematic differences were observed among the sampling days or locations (Figure 32). An overview of the DNA concentration is provided in Appendix H.

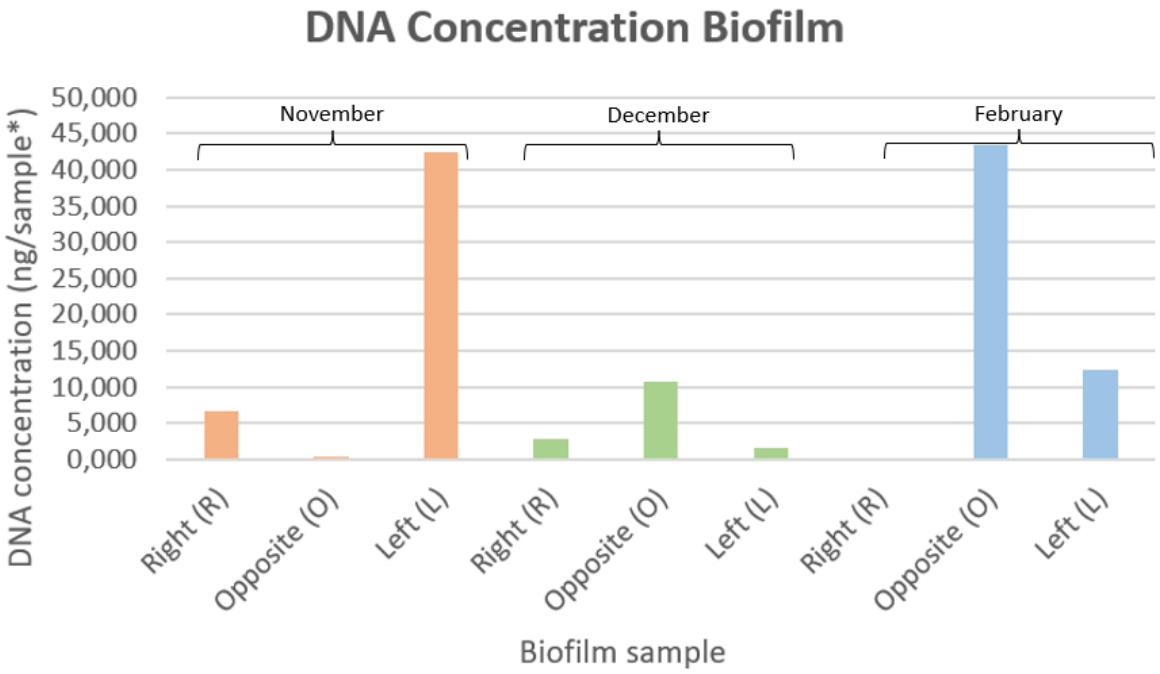

*Figure 32: DNA concentration measured with Quant-iT™ DNA Assay Kit from Biofilm (N=9, n=1), collected at the closed aquaculture system, Egget® at three sampling dates in 2022/2023. * Each sample contained biofilm from swabbing with a 4 cm sponge 3-4 times at the inside wall of Egget®.*

3.9 Bacterial Concentration in Water

The mean bacterial concentrations in the water samples collected during November were measured to be 5.55×10^5 cells/ml^{-1} for the inlet water and 5.88×10^5 cells/ml^{-1} for the outlet water. In December, the inlet water exhibited a mean concentration of 3.58×10^5 cells/ml^{-1}, while the outlet water showed a concentration of 3.65×10^5 cells/ml^{-1}. Furthermore, for February, the inlet water had a mean concentration of 3.03×10^5 cells/ml^{-1}, whereas the outlet water had a mean concentration of 2.92×10^5 cells/ml^{-1} (Figure 33). An overview of mean concentration ± standard error (SE) is provided in Appendix I.

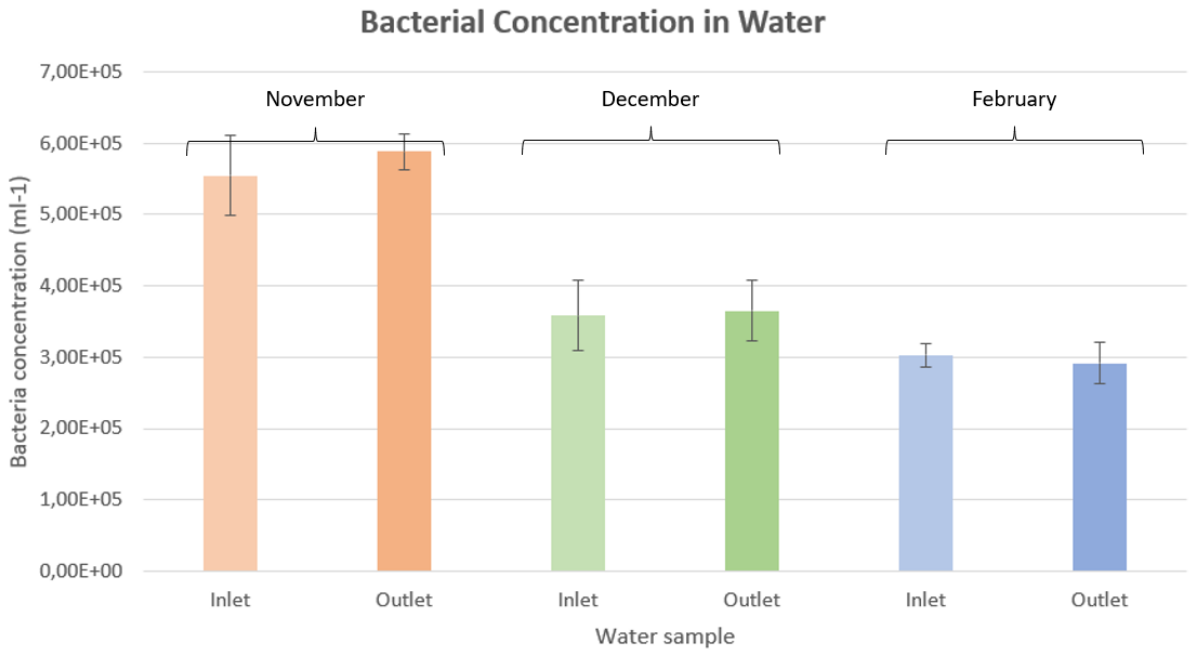

Figure 33: Mean bacteria concentration measured with flow cytometer from inlet and outlet water (N=18, n=3) collected at the closed aquaculture system, Egget®, at three sampling dates in 2022/2023.

3.10 Novogene Sequencing

3.10.1 Alpha Diversity Indices

The observed variations in alpha diversity indices, Shannon, Simpson, Chao1, and Observed_species are shown in Figure 34. The biofilm samples exhibit higher average indices in both Shannon and Simpson analyses than the water samples (inlet and outlet water samples). Specifically, the Shannon analysis reveals an index of approximately 7.3 for biofilm samples, while the water samples exhibit an index slightly lower than 6.0. In the Simpson analysis, the biofilm samples show indices around 0.98, whereas the water samples display an index of approximately 0.92. Regarding the Chao1 and Observed_species indices, the inlet, outlet, and biofilm samples demonstrate relatively similar distributions, with mean indices of approximately 1700 and 1550, respectively.

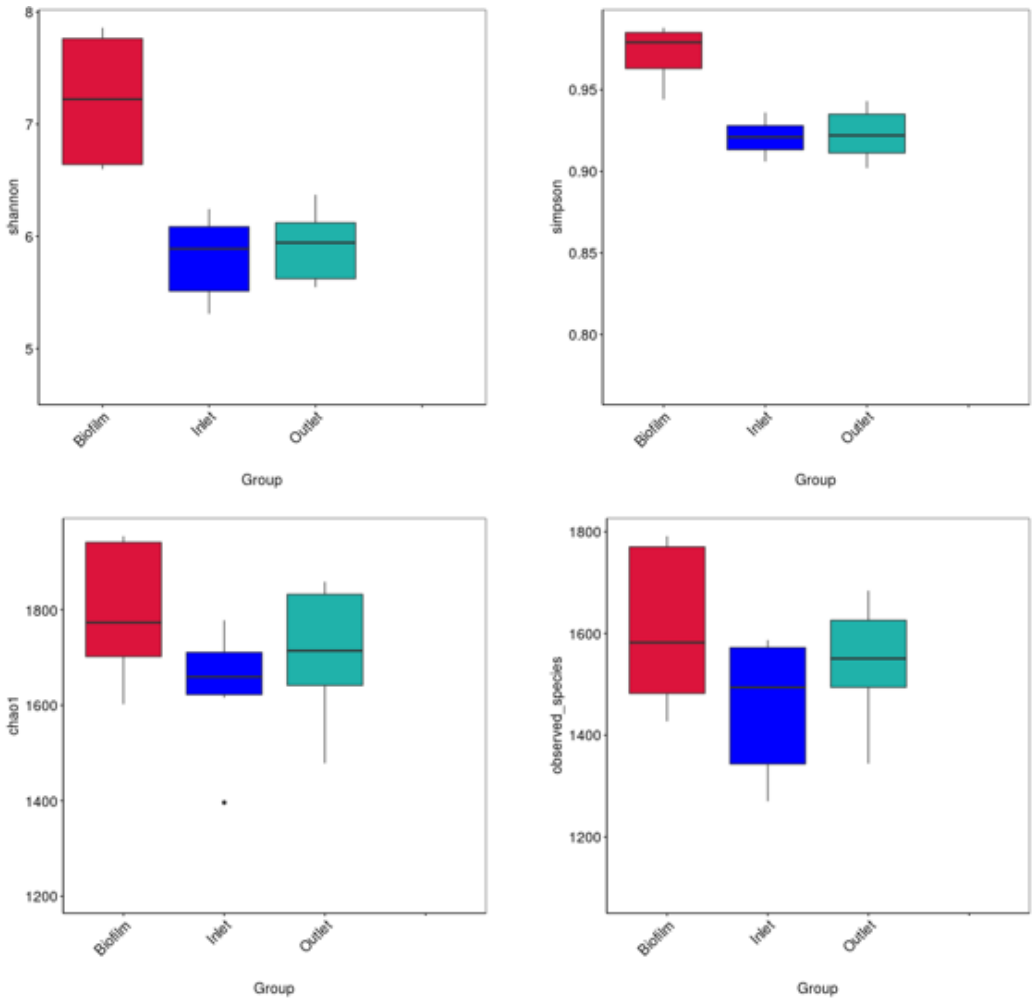

Figure 34: Box plot of four different alpha diversity indices, Shannon, Simpson, Chao1 and Observed_ species from microorganisms in the inlet (N=6), outlet (N=6), and biofilm (N=9) samples collected at the closed aquaculture system, Egget®.

3.10.2 Beta Diversity Indices

The Unweighted Pair-group Method with Arithmetic Mean (UPGMA) tree analysis revealed the clustering of samples into two distinct groups, as depicted in Figure 35. The inlet and outlet water samples formed a cluster, while the biofilm samples formed a separate and distinguishable cluster.

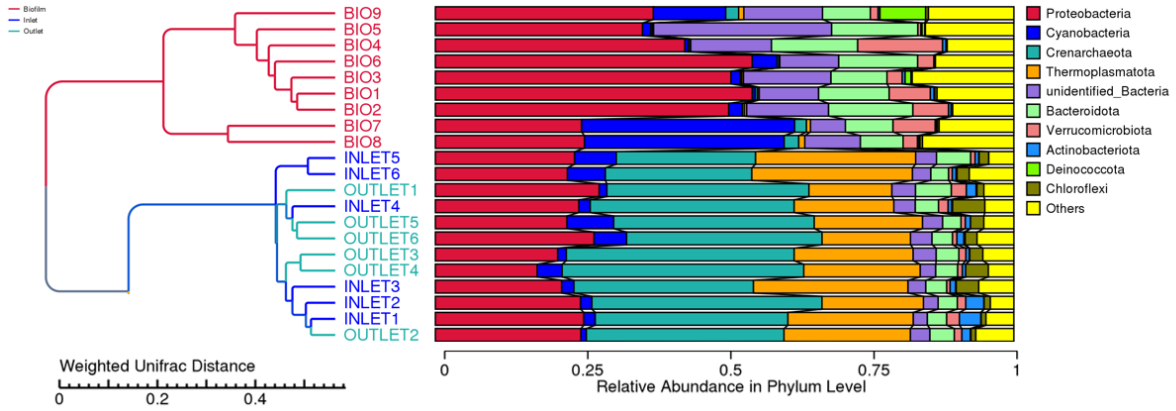

Figure 35: Unweighted Pair-group Method with Arithmetic Mean (UPGMA) tree plotted with relative abundance in phylum from microorganisms in the inlet (N=6), outlet (N=6) and biofilm (N=9) samples collected at the closed aquaculture system, Egget®.

3.10.3 Bacterial Community Composition

The top 10 taxa at the phylum, family and genus level, illustrating the microbial community at the taxa in the biofilm, inlet-, and outlet water samples. The biofilm samples labelled BIO1–9 are organised chronologically according to their sampling locations: right, opposite and left from the main entrance. BIO1–3 corresponds to the samples collected in November, BIO4–6 to December, and BIO7–9 to February.

A total of 35,927 operational taxonomic units (OTUs) were identified, primarily comprising bacterial taxa. These OTUs were classified into different phyla, dominated by Proteobacteria (red colour) (18–55%), Cyanobacteria (dark blue colour) (0.6–37%) and Bacteroidota (light green colour) (3–15%), Verrucomicrobiota (pink colour) (0.5–15%), which all were detected to varying degrees across all samples (Figure 35). Crenarchaeota (turquoise colour) (0.3–42%) and Thermoplasmatota (orange colour) (0.1–28 %) were also observed, with the most abundant in the water samples. Within these phyla at the family level (Figure 36), the presence of Alteromonadaceae (purple colour) (0.4–20%) and Rhodobacteraceae (light green colour) (2–17%) were detected in all samples. Nitroscopumilaceae (dark blue) (0.3–42%) was primarily detected in the water samples but to some degree in some biofilm samples. In water samples, SAR86_clade (pink colour) (1–3%) and Clade_I (light blue) (1.5–4%) were also observed. At the genus level (Appendix J), Glaciecola (orange colour) (0.3–16%) was observed in all samples in different abundance, while Candidatus Nitrosopumilus (dark blue colour) (0.2–41%) was most abundant in water samples.

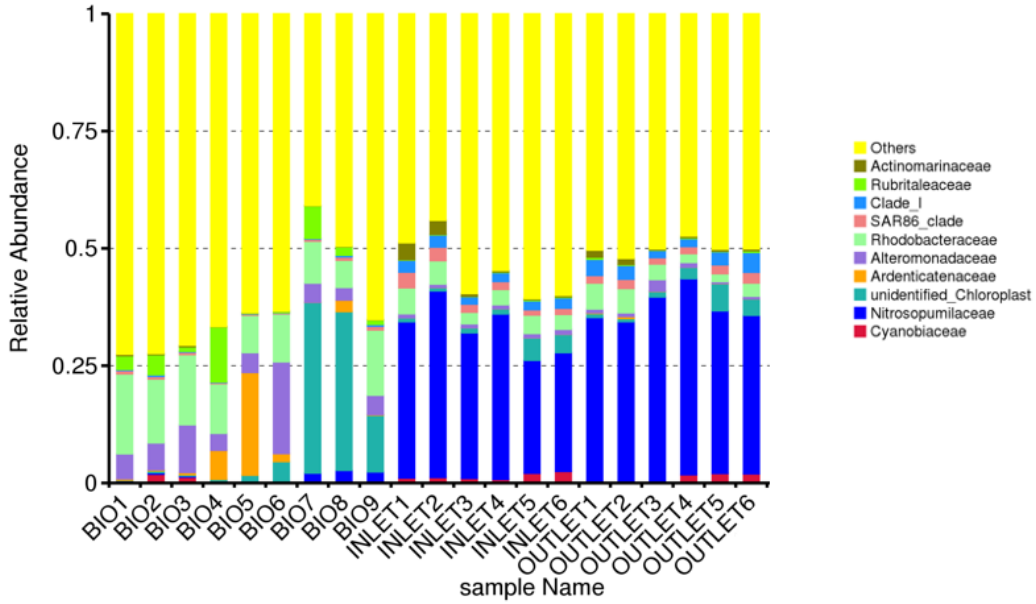

Figure 36: Bar plot at family level of microorganisms in the inlet (N=6), outlet (N=6) and biofilm (N=9) samples collected at the closed aquaculture system, Egget®.

3.10.4 Heatmap

The heatmap revealed clustering patterns at the genus level, revealing the top 35 genera and their corresponding phylum in the water and biofilm samples (Figure 37). The biofilm samples exhibited a greater diversity of genera, with higher abundances indicated by red-coloured squares on the heatmap. The genera Emticicia, Rubidimonas and Truepera were found to dominate the biofilm samples. Additionally, the biofilm samples showed high diversity within the Proteobacteria phylum, as evidenced by several light red squares in the heatmap. The water samples displayed lower abundances than the biofilm samples. The genera Pseudohongiella, SUP05_cluster, Amylibacter and Candidatus_Nitrosopumilus were found to have the highest abundances in the water samples.

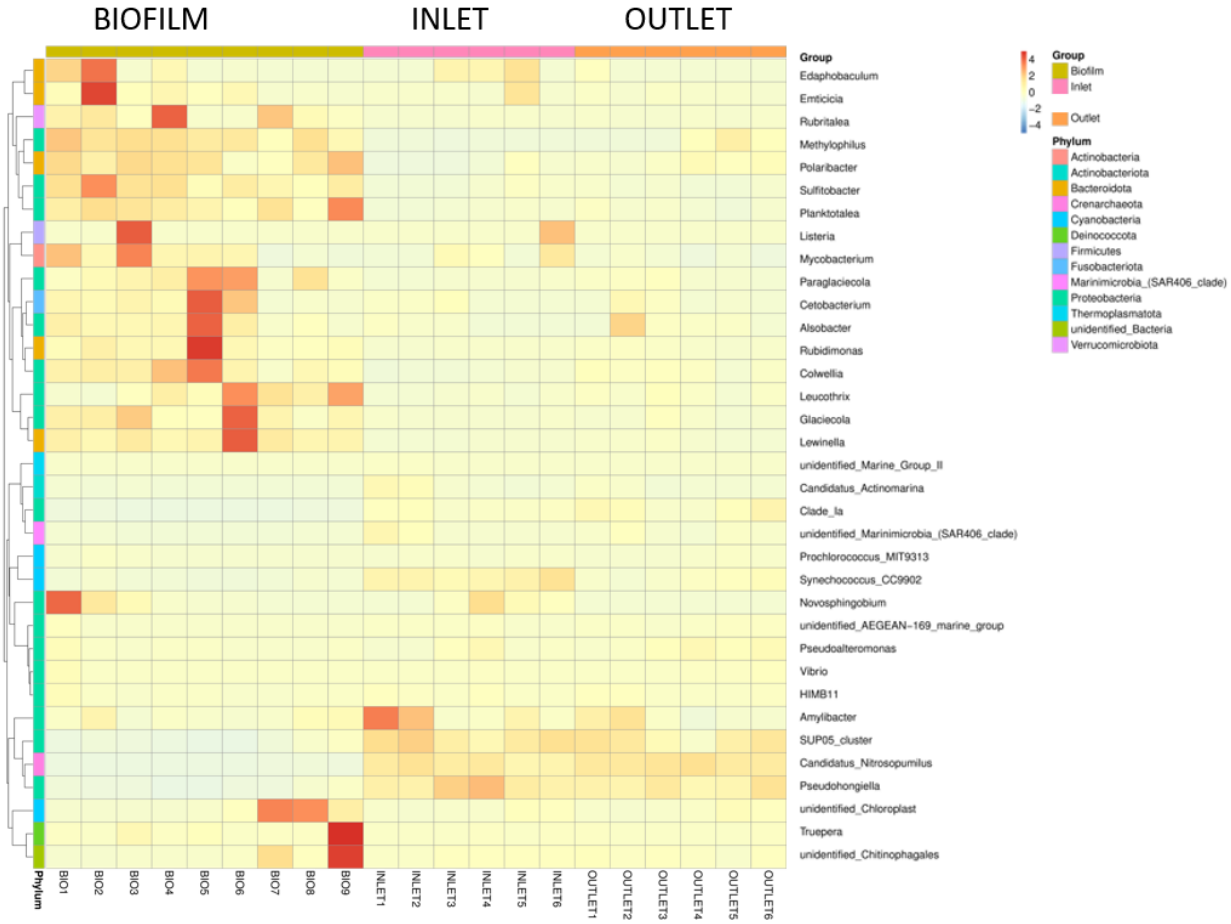

Figure 37: Heatmap of taxonomic genus abundance from the inlet (N=6), outlet (N=6) and biofilm (N=9) samples collected at the closed aquaculture, Egget®.

3.10.5 Venn diagram

The Venn diagram visually represented the shared and distinct information derived from the feature sequences of the various samples, inlet, outlet, and biofilm (Figure 38). Specifically, it revealed that among these sequences, 1981 was found to be shared across all samples. In addition, there were 611 unique sequences exclusively identified in the inlet, 758 unique sequences exclusive to the outlet, and a higher number with 1646 unique sequences in the biofilm.

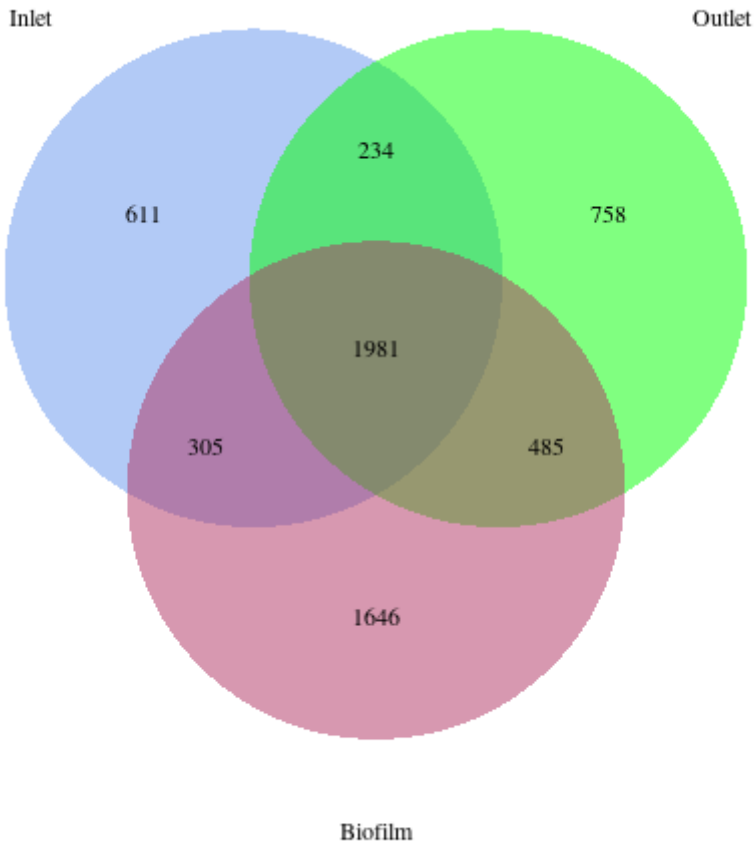

Figure 38: Venn diagram with shared and distinct sequences in the inlet (n=6), outlet (n=6), and biofilm (n=9) samples collected at the closed aquaculture system, Egget®.

4. Discussion

4.1 Evaluation of Methods

4.1.1 Fish Sampling Evaluation

The fish sampling method may have been affected by various factors, such as the fish's attraction to feed pellets and potential fluctuations in their weight measurements. Despite being randomly sampled, fish attracted to the feed were more likely to be collected. The first sampling experienced weight issues, likely due to water interference, necessitating a different weight in subsequent samplings. This is not considered to have influenced the results to a large extent. The fish weight was estimated by the camera in the cage and measured from the sampling (Table 5). The data supported the assumption of measurement errors in the initial sampling, but this relatively minor error was not further considered in subsequent samples. Additionally, sampling time was a crucial aspect of the study, as more prolonged anaesthetic exposure and euthanasian time cause degenerative tissue, particularly the gill tissue, and could cause mucous cell swelling, affecting the results (Furnesvik et al., 2022; Munday & Jaisankar, 1998). The samplings were coordinated with other sampling needs to minimise the number of fish euthanised. This resulted in a longer anaesthetic exposure time than preferred for some fish. Although increasing the number of fish could have given more credibility to the data, 30 fish was considered enough in this study, and it was not desirable to euthanise more individuals than necessary. A method with randomised frames was employed to avoid human errors in selecting parts of the tissue for analysis. Two trials of the histological analysis were conducted, one as practice with guidelines from Grigory Merkin from Quantidoc and one valid analysis to ensure a precise, representative, and objective evaluation of the tissue.

Certain requirements must be met to conduct statistical analyses on the obtained observations. In this study, the observations are considered independent despite the fish being housed together in a closed environment. Although removing some fish may have slightly impacted the remaining individuals and resulted in a slight reduction in density in the system, this is considered negligible in this study. The normality assumption of the observations was assessed using a QQ plot (Quantile-Quantile plot) generated in R (Appendix K). The QQ plot indicated a moderate deviation from a perfectly normal distribution. Additionally, the equality of variances among groups was assessed using Levene's test for homogeneity of variances (Appendix L). This test examined whether the variance within each group was approximately equal. The parametric test, ANOVA (Analysis of Variance) and Tukey's HSD (honest significant difference) post hoc-analysis were employed in this study on gill lamellae mucous cell area (MCA), mucous cell density (MCD) and defence activity (DA) over time (Appendix M). ANOVA is known to tolerate moderate deviations from a normal distribution and is more robust to violations of

homogeneity of variances compared to other tests, such as the t-test. However, it is essential to acknowledge the limitations of the study. The sample size in each group was limited to ten individuals, which may impact the statistical power and generalizability of the findings. Additionally, since the samples were collected within a single system without a control group, there is a possibility of confounding effects, potentially influencing the observed outcomes.

Table 5: An overview of the average weight measured for Atlantic salmon in the closed aquaculture system, Egget®. The data was obtained from individual fish through sampling and estimated weight by a camera integrated into the system.

Average weight (g) camera	Average weight (g) sampling
342	235
640	602
1002	1157

4.1.2 Microbial Growth and Detection Method

Bacterial growth limitations arise due to the specific suitability of marine broth agar (MBA) as a growth medium, as not all microorganisms thrive under identical conditions (Juni et al., 1986). This could potentially lead to an incomplete representation of the microbial community. The work adhered to aseptic conditions to reduce the chance of contamination by other microorganisms, which might compromise the overall representation of microbial diversity. Polymerase chain reaction (PCR) was employed for microbial identification, a robust method for identifying specific bacteria. However, it is important to acknowledge that errors introduced during the initial PCR cycles can have significant consequences. Nevertheless, PCR is recognised for delivering relatively precise results (Polz & Cavanaugh, 1998). To mitigate contamination during PCR, rigorous adherence to laboratory protocols, employment of sterile techniques, and utilisation of positive and negative controls for quality assurance were implemented. Sequence trimming parameters were determined based on sequence quality and subjective evaluation, which could benefit from more experience and training.

4.1.3 Sources of Error in Measuring DNA and Bacterial Concentration

Despite following standardised protocols for sample collection, deviation and errors could have occurred during the process. Two litres of water were collected from the CCS, Egget®, to represent the

surroundings. This may appear insignificant compared to the quick water turnover in the system. However, this method is intended to provide information regarding the entire microbial community.

Biofilm within the system was sampled following the same protocol each day and as the last sampling each time. Factors such as the manually driven ROV-brusher could affect how the sampling spot was "cleaned" on that specific sampling day. How much fouling was transferred to the Eppendorf tube is somewhat unclear and could be considered another source of error. Factors such as incomplete lysis of microorganisms and variations in elution efficiency can influence the amount of DNA yield. Acknowledging that slight variations in laboratory techniques, equipment, and experience could have contributed to overall result variability is essential.

Flow cytometry (FCM) assesses microbial cells based on their light scattering and fluorescence properties. Keeping FCM samples at low temperatures is essential to slow down degradation, maintain the stability of fluorescent markers, and ensure precise measurements. Differences in the schedules for sampling days affected how quickly the samples were stored in the -80°C freezer, potentially influencing the results.

4.2 Discussion of Results

This master's thesis aims to present the pioneering investigation of the effects on young Atlantic salmon within the novel floating closed containment system (CCS), Egget®. The findings from this comprehensive investigation collectively attest to the robust health of the fish reared within Egget®. The fish exhibited a low mortality rate and demonstrated favourable growth throughout their five months in Egget®. Notably, this study documents the increase in mucous cells in the gill lamellae, probably as a response to an outbreak of amoebic gill disease (AGD). A consistent and unaltered state of skin mucous was observed, correlating with the absence of treatments and handling procedures. The bacteria extracted from the fish gills and the inlet and outlet water displayed a composition in line with established aquaculture bacterial communities, indicative of a healthy microbial balance within the system. Furthermore, the DNA and bacterial concentration in the water and biofilm did not reveal any indications of microbial accumulation within the system. Additionally, samples collected from the biofilm and inlet/outlet water had similarities yet exhibited distinct categorisations into two primary groups: biofilm and water samples. These groups displayed noticeable differences, which can be attributed to biofilm offering a distinct habitat characterised by unique chemical and physical conditions that attract and facilitate the accumulation of microorganisms better than water. However,

minimal variation was observed within water and biofilm samples during the sampling period. This suggests stability in the microbial communities within Egget®, which the healthy fish also indicated.

4.2.1 Fish Samples

This study aimed to document the mortality rates, as the most commonly used method for measuring welfare, for fish reared in Egget® compared to traditional farming of salmon in Norway. The mortality rate of fish during production in Egget® was observed to be extremely low, recorded at only 1.2% (Table 3). While not a direct comparison, in order to gain an understanding of losses in the industry, the median mortality rate for the entire sea phase of a production cycle spanning 12 months or more is 16.6% and 17.7% (Sommerset et al., 2023) (Table 6) in the specific production zone were Egget® is sited, extending from Stad to Hustadvika. The zone is relevant as it reflects the conditions and challenges where Egget® is positioned. However, it is essential to note that the fish were only held in Egget® for approximately five months and not throughout the entire sea phase as in regular production. This difference could have led to an increased total mortality rate for the sea phase, only visible later. More comparable are Egget®'s genetically similar sibling fish at the location Urdaneset. They were farmed in an open net pen in the same production zone and transferred to sea three weeks before the fish in Egget®, and they experienced a mortality rate of 20.4% (Table 6). However, the mortality observed in Egget® is in line with another CCS called FishGLOBE, which registered a mortality of 1.4% after 11 weeks of production (Espmark et al., 2020) (Table 6). This indicates that Egget® and closed systems can provide a more protected environment where notably more fish survive the first part of the sea phase.

In FishGLOBE, a particularly higher number of cases of external injuries were recorded (Espmark et al., 2020) compared to those in Egget®. In Egget®, there were 0 wounds and very few cases of external injuries registered. However, analysis of the dead fish revealed that 80% of the 1.2% dead fish died of cerebral haemorrhage, presumably from jumping into the wall/eggshell. The differences in the degree of external injuries observed between the two CCSs could be explained by several factors, including the potential influence of great genetics, feed quality, or water conditions (Espmark et al., 2020; Sambraus et al., 2017). Alternatively, it may be attributed to the design or routines for fish rearing in the systems. Egget® consists of a smooth surface with few edges that undergoes daily brushing, unlike FishGLOBE. This may contribute to the reduced risk of external injuries and the accumulation of pathogenic organisms that pose a threat to the fish. Considering that a significant portion of the fatalities in Egget® were due to internal bleeding, there is room for improvement in the design of Egget®, although there was minimal external damage.

Table 6: Overview of various salmon farming systems and essential parameters, including mortality rates, production times, delousing treatments, and size range during the seawater phase. Urdaneset is the farming location of Egge's genetically sibling fish in standard open net pens.

System	Type of system	Mortality %	Production time (weeks)	Delousing	Fish size (g)	Reference
Egget® [1]	CCS	1.2	21	0	≈150–1100	Ovum AS
FishGLOBE®	CCS	1.4	11	-	≈250–750	(Lazado et al., 2022a)
Urdaneset, Egget®'s genetically sibling fish	Standard open net pen	20.4	24	3	≈100–900	Urdaneset/Ovum AS
Average Norwegian Salmon Industry 2022	Standard open net pen	16.6[2]	≥52[3]	3	≈150–4000/6000	(Sommerset et al., 2023; Thorland et al., 2020)
Average production zone 5	Standard open net pen	17.5	≥52	-	-	(Sommerset et al., 2023)

The study aimed to investigate the infestation of sea lice on salmon, compared to traditional salmon farming. Remarkably, both CCSs, FishGLOBE and Egget®, exhibited minimal sea lice infestations, primarily due to a controlled water supply from beneath the lice barrier in the water column. Furthermore, it is worth noting that there was no reproduction of louse in Egget®, and delousing or other treatments were unnecessary. At the same time, its genetic sibling fish in open net pens underwent three separate delousing treatments concurrently (information given by the farm at Urdaneset to Ovum AS) (Table 6). Given that delousing procedures are stressful for the fish (Øvrebø et al., 2022), the absence of these treatments in Egget® may substantially contribute to the observed robustness of the fish and the low mortality in Egget®.

Robust fish health can be reflected in the fish's growth, and this study aimed to document the growth of fish cultured within Egget® compared to conventional salmon farming practices. Within the scope of this thesis, the fish sampled from Egget® exhibited a notably high specific growth rate (SGR), factoring in temperature and size considerations, registering at 1.6% over the sampling period of 14

[1] The entire production cycle in Egget® from October 9th, 2022, to February 22nd, 2023.
[2] The median mortality rate per completed production cycle in the sea phase in 2022 (Sommerset et al., 2022).
[3] One production cycle, 52 weeks/12 months or longer.

weeks (Table 7). This growth performance exceeds the 1.2% SGR observed in genetically similar sibling fish over 24 weeks and the results reported by FishGLOBE, which documented a SGR of 1.3% over 11 weeks (Espmark et al., 2020) (Table 7). The condition factor (CF) ranged from 0.3 to 1.3 in Egget®, correlating with CF in FishGLOBE at 1 to 1.3. This growth can be considered healthy, even for farmed salmon from selected genetic lines, which exhibit faster growth rates and higher CF values than wild and hybrid salmon lines (Glover et al., 2009; Thodesen et al., 1999). CF values are positively correlated with fat content, and values exceeding 0.9 are typically desirable, making the observations in both CCSs highly favourable (Noble et al., 2018). It should be noted that several factors influence growth, including genetics, diet, and the health of the fish (Aas et al., 2019), this may contribute or be part of the explanation for the observed results. While low mortality and fast growth provide short-term benefits, it is crucial to consider the long-term consequences, such as their impact on development and the risk of premature maturation, especially when transferring fish from Egget® to open net pens. Aquaculture aims to sustain low mortality rates, vigorous growth, and good welfare until harvest, often at 4–6 kg, and not only the first sea phase.

Results from Egget® revealed a trend wherein the SGR increases from the first to the second sampling, followed by a decrease during the third sampling. The CF in December and February were clustered around 1.1 and 1.2 and showed higher values than in November, where more variation in CF was observed. It appeared that the CF reached a gradual stabilisation or flattening by the third sampling. One natural explanation for the observed trend is that SGR typically decreases when body size increases (Austreng et al., 1987). Another parameter that could explain this is the environmental conditions, particularly temperature. Fish thrive and grow faster within their ideal temperature range; for Atlantic salmon, 8–14°C, they can experience reduced growth rate and welfare when temperatures deviate from this range (Falconer et al., 2020; Johansson et al., 2009). Decreasing temperatures correlate with reduced growth rates from 16°C and below (Austreng et al., 1987; Nordgarden et al., 2003; Thorarensen & Farrell, 2011). The sea temperature declined from approximately 11 to 6°C throughout the sampling period from November to February. The present study aligns with prior research (Austreng et al., 1987; Falconer et al., 2020), indicating that the decline in SGR between December and February, along with the stabilisation of CF within Egget®, is likely influenced by increased fish body size and decreased temperature.

Table 7: Overview of various salmon farming systems and their differences in production times, specific growth rate % (SGR), Condition factor (CF), fish size and sea temperature during the sea phase. Urdaneset is the location farming Egge's genetically sibling fish in standard open pens.

System	Type of system	Production time (weeks)	SGR (%)	CF	Fish size (g)	Sea temperature (°C)	Reference
Egget® [4]	CCS	21	1.4	-	≈150–1100	13–6	Ovum AS
This study in Egget®[5]	CCS	14	1.6	0.3–1.3	≈200–1100	11–6	
FishGLOBE®	CCS	11	1.3	1–1.3	≈250–750	8.5[6]	(Espmark et al., 2020)
Urdaneset, Egget®'s genetically sibling fish	Standard open net pen	24	1.2	-	≈100–900	13–6	Urdaneset/Ovum AS
Average Norwegian salmon industry	Standard open net pen	≥52[7]	1.3 1 0.5	-	150–600 150–600 600–2000	11 8 6	(Sommerset et al., 2023) (Austreng et al., 1987)

4.2.2 Mucous Dynamics in Gills and Skin

The study aimed to investigate the potential mucous cell dynamics in the gill lamellae and the skin of the salmon as a welfare indicator. Mucins are the main product of mucus cells and are produced in the cytosol. Therefore, the cell size (amount of cytosol) determines the amount and type of proteins produced by the cell and the surface area for trans-membrane mucins, which signal to the outside environment (Fast et al., 2002). Small gill mucous cells can have an average size of 20 μm^2, whereas larger skin mucous cells can be 300 μm^2, which gives 15 times more surface area and 58 times more volume and cytosol (Table 8). These two tissues have, therefore, varying capacities for producing antimicrobial proteins, surface receptor exposure, and both external and internal communication. This

[4] The entire production cycle in Egget® from October 9th, 2022, to February 22nd, 2023.
[5] The period of this study, November 10th, 2022, to February 16th, 2023.
[6] Only measured at first sampling in FishGLOBE, January 9th, 2020.
[7] One production cycle, 52 weeks/12 months or longer.

suggests the potential for distinct mechanisms in each tissue. Only reporting the number of mucous cells can obscure this fundamental aspect of tissue activity.

Table 8: Mathematical overview of the effect of cell size on volume (cytosol) and surface area (Pittman, 2021, with permission).

	AREA (μ^2)	RADIUS µ	Surface area sphere (μ^2)	Volume sphere (μ^3)	NUMBER OF CELLS	Surface area in unit (µ2)	Volume in unit (µ3)
	20	2.52313252	80	67,2835339	5	400	336,41767
			80	67,2835339	10	800	672,83534
Gills	50	3,9894228	200	265,96152	5	1000	1329,8076
			200	265,96152	10	2000	2659,6152
	100	5,64189584	400	752,252778	5	2000	3761,2639
			400	752,252778	10	4000	7522,5278
	150	6,90988299	600	1381,9766	5	3000	6909,883
Guts			600	1381,9766	10	6000	13819,766
	200	7,97884561	800	2127,69216	5	4000	10638,461
			800	2127,69216	10	8000	21276,922
Skin	250	8,92062058	1000	2973,54019	5	5000	14867,701
			1000	2973,54019	10	10000	29735,402
	300	9,77205024	1200	3908,8201	5	6000	19544,101
			1200	3908,8201	10	12000	39088,201

The gill lamellae mucosa displayed significant differences in the mean mucous cell area (MCA), mean mucous cell density (MCD) and defence activity (DA) over time in Egget®. Before the second sampling, an outbreak of amoebic gill disease (AGD) was detected in fish in Egget® with an average AGD score of 1.6 on November 29th (n=10). The mean MCA in gill lamellae decreased from November (74 µm^2) to December (64 µm^2) but increased significantly from December to February (79 µm^2). Mean MCD and DA gradually and significantly increased during all three months, from 6–14% and 0.8–1.8. The mucous cells in the lamellae became smaller in size and increased in density from 1st to 2nd sampling. The mean mucous cells grew in size from December to February, and more cells were produced. This dynamic indicates an active and dynamic mucous layer reacting to something in the environment. The presence of *Paramoeba peruans,* the parasitic amoeba causing AGD, in Egget® was not unexpected as these amoebas are naturally present in seawater. Due to concerns related to AGD in CCSs and the outbreak of AGD during sampling, AGD scores were recorded for each fish. At the 1st sampling in mid-November, the average score was 0, but it increased to 1.3 in December before decreasing to 0.3 in February (Table 9). Minimal outbreak progression was registered, the fish effectively combated the pathogen, and the AGD score declined without human intervention. As the amoeba targets the fish's gills, the AGD score registered may be reflected in the mucous mapping pattern in the gill lamellae as it reacts to environmental challenges.

Table 9: Overview of average amoebic gill disease (AGD) score from 0–5 and mucous cell area (MCA), mucous cell density (MCD) and defence activity (DA) in the gill lamellae of fish in Egget® (n=10).

Date 2022/2023	Average AGD gill score (n=10)	MCA (µm²)	MCD (%)	DA
November 10th	0	74	6	0.8
November 29th	*1.6*	-	-	-
December 12th	1.3	64	9	1.4
February 16th	0.3	79	14	1.8

In aquaculture, treatments like hydrogen peroxide (H_2O_2) can be used to combat skin parasites like sea lice, but it can have unintended consequences on fish gills. These consequences include lifting, hyperplasia (increased cell production), fusion of lamellae, and necrosis (tissue damage) (Tort et al., 2002). When addressing AGD, peracetic acid (PAA) is one recommended treatment. Nevertheless, the PAA may also lead to unintended outcomes, such as developing lesions on the lamellae and lamellae fusion (Lazado et al., 2022b). Both treatments, H_2O_2 and PAA, have been linked to elevated mortality rates (Lazado et al., 2022b; Tort et al., 2002). For instance, gill lifting results in fluid accumulation within the gill epithelium, while necrosis renders the gills more vulnerable to infections from surrounding microorganisms. These effects do not only compromise the respiratory functions of the fish's gill but also the overall health of the fish and their welfare. Physical abrasions and stressors can compromise the natural defence mechanisms of fish under intensive aquaculture conditions. This affects the mucus and epidermal barriers of the fish, making it easier for pathogens to cause infections (Ellis, 2001; Fæste et al., 2020; Raj et al., 2011). This reduced health status of the fish requires activation of the immune system and energy to repair and reconstruct the gill barrier. Additionally, stress reduces welfare and can further weaken the fish and the tissue's ability to protect against diseases. However, in Egget®, no delousing, AGD treatment or other handling procedures stressing the fish were carried out due to operational decisions and the low extent of the AGD outbreak.

Tissue exposed to unfavourable conditions has exhibited elevated hyperplasia in the lamellae and an increase in the number of mucous cells, as indicated by several prior studies (Dang et al., 2019; Gjessing et al., 2019; Khan & Kiceniuk, 1984; Vatsos et al., 2010). Furthermore, previous research has observed an increase in the volumetric mucous density of salmon a few days after exposure to PAA or H_2O_2 (Haddeland et al., 2021; Rantty, 2016) (Table 10). It is worth noting that these studies generally reported smaller mucous cell sizes compared to those observed in this investigation. The extended perimortem period in this study likely caused gill swelling, explaining this discrepancy. Beyond the differences in mucous cell sizes, there are similarities in the observed trends. In the study by Haddeland et al. (2021), repeated exposure of PAA on gill lamellae at doses of 1.2 and 0.6 ppm resulted in increased MCA both two hours and two days after exposure. Similarly, in Rantty's study in 2016, an increase in MCD was observed after a delousing treatment with H_2O_2, persisting until 18 days after

exposure. The same pattern is registered in this study. An increase in both MCA and MCD persisted despite the decline in the AGD score, leaving the state higher than before the AGD outbreak (Table 9).

Table 10: Overview of changes in mucous dynamic, mucous cell area (MCA) and mucous cell density (MCD) in gills and skin in different studies.

Tissue sampled	Species	Challenge	Mucous reaction	Study
Gills	Atlantic salmon	Amoebic gill disease (AGD)	Firstly, decreased mucous cell area (MCA), followed by increased MCA. Increase mucous cell density (MCD)	Egget® (Current study)
Gills	Atlantic salmon	Peracetic Acid (PAA)	Non-naïve mucous cells exposed 2nd time for PAA, 0.6 and 1.2 ppm PAA significantly increased in MCA in reference to the control group	(Haddeland et al., 2021)
Gills	Atlantic salmon	Delousing with hydrogen peroxide (H_2O_2)	Increased mucous cell density until returning to baseline	(Rantty, 2016)
Gills	Atlantic salmon	AGD	Increase in MCA and MCD 24 hours and four weeks after exposure	(Lazado et al., 2022b)
Gills	Atlantic salmon	PAA treated fish with AGD	Increase in MCA and MCD 4 weeks after exposure	(Lazado et al., 2022b)
Gills	Atlantic salmon	Heavy metal exposure	Gill lamella MCD followed a toxicity gradient, highest at the most contaminated station and lowest in the least contaminated area	(Dang et al., 2019)
Skin	Atlantic salmon	AGD	Unchanged	Egget® (Current study)
Skin	Atlantic salmon	Delousing with H_2O_2	Increased MCD and slightly decreased MCA after exposure	(Rantty, 2016)
Skin	Atlantic salmon	Skin parasite	Smaller mucous cells in the skin in areas with the highest skin parasitic pressure	(Dang et al., 2019)
Skin	Sea bass	Unfavourable conditions of oxygen	Increased mucous cells per skin area	(Vatsos et al., 2010)
Skin	Sea bass	Unfavourable conditions of nitrate	Increased mucous cells per skin area	(Vatsos et al., 2010)
Skin	Sea bass	Supplementation in dietary oil source	No change	(Torrecillas et al., 2015)

Lazado et al. (2022b) reported that salmon infected with AGD exhibited larger (MCA) and denser mucous cells (MCD) in gill lamellae after seven weeks, irrespective of whether they were treated with PAA or not, in comparison to the observations made three weeks after exposure. The fish in the

present study were sampled two weeks (December) and 11 weeks (February) after the onset of AGD in the system. Also, they exhibited larger and denser mucous cells in February compared to December. Experiments involving PAA and H_2O_2 have shown an initial increase in MCD following exposure to environmental stress, followed by a return to baseline levels (Haddeland et al., 2021; Rantty, 2016). The absence of a subsequent decrease in MCD in this study, as also observed in Lazado's research, could be attributed to differences in sampling timing or variations in study design. A more likely explanation could be that the cells have established a new setpoint, remaining non-naïve and ready to respond to the naturally occurring and chronically present *Paramoeba perurans* amoeba in aquatic environments. This supports the cells' reactions as an adaptive defence mechanism (Strzyżewska-Worotyńska et al., 2017).

Unlike experimental systems, fish in this study were continuously and naturally exposed to the amoeba in the aquatic environment, without any treatment. These observations suggest that mucous cells continue to grow and produce mucus after the pathogen or other environmental challenges have declined. Demonstrated as the MCA and MCD continued to increase even after the decline in AGD scores. This indicates a latent response, prolonged vigilance of non-naïve cells to the chronic presence of amoeba and a new homeostasis. It is uncertain whether this study's changes in mucous cells are due to the amoeba, the aquaculture system itself, or unexamined variables. Nonetheless, the analogous pattern observed by Lazado et al. (2022b) suggests that the alterations in gill mucosal cells in this study can be caused by the AGD infection rather than the new aquaculture system itself.

The results obtained from mucosal mapping in gill lamellae in this study revealed an increased volumetric density of mucous cells and alterations in cell size. These results align with Dang et al.'s 2019 study, showing a gradient of MCD corresponding to pollution levels, with the highest MCD at the most polluted station and the lowest at the least polluted one. Additionally, smaller MCA was noted in the skin with high parasitic pressure (Dang et al., 2019). These findings align with the hypothesis that a reduction in cell size could coincide with increased cell density in tissue, potentially serving as a defence mechanism against parasites and environmental challenges. Smaller mucous cells may facilitate rapid cell filling and movement to the tissue surface, thereby promoting the efficient removal of pathogens through a washing-off process (Dang et al., 2019; Torrecillas et al., 2015). The phenomenon known as the “pathogenesis model” proposed by Arthur Lyngøy (A. Lyngøy, Fiskr, pers.comm.) suggests that the increased presence of smaller-sized mucous cells, enhancing faster filling and migration to the surface, is an acute protective response in fish against environmental stressors or pathogens. When the stressor persists, fish may adapt by increasing cell size and density to augment cytosol for mucin production before returning to a baseline level. In cases of prolonged stressor exposure, a new homeostatic baseline can be established to better respond to the ongoing

challenge. The results of the mucous mapping of gill lamellae in this study coincide with the hypotheses of Dang et al. 2019, A. Lyngøy and the pathogenesis model that a parasite or environmental challenges result in a reduction in MCA as a first response to the attack, followed by an increased adapted baseline in MCD.

The observations of the dynamic change in mucous cells in the gill lamellae of salmon in Egget® (Figure 20) suggest that the fish in Egget® were robust, healthy, minimally stressed, and grew well while still having surplus energy to combat the pathogen. Little to no handling and a stable environment have previously been connected with healthy individuals capable of combatting pathogens without external treatment (Grefsrud et al., 2022b). Mortality rates remained consistently low throughout the production period in Egget® (Table 3), with no evidence indicating that the AGD outbreak had increased fish mortality. While CCSs have raised concerns about pathogen accumulation, particularly AGD (Hjeltnes et al., 2019; Riksrevisjonen, 2023; Sommerset et al., 2023), this pilot project of Egget® did not observe AGD accumulation. It has been documented that *Paramoeba peruans* amoebae thrive in high salinity environments (Sommerset et al., 2023). The slightly higher salinity levels in the lower depths of the water column (where CCSs apply water) may pose an increased pathogen risk. Further research is needed to investigate 1) if there is an increased presence of *Paramoeba peruans* in CCSs and 2) whether it is related to the water source location in CCSs.

The skin mucosa showed no significant differences over time in either MCA, MCD or DA (Figure 24). Skin, as an important mucus barrier together with the gills, reflects the fish's physiologic status and environmental conditions as multiple factors influence the mucous dynamic (Cabillon & Lazado, 2019; Dash et al., 2018). Besides the *Paramoeba peruans* amoeba, no parasites or other environmental challenges were recorded in Egget®, and the consistent condition of the skin throughout the observed period suggests a stable equilibrium between the environment and the fish. Given the absence of environmental challenges, the skin has retained balance and protection, indicated by no change in mucous cells on the skin. In Rantty's study (2016), the skin mucous cells were denser after H_2O_2 delousing. In previous studies, unfavourable environmental conditions with O_2 and nitrate have shown increased skin mucous (Vatsos et al., 2010) (Table 10). Additionally, Dang et al. 2019 documented changes in the mucous dynamic in both gills and skin corresponding to toxicity and parasitic pressure.

The database of Quantidoc includes mucous mapping data of 1543 salmonids from different rearing conditions in seawater environments, encompassing both farmed and wild populations (Figure 25). The skin mucous samples from this study (the coloured dots in Figure 25) were clustered in the green centrum of average healthy mucous dynamics. This suggests a normal and healthy skin condition (Quantidoc database) of the fish sampled in Egget®.

4.2.3 Bacteria in Gills and Water

This study used a phylogenetic analysis on 82 bacterial colonies isolated from fish gill and water samples and 25 reference bacteria to construct a Neighbour-joining phylogenetic tree. The results revealed five dominant genera isolated from growth on marine broth agar (MBA): *Pseudoalteromonas, Psychrobacter, Colwellia, Flavobacterium, and Polaribacter*. The most dominant genera were *Pseudoalteromonas*, a well-known psychrophilic bacterium, previously detected in biofilm fouling in sediments in connection to fish farms (Iijima et al., 2009; Parrilli et al., 2021; Pujalte et al., 2007). *Psychrobacter* is another psychrophilic, and together with *Pseudoalteromonas* is considered to mostly consist of non-pathogenic species (McCarthy et al., 2013; Pujalte et al., 2007). Both genera have been isolated from Atlantic salmon and are considered part of the normal microflora of salmon (Bakke-McKellep et al., 2007; Kristiansen et al., 2011; McCarthy et al., 2013; Ringø et al., 2008). Additionally, *Colwellia*, *Flavobacterium*, and *Polaribacter* were also identified as major genera in the samples and are also psychrophilic and naturally abundant in cold marine environments (Ramírez et al., 2022; Roalkvam et al., 2019; Wang et al., 2018). *Colwellia* sp. was detected in both the inlet/outlet water and on the fish in the present study. Previous research has shown a strong correlation between the presence of *Colwellia* sp. and the absence of melanin deposits in the gills of Atlantic salmon during the early stages of farming in recirculating aquaculture systems (Quezada-Rodriguez et al., 2023), suggesting a potentially beneficial role for this bacterium in promoting a healthy microbiota. *Flavobacterium* and *Polaribacter*, both members of the phylum Bacteroidetes, have been associated with various functions in marine ecosystems and have been previously detected concerning fish farm environments, most non-pathogenic species (Duchaud et al., 2007; Roalkvam et al., 2019; Wang et al., 2018). These genera indicate a natural and diverse composition of bacteria in Egget® that naturally inhabit cold waters; other studies have associated them with aquaculture systems. The results indicate no accumulations of specific bacteria in the system, as there was no to little clustering within the sampling dates. However, acknowledging that this analysis is limited by several factors, such as the assumption that bacteria grow on MBA, is essential. Further investigations are needed to understand the microbial dynamics in Egget® fully.

4.2.4 Microorganisms in Water and Biofilm

The mean DNA concentrations observed in the inlet and outlet water of Egget® ranged between 2–6 ng/sample, which is in line with other observations of clear ocean water filtered with the same pore size, ranging from 1–7 ng/µl (Kumar et al., 2022). One explanation for the slightly higher DNA

concentrations observed in water samples in November may be attributed to the slightly higher temperature, which can affect the DNA concentration in aquatic environments (Jo et al., 2019; Lacoursière-Roussel et al., 2016). Since feed and faeces are associated with higher DNA concentrations (Turner et al., 2015), the somewhat increased DNA concentration in the outlet water can be considered a natural fluctuation.

The DNA concentration in the Biofilm exhibited a broader range from less than 1 µg/sample to more than 43 µg/sample. There was no systematic pattern observer between the sampling days or locations. A linear relationship between alpha diversity and richness in biofilm over time has been identified in prior research (Karačić et al., 2022; Veach et al., 2016). This differs from our results but could be attributed to the daily brushing of surfaces within Egget®, which hinders the typical development of the biofilm. While biofilm brushing is beneficial, it releases microorganisms and particles into the water, potentially reducing water quality and causing fish stress. This is especially pertinent in larger-scale systems, where increased surface area may foster more biofilm formation. Balancing biofilm management and water quality is critical for fish well-being in such environments. The absence of a systematic increase in DNA concentration in the biofilm or greatly elevated concentrations in the outlet water supports the effectiveness of the water flow and sludge collection system. This is particularly evident as no accumulation occurred within Egget®, and the DNA concentration decreased despite increased biomass.

The bacterial concentrations observed in the inlet and outlet water samples with flow cytometry (FCM) exhibited a relatively consistent pattern across all sampling ranging from 3–6 $*10^5$ per /ml and are in line with other concentrations reported in seawater from 2–6 $*10^5$ cells/ml (Borsheim et al., 1990; Marie et al., 1999; National Research Council (US) Steering Group for the Workshop on Size Limits of Very Small Microorganisms, 1999). Environmental factors such as sufficient and appropriate nutrients, oxygen levels, light availability, and temperature can collectively affect bacterial populations. Notably, an elevation in temperature tends to augment enzyme activity, promoting bacterial growth (Qiu et al., 2022; Wei, 2020). The observed trend of declining bacterial concentrations within and outside Egget® may be attributed to the concurrent reduction in seawater temperature. The minimal discrepancies between the inlet and outlet water strengthen the theory of an absence of significant bacterial accumulation within Egget®.

Analyses for the Novagene report revealed distinguished dynamics in biofilm samples compared to the water samples containing inlet and outlet water. Biofilm exhibited higher values across all alpha indices compared to the water samples. A particularly pronounced difference was observed in the Shannon index, indicating increased species richness, and the Simpson index, indicative of species dominance.

Building upon these findings, the Unweighted Pair-group Method with Arithmetic Mean (UPGMA) results from the beta indices revealed these two distinct clusters. This outcome is expected and demonstrates the capability of microorganisms in seawater to attach, colonise, and form biofilms, fostering a different dynamic than in seawater (Rud et al., 2017). Previous studies have indicated through alpha and beta diversity analysis that the biofilm exhibits higher bacterial community richness and diversity compared to water samples in aquaculture settings. Additionally, biofilm encompasses shared and significantly distinct bacterial compositions compared to water samples (Kalmbach et al., 1997; Martiny et al., 2005; Roquigny et al., 2021; Rud et al., 2017). This pattern echoes broader findings in Egget®, including the concentration of DNA and bacteria in water and biofilm samples, alpha and beta diversity, relative abundance, heatmap and the Venn diagram. These collective observations underscore the distinctive microbial landscape supporting the natural state within Egget® regarding water-biofilm dynamics.

5. Conclusion

This study explored fish health and welfare conditions, with mucosal dynamics in the gills and skin, microbiota in the gills, inlet/outlet water, and biofilm within the pioneering closed containment aquaculture system prototype, Egget®. The rearing practices for Atlantic salmon (Salmo salar) in Egget® featured a controlled and stable environment, the utilisation of unfiltered, untreated seawater from a depth of 17 meters, daily brushing of system walls to prevent biofouling, and no handling or treatment applied to the fish. These practices conferred numerous advantages over conventional open net pen farming.

The findings in this study demonstrated that these practices minimised fish handling, thereby reducing stress and optimising growth potential while maintaining fish welfare. This was demonstrated by a low mortality rate of 1.2% and a high specific growth rate of 1.6%, surpassing industry standards and Egget®'s genetic sibling fish reared in the same production zone. Moreover, fewer external injuries were observed in comparison to another closed system. During the sampling period the amoeba *Paramoeba peruans*, responsible for amoebic gill disease (AGD), was detected (November 29th). However, the adoption of a non-interventionist approach allowed for the natural adaptation of the gills to the presence of the amoeba. Initially, an acute response with a decrease in mucous cell size in gill lamellae was observed, followed by establishing a new homeostatic state adapted to the environment in the presence of the amoeba. In contrast, the skin mucous layer, less affected by the gill parasite and had no interventions with handling, maintained an unaltered state throughout the sampling period. Bacterial sequencing from the fish's gills and the water confirmed that the bacteria belonged to the salmon's normal flora and the cold marine aquatic environment. Consequently, Egget®, the innovative floating closed containment system, positively influences fish welfare within the system, and the **HA_1** hypothesis is **accepted**.

This study found no evidence of bacterial accumulation within the system. Inlet and outlet water exhibited similar DNA and bacterial concentrations and dynamics, and the absence of adverse accumulations or heightened severity of the AGD outbreak. Similarly, no biofilm accumulation was recorded. These findings suggest that Egget®'s technical system and rearing procedures can effectively maintain the stability of fouling organisms, likely achieved through regular surface brushing and frequent water turnover. Consequently, the **$H0_2$** hypothesis, positing that the routines for fish rearing in Egget® provide no improvement in resilience to ambient (pathogenic) organisms, is **accepted**.

Nevertheless, it is crucial to acknowledge unexamined variables such as other factors in the fish's immune system, genetics, and feed quality, which were not directly assessed in this study and may

have contributed to these outcomes. Factors like location microbiota, water or fish treatments, antifouling procedures, etc., can all influence the holistic health of fish barriers. It is noteworthy that this study marks the first-ever production cycle in the first Egget® prototype at one location, representing very low mortality, robust growth, an active and adaptive immune system, and a healthy ecosystem free of accumulations within the system and the water, with a beneficial effect on fish welfare.

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Appendix

Appendix A: Standard Quantidoc sampling protocol.

Verification of Barriers - Sampling of skin, gills and guts

Revised March 2019

NOTE: The barriers of fish have living cells on their surfaces.

Tools: plastic histocassettes, scalpel, extra scalpel blades, forceps, scissors, pencil for labels, about 2 L

of 10% phosphate buffered formalin (Merck®, HT501320); pre-labelled wide-mouth jars for storing formalin or biopsy containers (Biopsafe® contains formalin) and histocassettes with samples; notebook.

Sampling: The salmon should be only very freshly dead or terminally anesthetized (waiting time makes

the tissue degrade, as can be quickly seen on the gills). Do not touch the area to be sampled because the mucous cells will wipe off. Carry the anesthetized fish carefully to the sampling table, avoiding areas to be samples – we usually carry flat and hold with open hands on the flank side which will not be sampled.

Overview of Sampling procedure:

1. Fill in Project form (see page 4).

2. Pre-label histocassettes with fish nr, and tissue (pencil on sloping end* See Appendix for guidelines on labelling and shipping.

3. Pre-label widemouth 90 ml jars or biopsy containers with project name and date (fish number(s)

and tissue).

4. Cut out the desired tissues section using a scalpel (see below for details)

5. Transfer tissues to individual histocassettes by touching only the ends of the section. If the

section is too broad to fit in without crushing, cut away some tissue or gill arch. Remember one

sample is for one histocassette.

6. With pre-labelled wide-mouth jars: Place the labelled histocassettes containing all tissues for

an individual fish into labelled jar and fill with 10% buffered formalin. Rule of thumb: fill to

the top. Tape jar closed to prevent leakage.

With pre-labelled biopsy containers (read Biopsafe® instructions): a) Unscrew the lid. Place

3 labelled histocassettes with samples for an individual fish into labelled containers. b) Close

Biopsafe by screwing the lid onto the container. The lid must be screwed all way on. c) Release

the formalin by pressing the top of the lid.

7. Fill in Fish identification form (see page 5).

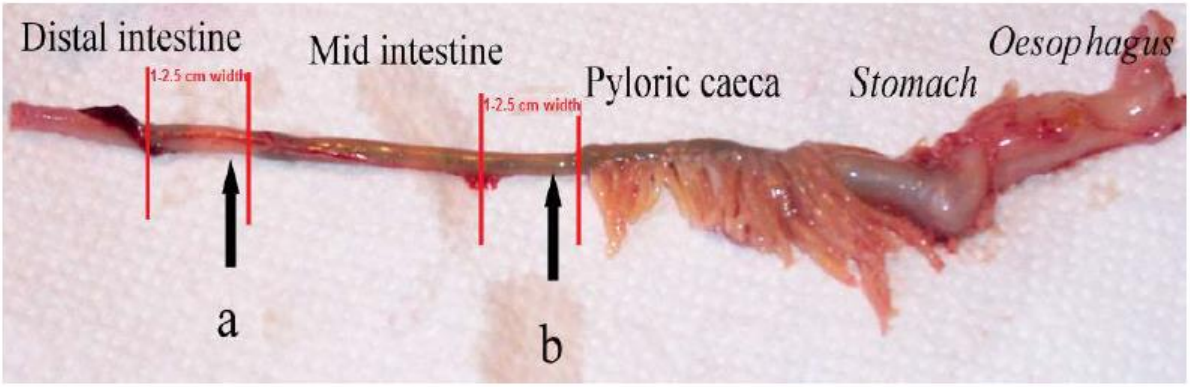
8. Pack securely and ship to Quantidoc (*see Appendix for details).

Left: an example of histocassette size and labelling for gills, dorsal skin, hindgut and foregut from one fish (number 12). Middle: an example of labelled formalin jar for multiple samples of dorsal skin from 5 fish. Right: an example of Biopsy container (Biopsafe®) with 3 histocasettes.

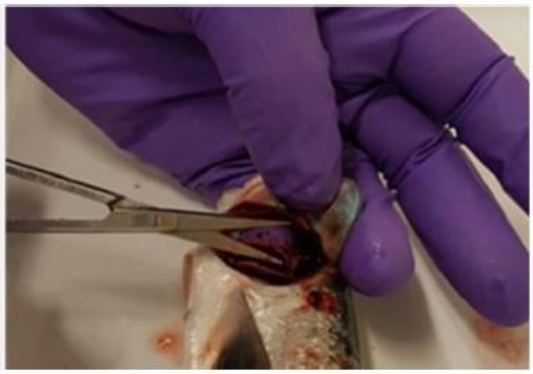
For the SKIN: using a sharp scalpel, make a rectangle about 1-2 cm x 1-2.5 cm just below the dorsal fin (line up with the front edge of the dorsal fin, see figure). Lift one edge carefully and remove the skin square WITH a bit of the underlying muscle. Place carefully into marked histocassette, close cassette and pop into Biopsafe container or a jar with 10% phosphate buffered formalin. More skin sections can be cut, according to need. The head and tail skin sections are taken in that order because the tail is hardest to hold still. It is unlikely that you will get more than about 1.5 x 1.5.cm slices from head and tail. Put head and tail skin sections carefully into properly labelled histocassettes and fix in buffered formalin.

If the sample flips over on itself or wipes along the table or body, the mucous cells will be disturbed, so start again. A few practice rounds are useful. If nonetheless the sample is potentially disturbed, make a note of this in the comments section of the overview.

For the GUT: Cut a piece of the hindgut after about “a” of the picture and a piece of the foregut at “b”of the picture. Flush it with seawater if necessary to get out any remaining food but do not cut it open. Place in marked histocassette and pop into 10% phosphate buffered formalin. At this point, note fish gender by checking gonads (sex makes a difference). It is important that all samples are from standardized places because the mucous epithelium has zones which are anatomically distinct.



For the GILLS: remove much of the second gill arch (the one below the outermost one) with the gill filaments and lamellae, without touching the soft tissue. It is often easiest to hold the arches spread by going through the mouth. Cut the arch at the top and bottom of the "V" of the gill arch, leaving the outermost soft parts untouched by you and intact. Lift by the bony arch, place carefully in marked histocassette and pop into 10% phosphate buffered formalin.



Appendix B: Overview of the incubation period of bacteria collected from Atlantic salmon's gills and water samples at Egget® (Table 11).

Table 11: Overview of the incubation period[8] of colony cultivated on marine broth agar (MBA).

	MBA-Plates from 1. Sampling	MBA-Plates from 2. Sampling	MBA-Plates from 3. Sampling
1. Inoculated dishes	2022-11-10	2022-12-12	2023-02-16
Incubated[9]	10-11 days	6 days	10 days
2. Inoculated dishes	2022-11-24/25	2022-12-19	2023-02-27
Incubated	12 days	38 days	14 days
3. Inoculated dishes	2022-12-06/07	2023-01-26	
Incubated	-	22 days	
Colonies picked for PCR[10]	2022-12-06/06	2023-02-17	2023-03-13
Started PCR	2023-01-10	2023-02-17	2023-03-14

Appendix C: Picture of the GeneRuler 1 kb DNA ladder used in the 1% gel electrophoresis (Figure 39).

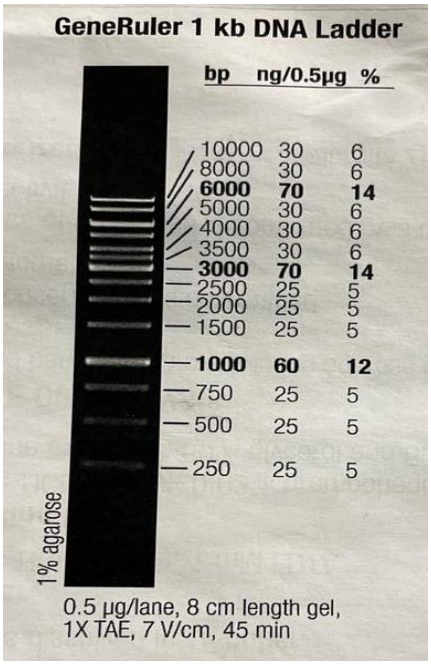


Figure 39: The GenRuler 1 kb DNA Ladder used in the 1% gel electrophoresis. Source: Picture taken by Andrea C. Opshaug, content from supplier of equipment Invitrogen, Thermo Fisher Scientific.

[8] Attempts with error or contamination are not included in the table and are some of the reasons for uneven incubation times. The petri plates were kept an eye on during the incubation. Morphological changes were recorded several times for each dish.

[9] Incubated in a ventilated cooling room at 4°C.

[10] Some samples were stored in a freezer after being picked and diluted in sterile water until further PCR procedure. Growth determined which plates the colonies were picked from.

Appendix D: Protocol on how to construct a phylogenetic tree.

Steps to Construct Phylogenic Tree using MEGAX:

Save all your fasta sequences in notepad or textpad file with (.fas) extension (notepad symbol will change to mega), because MEGA can read this *.fas* files)

Open MEGAX and follow steps given below:

1. Select **Align** option
2. select **Edit/Build alignment,** then Alignment Editor will open
3. select Create a **New Alignment** and OK
4. Select **DNA** (depending on type of sequences) then a New MX: Alignment Explorer will open
5. Go to Edit dropdown, select **Insert sequences from file**
6. Open your .fas file (having all your required sequences)
7. Using CtrlA select all sequences and open Alignment dropdown, Select **ClustalW**
8. Now **ClustalW options** box will appear
9. Go to **Matrix** and from **DNA Weight Matrix** select ClustalW and OK
10. Now, your sequences are aligned and you can see *** (asterisk\star)** for conserved nucleotides in al sequences.
11. Always select sequences from where **first * star** appears and **delete** previous nucleotides from all sequences and delete sequences appearing after last star* so that you have all conserved sequences aligned from all samples.
12. In **Data** dropdown, select **Export Alignment** (to save), (always save in two formats)
    (i) MEGA format
    (ii) FASTA format
        1. Save to your folder, input name is not necessary anyways you can put whatever you like, select NO when for asked for Protein-coding nucleotide sequence data.
13. Close Alignment window and go back to home page of MEGAX
14. Steps **to select best Model** to construct phylogenic TREE using aligned sequences:
    (i) In MODEL menu
    (ii) Select Find Best DNA/Protein model

(iii) Result will appear in table format, always select first model or decide it depending on BIC ((Bayesian Information Criterion)) value, usually lowest score is considered and it is always the first one.

(iv) Save these details for later use in literature, either in excel format or txt, select options from files.

(v) Close the table and go back to MEGAX home.

15. Steps to construct Phylogenic TREE:

(i) In PHYLOGENY menu

(ii) Select **Neighbor-joining** (it works fast and gives overview about sequences, later you can re-construct using Maxmimun-liklihood, it takes few hours depending on number of sequences)

(iii) Analysis preference box will appear

(iv) Select **Bootstrap method** to 1000

(v) **Kimura model** if model shown in your table is not found

(vi) In Gaps\Missing data treatment select **Pairwise Deletion** (this option will prevent loss of gaps which are valid, specially when using 16S rRNA gene sequences which are conserved and each nucleotide is essential for comparative studies)

After selecting OK, the program will construct a tree for you which will open in new tab.

16. Here, you can edit the tree depending on your requirement.
17. To save tree, from **Image menu** select **copy tree to clipboard to edit in Powerpoint** and also save in other given formats.

Note: Parameters can be changed at steps depending on your sequence data, but these steps and parameters are general.

Appendix E: Sampling protocol of prokaryotes, Lise Øvreås.

Sampling protocol: Metabarcoding (DNA and RNA) of prokaryotes One Ocean

A) **Parameter definition:** Cells collected on filter, to be used for DNA and RNA isolation.
B) **Method responsible:** Lise Øvreås (UiB)

C) **Special requirements:**
- Clean area for filtration/sample handling

D) **Sampling depth(s):**
- Surface and at Chl max if possible

E) **Equipment**

- 0.2 µm Sterivex filters, SVGPL10RC
- Syringe (50 mL)
- Inlet and outlet caps for Sterivex filters
- Sterile lab gloves (Medium)
- 2 liter container for collection of seawater
- 2 liter mug to record water flow through
- Measuring cylinder (500 mL))
- Labelling pens
- 60 ml RNA-later
- 70% Ethanol in squeeze-bottle

E) **Methodological description:**

Important:
- Always use lab gloves (also when handling equipment, e.g. washing, storing).
- RNA degrades quickly due to hydrolysis and RNAses (which are everywhere!). Change gloves after touching surfaces, work quickly. Never leave RNA at room temperature.
- DNA is very stable. Cross-contamination of samples is thus an issue. Clean tweezers with ethanol before use. Change gloves between samples.
- Immediately after use, rinse all containers, tubes and filtration equipment with distilled water and leave to dry in clean area.

1. Prior to sampling: Label and number Sterivex filters
2. Collect 2l of water into a 2L clean carboy (labelled with depth).
3. Fill the 50 ml syringe with seawater from the carboy
4. Connect the Sterivex filter unit to the Luer-Lok fittings on 50 ml syringe (with a twist). The female Luer-lok side is the inlet.
5. Push the 50 ml water through the Sterivex (avoid bubbles on the filter)
6. Disconnect the filter from the syringe
7. Fill the syringe with another 50 ml of seawater
8. Push the 50 ml water through the Sterivex (avoid bubbles on the filter)

9. Repeat step 4 – 8 so that you have at least 1 liter of seawater through the filter. (If possible, you may want to filter up to 2 liters. This depends on how fast the water goes through the filter and how much water you have available. If it gets to difficult to push the water through then you stop).
10. Use a 10/20 ml syringe to push out the remaining water in the Sterivex.
11. Take up 2 ml of *RNA later* in a 2 ml syringe
12. Connect the 2 ml syringe to the female Luer-Loc and push the RNA later into the filter very slowly and avoid bubbles.
13. Cap the Sterivex with caps (2 different types)
14. Mark the filters accurately and store it in a plastic bag.
15. Measure the volume of filtered water from buckets, using measuring cylinders.
16. Repeat procedure with next Sterivex filter.
17. For each filter, note down time of sampling (UTC), filtration volume, filtration time, size fraction, any deviation from protocol, observation (e.g. colour on filter) in log-sheet.

Amount of water needed:
1 - 2000 mL

Sampling time:
appr. 1 hour per sample

Sample labelling:
Station, date, depth, volume

Parameters to be recorded:
Station, date, time of sampling (UTC), depth, size-fraction, volume, filtration time, protocol used (ID nr?), any deviations from protocol, filter colour, buffer added (volume and type), storage (i.e. LNG), operator, sample responsible

Appendix F: Overview of DNA sequences from salmon gills and water samples collected at Egget® (Table 12).

Table 12: Overview of DNA sequences with the best hit in Basic Local Alignment Search Tool (BLAST), sequences from isolated colonies from Atlantic salmon gills and water samples collected in the closed aquaculture system, Egget®.

Fish and water sample	Best BLAST hit	BLAST accession number	Sequence Similarity %	Base identities
Fish 1.1	Pseudoalteromonas nigrifaciens	MN220613.1	99.22	892/899
Fish 1.2	Pseudoalteromonas sp.	KM979160.1	99.88	801/802
Fish 2.1	Pseudoalteromonas sp.	EU365532.1	92	202/219
Fish 2.2	Pseudoalteromonas translucida	NR_025655.1	99.63	812/815
Fish 3.2	Flavobacterium frigidarium	AY771722.1	99.75	807/809
Fish 3.4	Shewanella livingstonensis / vesiculosa	CP034015.1/ CP073588.1	100	584/584
Fish 4.1	Psychrobacter piscatorii/ proteolyticus	MN220600.1/ LS483016.1	99.25	527/531
Fish 4.2	Polaribacter sp	KC462920.1	99.87	759/760
Fish 5.1	Pseudoalteromonas distincta/ elyakovii/ agarivorans	OP716198.1/ OP716196.1/ON908607.1	99.74	378/379
Fish 5.3	Gillisia myxillae	MK131342.1	99.60	492/494
Fish 6.1	Pseudoalteromonas neustonica/ prydzensis/ rhizosphaerae	MN220612.1/ KX417181.1/ CP102371.2	99.45	728/732
Fish 6.2	Pseudoalteromonas sp.	MN889237.1	99.49	590/593
Fish 6.3	Pseudoalteromonas nigrifaciens	MH550147.1	99.57	688/691
Fish 6.4	Winogradskyella rapida/ pacifica	NR_118846.1/ LR745711.1	97.88	830/848
Fish 7.1	Pseudoalteromonas neustonica/ prydzensis	MK955337.1/ KX417143.1	99.68	623/625
Fish 7.3	Shewanella livingstonensis/ vesiculosa	CP034015.1/JQ762254.1	100	663/663
Fish 7.4	Flavobacterium frigidarium	MG780342.1	99.54	863/867
Fish 8.1	Pseudoalteromonas prydzensis/ mariniglutinosa	KX417181.1/ JQ867499.1	99.21	625/630
Fish 8.2	Pseudoalteromonas distinct	CP040559.1	100	754/754
Fish 8.4	Gillisia mitskevichiae	NR_113917.1	98.94	841/850
Fish 9.2	Pseudoalteromonas sp	DQ789376.1	99.75	396/397
Fish 9.3	Shewanella sp.	LC460999.1	99.26	536/540
Fish 9.4	Gillisia myxillae	MK131342.1	97.63	371/380
Fish 10.1	Pseudoalteromonas nigrifaciens/ distincta	MN220613.1/ CP040559.1	99.78	888/890

Fish 10.2	Pseudoalteromonas distincta/ elyakovii/ hodoensis	OP716198.1/ OP716196.1/OP521977.1	99.11	556/561
Fish 10.4	Salegentibacter sp./ Mesonia sp.	KT429723.1/MK780012.1	99.77/99.65	857(855)/859
Water 1 Inlet 1	Neptunomonas sp.	AB742372.1	94.84	607/640
Water 1 Outlet 1.1	Pseudoalteromonas citrea	EU919201.1	99.20	621/626
Water 1 Outlet 2.2	Mesonia sp.	MW580036.1	100	721/721
Fish 2.1	Pseudoalteromonas distincta	CP040559.1	99.80	1001/1003
Fish 2.3	Salegentibacter sp.	KT429723.1	99.90	973/974
Fish 3.1	Pseudoalteromonas elyakovii	OP716196.1	99.90	1009/1010
Fish 3.3	Pseudoalteromonas nigrifaciens	MN220613.1	99.89	951/952
Fish 4.4	Planococcus halocryophilus	MH929579.1	99.52	831/835
Fish 5.2	Pseudoalteromonas arctica	MK439562.1	99.35	761/766
Fish 7.2	Pseudoalteromonas prydzensis	MG681177.1	99.90	958/959
Fish 8.3	Shewanella ulleungensis	NR_179059.1	99.89	914/915
Fish 10.3	Pseudoalteromonas atlantica	MK439567.1	99.19	735/741
Water 1 Inlet 3.1	Poseidonibacter antarcticus	MH473590.1	100	1001/1001
Water 1 Inlet 3.2	Uncultured bacterium clone	HQ203898.1	99.36	937/943
Water 1 Inlet 3.3	Winogradskyella algae	OP623387.1	98.48	972/987
Water 1 Inlet 3.4	Colwellia sp.	MW580204.1	99.52	1027/1032
Water 1 Outlet 2.1	Moritella sp.	MW579993.1	98.11	986/1005
Water 1 Outlet 3.3	Polaribacter irgensii	AY771779.1	99.80	1008/1010
Fish 11.2	Pseudoalteromonas sp.	FJ205743.1	99.90	979/980
Fish 13.1	Pseudoalteromonas sp.	MN889137.1	99.80	979/981
Fish 14.2	Pseudoalteromonas translucida	MG681182.1	99.22	895/902
Fish 19.2.1	Pseudoalteromonas arctica	HG795046.1	99.90	1008/1009
Water 2 Outlet 2.1	Aliivibrio logei isolate	EU257749.1	99.69	957/960
Water 2 Outlet 3.1 U2.3.1	Colwellia sp.	DQ520892.1	100	954/954
Fish 21.1	Psychrobacter okhotskensis	LT852800.1	99.80	990/992
Fish 21.2	Flavobacterium gelidilacus	KJ475154.1	99.09	983/992
Fish 22.1	Psychrobacter nivimaris	MN062081.1	99.51	1008/1013
Fish 22.2	Planococcus kocurii	CP013661.2	95.17	867/911
Fish 22.3	Flavobacterium gelidilacus	KJ475154.1	89.80	990/1002

Fish 23.1	Psychrobacter nivimaris	MN062081.1	99.51	1008/1013
Fish 23.2	Pseudoalteromonas tetraodonis	KC178954.1	92.39	899/973
Fish 23.3	Flavobacterium frigidarium	MG780342.1	99.20	987/995
Fish 24.1	Psychrobacter aquimaris	EF101545.1	99.31	1006/1013
Fish 24.2	Gillisia myxillae	MK131342.1	99.50	987/992
Fish 25.1	Psychrobacter piscatorii	KC534182.1	99.32	1028/1035
Fish 25.2	Moritella marina	NR_040842.1	99.50	998/1003
Fish 25.3	Polaribacter irgensii clone	AY771779.1	98.90	989/1000
Fish 26.1	Psychrobacter cryohalolentis	LT852801.1	99.41	1012/1018
Fish 26.2	Planococcus donghaensis	LT852802.1	99.02	1015/1025
Fish 26.3	Polaribacter irgensii clone	AY771779.1	98.30	984/1001
Fish 27.1	Psychrobacter piscatorii	KC534182.1	99.60	1008/1012
Fish 27.2	Cognaticolwellia mytili	NR_156046.1	97.77	963/985
Fish 27.3	Polaribacter staleyi	NR_159336.1	98.71	996/1009
Fish 27.4	Flavobacteriaceae bacterium	AY285943.1	99.70	996/999
Fish 28.1	Alteromonas naphthalenivorans	MN746143.1	99.39	985/991
Fish 28.2	Shewanella frigidimarina	KU204882.1	99.60	997/1001
Fish 28.3	Uncultured bacterium clone	HQ203898.1	99.19	984/992
Fish 29.1	Psychrobacter glacincola	KM281942.1	99.00	991/1001
Fish 29.2	Cognaticolwellia mytili	NR_156046.1	97.53	947/971
Fish 29.3	Flavobacterium frigidarium gene	MG780342.1	99.70	1008/1011
Fish 30.1	Psychrobacter glaciei	MN080182.1	99.80	1011/1013
Fish 30.2	Cognaticolwellia mytili	NR_156046.1	98.35	895/910
Fish 30.3	Flavobacterium gelidilacus	KJ475154.1	98.81	1000/1012
Water 3 Inlet 1.1	Colwellia psychroerythraea	AF173964.1	99.36	935/941
Water 3 Inlet 2.1	Thalassomonas sediminis	DQ660392.1	98.11	936/954
Water 3 Inlet 2.2	Colwellia psychrerythraea	MK439533.1	97.74	952/974
Water 3 Outlet 1.1	Cognaticolwellia mytili	NR_156046.1	98.40	925/940
Water 3 Outlet 1.2	Tenacibaculum dicentrarchi	CP013671.1	97.63	949/972
Water 3 Outlet 2.1	Moritella marina	AB121097.1	97.81	892/912
Water 3 Outlet 2.2	Polaribacter irgensii clone	AY771779.1	97.64	951/974
Water 3 Outlet 3.2	Tenacibaculum ovolyticum	LC144619.1	97.46	960/985

Appendix G: DNA concentration in water samples (Table 13).

*Table 13: Mean DNA concentration from inlet and outlet water + standard error (SE), collected at the closed aquaculture system, Egget®, at three sampling dates (N=12, n=2). *Each sample contains two filters filtering one litre of seawater, each with a pore size of 0.22 µm, a total of 2 litres of filtered seawater in each sample.*

Sample 2022/2023	**DNA concentration (ng/sample*) + SE**
November 10th Inlet water	6.15 + 1.80
November 10th Outlet water	4.87 + 0.22
December 12th Inlet water	1.94 + 0.45
December 12th Outlet water	3.79 + 0.85
February 16th Inlet water	3.47 + 0.09
February 16th Outlet water	4.15 + 0.16

Appendix H: DNA concentration in biofilm (Table 14).

*Table 14: DNA concentration from biofilm, collected at the closed aquaculture system, Egget®, at three sampling dates. *Each sample contained biofilm from swabbing with a 4 cm sponge 3-4 times at the inside wall of Egget®.*

Sample 2022/2023	**DNA concentration (µg/sample*)**
November 10th Location 1	6,69
November 10th Location 2	0,41
November 10th Location 3	42,50
December 12th Location 1	2,74
December 12th Location 2	10,73
December 12th Location 3	1,53
February 16th Location 1	0,06
February 16th Location 2	43,50
February 16th Location 3	12,31

Appendix I: Bacterial concentration in water (Table 15).

Table 15: Mean bacterial concentration + standard error (SE) in inlet and outlet water from the closed aquaculture system, Egget®, at three sampling dates (N=18, n=3).

Sample 2022/2023	**Bacterial concentration (ml^{-1}) + SE**
November 10th Inlet water	$5{,}55*10^5 + 5{,}59*10^4$
November 10th Outlet water	$5{,}88*10^5 + 2{,}56*10^4$
December 12th Inlet water	$3{,}58*10^5 + 4{,}97*10^4$
December 12th Outlet water	$3{,}65*10^5 + 4{,}24*10^4$
February 16th Inlet water	$3{,}0*10^5 + 1{,}62*10^4$
February 16th Outlet water	$2{,}92*10^5 + 2{,}92*10^4$

Appendix J: Relative abundance at the genus level, from the Novogene report (Figure 40).

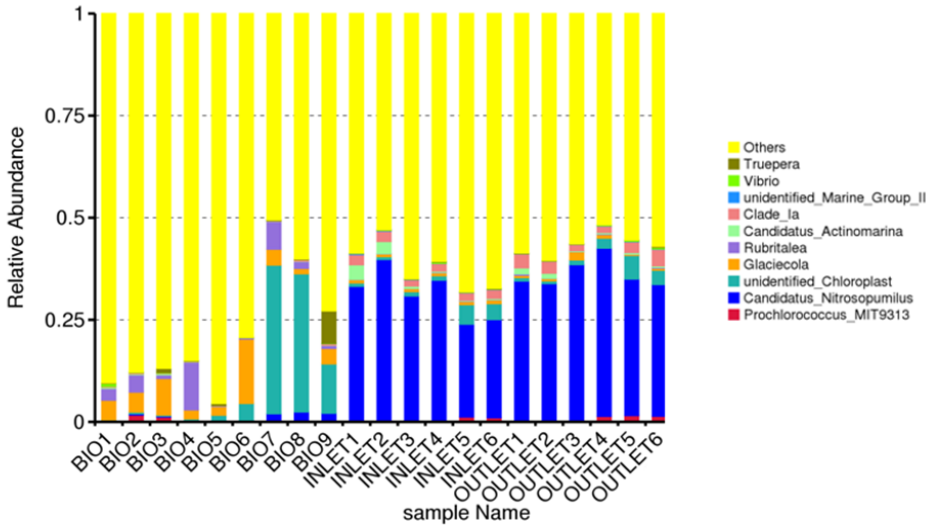
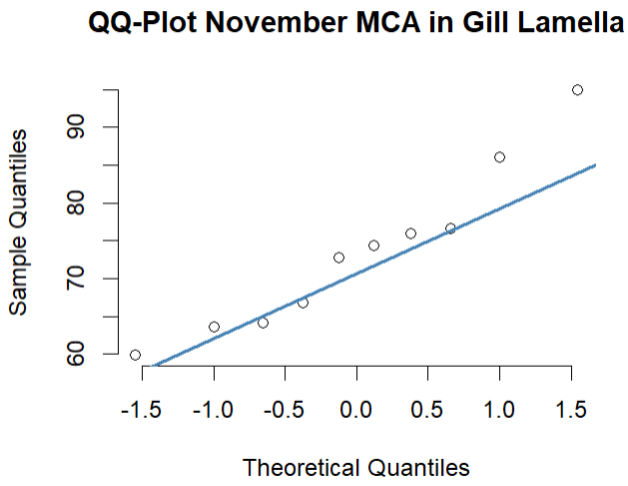
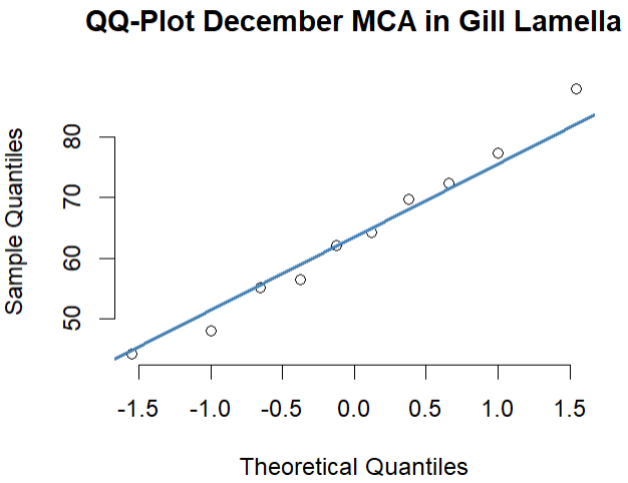
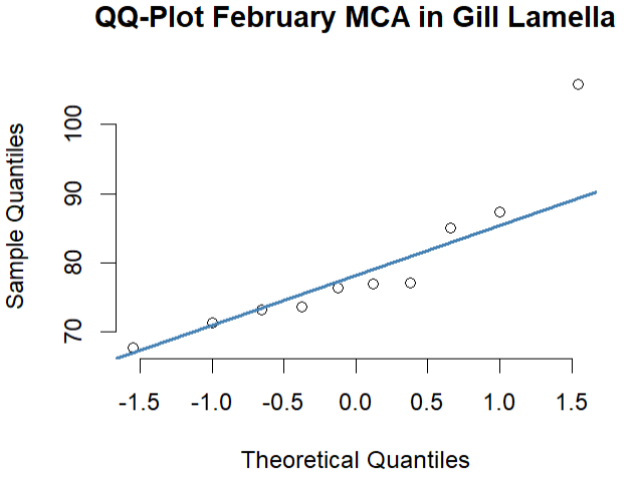


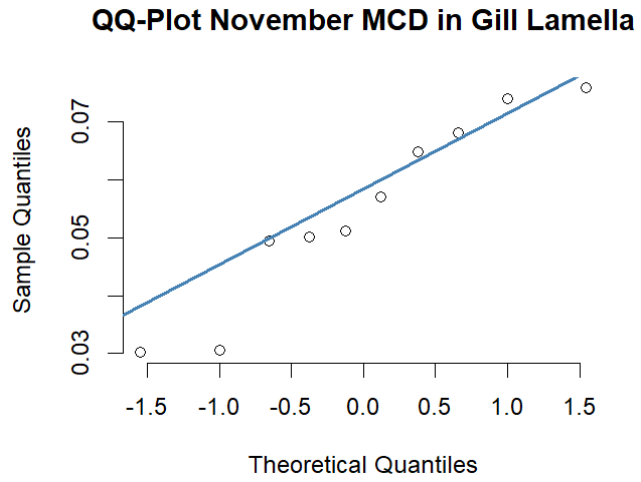
Figure 40: Bar plot at genus level of microorganisms in the inlet (N=6), outlet (N=6) and biofilm (N=9) samples collected at the closed aquaculture system, Egget®.

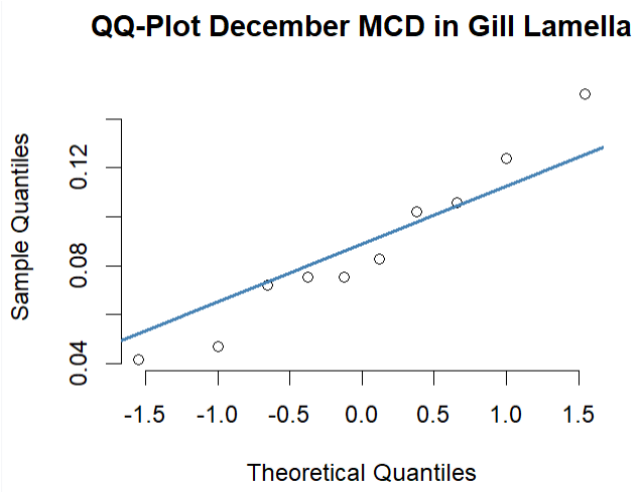
Appendix K: Overview of approximately normal distribution between the different mucous samples, mucous cell area (MCA), mucous cell density (MCD) and defence activity (DA) in gill lamellae against the three sampling months. It is shown with Quantile-Quantile plots (QQ plots).

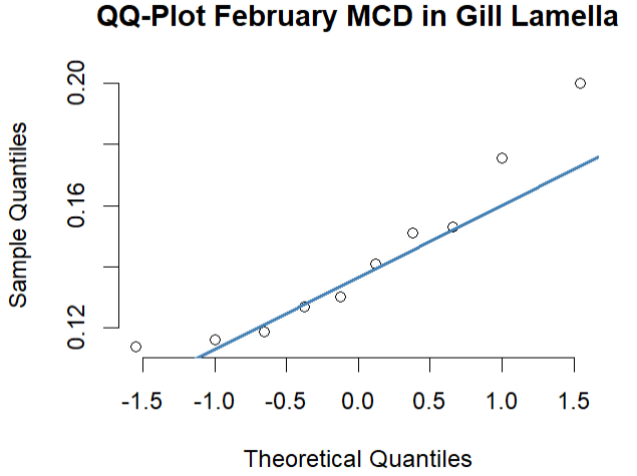


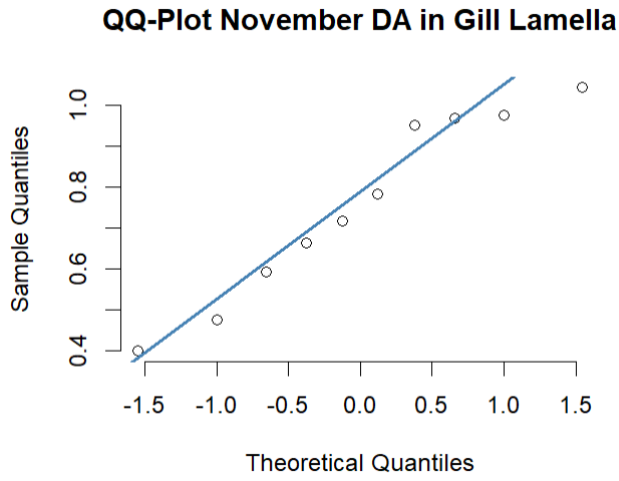


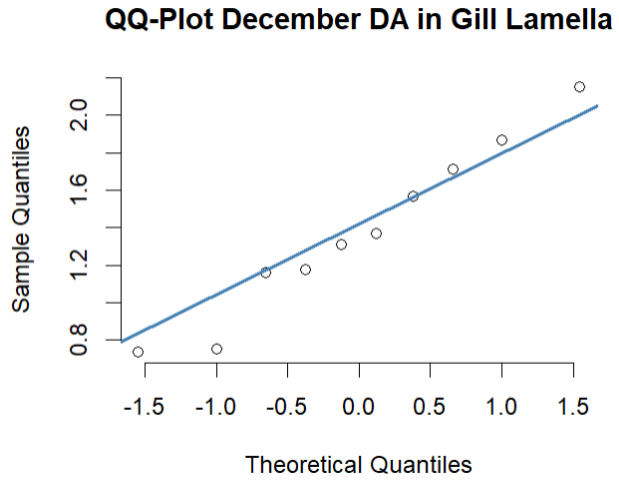


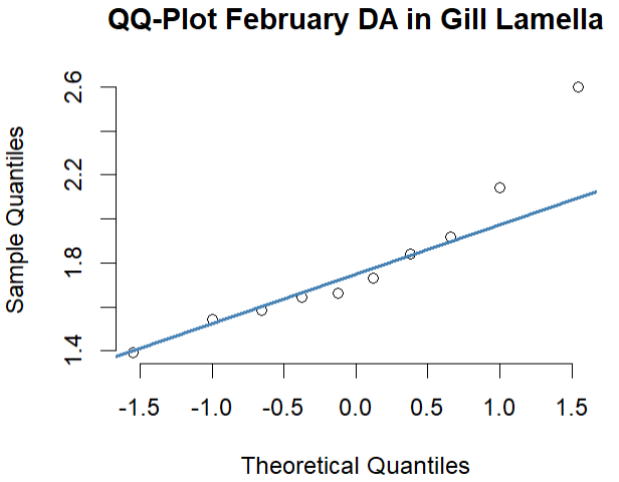

QQ-Plot November MCD in Gill Lamella
Sample Quantiles
0.03
0.05
0.07
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Theoretical Quantiles


QQ-Plot December MCD in Gill Lamella
Sample Quantiles
0.04
0.08
0.12
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Theoretical Quantiles


QQ-Plot February MCD in Gill Lamella
Sample Quantiles
0.12
0.16
0.20
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Theoretical Quantiles


QQ-Plot November DA in Gill Lamella
Sample Quantiles
0.4
0.6
0.8
1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Theoretical Quantiles


QQ-Plot December DA in Gill Lamella
Sample Quantiles
0.8
1.2
1.6
2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Theoretical Quantiles


QQ-Plot February DA in Gill Lamella
Sample Quantiles
1.4
1.8
2.2
2.6
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Theoretical Quantiles

Appendix L: Overview of variance tested within November, December, and February in gill lamellae, mucous cell area (MCA), mucous cell density (MCD) and defence activity (DA) with Levi's test. No significant variance in the gill samples over time. Analyses conducted in R.

```
> leveneTest(MeanMCArea ~ Month, data = df.projectGill)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  2   0.556 0.5799
      27
> leveneTest(MeanMCDensity ~ Month, data = df.projectGill)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  2  1.3624 0.2731
      27

> leveneTest(DefenseActivity ~ Month, data = df.projectGill)
Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  2  1.3822 0.2682
      27
```

Appendix M: Overview of ANOVA analyses and Tukey's HSD posthoc test between the three sampling months of November, December and February in gill lamellae, mucous cell area (MCA), mucous cell density (MCD) and defence activity (DA). Significant differences were observed. Analyses conducted in R.

```
> anova_MCA_gil <- aov(MeanMCArea ~ Month, data = df.projectGill)
> summary(anova_MCA_gil)
            Df Sum Sq Mean Sq F value Pr(>F)
Month        2   1263   631.7   4.533 0.0201 *
Residuals   27   3763   139.4
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> # Tukey's HSD (honest significant difference), post hoc-analyse:
> posthoc_MCA_gil <- TukeyHSD(anova_MCA_gil)
> print(posthoc_MCA_gil)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = MeanMCArea ~ Month, data = df.projectGill)

$Month
                       diff        lwr      upr     p adj
December-November -9.826577 -22.916174  3.26302 0.1694005
February-November  5.908073  -7.181524 18.99767 0.5108635
February-December 15.734651   2.645054 28.82425 0.0160796
```

```
> anova_MCD_gil <- aov(MeanMCDensity ~ Month, data = df.projectGill)
> summary(anova_MCD_gil)
            Df  Sum Sq  Mean Sq F value   Pr(>F)
Month        2 0.03904 0.019518      27 3.62e-07 ***
Residuals   27 0.01952 0.000723
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> posthoc_MCD_gil <- TukeyHSD(anova_MCD_gil)
> print(posthoc_MCD_gil)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = MeanMCDensity ~ Month, data = df.projectGill)

$Month
                         diff         lwr        upr     p adj
December-November 0.0324242 0.002611961 0.06223644 0.0309853
February-November 0.0873936 0.057581361 0.11720584 0.0000002
February-December 0.0549694 0.025157161 0.08478164 0.0002758

> anova_DA_gil <- aov(DefenseActivity ~ Month, data = df.projectGill)
> summary(anova_DA_gil)
            Df Sum Sq Mean Sq F value   Pr(>F)
Month        2  5.570  2.7851   21.91 2.22e-06 ***
Residuals   27  3.432  0.1271
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> posthoc_DA_gil <- TukeyHSD(anova_DA_gil)
> print(posthoc_DA_gil)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = DefenseActivity ~ Month, data = df.projectGill)

$Month
                         diff        lwr       upr     p adj
December-November 0.6239536 0.22864878 1.0192584 0.0015624
February-November 1.0492339 0.65392905 1.4445387 0.0000014
February-December 0.4252803 0.02997545 0.8205851 0.0330874
```