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Mucosal immune responses of Atlantic salmon parr following a pathogen breach in a recirculating aquaculture system

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Foreword

This thesis was written for my master's degree in Fisheries and Aquaculture science at the Arctic University of Tromsø (UiT), Norway. The purpose of this study was to get a better understanding on how a biosecurity breach in a recirculating aquaculture system (RAS) will affect Atlantic salmon parr if a pathogen enters the system using two different ways of entry. This is relevant as the use of RAS in aquaculture is increasing, with the knowledge on how a biosecurity breach affects the system still being unknown. This study is part of the Research Council of Norway project RASHealth – 302767 "Water disinfection strategies to improve Atlantic salmon parr production".

To my family and friands, thank you for the constant support throughout this whole process, especially when I felt overwhelmed at times.

To my good friend Simen Jensen, a very special thank you for letting me borrow your computer for weeks when I needed.

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Lena Hovda Aas

Abstract

With the increase in the use of recirculating aquaculture system (RAS) in the Norwegian Atlantic salmon *(Salmo salar)* aquaculture industry, the importance of understanding how pathogen enters and spreads in the system is crucial for its further development. *Yersinia ruckeri* is a pathogen known for infecting salmonid fish in freshwater stage and it has been shown that the RAS environment favors its proliferation. This study simulated two biosecurity breach scenarios, where pathogen entered the system and triggered a disease outbreak. The first sub-trial simulated a breach where the pathogen entered the RAS facility via the makeup water while the second sub-trial used fish as a vector for the pathogen. Sub-trial one consisted of 3 treatment groups - control, uninfected group, and 2 infected groups, where one group was infected by a single entry (SE) and the other group was infected via a multiple entry (ME). Sub-trial 2 also consisted of three treatment groups - control, uninfected group, and 2 infected groups, where one group was stocked with 10% of the population was infected (i.e., low, 5/50 fish infected) while the other group was stocked with 40% of the population was infected (i.e., high, 20/50 fish infected). For both trials, 3 replicate RAS units were used for each treatment group. Disease development and survival was followed for 14 days, and samples were collected on day 1 and 14 following the biosecurity breach. The innate immune responses were studied in the gills, olfactory organ and distal gut of salmon parr by molecular and histological evaluations. In addition, water quality was monitored during the trial. The results showed that during sub-trial 1, the survival was not dependent whether *Y. ruckeri* was introduced into the system once or three times. Significant alterations in the expression of immune genes were registered at T14. The genes *Lysozyme* and *Cathelicidin* were the genes most heavily affected, and they are both antibacterial defense genes. Sub-trial 2 showed that the mortality among the already infected fish were high compared to the rest, the changes in all genes were also registered at T1. The genes that were affected the most were *Interleukin 1β* and *Interleukin 10*, which are both cytokines. The water quality was not affected by pathogen breach in either of the two trials.

This study revealed that the way the pathogen enters the RAS affected the outcome of disease progression and immune response in salmon parr. Biosecurity breach via makeup water appeared to have a greater impact in parr on health than introducing an infected fish to the system in this study. These results will be valuable in improving biosecurity and developing disinfection protocols in RAS.

Keywords: Atlantic salmon, recirculating aquaculture system, biosecurity, *Yersinia ruckeri*

1 Introduction

The world's population is increasing at a rapid pace and is estimated to reach 9.7 billion by 2050 (FAO, 2020). With the increasing population comes an increasing demand for food, especially protein (Kumar et al., 2015; Roques et al., 2021). Aquaculture is the fastest growing food production sector in the world and this increase in production is needed to meet the growing demand from the consumers. Food and Agriculture Organization of the United Nations (FAO) has estimated the aquaculture production to reach 109 million tonnes in 2030 and this would be an increase of 32 percentage since 2018, with Asia being the largest producer (FAO, 2020). The growing demand from the consumers is not just regarding the access to product for consumption, but also when it comes to the sustainability of the production for the different products.

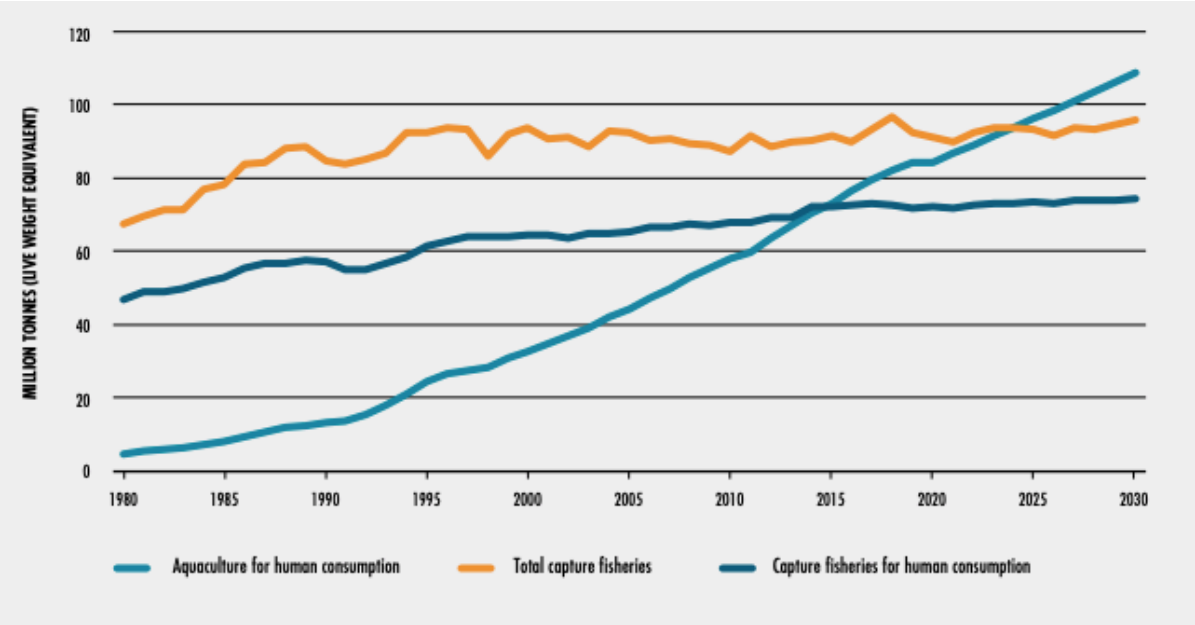


Figure 1: FAOs estimations of the global capture fisheries and aquaculture production from 1980-2030. Retrieved from FAO, 2020.

1.1 Norwegian aquaculture

Norway as the largest producer of Atlantic salmon (*Salmo salar*) has a responsibility to evolve in a sustainable direction when it comes to the production. The public is becoming more aware of sustainability and the impacts the products consumed has on the environment. From the Norwegian aquaculture industry, most of the environmental concerns escapees and sea lice infestation (Ellingsen et al., 2009). Sea lice and the removal of sea lice is considered the biggest health hazards within the Norwegian aquaculture industry (Walde & Sommerset, 2021). In Norway, an increasing number of the hatcheries and smolt production facilities are land based

and using recirculating aquaculture systems (RAS) (Lewin et al., 2020). Production of smolts requires a lot of water and by having the smolt production in RAS facilities the amount of water needed for the production is being drastically reduced. The earlier life stages and the transformation to smolt are more fragile stages for the salmon, this makes the adoption of RAS technology favorable for an increased production with a more stable and controlled environment for the fish (Gåsnes et al., 2021). This is favorable for the growth of the fish. Because of this the production cycle is shortened and the fish are ready for net pens earlier than in flow-through (FT) systems (Bergheim et al., 2009). During the parr phase, the biggest challenge over the years has been disease, this is both in traditional FT systems and RAS. There are now a number of effective vaccines and the fish are getting these before they are transferred to the grow-out farms (Munang'andu et al., 2016). The use of more RAS facilities in the aquaculture industry is also in line with UNs sustainability development goals, 2. *Zero hunger* with increasing the food produced to feed more people, 12. *Responsible consumption and production* by producing and consuming products that are produced in a more sustainable way, 13. *Climate action* by reducing the impact production has on the environment and 14. *Life on water* by reducing the impact production has on the oceans and reducing the stress on wild stocks.

1.2 Recirculating aquaculture system

RAS offers a better control of the conditions that the fish are living in and limit the outside environmental factors, such as the temperature, light and weather (Kolarevic et al., 2014). In a RAS loop (**Figure 2**) the water flows from the outlet of the fish tank to a mechanical filter where solids are removed, before it further continues to a biofilter where nitrifying bacteria are converting potentially toxic ammonia to nitrite and further to nitrate, on to a degasser where the water is aerated and stripped for carbon dioxide. Before the water is returned to the fish tanks it is oxygenated. Other components can be added onto the system to meet the exact requirements of the organisms cultured as well as the location where they are reared (Bregnballe, 2015). Being able to provide the optimal water quality for the aquatic organisms is essential for both growth, survival and welfare (Bugten et al., 2022). The Norwegian Food Safety Authority are providing the minimal and maximum recommended concentrations for the most important water quality parameters. The welfare of fish is closely related to the environment, as they are always living in it. This is not just water quality, but a large part of it. There are different welfare indicators that are water quality based such as temperature, salinity, oxygen, pH, ammonia (NH_3), nitrite, nitrate and turbidity. Atlantic salmon in the parr stage has

its own range of preferred values in all these parameters. For temperature, the preferred range is from 12-14 °C as parr naturally lives in rivers, with this the preferred salinity is from 0-10 ppm, as it is in fresh water. For oxygen in the parr stage the if the oxygen saturation is below 39% it has been found to be limiting for the fish. If parr are exposed to low (4,2-4,7) pH over a longer period it can affect the growth performance and smoltification later, having the pH between 6,5 and 7 is the preferred range. The total ammonia refers to the sum of NH_3 and ion ammonium (NH_4^+), as NH_3 reacts with water to create NH_4^+. This reaction goes both ways and how much of the ammonia that ends up as ammonium is primarily dependent upon the pH, with salinity and temperature also affecting it. If the pH, salinity and temperature is decreasing, so it the conversion of ammonia to ammonium. The exposure of salmon to ammonia is recommended for short term to stay below 0,1 mg L^{-1} and long term below 0,012 mg L^{-1}. Nitrite is converted to nitrate in the biofilter by the Nitrobacter bacteria, as nitrate is less toxic to fish, the recommended value for nitrite is 0,1 mg L^{-1} and for nitrate 100 mg L^{-1}. Turbidity is the clarity of the water and with a higher turbidity, it can be harder to observe the fish health and to see how they are feeding (Noble et al., 2018).

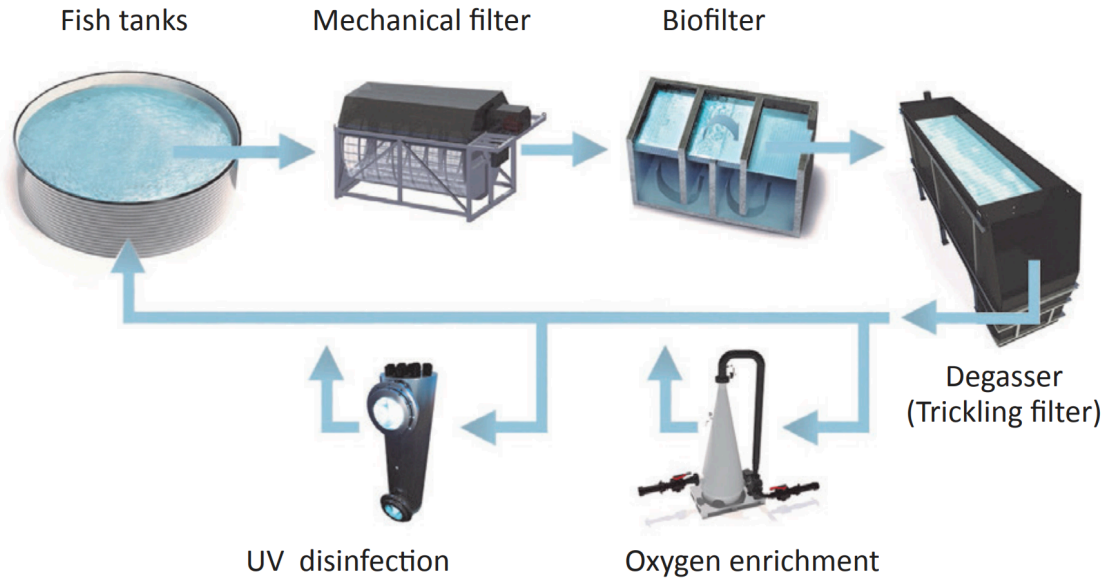


Figure 2: A simple RAS loop illustrating how the water moves through the different components. Retrieved from Bregnballe, 2015

Preventing disease causing agents or other agents that can pose a risk to both the organisms cultured and the outside environment is the biosecurity of a facility. In RAS as in any other aquaculture system is crucial to secure optimal health, welfare and production, there are however differences between system types. Even though RAS has a tighter biosecurity than

traditional FT systems, there are still challenges, such as in the system components (filtration), system inputs (water, fish, feed, chemicals) and the disease management. The filtration components must work properly - the mechanical filter removes solids and the biofilter transforms the wastes, these are dependent on the species and stocking density of the system. These components are working to keep the water quality at an optimal level for the fish in the system and reduce the risks of disease outbreaks.

The foot traffic within the facility, how things and people are being moved within a facility should be carefully considered, when it comes to shoes in certain areas and clothes. This is to prevent any potential cross contaminations between areas that would not be in contact if it weren't for the foot traffic. When it comes to animals carrying disease, they should be in isolation from the rest of the facility, this also includes new animals that could carry disease without it being noticeable. Health screening should be done on a regular basis at all life stages to prevent the development and spread of disease. The systems should also be housed in secure structures with doors that protects it from weather and keeps dirt out. Dirt, rainwater, pests such as rodents, insects and other animals could carry and spread disease and should not have access to a RAS facility. All the factors mentioned above are measurements taken to provide better biosecurity for RAS facilities and if they are working as they should the risk for disease to enter the system is minimized (Yanong, 2012).

RAS is ecosystem on its own, there are microorganisms present in all parts of the system, with the biofilter being where the concentration of microorganisms are the highest (Rutangwa & Verdegem, 2015). The microbial water quality all depends on the number and types of microorganisms and organisms present in the system, microbial control is focusing on keeping the numbers of bacteria low and preventing specific pathogens from entering the system. This is especially important when it comes to aquatic organisms as they are present in the water at all times (Fossmark et al., 2020).

1.3 Fish mucosal immune system

Fish are living in the water and are always surrounded by it, because of this the immune system of fish are different compared to terrestrial animals. The first line of defense is the gills, skin, intestine and the olfactory organ (Ringø et al., 2010) which are called the mucosal tissues. The mucosal tissues have other physiological roles and not just the first line of defense for fish. The gills are responsible for the osmotic, ionic and acid-based regulations, the skin has osmotic balance and sensory reception and the gut has catabolism and nutrient uptake (Cabillon &

Lazado, 2019). The mucosal health of fish is especially important as the mucosal surfaces is the barrier protecting them against the environment, they are living in. The barrier is important when it comes to the maintenance of homeostasis in both the tissue and cells (Gomez et al., 2013; Marjoram et al., 2015). When a pathogen first comes in contact with a fish, it first encounters the mucosal surfaces; the mucus, scales and epithelium limit provide a physical and biological barrier. The skin, gut and the gills have some functional and structural differences, but there are certain structures that they share in common as mucosal tissues.

The mucus layer has a key defense function, including trapping of the pathogen and direct action through various antimicrobial factors. The antimicrobial factors present in the mucus include lectins, lysozymes, immunoglobulins and anti-bacterial peptides. (Peatman & Beck, 2015). Fish like any other animal is sensitive to factors such as pathogens and bacteria that can cause cellular damage (Løkka & Koppang, 2016). Compared to terrestrial animals, this is very different in fish and the surroundings will affect them more as they are in the water at all times (Austin, 2006). The most common factor for causing damage on the intestinal mucosal barrier of Atlantic salmon are pathogens (Zhang et al., 2020). In RAS especially, where the water is reused to a large extent, pathogens in the system are a serious concern. Pathogens can be introduced to RAS a number of different ways, fish, water, staff and feed are some of the more common ones (Blancheton et al., 2013). Therefore, effective biosecurity measures are necessary.

1.4 Yersinia ruckeri

The pathogen *Yersinia ruckeri* causes enteric red moth disease (ERM) and has been shown to thrive well in a RAS environment, especially in the biofilter (Glenn et al., 2011). *Y. ruckeri* is a Gram-negative rod-shaped enterobacterium which is known to infect salmonids at all life stages, with the earlier life stages being more susceptible to infection. It was first discovered in farmed rainbow trout (*Oncorhynchus mykiss*) in Idaho, USA. It has become one of the most common pathogens in farmed salmonids and is responsible for great economic losses in the industry (Pajdak-Czaus et al., 2019). Fish infected by *Y. ruckeri* often have change in their behavior such as swimming near the surface and loss of appetite. When taking a closer inspection of the fish there can often be observed darkening of the skin, red hemorrhages in and around the mouth and throat is also common. There are two known biotypes of *Y. ruckeri*, biotype 1 (BT1) and biotype 2 (BT2). The main differences between the two is that BT1 are motile and has a detectable flagella, BT2 does not have that (Davies & Frerichs, 1989). The

ERM outbreaks in the aquaculture industry is often associated with the BT2 strains. It is thought that the BT2 strains are lineages that has arisen individually multiple times from BT1, this suggests that there is an advantage to the BT2 strains in the aquaculture industry (Wheeler et al., 2009; Wiens & Vallejo, 2010). The gills are known for being the main entrance of *Y. ruckeri,* the pathogen spreads via the bloodstream to the other organs. It has been shown that shortly after fish is infected by *Y. ruckeri* the pathogen can be detected in the intestine (Ohtani et al., 2014). Outbreaks of *Y. ruckeri* is often related to either poor water quality, high stocking densities, other environmental stressors or a combination of the different factors (Tobback et al., 2007).

Figure 3: Fish infected with Yersinia ruckeri. A: exophthalmia, B shows the enlarged spleen and C: shows some hemorrhages on the skin. Credit: Carlo Lazado.

1.5 Objective

With the increasing use of RAS in Norwegian aquaculture sector, there is a need for a greater understanding on how pathogens breach the biosecurity protocol, enter the system, and cause disease outbreaks. The main aim of this study was to simulate a biosecurity breach in RAS using *Yersinia ruckeri* as a model pathogen and investigate how this affected Atlantic salmon parr survival and mucosal immune responses. In sub-trial 1, *Y. ruckeri* is introduced to the system via the makeup water, making the water as the vector for the pathogen. In sub-trial

2, the pathogen was introduced to the system via fish that had been infected prior to stocking them to the RAS units.

2 Materials and methods

2.1 Ethical statement

The trials were approved by the Norwegian Food Safety Authority (Mattilsynet) under FOTS ID number 26793 and was conducted having the 3R´s in mind. The number of fish used were minimized and the infection was not allowed to progress severely.

2.2 Fish source

Atlantic salmon (*Salmo salar*) produced at Tromsø Aquaculture Research Station was hatched and raised in freshwater flow-through system under constant daylight (LD 24:00) before they were transferred to the Fish Health laboratory. The parr had starting weight of 12.2 ± 2.3 g in Sub-trial 1 and 11.9 ± 2.5 g in Sub-trial 2. The water quality was kept at an optimal level and the fish were fed a commercial diet to apparent satiation.

2.3 Experimental design

The experiment was conducted at the fish health laboratory of the Tromsø Aquaculture Research Station in Kårvika. It included nine separate RAS in the same room all consisting of individual 500-liter tanks, drum filter, moving bed biofilter (MBBR), CO_2 degasser, down-flow bubble contactor, air to water heat pump (for cooling and warming the water), flowmeter, oxygen-, pH- and water level probes. **(Figure 4)** (Mota et al., 2022). **Figure 4A** illustrates the whole room with the 9 RAS systems and **Figure 4B** illustrates one individual with the different components.

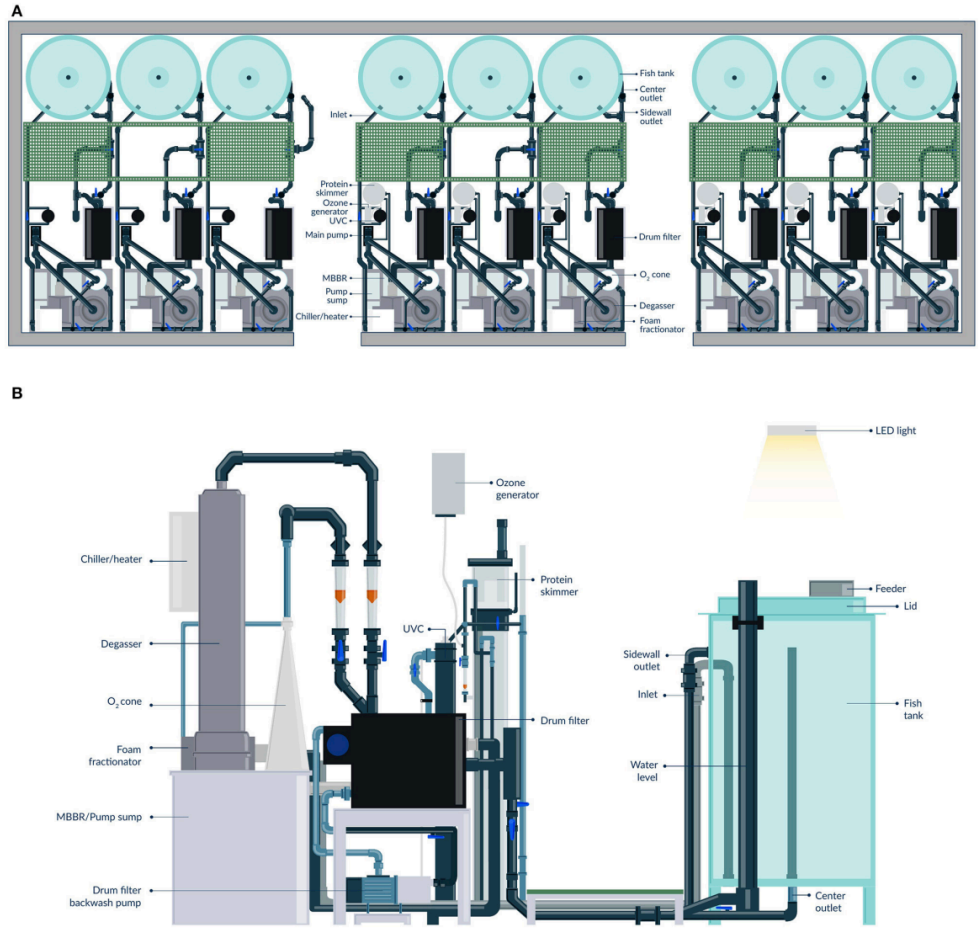


Figure 4: Illustration of the RAS systems in Kårvika, A: illustrates the room with the nine different systems, B: is an up-close illustration of the system with all the components. Retrieved from Mota et al., 2022.

The trial consists of two sub-trials where *Y. ruckeri* is introduced to the system in two different ways, during the first sub-trial the water acts as a vector for the pathogen and during the second sub-trial infected fish will work as a vector. The two ways for the pathogen to enter the system illustrates two realistic ways that a pathogen may enter and spread in a RAS facility. By having the two trials done in the same system and on the same species of fish, it is possible to compare how the different entry ways affect the mucosal immune system of Atlantic salmon parr.

2.4 Culture of Yersinia ruckeri

The *Yersinia ruckeri* strain used during the trials were: *Yersinia ruckeri* 2014-70 646 (serotype O1), which was originally received from the Veterinary Institute in Harstad 20.02.15. Media: Blood agar (BA) without NaCl. Frozen cultures were put on BA and checked after 2-3 days; single colonies were then picked for further culturing in liquid media. The isolate was cultured in Brain Heart Infusion broth at 12-15℃ for 24 hours with constant agitation.

2.5 Sub-trial 1, water as a vector for pathogen entry to RAS

Sub-trial 1 had *Y. ruckeri* introduced to the system through the water, with 3 control systems, 3 systems with single entry (SE) and 3 systems with multiple entry (ME). There were fifty (50) fish stocked in each individual RAS (**Figure 4**). The fish were stocked in the individual RAS for acclimation 7 days before the trial started. For the control group, no *Y. ruckeri* was added. The 24-hour culture of *Y. ruckeri* with a concentration of about 10^8 cfu/ml was introduced via the make-up water for the SE tanks at 1% (v/v) per total daily volume (ca. 20 L/day) on day 0. For the ME tanks the 24-hour culture of *Y. ruckeri* (ca. 10^8 cfu/ml) was introduced at three different time points, during day 0,1 and 2 via the make -up water at 1% (v/v) per daily volume of ca. 20 L/day. The trial ran over a span of two weeks (**Figure 5**). During this sub-trial, the water exchange rate for the systems started at 0 L for all systems at T0, at T14 all the system had less than 400L of water exchanged throughout the trial period.

2.5.1 Sampling

Different samples from the system and the fish were collected during the trial. The fish sampling and the swabs from the systems were sampled on the same day while the water quality measurements were done on the days prior to fish sampling, this to provide more accurate water quality measurements as the fish were not fed during the sampling days. Control tanks were sampled first, before the ones with low infection and the ones with the high infection were sampled last (**Figure 5**).

2.5.1.1 Fish sampling

Fish were sampled on two days during the two-week period, at day 1 (T1) and day 14 (T14). Five random fish were taken from each tank and were humanely euthanized with an anesthetic overdose using Benzocaine (Benzoak vet, 200 mg/ml, EuroPharma, Leknes, Norway). The fish were then given a running number, length and weight were also recorded. Samples for histology in 10% formalin and samples in RNA-later for qPCR were collected (**Table 1**). The tissues collected were skin from below the dorsal fin on the left-hand side of the fish, the first two gill arches on the left, both olfactory organs, spleen and the distal gut for both formalin and RNA-later. During the sampling, scalpels and gloves were changed with every fish and all the other equipment were wiped down with alcohol in between the different fish to prevent any contamination.

Table 1: Summary of the samples, from where they are collected and what they are used for.

Tissue/organ	RNAlater	Formalin
Skin	1x1 cm skin from the left side under the dorsal fin	1x1 cm skin from the left side under the dorsal fin
Gills	Fist gill arch from the front, left side	Second gill arch from the front, left side
Distal gut	The ½ distal gut, right in front of the anus	The ½ distal gut, right in front of the anus
Olfactory organ	1 olfactory organ, the whole nostril with bone was cut out	1 olfactory organ, the whole nostril with bone was cut out
Spleen	½ of the spleen	½ of the spleen

2.5.1.2 Surface swabs from the RAS

Swabs from different parts of the system were collected on the same days as the fish were sampled (T1 and T14). Swabs were collected from 4 different parts of the system. The intake water chamber, in the degasser, tank wall and wall of the water outlet pipe. Bio filter media were also collected on the same days. The swabs were put into RNA-later and the bio filter media were collected in sterile bags, put on ice and stored at -70°C upon arrival in the laboratory.

2.5.1.3 Water quality sampling

Water quality was measured at four days during the trial, on the day before *Y. ruckeri* were introduced (T-1) and one day prior to the end of the trial (T13). The measurements taken were salinity, pH, dissolved oxygen (O_2), temperature, ammonium (NH_4), nitrite (NO_2), nitrate (NO_3), total alkalinity (as $CaCO_3$) and turbidity. The water quality was measured both from the inlet and the outlet water (**Figure 5**). A probe was used to measure salinity, pH, dissolved oxygen and temperature. Prior to the first measurements the pH probe was calibrated. For NH_4, NO_2, NO_3 and $CaCO_3$ water quality kits from VWR was used according to the manufacturer's recommendations. Turbidity was measured using a turbidity meter.

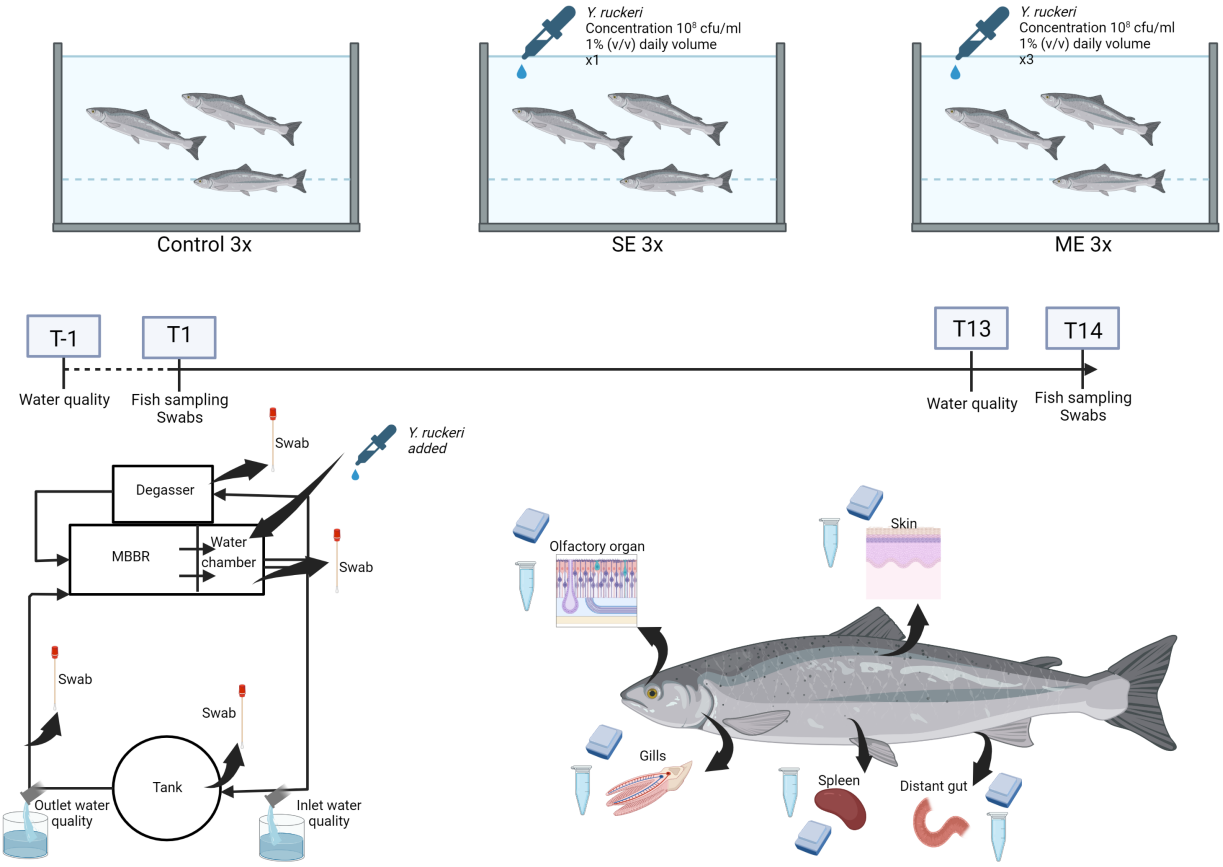


Figure 5: Sub-trial 1: Illustration of the sampling timepoints with the location of the water quality samples, swab samples and the tissue samples from the fish collected. Created with BioRender.com

2.6 Sub-trial 2, fish as a vector for pathogen entry to RAS

Sub-trial 2 has fish as the vector for introduction of *Y. rukceri* to RAS: 9 systems were used, 3 control, 3 low infection and 3 high infection systems. There were 50 fish in each tank, for the control group 0 out of the 50 fish were infected, for the low infection tanks 5 out of the 50 fish were infected and for the high infection tanks 20 out of the 50 fish were infected with *Y. ruckeri*. The fish prior to the start of the trial were divided into two groups, group 1 was the uninfected group with 375 fish and group 2 the infected group with 75 infected fish. The fish were reared in 1000L tank flow-through system prior to the transfer. Three days before infection, the fish from group 2 were sedated and tagged. The fish were tagged with Visible Implant Elastomer (VIE) tags near the dorsal fin. After the tagging, the fish were returned to the tank and allowed to recover for three days before the bath exposure to *Y. ruckeri.* The fish from group 2 were infected with *Y. ruckeri* using a bath exposure, the fish was transferred to 20L tanks for seven hours with 5 L of water that had a temperature of 15 °C. The fish had

constant aeration and it contained 6.33x10^8 CFU/ml *Y. ruckeri.* This infection protocol was according to Villumsen et al., 2014 . After the bath exposure of group 2, both the infected and uninfected fish were transferred to the RAS units according to the distribution plan (**Figure 6**). The trial ran for 14 days.

2.6.1 Sampling

The sampling regime for the second sub-trial were the same as sub-trial 1 when it came to the timepoints, samples collected and from where they were collected (**Figure 6**).

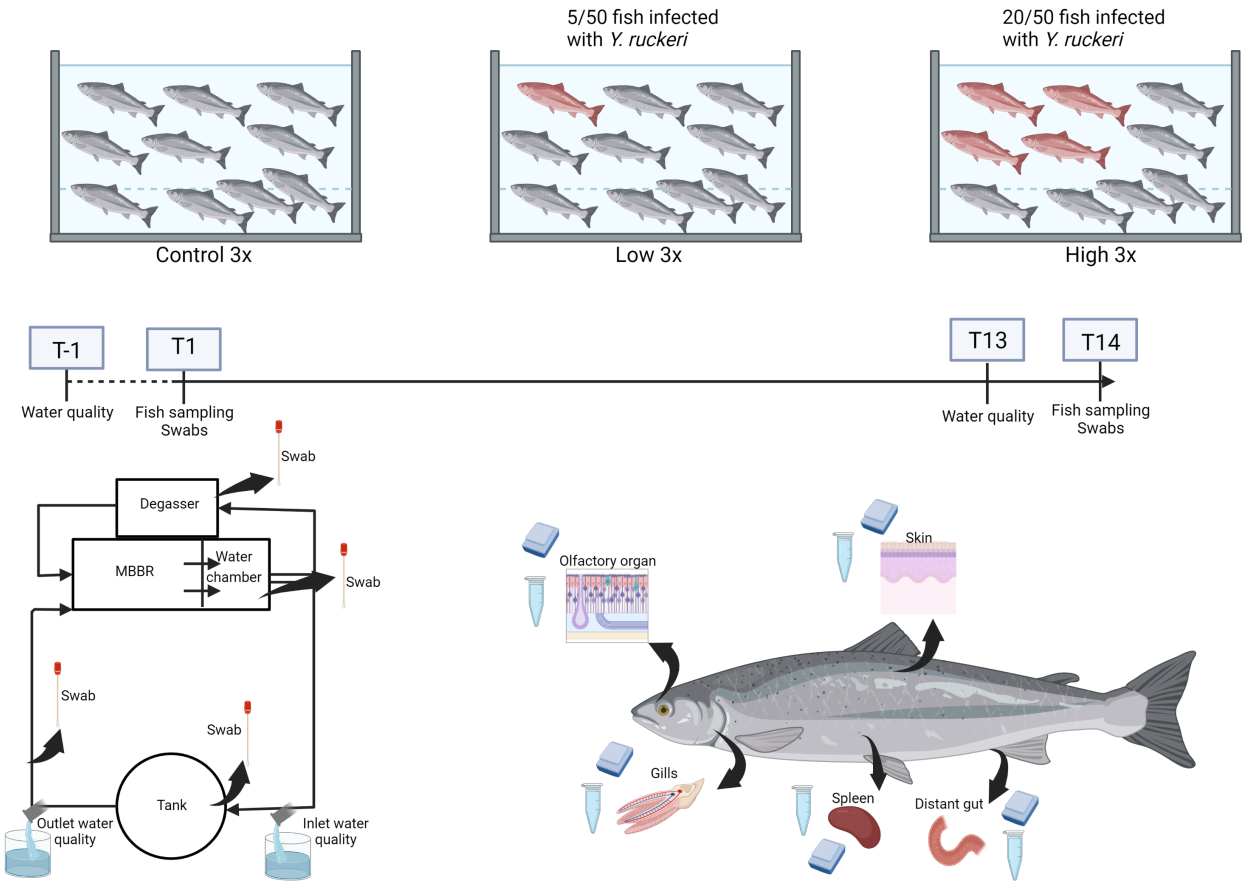


Figure 6: Sub-trial 2: Illustration of the sampling timepoints with the location of the water quality samples, swab samples and the tissue samples from the fish collected. The red fish illustrates the percentage of infected fish per system. Created with BioRender.com

2.7 qPCR and histology

The tissues that are being analyzed were the olfactory organ, gills and the distal gut. These tissues collected at T1 and T14 for both sub-trials were used for the qPCR and histology.

2.7.1 Quantitative polymerase chain reaction (qPCR)

Prior to the qPCR lab work, all the samples were entered into a template to get a good overview when working with the sample plates. qPCR process started with the RNA from the sample being isolated, the first step was to homogenate the tissues.

Homogenization of tissues:

1. 2x 2,8mm metal beads per 1 ml tubes in strips.

2. Added lysis buffer from the mirVana kit (27828 applied biosystems by Thermo Fisher Scientific, Vilnius, Lithuania), the concentration for the different tissues is stated in table 1.

3. Turned on the heating cabinet (37 C)

4. Strips lid were added to the tubes.

5. The tissues were homogenized using a FastPrep (Precyllys 24, FastPrep96, MP Biomedicals) for time stated in table 1 for the different tissues.

6. the samples were spun down.

7. Incubated the samples on 37 degrees Celsius from 25 minutes till 1,5 hours.

8. The samples were then transferred to another tray for storage at -80 degrees.

Table 2: Tissue concentration for the homogenization, the time and rpm used to fully homogenize the different tissues, the number of metal beads used.

Tissue	Tissue concentration	Homogenization	Beads
Gills	20 mg tissue/ 400 µl lysis buffer	2x 120 sec. x 1800 rpm	2 metal 2,8 mm
Distal gut	20 mg tissue/ 400 µl lysis buffer	2x 120 sec. x 1800 rpm	2 metal 2,8 mm
Olfactory organ	20 mg tissue/ 400 µl lysis buffer	1x 120 sec. x 1800 rpm	2 metal 2,8 mm

After finishing the homogenization of all the tissue samples, RNA was isolated from the homogenate. This was done using the mirVana kit (27828, applied biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) in the MagMax-Express 96 (applied biosystems by Thermo Fisher Scientific).

1. The samples were taken out from the -80-degree freezer and thawed at room temperature.

2. All the plates for the MagMAX96 were prepared according to the instructions for the machine (appendix: Plates and content for MagMAX96). The sample plate has several steps firstly the homogenate and lysisbuffer was added and they were mixed at 300 rpm for 5 minutes before 100 µl isopropanol was added to each well and mixed for another 2 minutes. Finally, the binding bead mix was added to the filled wells and mixed for another 5 minutes at 300 rmp.

3. After preparing all the plates, they were run in the MagMAX96 according to the instructions from the machine.

4. When the plates were done at the MagMAX96, the samples were transferred to new plates and spun down.

The RNA quality from the isolation were checked and determined using a NanoDrop8000 (Thermo Scientific), a selection of the samples was also analyzed using a Bioanalyzer (2100 Bioanalyzer, Serial no. DE13806315, Agilent). After getting the RNA quality, this is entered into the same template from earlier to calculate the water:RNA ratio needed to get an RNA concentration of 22,2 ng/µl for all the samples. The samples were then diluted to have a concentration of 22,2 ng/µl.

cDNA was synthesized using the High-Capacity RNA-to-cDNA™ kit (4387406, applied biosystems by Thermo Fisher Scientific, Vilnius, Lithuania). The mastermix for cDNA was prepared according to the packaging. 9 µl of the 22,2 ng/µl RNA samples was added to the plate with the mastermix. The plate was then spun down and put on a Thermocycler (GeneAmp®, PCR system 2700, applied biosystems by Thermo Fisher Scientific) with the following program: 37°C for 60 minutes, 95°C for 5 minutes and then down to 4°C ∞. The plate was then spun down again. After following the protocol for the cDNA synthesis, the cDNA was diluted 1:10, with 20 µl cDNA +180 µl H_2O, on the same plate as the cDNA was

synthesized on. After H_2O was added the plate was mixed and spun down before the cDNA was further diluted till 1:40 on a new plate. 500 µl cDNA at 1:40 was prepared for all the samples. A positive control was made using a mix of different samples from the different tissues. The trays were then kept at -20℃ for storage.

Forward and reverse primers were prepared according to the instruction on the packages to make a concentration at 100 µM this was then further diluted to a working solution at 5 µM. The primers have been used on Atlantic salmon tissue before and did not need any testing to determine if they would work on salmon or not. Three housekeeping genes were used, 18s, elfa and β-actin. The housekeeping genes and the other genes used can be found in table 3 below. The primers were provided by invitrogen by Thermo Fisher Scientific. The source for the primers used are what articles where they have been used earlier, all the primers had been used on Atlantic salmon prior (Eslamloo et al., 2020; Jenberie et al., 2018; Mutoloki et al., 2010), therefore there was no need for testing primers.

Table 3: Genes used for qPCR with the sequence for both the forward and reverse primers used. The source is from what articles the primers used has been found in.

Gene qPCR		Primer sequence 5' to 3'	Source
Hepcidin (hamp)	Forward	ATGAATCTGCCGATGCATTTC	Eslamloo et al., 2020
	Reverse	AATGGCTTTAGTGCTGGCAG	
Cathelicidin (camp)	Forward	AAGCCAGAAAATGCTCCAGA	Eslamloo et al., 2020
	Reverse	ACCCTCAGGACGACCAATTA	
Interleukin 1 β (il1b)	Forward	GTATCCCATCACCCCATCAC	Eslamloo et al., 2020
	Reverse	TTGAGCAGGTCCTTGTCCTT	
Interferon regulatory factor 1 (irf1)	Forward	GCAATGAAGTAGGCACAGCA	Eslamloo et al., 2020
	Reverse	CGCAGCTCTATTTCCGTTTC	
Interleukin 10 (il10)	Forward	CGCTATGGACAGCATCCT	Mutoloki et al., 2010
	Reverse	AAGTGGTTGTTCTGCGTT	
Interferon gamma (ifng)	Forward	CCGTACACCGATTGAGGACT	Eslamloo et al., 2020
	Reverse	GCGGCATTACTCCATCCTAA	
Lysozyme (lys)	Forward	CACCGACTATGGCATCTTCC	Mutoloki et al., 2010
	Reverse	CTGACCGCCACTGTGATGTC	
Cathepsin D (ctpd)	Forward	CAGGCTGGTAAGACCATCTGC	Mutoloki et al., 2010
	Reverse	TGTTGTCACGGTCGAACACAG	
Mucin 5AC (muc5ac)	Forward	GACCTGCTCTGTGGAAGGAG	Jenberie et al., 2018
	Reverse	AGCACGGTGAATTCAGTTCC	
Mucin 5B (muc5b)	Forward	ATTAAGAGCGATGTCTTCACAGC	Jenberie et al., 2018
	Reverse	AAGCACATGAGTCTCTCACACAA	
Mucin 2 (muc2)	Forward	GAGTGGGCTCTCAGATCCAG	Jenberie et al., 2018
	Reverse	GATGATGCGGACGGTAGTTT	
18s	Forward	TGTGCCGCTAGAGGTGAAATT	Kileng et al., 2007
	Reverse	GCAAATGCTTTCGCTTTCG	
β-actin	Forward	CAGCCCTCCTTCCTCGGTAT	Julin et al., 2009
	Reverse	CGTCACACTTCATGATGGAGTTG	
Elongation factor 1 alpha (elfa)	Forward	CGCCAACATGGGCTGG	Kileng et al., 2007
	Reverse	TCACACCATTGGCGTTACCA	

The different genes were run on all tissues and timepoints for both sub-trials on the QuantStudio5 (applied biosystems by Thermo Fisher Scientific), the data was all collected in QuantStudio™ Design & Analysis Software. Positive control and NTC were checked to see if there were any abnormalities or if the data could be accepted.

All the equipment and solutions used for the preparation and running of the qPCR are listed in the appendix.

2.7.2 Histology

The same three tissues as for the qPCR was used for the histology (gills, olfactory organ and distal gut). 24 hours and 2 weeks post infection were the timepoints used. The gills and olfactory organ samples were taken from the formalin and placed in the embedding cassette,

they were labeled according to the tank and timepoint sampled, 5 and 5 samples from the same systems and timepoints were placed together. The gills and olfactory organ were decalcified for 3 days in a solution of 10 10% Triplex 3X (Sigma Aldrich). The samples (olfactory organ gills and distal gut) were then bathed in 70% ethanol and kept there until being transferred into the automated tissue processor (TP1020, Leica biosytems, Nussloch, Germany), dehydration, clearing and paraffin infiltration was preformed according to the table below.

Table 4: Protocol steps used for the histopathology tissue processing.

Step	Solution	Time	Step	Solution	Time
1	70% EtOH	~	8	Xylen	30 min
2	90% EtOH	1h	9	Xylen	30 min
3	90% EtOH	1h	10	Xylen	30 min
4	96% EtOH	1h	11	Paraffin	1h
5	100% EtOH	30 min	12	Paraffin	2h
6	100% EtOH	30 min			
7	100% EtOH	30 min			

The samples were embedded 5 and 5 together (same tissue, tank and timepoint) using a heated paraffin module (Leica EG1150H, Leica biosystems, Nussloch, Germany). After the paraffin had solidified the samples were ready to be sectioned, this was done using a rotatory microtome slider (Leica RM2165, Leica biosystems, Nussloch Germany): the samples were cut in sections at 5µm and placed on a microscope slide (Surgipath, Leica Biosystems, Illinois, USA), then transferred into the oven at 60°C for the heat fixation and dehydration to remove the excess paraffin. The staining was done using an automated stainer (ST5010, Leica Biosystems) with Periodic Acid Schiff-Alcian Blue (AB-PAS) according to the protocol below. The slides were after that covered with an automated cover-slipper (Leica CV5030 Robotic Cover-slipper, Leica Biosystems). The slides were after this scanned using a slide scanner (Aperio CS2 slide scanner, Leica Biosystems)

Table 5: Protocol for Periodic Acid Schiff-Alcian Blue (AB-PAS) staining.

Step	Solution	Time	Step	Solution	Time	Step	Solution	Time
1	Xylen	5 min	9	Alcian Blue	15 min	17	Ammonia	1 min
2	Xylen	5 min	10	Wash	3 min	18	Wash	30 sec
3	Abs	3 min	11	Periodic Acid	10 min	19	70% EtOH	15 sec
4	Abs	3 min	12	Wash	3 min	20	96% EtOH	15 sec
5	96% EtOH	2 min	13	Schiffs	15 min	21	Abs	30 sec
6	96% EtOH	2 min	14	Wash	5 min	22	Abs	1 min
7	70% EtOH	2 min	15	Haemato xylin	30 sec	23	Xylen	1 min
8	Wash	3 min	16	Wash	2 min	24	Xylen	1 min

2.7.3 Swabs and external analysis

The swabs taken from the system and the collected biofilter media were sent to an external lab for detection of the pathogen in the system, further this will not be a part of this thesis. Tissue samples (gills, distal gut, olfactory organ and spleen) from both sub-trials at T14 was sent to PHARMAQ for detection of the pathogen *Yersinia ruckeri*. qPCR was performed and the results came back with ct-values and the level of detection. Two samples per system for each tissue was sent to PHARMAQ a total of 18 samples per tissue per sub-trial.

2.8 Statistical analysis and histological assessment

Excel was used to do simple statistics, mean and standard deviation for all the data collected. For the qPCR data the ΔΔCt method was used to calculate the Ct-values used later. The survival percentage was also calculated for both trials, for sub-trial 2 both with and without

the already infected fish. The mean and standard deviation for the water quality data was also calculated to determine if there were any significant differences. Excel was also used for the results from PHARMAQ to sort the results. SPSS was used to perform the two-way ANOVA to determine if there were differences at the different timepoint when comparing the control group to both high and low infection. Prior to the two-way ANOVA the data was checked for normality and any outliers was removed if they were significantly different compared to the rest of the samples. R studio was used to perform a PCA analysis using the script from the appendix looking at all the tissues, treatments and genes for both sub-trials at the beginning and end timepoint.

Histology plates were analyzed using scales that are tissue specific from 0-3, where 0 are healthy tissue and 3 is tissue that has clearly been affected to a large extent by disease. All the plates were scored two times and if there were any differences between the two scorings the plate was scored a third time to determine the final score of the plate. The scoring was based on the appearance of the tissue, the percentage of tissue loss, how defined the structures were and the smoothness of the surface. Histology scoring all depends on the person scoring and how they interoperate the scans, because of this multiple scoring were done to get as accurate results as possible. The different scorings were done with days a part to ensure that they were done with less bias. The scoring schemes used for the different tissues is found in the appendix.

3 Results

3.1 Sub-trial 1, water as vector for pathogen entry to RAS

The water quality measurements were done at both the inlet and outlet water of the tank. When comparing the measurements form the systems there was no significant difference between the 9 different systems when it came to the water quality measurements taken. The 9 inlet values were compared to each other, and the 9 outlet values were compared to each other. For the O_2 in the water quality table all values were also above 98%. The total alkalinity for all systems were too low to be measured and therefore for all systems the results were just LO.

Table 6: Average measured (± STDEV) water quality for the different treatment groups at the first measuring point (T-1), with both inlet (I) and outlet (O) water.

	T-1					
Water quality parameters	Control		SE		ME	
	I	O	I	O	I	O
NH4	0,01±0,11	0,05±0,03	0,01±0,06	0,02±0,01	0±0,02	0,01±0,02
NO2	0,04±0,01	0,04±0,01	0,02±0,01	0,02±0,01	0,03±0,01	0,02±0,01
NO3	1,37±1,01	1,23±0,92	1,83±1,04	1,07±0,53	1,53±0,87	2,13±1,31
Total alkalinity	LO	LO	LO	LO	LO	LO
Turbidity	1,52±0,81	2,14±0,42	1,03±0,46	1,37±0,74	1,31±0,38	1,35±0,56
pH	6,63±0,32	6,81±0,53	6,83±0,00	6,76±0,05	6,82±0,05	6,82±0,05
Salinity	0	0	0	0	0	0
O2	10,13±0,2	9,94±0,18	10,21±0,1	10,34±0,12	10,30±0,00	10,31±0,05
Temp	12,81±0,05	12,82±0,15	12,5±0,17	12,53±0,21	12,51±0,17	12,51±0,23

Table 7: Average measured (± STDEV) water quality for the different treatment groups at the last measuring point (T13), with both inlet (I) and outlet (O) water.

	T13					
Water quality parameters	Control		SE		ME	
	I	O	I	O	I	O
NH4	0,01±0,07	0,02±0,03	0,01±0,01	0,01±0,03	0,02±0,02	0,01±0,05
NO2	0,04±0,05	0,03±0,01	0,02±0,05	0,017±0,05	0,02±0,00	0,02±0,00
NO3	5,31±0,95	5,10±0,52	4,73±1,03	4,83±0,59	3,44±1,15	4,23±0,35
Total alkalinity	LO	LO	LO	LO	LO	LO
Turbidity	1,31±0,41	1,23±0,71	1,06±0,28	2,02±1,26	1,28±0,19	1,73±0,76
pH	7,12±0,21	6,91±0,06	6,94±0,06	6,69±0,26	6,84±0,31	6,52±0,67
Salinity	0	0	0	0	0	0
O2	10,61±0,34	10,33±0,44	10,45±0,12	10,17±0,05	10,45±0,40	10,31±0,29
Temp	12,73±0,06	12,82±0,06	12,71±0,36	12,72±0,31	12,71±0,32	12,73±0,23

The survival rate for the control tanks were at 100%, there was no mortality in either of the three tanks throughout the 14-day trial. For the SE systems (nr 4, 5 and 6) the survival rates were at 89,3%, 82,1% and 75,4% having an average survival rate of 82,3% for all the SE systems. The ME systems (nr 7, 8 and 9) had survival rates at 84,5%, 73,8% and 91,1% with an average of 83,1% survival during the trial. **Figure 7** illustrates the average survival per treatment group. The mortalities started at T7 after *Y. ruckeri* was introduced to the systems for both the SE and the ME systems (**Figure 8)**. All the infected systems had mortalities on T7 except for system nr 9 which did not have any mortalities until T10.

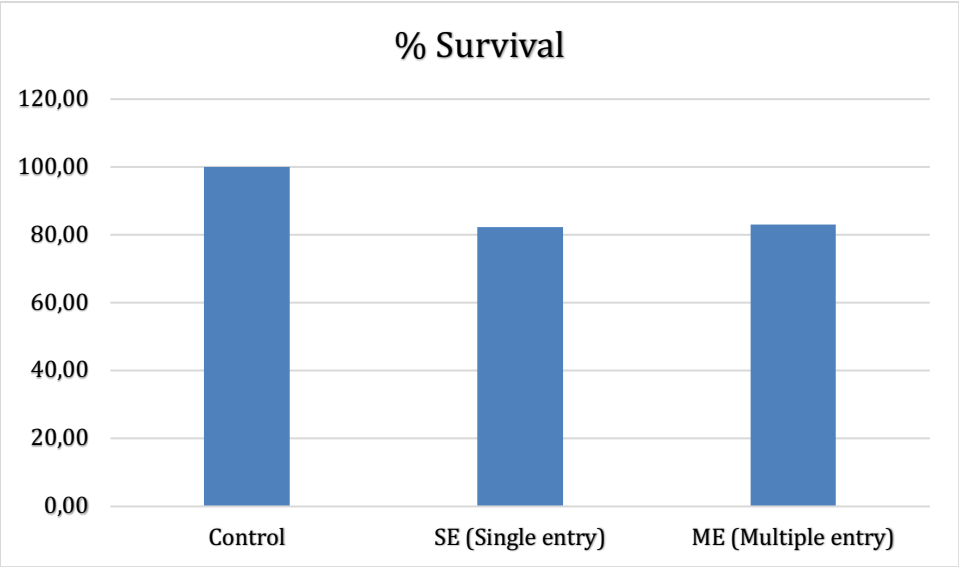


Figure 7: The average survival percentage for the different treatments.

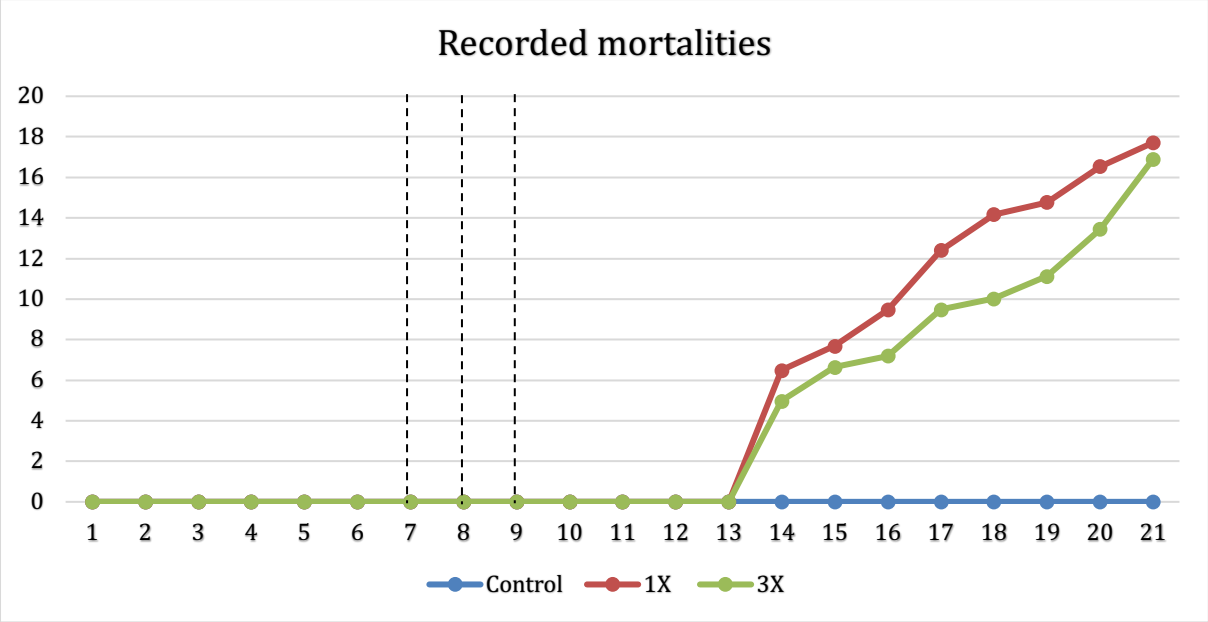


Figure 8: The recorded mortalities for the different treatments. The dotted lines illustrate the days Y. ruckeri was introduced to the system. The days prior to day 7 is the acclimation period. Note: 1X= SE and 3X=ME

The samples sent to PHARMAQ for detection of *Y. ruckeri* included some samples from the spleen (**Table 8**). There were in total 18 (3 per treatment) samples per tissue sent for analysis from T14, at the end of the trial. There were 6 samples per treatment group and 2 from each tank. There was no detection in any of the samples from the control group for either of the tissues. ½ of the samples from the SE and ME treatment groups came back positive for the gills, in 2/6 samples the detection of the pathogen was low and 4/6 the detection was moderate. For the spleen there were one more positive detection than in the gill samples, out of the 7 positive, one had low detection and the other six had moderate detection the pathogen. The distal gut

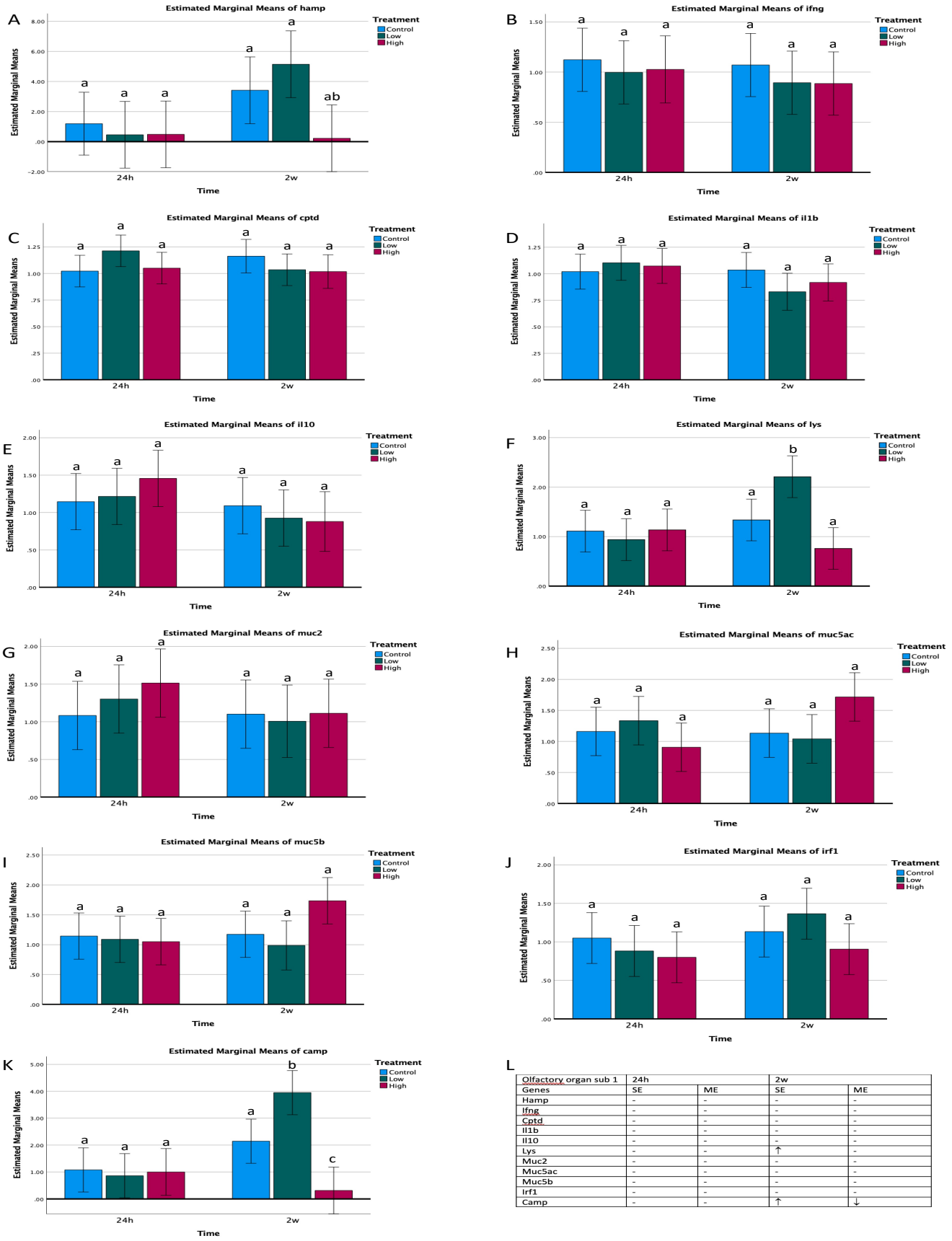
only had one positive sample from the 18 sent in for detection, the positive sample was moderate and from the ME infection group.

Table 8: Number of samples sent to PHARMAQ per tissue and the percentage of positive and negative per treatment group.

Tissue	Total number of samples			Positive (%)			Negative (%)		
	Control	SE	ME	Control	SE	ME	Control	SE	ME
Olfactory organ	6	6	6	0%	0%	0%	100%	100%	100%
Gills	6	6	6	0%	50%	50%	100%	50%	50%
Distal gut	6	6	6	0%	0%	16,7%	100%	100%	83,3%
Spleen	6	6	6	0%	66,7%	50%	100%	33,3%	50%

3.1.1 Olfactory organ

Comparing the SE and ME groups to the control group, there were no genes with any significant difference 24 hours post infection. At T14 the ct-values for the SE groups were an upregulation for both *lysozyme (lys)* and *cathelicidin (camp)*. Comparing the ME groups to the control, camp was downregulated at T14 (**Figure 9**).



L

Olfactory organ sub 1	24h		2w	
Genes	SE	ME	SE	ME
Hamp	-	-	-	-
Ifng	-	-	-	-
Cptd	-	-	-	-
Il1b	-	-	-	-
Il10	-	-	-	-
Lys	-	-	↑	-
Muc2	-	-	-	-
Muc5ac	-	-	-	-
Muc5b	-	-	-	-
Irf1	-	-	-	-
Camp	-	-	↑	↓

Figure 9: Expression of immune genes (A: hamp, B: ifng, C: cptd, D: il1b, E: il10, F: lys, G: muc2, H: muc5ac, I: muc5b, J: irf1, K: camp) in the olfactory organ. Values are presented as mean ± SD of 9 individual fish per treatment group. L: The upregulation and downregulation of the different genes in a table. Note: Low=SE and High=ME

The histology scoring for olfactory organ was 0 for all the control groups at both T1 and T14. For the SE groups the score was 1 for all the samples at T1, at T14 the score was still 1 for all the systems except for nr 6 where the score was 2 at the end of the trial. The ME group had one system with the score 0, one with 1 and one with 2 at T1. At T14 both the samples from system nr 7 and 8 got the score 1, while the samples from system 9 got a score of 2. **Figure 10** shows three different olfactory organs from **Figure 10A** from the control group at T14, **Figure 10B** is from the SE treatment group at T14 and **Figure 10C** is from the ME treatment group at T14. The blue dots are the mucosal cells, they have been stained by the AB-PAS. Magnitude on the **Figure 10** are 300 um

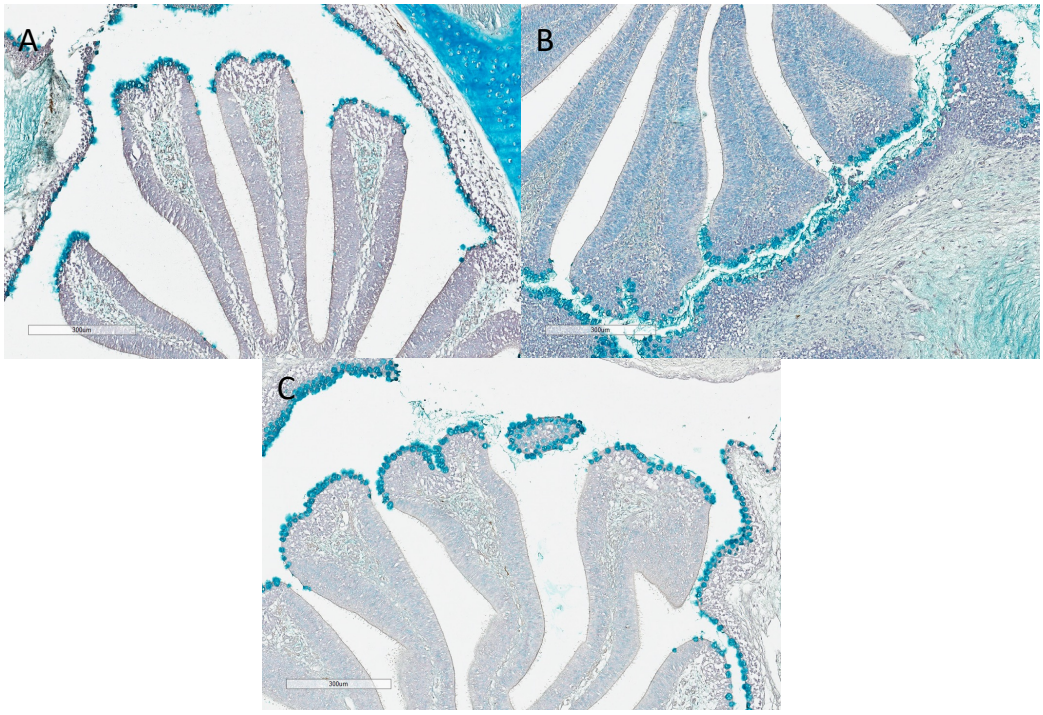
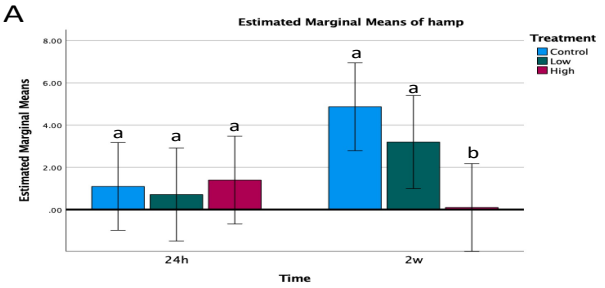


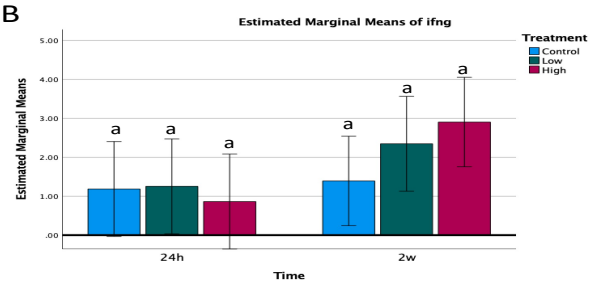
Figure 10: Olfactory organ histology slides. A: a healthy olfactory organ from the control group, B: olfactory organ from the SE treatment group showing the structure not as well defined, C: olfactory organ from the ME treatment group also showing loss of the well-defined structure.

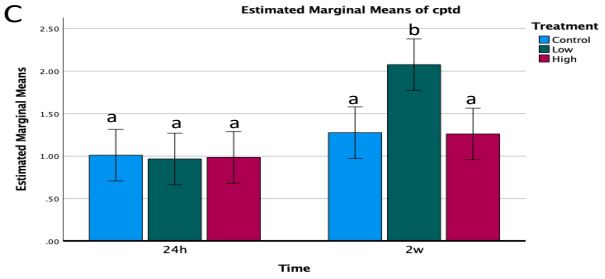
3.1.2 Gills

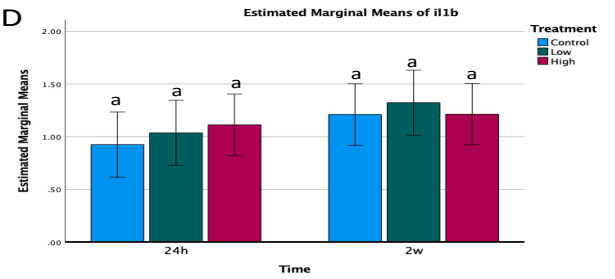
The results for the two-way ANOVA for the gene expression in the gills showed no significant difference 24 hours post infection. At T14, 7 out of the 11 genes had significantly different expression when comparing either the SE or the ME to the control groups (**Figure 11**L). Comparing the SE groups to the controls there were 3 genes with an upregulation compared to the control, this was for the genes *lys*, *cathepsin d (ctpd)* and *interferon regulatory factor 1 (irf1)*. Looking at the ME groups compared to the control there were two genes that

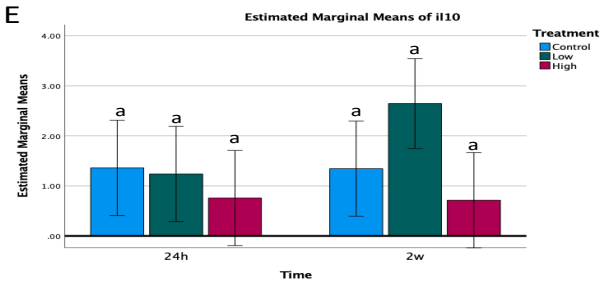
were upregulated, *mucin 5AC* (*muc5ac*) and *mucin 5B* (*muc5b*). Contrary to those, two genes, camp and *hepcidin* (*hamp*) were downregulated.

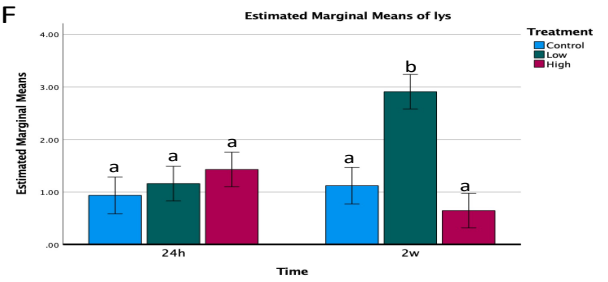


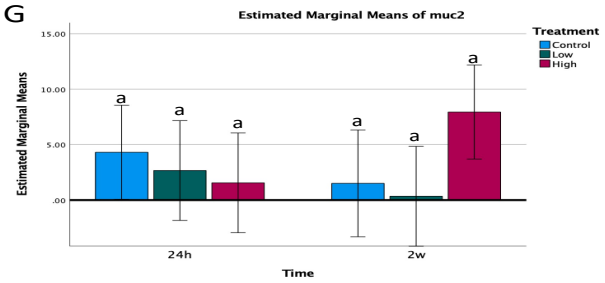


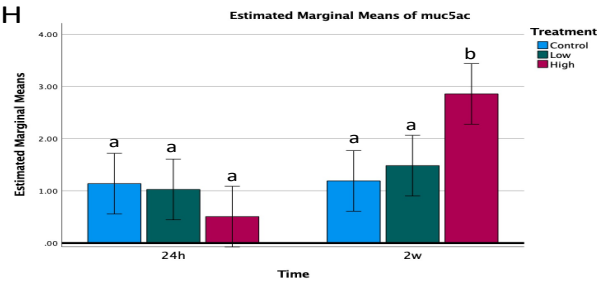


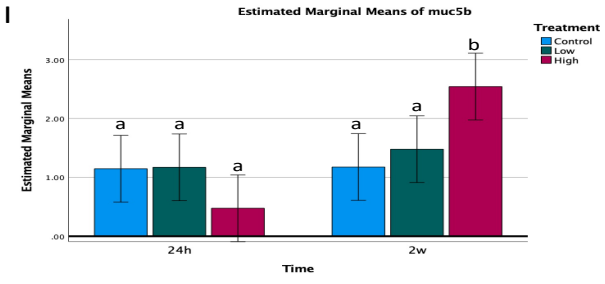


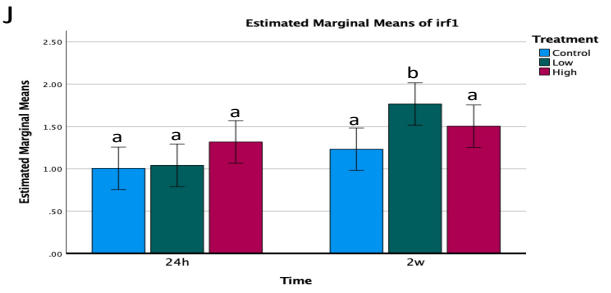


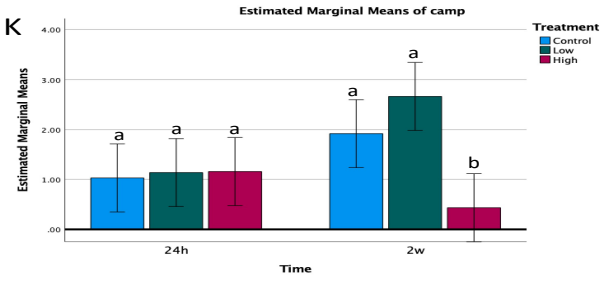












L

Gills sub 1	24h		2w	
Genes	Low	High	Low	High
Hamp	-	-	-	↓
Ifng	-	-	-	-
Cptd	-	-	↑	-
Il1b	-	-	-	-
Il10	-	-	-	-
Lys	-	-	↑	-
Muc2	-	-	-	-
Muc5ac	-	-	-	↑
Muc5b	-	-	-	↑
Irf1	-	-	↑	-
Camp	-	-	-	↓

Figure 11: Expression of immune genes (A: hamp, B: ifng, C: cptd, D: il1b, E: il10, F: lys, G: muc2, H: muc5ac, I: muc5b, J: irf1, K: camp) in the gills. Values are presented as mean ± SD of 9 individual fish per treatment group. L: The upregulation and downregulation of the different genes in a table. Note: Low=SE and High=ME

For the control samples, the histology scores were 0 at both T1 and T14. All three SE samples scored 0 at T1 and had an increase in score till 1 at T14. For the ME samples 2/3 had a score of 0 at T1 and the last one had 1 in score. At T14 samples from two systems had the same score as at T1 (score 0 and 1) while the third system score increased from 0 till 2. **Figure 12** below shows gills from the different treatment groups. **Figure 12A** is from the control group and the gills are well defined, **Figure 12B** SE treatment group at T14 and the arrow can be seen pointing towards where there is observed some lesions in the gills the gill filaments not being separate. **Figure 12C** is from the ME treatment group and the lesions here is where the arrows are pointing, and the gills are much more clustered together.

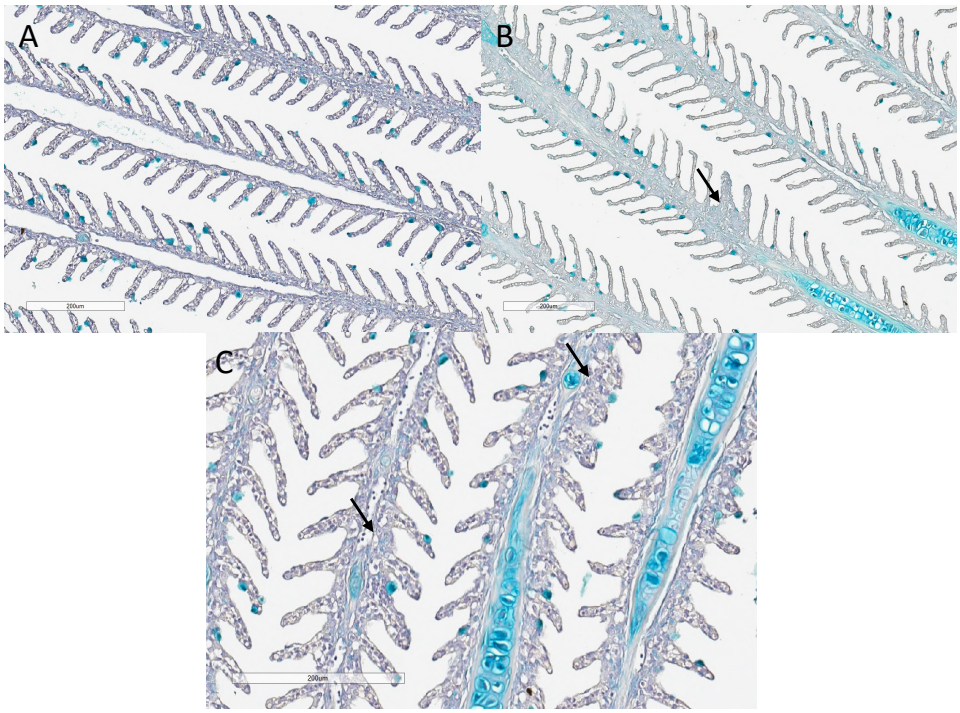
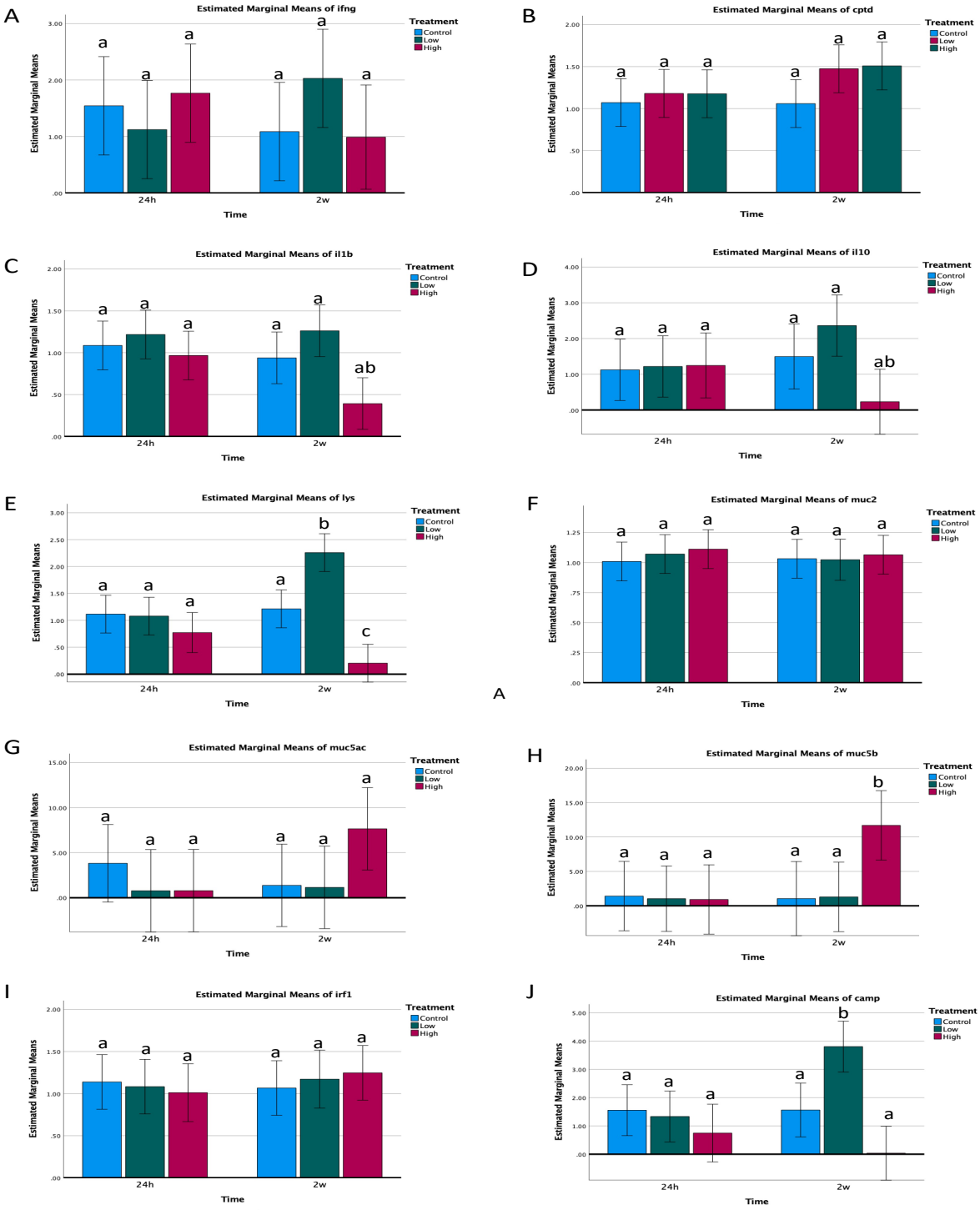


Figure 12: Histology slides from the gills. A is healthy gill filaments from the control group, B is from the SE treatment group, the arrow is pointing towards clubbing. C is from the ME treatment group at T14 and the arrows are pointing towards the lesions.

3.1.3 Distal gut

The two-way ANOVA showed no significant differences between the control groups and the SE and ME groups 24 hours post infection (**Figure 13**). T14 showed differences in 3 genes, SE had an upregulation in both *lys* and *camp*. The ME treatment group had an upregulation in muc5b and a downregulation in *lys*. The *hamp* gene were ran for distal gut as well, but the gene had no expression in the distal gut.



K

Distal gut sub 1	24h		2w	
Genes	SE	ME	SE	ME
Ifng	-	-	-	-
Cptd	-	-	-	-
Il1b	-	-	-	-
Il10	-	-	-	-
Lys	-	-	↑	↓
Muc2	-	-	-	-
Muc5ac	-	-	-	-
Muc5b	-	-	-	↑
Irf1	-	-	-	-
Camp	-	-	↑	-

Figure 13: Expression of immune genes (A: ifng, B: cptd, C: il1b, D: il10, E: lys, F: muc2, G: muc5ac, H: muc5b, I: irf1, J: camp) in the distal gut. Values are presented as mean ± SD of 9 individual fish per treatment group. K: The upregulation and downregulation of the different genes in a table. Note: Low=SE and High=ME

The histology scores for all the control groups were 0 for both T1 and T14. 2/3 in the SE group had a score of 0 at T1 and 1/3 had a score of 1. At T14 for the SE one of the two groups that scored 0 at T1 had an increase in score till 2, the other one had an increase till 1. The last SE group that scored 1 at T1 scored 1 at T14 as well. For the ME groups they all started with a score of 1 at T1 and 2/3 groups had an increase till 2 at T14. **Figure 14** below illustrates the changes that can be observed between the different treatment groups, for **Figure 14A** simple and complex folds appear long and thin. Comparing **Figure 14A** to **Figure 14B** can be observed that the complex folds appear to be thicker, but still long in **Figure 14B**.

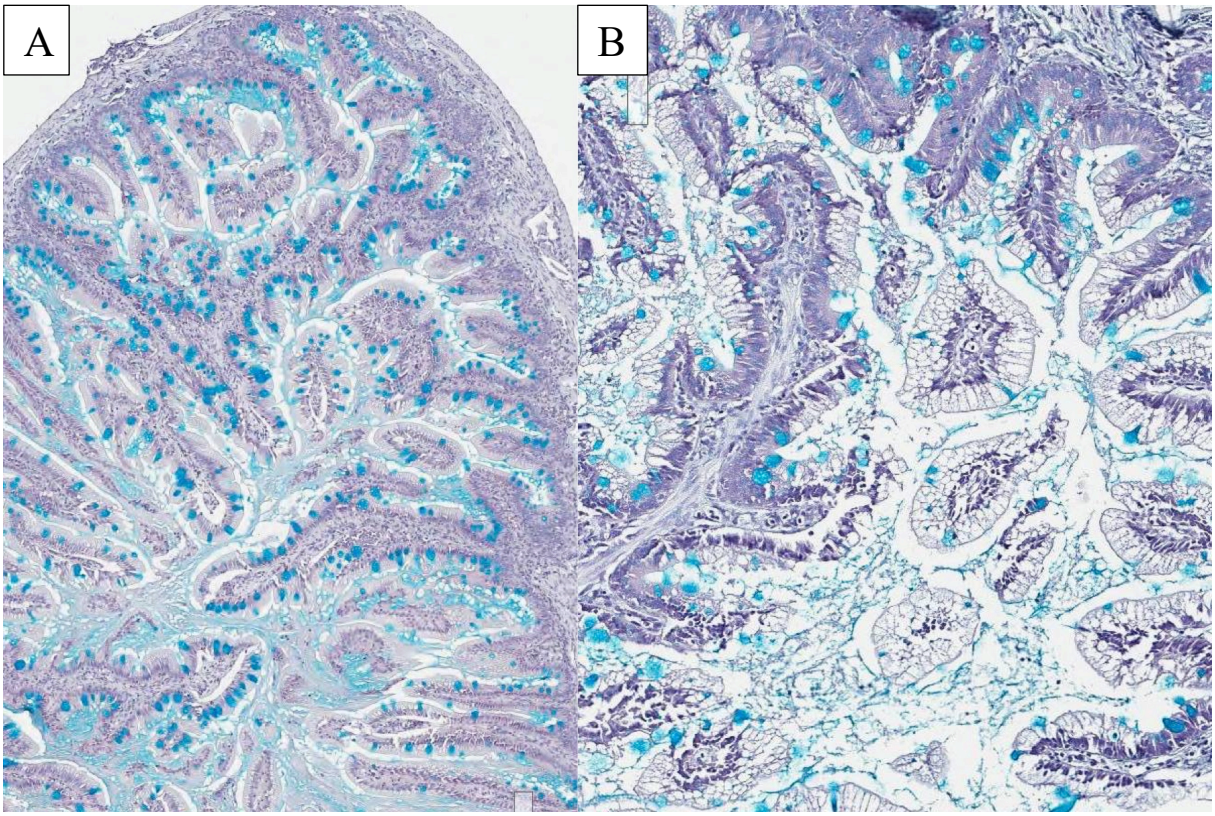


Figure 14: A is a distal gut from the control group with score 0 and B is a distal gut from the ME group with a collected score of 1 at T14.

3.1.4 PCA

The *hamp* gene was not included for any of the tissues in the making of the PCA plots because there was no data for the distal gut on *hamp* and R studio does not accept any columns without numbers in them when creating PCA plots. Except for that the rest of the genes and tissues was included in the PCA plots.

3.1.4.1 Treatment

Figure 15 are the PCA plots grouped by the different treatments. As **Figure 15A** shows, control overlaps both the SE and the ME treatment groups after 24 hours. It can also be observed that most of the genes are pulling towards a positive direction, except for *cptd.* The direction of the arrows allows for interpretation of the genes that captures the most variation, most of the genes have arrows that are close to the same length, none of them stands much out.

When comparing the 24-hour PCA plot (**Figure 15A**) to the 2 weeks one (**Figure 15B**) the direction for most of the genes have shifted, except for *muc5ac* and *muc5b* which are pulling towards the same direction as earlier. The grouping has also changed as in **Figure 15B** it can be observed that the control group is no longer overlapping the others and the ME group is the one with the biggest spread. The control and SE group can be observed to be closer compared to the ME treatment group which is not clustered together with the two others as closely. Most of the outliers in **Figure 15A** was from the control group, in **Figure 15B** it is mostly from the ME group, the control group is clustered more together at 2 weeks compared to at 24 hours. Looking at the arrows for the genes at **Figure 15A**, *ifng* is visibly longer than the rest. From this *ifng* is the reason for the most variation in the data.

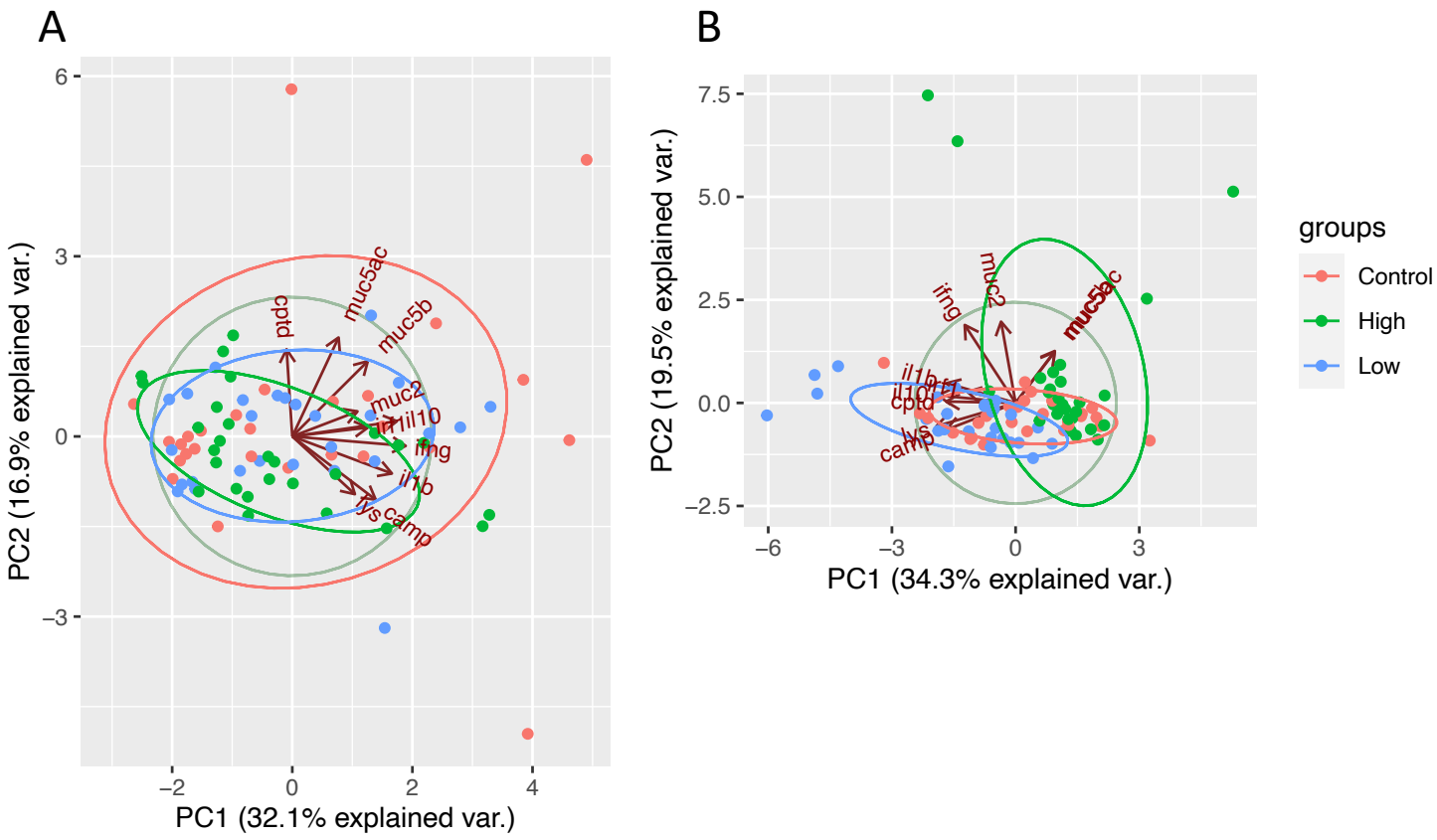


Figure 15: PCA-plots grouped by treatment, A= T1, B= T14. Note: Low is SE and High is ME

3.1.4.2 Tissue

At 24 hours (**Figure 16A**) it can be observed that the distal gut is the one of the tissues with the least clustering among the tissues and that it is the tissue with the most outliers. Looking at gills and olfactory organ, they are clustered more closely together and do not have the same widespread as the distal gut.

Comparing **Figure 16A** and **16B** it can be observed that there has been a change in the spread among the different tissues. Gills are now the one overlapping the olfactory organ and most of the distal gut, it is also now this tissue that has most of the outliers compared to the distal gut at 24 hours post infection. The olfactory organ is the tissue group that is the closest clustered together within both the distal gut and gills.

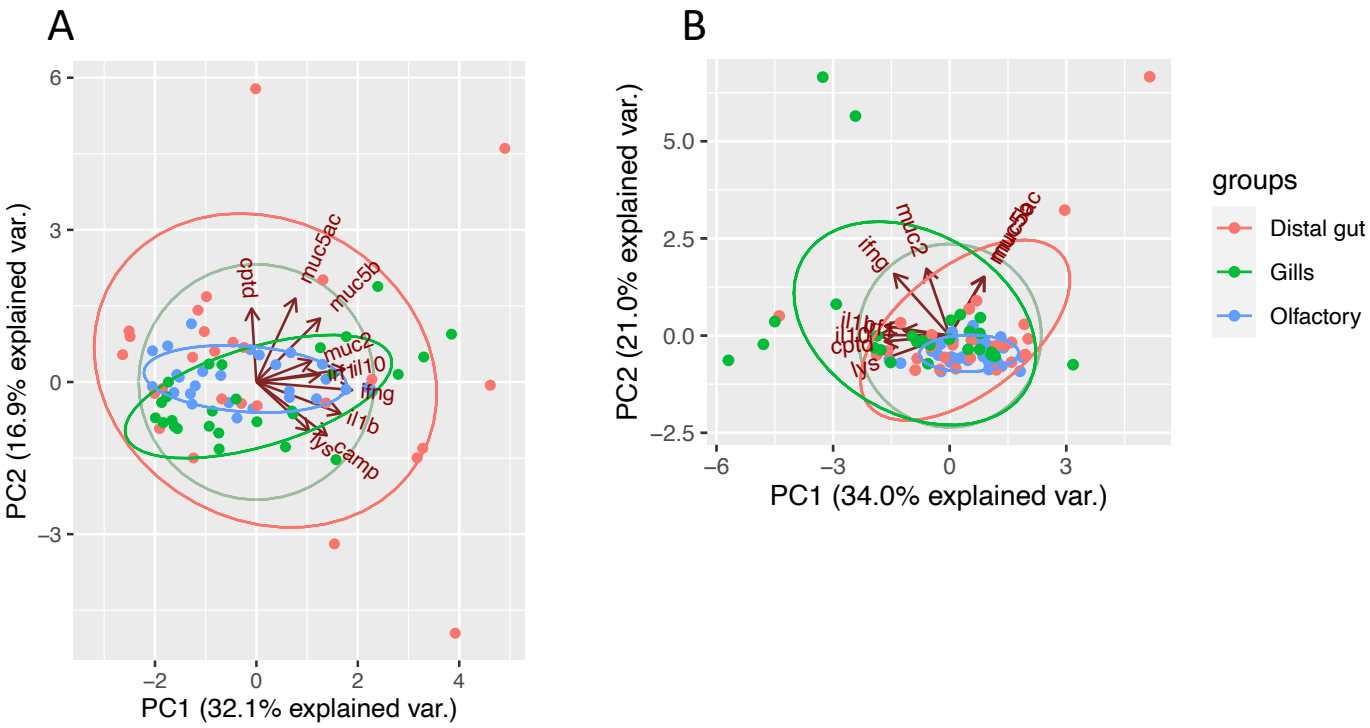


Figure 16: PCA-plots grouped by tissue, A= T1, B= T14

3.2 Sub-trial 2, fish as vector for pathogen entry to RAS

The water quality measurements were done at both the inlet and outlet water of the tank. When comparing the measurements form the systems there was no significant difference between the 9 different systems when it came to the water quality measurements taken, the standard deviation for all systems overlapped each other. For the O_2 in the water quality table all values were also above 98%. The total alkalinity for all systems were too low to be measured and therefore for all systems the results were just LO.

Table 9: Average measured (± STDEV) water quality for the different treatment groups at the first measuring point (T-1), with both inlet (I) and outlet (O) water.

	T-1					
Water quality parameters	Control		Low		High	
	I	O	I	O	I	O
NH4	0,02±0,02	0,01±0,01	0,00±0,04	0,04±0,02	0,03±0,03	0,02±0,02
NO2	0,03±0,01	0,01±0,00	0,01±0,01	0,01±0,01	0,01±0,01	0,02±0,01
NO3	0,67±0,06	0,67±0,06	0,80±0,017	0,76±0,06	0,83±0,15	0,96±0,29
Total alkalinity	LO	LO	LO	LO	LO	LO
Turbidity	0,85±0,05	0,96±0,32	0,78±0,18	1,02±0,15	0,94±0,16	0,91±0,25
pH	7,61±0,59	7,24±0,15	7,32±0,12	7,23±0,12	7,25±0,01	7,15±0,06
Salinity	0	0	0	0	0	0
O2	10,33±0,06	10,19±0,14	10,41±0,08	10,08±0,16	10,4±0,07	10,16±0,06
Temp	12,72±0,29	12,73±0,23	12,68±0,10	12,65±0,15	12,43±0,20	12,61±0,21

Table 10: Average measured (± STDEV) water quality for the different treatment groups at the last measuring point (T13), with both inlet (I) and outlet (O) water.

	T13					
Water quality parameters	Control		Low		High	
	I	O	I	O	I	O
NH4	0,05±0,02	0,08±0,01	0,10±0,06	0,05±0,03	0,06±0,02	0,08±0,03
NO2	0,04±0,01	0,05±0,01	0,05±0,01	0,06±0,01	0,05±0,00	0,05±0,01
NO3	5,06±0,64	5,40±0,50	4,90±0,21	4,90±0,29	4,20±0,78	3,80±0,60
Total alkalinity	LO	LO	LO	LO	LO	LO
Turbidity	1,16±0,12	1,35±0,33	1,14±0,22	1,28±0,29	1,19±0,16	1,4±0,29
pH	7,81±0,58	7,53±0,16	7,45±0,12	7,33±0,16	7,21±0,06	7,22±0,18
Salinity	0	0	0	0	0	0
O2	10,40±0,04	10,20±0,32	10,40±0,06	10,25±0,11	10,40±0,02	10,30±0,22
Temp	12,60±0,15	12,70±0,15	12,70±0,10	12,60±0,10	12,60±0,20	12,70±0,23

The survival rate for the control tanks were at 100%, there was no mortality in either of the three tanks throughout the 14-day trial. For the low infection group (system nr 4, 5, and 6) where 10% of the fish were infected with *Y. ruckeri* the survival was at 94%, 96% and 90% having an average survival rate at 93,3% for the low infection group. **Figure 17A** illustrates the average survival percentage per treatment group. For system 4 none of the fish that died were already infected, for system 5 50% of the fish that died during the trial were already infected prior to the trial start and for system 6 40% of the fish that died during the trial wereas already infected prior to trial start. The high infection group (systems nr 7, 8 and 9) where 30% of the fish in the systems were infected the survival rates were 78%, 90,2% and 84,0% with the

average survival for the high dose systems being 84,1%. In system 7 55% of the mortalities were fish previously infected. In system 8 all the mortalities were fish that were already infected as well as 87% of the mortalities in system 9. In **Figure 17B** the numbers of dead fish per system is illustrated, distinguishing between the mortalities of the already tagged fish and the ones that were introduced to the systems healthy.

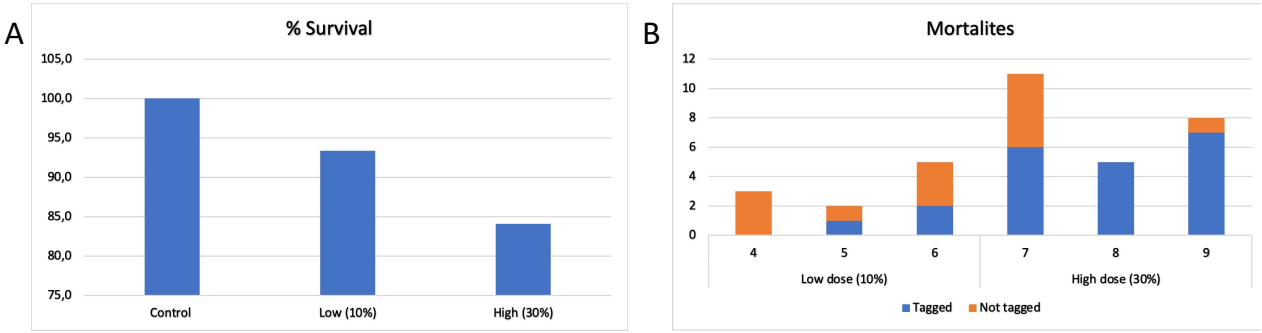


Figure 17: A: average survival rate for the different treatments, B: the mortalities, divided into tagged and not tagged.

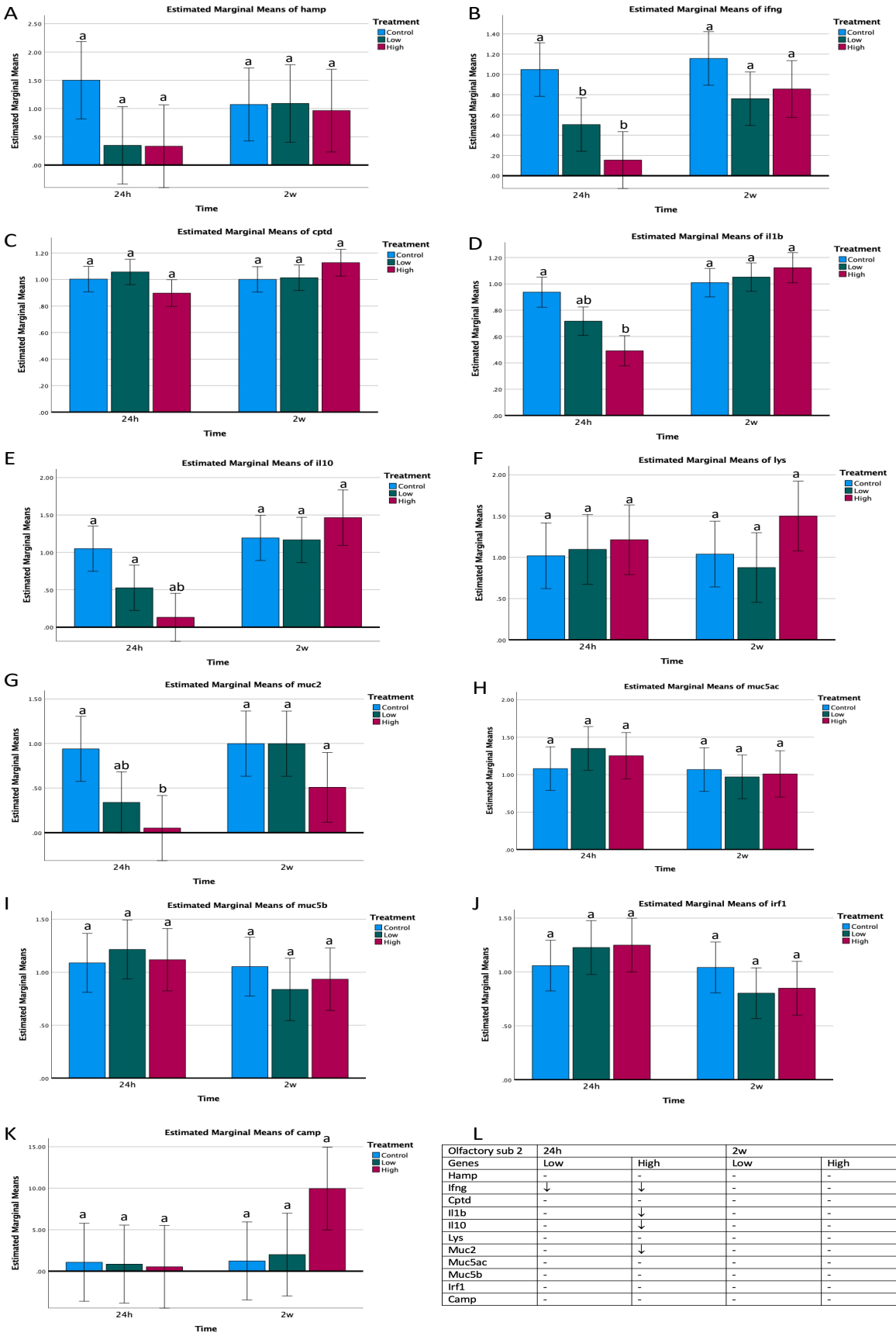
The samples sent to PHARMAQ for detection of *Y. ruckeri* included some samples from the spleen. There were in total 18 (3 per treatment) samples per tissue sent for analysis from T14, at the end of the trial. There were 6 samples per treatment group and 2 from each tank. There were no detection of *Y.ruckeri* in any of the samples from the control group for either of the tissues. No detection was made in either of the samples from the olfactory organ or the distal gut. There was one positive detection from the same fish for both the gills and spleen, where the detection of the pathogen was moderate for both of the positive samples.

Table 11: Number of samples sent to PHARMAQ per tissue and the percentage of positive and negative samples.

Tissue	Total number of samples			Positive			Negative		
	Control	Low	High	Control	Low	High	Control	Low	High
Olfactory organ	6	6	6	0%	0%	0%	100%	100%	100%
Gills	6	6	6	0%	0%	16,7%	100%	100%	83,3%
Distal gut	6	6	6	0%	0%	0%	100%	100%	100%
Spleen	6	6	6	0%	0%	16,7%	100%	100%	83,3%

3.2.1 Olfactory organ

4 genes showed to be significantly different when compared to the control group 24 hours after infection. For the low dose (10% infection) there was only one gene, interferon gamma (ifng) which were downregulated compared to the control group. For the high infection group (30%) there were 4 genes that were downregulated compared to the control group 24 hours after introducing the infected fish to the systems. These were *ifng*, *interleukin 1B* (*il1b*), *interleukin 10* (*il10*) and *mucin 2* (*muc2*). There were no significant differences at T14 when comparing either of the infected groups to the control.



L

Olfactory sub 2	24h		2w	
Genes	Low	High	Low	High
Hamp	-	-	-	-
Ifng	↓	↓	-	-
Cptd	-	-	-	-
Il1b	-	↓	-	-
Il10	-	↓	-	-
Lys	-	-	-	-
Muc2	-	↓	-	-
Muc5ac	-	-	-	-
Muc5b	-	-	-	-
Irf1	-	-	-	-
Camp	-	-	-	-

Figure 18: Expression of immune genes (A: hamp, B: ifng, C: cptd, D: il1b, E: il10, F: lys, G: muc2, H: muc5ac, I: muc5b, J: irf1, K: camp) in the olfactory organ. Values are presented as mean ± SD of 9 individual fish per treatment group. L: The upregulation and downregulation of the different genes in a table.

The histology results showed a scoring of 0 for all the control systems for both T1 and T14, the score reveal healthy olfactory organ where the mucosal structures are well defined. The low infection group had one system with the score 0, one with 1 and the last one with 2 for T1, at T14 the only change in scoring was for system 6 which went from 0 till 2 in score over the 14 days. For the high infection systems system 7 had the score 1 for both T1 and T14, system 8 had a change in score from 0 at T1 till 1 at T14 and system 9 had a change in score from 1 at T1 till 2 at T14. **Figure 19** shows three different olfactory organs where **Figure 19A** is from the control group, while **Figure 19B** is from the low infection group and **Figure 19C** is from the high infection group. In both **Figure 19B** and **Figure 19C** it can be observed that the olfactory organs structure is not as intact as in **Figure 19A**.

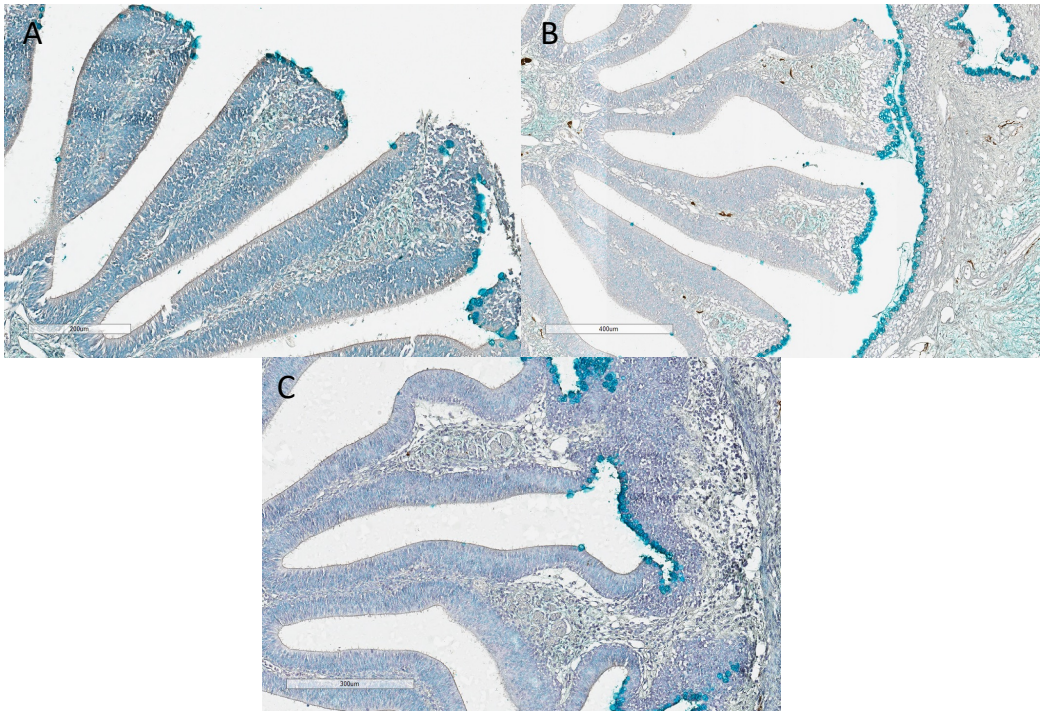
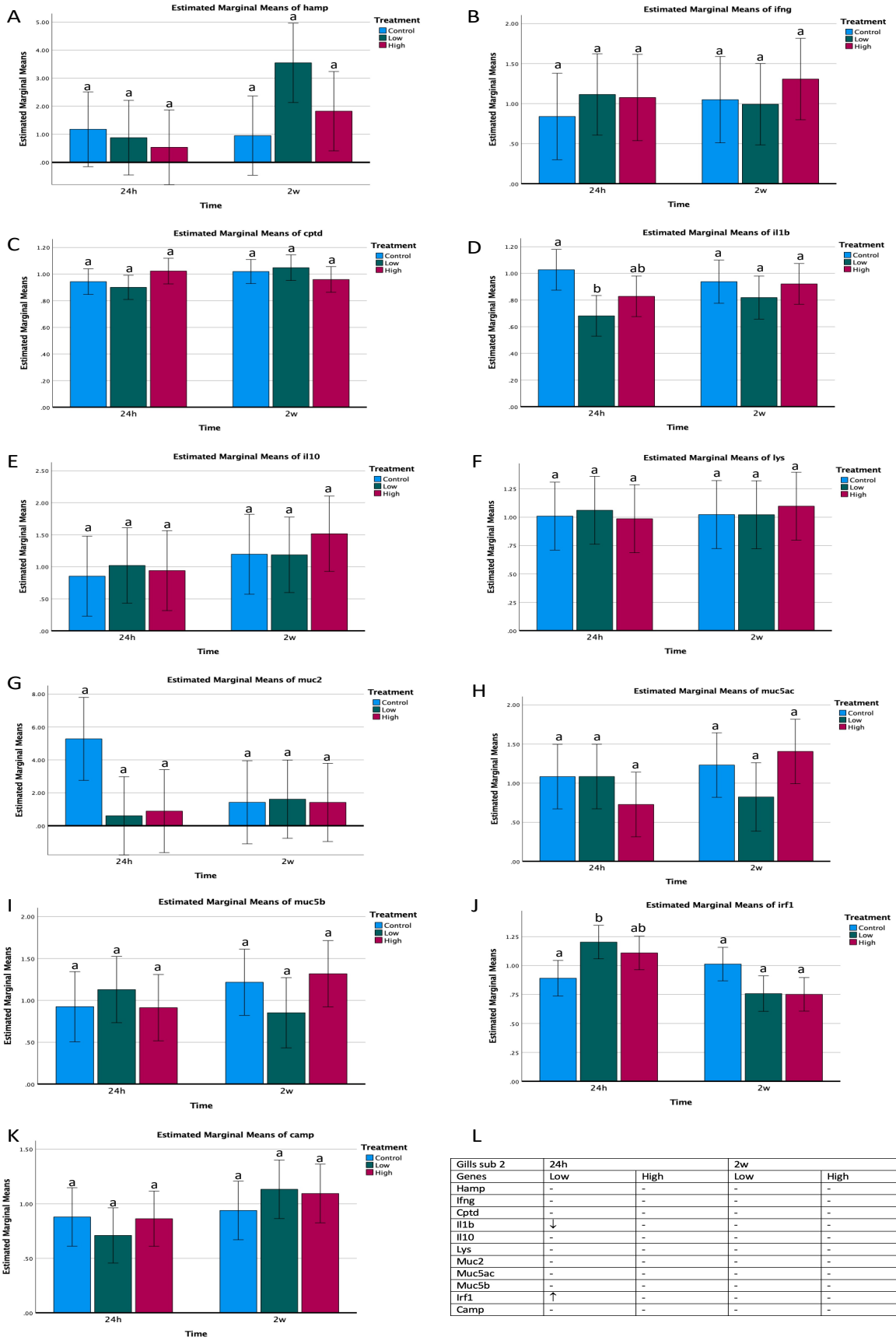


Figure 19: Histology from three different olfactory organs. A: olfactory organ form the control group, B: olfactory organ from the low infection group, C: olfactory organ from the high infection group.

3.2.2 Gills

For the gills there was only two genes that showed to have a significant difference compared to the control group at T1 and both in the low infection group. *Il1b* had a downregulation in the expression compared to the control group and *irf1* had an upregulation compared to the control group. There were no gens in the high infection group that had any significant differences compared to the control. At T14 there was no significant difference for either of the infected groups compared to the control.



Gills sub 2	24h		2w	
Genes	Low	High	Low	High
Hamp	-	-	-	-
Ifng	-	-	-	-
Cptd	-	-	-	-
Il1b	↓	-	-	-
Il10	-	-	-	-
Lys	-	-	-	-
Muc2	-	-	-	-
Muc5ac	-	-	-	-
Muc5b	-	-	-	-
Irf1	↑	-	-	-
Camp	-	-	-	-

Figure 20: Expression of immune genes (A: hamp, B: ifng, C: cptd, D: il1b, E: il10, F: lys, G: muc2, H: muc5ac, I: muc5b, J: irf1, K: camp) in the gills. Values are presented as mean ± SD of 9 individual fish per treatment group. L: The upregulation and downregulation of the different genes in a table

The histology results for the gills had scores of 0 for both T1 and T14 for the control groups, for the low infection group 2/3 scored 0 for T1 and one scored 1. At T14 the group that scored 1 had still a score of 1 and one of the other groups had increased the score from 0 till 1. For the high infection group 2/3 of them had a score of 1 at both T1 and T14 while the last group had a score of 1 at both timepoints. **Figure 21** is gills from the three different treatment groups and the gills in **Figure 21A** is well defined and they are separated in an orderly manner. **Figure 21B**, from the low infection treatment group has an arrow pointing towards where the gill filament is not as well defined and in **Figure 21C**, from the high infection treatment group this is even more clear, where the gill filaments has lost its very distinct structure.

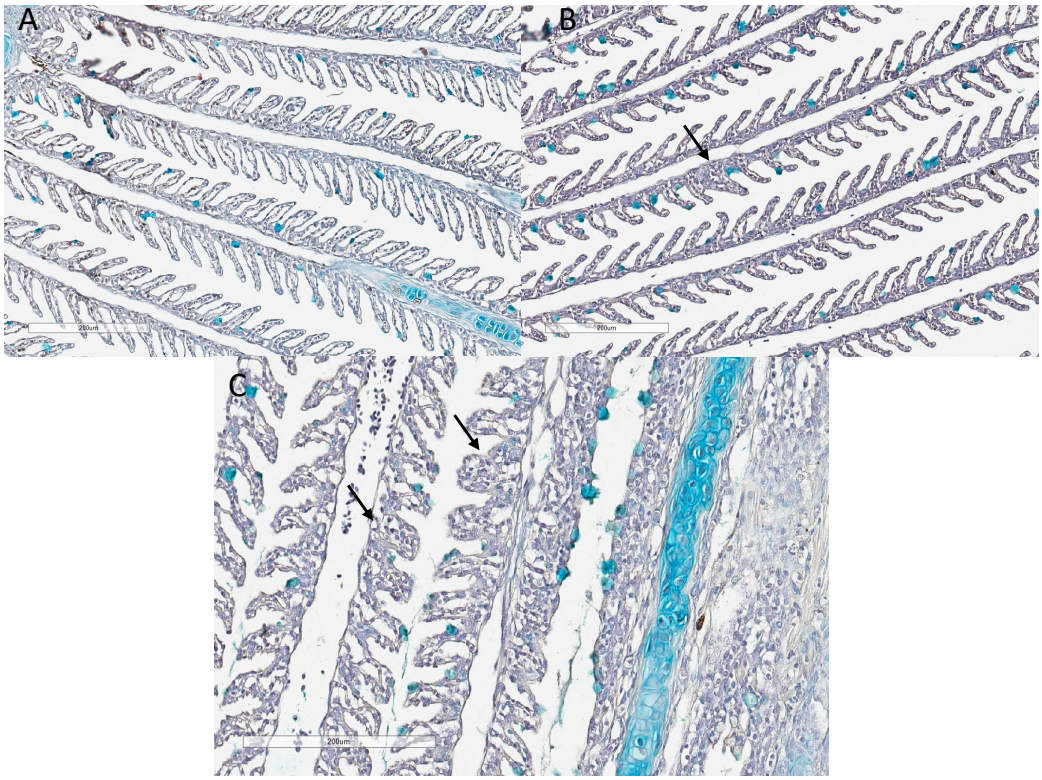
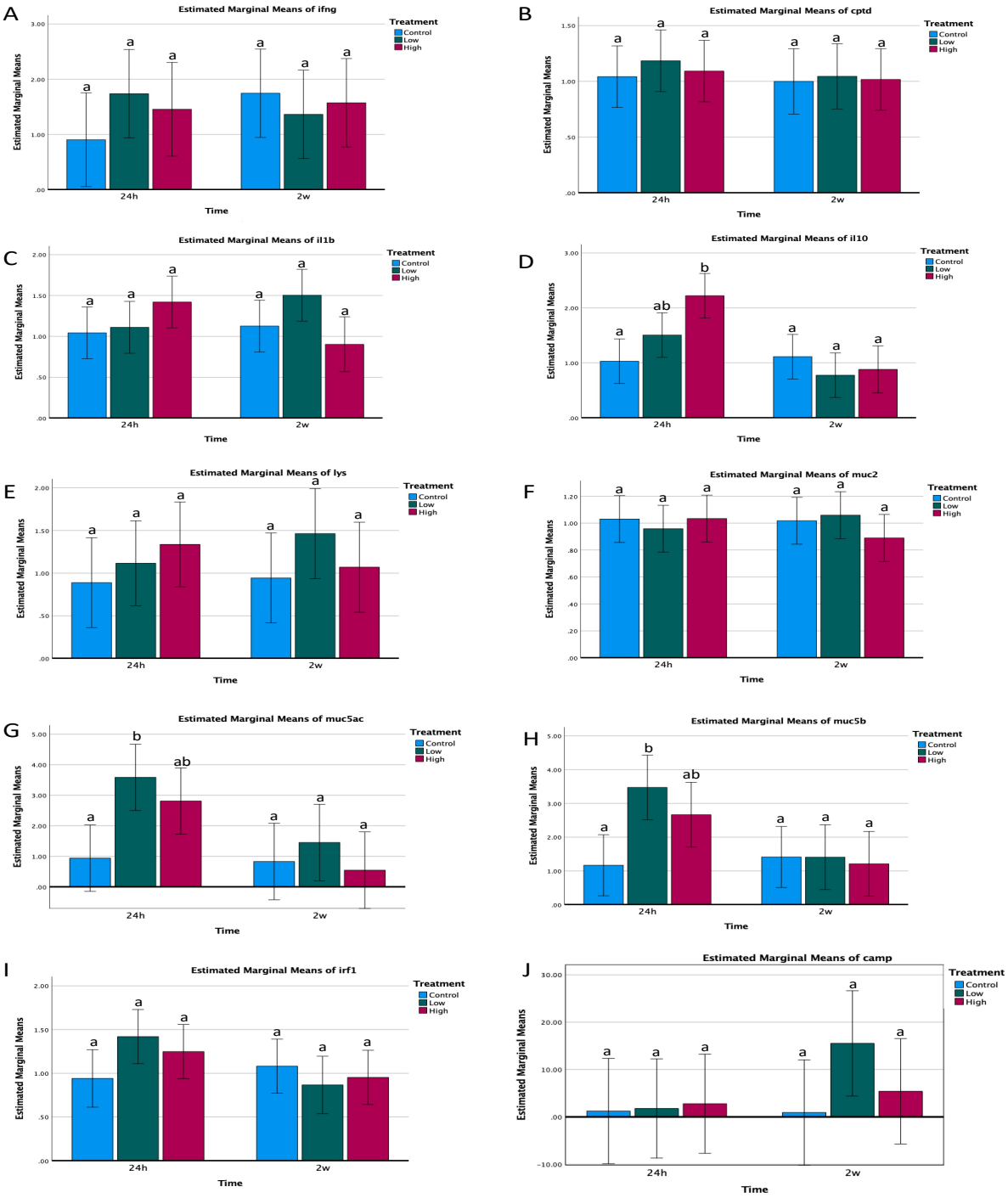


Figure 21: Histological section of the gills for the three different treatment groups. A: healthy gill filaments from the control group. B: gill filaments from the low infection group, the arrow is pointing towards lesions. C: gill filaments from the high infection group, both arrows are pointing towards lesions in the filaments.

3.2.3 Distal gut

3 genes showed to have significantly different ct-values compared to the control group at T1. Both *muc5ac* and *muc5b* were upregulated in the low infection group compared to the control. *Il10* in the high infection group was upregulated when compared to the control group. There

was no difference between the three treatment groups at T14. The gene was run for the distal gut, there were no expression for it and therefore it has not been included.



K

Intestine sub 2	24h		2w	
Genes	Low	High	Low	High
Hamp	N/A	N/A	N/A	N/A
Ifng	-	-	-	-
Cptd	-	-	-	-
Il1b	-	-	-	-
Il10	-	↑	-	-
Lys	-	-	-	-
Muc2	-	-	-	-
Muc5ac	↑	-	-	-
Muc5b	↑	-	-	-
Irf1	-	-	-	-
Camp	-	-	-	-

Figure 22: Expression of immune genes (A: ifng, B: cptd, C: il1b, D: il10, E: lys, F: muc2, G: muc5ac, H: muc5b, I: irf1, J: camp) in the distal gut. Values are presented as mean ± SD of 9 individual fish per treatment group. K: The upregulation and downregulation of the different genes in a table.

The histology results for the control groups had scores of 0 for all three at both T1 and T14, for the low infection groups the score was 1 for all three groups at T1 and for T14 for 2/3 groups the scored 1 and the last group had an increase from 1 to 2. The high infection groups all had the same score of 1 at T1 and an increase to 2 at T14. The picture below shows on the left hand side the distal gut from the control group with score of 0 and on the right hand side distal gut form the high infection group with a score of 2. Looking at the distal gut from the control group the simple and complex folds appear to be long and thin, especially when comparing them to the right hand side where they are more stubby and the structure is not as clear.

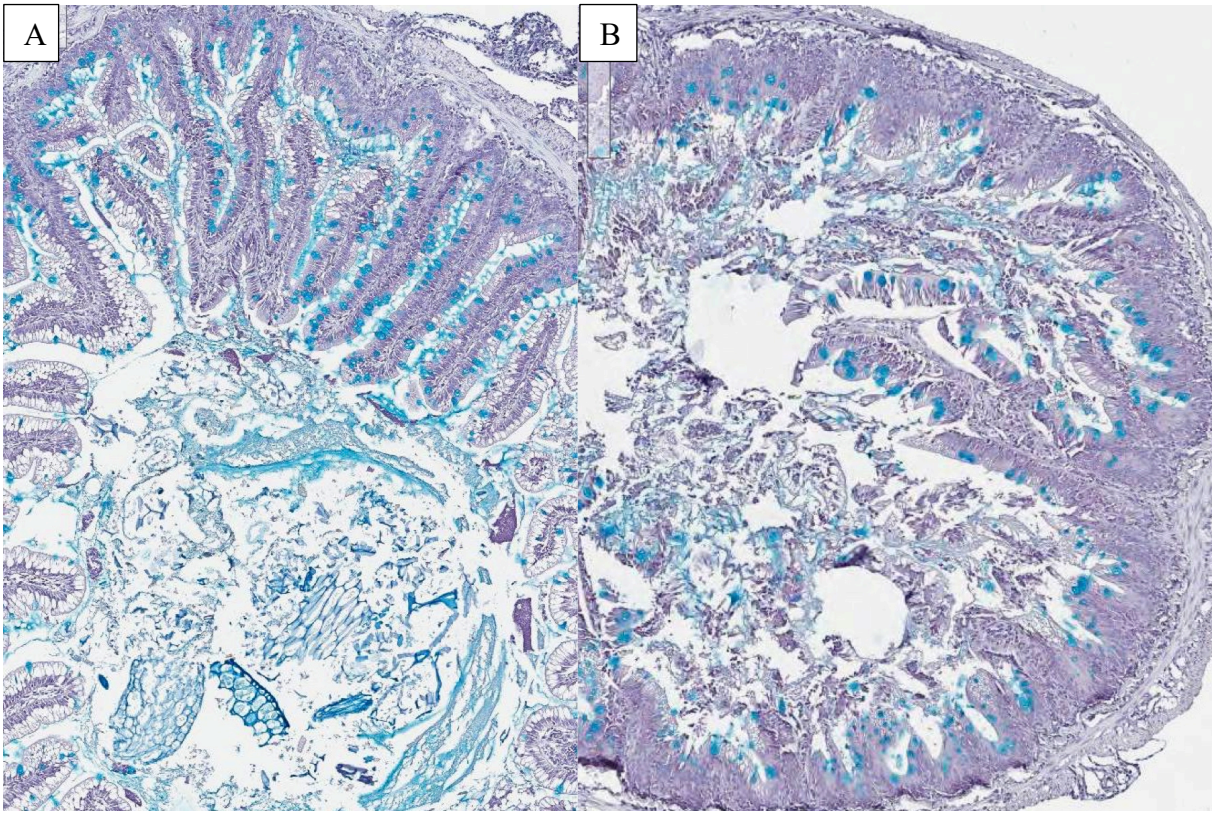


Figure 23: A- is the distal gut from the control group with score 0 and B- is the distal gut from the high infection group with a score of 2.

3.2.4 PCA

The *hamp* gene was not included for any of the tissues in the making of the PCA plots because there was no data for the distal gut on *hamp* and R studio does not accept any columns without numbers in them when creating PCA plots. Except for that the rest of the genes and tissues was included in the PCA plots.

3.2.4.1 Treatment

PCA plot for the treatments in sub-trial 2 at 24 hours (**Figure 24A**) shows that 24 hours after infection the control group was very clustered together compared to both the low and the high treatment groups which are overlapping each other mostly. Looking at the arrows of **Figure 24A** *muc5b* and *muc5ac* are the genes who seem to capture most of the variation on the data as they are longer than the other genes arrows.

2 weeks after infection it (**Figure 24B**) shows a bigger spread in the control group and the direction of the genes has shifted. *Lys* and camp are now the genes who seem to capture most of the variation of the data. At 24 hours (**Figure 24A**) both *lys* and *camp* were clustered together with most of the other genes and did not stand out in the same way they are in **Figure 24B**.

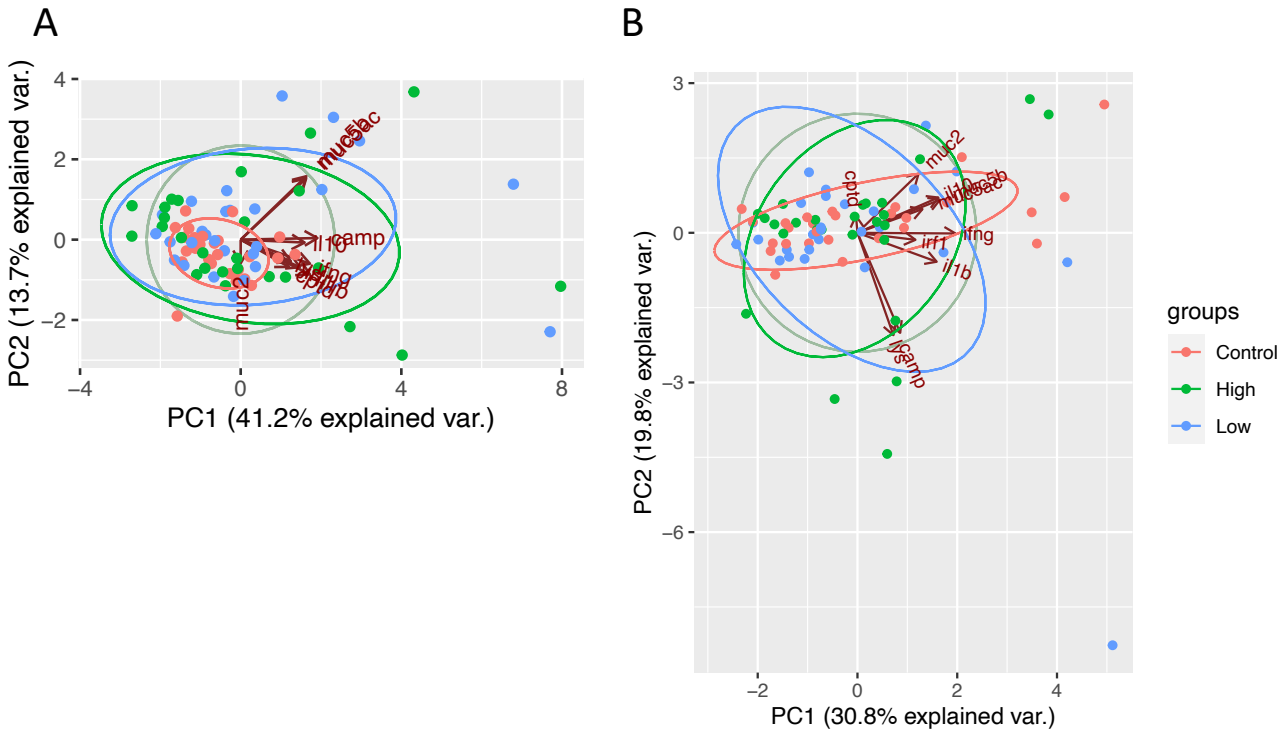


Figure 24: PCA-plots grouped by treatment, A= T1, B= T14

3.2.4.2 Tissue

The PCA plot grouping the tissues at 24 hours (**Figure 25A**) shows that the distal gut is the tissue with the biggest variation and that both gills and olfactory organ is more clustered together. For the genes that captures the most variation this is the same as when the grouping is treatment, the genes *muc5b* and *muc5ac* are the genes at 24 hours for both tissue and treatment grouping that captures the most variation.

At 2 weeks (**Figure 25B**) both olfactory organ and gills are not as much clustered together as at 24 hours (**Figure 25A**). There is a bigger variation and looking at the direction the oval for both olfactory organ and the distal gut is extended they are heavily affected by the genes *lys* and *camp* which are the genes that captures the most variation at 2 weeks. Comparing it to the gills, this tissue is not as heavily affected as the gills oval is not extended towards the same direction.

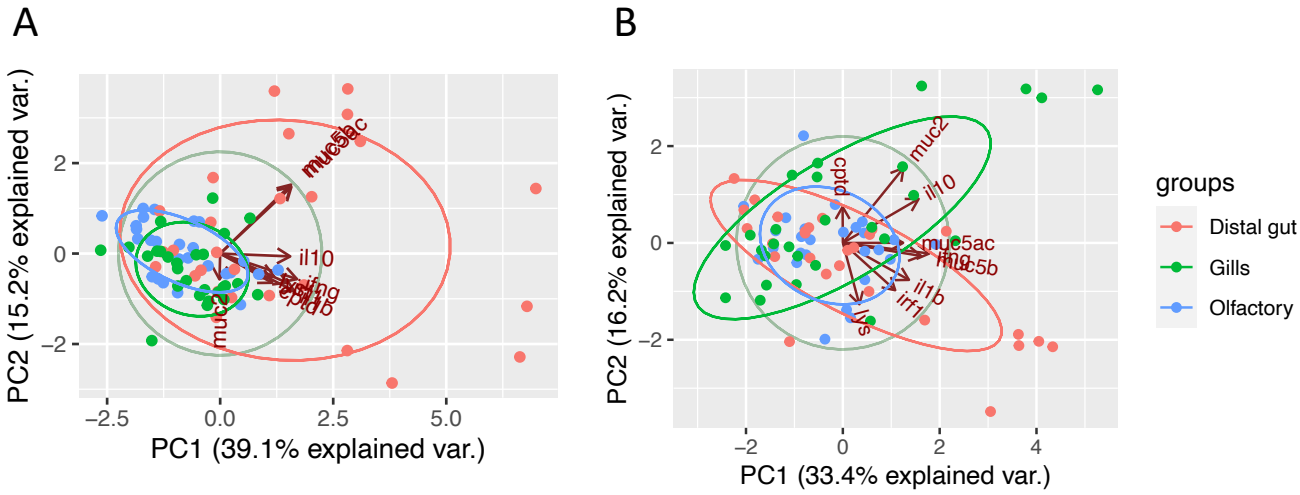


Figure 25: PCA-plots grouped by tissue, A= T1, B= T14

4 Discussion and conclusion

To date the way a pathogen spreads in a RAS compared to other aquaculture systems is largely unknown. This thesis addressed this by simulating a pathogen breach in RAS using *Yersinia ruckeri* as a model. Despite the vaccines and broad understanding of the virulence of *Y. ruckeri* especially when it comes to *Y. ruckeri* (Bridle et al., 2012; Wrobel et al., 2019), outbreaks still occur. This is quite relevant in new production systems such as RAS in salmon farming, where conditions in the system play a significant role in influencing how the pathogen

enters, proliferates and causes diseases to the fish. Looking at two different ways of entry for the pathogen into the system, entry through make-up water and through infected fish, some clear differences were observed. The mucosal organs were the only one assessed because the trials are focusing on the innate immune responses to pathogen breaches. For the same reason, this is also why the separate trials only ran over the span of 14 days. The genes analyzed are genes that are known to affect the early innate immune response in fish. The histology visualize some of the affects that an infection of *Y. ruckeri* can have on the different tissues analyzed.

4.1 Sub-trial 1

Firstly looking at the results from sub-trial 1, the qPCR results showed that the main differences between the treatment groups were found in the gills. Tthis can be explained with the fact that when the water is being moved across the gills when the fish is breathing and when the pathogen is in the water it will be in constant contact with the gills. When given the opportunity *Y. ruckeri* will bind more efficiently to the gills compared to other organs (Koppang et al., 2015). From earlier studies of *Y. ruckeri* two of the most common genes to have an upregulation are *Lysozyme* (*lys*) and *Cathelicidin* (*camp*) (Bridle et al., 2011; Bridle et al., 2012; Kumar et al., 2018) This is also the case with the expressed in sub-trial 1 where for all the tissues there is either an upregulation or downregulation in both *lys* and *camp* for both the SE and ME treatment groups compared to the control. *Lys* is an important gene in the innate immune response in fish, and can be found in the skin, gills and intestine of fish, *lys* is the first line of defense against pathogens in fish. *Lys* had an upregulation for the single entry (SE) treatment groups for both olfactory organ, gills and distal gut (**Figure 9L, 11L and 13K)**, as well as a downregulation in the multiple entry (ME) treatment group in the distal gut (**Figure 13K)**. *Lys* can be effective against gram negative bacteria such as *Y. ruckeri*, when the inner layer of peptidoglycan of the bacteria is exposed (A.Costaa et al., 2011). This can explain why there was neither downregulation nor any upregulation in *lys* at T1. Earlier studies have shown that antibacterial defense genes such as *camp* takes more than 24 hours before it is expressed in the gills (Bridle et al., 2011), this might explain why there was no detection of any upregulations compared to the control group at T1. *Camp* was however one of the genes that showed to have an upregulation or downregulation in all tissues from either of the treatment groups. *Camp* is an antimicrobial peptide that acts like an effector molecule for the innate immune system. It will be a mediator between the innate and adaptive immune system in fish, this by releasing cytokines. When cytokines are released the response of the fish to invading pathogens will be more effective, this will be on the site of the pathogen invasion, this is where

camp will have the primary antimicrobial action (Bridle et al., 2011). Therefore, *camp* was found in all the tissues on T14. The only cytokine that had an upregulation was *Interferon regulatory factor 1* (*irf1*) in the gills of the SE treatment group **(Figure 11L)**. The role of *irf1* is to regulate the cellular immune response upon an infection, as it regulates the early phase of various stress responses. It is produced by T lymphocytes that has been stimulated either by mitogens or antigens (Bergan et al., 2010).

Except for *lys* and *camp*, two other antibacterial defense genes had changes in the gills, these were *Cathepsin D* (*ctpd*) with an upregulation in the SE group and *Hepcidin* (*hamp*) with a downregulation in the ME group **(Figure 11L)**. *Cptd* is involved in a variety of immune processes, where the antimicrobial activity is included. Plays an important role in in the innate immune response of the fish and the defense against disease (Sathyamoorthi et al., 2017). *Cptd* has been reported to be an essential regulator of other immune genes in the immune tissues, the increased production of *cptd* is increasing the protection the fish has against disease. It has a knockdown effect, with the production of *cptd* effecting how other immune associated genes are transcribed (Yu et al., 2020). *Hamp* is an antimicrobial peptide that fights the bacterial outbreaks of disease such as with *Y. ruckeri.* The regulation of *hamp* is tissue specific and it is a big part of the innate immune response in fish, as the other genes. *Hamp* is regulating the iron, an upregulating of *hamp* results in an increased level of iron, this is an innate response to infection and inflammation (Bao et al., 2005).

There were also two mucins that were upregulated the ME group; two had an upregulation in the gills and one in the distal gut (**Figure 11L and figure 13K)**. *Mucin 5 B* (*muc5b*) in the distal gut and gills, *Mucin 5 AC* (*muc5ac*) in the gills. Mucins are present in the mucosal surfaces and their response will differ depending on the surface, but they are limiting the ability for the pathogen to adhere to the tissue (Minniti et al., 2019). The transcription of the mucins responds to parasites and pathogens. Both *muc5b* and *muc5ac* are a part of the secreted gel-forming mucins, they are increasing the production of the layer that is between the environment and the fish (Sveen et al., 2017). The gills and distal gut are seen more affected by the pathogen compared to the olfactory organ and this might explain why the mucins are expressed in the distal gut and gills. The PCA plots for sub-trial 1 (**Figure 15**) showed that at the beginning of the trial (**Figure 15A**) the control treatment group overlapped the SE and ME groups, there were no differences between the three groups, but as the trial progressed at the end of the trial (**Figure 15B**) the treatment groups are not overlapping each other as they were in the beginning.

The two figures illustrate that there were no registered differences in the beginning of the trial, only in the end.

Looking at the mortalities recorded during sub-trial 1 (**Figure 8**) they started for both treatment groups on the same day and in total there were more mortalities in the SE treatment group compared to the ME treatment group. These results indicate that the amount of times *Y. ruckeri* is introduced to the system does not affect the survival rate. The histology data backs up that there control group has not been exposed to any infection and that the tissue is healthy.

4.2 Sub-trial 2

When looking at the results from sub-trial 2 the changes in genes are just in the cytokines and mucins, no antibacterial defense genes as observed in sub-trial 1. All the upregulation or downregulation happened at T1 compared to sub-trial 1 where they were all in T14. The cytokine *Interleukin 1 β (il1b)* has been one of the genes in earlier trials that has showed upregulation in Rainbow trout exposed to *Y. ruckeri* (Raida & Buchmann, 2008). *Il1b* is fundamental in the immune response to pathogens and disease in fish, it is considered one of the best pro-inflammatory genes in Rainbow trout and is especially important in the early immune response (Fajardo et al., 2022; Raida & Buchmann, 2008). *Il1b* is triggering the inflammatory and immune response in fish (Morrison et al., 2012). *Il1b* has been described as the gatekeeper for inflammation because it is the primary effector of the whole inflammatory cascade in the immune response. It binds to the *interleukin 1 receptor* (*il1r*) to simulate a downstream to create a proinflammatory pathway (Metz et al., 2006). This explains why *il1b* was downregulated in both the olfactory organ for the high infection group and in the gills for the low infection group. *Irf1* which was upregulated in the gills in sub-trial 1 in the SE group was also upregulated in the gills for the high infection group. This as mentioned regulates the immune response upon infection and the early phase of different stress responses (Bergan et al., 2010). Two other cytokines were also expressed in sub-trial 2 *Interferon gamma* (*ifng*) and *Interleukin 10* (*il10*). *Ifng* were downregulated in the olfactory organ for both the high and the low treatment group, while *il10* were upregulated in the distal gut for the high treatment group and downregulated in the olfactory organ for the high treatment group. *Ifng* is a single protein produced by natural killer cells (NK cells) and T lymphocytes as a response to antigens or mitogens. It regulates both the adaptive and innate immune response. It is a key activator of macrophages, to increase the killing of pathogens. *Ifng* has the ability to modulate how the immune response of the fish is (Robertsen, 2006). *Il10* is considered the most important

cytokine that has anti-inflammatory properties, it is produced by activated immune cells such as monocytes/macrophages and t cells. It prevents and limits an over-whelming both specific and nonspecific immune reaction to bacteria and pathogens (Sabata et al., 2010).

The other group of genes that had either an up or downregulation in sub-trial 2 were the mucins, *muc5ac*, *mub5b* and *mucin 2* (*muc2*). *Muc2* was downregulated in the high infection group in the olfactory organ, *muc5ac* and *muc5b* were upregulated in the low infection group in the distal gut. The olfactory organ was when looking at the upregulation and downregulation of genes the tissue during sub-trial 2 with the most differences (**Figure 18L**) compared to the control group.

The survival for the control group were 100%, while for the low treatment group it was right below 95% and right below 85% for the high infection group **(Figure 17A)**. This shows that with the increase of infected fish the morality was higher. But looking at **Figure 17B** the number of already infected fish (tagged) that died in the high infection group during the trial were quite high, over 50 % of the mortalities were from the already infected fish. This was not as high for the low infection group. This can indicate that with a higher density of infected fish, this will affect the already infected fish more heavily compared to the fish that are not infected, in this trial. The histology data backs up that there control group has not been exposed to any infection and that the tissue is healthy.

4.3 Comparing the two sub-trials

The mortalities started at day 7, this is around the same as for other trials done on Rainbow trout which some started having mortalities at day 5 after infection (Chettri et al., 2012; Ohtani et al., 2019). Comparing the two sub-trials the survival percentage in sub-trial 1 was lower than in sub-trial 2, especially if we do not take into consideration the already infected fish. The higher mortality in sub-trial 1 might be explained by that because of the entry way, the gills were more effected in sub-trial 1 compared to sub-trial 2 and disease associated with the gills will have higher substantial losses in most cases (Koppang et al., 2015). The outcome from the two sub-trials differs from each other and this suggests that the route of infection effects the outcome largely. Earlier trials in flow through systems have found that the route of infection has profound effects on the outcome of the disease (Ohtani et al., 2019) and with these results now from RAS it does not suggest that it is any different. The way of entry for the pathogen looks to affect the course of the disease heavily. Another interesting observation when comparing the results of the two sub trials is that they showed differences in the genes at the

two different time points, sub-trial 1 had all the differences registered at the 2-week time point and sub-trial 2 had them registered at the 24-hour time point.

When looking at the results of the two trials, when the water acts as a vector it takes more time before the immune response of the fish are responding as there were no changes at T1, while for the second sub-trial with fish as a vector it seems like the immune response of the fish kicks in almost straight away. The results indicate that when the water is a vector the results are more fatal for the fish compared to if the pathogen spreads via fish. Most of the fish that died during sub-trial 2 were already infected fish especially in the high infection group. This can indicate that the fish that are already sick will be affected more by having other sick fish around compare to the low infection group where not that many already infected fish died **(Figure 17B)**. The results are limited to this trail and the outcome might be different if it was in a commercial RAS facility.

4.4 Limitations and critical assessment

When it comes to the study design all the nine RAS were placed in the same room, gloves were changed between the tanks and when handling the samples everything was wiped down to prevent cross contamination. There was not found any pathogen in the control groups for either of the trials, during these trials cross contamination is therefore excluded. This however could be a limitation to the study design as if there were to be any cross contaminations that would not be noticed until the trials were done and the samples assessed. Before getting the final qPCR, values there were several steps throughout the process to prevent any limitations to the results, checking the RNA quality on the NanoDrop8000/1000 and running random samples from the different tissues on the Bioanalyzer to check that the values from the NanoDrop8000/1000 were correct. The final qPCR results from the QuantStuido5 were also compared to positive control samples and water samples before they were accepted to make sure that the results could be accepted and used in the further analysis.

Especially when it came to the histology results, there would always a possibility for some bias as the scoring is done by a person and not a machine, if the scoring were done by someone else the results might be completely different. Scoring and analyzing histology slides is something that takes practice and someone analyzing it for the first time will have a different understanding compared to someone with more experience that knows what to look for. Because of this in the histology findings there might be more bias compared to the other results. Being the first time the histology was done the main information to get out of this was that the

control group was not affected, except for that to compare the histology results to any of the other treatment groups did not say more. Looking at the histology slides that were scanned, both for the olfactory organ and the gills they should ideally be done again as the final results after the staining was not the best, cutting the gills were also not easy and one sample had to be over several slides as they were split up. There were not enough time to do both the olfactory and the gill staining again. It was still possible to evaluate the slides and see some clear differences between the infected and non-infected fish.

Other limitations to the sub-trials are the water exchange rates in the individual RAS systems, which now were kept below 400L per system over the 14 days trial period, this may be especially applicable to the first sub-trial where the *Y. ruckeri* was introduced into the water. If the water was exchanged at a higher or lower rate how would this affect the outcome of the trials, this might also affect the second sub-trial. Other factors that can affect the results heavily is the stocking density in the different systems, as the stocking densities during the two trials were kept low. The research on how pathogens spread in RAS in the aquaculture industry has been limited and there has not been a project like this performed on RAS before in Norway. The assumptions made based on the results in these two sub-trials are made comparing the results to other trials that are mostly done in flow-through systems. Because of this what is common for RAS when it comes to how *Y. ruckeri* spreads depending on the entry way is not known and the results can only be compared to earlier trials done on mostly flow-through systems. The results from the trials are limited to how the different pathways can evoke the innate immune response.

4.5 Further research

There is still a need for understanding how pathogens spread in RAS, especially when there is an increase in the use of RAS in the aquaculture sector. When it comes to understanding how *Y. ruckeri* spreads it will be interesting to look at the swabs collected during these two sub-trials, especially since *Y. ruckeri* is known to adhere to solid surfaces (Wrobel et al., 2019) which there is found a lot of in RAS. Also looking at other tissues such as the spleen where it earlier has been found to be three times as likely to detect differences compared to other tissues (Sibinga & Marquis, 2021). Not only looking at the spleen but collecting data at more timepoints from the day of infection and through the trial could also have interesting results as it has been seen earlier that pro-inflammatory genes will peak at day 3 (Harun et al., 2011), for

other antibacterial defense genes and cytokines the symptoms was not detected in the intestine until two days post infection (Ahmed et al., 2021).

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6 Appendix

6.1 Equipment used for water quality measurements

Name	Supplier
Nitrate test in fresh water	Vwr
Nitrite	Vwr
Ammonium	Vwr
Total alkalinity	Vwr
Multi 3630 IDS SET (Probes)	WTW, Weilheim Germany

6.2 Plates and content for MagMAX96

Plate	**Solution**	**Plate position**	**Volum/well**	**Tray type**
Sample plate	Sample (homogenate) Lysisbuffer Isopropanol-2 Binding bead mix	1	220 µl 50 µl (100 µl for olfactory organ) 50µl (none for olfactory organ) 100 µl 20µl	Deep Well (DW)
Wash plate1	Wash solution 1	2	150 µl	DW
Wash plate 2	Wash solution 2	3	150 µl	DW
DNase plate	Turbo DNase solution	4	50 µl	DW
Wash plate 3	Wash solution 2	5	150 µl	DW
Wash plate 4	Wash solution 2	6	150 µl	DW

Elution plate	Elution Buffer	7	50 µl	Elution plate
Tip comp		8		DW

6.3 Equipment lists qPCR

Reagents & equipment	Supplier
RNaseZap™	invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania
Centrifuge 5804	Eppendorf

6.3.1 Equipment used for the homogenization

Reagents	Cat nr. & Supplier
MagMAX™ *mir*Vana™ Total RNA Isolation Kit, 96 rx.	27828, applied biosystems by Thermo Fisher Scientific, Vilnius, Lithuania

Equipment	Name	Cat nr. & Supplier
Metall beads	Matrix S, 3,175 mm stainless-steel grinding beads	116925000 MP Biomedicals
Tubes for FastPrep (96-format)	Collection Microtubes (racked, 10x96) (1.2ml)	(Cat.19560) Qiagen, Hilden, Germany
Lids for FastPrep-tubes	Collection Microtube Caps (120x8)	(Cat.19566) Qiagen, Hilden, Germany
Homogenizer	Precellys 24, FastPrep96	MP Biomedicals

Plate covers	Plate covers	applied biosystems by Thermo Fisher Scientific, Vilnius
96 plates for storage	Processing plate	applied biosystems by Thermo Fisher Scientific, Vilnius

6.3.2 Equipment used for RNA-isolation

Reagents & equipment	Cat nr. & Supplier
MagMAX™ *mir*Vana™ Total RNA Isolation Kit, 96 rx.	A27828, applied biosystems by Thermo Fisher Scientific, Vilnius, Lithuania
Isopropanol-2, 100 %	I9516 Sigma, Merck
Etanol, 100 % (absolutt alkohol prima)	Kremetyl
Deep Well plates	applied biosystems by Thermo Fisher Scientific, Vilnius
Eluation plate (MagMax Express plate, 200 μl)	applied biosystems by Thermo Fisher Scientific, Vilnius, Lithuania
MagMax Express Tip Comp for Deep Well Magnets	applied biosystems by Thermo Fisher Scientific, Vilnius
MagMAX™ Express 96	applied biosystems by Thermo Fisher Scientific
Plate covers	applied biosystems by Thermo Fisher Scientific, Vilnius

6.3.3 Equipment used for controlling the RNA quality

6.3.3.1 NanoDrop8000/1000

Reagents & equipment	Cat nr. & Supplier
NanoDrop 8000	Thermo Scientific
Nuc free water	invitrogen by Thermo Fisher Scientific, Austin, USA

6.3.3.2 Bioanalyzer

Reagents & equipment	Cat nr. & Supplier
2100 Bioanalyzer	Serial No. DE13806315, Agilent
RNA 6000 Pico kit	Agilent

6.3.4 Equipment used during the cDNA synthesis

Reagents & equipment	Cat nr. & Supplier
High-Capacity RNA-to-cDNA™ kit, 2-KOMPONENT	4387406, Thermo Fisher Scientific, Vilnius, Lithuania
Already prepared RNA- samples	
RP, LF, SUB Sk, 96 well plate, designed to fit ABI/Life ThechnologiesÒ Cyclers	BIOplastics, Landgraaf, The Neherlands
PCR® Strip Caps	Axygen Scientific, California, USA
Nuclease-free water	invitrogen by Thermo Fisher Scientific, Austin, USA
Plate covers	applied biosystems by Thermo Fisher Scientific, Vilnius

PCR-maskin, PCR system 2700, GeneAmpÒ	applied biosystems by Thermo Fisher Scientific

6.3.5 Equipment used for preparation of Primers

Reagents & equipment	Cat nr. & supplier
Micro tube 1,5 ml DNA LowBind	Sarstedt AG & Co, Numbrecht, Germany
Nuclease-free water	invitrogen by Thermo Fisher Scientific, Austin, USA

6.3.6 Equipment used for qPCR analysis

Reagents & equipment	Cat nr. & supplier
PowerUp™ SYBR™ Green Master Mix	A25742, Thermo fisher, Vilnius, Lithuania
Nuclease-free water for NTC control	invitrogen by Thermo Fisher Scientific, Austin, USA
QuantStudio5	applied biosystems by Thermo Fisher Scientific
384-plate	applied biosystems by Thermo Fisher Scientific, Vilnius
Plate covers	applied biosystems by Thermo Fisher Scientific, Vilnius

6.4 R Studio PCA script

```
library(readxl)
X24h_sub_1 <- read_excel("Documents/UiT master/RASHEALTH
 masteroppgave/24h sub 1.xlsx")

pc.plot<-X24h_sub_1
library(ggbiplot)
library(devtools)
install_github("vqv/ggbiplot", username = "vqv")
library("ggbiplot")
ir.pca <- prcomp(pc.plot[, 3:12], center = TRUE, scale. = TRUE)
g <- ggbiplot(ir.pca, obs.scale = 1, var.scale = 1,
              groups = pc.plot$Treatment, ellipse = TRUE,
              circle = TRUE)
plot(g)

install.packages("devtools")

ir.pca <- prcomp(pc.plot[, 3:12], center = TRUE, scale. = TRUE)
g <- ggbiplot(ir.pca, obs.scale = 1, var.scale = 1,
              groups = pc.plot$Tissue, ellipse = TRUE,
              circle = TRUE)
plot(g)
```

6.5 Histology scoring schemes

6.5.1 Distal gut scoring scheme

Score	Description
0	Large vacuoles occupy almost the entire apical part of the enterocyte. Simple and complex folds appear long and thin.
1	Medium-sized vacuoles, which occupy less than half of the enterocytes, are present. Simple mucosal folds have medium length. Complex folds are still long but appear thicker.
2	*At least 2 of these changes are observed:* Sparsely scattered vacuoles. Some mucosal folds appear stubby. Pycnotic cells are found sporadically. Cases of widened *lamina propia* due to cellular infiltration and increased numbers of intra-epithelial lymphocytes are observed.
3	*At least 3 of these changes are observed:* Almost no supranuclear vacuoles are present. Epithelial structure is severely compromised. Widespread pycnotic cells are found. Increased cases of widened *lamina propia* due to cellular infiltration and increased numbers of intra-epithelial lymphocytes are observed. Necrosis and multifocal inflammation are observed.

6.5.2 Gills scoring scheme

Score	Description
0	Gill structures (filament and lamella) are well-defined, non-specific pathologies such as lifting, hyperplasia and clubbing account for <5% of the evaluated lamella
1	5 to 10% of the microscopic field show lesions, including increased cases of lifting, hyperplasia and clubbing. Lamellar fusion can be observed sporadically.
2	11 to 20% of the microscopic field show lesions, including increased cases of lifting, hyperplasia, clubbing and lamellar fusion. Sporadic cases of lamellar bleeding and aneurisms.
3	More than 20% of the microscopic field show lesions, including increased cases of lifting, hyperplasia, clubbing and lamellar fusion. Cases of bleeding and aneurism increase. Widespread multifocal proliferative gill inflammation is observed. Necrosis is observed. Severe filamental and lamellar congestion are observed.

6.5.3 Olfactory organ scoring scheme

Score	Description
0	Structure is intact and well-defined, epithelial surface is smooth and mucosal cells are evenly distributed on the mucosal tip
1	10-20% show tissue damage including loss of well-defined structure, epithelial surface shows roughness; sporadic vacuolisation is observed. Mucosal cells become denser on the mucosal tip.
2	>20-40% show tissue damage including loss of well-defined structure, epithelial surface shows roughness. Mucous cells become denser in some olfactory organ lamella or loss of mucous cell layer can also be observed. Focal bleeding is observed.
3	>40% or complete loss of tissue structure including severe signs necrosis and bleeding. Multifocal inflammation is observed.