Histological investigation of gill health responses to recirculating aquaculture system (RAS) water quality in apparently healthy Atlantic salmon (*Salmo salar*)

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Abbreviations

AB-PAS	Alcian Blue - Periodic Acid-Schiff
BW	Brackish water
CA	Carbonic anhydrase
DOC	Dissolved organic carbon
EGC	Eosinophilic granule cells
FW	Freshwater
HE	Haematoxylin and Eosin
ILU	Interlamellar unit
IQR	Interquartile range
MCH	Major histocompatibility complex
MTB	Maximum allowable biomass
NCC	Nonspecific cytotoxic cell
NK-cell	Natural killer-cell
PMN	Polymorphonuclear cell
POC	Particulate organic carbon
RAS	Recirculating aquaculture system
SW	Seawater
TAN	Total ammonia nitrogen
T_c-cell	T-cytotoxic cell
T_h-cell	T-helper cell
TOC	Total organic carbon
TSS	Total suspended solids

Abstract

Recirculating aquaculture system (RAS) technology has become more widespread in Atlantic salmon farming, and the need for knowledge about operating such a system has been highlighted in recent years. Gill pathology has been identified by fish health personnel as a bigger problem in RAS than in flow-through systems, and this thesis has sought to investigate potential factors influencing gill health in RAS. This was done by following a production cycle in three different RAS, where two were of commercial scale, and one was operated at a much lower stocking density. The difference in stocking density was included to illustrate potential differences in the organic loading of the systems. Total organic carbon and bacterial numbers were investigated on a biweekly basis, and regularly monitored water quality data was obtained from the systems.

Apparently healthy fish were sampled, and throughout production an increase in clubbing, inflammatory cell infiltration in the clubbing, and inflammatory cell infiltration of the secondary lamellae showed a significant increase in all three systems.

Bacterial numbers and TOC did not correlate, but the concentration of TOC in the system did increase in all three RAS. Potential interactions between TOC and the microbial community, as well as several microbial interactions which may affect gill health are discussed in this thesis.

For posterity: COVID-19 interfered with the final stages of lab work for this thesis. Therefore, some of the work that was planned, was not completed. The university of Bergen has recognised that some of the results may not be as complete as one would like but decided that master students should complete their theses in a manner which allows them to progress as normally as possible.

1. Introduction

1.1 Aquaculture and RAS in Norway

Consumption of fish has been increasingly important in feeding the world's population, and made up 17 % of the animal protein consumed in 2015 (FAO, 2018). Norway's 1 326 thousand tonnes in 2016 made up 1.7 % of the worlds aquaculture food fish production, and made Norway the largest producer in Europe (FAO, 2018). Salmonids are high-valued fish, and in 2019 the total value of slaughtered fish in Norway was almost 72 billion NOK (Fiskeridirektoratet, 2020).

The advantageous natural conditions of the Norwegian coastline, has enabled Norway to produce Atlantic salmon (*Salmo salar,* Linnaeus 1758) and rainbow trout (*Oncorhynchus mykiss,* Walbaum 1792) at a relatively low cost, despite high salary expenses (Holm, 2015).

The goal of the Norwegian government has been to increase aquaculture production substantially, while maintaining a sustainable environmental impact on natural species (Meld. St. 16 (2014-2015)). Therefore, production growth regulations based on aquaculture impacts of salmon lice (*Lepeoptheirus salmonis*) on the wild salmon and trout smolt population have been implemented (Produksjonsområdeforskriften, 2017 §8). In marine production areas unable to fulfil the criteria set in the regulatory system, production growth has been restricted, or reductions have been proposed.

In contrast, the production of smolt in hatcheries was earlier seen as the largest bottle-neck for continued growth in Norwegian aquaculture (Kittelsen *et al.*, 2006). Increased production with flow-through hatchery technology would have exceeded an acceptable load on available fresh water resources, therefore recirculating aquaculture systems (RAS) were presented as a solution for utilizing existing water resources and reach the long term rise in smolt demand (Kittelsen *et al.*, 2006). In 2006, 3 facilities in Norway used RAS technology on parts of or all their production. There are no official statistics of how many RAS-facilities exist in Norway

today, but there are at least 48 RAS hatcheries (Nistad, 2020), and counting other kinds of RAS facilities there are over 50 (Nistad, *pers. com*.).

There is an increasing interest in the on-growing of post-smolts in marine aquaculture facilities on land (Holm, 2015). Previously, a hatchery could only produce fish up to 250 g, but since 2012, changes in Norwegian law allowed the production of fish up to 1000 g with a hatchery permit (Holm, 2015; Gorle *et al.*, 2018). The revised permission to run a land-based grow-out facility was decided to be without extra licencing costs (Holm, 2015), unlike developing permits for grow-out facilities at sea. Ordinary permits for aquaculture at sea are limited, and when they are available, they come with high fees or are auctioned off (Holm, 2015).

The increasing treatment cost and regulations regarding sea lice, low availability, and high cost of sea cage permits, along with land-based permits without extra fees, has given the aquaculture industry strong incentives to place larger parts of their production cycle on land. In many cases, this is done by keeping fish in post-smolt facilities until they are 300-500g, and then moving them to sea. This post-smolt production allows for a more efficient use of the maximum allowable biomass (MTB) each company has available in the marine phase, by shortening the phase that fish are kept at sea.

1.2 Recirculating aquaculture technology

A RAS can be defined in different manners, but a widely accepted definition is a system which recirculates more than 90% of its volume, and exchanges less than 10% of the volume per unit time (Timmons and Ebeling, 2010c). Facilities which exchange more than 10% of its volume, would be termed a re-use system. There are several ways to define this percentage, but the most typical ways of defining the recirculation in a system is: the degree of recirculation (%) (Eq. 1), water exchange per day (%) (Eq. 2) or water exchange per day per kg of feed (L kg^{-1} feed) (Eq. 3) (Holan, Good and Powell, 2020)

$$\frac{Water\ flow\ to\ the\ tank\ per\ hour}{New\ water\ per\ hour + Water\ flow\ to\ the\ tank\ per\ hour}\ x\ 100 \qquad (1)$$

$$\frac{Water\ exchange\ per\ day\ (m^3 day^{-1})}{Total\ water\ volume\ in\ the\ system}\ x\ 100 \qquad (2)$$

$$\frac{Water\ exchange\ per\ day\ (m^3 day^{-1})}{Feeding\ per\ day\ (kg\ day^{-1})}\ x\ 100 \quad (3)$$

An increase in the use of RAS technology was thought to help control disease, allow for optimal production by controlling more of the fish environment, as well as increasing growth rates. However, the increasing interest in utilizing RAS technology has exposed the need for knowledge about running such a system (Hjeltnes *et al.*, 2012). One of the biggest obstacles for RASs in Norway today, is a lack of qualified personnel and exchange of knowledge about optimal conditions in different systems (Badiola, Mendiola and Bostock, 2012). To run a RAS, one needs both technical and biological insight, as well as a good understanding of chemical processes.

1.2.1 Basic outline of a RAS

A RAS is made up of two main components, the production tank(s), and the water treatment system. The recirculation of water leads to a build-up of certain compounds, and a higher degree of recirculation leads to a higher degree of accumulation, as less water is exchanged (Colt, 2006). To achieve a high degree of recirculation, the system needs to control the amount of fine solids, surface-active compounds, metals and ammonia (Colt, 2006). There will also be a need to closely monitor and adjust important water quality parameters, such as oxygen (O_2), carbon dioxide (CO_2), total gas pressure (TGP), pH, alkalinity and temperature (Colt, 2006).

The tank itself has to be built and operated to allow for currents to effectively clean the tanks, and prevent biofilm build-up (Gorle *et al.*, 2018). Control of solids, like feed waste and faeces, is in most facilities controlled by a mechanical filter, which removes the large particles from the water and sends it to waste water treatment (Timmons and Ebeling, 2010b). Some systems also have a swirl separator, where the settleable particles are removed before the water goes through the mechanical filter (Davidson and Summerfelt, 2005). In order to remove dissolved organic components, a foam fractionator may be used (Barrut *et al.*, 2013). These different means of filtering aim to remove as much organic matter as possible, to avoid affecting the bacterial dynamic in the biofilter (Chen, Ling and Blancheton, 2006; Blancheton *et al.*, 2013).

The biofilter is made up of bacteria growing on a form of media, often made of plastic. Several different kinds of biofilter exist, but they all aim for bacteria to convert the nitrogenous waste of the fish from substances toxic to fish into less toxic forms (Timmons and Ebeling, 2010a).

Fish excrete most of their nitrogenous waste as ammonia, to a large degree across the gills. In aqueous solution, ammonia exists in two forms: NH_3 and NH_4^+., where NH_3 is toxic for fish at low concentrations (Evans, Piermarini and Choe, 2005).

The main kinds of bacteria in the biofilter are autotrophic nitrifiers, which oxidise ammonia into nitrate (NO_3^-) , and heterotrophic bacteria, which oxidise organic matter into ammonia (Blancheton *et al.*, 2013). The microbiota in the biofilter is an ecosystem, which changes its composition at different times in the production cycle, from system to system and under different operating parameters, like salinity or temperature (Bakke *et al.*, 2017). Even though the bacterial community may differ, in a simplified nitrification process, NH_4^+ is oxidized into nitrite (NO_2^-), mainly by *Nitrosomonas* sp. and *Nitrosococcus sp.* (Eq. 4), before nitrite is oxidised into NO_3^-, mainly by *Nitrobacter* sp. and *Nitrospira* sp. (Eq. 5). Equation 6 may be used to calculate consumption of O_2 and alkalinity, and production of bacterial biomass ($C_5H_7O_2N$) (Chen, Ling and Blancheton, 2006; Holan, Good and Powell, 2020).

$$NH_4^+ + 1{,}5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+ \quad (4)$$

$$NO_2^- + 1{,}5\ O_2 \rightarrow NO_3^- \quad (5)$$

$$NH_4^+ + 1{,}83O_2 + 1{,}98HCO_3^- \rightarrow 0.021\ C_5H_7O_2N + 0.98\ NO_3^- + 1.041\ H_2O + 1.88\ H_2CO_3^- \quad (6)$$

The nitrification process is dependent on oxygen being available (Eq. 4 and 5), thus being competed with by heterotrophic bacteria's oxidation of organic waste. This may limit the efficacy of the nitrification process, as fast-growing heterotrophic bacteria outcompete nitrifiers in consuming oxygen. In addition, the heterotrophic bacteria produce more ammonia, CO_2 and sludge, adding to the waste load in the system, showing the importance of limiting the amount of organic waste entering the biofilter (Blancheton *et al.*, 2013). However, heterotrophic bacteria are important as they fill several niches, thus reducing available resources for opportunistic, possibly pathogenic, bacteria. It has been suggested that this ecosystem quality may supress pathogens from developing disease in the fish, even though they are present in the system (Blancheton *et al.*, 2013; Garcia-Mendoza *et al.*, 2019). The pathogen-suppressive qualities of well-established microbiotas is being explored and documented in several different environments, like plants (Vannier, Agler and Hacquard, 2019), soil (Toyota and Shirai, 2018) and in humans (Kho and Lal, 2018; Khan, Petersen and Shekhar, 2019), and may be the case in RAS environments as well.

Some RASs include a denitrification system, where facultative aerobic bacteria may utilize nitrate or nitrite as a substitution for O_2 in anaerobic respiration (Gutierrez-Wing and Malone, 2006; Timmons, van Rinj and Ebeling, 2010). Including this step allows for a higher degree of recirculation as it converts nitrate into nitrogen gas, instead of relying on water exchange to avoid accumulation of nitrate. However, this technology is not yet commercially available, because of the cost and complexity of the process (Timmons, van Rinj and Ebeling, 2010; Müller-Belecke *et al.*, 2013).

Both fish and heterotrophic bacteria produce CO_2, which affects the pH and alkalinity of the system (Colt, 2006; Blancheton *et al.*, 2013). A RAS therefore needs CO_2-aeration to allow CO_2-gas to escape the water (Moran, 2010). Addition of buffer also helps keep pH and alkalinity at acceptable and stable levels (Fjellheim *et al.*, 2006). Maintaining the alkalinity is necessary, as the nitrifying bacteria use HCO_3^- in their oxidation process (Eq. 6) (Chen, Ling and Blancheton, 2006). Oxygenation is often the last step of water treatment before the water goes back into the production tank. Some facilities have in-stream disinfection, where the water is disinfected by UV or ozone as a part of the recirculatory water treatment, to keep bacterial levels in the system low. Others choose to only disinfect the intake water, to not affect the microbial community in the system (Blancheton *et al.*, 2013).

1.3 Gills

1.3.1 Gill anatomy and physiology

The gill is the main respiratory organ for most fish, and is also the site for osmoregulation, pH regulation, hormone production, and the aforementioned excretion of nitrogenous waste, by Na^+/NH_4^+, Na^+/H^+ and Cl^-/HCO_3^- coupled exchange mechanisms (Evans, Piermarini and Choe, 2005; Speare and Ferguson, 2006). Gills are sensitive to their surrounding environment, with an epithelial layer only one cell thick and with a large surface area that scales logarithmically with body mass (Palzenberger and Pohla, 1992). Gills are therefore good candidates for detecting poor water quality or conditions which may affect fish health (Flores-Lopes and Thomaz, 2011; Gomes *et al.*, 2012; Dang *et al.*, 2020).

Each fish has four gill arches on each side of its head, covered by an operculum, which provides some coverage from the surrounding environment. A gill arch is covered by horizontal gill filaments, the filaments are in turn covered by vertical lamella, which are the site of exchange.

One gill arch has two rows of filaments, called a holobranch, while a single row of filaments is called a hemibranch (Kryvi and Poppe, 2016). In between two hemibranchs, and along the trailing edge of the gill, the interbranchial lymphoid tissue complex (ILT) is located. The ILT is mainly associated with the presence of T-lymphocytes (Dalum *et al.*, 2015, 2016).

A healthy gill would have lamellae consisting of a structural core of pillar cells, which make up lacunar spaces through which blood flows (Kryvi and Poppe, 2016). The epithelium of the gill is made up of a single layer of pavement cells, resulting in a short distance of 0,5-4 µm between water and blood (Evans, Piermarini and Choe, 2005; Kryvi and Poppe, 2016). This short distance is ideal for physiological processes, such as diffusion of O_2 and CO_2, but can be problematic in terms of pathogens and other irritants, as they may easily enter the blood stream (Koppang, Kvellestad and Fischer, 2015).

O_2-uptake relies on diffusion across the gill epithelia into the blood, which transports O_2 for respiration around the body. The respiratory by-product carbon dioxide (CO_2) is brought back to the gills in the form of bicarbonate (HCO_3^-) in the blood, which is converted into CO_2 using carbonic anhydrase (CA) in the red blood cells. CO_2 then diffuses out into the water (Evans, Piermarini and Choe, 2005). Gills are also an important site for osmoregulation, with different specialised cells called mitochondrion-rich cells, ionocytes, or commonly referred to, "chloride cells", responsible for ion-exchange (Hiroi and McCormick, 2012). The physiological salinity of Atlantic salmon is approximately 10 ‰, and thus different from that of both freshwater and seawater (Nilsson *et al.*, 2018). In freshwater, fish are in an hypoosmotic environment and need an active uptake of ions to maintain their ion levels. In saltwater, the environment is hyperosmotic, meaning that the fish needs to excrete ions (Evans, Piermarini and Choe, 2005). The ionocytes responsible for ion-excretion in saltwater have been suggested to also play a part in acid-base regulation (Hiroi and McCormick, 2012). In fish tissue, ammonia (NH_3) mostly exist as its ionized form ammonium (NH_4^+) because of the low physiological pH. Some ammonia is excreted by the kidney, but excretion occurs mainly across the gills, by NH_3-diffusion across epithelia, by NH_3-diffusion and simultaneous excretion of H^+ by Na^+/H^+-exchange, or by active transport of NH_4^+ in exchange for Na^+ (Evans, Piermarini and Choe, 2005).

1.3.2 Gill defence mechanisms

To protect the gill epithelia, fish have a mucosal barrier coating the entire surface of the gill. This mucus layer works both as a physical barrier thanks to its viscosity, and as an active biological defence which is part of the fish immune system (Uribe *et al.*, 2011). Increases in number, size, distribution and histochemical staining characteristics are often reported during several types of gill disease, exposure to irritants, or parasites (Ferguson *et al.*, 1992; Roberts and Powell, 2005; Speare and Ferguson, 2006). Therefore, the number of mucous cells, along with other non-specific gill pathologies are often seen as stereotypical responses, and used as an indicator for gill health (Speare *et al.*, 1997; Speare and Ferguson, 2006; Gomes *et al.*, 2012; Good *et al.*, 2017). This increase in secreted mucus may trap the pathogens, and the increased turnover in the mucus layer may also increase the rate that unwanted particles are shed (Speare and Ferguson, 2006).

Mucus contains several factors meant to stop pathogens from entering the body, including lectins, lysozyme, pentraxins, complement proteins, antibacterial peptides and immunoglobulins (Uribe *et al.*, 2011; Castro and Tafalla, 2015). The first line defence detect and eliminate pathogens in a non-specific manner (Kelly and Salinas, 2017).

Bacteria contain pathogen associated molecular patterns (PAMPs), like lipopolysaccharides (LPS), peptidoglycan or bacterial DNA. These do not occur regularly in multicellular organisms, and are recognized by pattern recognition receptors (PRRs) of the fish immune system (Uribe *et al.*, 2011; Gomez, Sunyer and Salinas, 2013; Grayfer *et al.*, 2018). This recognition is the first step in a multitude of different activation routes, and may stimulate production of cytokines, such as chemokines and interleukins. These are soluble factors responsible for regulating the immune response by mediating cell signalling (Castro and Tafalla, 2015), and may be both pro-inflammatory (e.g. IL-1, IL-12, IL-18 and TNF-α) and anti-inflammatory (e.g. IL-10) (Koppang, Kvellestad and Fischer, 2015).

The cells of the immune system are lymphocytes, monocyte/macrophage, polymorphonuclear (PMN) cells (neutrophils, eosinophils and basophils), eosinophilic granule cells (EGC), dendritic cells, and thrombocytes (Lovy, Wright and Speare, 2008). Lymphocytes are divided into B-cells, T-cells, and natural killer (NK)-cells, often termed nonspecific cytotoxic cells (NCC) in fish. T-cells may be divided into T cytotoxic (T_c)-cells ($CD8^+$) and T helper (T_h)-cells ($CD4^+$), and they need antigens presented to them on a major histocompatibility complex (MHC), either class I

or class II. MHC I is present on nearly every nucleated cell, and presents intranuclear proteins to T_c-cells, which destroys the cell if non-self-antigens are detected. MHC II is only present on antigen-presenting cells, or phagocytes, which ingest extracellular material and present them to T_h-cells. Monocytes, macrophages, PMN cells, dendritic cells and B-cells in fish are phagocytic (Haugland, Jordal and Wergeland, 2012; Castro and Tafalla, 2015).

The interbranchial lymphoid tissue is a diffuse mucosal lymphoid tissue, indicated to have a role in maintaining immune tolerance and homeostasis in the gill, to avoid a continuous immune stimulation by harmless antigens (Aas *et al.*, 2017). It consists of T-cells, most of which have been identified as $CD4^+$, along with major histocompatibility complex class II (MHC II)-cells (Dalum *et al.*, 2015). CD4 and MHC II are associated with the T-helper (T_h)- cell response to extracellular peptides (e.g. bacteria). The T_h-responses are divided in mainly three different kinds: T_h1 and T_h17, which are associated with pro-inflammatory responses, and T_h2, where high levels are associated with a down-regulation of the T_h1 and inflammatory response (Fischer, Koppang and Nakanishi, 2013). The gill appears to have a Th2-skewed environment, which would prevent the gill from developing a highly inflammatory environment against harmless antigens (Takizawa *et al.*, 2011; Koppang, Kvellestad and Fischer, 2015). Fish are also less susceptible to LPS-stimulation than mammals, which suggests that fish sense LPS differently than mammals, caused by their close interactions with a highly microbial environment (Castro and Tafalla, 2015).

Despite the defence mechanisms of the fish immune system, some bacteria are able to form a commensal microbiota on mucosal surfaces (Musharrafieh *et al.*, 2014; Lowrey *et al.*, 2015). In skin, this microbiota has shown advantageous traits for the fish, like antifungal or antibacterial effects (Boutin *et al.*, 2014; Lowrey *et al.*, 2015). The innate immune system may even be shaped by its commensal bacteria (Kelly and Salinas, 2017).

This vast number of factors makes fine-tuning the response to a challenge possible, however, it also means that mapping out the fish immune responses is difficult. There is, for example, little information linking the immune response to specific pathological changes (Koppang, Kvellestad and Fischer, 2015). The mechanisms for tolerating harmless or advantageous bacteria, but not pathogens, are poorly understood, but stress or environmental changes may result in loss of homeostasis and lead to a shift in what is pathogenic for the fish (Gomez, Sunyer and Salinas, 2013; Kelly and Salinas, 2017).

1.4 Water quality

Specific operating water quality recommendations for hatcheries (Table 1.1) were removed from regulations in 2018 (Forskrift om endring i forskrift om drifta av akvakulturanlegg, 2018), as the industry relies increasingly on RAS technologies. Water quality in land-based facilities in Norway are today regulated as *" (…) giving the fish good living conditions, based on fish species, age, state of development, weight, and physiological and behavioural needs*", and "*water quality and the effects between different water parameters should be monitored based on the risk of poor fish welfare*"(Akvakulturdriftsforskriften, 2008 §22).

In recent years, RAS-facilities are no longer purely freshwater or sea water. Many systems run on some degree of brackish water when producing smolt and post-smolt, and in grow-out facilities. Research studying water quality in aquaculture has mostly been performed in flow-through systems. Studies performed in closed containment systems, such as RAS, during treatments for amoebic gill disease (AGD) or sea lice, or during transport, are mostly performed in fresh water, or seldom verified for different salinities.

Salinity affects several water chemistry parameters, and salinity-levels in brackish systems may vary, leading to an unstable environment. When operating a RAS, the impacts on the nitrification process in the biofilter, not only effects on the fish, need to be taken into account (Table 1.1) (Colt, 2006). Ranges established for freshwater can therefore not be directly transferred to seawater, or from flow-through systems to RAS. A system will also need to adjust its operating parameters throughout production, as production conditions are constantly changing. Raw water quality, system design, and biology all vary between facilities, making it difficult to conduct studies which evaluates all possible mixing effects.

Table 1.1: Recommended ranges for important water quality parameters when operating land-based aquaculture for salmonids in RAS. Values for biofilter are based on what range give an optimal nitrification process. FW = freshwater, SW = seawater. Source indicated by subscript in table. 1) Recommendations from the Norwegian Food Safety Authority (NFSA), removed 2018 (Fjellheim *et al.*, 2016; Kolarevic *et al.*, 2018). *2)* (Chen, Ling and Blancheton, 2006) *3)* (Thorarensen and Farrell, 2011) *4)* (Timmons and Ebeling, 2010c)

Water quality parameter	**NFSA 2018**	**RAS**	**Biofilter**
pH	6,2 - 7,8 [1)]	6,5-8,5 [4)]	7,5-9 [2)]
Oxygen saturation in tank	≤ 100% [1)]	85-120 % [3)]	4 mg/l [2)]
Oxygen (drain)	> 80% [1)]	6-8 mg/L [4)]	
Total Gas Pressure (TGP)	≤ 100% [1)]	≤ 100 % [3)]	
Carbon dioxide	< 15 mg/l [1)]	<20 mg/l [4)]	
Nitrite	< 0,1 mg/l (FW) [1)]	<1 mg N/l, 0,1 in soft water [4)*]	
	< 0,5 mg/l (SW) [1)]		
Total ammonia-N (TAN)	< 2 mg N/l [1)]	<1 mg N/l [4)]	Limiting [2)]
Nitrate		0-400 mg N/l [4)]	
Ammonia (unionized)		<0,0125 mg N/l [4)]	
Total organic carbon (TOC)	< 10 mg/l [1)]		Minimized [2)]
Alkalinity		50-300 mg/l (as $CaCO_3$) [4)]	> 200 mg/l [2)]
Temperature		15°C [4)]	14-27 °C [2)]
Total suspended solids (TSS)		15 mg/l [3)]	

[*] Reduced toxicity by maintaining a Cl^-:NO_2^- -N-ratio of 104:1 (Gutiérrez *et al.*, 2019)

1.4.1 Water quality in a system

Temperature and salinity

Several water quality parameters are affected by temperature and salinity. How the different parameters are affected will be mentioned in each passage. An optimal temperature for Atlantic salmon will be around 15 °C (Timmons and Ebeling, 2010c). Temperatures below 14°C limit nitrification, but with higher temperatures the diffusion layer of a fixed biofilm may cause limited O_2 availability. This can, however, be counteracted by sufficient turbulence and mixing of the water (Chen, Ling and Blancheton, 2006).

High salinity systems have a lower rate of nitrification compared to a freshwater system (Chen, Ling and Blancheton, 2006; Díaz *et al.*, 2012). A sea water biofilter also needs a longer start-up period to reach stable nitrification (Navada, Sebastianpillai, *et al.*, 2020). Rapid changes to salinity in filters matured in freshwater show a pronounced effect on nitrification (Kinyage, Pedersen and Pedersen, 2019; Navada *et al.*, 2019), however, salt-primed freshwater biofilters or brackish water biofilters have shown less reduction in nitrification when exposed to increased salinity (Gonzalez-Silva *et al.*, 2016; Navada, Vadstein, *et al.*, 2020).

Oxygen (O_2), carbon dioxide (CO_2) and partial pressure

The O_2 saturation varies depending on both temperature and salinity. Warm saltwater contains less O_2 when fully saturated compared to cold freshwater (Thorarensen and Farrell, 2011), this fact is important to keep in mind when monitoring O_2 by saturation (%). When adding oxygen, one should keep control of the total gas pressure in the tank, as this may contribute to supersaturation and gas bubble disease (Thorarensen and Farrell, 2011). Hyperoxia may reduce the fish respiration frequency, cause retention of CO_2 in the blood, and result in respiratory acidosis (Powell and Perry, 1997; Thorarensen and Farrell, 2011).

In activated sludge, the optimal amount of dissolved oxygen for nitrification was found to be 4 mg/l. Other studies have investigated values limiting the nitrification rate, and indicates that the oxidation of nitrite to nitrate is more sensitive to low oxygen-levels than ammonia to nitrite (Chen, Ling and Blancheton, 2006).

For both O_2 and CO_2, partial pressure is an important factor for uptake and excretion, as they both diffuse over the gill. The solubility of CO_2 varies with both temperature and salinity, meaning that the same concentration of CO_2 can differ with 40% partial pressure between 5 °C and 15 °C (Thorarensen and Farrell, 2011). As well as CO_2 reducing the growth rate of fish,

high levels have an anaesthetic effect. High CO_2-levels also reduces water pH, some may therefore recommend a cautious approach, and keep levels below 10 mg/l (Thorarensen and Farrell, 2011). However, recommendations differ from 9 to 30 mg/l as an upper safe limit of CO_2 (Timmons and Ebeling, 2010c). Most research is performed on steady state concentrations, however Thorarensen and Farrell (2011) raises the issue of lack of knowledge about the spatial variations of CO_2-consentrations within the tank, as the fish pH compensation system may take days to responds to changes.

pH

High and low pH-levels can cause stress or lethality in fish, however, the effects of pH-levels on other water quality parameters are more often a problem than pH itself (Timmons and Ebeling, 2010c). The continuous production of CO_2 by fish and bacteria, as well as the continuous release of H^+ from the nitrification process (Eq.4) will lower pH in the system, making addition of buffer necessary to maintain desired pH-levels (Romano and Sinha, 2020). An increase in pH will lead to a higher degree of TAN as NH_3 and may become toxic to fish. Low pH will affect the nitrification process, which may lead to an accumulation of TAN or nitrite (Chen, Ling and Blancheton, 2006). Optimum pH-levels in a biofilter varies with what bacteria are present, but a pH below 5,7 have shown an inhibitory effect on nitrification (Chen, Ling and Blancheton, 2006). Rapid changes in pH (>0,5-1 units) may stress the biofilter, and cause reductions in nitrification until the bacteria are able to adapt (Timmons and Ebeling, 2010a), or cause toxicity of heavy metals for fish (Skjelkvåle *et al.*, 2007). Alkalinity may be increased to buffer pH-fluctuations (Davidson *et al.*, 2009).

Alkalinity

Alkalinity is a measure of pH-buffering capacity, often expressed as $CaCO_3$-equivalents. Alkalinity changes the relationship between pH and CO_2, and needs to be monitored and adjusted in order to keep pH and CO_2 at desired levels (Timmons and Ebeling, 2010c). Low alkalinity levels reduce the waters ability to buffer heavy metal, pH and CO_2-toxicity (Davidson *et al.*, 2009). Levels are adjusted by adding a buffer, commonly sodium bicarbonate ($NaHCO_3$) (Timmons and Ebeling, 2010c). This needs to be regularly added, as nitrifying bacteria consume carbonate or bicarbonate in the nitrification process (Eq. 6) (Chen, Ling and Blancheton, 2006).

Ammonia

Ammonia is excreted by the fish as metabolic waste and will accumulate in the system if water exchange is low or the nitrification in the biofilter is not working optimally. The relationship between the unionized (NH_3) and ionized (NH_4^+, ammonium) form of ammonia changes with pH, salinity, and temperature, therefore recommendations for total ammonia-nitrogen (TAN) (NH_3 + NH_4^+) are given (Table 1.1). Higher pH and temperature increases the percentage of TAN in the form of NH_3 (Chen, Ling and Blancheton, 2006), and increasing salinity reduces the amount of TAN as NH_3 (Nilsson *et al.*, 2018). Nitrogen compounds are often expressed as the nitrogen they contain, e.g. ammonia-nitrogen (NH_3-N). TAN can then easily be calculated from NH_4^+-N + NH_3-N (Timmons and Ebeling, 2010c). Conversion between NH_3 and NH_3-N, as well as other nitrogen compounds, is done by simple multiplication or division (see table 7.1 in Timmons and Ebeling (2010a)).

NH_3 is toxic to fish, as it is able to cross cell membranes and impair cerebral energy metabolism or cause cerebral hypoxia, therefore levels are recommended to be kept as low as 0,0125 mg-N/l (Table 1.1) (Arillo *et al.*, 1981; Timmons and Ebeling, 2010c).

In terms of the biofilter, TAN is the limiting substrate for nitrification. A study described in Chen, Ling and Blancheton (2006) found the minimum concentration of TAN to be 0,07 ± 0,5 mg/l at 27,2 °C.

Nitrite

Nitrite (NO_2^-), or nitrite-nitrogen (NO_2^-–N), will quickly be converted into nitrate if conditions are ideal in the biofilter, however, this process is more sensitive to sub-optimal water quality or changes in operation than conversion of ammonia to nitrite (Chen, Ling and Blancheton, 2006). Nitrite can compete with chloride (Cl^-) in the Cl^-/HCO_3^- - gill exchange, therefore, when the Cl^-/NO_2^- - ratio is low, nitrite may reach toxic levels in fish (Jensen, 2003; Timmons and Ebeling, 2010c; Gutiérrez *et al.*, 2019). Acute toxicity of nitrite is caused by its ability to oxidise the iron in haemoglobin, forming methaemoglobin, which has a lower affinity for O_2 (Jensen, 2003). Methaemoglobin gives blood a brown colour, and is therefore called “brown blood disease” (Timmons and Ebeling, 2010c; Romano and Sinha, 2020). To avoid toxicity, it has been recommended to keep the Cl^-:NO_2^- -N-ratio above 20:1 (Timmons and Ebeling, 2010c), however, an increase in the recommended ratio to 104:1 has recently been proposed (Gutiérrez *et al.*, 2019).

Nitrate

Nitrate (NO_3^-), or nitrate-nitrogen (NO_3^-–N), is less toxic to fish than both nitrite and ammonia, and fish entry has been proposed to occur through passive diffusion (Freitag *et al.*, 2015). Most facilities rely on water exchange to keep nitrate from accumulating to high levels (Timmons and Ebeling, 2010c). How well fish adapt to chronic high levels of nitrate, is not sufficiently researched to set a recommended limit, but levels ≤ 100 mg N/l did not show detrimental effects of the health of Atlantic salmon in freshwater (Freitag *et al.*, 2015; Davidson *et al.*, 2017; Good *et al.*, 2017). Harder water has been shown to reduce the toxicity of nitrate for juvenile rainbow trout (Baker *et al.*, 2017).

Heavy metals

Accumulation of heavy metals, such as, copper, zinc and cadmium may occur in a RAS. Safe levels heavily depend on water chemistry, but should be kept below 3,1 µg/l, 81 µg/l and 8,8 µg/l, respectively, when in seawater (Timmons and Ebeling, 2010c). Some heavy metals have been shown to accumulate in the gills, as negatively charged components in mucus may bind to cations, such as mercury, zinc, and copper. However, 80% of these accumulated metals were found to reside in the mucus (Speare and Ferguson, 2006).

Total suspended solids (TSS)

In RAS, suspended solids are mainly generated from faeces, microfloc, and feed remains (Timmons and Ebeling, 2010b; Schumann and Brinker, 2020), and recommendations of an upper level of 15 mg/l exists (Thorarensen and Farrell, 2011).The type of suspended solid in a system is important, as the shape, size and hardness of the particle affects the impact it has on the gill, i.e. blasting dust provides more damage than soil particles (Skjelkvåle *et al.*, 2007). Several studies have not been able to demonstrate an effect of increased levels of suspended solids on gill health, using kaolin clay or resuspended particles from RAS (Goldes *et al.*, 1988; Becke *et al.*, 2017, 2018, 2019). A possible explanation to this may be that the majority of particles in a RAS will be of organic nature, and thus softer in structure particles found in natural rivers (Schumann and Brinker, 2020). RAS systems facilitate an accumulation of fine particles, caused by the difficulty of removing particles of small sizes (Schumann and Brinker, 2020).

Total organic carbon (TOC)

Total organic carbon is rarely measured as part of monitoring aquaculture production, and its effect on fish is not known. Levels of TOC in intake water have been interesting for freshwater flow-through hatcheries, as it binds metal ions and thus decreases metal toxicity (Skjelkvåle *et al.*, 2007).

The rate of nitrification is affected by the ratio of C/N; As the ratio increases, nitrification decreases because of competition between the heterotrophic and autotrophic bacteria (Chen, Ling and Blancheton, 2006). An experiment showed a 40% decrease in nitrification rate when the amount of particulate organic matter was increased from 0,2 to 10 mg/l (Chen, Ling and Blancheton, 2006). The amount of TOC in the system should therefore be as low as possible in terms of nitrification efficiency.

Bacterial community

The bacterial community is not regularly monitored in aquaculture production, but is affected by most water quality parameters (Chen, Ling and Blancheton, 2006). With biofilters and a low exchange of water, the system establishes a more stable microbial community in the production tanks than in a flow-through system (Attramadal *et al.*, 2012; Rojas-Tirado *et al.*, 2018, 2019). Fish microbiota affects the tank microbial community, and shows large variations between individuals (Kelly and Salinas, 2017). The microbial community changes with time, and salinity, and the biofilter community differs from the tank community (Bakke *et al.*, 2017). With an increase of available particle surface area in a system, there is an associated increase in bacterial activity (Pedersen *et al.*, 2017).

1.5 Aims and objectives

This thesis is part of a strategic initiative at the Institute for Marine Research (IMR) looking at gill and heart health, project number 15555-03, and was intended as a pilot study with the goal of identifying factors relating to gill health in RAS. The goal was to narrow down the scope and identify interesting targets for following studies at IMR.

Non-specific gill pathology has been categorized as a bigger problem in RAS than in flow-through hatcheries by fish health personnel in Norway (Hjeltnes *et al.*, 2019, chap. 8.1). Gill diseases are often complex and multifactorial, and identifying as many pre-disposing factors as possible should be sought after and prioritized, as prevention is better than treatment after gill have deteriorated and exposed fish to stress.

The role of TSS in the development of gill pathology has been investigated, but has not shown an effect, in rainbow trout (Goldes *et al.*, 1988; Ferguson *et al.*, 1992; Becke *et al.*, 2017, 2018). Others are also investigating the effects of particulate factors in RAS (Holan *et al.*, 2016). Both carbon and particle surface area may impact the bacterial community, and activity, in a RAS (Chen, Ling and Blancheton, 2006; Pedersen *et al.*, 2017). The goal of this thesis was to investigate what gill pathologies may be observed in apparently healthy Atlantic salmon in RAS, and if the organic loading of the system, hereunder TOC and bacterial numbers, may be connected to these.

Objectives

1. To quantify the occurrence of gill lesions and mucous cell numbers in different RAS over time, in systems with different fish densities.
2. To investigate the relationship between TOC and bacterial numbers in different RAS, and how levels change throughout production.
3. To discuss the possibility of TOC, bacterial numbers, and water quality regularly measured at each site, as potential causes of observed gill changes.

2. Material and methods

2.1 Sampling locations

2.1.1 Location 1

Location 1 was a commercial RAS for the purpose of growing post-smolts, with the main water intake from seawater at 70 m depth, and freshwater added from a nearby lake. Salinity varied from 12,2 ‰ to 22,2 ‰, and the system was buffered using sodium bicarbonate ($NaHCO_3$). The post-smolt module was comprised of two separate RAS, consisting of a total of four tanks (Fig. 2.1). Each system had an intake of approximately 200 litres of water per minute, which was UV-treated before entering the system. Each production tank (1150 m^3) had an exchange rate of about 15 000 l/min, and a recirculation degree of 95 %. The water went through a water treatment consisting of a drum filter, a moving bed biofilter (300 m^3, 1500 kg feed/day), CO_2-aeration and into the pump sump. Water was then pumped from the pump sump back into the production tank. About 10 % of the water added to the tank was diverted from the pump sump and oxygenated in oxygen cones before it entered the production tanks through separate pipes.

As the intake water and system design were approximately the same, both systems were sampled; RAS1 and RAS2. Fish from the same generation was moved from a RAS at the same location and placed in tank A, B and D, while tank C had 50 000 fish left from last cycle. Fish were subjected to normal handling at a commercial facility. Samples were taken from tank B (RAS 1) and tank D (RAS 2), in order to monitor fish from the same generation. The fish density in tank B started at 16,3 kg/m^3 and increased to 56,4 kg/m^3. In tank D, production started at 17,8 kg/m^3, and ended at 55,2 kg/m^3. RAS2 were fed commercial feed from Biomar throughout the entire cycle. RAS1 was fed a RAS-specialized feed from Alltech, however, halfway through the cycle, the feed in tank B was changed to the Biomar feed.

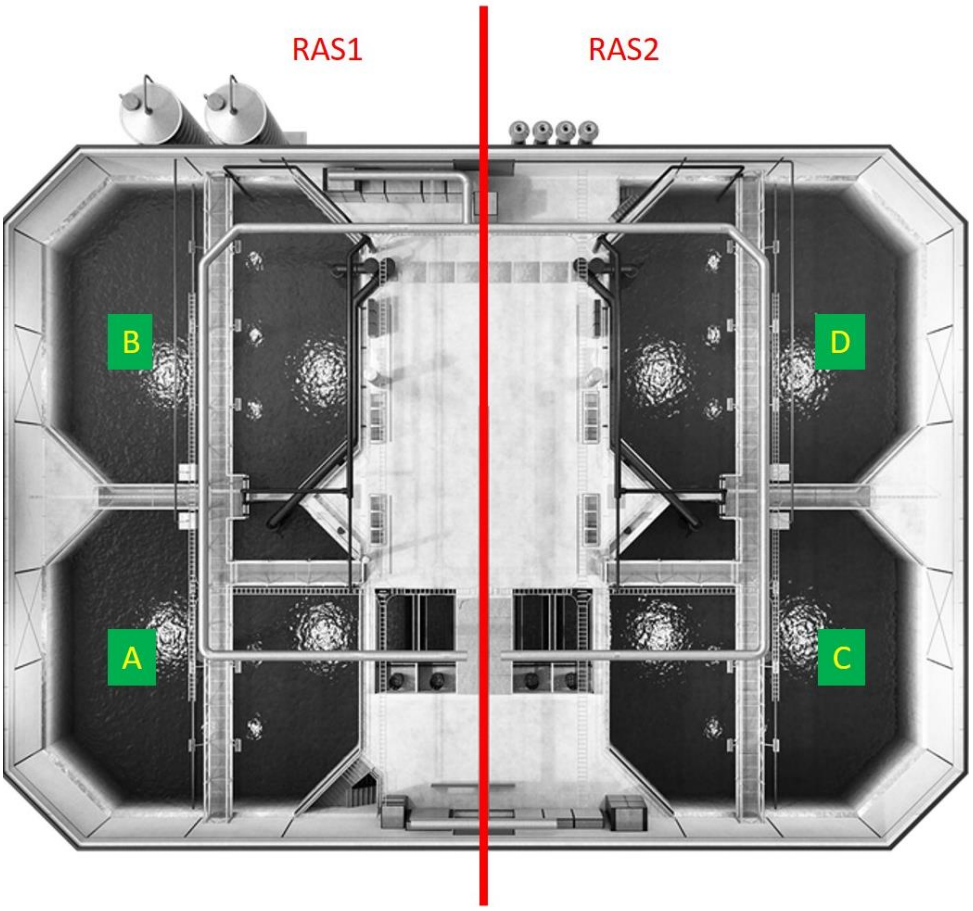


Figure 2.1: Schematic overview of the tank setup at location 1. The red line shows the separation between the two RAS, each consisting of two tanks of 1150 m3. Tank B and D were sampled. Figure modified from (Nofitech, 2019).

2.1.2 Location 2

Location 2 was a RAS growing fish from 3,0 ± 0,39 g to 21,3 ± 6,73 g, and then selling them for research purposes. The facility had an intake of freshwater, and then added sea salt (Sjøsalt Fiskeri, Saltimport AS, Bergen) to a salinity of 1-3 ‰. The system was buffered using $NaHCO_3$, and calcium chloride (CaCl) was added to elevate Ca^{2+}-levels. During the sampling period, fish density increased from 2,6 kg/m^3 to 18,8 kg/m^3. The system was made up of 8 tanks of 2000 l (2 m^3), with 1500 fish in 7 of the tanks. Outlet water from the production tank went through a swirl separator, a drum filter, a biofilter (16 kg feed/day) before aeration, O_2-addition and into the pump sump. The fish were moved from a flow-through system, and then not handled through the entire sampling period. The system will be called RAS3 from this point.

2.2 Sampling

2.2.1 Location 1: RAS1 and RAS2

The first sampling was performed the day before the fish were moved into RAS1 and RAS2. Ten fish were taken as zero samples for both RAS1 and RAS2. The two systems were sampled approximately every two weeks from the 21st of October until the 8th of January (Fig. 2.2). Ten apparently healthy individuals per system were euthanized using an overdose of Benzoak™ (>100 mg/l) and then sampled.

Length and weight were recorded. The second gill arch on the left side was collected for histology and placed on 10 % neutral buffered formalin. More samples were collected, but not processed for this thesis; for full sampling protocol, see Appendix I.

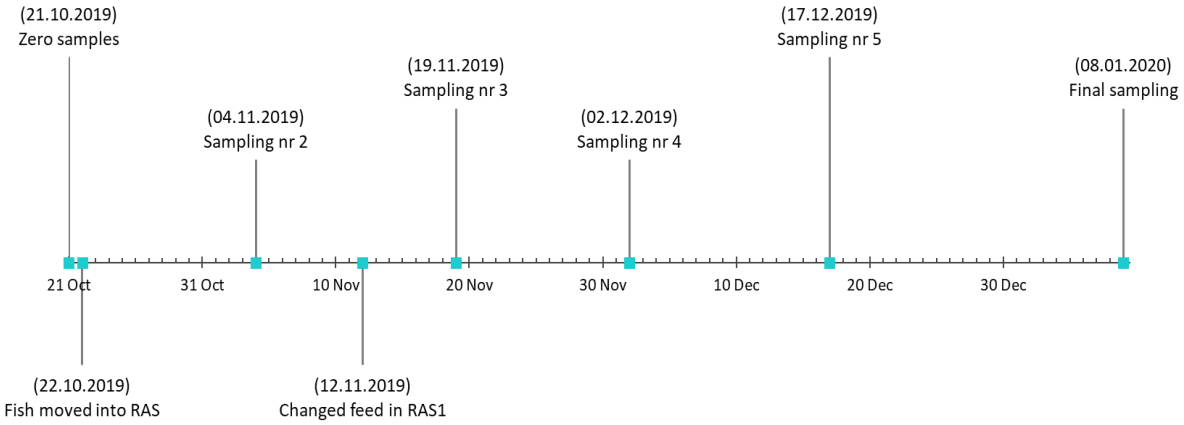


Figure 2.2: Dates of sampling events at location 1, indicated above the line, with important events in production below the line

2.2.2 Location 2: RAS3

The first sampling took place the day after the fish were moved into the RAS. Ten apparently healthy individuals were taken per sampling event, all from the same tank in the facility. The facility was sampled every two weeks from the 10th of September to the 18th of December, making for a total of 8 sampling events (Fig. 2.3). Fish were euthanized using an overdose of Finquel® vet. (200 mg/L), buffered with $NaHCO_3$ (2:1).

Length, weight, and gill arch for histology were sampled following the same procedure as in RAS1 and RAS2.

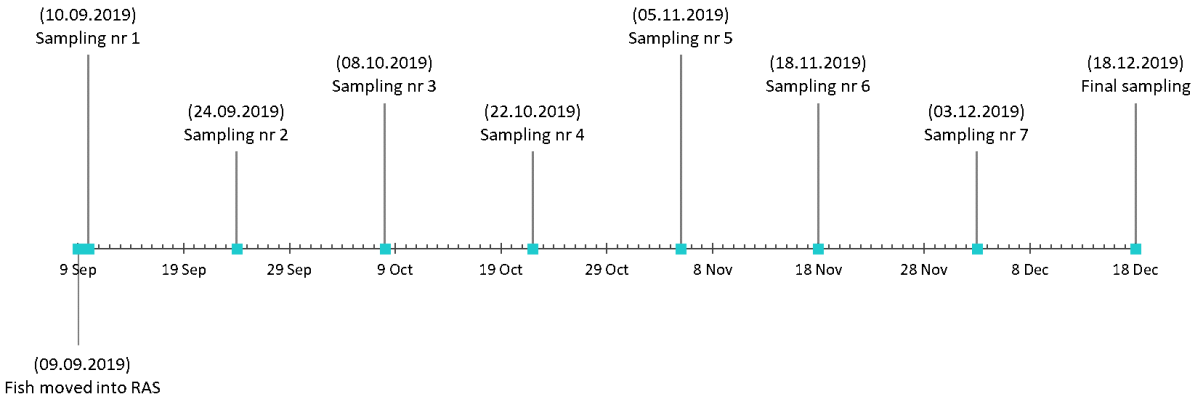


Figure 2.3: Timeline of sampling dates at location 2, with important events indicated below the line.

2.3 Water quality

2.3.1 Raw water samples

At the start and end of each production cycle, raw water samples were taken from both locations, to get a characteristic of each system. Samples were collected using 0,5 l bottles, from the intake water of the facility; for RAS1 and RAS2, both the freshwater- and seawater intake were sampled. Additionally, the outlet water from the production tank (point A, section 2.3.2.) was sampled. Samples were sent to the Norwegian Institute for Water Research (NIVA) for analysis (table 2.1).

Table 2.1: Methods used, and substances analysed in water chemistry samples by NIVA. Freshwater intake from RAS1 and RAS2 was analysed as FW, all other samples were analysed as SW. All samples from RAS3 were analysed as FW. FW: Freshwater, SW: Seawater, NA: Not Analysed

Substance	**Method of analysis**	
	FW	**SW**
Turbidity	NS-EN ISO 7027-1	NS-EN ISO 7027-1
Alkalinity	Internal method based on NS 4754-1	Internal method based on NS 4754-1
Conductivity at 25 °C, measured at 22 ± 2°C	NS-EN ISO 7888	NS-EN ISO 7888
pH, measured at 21 ± 2°C	NS-EN ISO 10523	Internal method
Total Nitrogen	NS 4743	Internal method 6
Nitrate	NS-EN ISO 13395 (as NO_3-N)	Internal method 6
Total Organic Carbon	NS-EN 1484	NS-EN 1484
Sulphate (SO_4)	NS-EN ISO 10304-1	NS-EN ISO 10304-1
Silicate (SiO_2)	NA	Internal method
Silicon (Si)	According NEN EN ISO 17294-2	According NEN EN ISO 17294-2
Copper (Cu)	ICP-MS - EN ISO 17294-2	ISO 17294m:2016
Zinc (Zn)	ICP-MS - EN ISO 17294-2	ISO 17294m:2016
Aluminium (Al)	ICP-MS - EN ISO 17294-2	ISO 17294m:2016
Aluminium - illabile	Internal method	NA
Aluminium - reactive	Internal method	NA
Iron (Fe)	ICP-MS - EN ISO 17294-2	ISO 17294m:2016
Potassium (K)	According NEN EN ISO 17294-2	ISO 17294m:2016
Calcium (Ca)	According NEN EN ISO 17294-2	ISO 17294m:2016
Magnesium (Mg)	According NEN EN ISO 17294-2	ISO 17294m:2016
Chloride (Cl)	NS-EN ISO 10304-1	NS-EN ISO 10304-1
Sodium (Na)	According NEN EN ISO 17294-2	According NEN EN ISO 17294-2
Manganese (Mn)	ICP-MS - EN ISO 17294-2	ISO 17294m:2016

2.3.2 Total organic Carbon (TOC) and bacteria numbers

Every two weeks, coinciding with fish sampling, water samples for total organic carbon (TOC) and bacteria numbers were collected at three points in the system.

A. The water leaving the production tank, before any water treatment
B. After the drum filter, before the biofilter
C. After all water treatment, before the water was sent back into the production tank

TOC-samples were processed by NIVA (table 2.1). Samples for bacterial numbers were kept overnight in a fridge, plated for bacterial colony counts after 24 h. 100 µl of each sample was serial diluted 1:10, using sterilized water from respective production tanks, and plated in duplicate on agar. In RAS3, the samples were plated on nutrient agar (5‰ NaCl). From sampling number three and onwards, both nutrient agar (5‰ NaCl) and blood agar was used. Samples from RAS1 and RAS2 were plated on marine agar. Marine agar and nutrient agar were produced following standard protocols at the Institute of Marine Research (IMR) (Appendix II), while the blood agar was ordered from Haukeland University Hospital.

Agar plates were incubated at 15°C for 48 h before counting (Stuart® SC6 Colony counter). The bacterial counts were then calculated into colony forming units per µl (cfu/µl). Outliers were identified using the interquartile range (IQR). A major outlier was classed as the IQR*3. To decide whether to keep the major outlier in the dataset, it was evaluated against its duplicate. All remaining bacterial counts were then calculated as an average number (cfu/µl) for each sampling point.

2.3.3 Water quality data monitored on location

Each location monitored their water quality as a part of their production.

Location 1 (RAS1 and RAS2) measured temperature, O_2, CO_2, salinity, pH, alkalinity, nitrite, nitrate, and total ammonia nitrogen (TAN). Temperature and oxygen were measured daily in the production tank. Salinity, pH, and CO_2 was measured daily in water from the pump sump, just prior to returning the water to the tanks. Alkalinity, nitrite, nitrate, and TAN were measured in water taken from point B, as described in section 2.3.2. Alkalinity was measured daily. Nitrite, nitrate, and TAN was measured 2 times per week.

Water quality monitoring data from location 2 (RAS3) was not available.

2.4 Histology

Tissue was kept on 10 % neutral buffered formalin for at least 48 h, and maximum of 7 days. Tissue samples were dehydrated and embedded in paraffin using standard histological equipment (TP1020, Leica, Buffalo Grove, United States). Tissue from sampling nr 1, 4 and 6 in RAS1 and RAS2 and sampling nr 1 and 8 in RAS3, were sectioned (Microm HM354S, Thermo Fisher Scientific, Oslo, Norway), making sections at 3 µm. Sections were placed on glass slides and heat treated in a Labnet Mini Incubator at 55°C for 30 minutes.

After staining, the sections were digitally scanned (NanoZoomer™ S60, Hamamatsu Photonics K.K., Hamamatsu City, Japan), and viewed using the software NDP view 2.7.52 (Hamamatsu Photonics K.K., Hamamatsu City, Japan)

2.4.1 Gill lesion characterization and quantification

Sections were stained with Haematoxylin & Eosin (HE) (Appendix III).

Lesions were quantified by first counting the number of well oriented filaments (Speare *et al.*, 1997), and excluding filaments with more than 70% artifacts. When there was a choice between several sections, and the number of adequate filaments were approximately the same, the section with the best area C (Fig. 2.4) was chosen.

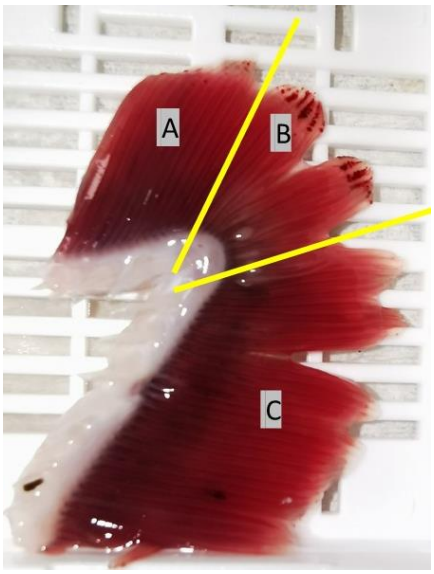


Figure 2.4: Different areas of gill tissue.

As the filament orientation and small size of the RAS3-gills made sectioning difficult, these gills were evaluated using well-oriented stretches of 10 ILU instead of entire filaments. These stretches will be included in the term filament, as the lesion quantification was performed in the same way.

Quantification of gill lesions was done by calculating the percentage of affected filaments for each lesion. The total number of approved filaments was counted per gill arch, before the number of affected filaments was counted for each of the following lesions:

1) *Epithelial lifting*: the gill epithelia had detached from the pillar cells (Fig. 2.5 a) (Kryvi and Poppe, 2016).

2) *Hyperplasia*: an increase in the number of cells (Bruno, Noguera and Poppe, 2013). To differentiate between severity two values were recorded*: complete hyperplasia:* fusion of two or more lamellae(Fig. 2.5 b), and *incomplete hyperplasia*: an increase in the number of cells between two lamellae or an increased number of cells on a single lamella (Fig. 2.5 c, h).

3) *Clubbing*: a swelling, or increase in number, of cells at the distal end of the lamella, making it appear like a club (Fig. 2.5 d). In addition to clubbing, the number of filaments with inflamed clubbing was recorded. *Inflamed clubbing* was differentiated by the infiltration of inflammation cells in the clubbed part of the lamellae (Fig. 2.5 e, h). If the scoring was not conclusive, the filament was recorded as not affected.

4) *Telangiectasia*: widening of lacunal spaces in the lamellae (Fig. 2.5 f) (Bruno, Noguera and Poppe, 2013).

5) *Bleeding*, other than telangiectasis. As there is some disagreement about the definition and use of these expressions (Kryvi and Poppe, 2016), blood contained within the lamellar epithelia but not the blood vessel was classified as bleeding, not telangiectasia (Fig. 2.5 g).

6) *Synechia*: adhesion of adjacent lamellae, usually at the distal end (Fig. 2.5 i). If two or more lamellae were fused, the lesion was recorded as present on that filament.

7) *Inflammation*: the infiltration of inflammatory cells in the filaments (Fig. 2.5 c, e, h). If a lamellae had a cluster of inflammation cells, the filament was recorded as positive (Speare *et al.*, 1997).

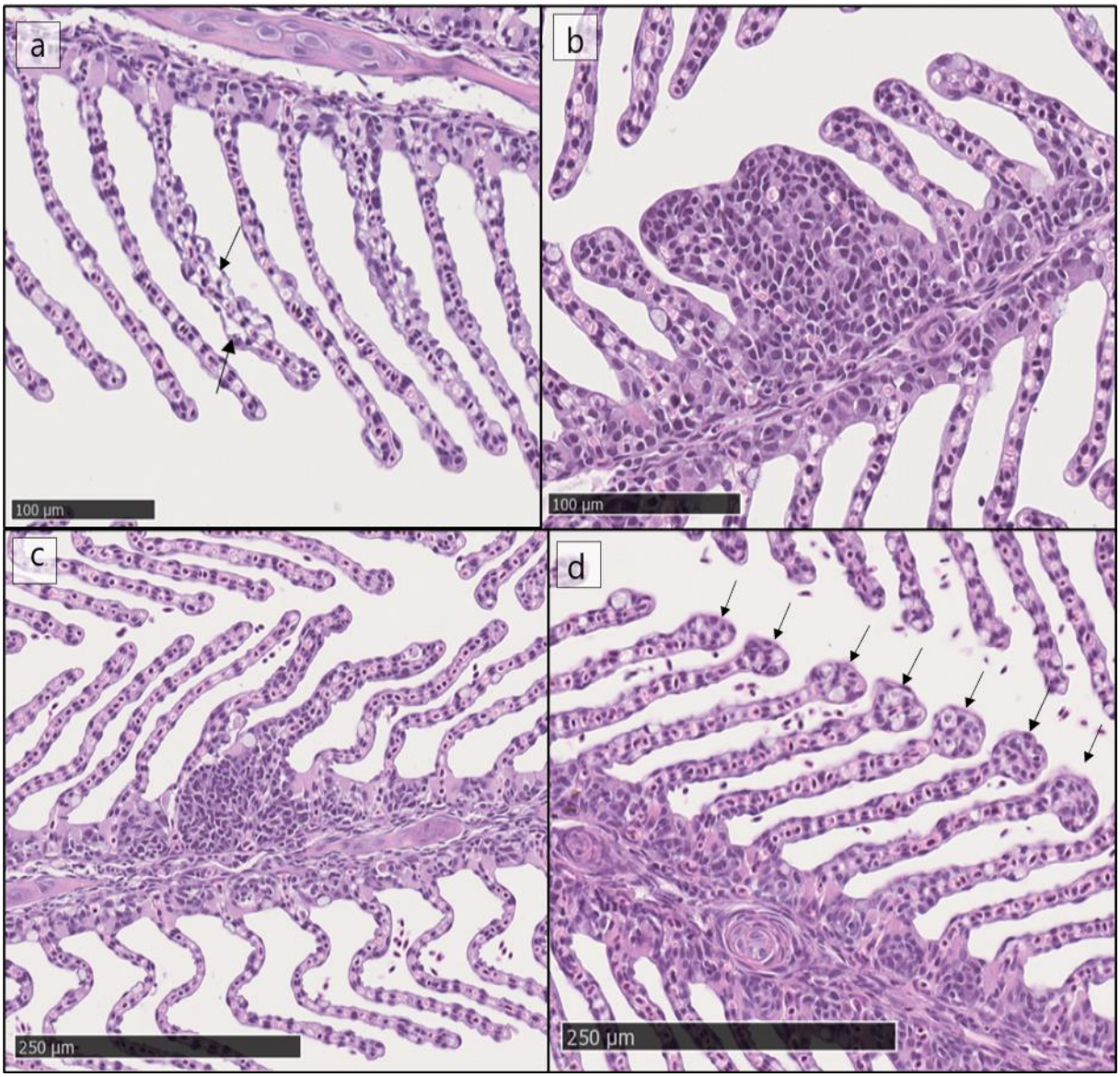
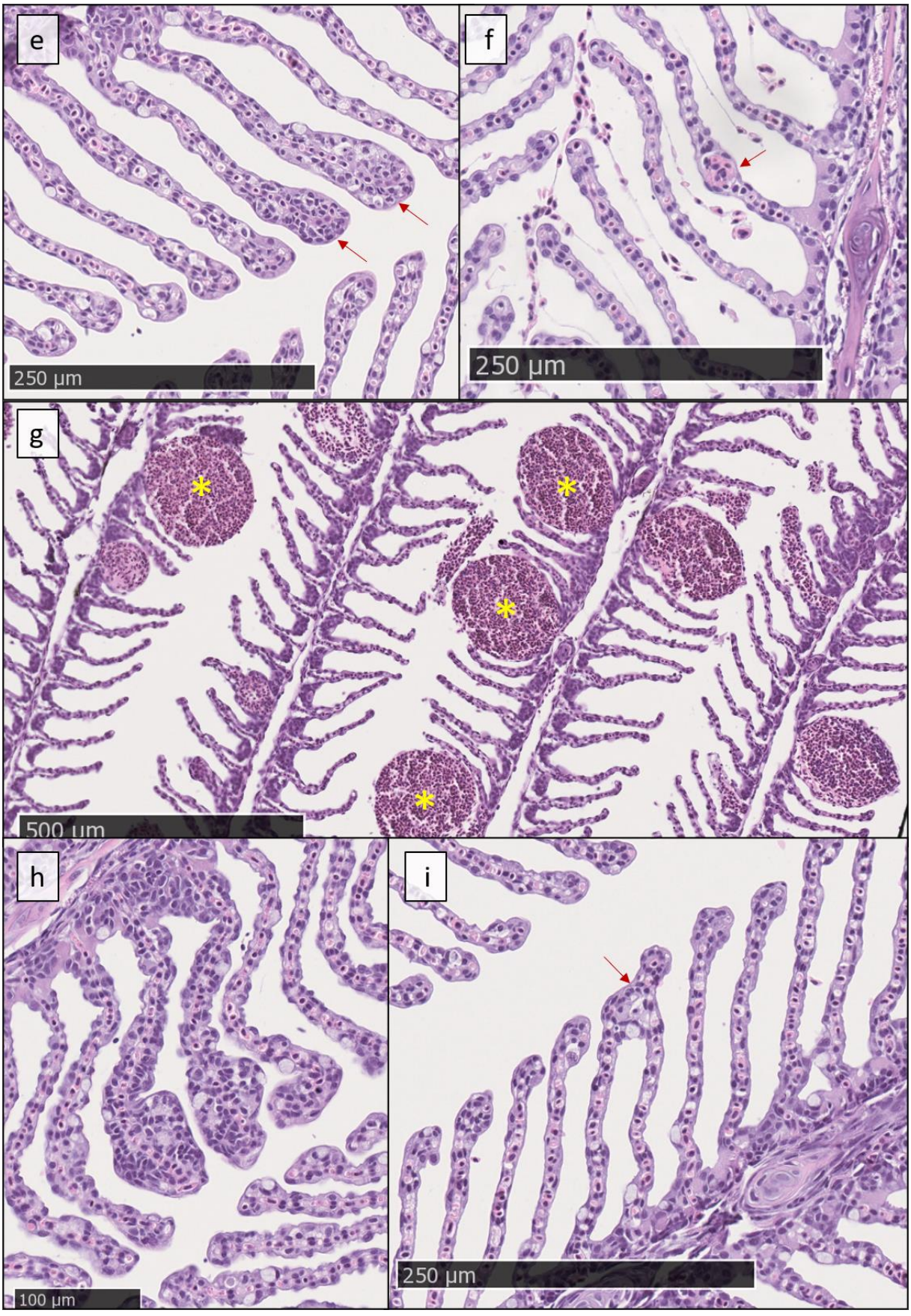


Figure 2.5: Examples of the lesions scored from histology sections, stained with HE. a) Epithelial lifting, indicated by arrows, b) Complete hyperplasia between two lamellae, c) incomplete hyperplasia in the interlamellar space, d) clubbing at the distal end of lamella, indicated by arrows, e) inflamed clubbing, characterized by the infiltration of mononuclear cells, indicated by red arrows, f) telangiectasia, widening of lacunal space in pillar cell, indicated by arrow, g) bleeding, here contained within lamellar epithelia, some are indicated by yellow star, h) infiltration of inflammatory cells, inflamed clubbing and incomplete hyperplasia, i) synechia, adhesion of distal end of lamellae, indicated by arrow.


e
250 µm
f
250 µm
g
500 µm
h
100 µm
i
250 µm

2.4.2 Gill mucous cell counting

Sections were stained using combined Alcian Blue - Periodic Acid-Schiff (AB-PAS) for acid and neutral mucins (Mowry, 1956 in (Cook, 1977)). The mucous cells were counted on a total of 12 well-oriented filaments (Speare *et al.*, 1997). When possible, the counted filaments were equally distributed between the dorsal, middle, and ventral part of the gill arch (Ferguson *et al.*, 1992). Ten interlamellar units (ILU) (Fig. 2.6 A) were counted on both sides of the lamellae (Fig. 2.6 B). After counting the total number of lamellae, the starting points of the 10 ILU were decided by a random number generator. For RAS3, fish 1-10, 5 ILU were counted because of the small size and difficulties with orienting the gills during sectioning. Based on the counts, an average for 1 ILU was calculated per fish.

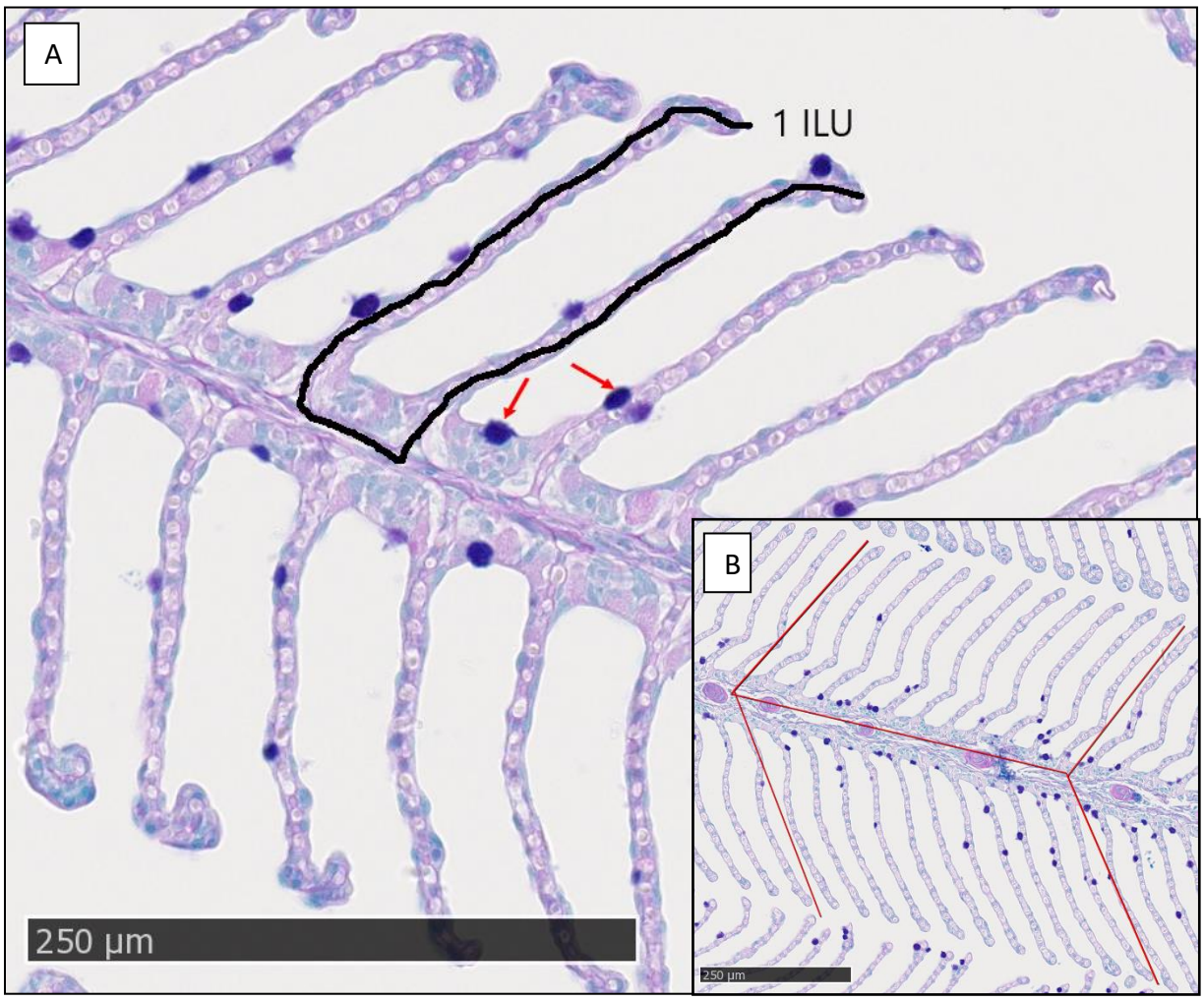


Figure 2.6: Histological section stained with AB-PAS. A) Black lines mark 1 interlamellar unit (ILU), some mucous cells are indicated by red arrows. B) Red lines indicate the area that represents one count of 10 ILU on both sides of the filament. Twelve areas like this were counted per gill arch, each on individual filaments.

2.5 Data analysis

Data processing was performed using R version 3.6.3 in RStudio version 1.2.5042. Some calculations were done in Microsoft Excel.

2.5.1: Lesions

To check if there was a statistically significant change in the percentage of affected filaments over time in a system, tests were performed on each individual lesion. The lesions were the response variable, and the sampling events were the predictor variable. To control for normality, a Shapiro-Wilks test was conducted. As the data was not normally distributed, a non-parametric test was used. For the data from RAS1 and RAS2, a Kruskal Wallis-test was performed, as the data contained three sampling points. Since RAS3 only had two sampling points to compare, a two-sample Wilcoxon rank sum test was performed

The significance level was set to $P \leq 0,05$. A Dunn's Test of Multiple Comparisons Using Rank Sums was used as a post-hoc for lesions with a significant result in the Kruskal-Wallis test, to correct for testing multiple sampling points. Dunn's Bonferroni adjustment was used for adjusting the p-values in the post hoc-test, which changed the significance level to $p \leq 0,025$.

Kruskal Wallis-test

```
kruskal.test (Response ~ Predictor, data= x.df)
```

Two-sample Wilcoxon rank sum test

```
wilcox.test (Response ~ Predictor, data=x.df)
```

Dunn's Test of Multiple Comparisons Using Rank Sums

```
dunn.test (x = dataset$Variable, g = dataset$Group, method= "bonferroni)
```

2.5.2: Mucous cell counts

The mucous cell counts were calculated into an average number per 1 ILU for each fish using Excel. The distribution of the data was checked using a Shapiro-Wilks test. The data was normally distributed, therefore a One-Way Analysis of Variance (One-Way ANOVA) was used for RAS1 and RAS2. As RAS3 only had two sampling points, a Welch Two Sample t-test was performed.To investigate if a relationship between the number of mucous cell densities and gill surface area, a correlation was performed. Palzenberger and Pohla (1992) showed that gill surface area scales logarithmically with body mass, therefore mucous cell densities were

correlated with $\log_{10}$(body mass). A Shapiro-Wilks test indicated that the data was not normally distributed, and a Spearman rank-order correlation was performed.

One-Way ANOVA

```
model.lm <- lm (Mucus_count ~ Sampling, data=x.df)

anova(model.lm)
```

Welch Two Sample t-test

```
t.test (Mucus_count ~ Sampling, data = x.df)
```

Spearman rank correlation coefficient

```
cor.test (dataset$variable1, dataset$variable2, method = "spearman")
```

2.5.3: Water quality data

For bacterial numbers and TOC, the samples collected at point A were used for the statistical analyses, as this represented the water the fish were in.

To investigate if there was a correlation between the values for TOC and bacterial numbers, and, in RAS3, the bacterial numbers from blood agar and nutrient agar, a correlation test was performed. A preliminary Shapiro-Wilks test was used to test the assumption of normally distributed data. P-values greater than 0,05 showed that the data was significantly different from a normal distribution, and non-parametric tests had to be used, in this case a Spearman rank-order correlation. The significance level was set at $p \leq 0{,}05$.

For the regularly measured water quality data, linear regressions were performed to see if the values showed a trend over time in the different systems. For RAS1 and RAS2, day 0 was set as 21.10.2019, the date the zero samples were collected and the day before fish were moved in. The production data was not analysed beyond the final sampling, so the final datapoint was day 79, 08.01.2020. In RAS3, day 0 was the day the fish were moved into the system, 09.09.2019, and the final sampling point was used as the cut-off, 18.12.2019.

A change in makeup water in early January in RAS1 and RAS2 was suspected. Regression models for TOC and bacterial numbers were therefore performed both with and without the final sampling point (08.01.2020).

Linear model and output type used:

```
summary (lm (Water quality parameter ~ Days, data = System.df))
```

3. Results

3.1 Water quality

3.1.1: Raw water analysis

At location 1, the parameters tested in the freshwater and sea water intake were mostly the same between the start and the end of sampling (Table 3.1). In freshwater, there was an increase in manganese (43%), chloride (41%), conductivity (23%), sulphate (17%) and total nitrogen (17%), and a decrease in zinc (33%), magnesium (29%), sodium (28%), TOC (27%), potassium (26%), calcium (22%) and alkalinity (16%). In sea water, the only increase was 1,6 µg/l of copper (320%), while there was a decrease in total nitrogen (60%), silicate (57%), silicon (56%), nitrate (32%), turbidity (27%), as well as TOC (25%). Remaining parameters were below a 10% change.

The tank water in both RAS1 and RAS2 showed an increase in excess of 100% for turbidity, copper, zinc, manganese, nitrate, total nitrogen, and TOC (Table 3.1). TOC increased by 15,5 mg/l and 10,8 mg/l in RAS1 and RAS2, respectively, while turbidity increased by 2,04 FNU in RAS1 and 1,15 FNU in RAS2. Nitrate levels increased by 432,2 mg/l, and total nitrogen increased by 108,6 mg/l in RAS1, and in RAS2, nitrate increased by 365,7 mg/l, while total nitrogen increased by 82,8 mg/l. Copper, zinc and manganese increased by 5,3 µg/l, >61 µg/l, and >0,405 µg/l in RAS1 and >3,6 µg/l, 38,8 µg/l, and >0,052 in RAS2, respectively. Potassium, calcium, magnesium, chloride, conductivity, sodium, pH, and sulphate all decreased by less than 21% in both RAS1 and RAS2, while silicate and silicon decreased by 100 % and >76%, respectively, in RAS1 and they both decreased by 75% in RAS2. Alkalinity decreased in the tank water of RAS1 (22%) and increased in the tank water of RAS2 (86%) (Table 3.1).

Table 3.1: Raw water analysis results from location 1 (RAS1 and RAS2). Start: 21.10.2019, end: 08.01.2020. NA: not analysed. 1) direct measurements, 2) ICP-MS, 3) NO3-N

		Freshwater intake		Saltwater intake		RAS1 tank water		RAS2 tank water	
Analysis	**Unit**	**Start**	**End**	**Start**	**End**	**Start**	**End**	**Start**	**End**
Turbidity	FNU	0,6	0,58	0,51	0,37	0,66	2,7	0,85	2,0
Alkalinity	mmol/l	0,189	0,159	2,22	2,22	1,34	1,05	0,891	1,66
Copper (Cu)	µg/l	0,95[2)]	1,0[2)]	0,5	2,1	0,5	5,8	<0,5	4,1
Zinc (Zn)	µg/l	3,6[2)]	2,4[2)]	<2	<2	<2	63	9,2	48
Aluminium (Al)	µg/l	68[2)]	70[2)]	<4	<4	<4	<4	<4	<4
- Aluminium - illabile	µg/l	7,1	6,6	NA	NA	NA	NA	NA	NA
- Aluminium - reactive	µg/l	14	13	NA	NA	NA	NA	NA	NA
Iron (Fe)	mg/l	0,31[2)]	0,32[2)]	<0,05	<0,05	<0,05	<0,05	<0,05	<0,05
Potassium (K)	mg/l	0,57[1)]	0,42[1)]	420	420	230	210	220	210
Calcium (Ca)	mg/l	11[1)]	8,6[1)]	470	470	250	200	240	190
Magnesium (Mg)	mg/l	1,4[1)]	1,0[1)]	1400	1400	720	630	690	620
Manganese (Mn)	mg/l	0,091[2)]	0,13[2)]	<0,005	<0,005	<0,005	0,41	<0,005	0,057
Chloride (Cl)	mg/l	8,11	11,4	22200	21000	12600	10000	10800	9800
Conductivity at 25°C (22 ± 2°C)	mS/m	10	12,3		4380	2840	2430	2660	2430
Sodium (Na)	mg/l	8,1[1)]	5,8[1)]	11000	11000[1)]	6200	5100[1)]	6300	5400[1)]
Nitrate	mg/l	0,21[3)]	0,2[3)]	0,4223	0,2889	12,97	445,2	61,04	426,7
pH, measured at 21 ± 2°C		7,3	6,7	7,9	7,9	7,6	6,6	7,3	6,8
Silicate (SiO2)	mg/l	NA	NA	1,7	0,73	3,6	0,0	3,0	0,75
Silicon (Si), direct	mg/l	1,4	1,3	0,78	0,34	1,7	<0,40	1,4	0,35
Sulphate (SO4)	mg/l	18,3	21,5	2740	2690	1380	1230	1370	1180
Total Nitrogen	mg/l	0,23	0,27	0,23	0,0923	3,7	112,3	15	97,83
Total Organic Carbon (TOC/NPOC)	mg/l	4,1	3,0	0,8	0,6	2,7	18,2	3,9	14,7

In RAS3, the freshwater intake showed a slight increase in pH (0,1), turbidity (0,3 FNU), and chloride (1,47 mg/l) from start to end, while TOC showed a decrease (2,2 mg/l) (Table 3.2). In the tank water, there was an increase in pH (0,2) and turbidity (1,81 FNU), and a decrease in TOC (0,8 mg/l). Comparing the tank water to the intake water at the end sampling, the tank water showed a great (>1000%) increase in chloride, sulphate, potassium, calcium, sodium, and conductivity. Levels of turbidity, TOC, alkalinity, total nitrogen and nitrate-N was, respectively, 2,18 FNU, 2,6 mg/l, 0,233 mmol/l, 9,5 mg N/l and 8,2 mg N/l, higher in the tank water than in the intake water. Copper increased by 1,45 µg/l and magnesium by 1,48 mg/l. Meanwhile, the tank water had reduced levels of aluminium (44%), iron (20%) and manganese (18%), compared to the freshwater intake water (table 3.2).

Table 3.2: Raw water analysis from RAS3. Start: 10.09.2019, end: 18.12.2020. NA: not analysed

		Freshwater intake		**RAS3 tank water**	
Analysis	**Unit**	**Start**	**End**	**Start**	**End**
pH measured at 21 +/- 2°C		6,6	6,7	6,6	6,8
Turbidity	FNU	0,28	0,52	0,89	2,7
Alkalinity	mmol/l	NA	0,062	NA	0,295
Chloride (Cl)	mg/l	3,93	5,4	NA	1230
Sulphate (SO4)	mg/l	NA	1,72	NA	36,9
Total nitrogen	mg/l	NA	0,077	NA	9,6
Nitrate (NO3-N)	mg/l	NA	0,072	NA	8,3
Total Organic Carbon (TOC/NPOC)	mg/l	4,4	2,2	5,6	4,8
Copper (Cu) ICP-MS	µg/l	NA	0,35	NA	1,8
Aluminium (Al) ICP-MS	µg/l	NA	78	NA	44
- Aluminium - illabile	µg/l	NA	26	NA	
- Aluminium - reactive	µg/l	NA	34	NA	
Iron (Fe) ICP-MS	µg/l	NA	15	NA	12
Manganese (Mn) ICP-MS	µg/l	NA	1,1	NA	0,9
Potassium (K), direct	mg/l	NA	0,27	NA	6,6
Calcium (Ca), direct	mg/l	NA	0,96	NA	13
Conductivity at 25 °C (measured at 22± 2°C)	mS/m	NA	3,01	NA	359
Magnesium (Mg), direct	mg/l	NA	0,42	NA	1,9
Sodium (Na), direct	mg/l	NA	4,3	NA	740
Silicon (Si), direct	mg/l	NA	2,9	NA	2,9

3.1.2: Regularly measured water quality data from Location 1

Total organic carbon and bacterial numbers were analysed both with and without the final sampling point. With the final sampling point included, RAS1 had a significant increase ($F_{1, 4}$ = 14.72, p = 0.019), while RAS2's model was not significant ($F_{1, 4}$ = 5.62, p = 0.077). Without the final sampling point (Fig. 3.1 A), TOC showed a significant increase in both RAS1 ($F_{1, 3}$ = 46, p = 0.007) and RAS2 ($F_{1, 3}$ = 72.79, p = 0.003).

Bacterial numbers did not show any significant trends in RAS1 ($F_{1, 4}$ = 0.136, p = 0.731) or RAS2 ($F_{1, 4}$ = 0.286, p = 0.624). When excluding the final sampling point, RAS1 showed a significant increase ($F_{1, 3}$ = 10.84, p = 0.046), while RAS2 did not show a significant trend ($F_{1, 3}$ = 2.781, p = 0.194) (Fig. 3.1 B)

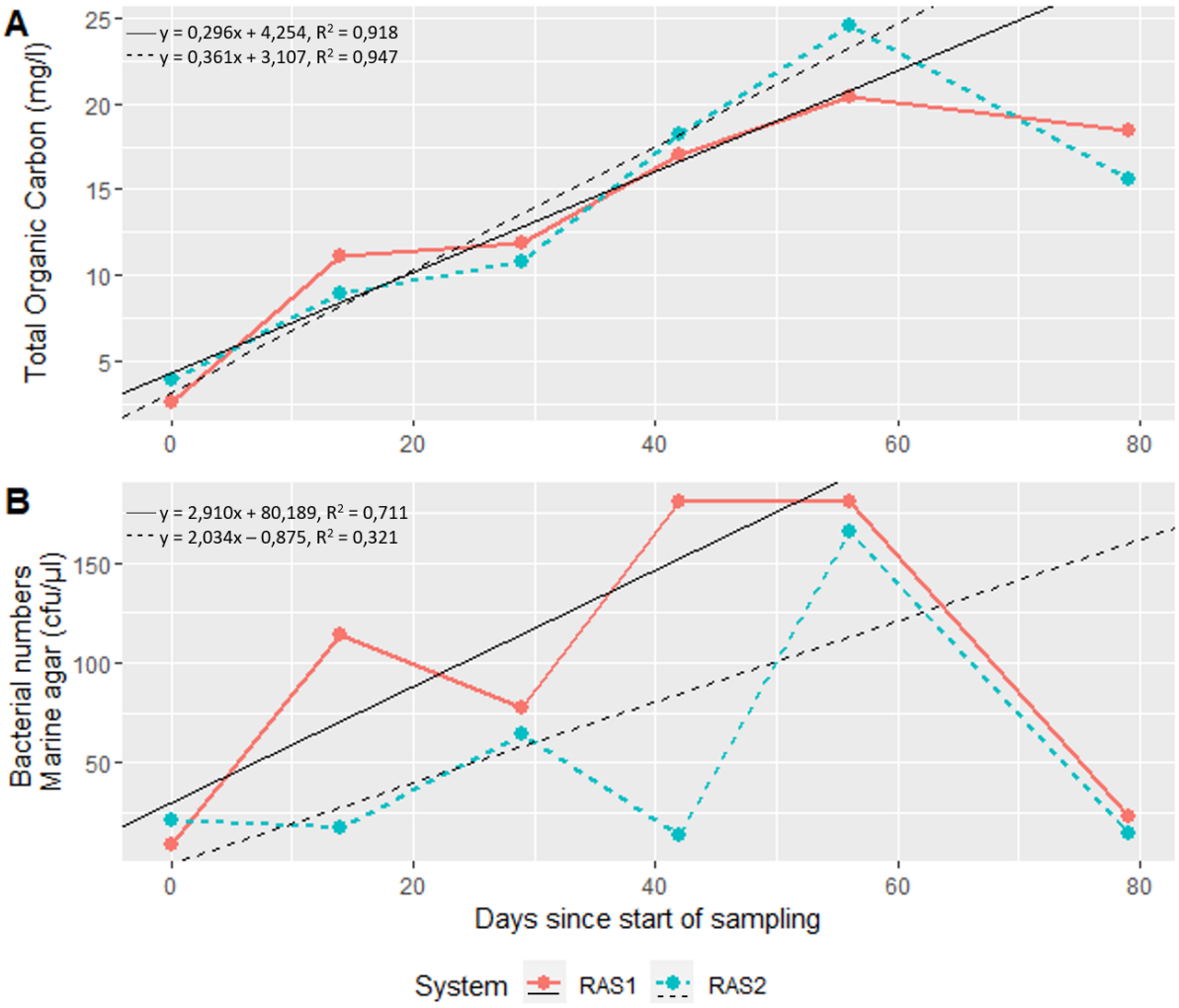


Figure 3.1: Water quality parameters measured every two weeks at point A in the system (described in section 2.3.2), as part of project sampling in RAS1 and RAS2. Note that linear regression models added to figure are excluding the final sampling point, and that R^2 is "adjusted R^2". A) Total organic carbon levels B) Bacterial densities using marine agar.

Temperature varied greatly throughout the production cycle, with a mean of 14,1 ± 1,02° C [min: 11,7, max: 15,7] in RAS1 and a mean of 14,5 ± 0,98°C [min: 12,9, max: 16,7] in RAS2 (Fig. 3.2 A). O_2-levels remained between 95% and 90% during the entire sampling period. A significantly increasing trend was seen for salinity (RAS1: $F_{1, 78} = 58.88$, $p < 0.001$; RAS2: $F_{1, 78} = 48.23$, $p < 0.001$)(Fig. 3.2 B), total ammonia nitrogen (RAS1: $F_{1, 21} = 60.73$, $p < 0.001$; RAS2: , $F_{1, 21} = 32.63$, $p < 0.001$) (Fig. 3.3 A), nitrite (RAS1: $F_{1, 21} = 70.06$, $p < 0.001$; RAS2: $F_{1, 21} = 117.5$, $p < 0.001$) (Fig. 3.3 B), nitrate (RAS1: $F_{1, 20} = 31.66$, $p < 0.001$; RAS2: $F_{1, 20} = 12.53$, $p = 0.002$) (Fig. 3.3 C), and CO_2 (RAS1: $F_{1, 78} = 357.7$, $p <0.001$; RAS2: $F_{1, 78} = 465.9$, $p<0.001$) (Fig. 3.4 A) for both RAS1 and RAS2. pH (RAS1: $F_{1, 78} = 157.5$, $p < 0.001$; RAS2: $F_{1, 78} = 19.15$, $p < 0.001$) (Fig. 3.4 B) showed a significantly decreasing trend in both systems. Alkalinity (Fig. 3.4 C) had a significantly decreasing trend in RAS1 ($F_{1, 73} = 6.161$, $p = 0.015$). However, in RAS2, the linear regression model was not significant ($F_{1, 73} = 0.247$, $p = 0.620$).

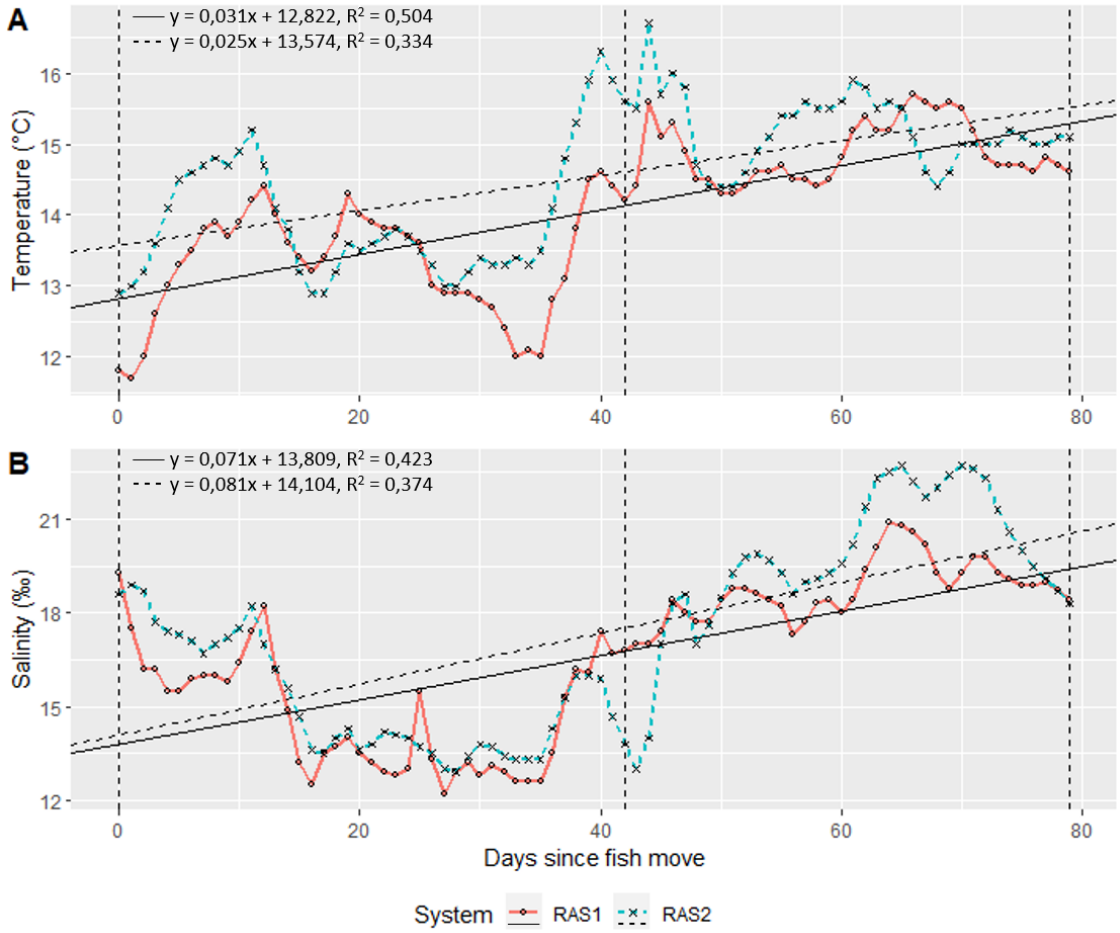


Figure 3.2: Daily measured production data from RAS1 and RAS2, from the first to final sampling day. Dotted vertical lines mark the three samplings where histological lesions were scored. Linear regression models for each system are included. Note that R^2-values are "adjusted R^2". A) Temperature data; measured in the production tank. B) Salinity data; measured at point C, described in section 2.3.2

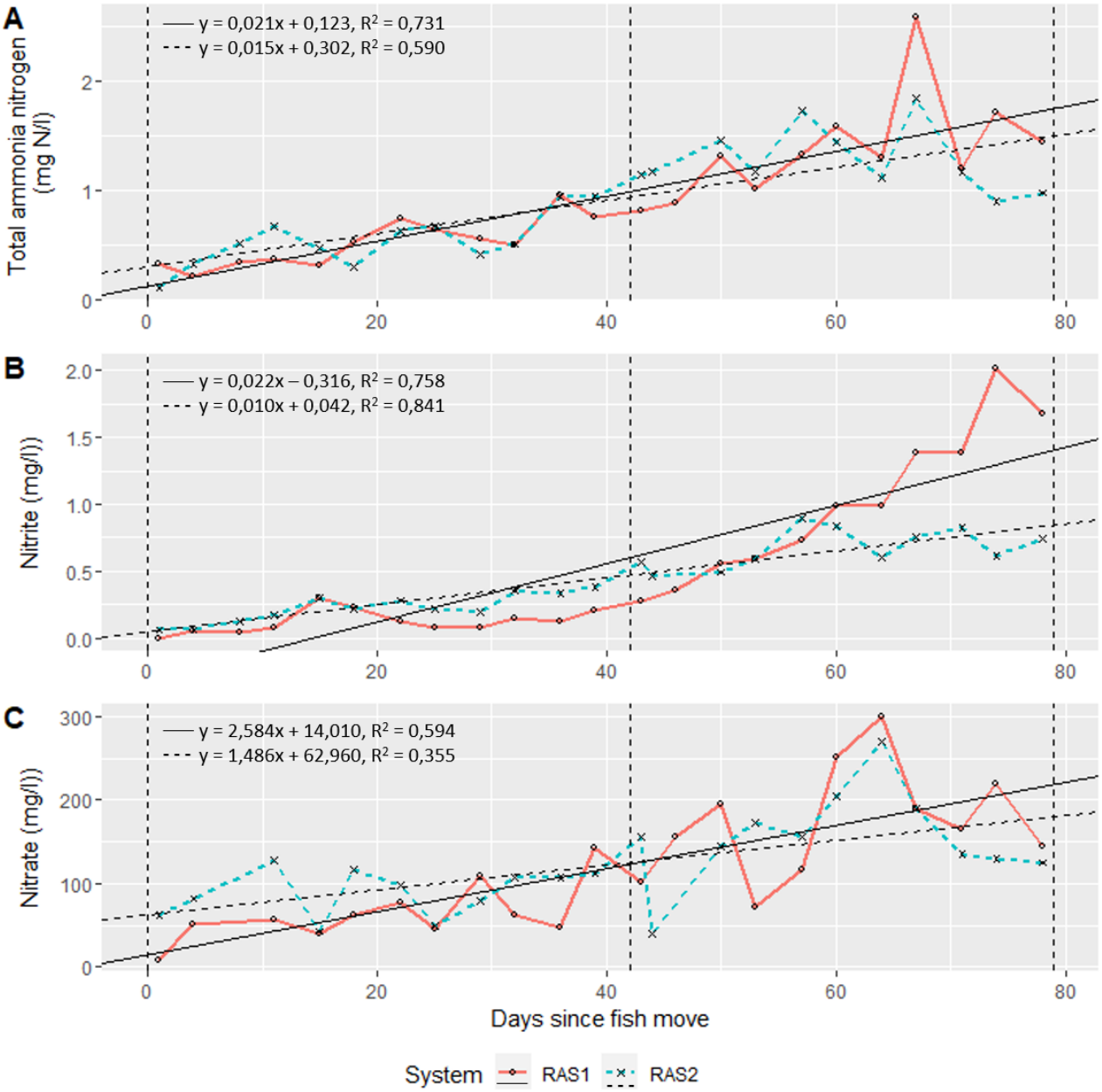


Figure 3.3: Levels of nitrogen compounds measured two times a week as part of production in RAS1 and RAS2. All measurements were performed on water from point B in the system described in section 2.3.2. Dotted vertical lines mark the three samplings where histological lesions were scored. Linear regression models for each system are included. Note that R^2-values are "adjusted R^2". A) Total ammonia nitrogen data B) Nitrite (NO_2^-) data C) Nitrate (NO_3^-) data

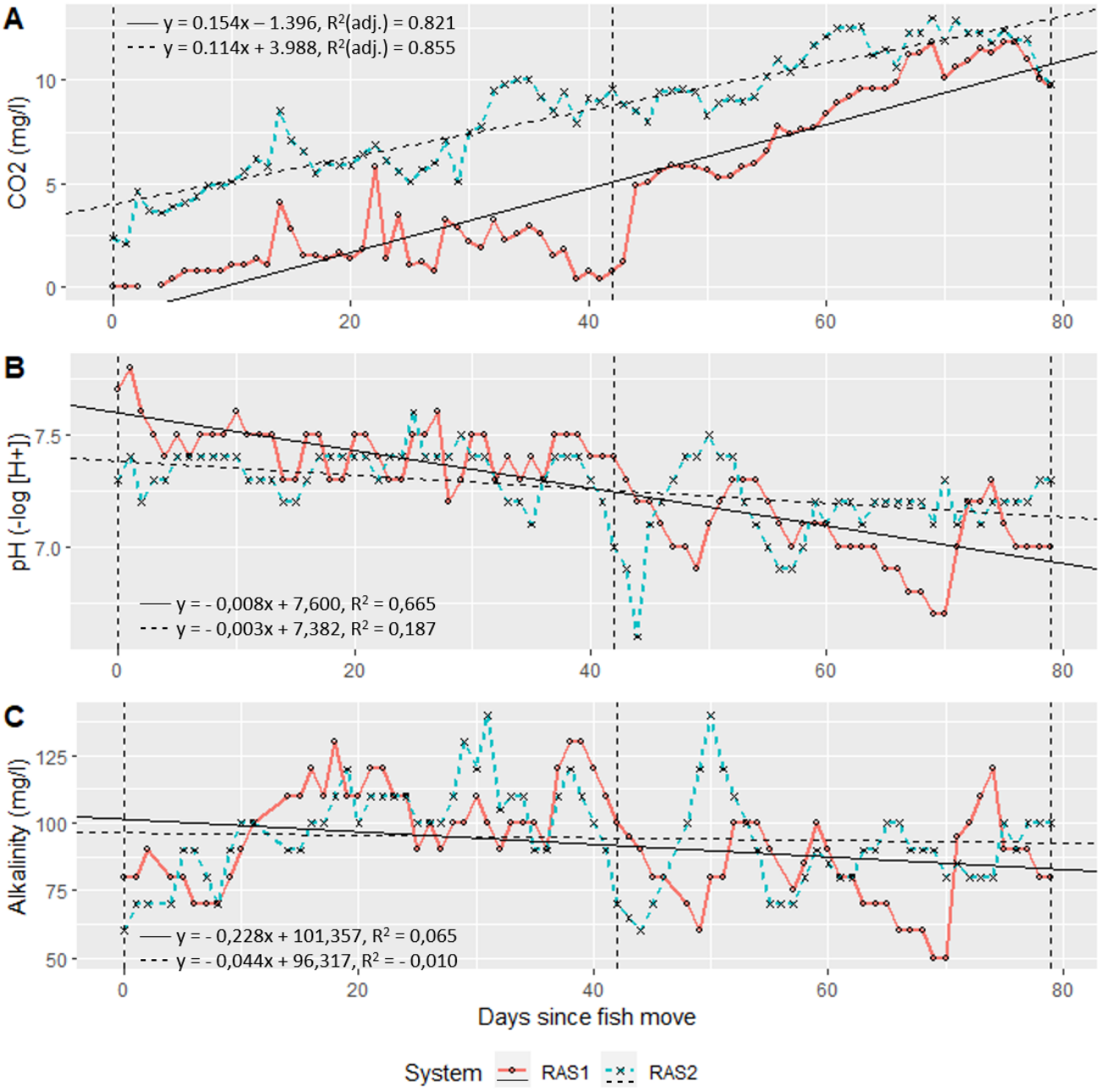


Figure 3.4: Regularly measured production data from RAS1 and RAS2, from the first to final sampling day. Dotted vertical lines mark the three samplings where histological lesions were scored. Linear regression models for each system are included. Note that R^2-values are "adjusted R^2". A) CO_2 levels; measured daily at point C in the system, described in section 2.3.2. B) pH levels; measured daily at point C in the system, described in 2.3.2. C) Alkalinity levels; measured daily, at point B, described in section 2.3.2.

3.1.3: Regularly measured water quality data from Location 2

There was no significant trend in TOC over time ($F_{1, 6} = 4.797$, $p = 0.071$) (Fig. 3.5 A). Similarly, the bacterial numbers, neither for blood agar ($F_{1, 6} = 3.245$, $p = 0.146$) nor nutrient agar ($F_{1, 6} = 3.513$, $p = 0.11$), showed a significant trend over time (Fig. 3.5 B-C).

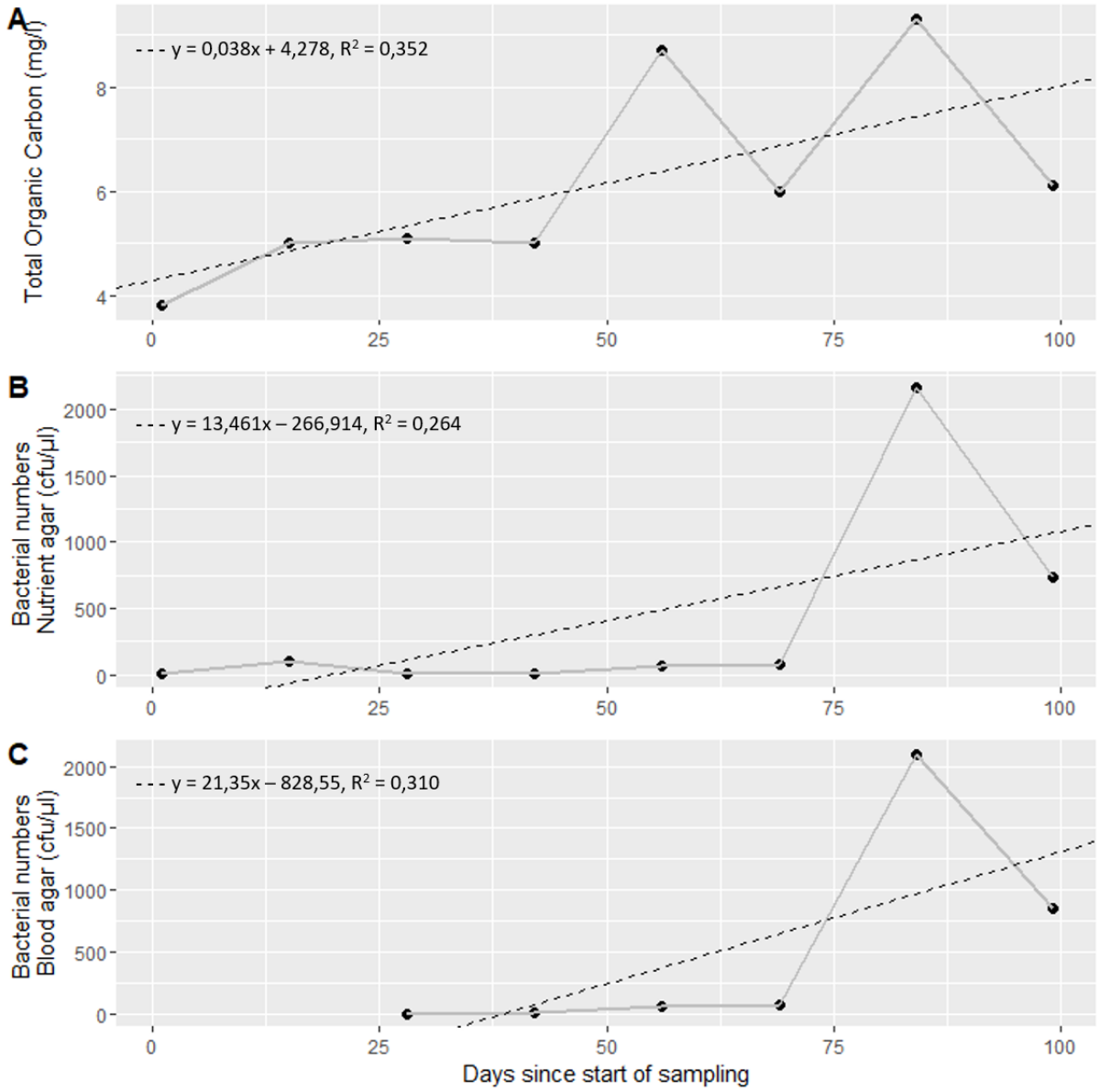


Figure 3.5: Water quality parameters measured every two weeks as part of project sampling in RAS3, at point A in the system, described in section 2.3.2. Linear regression models added to figure. A) Total organic Carbon levels B) Bacterial density from nutrient agar counts C) Bacterial density from blood agar counts.

3.1.4: Correlations

TOC and bacterial numbers were not significantly correlated, neither for RAS1 (rho = 0.6, p = 0.242), RAS2 (rho = 0.029, p = 1), nor RAS3 (Fig. 3.6 A-D). For RAS3, neither bacterial numbers from the nutrient agar (rho = 0.611, p = 0.108) nor the blood agar (rho = 0.771, p = 0.103) showed a significant correlation with TOC. RAS3's bacterial numbers from blood agar counts and nutrient agar counts showed a significant correlation (rho = 1, p = 0.003), which may also be described by a linear regression ($F_{1, 4} = 873.3$, $p < 0.001$) (Fig. 3.6 E).

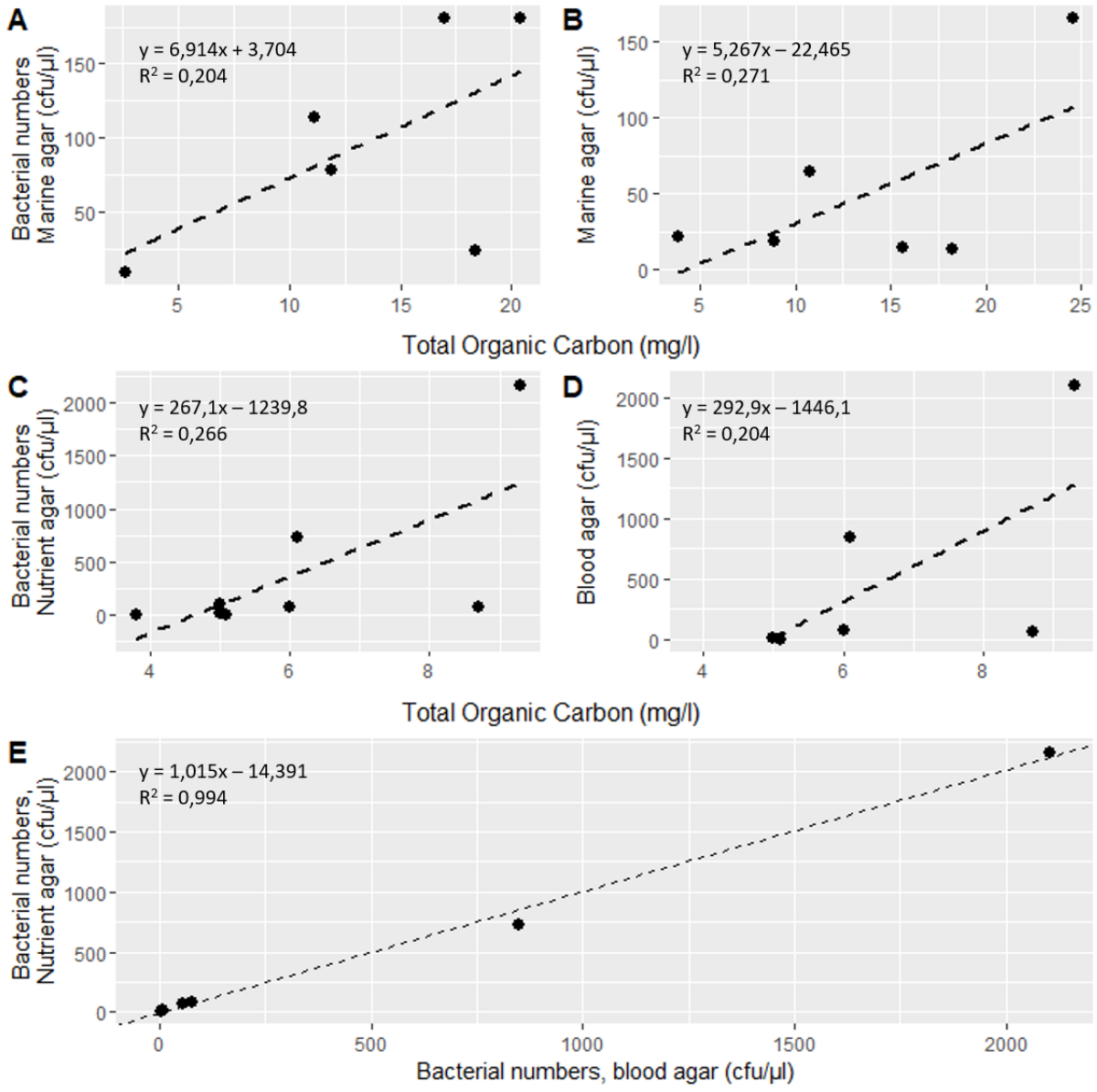


Figure 3.6: Correlation scatter plots, including linear regression models to illustrate the tested correlation relationship. A-D show bacterial densities correlated with TOC for A) RAS1, using marine agar B) RAS2, using marine agar, C) RAS 3, using nutrient agar (5‰ NaCl) D) RAS3, using blood agar. E) Correlation of RAS3 bacterial densities from nutrient agar with RAS3 bacterial densities from blood agar.

3.2: Gill lesions

3.2.1 Location 1

Epithelial lifting (H_2 = 5,886, p= 0,053), clubbing (H_2 = 19,029, p < 0,001), inflammatory clubbing (H_2 = 22,546, p < 0,001), synechia (H_2 = 11,478, p= 0,002) and inflammation (H_2 = 18,578, p <0,001) all showed a significant increase in percentage of affected filaments in RAS1. Clubbing showed a significant increase from the start to the middle sampling (Dunn's test, p = 0,011), while inflamed clubbing, inflammation and synechia showed significant increases (Dunn's test, p <0.001 - 0.005) from the start to the final sampling (Fig. 3.7)

In RAS2, bleeding (H_2 = 6,215, p= 0,045), clubbing (H_2 = 19,226, p < 0,001), inflammatory clubbing (H_2 = 24,205, p < 0,001), synechia (H_2 = 15,677, p < 0,001), and inflammation (H_2 = 16,795, p<0,001) showed a significant increase in percentage of affected filaments (Fig. 3.15). Clubbing, inflamed clubbing and inflammation all showed significant increases from the start to the middle sampling (Dunn's test, p = 0,021-0,0006), while synechia showed a significant increase from the start to the end sampling (Dunn's test, p <0,001).

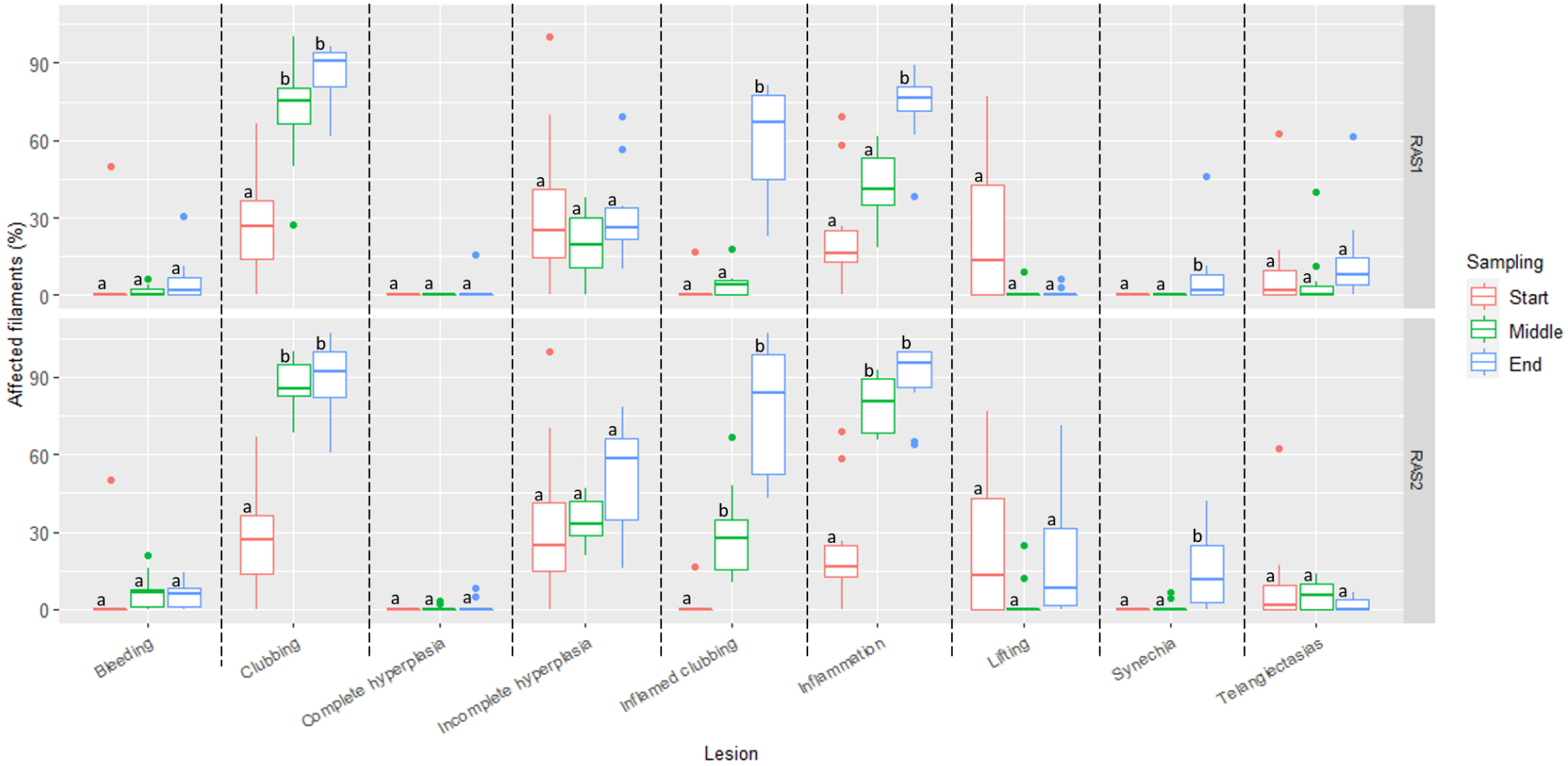


Figure 3.7: Percentage of affected filaments by gill lesions, quantified from histological sections stained with HE from RAS1 and RAS2. "Start" = first sampling (21.10.2019; zero samples, from the day before fish were moved into RAS, and therefore are the same for both RAS1 and RAS2) (n=10), "Middle" = sampling nr. 4 (02.12.2019) (n=10), "End" = final sampling (nr 6, 08.01.2020) (n=10). The line inside the boxplot shows the median value. Letters indicate significant difference between sampling points in the Dunn's test (p ≤0,025).

3.2.2 Location 2

Incomplete hyperplasia (W= 0, p < 0,001), inflammation (W= 12, p = 0,007), clubbing (W= 0, p < 0,001) and inflammatory clubbing (W= 22,5, p= 0,034) had a significant increase in percentage of affected filaments between the start- and end sampling in RAS3 (Fig. 3.8).

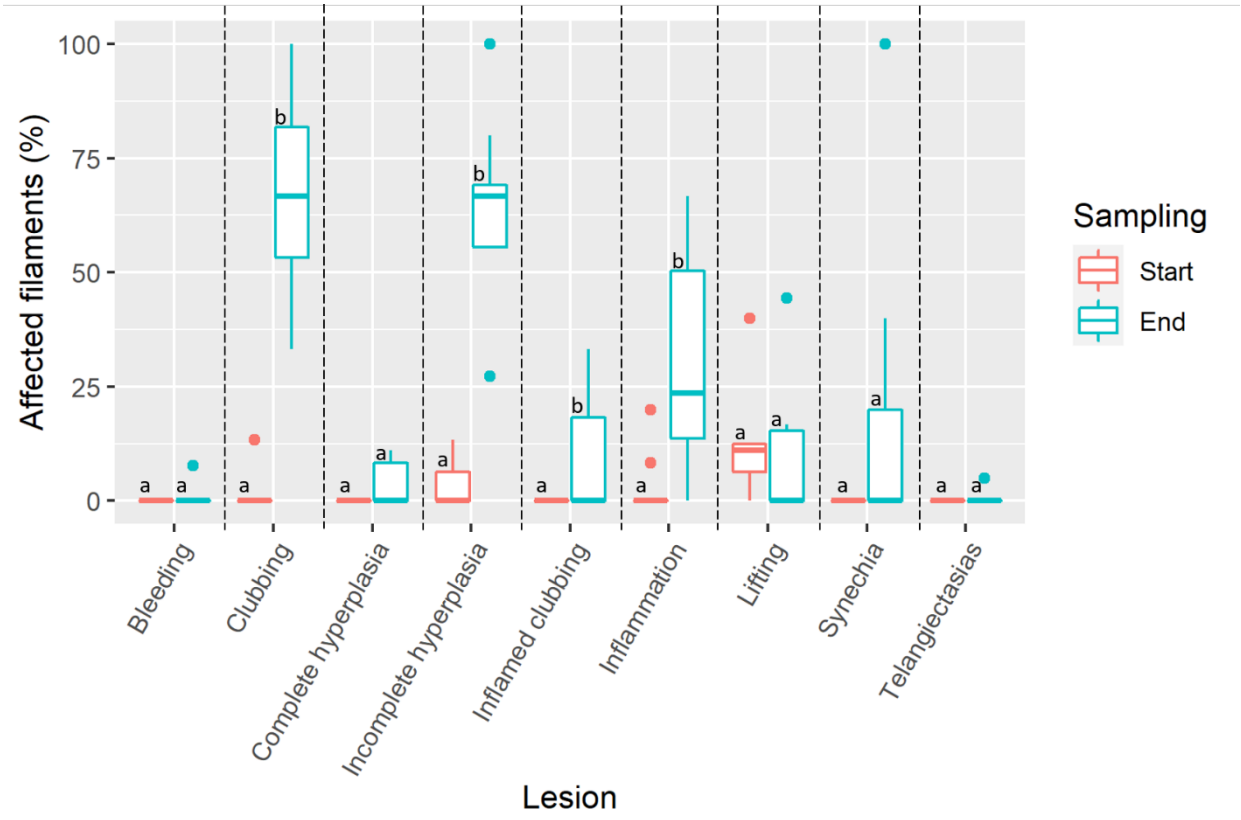


Figure 3.8: Percentage of affected filaments by gill lesions quantified from histological sections stained with HE from RAS3. "Start" = first sampling event, 10.09.2019 (n=9), "End" = final sampling event, 18.12.2019 (n=9). The line inside the boxplot shows the median value. Letters indicate significant difference between sampling points (p < 0,05).

3.2.3: Mucous cell densities

In RAS3, the number of mucous cells showed a significant difference between the start and the end sampling ($p < 0.001$) (Fig. 3.9). However, no such difference was shown in neither RAS1 ($F_2 = 0.320$, $p = 0.73$) nor RAS2 ($F_2 = 0.008$, $p = 0.993$). A correlation of all three systems gave a significant correlation of mucous cell densities and $\log_{10}$(body mass) (rho= 0,393, p = 0,003). Performing the same correlation for RAS3, where a significant difference in mucous cell density was observed in the One-Way ANOVA, the relationship explains more of the variance (rho = 0,737, p = 0,002).

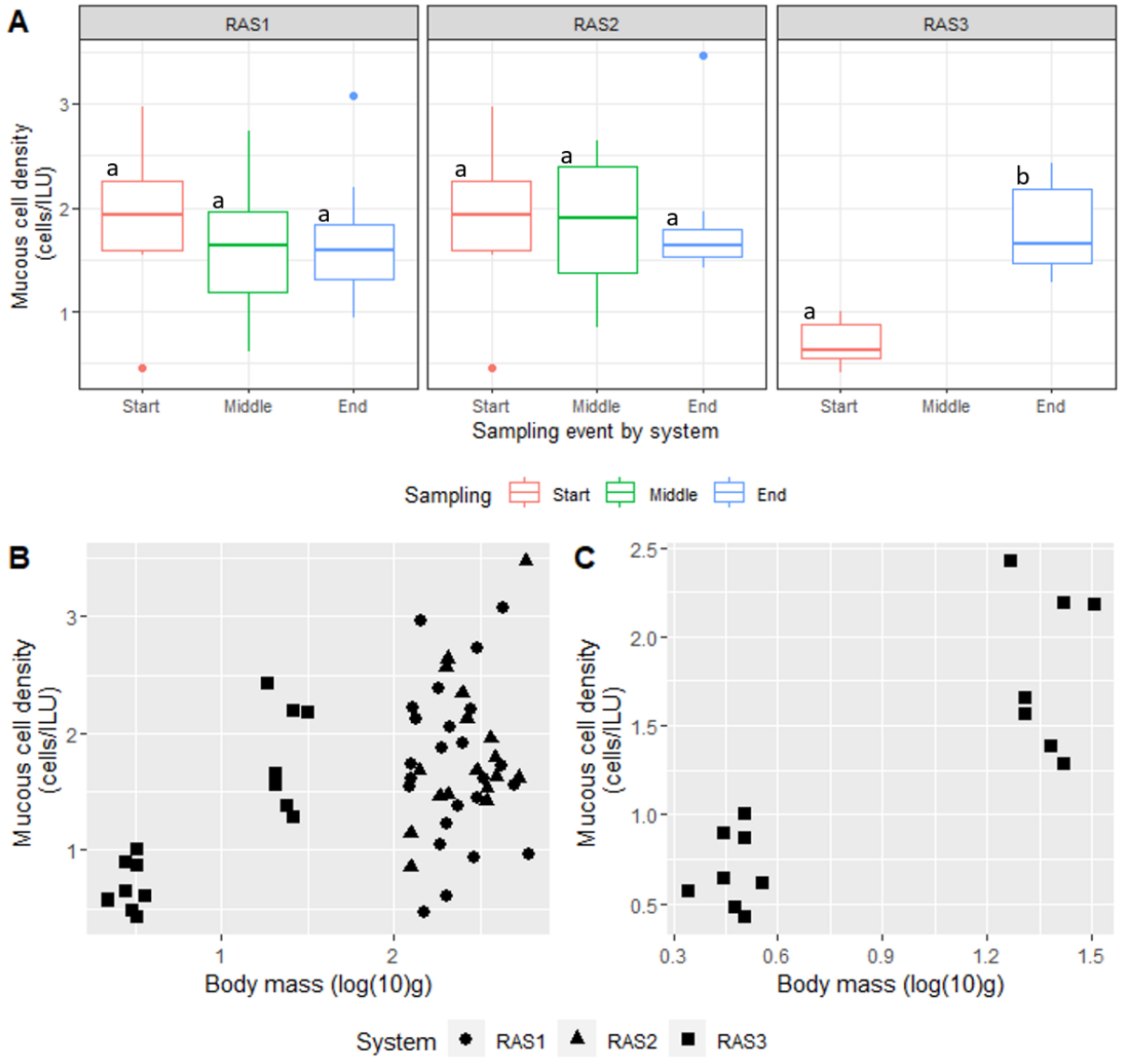


Figure 3.9: A) Mucous cell density in gills per ILU from AB-PAS-stained histology sections. Values for "start" in RAS1 and RAS2 are zero samples, thus the same (n=8). RAS1: middle sampling (n=8), end sampling (n=8), RAS2: middle sampling (n=8), end sampling (n=9), RAS3: start sampling (n=8), end sampling (n=7). Letters indicate significant change between sampling events (p ≤ 0,05) B) Scatter plot of the correlated relationship of log (body mass) and mucous cell densities in RAS1, RAS2 and RAS3. C) Scatter plot of the correlated relationship between log(body mass) and mucous cell density in RAS3.

4. Discussion

In all three facilities examined, a significant increase ($p < 0,05$) in the frequency of the same lesions were observed; lamellar infiltration of inflammatory cells, clubbing of the secondary lamellae and infiltration by inflammatory cells in clubbing of the secondary lamellae. An increase in TOC was observed throughout production, although levels did not correlate with bacterial numbers. Even though bacterial numbers did not show a significantly increasing trend when performing a linear regression, the number of cells per ml varied considerably throughout production, where the highest values occurred towards the end of the cycle (Fig. 3.1B, 3.5 B-C). The method used for quantifying bacteria was not sensitive enough to exclude system bacteria as a reason for reduced gill health and will therefore be discussed as a potential reason below. TAN, nitrite, nitrate, and CO_2, all significantly increased throughout production, and will therefore be evaluated as potential influencers of gill health.

Since no facility-monitored water quality data was available from RAS3, the trends of water quality parameters in this system cannot be discussed in depth. It is, however, interesting to note that the increases for the same lesions are seen in all three systems, despite different salinity, fish size and significantly different stocking densities.

4.1 Gill lesions

Clubbing of the secondary lamellae appeared early in the production cycle, while the infiltration of inflammatory cells in the clubbing and secondary lamellae, occurred at a later stage (Fig. 3.15). The inflamed clubbing and inflammation indicated an inflammatory response by the fish. Based upon morphological classification, the infiltrating cells were likely to be mononuclear cells, as they had a dense nucleus, surrounded by a narrow cytoplasm (Speare and Ferguson, 2006; Dalum *et al.*, 2015). Mononuclear cells (lymphocytes, monocytes and macrophages), are often associated with an inflammatory response, and eosinophilic granule cells (EGCs) can be recruited to sites with chronic inflammation (Koppang, Kvellestad and Fischer, 2015). EGCs were not observed in the lesions.

Epithelial lifting may be an artifact, as fish gills deteriorate quickly after fish have been euthanized, and lamellar oedema develops rapidly (Speare and Ferguson, 2006). Lamellar synechia, where parts of two adjacent lamellae fuse together, is a more reliable lesion less prone to be an artifact (Speare and Ferguson, 2006). This lesion is commonly associated with

heavy metal toxicity, e.g. copper, as well as in response to parasitic infections with *Ichtyobodo* sp. (Speare and Ferguson, 2006; Bruno, Noguera and Poppe, 2013). The mechanism behind the lesion is not known, however, it has been proposed that the toxicant alters the glycoprotein of the mucus, leading to a change in polarity and a favouring of fusion to adjacent lamellae (Speare and Ferguson, 2006). Cu-levels in the tank water of RAS1 and RAS2 were 2,7 µg/l and 1 µg/l, respectively, above the recommended limit for seawater of 3,1 µg/l (table 3.1) (Timmons and Ebeling, 2010c), and may have been the cause of the slight increase of these lesions. In RAS3, the alkalinity was 15 mg/l (as $CaCO_3$), and Cu-levels in the tank water were 0,5 µg/l above the recommended limit for freshwater at 10 mg/l (as $CaCO_3$) alkalinity (table 3.2) (Timmons and Ebeling, 2010c). As alkalinity in the system was slightly higher than the alkalinity-levels used in the recommendations, this may have led to a higher Cu-tolerance in RAS3, as increased alkalinity decreases the toxicity of Cu (Timmons and Ebeling, 2010c).

Although epithelial lifting and lamellar synechia showed a significant change in the Kruskal-Wallis-test, they did not show a significant change between sampling events in the post-hoc test. This difference in significance is caused by the effects of testing several hypotheses at once in the Kruskal-Wallis, which can give false positives (Li *et al.*, 2017). This was corrected by using the Bonferroni-adjustment of the p-values, leading to a non-significant result in the post-hoc test. Identifying a potential cause of incomplete hyperplasia in RAS3 was not possible since limited data was available.

Histology can be a very subjective quantification of lesions, therefore it is important to define what lesions are being observed, as different scientific communities may use different names for the same lesion, e.g. “clubbing” being used for clubbing of a filament, or referring to clubbing of secondary lamellae; also called tip thickening and clavate lamellae (Clark *et al.*, 1997; Sanchez, Speare and Johnson, 1997; Roberts and Rodger, 2012; Becke *et al.*, 2018, 2019). Results obtained by visually quantifying lesion may become more resilient by having more than one person evaluating the sections (Gibson-Corley, Olivier and Meyerholz, 2013).

Very few descriptions of the lesion clubbing exists, it is mostly mentioned as a potential gill change, and sometimes only illustrated by pictures (Sanchez, Speare and Johnson, 1997; Roberts and Rodger, 2012). The mechanisms of this lesion are therefore not known. It can, however, be hypothesised that clubbing is a result of a hyperplastic reaction in the gills. The

gill epithelial cells origin at the base of the secondary lamellae, and as they mature, move further out along the lamellae, before being shed or apoptosis occurs at the tip (Speare and Ferguson, 2006). It may be proposed that if the mitotic rate of the epithelial cells increases disproportionately to the apoptotic rate, epithelial cells may accumulate at the tip, making it appear as a club; “clubbing”. It may therefore be a sign of a shift in the gill homeostasis, and an early indication of reduced gill health.

4. 2 Mucous cell densities

Mucous cell densities did not change in RAS1 and RAS2. This could suggest that the fish gills were not irritated in a way that would illicit a mucous response. The increase in mucous cell density seen in RAS3 may be related to the increase in gill surface area in early stages of life (rho = 0,737), as a correlation coefficient higher than 0,7 may indicate a relationship (Fig. 3.9 C). However, the high unexplained variance when correlating all three systems (rho = 0,393) indicates that other factors are involved in determining the mucous cell densities in the gills.

It may be noted that the acidification of mucous cells was not quantified in this study. A change in acidification has been observed in other studies where the total number of mucous cells remained the same, indicating a change in mucus viscosity or production as a potential protective response to both environmental conditions and infection (Ferguson *et al.*, 1992; Sanchez, Speare and Johnson, 1997; Roberts and Powell, 2005, 2008). Changes in the mucus layer cannot be easily identified in histological sections, as the mucus layer coating the gills is removed by fixatives (Speare and Ferguson, 2006)

Other potential reasons for the increase of mucous cell numbers in RAS3 are difficult to verify, as this can be attributed to a variety of reasons. High concentrations of NH_3 may cause an increase in the number of mucous cells (Ferguson *et al.*, 1992), unfortunately this cannot be investigated, as data from the facility was not available. Mucous cell counts have been shown to be higher in fish in sea water compared to fish in freshwater (Roberts and Powell, 2003). The fish in RAS3 were moved from a freshwater flow-through system to a RAS system with 1-3 ‰ salinity. It is unclear how big the change in salinity needs to be before a change in mucous cells occur.

Becke *et al.* (2018) showed an increase in mucous cells in a RAS with increased levels of TSS and turbidity. In the present study, only turbidity was measured, and levels in RAS1, RAS2 and RAS3 increased from 0,66, 0,85 and 0,89 FNU to 2,7, 2,0 and 2,7 FNU, respectively. As the changes were approximately the same in all three systems, this does not indicate that turbidity was the factor causing an increase in mucous cells in RAS3. An unpublished student paper from NMBU could not correlate turbidity and mucous cell counts either, although this study was methodologically problematic.

The method of counting mucous cells in histological sections has been validated as not significantly different from more sophisticated scientific methods for quantifying mucous cells (Quantidoc) (Dang *et al.*, 2020). However, histology can be quite subjective, therefore sufficiently describing the criteria on which scoring, or quantification is performed is important for reproducibility (Gjessing *et al.*, 2019). Mucous cell quantification may be affected by factors such as tissue depth, orientation of the gill arch, or location on the gill (Dang *et al.*, 2020).

4.3 Raw water samples

As expected, in RAS1 and RAS2, metals zinc, copper and manganese accumulated in the recirculated tank water over time, and may enter the system through the feed or make-up water, or leach from pipes and fixtures (table 3.1) (Davidson *et al.*, 2009). In the intake water, start and end values for seawater were similar. The increase in copper in the intake water from 0,5 to 2,1 µg/l was, however, unexpected. A possible reason could be rain runoff from land, as there was construction work close to the facility as the samples were taken (Davis, Shokouhian and Ni, 2001). The difference in alkalinity between the raw water in RAS1 and RAS2 reflect that alkalinity has shown a huge variation throughout production with no clear trends (Fig. 3.4 C). Alkalinity was adjusted by addition of sodium bicarbonate to the system; therefore, this would be expected.

When comparing the start and end raw water samples of RAS1 and RAS2, nitrate, total nitrogen and TOC showed an increase over time, as seen in the production and biweekly sampling data (table 3.1, Fig. 3.1 A, 3.3 A, C). In RAS3, trends in turbidity, total nitrogen and nitrate cannot be confirmed by monitoring data from production, but levels were higher in the tank than in the freshwater intake (table 3.2). The TOC values from the raw water analysis

in RAS3 are in the same range as the biweekly samples (Fig. 3.5 A). Although the linear regression of TOC-values did not show a significant trend, the highest values occurred in the last half of the sampling period (Fig. 3.5 A). Also, since the raw water samples showed that TOC values in the intake water decreased, the higher values in the tank may still indicate an accumulation of TOC in the system.

The increase in pH, alkalinity, chloride, sulphate, copper, potassium, calcium, magnesium and sodium between the intake water and the tank water in RAS3can be explained by the sea salt, $NaHCO_3$, and CaCl added to the system. The increase in ions leads to an increase in the conductivity. The decrease in aluminium, iron and manganese between the intake and the tank water may be explained by the increased levels of TOC in the tank water, as organic matter has been shown to bind and form complexes with these metals (Skjelkvåle *et al.*, 2007).

4.4 Water quality impact on gill health

The gill lesions associated with high levels of NH_3 include lamellar hypertrophy and hyperplasia with lamellar fusion (summarised by (Daoust and Ferguson, 1984)). However, the methods for reaching high levels of NH_3 in these experiments were, in several cases, a reduction of water exchange or overcrowding, and effects of high levels of NH_3 may therefore have been confounded (Daoust and Ferguson, 1984). Becke *et al.* (2019) showed an increase of cell infiltration and clubbing when combining high TSS-load with high levels of NH_3, however, another potential reason for this observation will be discussed below. TAN-levels observed in RAS1 and RAS2 accumulated to above recommended levels (table 1.1). However, the highest measured TAN in RAS2 (2,84 mg/l) coincided with a decrease in pH (Fig. 3.3 A, 3.4 B), thus the amount of NH_3 would be 0,004 mg/l in freshwater (0,17 % at pH 6,8, 15°C); salinity-levels in the system would further decrease the amount of NH_3 (Timmons and Ebeling, 2010c; Nilsson *et al.*, 2018). Total ammonia nitrogen and NH_3 was therefore unlikely to have caused the observed gill lesions.

With nitrite toxicity, chloride cell degeneration and necrosis has been reported (Speare and Ferguson, 2006). Since such lesions specifically were not quantified in this thesis, they may have occurred to some extent. However, the highest nitrite concentrations observed in production were 0,61 mg N/l in RAS1 and 0,27 mg N/l in RAS2, which was below the recommended levels for nitrite-N in seawater (table 1.1).

Nitrate concentrations were below recommended limits of 400 mg N/l (table 1.1), which correspond to 1772 mg/l (Timmons and Ebeling, 2010a), for the entire production cycle. When comparing the monitoring data from production and the end-sampling water analysis performed by NIVA, a 300 mg/l difference in levels of nitrate was discovered. The nitrate concentrations (as NO_3^-) measured at the facility 07.01.2020 were 145 mg/l in RAS1 and 125 mg/l in RAS2 (Fig. 3.5). Meanwhile, NIVA measured 445,2 mg/l in RAS1 and 426,7 mg/l in RAS2 the 08.01.2020 (table 3.1). This suggested that there may have been a difference in method, or that there was a difference caused by sampling location. Based on the location of the sampling, the production data sampling was performed after the drum filter, and before the biofilter, thus after makeup water dilution and before additional nitrate production in the biofilter. The samples analysed by NIVA were taken before make-up water was added. If this caused the difference, it demonstrates the importance of measuring water quality parameters at appropriate locations in the system, to best evaluate and maintain a healthy fish environment.

However, even the higher values of nitrate were below levels tested in other studies, where no negative impact on gill health was observed (Freitag *et al.*, 2015; Davidson *et al.*, 2017; Good *et al.*, 2017). RAS1 and RAS2 also used brackish water, and a higher ionic strength of the water has been shown to decrease the toxicity of nitrate in rainbow trout (Baker *et al.*, 2017). The concentrations investigated in the previously mentioned studies, of 100 mg N/l are, however, four times lower than the upper recommended limit of nitrate-N (table 1.1). This illustrates the need for verifying safe limits and investigating sublethal effects to fish at high levels of nitrate, as well as effects of nitrate in varying salinities.

The accumulation in TAN, nitrite and nitrate observed (Fig. 3.3 A-C) may be partially explained by the variable, yet increasing, salinity observed in the systems (Fig. 3.2 B). Biofilters, matured in brackish water or freshwater, have shown that increasing salinity may reduce the rate of nitrification (Gonzalez-Silva *et al.*, 2016; Navada *et al.*, 2019). The increase in TOC may also be partially responsible, as nitrification has been shown to decrease when the C:N-ratio is increase due to competition for oxygen between the bacteria (Chen, Ling and Blancheton, 2006).

Carbon dioxide concentrations were kept below recommended values for the entire production cycle (Fig. 3.4 A, table 1.1). High levels of CO_2 are not associated with lesions in the gills, but an increase in chloride cells has been reported in catfish (*Ictalurus puncttatus*) (Laurent and Perry, 1991). Exposure to CO_2-levels twice as high as what was observed in RAS1 and RAS2, did not show effects of high CO_2-exposure on the gill tissue (Good *et al.*, 2010), thus, CO_2 is unlikely to have caused the gill lesions observed in this study.

Total organic carbon was shown to accumulate in RAS1, RAS2, and possibly RAS3, over time, and may have caused a shift in the C:N-ratio, which could alter the microbial community in the biofilter, or ultimately in the production tank (Chen, Ling and Blancheton, 2006). A recent study has shown that reducing the TOC in RAS by using a membrane filtration lead to a more stable microbial community, with lower bacterial numbers and a more diverse microbial community (Fossmark *et al.*, 2020). Bacterial numbers obtained in this study showed large variations over the sampling period (Fig. 3.1 B, 3.5 B-C), with the highest variations in the numbers of bacteria when there were high levels of TOC (Fig. 3.1, 3.5). The TOC-reductions observed by Fossmark *et al.*(2020) were mainly in particulate organic carbon (POC), not in dissolved organic carbon (DOC). Pedersen *et al.* (2017) has also observed that reducing the available particle surface area reduces bacterial activity.

A lack of correlation in TOC and bacterial numbers in the tank water was consistent with other findings, where it has been suggested that the bacterial community in the biofilter was able to absorb most of the changes in organic carbon, thus not changing the bacterial numbers in the production tank (Rojas-Tirado *et al.*, 2018, 2019). However, the fact that TOC-levels increased in RAS1, RAS2, and possibly, RAS3, indicates that the bacteria in the biofilter did not absorb all of these changes, and may have caused the bacterial numbers in the production tanks to become less stable (Fig. 3.1 B, 3.5 B-C) (Fossmark *et al.*, 2020).

Regarding the bacterial numbers observed, it is important to note that culturing and counting colonies on a media may not represent the actual conditions in the tanks. The numbers obtained may only be related to each other over time, as different bacteria from each system may be the dominating species on the growth media used. The media chosen was non-specific, and this may have caused fast-growing opportunistic bacteria to be dominating and not represent the bacterial population in the system.

Becke *et al.* (2019) studied the combined effect of high levels of NH_3 and TSS on gill health in rainbow trout, and attributed the observed increase in cell infiltration and clubbing to high levels of NH_3. The study increased TSS by applying shear forces to particles initially removed by the drum filter, a process which reduces the size of the particles. Increasing the particle surface area has been shown to increase the bacterial activity in the water (Pedersen *et al.*, 2017), and in Becke *et al.* (2019) a higher level of bacterial activity was observed in the treatment tank than in the control tank. Increased cell infiltration and clubbing are not lesions normally associated with high levels of NH_3 (Daoust and Ferguson, 1984), therefore it may be suggested that the gill pathology observed by Becke *et al.* (2019) was caused by other factors, such as the increased levels of bacterial activity in the treatment tank.

Several different microbial effects may be suggested as a cause for the inflammatory response observed in the three systems observed in this study. Increases in bacterial activity or changes in the microbial communities may result in the homeostasis of the mucosal commensal bacteria to be lost, and niches may open to potential pathogens, or commensals may rise to levels which become pathogenic to the fish (Gomez, Sunyer and Salinas, 2013; Kelly and Salinas, 2017).

The microbial communities in RAS varies with salinity (Gonzalez-Silva *et al.*, 2016; Navada *et al.*, 2019), thus variations in salinity is a factor which may affect the biofilter homeostasis. In transitional phases this may also affect the tank microbiota.

Another potential microbial effect may be caused by the priming of the innate immune system. The fish mucosal immune system is known to be developed by the microbiome in its environment (Gomez, Sunyer and Salinas, 2013; Kelly and Salinas, 2017). Fish reared in one system, either flow-through or another RAS, may have an innate immune system primed for different microbial conditions (Rud *et al.*, 2017; Navada *et al.*, 2019). Therefore, the immune system may not recognize the environmental bacteria as harmless and mount an inflammatory response. However, in the systems observed in this study, inflammatory cells infiltrated the lamellae quite late in the production cycle, therefore, this cause may be a less likely alternative in this study.

The correlation between RAS3 bacterial numbers from nutrient agar (5 ‰ NaCl) and blood agar was interesting to note, as blood agar is used for growing pathogenic bacteria, and haemolytic colonies were observed throughout sampling. This may indicate the presence of possibly pathogenic bacteria, as previous studies have shown that pathogenic bacteria can be present without causing disease (Blancheton *et al.*, 2013; Rud *et al.*, 2017; Becke *et al.*, 2018). Based on this observation, the question may be raised of whether a low presence of pathogens was able to cause an inflammatory response in the fish gills without causing disease. However, this cannot be confirmed by the results in this thesis, and results from RAS1 and RAS2 cannot support the observations from RAS3 blood agar, as only marine agar was used.

To investigate the microbial effects on fish health further, other and preferably several methods for quantifying and characterizing bacteria should be included, like a hydrogen peroxide assay, Bactiquant ®, flow cytometry, or even deep-sequencing (Rud *et al.*, 2017; Rojas-Tirado *et al.*, 2018, 2019).

Three studies performed on the effects of exposure to increased concentrations of TSS on rainbow trout gills are interesting in relation to the findings of this thesis. Becke *et al.* (2017, 2018, 2019) has looked at short-term and chronic exposure to increased levels of TSS, as well as the combined effect of increased levels of TSS and NH_3. A similar increase in gill lesions to what was observed in this thesis, was observed in the studies by Becke *et al.* (2017, 2018, 2019). However, the arbitrary scoring system of no, mild, moderate, and severe changes makes potential differences in prevalence difficult to identify, and potential findings are difficult to compare with the results of this thesis. In Becke *et al.* (2018, 2019), a high background level of lesions was present when the study began, and by scoring lesion from 0-3, potential differences caused by the treatment may be hard to distinguish.

Additionally, changes in lesion severity were not evaluated over time, but by visually evaluating included and supplementary graphs, there appears to be a change in several of the lesions over time for both the control and treatment fish (Becke *et al.*, 2017, 2018, 2019). In Becke *et al.* (2019), a high level of background pathology may be observed in the supplementary figures. The fish had been in the RAS for 3 months before the study started to acclimatize. These lesions may therefore show the effect of other RAS-parameters.

Based on results presented in (Becke *et al.*, 2017, 2018, 2019), it would seem that both the control and treatment experienced effects on gill health unrelated to the parameters the studies sought to investigate. Although the histological scoring made it difficult to distinguish between effects, the RAS environment shows some of the same effects on gill health as was observed in this study. These studies illustrate the importance of describing the criteria for the scoring or quantification method used, to ensure that results may be used for comparison by other researchers. A scoring system (0-3) which sufficiently defined the different scores and may be replicated by others is given in Good *et al.* (2017), while a more in-depth scoring system for identifying causes of complex gill disease in research is given by Gjessing *et al.* (2019).

The need for continued investigation into the gill lesions in RAS, and how conditions compare to flow-through systems is illustrated in a study by Good *et al.* (2010). A flow-through system showed similar or higher prevalence of gill lesions compared to a reuse system, even though the flow-through system had lower levels of TAN, nitrite, TSS and total particle.

4.5 Conclusions

This thesis observed an inflammatory response in the gills of fish from three separate RAS facilities, all operating under different salinities, densities, and production conditions. It was also confirmed that TOC accumulates in a RAS over time, however, TOC-concentrations did not correlate with bacterial numbers obtained in this work. Further research on the interactions between TOC, gills, and the microbial community of the production tank in a RAS are needed, as well as defining safe limits for water quality operating parameters in a marine RAS.

4.6 Future perspectives

For future work, one should include other, and preferably several, methods for investigating the bacterial community both in the production tank and on the fish. This may help to investigate the gill commensal microbiota, and how it modulates, or otherwise affects the fish immune system. It is further suggested to perform PCR-analyses of cytokines in the gill to analyse the inflammatory response morphologically identified in this thesis. PCR-samples were collected as part of this project.

This thesis also highlights the need for confirming recommended water quality requirements in marine RAS, both for fish and the nitrifying bacteria.

Finally, the findings of this thesis could be further substantiated by investigating if similar changes normally occur in RAS, by investigating a higher number of systems. A comparison between the regularly occurring gill lesions in flow-through and RAS may help elucidate potential differences between the two.

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Appendix I: Sampling protocol

Sampling location 1

Mucus samples were collected first, using a slightly altered wiping method from Fæste *et al.* (2020). The fish was held by forceps above the water for 10 seconds to allow most of the water to run off, before the fish was placed on tissue paper. The mucus samples were collected from the unhandled left side of the fish, below the dorsal fin, using a Kimtech Science Precision wipe. The wipe was placed in an Eppendorf tube® containing the end of a pipette tip, and immediately placed on ice.

Second, length and weight were recorded. To avoid blood contamination of the histology samples, gill arches on the right side were cut and immediately placed with their right side down. The second gill arch on the left side was collected for histology using scissors and forceps, put in a pre-labelled histocasette and placed on 10 % neutral buffered formalin. Gill-PCR samples were collected from the medial apex (region B, figure 2.4) of the third gill arch using scissors and forceps and placed on RNA-later. Finally, kidney samples for histology were collected from the middle of the kidney using scalpel and forceps, put in a pre-labelled histocasette and placed on 10% neutral buffered formalin.

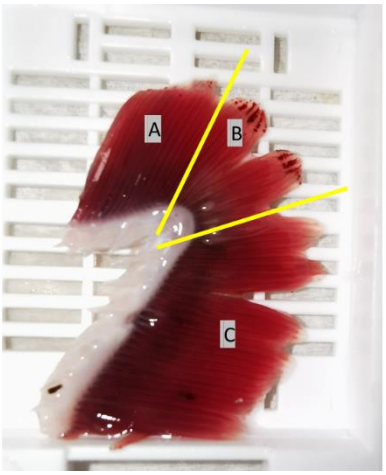

Figure 2.3: Overview of gill regions

Sampling location 2

Mucus samples were collected first, slightly altering the wiping method from Fæste *et al.* (2020). The fish was held by forceps above the water for 10 seconds so most of the water could run of, before the fish was placed on top of the plastic side of medical paper with a collection wipe on top. To collect the mucus, two pieces of Kimtech Science Precision wipe were used. Due to the small size of the fish, mucus was collected from both sides of the fish, and the collection wipes were placed in an Eppendorf tube® containing the end of a pipette tip. Mucus samples were immediately placed on ice.

Length, weight, and gill arches for histology and PCR were collected following the same procedure as in RAS1 and RAS2. The only exception was that right-side gill arches were not

cut to avoid blood contamination. Instead, the fish was cut at the caudal peduncle, because of its small size.

Processing of unused samples

Mucus samples were put directly onto ice. As soon as possible after sampling, the samples were centrifuged at 13000 rpm and 4°C for 10 minutes. The pipette tip allowed the mucus and collection wipe to separate, and the collection paper and filtering tip was removed from the tube using forceps. The remaining mucus was placed in a -80°C freezer to await further processing.

PCR-samples were kept on RNA-later in a fridge for at least 24 hours before they were frozen at -20°C.

Kidney samples from RAS1 and RAS2 were processed for histology following the same procedure as the gill tissue (section 2.6) and stained using HE.

Frequently occurring, as well as certain unusual, *bacterial colonies* were isolated and subcultured, and then frozen on growth medium (800 µl broth and 200 µl glycerol). Fresh water bacteria were put into nutrient broth, while saltwater bacteria were put into marine broth. Samples were frozen at -80 °C.

Water samples from all three sampling points (A, B, C) were filtered through a .45 µm filter using vacuum (Thermo Scientific Nalgene test filter funnels), filters were put into a 15 ml polypropylene tube and frozen at - 20°C.

Water samples were analysed for calcium (Ca^{2+})-content by NIVA (ISO 17294m:2016).

Mucus, PCR-samples, and kidney histology samples were not processed in this thesis but kept for future research.

Appendix II

MICROBIOLOGY

Marine agar - reagents

Marine Agar 2216 from Difco, One Med AS – 55,1 g

Distilled water – 1000 ml

Nutrient Agar 5‰ NaCl - reagents

Nutrient Agar from Oxid, Nerlien Kjemisk Tekniske – 28 g

Distilled water – 1000 ml

Agars - procedure

1. Boil until dissolved using a heated magnetic stirrer
2. Autoclave at 121 °C for 15 min
3. Cool to 55 °C in a water bath
4. Pour into petri dishes, let cool
5. Check pH at 25 °C (Marine agar – 7,6 ±0,2, Nutrient agar – 7,4±0,2)

Appendix III

HISTOLOGY - STAINING

Combined Alcian blue – PAS

1. De-wax sections and bring to water
2. Alcian blue solution – 5 min
3. Wash in water, then in distilled water
4. 1 per cent aqueous periodic acid – 2 min
5. Rinse well in distilled water
6. Schiff's reagent – 8 min
7. Wash in running tap water – 5-10 min
8. Rinse in absolute alcohol
9. Clear in xylene and mount

Results

Acid mucins – blue

Neutral mucins – magenta

Mixtures of the above – the colour will depend on the dominant entity and will range from blue-purple through purple to a violet or mauve colour

H&E staining

1. Distilled water – 1 min
2. Shandon instant haematoxylin – 3 min
3. Hydrochloric acid 0,1 %, aqueous – 2 sec
4. Running tap water – 3-5 min
5. Eosin Y-solution 0,5 %, aqueous, working solution – 3 min
6. Running tap water – 30 sec
7. Ethanol 70 % - 1 min
8. Ethanol 70 % - 1 min
9. Ethanol 96 % - 1 min
10. Ethanol 96 % - 1 min
11. Ethanol 100 % - 1 min
12. Ethanol 100 % - 1 min
13. Xylene or Neo-Clear® – 5 min
14. Xylene or Neo-Clear® – 5 min
15. Mount

Results

Nuclei – dark blue to dark violet

Cytoplasm, intercellular substances – pink to red

Erythrocytes – yellow to orange