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Effects of salmon lice treatment on the barrier properties of salmon skin mucus

Sushil Banjade

MSc in Biology
Submission date: June 2018
Supervisor: Ingrid Bakke, IBT
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Preface

It has been a valuable experience for me working with this master's thesis. I learned amazing experience during my Laboratory work and writing my thesis. I was able to understand the concept of nanoparticles, salmon skin mucus, salmon lice and confocal laser scanning microscopy which was new for me. The work has been interesting and challenging as well. In addition, I feel that I have learned a lot about the scientific writing process.

I would like to thank my supervisors, Associate professor Ingrid Bakke and Dr. Catherine Taylor Nordgård for their guidance throughout the entire journey. Catherine T. Nordgård has been immense help with everything. I highly appreciate her motivation, constructive guidance and wise suggestion. I am also obliged to Astrid Bjørkøy for training me on confocal microscope. I would like to thank everyone who has contributed to this project. I am extremely grateful for my family and friends for their endless efforts in every aspects of my life.

Trondheim, June 2018

Sushil Banjade

Abstract

Salmon skin mucus forms a thin physical barrier between the external environment and internal milieu acting as a first line of defence against infection through skin epidermis. In addition, mucus has role in defence mechanism of fish acting as a biological barrier. Reduced function of this barrier can cause threat to the health of fish. The current problem in the salmon farming industry is due to ectoparasitism with sea lice feeding off the flesh and skin of salmon fish. For the efficient control of sea lice, diverse treatment has been tried over the years. Despite the importance of the skin mucosa in the first line defence against environmental pathogens, the effect on barrier properties of fish skin mucus due to lice treatment have yet not been well characterized. The aim of this project was to investigate the effects of treatments applied against salmon lice on the mucus barrier properties.

In this master's thesis, variance in immobilization of 200nm diameter Carboxylate-modified microsphere in two different groups of samples i.e., in untreated samples and freshwater treated samples for sea lice were investigated and compared regarding the mucus thickness. The nanoparticles were placed on the top of salmon skin and the mucus ability to immobilize the given nanoparticles was characterized by using confocal laser scanning microscopy.

We found that there was considerable variability in the mucus and scales structure between two different groups of samples. The variability in mucus and scales was also observed in same fish skin samples and within the same treatment group samples as well. It can be concluded from this study that there was an effect of treatment on salmon skin mucus indicating that freshwater treatment can cause substantial increase in mucus thickness.

Abbreviations

µm	Micrometre
Å	Angstrom
CLSM	Confocal Laser Scanning Microscope
DNA	Deoxyribonucleic acid
FRAP	Fluorescence recovery after photobleaching
GABA	Gamma-amino butyric acid
GalNAc	N-acetylgalactosamine
GI	Gastrointestinal
GlcNAc	N-acetylglucosamine
LAS X	Leica Application Suite X
MALT	Mucosa-associated lymphoid tissue
nm	Nanometre
NPs	Nanoparticles
ppt	Parts per thousand
SIgA	Secretory Immunoglobulin A
UV	Ultraviolet

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1 Introduction

1.1 Background Introduction

1.1.1 Salmon Farming

Global health authorities are promoting the health benefits of sea food, and aquaculture is more sustainable than agriculture. As Salmon is rich in micronutrients, minerals, omega- 3 fatty acids, high-quality proteins and several vitamins salmon farming has become a flourishing component of aquaculture sector ("Salmon Farming Industry Handbook," 2017). Atlantic salmon accounts for 80% of total fish produced in European countries. Technical system and aquaculture infrastructure have greatly improved in the last few years. Salmon farming, which started in Norway in the early 1970s, has now become one of the largest export industries for Norway by economic value (Taranger et al., 2015). Salmon are anadromous fish that undergo smoltification. The farming cycle of salmon starts in freshwater. Fertilized eggs of salmon are kept in freshwater until they are hatched into tiny fish. When they weigh about 6 grams, the fish are transferred to larger freshwater tanks or open net cage in a lake. They are ready to move into the smolt stage when they weigh about 60-80 grams. In smolt stage, the fish undergo physiological change and they are kept in net pens in the sea until they are matured into adult salmon. They will remain in the sea over a year and when they reach the market weight (4.5-5.5 kg) they are then harvested, processed and distributed ("Salmon Farming Industry Handbook," 2017). The lifecycle of farmed salmon with different steps in production cycle is shown in figure 1.

In natural life cycle, salmon utilizes river for reproductive and nursery phase (Mills, 1991). The adult female lay eggs, which are then fertilized by males. About 90-95% of all Atlantic salmon die after spawning. Those that survive may spawn again. The eggs are hatched into alevins and when they reach about 3cm in length, the fish are known as fry. Then they grow into parr and smolt that begin to leave river for sea. They spend 1-4 years in the sea and again return to natal river for spawning (Hendry & Cragg-Hine, 2003). The natural life cycle of Atlantic salmon is shown in figure 2.

Salmon are exposed to mechanical and environmental stress through intensive farming. The stress can be acute or short term and chronic or long term. The acute stress occur during handling, netting, transport and confinement (Kubilay & Ulukoy, 2002) while the chronic stress occurs during feeding technique, diet, salinity, vaccination, stocking density, high

ammonia concentration and low oxygen concentration in the environment (Schreck, Olla, & Davis, 1997). These stress that are involved in industrial salmon farming processes can cause physical damage to skin defences and the unnatural environment may cause change in physiological response of salmon. So, farmers try to reduce the amount of handling that can cause damage fish health (Quantidoc, 2014). A major challenge to salmon farming in open pens is salmon lice. To overcome this, farming industry will go after multiple strategies to control salmon lice infection rates to allowable levels and the parasite will exhibit the capacity to adopt to these strategies. So there is a continuous need to manage salmon lice infestation (Ole Torrissen et al., 2013).

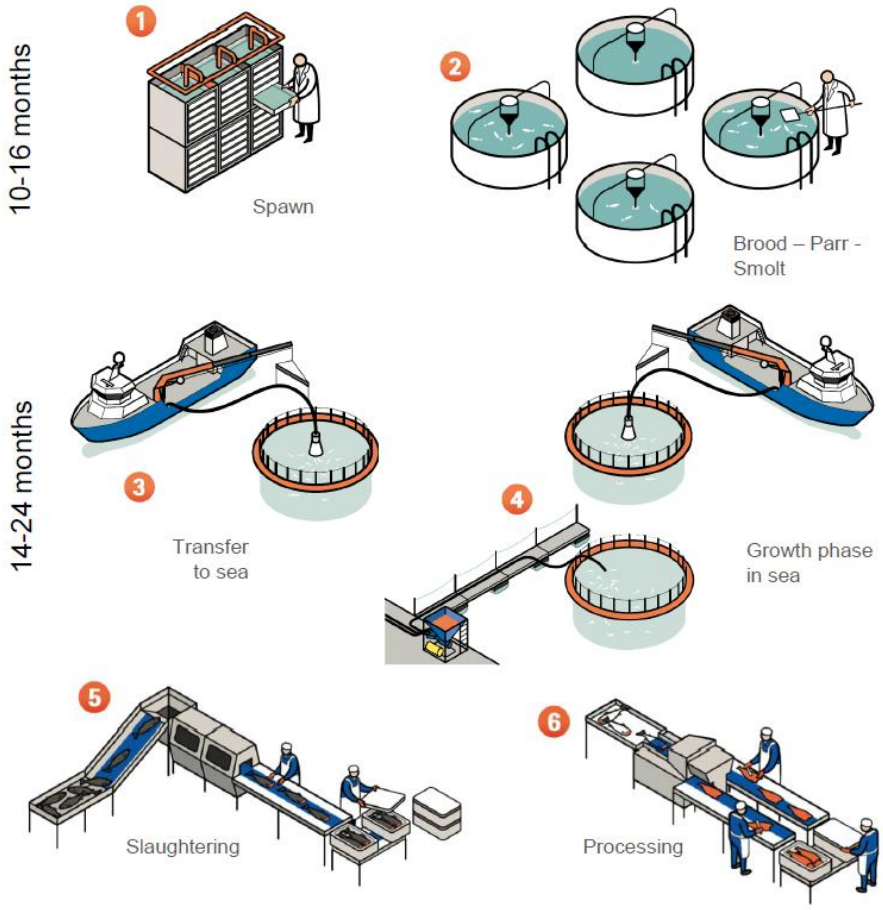

Figure 1.1: The cycle of farmed salmon with various steps (1) Eggs are kept in incubation tanks in freshwater (2) the tiny fish hatched are transferred to larger freshwater tanks (3) when they reach 60-80 grams are transferred to sea water in net pens (4) kept in net pens until they reach the market weight (5,6) finally the salmon are harvested, processed and distributed. Source: ("Salmon Farming Industry Handbook," 2017)

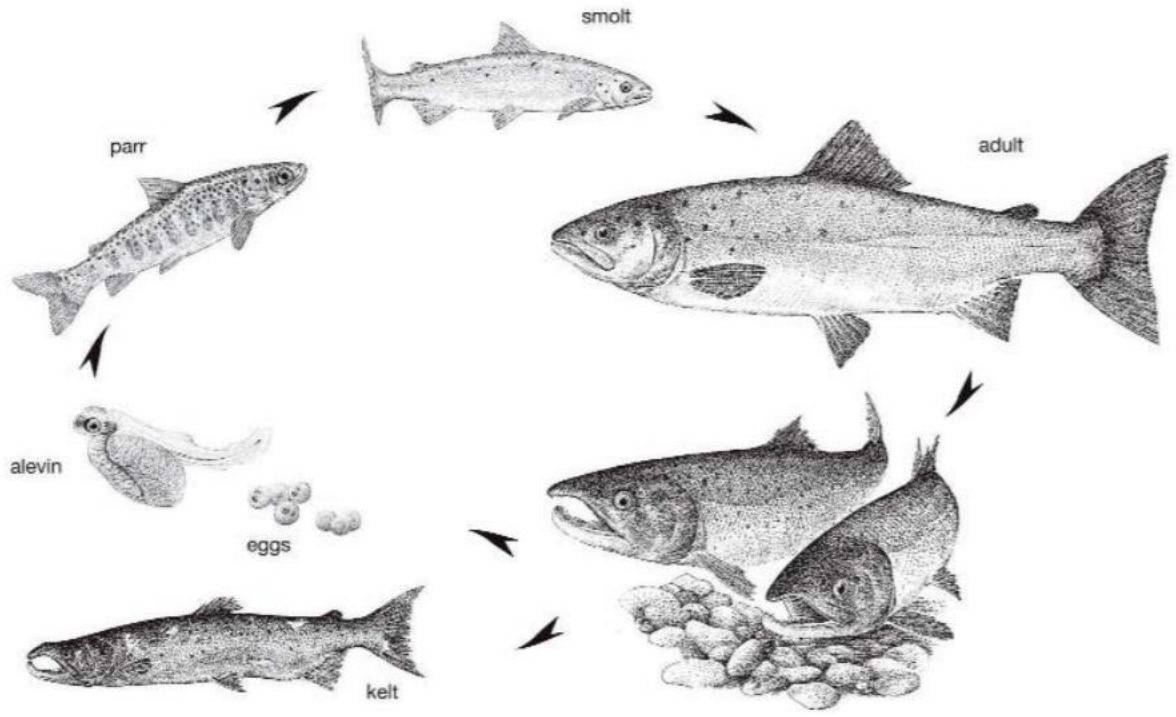

Figure 1.2: Showing several life stages in natural life cycle of Atlantic salmon. Source (Hendry & Cragg-Hine, 2003)

1.1.2 Salmon lice

Salmon lice (*Lepeophtheirus salmonis*) are external parasitic copepods in the marine environment that infest skin, mucus, and blood of the fish causing wound and infections (Frazer, Morton, & Krkošek, 2012). Secondary infection might occur at higher rates which leads to mortality if untreated (Grimnes & Jakobsen, 1996). The secondary infection is caused through stress associated immunosuppression induced by salmon lice or by serving as a vector for bacterial, viral and fungal pathogens. As the salmon lice reduces the physical wellbeing of Atlantic salmon, the fish are vulnerable to infection caused by bacteria and fungi. Salmon lice are one of the main problems in the aquaculture industry today which is responsible for causing a significant economic loss in this industry. It occurs throughout the North Pacific and Atlantic oceans. The life cycle of *L. salmonis* comprises a nonfeeding planktonic larval stage, infective free-living planktonic copepodites, immature chalimi and mobile preadults and adults. Various stages in the life cycle of sea louse *L. salmonis*is is shown in figure 1.3.

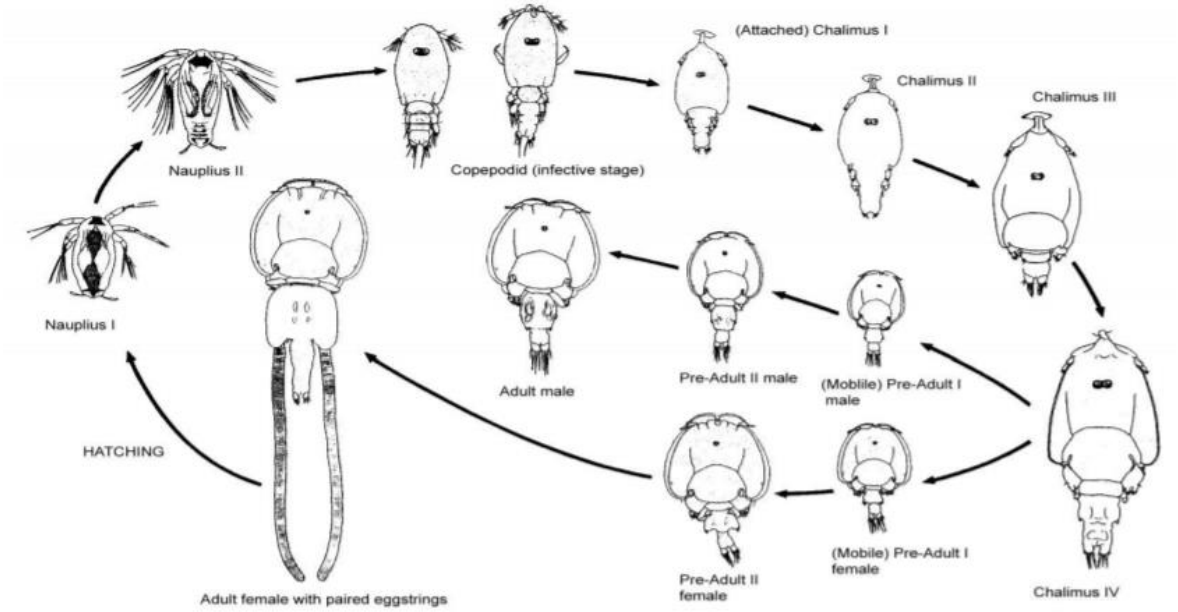

Figure 1.3: The molt stages and life cycle of *L. salmonis* (Whelan, 2018)

The eggs are carried by female sea louse in a pair of egg sacs extruding from abdomen. Over her lifetime the female sea louse can produce 6-11 broods. After release the eggs are hatched into naupilus which are the first larval stage. The louse has two initial larval stage, called nuplii as seen in figure 1.3, and both are non-feeding and planktonic. After 5-15 days depending on temperature the nuplii moults into infective copepodid stage. Free-living copepoid stage of salmon louse recognizes and respond to the physical and chemical environment for optimizing host finding and settlement. The copepodids after located to the host skin, moults into chalimus stage and attach by means of their antenna. There are four successive chalimus stage that feed on the host skin around their point of attachment. Followed by chalimus stage there are two further immature preadult stage in *L. salmonis*, which move freely over the host skin to feed. The feeding behavior of mobile preadult and adult stages cause damages to the host. The loss of epithelium, increased mucus discharge, alteration of mucus biochemistry and loss of physical and microbial protective function are the common impact on the host (Whelan, 2018). This increase the probability of bacterial and fungal infections and affects the osmotic balance to the fish. Sea lice have affected salmon farming for a long time and are todays one of the most important cause of mortality in farmed salmonids (*Sea Lice*, 2009). The combine effect of parasite includes reduced growth, reduced swim performance and increased stress (Brauner et al., 2012). The total cost on combating

sea lice infestation in 2015 at salmon fish farms in Norway surpassed $575 million (Ramsden, 2016).

Temperature is directly linked to the rate of development and metabolism of salmon lice. Cold temperature causes sea lice to live longer and grow larger. Also, high salinity sea water is most favorable for the survival and development of *L. salmonis*. In less susceptible species infections tend to be of lower intensity and reduced duration. Local inflammatory responses, vaccination, dietary immunostimulants etc. makes the salmon less susceptible to sea louse (Jones & Johnson, 2015).

1.1.3 Lice treatment

To ensure the health and welfare of farmed fish efficient control of salmon lice is crucial. Diverse treatment has been tried against salmon lice infestation over the years. Though there have been initiatives to develop integrated pest management strategies, commercial salmon farming is largely dependent on chemical treatment to control lice infestation (Roth, Richard, & Sommerville, 1993). The use of chemotherapeutics for lice treatment has increased significantly from 2009. Widespread use of chemotherapeutics to manage sea lice infections has led to reduced sensitivity or even drug resistant (Aaen, Helgesen, Bakke, Kaur, & Horsberg, 2015). At first, formaldehyde bath was used which have a controversial effect. Since large quantity ($400mgl^{-1}$) of formaldehyde was required for marginal efficacy, juvenile salmon could not tolerate at this concentration at 8.2°C. After that, organophosphates were launched as an oral treatment (Brandal & Egidius, 1977). Organophosphates act by inhibiting the enzyme acetylcholine esterase that is responsible for catalyzing the hydrolysis of the neurotransmitter acetylcholine. It has a quick response and the effect is best on pre-adult and adult parasites. Since the oral delivery medicines seem to have a low safety margin, bath application was introduced. Immersion bath treatments with organophosphates, hydrogen peroxide, or the synthetic pyrethroids are currently used if outbreaks of salmon lice occur. The pyrethroids act by interfering with nerve impulse that ultimately causes paralysis and death. The full effect of this compound can be detected after 1-2 weeks and is effective against all developmental stages of salmon lice. Hydrogen peroxide which is a powerful oxidizing compound induces mechanical paralysis caused by bubble forming in the body and hydroxyl radicals may lead to inactivation of enzymes and DNA replication (O. Torrissen et al., 2013) (Grant, 1997). These treatments cause substantial stress to the fish, are time-

consuming and labor intensive (J. Stone, I. H. Sutherland, C. S. Sommerville, R. H. Richards, & K. J. Varma, 1999).

To avoid the drawback correlated with bath applications that are only effective against preadult and adult stage of sea lice and allowing chalimus stage to survive , a treatment that is efficacious against all parasitic stage of sea lice could be administered in the feed (J. Stone et al., 1999). When fish is fed, the drug is absorbed from the gut and distributed to variety of tissue. When the sea lice feed on the mucus, blood, skin and muscle of host fish it is taken up into the tissue of louse. At present, there are two in feed treatments available in EU: Teflubenzuron (Calicide®, Nutreco) and emamectin benzoate (Slice®). Teflubenzuron, which is effective against moulting stage of lice seems to have no effect on adult lice and gives limited protection after the treatment period (Ritchie, Ronsberg, Hoff, & Branson, 2002). Emamectin benzoate which came in 1999 (J. Stone, Sutherland, Sommerville, Richards, & Varma, 2000) was better and effective for all developmental stages lasting up to 10 weeks. Emamectin benzoate modulates glutamate and gamma-aminobutyric acid (GABA) gated ion channel by binding to nerve cells and disrupts transmission of nerve impulse resulting in paralysis and death of parasites (J Stone, Sutherland, Sommerville, Richards, & Endris, 2000).

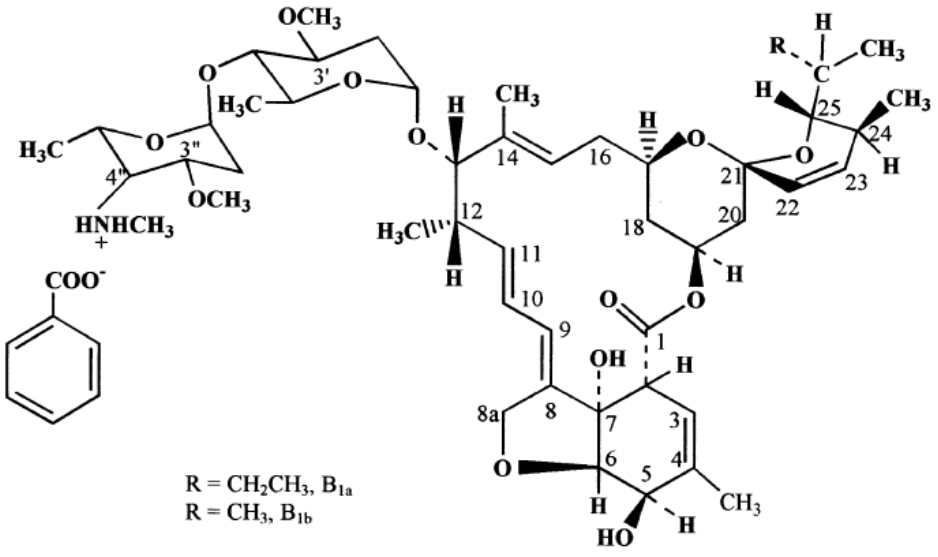

Figure 1.4: Chemical structure of Emamectin benzoate (MK-244, SCH 58854), 4”-deoxy-4” epimethylaminoavermectin B1 benzoate) (J. Stone, I. Sutherland, C. Sommerville, R. Richards, & K. Varma, 1999)

The primary method for treating salmon infected with sea lice is drug treatment (Mordue & Pike, 2002). This method is practice costly and may contribute to a diminished public image of salmon farming. Development of resistance in sea lice for the medication used to treat them has been proved e.g., for diclorvos (Tully & McFadden, 2000) and hydrogen peroxide (Treasurer, Wadsworth, & Grant, 2000). The biology and welfare of fish are also compromised by these treatments. To control sea lice infection, sustainable and cost-effective measures are needed.

1.1.3.1 Freshwater treatment of sea lice

The work done with respect to freshwater treatment of sea lice where water chemistry and its connection have been studied are less. Present day studies are undertaken to access the possibilities for using freshwater to remove attached sea lice from Atlantic salmon commercially. When the Atlantic salmon infested with sea lice are exposed to salinities below 29 ppt, gradual loss of sea lice occurs. *L. salmonis* is killed in freshwater rapidly (B. M. Connors, E. Juarez-Colunga, & L. M. Dill, 2008). The salmon is held in freshwater for about 3-4 hours before they are released back to the net pens. Freshwater treatment of Atlantic salmon infected with sea lice seems to be effective and possess a low threat to overall fish health and has good safety margin reducing residual discharge to the environment. In natural lifecycle of Atlantic salmon, returning to freshwater for wild fish results in the loss of attached and mobile stages of sea lice because of the incompetence of sea lice to sustain long term in freshwater (B. Connors, E. Juarez-Colunga, & L. Dill, 2008). In response to freshwater, adult female *L. salmonis* drop the egg string but depending on the temperature the attached calimus larval stage can survive few more days (Finstad, Bjørn, & Nilsen, 1995). The phenomenon of “premature migratory return” of sea lice infected fish to freshwater has been noted that has been responsible to be a behavioural response of host fish to extreme sea lice burdens. It has been interpreted that such behaviour of fish reduced the stress caused by the infection of *L. salmonis* and strengthen the survival of the fish (Bjørn, Finstad, & Kristoffersen, 2001).

The scale of Norwegian production and logistics to handle and treat large volume of fish should be considered in attributing best practice for freshwater treatment of sea lice (Powell, Reynolds, & Kristensen, 2015).

1.2 Scientific introduction

1.2.1 Salmon skin mucosa: the first line of defense

Vertebrates immune system incorporates specific mucosal defences. Mucosal surfaces are in constant contact with the external environment and are first point of contact for pathogens. The mucosal surface is a principal protective barrier against both primary and opportunistic pathogens (Gomez, Sunyer, & Salinas, 2013). Mucosal surface not only act as a physical barrier, also it is armed with cellular and humoral defense. Cellular response provides the defence barrier in the form of mucus and epithelial cells that line the skin and is responsible for preventing entry of pathogens into the body. While the humoral defence recruit's wide variety of proteins and glycoproteins capable of destroying the pathogens (Aoki, Takano, Santos, Kondo, & Hirono, 2008). Fish skin mucosa shares important functional and structural properties with fish gut mucosa. While the crucial importance of the gut microbiota to the mucosal barrier function is increasingly recognized, the role of the fish skin microbiota is little studied. Furthermore, despite the importance of the skin mucosa in the first line defense against environmental pathogens, the barrier properties of fish skin mucus have yet not been well characterized. The most abundant component of mucus is mucin which is strongly adhesive glycoproteins (Esteban, 2012).

The skin of salmon is different from that of mammals because it secretes mucus (Salinas, Zhang, & Sunyer, 2011) in contrary to terrestrial vertebrates which mainly have keratinized epidermis. Generally, the skin of salmon is divided into epidermis and dermis (fig. 1.5). Epithelial cells are the basic cellular element of the fish epidermis and are metabolically active (Elliott, 2000). The epidermis consists of three layers: stratum superficiale, stratum spinosum, and stratum basale. The epidermal layer thickness is generally 2-10 cell layers. The mucus producing goblet cells are scattered in stratum superficiale (Beck & Peatman, 2015). Goblet cell is a unicellular exocrine gland that is common to most animal groups. The dermis consists of two layers: outer stratum spongiosum and inner stratum compacticum. Stratum spongiosum is a loose network of connective tissue that contains fibroblast, nerves and pigment cells while the stratum compacticum is a denser layer consisting orthogonal collagen bands (Hawkes, 1974). A cellular basement membrane also called basal lamina separates the epidermis from the dermis (Elliott, 2011). Usually, the scales are covered by epidermal tissue. The scales of Atlantic salmon originate in scale-pocket from dermis anchored by collagen filaments (Beck & Peatman, 2015).

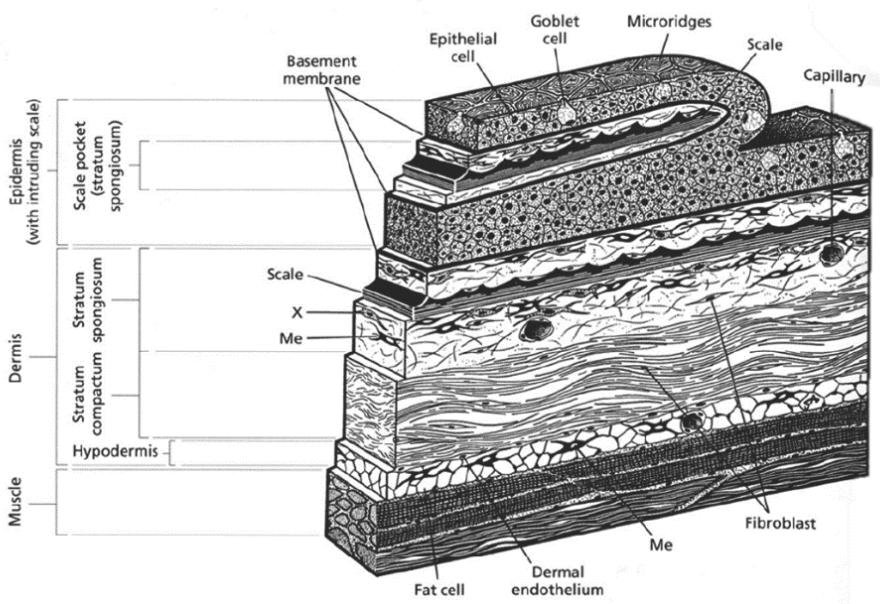

Figure 1.5: Longitudinal section of salmonid skin showing different skin layers and cell types of the epidermis (Elliott, 2000).

The skin of aquatic vertebrates is significantly important as a primary defense against pathogens since the aquatic environment is more rich in pathogens than terrestrial (Magnadottir, 2010). These surfaces are exposed continuously to the external environment and encounter microbes and stressors regularly. So, the defences are very important that contribute for fish health and survival. Mucus has specific functions which enable the fish to survive. The range of roles for mucus is large involved in respiration, ionic and osmotic regulation, reproduction, excretion, communication, disease resistance and protection (Shephard, 1994). The B cells and immunoglobulins that are contained in mucosa-associated lymphoid tissue (MALT) plays a central role in maintaining homeostasis. To maintain homeostasis two layers of adaptive anti-inflammatory defence has developed by the mucosal immune system: (a) immune exclusion provided primarily by SIgA antibodies to limit epithelial contact with dangerous antigens, and (b) immunosuppressive mechanisms to hinder overreaction against antigens (Brandtzaeg, 2009). Salmon skin mucus also possesses immunological properties including antimicrobial peptides, lysozyme, lipoprotein, cytokines, antibodies (Hatten, Fredriksen, Hordvik, & Endresen, 2001), complement factors and protease (Nigam, Kumari, Mittal, & Mittal, 2012). Alkaline phosphatase, a lysosomal enzyme in fish mucus is proposed to have protective roles during the first stage of wound healing (Iger & Abraham, 1990) and this enzyme level seems to be increased in mucus under parasitic infection in Atlantic salmon (Ross, Firth, Wang, Burka, & Johnson, 2000). Serine protease

which is one of the major mucus protease present in the salmon skin mucus act by cleaving the pathogen's protein (Subramanian, MacKinnon, & Ross, 2007) and hampering the colonization and invasive mechanisms.

Mucus secretion is significantly triggered by poisonous and irritating substances increasing the thickness of mucus while moving the irritants away from the epithelium. Mucus forms and maintains adherent unstirred layer and thus a diffusion barrier alongside the epithelial surface. So, the pathogens must migrate upstream to reach the epithelium for infection because of mucus secretion (Cone, 2009). There is limited knowledge about the defense mechanism of epidermal mucus of fishes at the present time.

1.2.2 Mucus composition and barrier properties

Mucus is a viscoelastic secretion that protects the most surface of the vertebrate's body especially the epithelia that line the lumen of all the organs and glands exposed to external environment. It has been described in all forms of life including bacteria, fungi, viruses, plants fish etc. The mucus layer is contained in organ system in vertebrates. In addition, most aquatic organisms possess mucus layer in their skin also (Probst, Gertzen, & Hoffmann, 1990). Mucus is continuously secreted and shed. It is secreted by specialized mucous and goblet cells in the columnar epithelia (Cone, 2009). The goblet cells are ample on almost all fish epidermal surfaces and particularly on gill surfaces (P. Laurent, 1984). Other cell types like secretory cells also have been identified in fish skin mucus that contribute to fish mucus and their secretions naturally mix with that of goblet cells (Mary Whitear, 1986).

The study done by Olmsted et al. (2001) stated two possible mechanism that hinders particles diffusing through the mucus gel. Either particle is stuck in the mucin fibres or stopped by the size of mesh spacing between the mucin fibres. Mucus is a sticky gel so many surfaces stick on it. It provides a barrier to toxic substances and pathogens by trapping them and obstructing the access to epithelia (Cone, 2009). Mucus gel efficiently trap foreign particles forming polyvalent adhesive interactions (Daniel A. Norris & Sinko, 1997). The mucin fibres that form mucus gel consist arrangement of alternating hydrophilic and hydrophobic regions. So, array of low affinity hydrophilic or hydrophobic bonds can form between the flexible fibres and incoming particle depending on the conformation of incoming particle. The mesh like structure of ovulatory cervical mucus can have an average mesh spacing of less than 1µ in diameter (Cone, 2009). Mesh size can increase with increasing concentration of mucus (Sanders et al., 2000). Fish skin mucus act as the natural, physical, biochemical, dynamic and semipermeable barrier enabling the exchange of nutrients, gas, water, hormones while being

impermeable to bacteria and most pathogens. Mucus has powerful barrier mechanisms that can stop pathogenic organisms and nanoparticles and hinder contact with the epithelial surface (Cone, 2009).

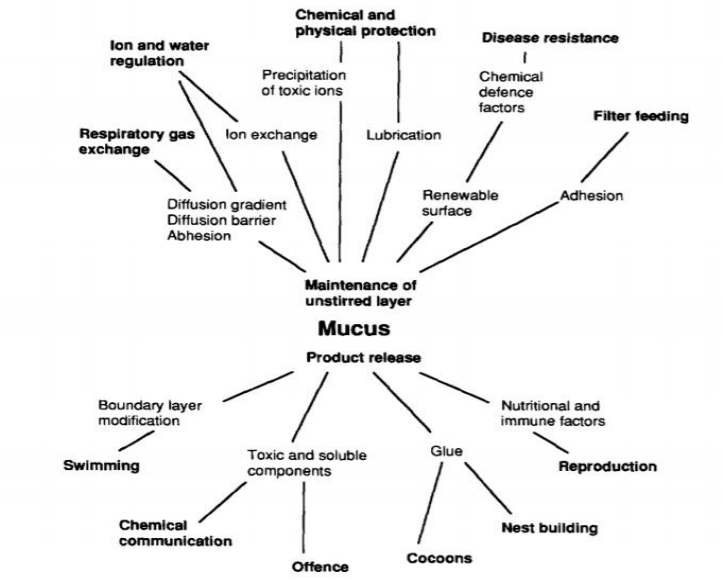

Figure 1.6: Various roles of fish mucus (Shephard, 1994)

Mucus composition: Mucus is a hydrogel consisting water and mucin as main components. Apart from that it is composed of electrolytes, lipids and various proteins. Water, which serves as the solvent and diffusion medium (allowing for the passage of certain molecules while rejecting many others), constitute about 90-95% of mucus. Mucins are high molecular weight glycosylated glycoproteins which constitute greater than 50% carbohydrates. The viscoelastic and rheological properties of mucus is due to this glycoprotein. Mucin possesses repetitive regions rich in serine, threonine, and proline where glycosylation takes place (Rose & Voynow, 2006). Mucin is present at the concentration of 1-5% in mucus. Mucin can be classified into secreted mucin and membrane-bound mucin. Secreted mucin is packaged in and secreted extracellularly from secretory granules and membrane-bound mucin is anchored by insertion through the plasma membrane. Membrane bound mucin and secretory mucin are highly glycosylated consisting of 50-80% carbohydrates as mentioned above. Carbohydrates are composed of N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, galactose and Sialic acid. The mucin monomer, where oligosaccharides are linked O-glycosidically consist of a linear protein core with a serine/threonone rich tendem repeats regions. The mucin monomer dimerizes through the formation of C terminal S-S bonds of cysteine groups. Mucin multimers are formed by further polymerization of these dimers (Bansil, Celli, Hardcastle, & Turner, 2013) as shown in figure 1.7. Since the glycosylated

regions are hydrophilic and the protein moieties are hydrophobic in nature, mucin is accessible to both hydrophilic and hydrophobic interactions (Bansil & Turner, 2006).

The secretory mucins in mammals include MUC 2, 5AC, 5B, 6, 7, 8, 9, 19 and membrane mucins include MUC 1, 3A, 3B, 4, 11, 12, 13, 15, 16, 17, 20, 21 (Andrianifahanana, Moniaux, & Batra, 2006) (Rose & Voynow, 2006). In the study done by Sveen, Grammes, Ytteborg, Takle, and Jørgensen (2017) seven unique mucins were identified as secreted gel forming mucins in the Atlantic salmon reference genome. The tissue specific transcription pattern of Atlantic salmon MUC 2 and MUC 5 families were similar to those of other species. Eleven different gel forming mucins have been found in Zebrafish (*Danio rerio*) (Lang et al., 2016).

Common mucus electrolytes are sodium and potassium chlorides, sodium bicarbonates, phosphate, magnesium, and calcium. They have roles in controlling mucus hydration and rheology (Verdugo, Deyrup-Olsen, Aitken, Villalon, & Johnson, 1987). Since salmon fish lives in saltwater (sea) and freshwater, balance of electrolytes is very crucial. Mucus helps to maintain constant condition inside the fish by partially blocking the movement of water into and out of fish body (Crampton, 2015). Lipid constitutes 1-2% of mucus that is covalently and non-covalently associated with mucus. The lipid affects the wettability, hydrophobicity and barrier properties of mucus (Lichtenberger, 1995). Various proteins that have an important immune function in fish can be found in fish mucus. For instance, the lactoferrin stimulates skin immunity and inhibit an allergic response (González-Chávez, Arévalo-Gallegos, & Rascón-Cruz, 2009).

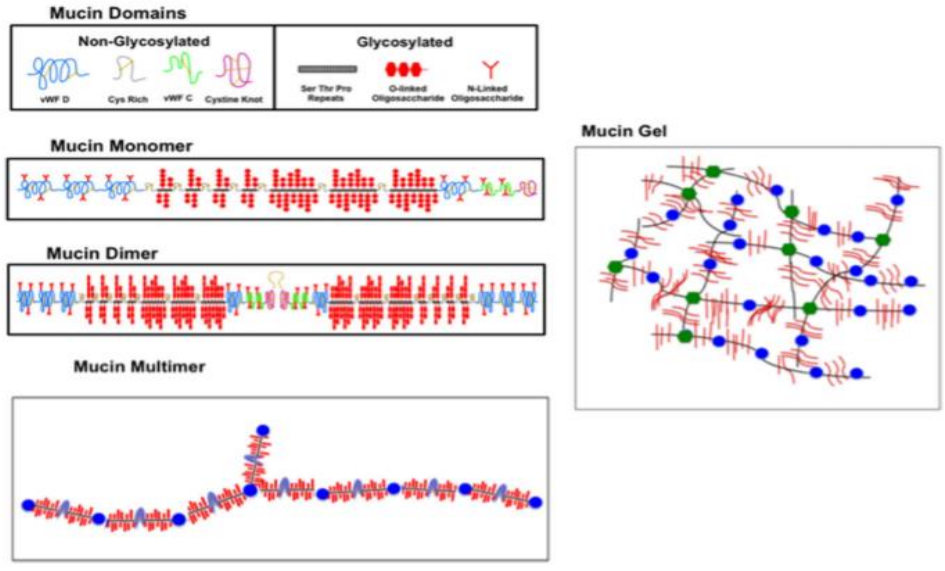

Figure 1.7: Hierarchal structure of mucin glycoproteins (Bansil et al., 2013).

1.3 Aim of thesis

Despite the importance of the skin mucosa in the first line defence against environmental pathogens, there is limited observation on barrier properties of fish skin mucus. It is a need for more knowledge on damage caused by sea lice on salmon skin and change in mucus after treatment of sea lice. Such knowledge is important to get a better understanding of salmon skin mucus for health and welfare of fish. This can only be achieved by appropriate and workable methods.

The aim of the thesis is to find out if microscopic assessment of nanobead penetration in fish skin mucus allows the measurement of a nanoparticle to scale distance (mucus thickness) and to test whether salmon lice treatment changes the mucus thickness measurement using this method.

1.4 Technical Introduction

1.4.1 The use of nanoparticles to evaluate mucus barrier properties

Nanoparticles (NPs) are microscopic particles which size are measured in nanometres (10^{-9} to 10^{-7} metres). They have a very high surface area to volume ratio. Nanoparticles are of prominent scientific interest due to a wide variety of application in research in the biomedical, optical and electronic field (S. Laurent et al., 2008). Some of the well-known nanoparticles based on physical and chemical characteristics are Carbon based nanoparticles, metal nanoparticles, ceramics nanoparticles, semiconductor nanoparticles, polymeric nanoparticles and lipid based nanoparticles (Khan, Saeed, & Khan, 2017). Because of the drug delivery research, a lot is known about nanoparticles and mucus barriers (Alexis, Pridgen, Molnar, & Farokhzad, 2008).

The dense network of mucin fibres that contain negatively charged segments is prominent for mucus barrier properties that shows a high affinity towards positively charged particles (Lai, Wang, & Hanes, 2009). The diffusion of nanoparticles in mucus is blocked by trapping and steric hindrances due to firm and adhesive network of mucin fibres (Liu, Zhang, Shan, & Huang, 2015). A dynamic series of interaction occurs between nanoparticles surface and biomolecular surface that are controlled by a variety of forces. The force can be long range arising from attractive van der Waals and repulsive electrostatic interactions, and short range arising from charge, steric and solvent interaction (Nel et al., 2009).

Advanced imaging technique based on fluorescent nanoparticles has provided distinctive tool for observing Nano-bio interactions with accuracy. The fluorescent nanoparticles in the sample can be easily visualized in x, y and z planes by confocal fluorescent microscopy without the need of sectioning the sample (Alvarez-Román, Naik, Kalia, Guy, & Fessi, 2004). Similarly, fish scales can be visualized in x, y and z planes by confocal reflectance microscopy. For the carboxylate modified nanoparticles used in the study, it is expected that if the mucus is 'good quality' then the nanoparticles will be immobilized at the mucus surface and the excluded distance between nanoparticles and scales will equal mucus thickness. For poor quality mucus or in case of damaged mucus the excluded distance will be reduced. The damage can be occurred by diseased condition, lice infection, during fish handling or chemical treatment.

1.4.2 Confocal laser scanning microscopy

For investigation in biological and medical sciences, confocal laser scanning microscope (CLSM) has become an extremely useful tool. This instrument can be used for imaging thin optical section of either living or fixed specimen that range in thickness up to 100 micrometers (S Claxton, Fellers, & Davidson, 2018)**.** Cutaneous cells from distinct epidermal layer can be distinguished in a magnificent way using confocal laser scanning microscopy (Meyer, Otberg, Sterry, & Lademann, 2006). Present day confocal microscopes consist of one or more electronic detectors, a computer for displaying images and several lasers system (Goldman, 2004). In standard microscopy, there is a problem of the image as "out of focus blur" if the specimen is not very thin. There are several advantages of confocal microscopy over ordinary widefield optical microscopy. It possesses the capability to control the depth of field, eliminating background information away from the focal plane and most importantly to collect serial optical section from the thick specimen. Sharply focused optical slice through the specimen is produced by using a pinhole between the specimen and detector, that selects information from the single focal plane. A three-dimensional data set is produced by taking a series of an optical slice from different focus level in the specimen (Dave Johnston).

Principle: Light from the excitation source passes through the pinhole aperture which is located in a confocal plane with a scanning point on the specimen and a second pinhole aperture placed ahead of the detector. When the laser is reflected by the dichromatic mirror and scanned across the specimen in a fixed focal plane, then secondary fluorescence emitted from specimen passes back through the dichromatic mirror and are focused as a confocal

point at the detector pinhole aperture (S Claxton et al., 2018). Diagrammatic presentation of the principle of confocal laser scanning microscope is shown in figure 1.8.

The barrier filter, excitation filters and dichromatic mirror of confocal microscopy have a similar function as other ordinary microscope (Rost, 1992). The pinhole aperture is one of the most crucial components of this microscopy. It is positioned directly in front of the photomultiplier and performs as a spatial filter at the conjugated image plane. The image of an extended specimen in laser scanning confocal microscopy is produced by scanning the focused beam across a defined area in a raster pattern that is controlled by two high-speed oscillating mirrors which are controlled by galvanometers motors (S Claxton et al., 2018).

The advantages of confocal microscopy are high resolution image, reconstruction of 3D images, the absence of artifacts (e.g., shrinkage) and in vivo microscopy to a depth of 200µm. Similarly, the limitations are photobleaching of fluorescent probes and phototoxicity of live samples, depth of imaging limited by optical penetration and signal to noise ratio and is expensive (Nwaneshiudu et al., 2012).

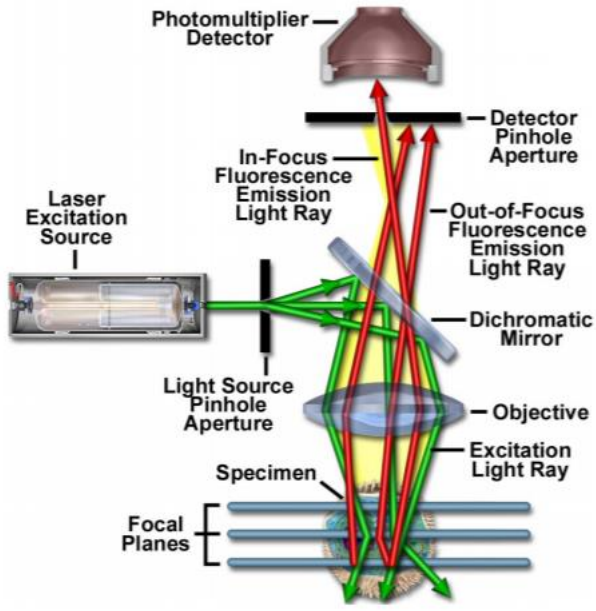

Figure 1.8: Basic illustration of confocal laser scanning microscope (S Claxton et al., 2018)

1.4.2.1 Reflectance and fluorescence

Reflectance and fluorescence are two modes that can be performed in confocal microscopy. Naturally occurring tissue components are demonstrated by reflectance mode which is based on contrast from variations in refractive indices of tissue microstructure. The contrast in

reflectance mode is associated with naturally occurring components like scales of fish. For *in vivo* reflectance mode measurements, a laser light with near infrared wavelengths is used. Reflectance mode is generally used for morphological studies. Fluorescence mode achieves contrast by the excitation of administered fluorescence dye that may be used to label specific structures for example by linking to an antibody. Reflectance and fluorescence allow distinct predication and information on the state of the tissue. Both scanning modes are consistent and can supplement each other to obtain additional information from the tissue (Hoffman, 2002) (Meyer et al., 2006).

Fluorescence is the property of absorbing light at a certain wavelength and subsequently emitting the light of longer wavelength after a certain interval by atoms and molecules. Fluorescence is normally emitted from fluorophores (*Microscopy: advances in scientific research and education*, 2014). When light is absorbed by the molecule the electrons are excited to the higher state due to the energy given by photons in the light. The absorption of light and excitation of electrons take place in very short time (10^{-15} seconds). Since the excited state is an unstable state, the electrons cannot stay there longer and return to the stable ground state. The electron emits energy in the form of photon while returning to the ground state, which is detected as luminescence. The use of this technology has been increased dramatically in multidisciplinary fields within the last three decades because of its high sensitivity towards detection (Lakowicz, 2006).

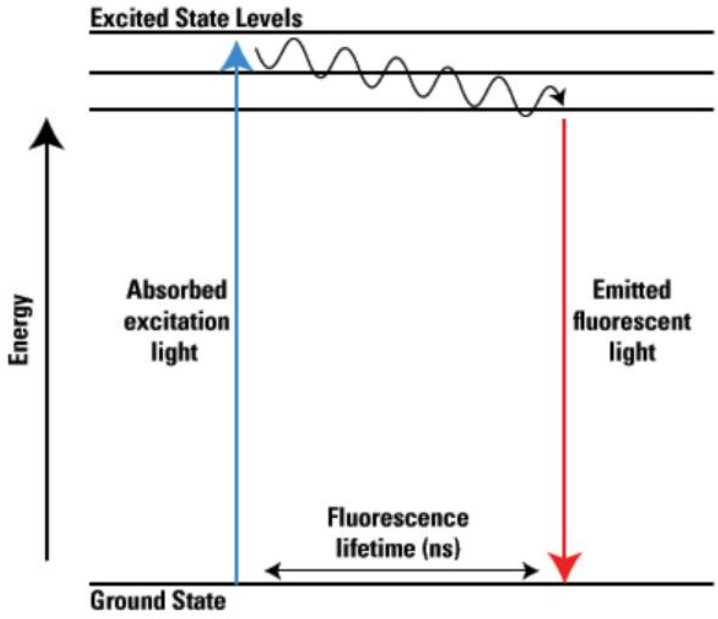

Figure 1.9: Jablonski energy diagram showing mechanism of fluorescence. The excitation and photon emission from a fluorophore is cyclical. Source: ("Fluorescent probes,")

1.4.2.2 Optical slices and Z stacks

Confocal microscopy allows looking at thin optical sections from a thick sample of cells or tissue without physically cutting the sample. In specimen that is more than 2 µm thick, secondary fluorescence can hinder the resolution of a feature of interest in conventional wide-field microscopy. This concern is addressed by placing a pinhole in front of the detector to remove out of focus light. The images are formed by scanning one or more focused beam of light from a laser across the specimen that provides a non-invasive way to produce optical sections at different depths (Boguslavsky, 2003). In confocal microscopy, the optical section can be generated in three different planes: xy, xz, and yz. Since the lateral spatial resolution (xy) is better than axial spatial resolution (xz and yz), sectioning in xy plane is common (Wright & Wright, 2002).

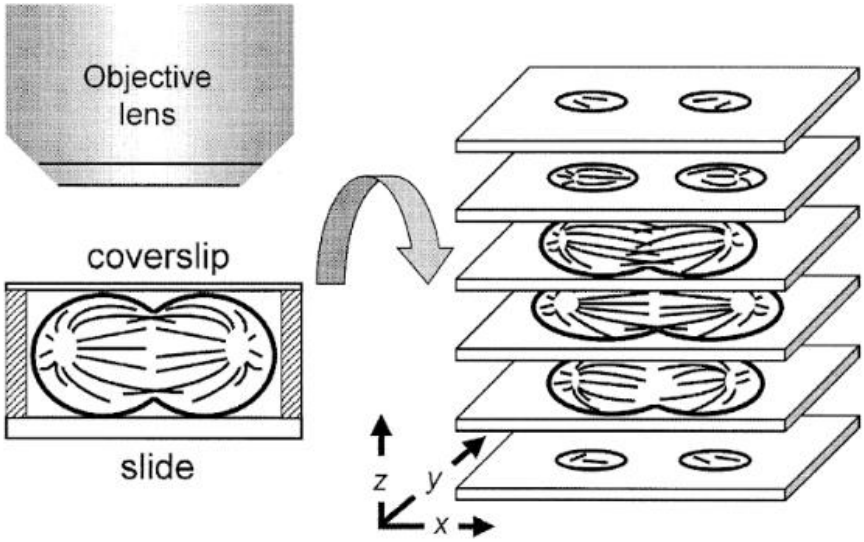

Figure1.10: Generation of the optical section in a confocal microscope. The optical sectioning shows only the structures in the focal plane leaving the sample undamaged (Wright & Wright, 2002).

Numerous optical sections are merged with software to rebuild the original 3D structure. A Z-series is a series of optical sections collected at different focal depth along the optical axis. For 3D visualization, the optical section can be digitally stacked which is useful to study the biological structure (Boguslavsky, 2003). The distance of objective, the thickness of the sample and penetration of fluorochrome in the sample determines the depth of field (Wright & Wright, 2002). The entirety of sample can be analyzed from Z stacks obtained through confocal microscopy. It is also possible to calculate what it will look like if sliced at any position.

2 Materials and Methods

2.1 Materials

2.1.1 Fish skin sample

The samples used in this experiment were from Åkerblå As, Trondheim. The sampling date was 20.12.2016 (week 51). Samples from sea cage 6 and sea cage 8 were taken for this project. Cage 6 fish were treated with freshwater in week 44/45 and cage 8 fish were left untreated. However, both cages fish were treated with slice in week 30-31. All the samples were kept in freezer at all the times before use. The sample thickness was more than 1 cm.

Figure 2.1: Fish skin samples from the freezer are placed on cutting board on lab bench for thawing and sample preparation.

The frozen fish skin samples taken from the freezer were thawed at room temperature. Thawing was done to remove the ice crystals formed during storage and some cellular debris collected on the skin surface during post mortem as seen in above figure will. Also thawing makes easier to dissect the sample without getting damage of scales and mucus.

2.1.1 Nanoparticles

Yellow green Carboxylate-Modified microsphere (FluoSphere®) of size 0.2 µm and 2% solids from Invitrogen were used in the experiment. These are made by grafting polymers containing carboxylic acid group to sulfate microspheres resulting in highly charged, hydrophilic and moderately porous surface layer microspheres. Since the external layer in this modification process is only a few Angstrom (Å) thick, it does not change the size of seed particles significantly. Because of high charge that reduce their attraction to cells, Carboxylate-modified microspheres are superior for applications in biological systems. They can also be covalently coupled to proteins, nucleic acids and other biological molecules. FluoSphere were stored in refrigerator prior to use.

2.1.2 Microdishes

The µ-Dish$^{35\ mm,\ high}$ from Ibidi® were used for the experiment. These microdishes have high optical quality of material like that of glass to perform fluorescence experiments with uncompromised resolution and choice of wavelength. It has the bottom thickness of 180µm (+10/-5 µm).

Figure 2.2: A 35 mm imaging dish from Ibidi®

2.2 Methods

2.2.1 Preparation of fish skin sample

The frozen fish skin samples as shown in figure 2.1 obtained from Åkerblå As, that were stored in the freezer were thawed to room temperature. A scalpel of size 24 from Swann-morton was taken and longitudinal skin samples (approx. 1.5cm ×1.5 cm) were dissected carefully without damaging the mucus layer. About 20 µl of 0.02% 0.2µm prepared fluospheres was placed on the top of skin with a pipette. After about 5 minutes, dissected samples were placed skin side down in labelled microdishes for microscopy.

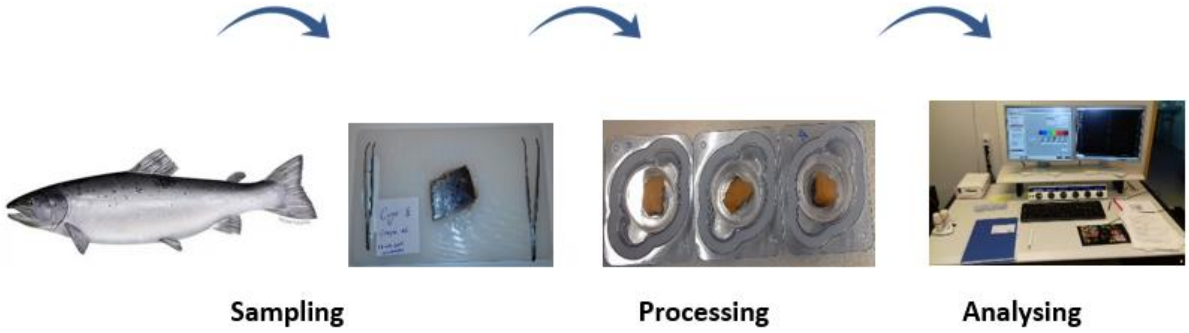

Figure 2.3: General work flow from sampling fish skin to microscopic analysis.

The work flow can simply be divided into three parts namely sampling, processing and analysing. As stated earlier sampling, was done by Åkerblå As, from where we obtained two different groups of salmon skin samples: Untreated samples and Fresh water treated samples. These samples were of size 4cm×5cm approximately and thickness of more than 1cm. The samples were then processed carefully by dissecting them into appropriate pieces and placing prepared Carboxylate modified fluospheres as described above and taken to the Confocal laser scanning microscopy lab for observation. Z-stack of the samples were captured and stored. Further analysis of captured Z-stacks was performed by using a software Leica Application Suite X (LAS X) from where z distance between nanoparticles to scales were obtained.

2.2.2 Preparation of fluospheres

0.02% of 0.02µm Carboxylate modified fluospheres were prepared by vortexing 10 µl of FluoSpheres® from Invitrogen in 990 µl of sea water. Prepared Fluospheres were stored in refrigerator prior to use.

2.2.3 Microscopy

All the microscopic assessment of prepared samples was carried out using confocal laser scanning microscope (Leica TCS SP5) from Leica Microsystems. The system provides the full range of scan speeds at highest resolution. The specimen can be imaged visually or using lasers to create a 3D image (optical sectioning). Since lasers provide single wavelength light and very bright light, they are used in confocal microscopy. The laser line chosen in our experiment was Argon accordingly to the excitation properties of the dye used together with 63.0×1.20 water UV objective. Argon ion lasers have trend for multi-line operation with concurrent output at various wavelength. The laser line of wavelengths 458nm and 496nm in the visible light spectral regions were utilized and were set at strength of 10% and 20% respectively. The strength of laser was set to 20% for all the measurements. The parameters for confocal laser microscope setup is given in appendix A.

Improved imaging of thicker sections of wide variety of specimen types is the major application of this microscopy. Individual optical sections at high resolutions in sequence through the specimen can be imaged using this approach. Confocal microscopy can be operated in dual mode namely reflectance mode and fluorescence mode. The reflection of fish scales provided the source of contrast in reflectance mode of confocal laser scanning microscopy. In fluorescence mode of confocal microscopy externally applied fluorescent nanoparticles excites with a laser light sources. Thereafter, the fluorescence signal can be detected. Carboxylate modified fluospheres were placed on the top of salmon skin mucus in this experiment.

Z-stack of the samples were captured by setting the upper and lower limits by marking the locations of the top and bottom of the specimen. In our experiment fish scales were marked as upper limits whereas the point from where nanoparticles start to visualize were marked as lower limits. Z stacks is a series of optical sections collected at different levels perpendicular to the z-axis with in a specimen. These Z stacks are generated by incrementally stepping through a sample using a focal drive. A program in the computer allows to acquire and save

an image, change the focus, acquire and save a second image and so on. The obtained Z series were then further analysed using special software called Leica Application Suite X (LAS X).

2.2.4 Data analysis

Data analysis have been a great part of this thesis. The obtained microscopic images-series (Z series) were analysed by using the software Leica Application Suite X (LAS X) from Leica Microsystem. This software integrates confocal, widefield, stereo, super-resolution, and light sheet instruments from Leica Microsystems. It is a computer three-dimensional reconstruction programme designed specifically for processing confocal images. This software enables to made length, depth and volume measurement in the specimen. The z-steps between the nanoparticles to scales were measured using this software to acquire dataset. The obtained z-steps were multiplied with slice depth (step size) to get nanoparticles to scales excluded distance. Slice depth is the thickness of the section of sample imaged by the microscope that depends upon the objective lens and the diameter of pinhole used. At least 30 different points (measurements) were taken for each sample. The obtained data were further analysed by using Microsoft office programme Excel to calculate the variance among the samples. Two sample t-test and ANOVA were performed to test the difference between the samples. Statistical significance was accepted at $p<0.05$.

3 Method Development

A method is a set of experimental conditions that is designed to generate good analysis of a sample. Method development has become the most important part of this study which has taken few months. The goal of this method was to detect and quantitate the mucus layer in the salmon skin mucus from two different group of samples. What detection technique can be used to analyse the sample and the way to quantify it was the prime challenge. As microscopic assessment of nanoparticles penetration was the detection technique planned, we must be sure about the number of samples we need, size of punch that can fit in the coverslip, type and concentration of nanoparticles and basic training of confocal laser scanning microscopy before starting the experiment. Several experiments were performed taking the extra samples from Ingrid's Lab before starting to work with experimental samples. Basic confocal laser scanning microscopy training was taken with the help of Astrid and Catherine. Two different biopsy punches of 6mm and 3mm diameter from Uni-Core^{TM} designed to eject cored samples from source material were tried to punch the skin samples. 6mm punch was preferred among them because of its larger size and more surface area. The size obtained from these biopsy punches were perfect to get placed in the chambered glass slides for microscopic observation and there was less damage in the sample as well. While observing the prepared samples under confocal laser scanning microscopy, variability in the mucus and scale structure were observed within the same fish samples and among the samples as well. Because of this variability, three punches were taken from each skin sample for analysis purpose to increase the chance that the overall data was representative. From each punch 30 nanoparticles scale distance measurement were made. Since aim was to study the effect salmon lice treatment on barrier properties of salmon skin mucus, we choose two different types of samples (treated and untreated). While taking punches from the original samples, the biopsy punch of both diameter did not work as expected instead scales and mucus layer were damaged. We assumed that was due to thickness of sample and tried to remove the flesh. It also did not work and finally decided to use the microdishes of 35 mm diameter for observation where larger punch dissected with scalpel can be placed without damaging the mucus. This idea worked, and final experiments were conducted.

4 Study challenges

Regardless of the importance of skin mucosa as first line of defence against environmental pathogens, the barrier properties of fish skin mucus are little studied. To study the barrier properties of mucus, choosing the right methodology was the first challenge. Right methodology is always needed in order to move forward in research. Doing a pilot study to test the methodology is extremely important while performing the novel research. We did the same to validate the methodology. The next challenge was to make sense of the data that have been collected because the data obtained need to be connected with the existing research. A body of literature existing on the topic of interest and methodology were lacking or insufficient. So direct comparison of the results was not possible though general conclusions are drawn.

5 Results

5.1 Microscopic assessment of nanobead penetration in fish skin mucus

By studying the nanoparticles placed on the top of salmon skin, the mucus ability to immobilize the given nanoparticles is investigated by using confocal laser scanning microscopy. The chosen objective of confocal microscopy was 63.0×1.20 water UV. The immobilization of nanoparticles in mucus will most likely have different property depending on the quality of mucus. Investigating the variance in immobilization of Carboxylate-modified microsphere of size 0.2µm in two different samples i.e., in untreated samples and freshwater treated samples for sea lice, a comparison can be made regarding mucus thickness in these two different groups of samples.

5.1.1 Scales reflection

Scales are highly refractive (bright) surface that covers the skin of most fishes including salmon. The reflection of fish scales can be visualized in x, y and z planes using confocal reflectance microscopy. While defining the volume of Z stacking in confocal laser scanning microscopy, the Z value for the end of sampling was set up to the scales by seeing the reflection of scales. The begin point was the value where nanoparticles get start to be visualized. Channel 1 was activated for seeing the reflectance image of scales. The fish surface is not uniform. The fish scale displays quasi-periodic pattern comprised of alternative rows of overlapping scales running over the length of fish. The same was seen while Z stack obtained was orthogonally sectioned using a special feature of CLSM and variation in distance from cover slip to scales was observed as shown in figure 5.1.

In the figure shown below the scales can be seen clearly. The difference in distance from coverslip (microdish surface) to scales is indicated by left right yellow arrow. The scales were not observed to be similar for all samples. Some damages in scales was seen that might be due to post mortem damage of skin samples or during sample preparation.

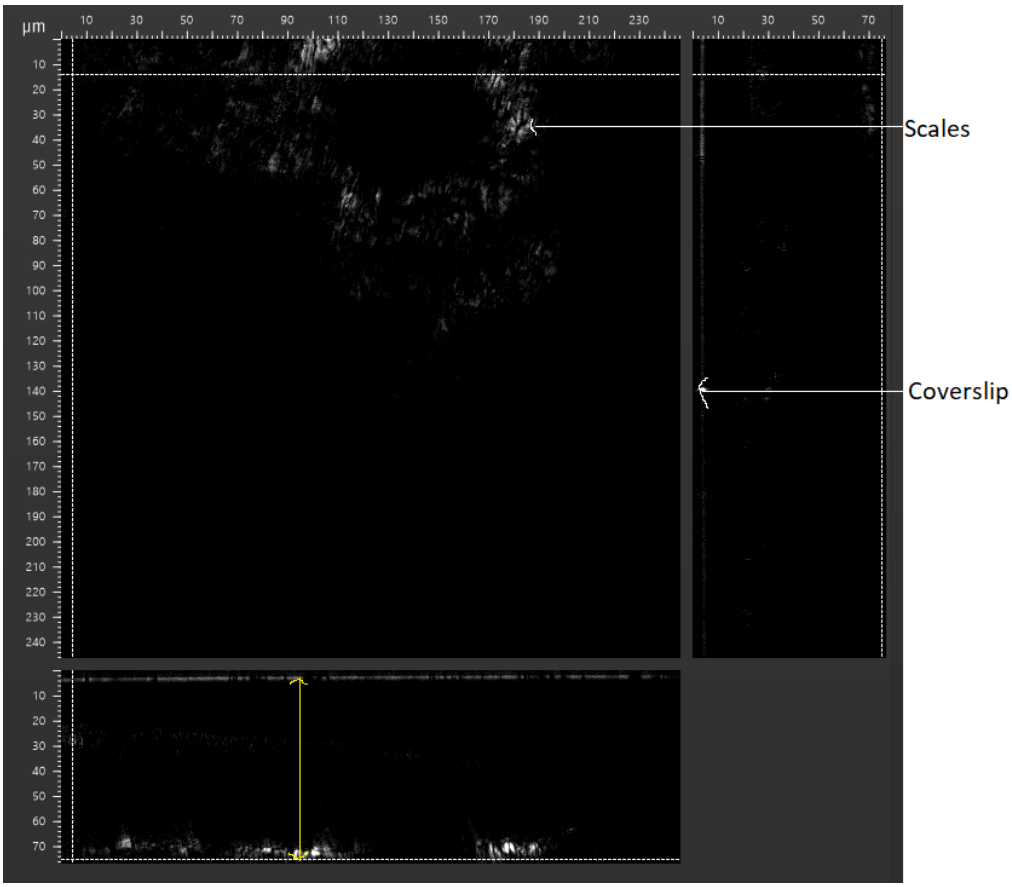

Figure 5.1: Reflectance image of fish scales obtained in confocal laser scanning microscopy. The main panel shows the enface image at the z-depth. The bottom and side panels show the x-z and y-z cross-sectional image respectively. The dashed lines represent orthogonal sections of z-series.

5.1.2 Nanoparticles fluorescence

The distribution of Carboxylate-Modified microspheres in the salmon skin mucus is an important part studying the barrier properties of mucus. Fluorescence nanoparticles that are placed on the top of salmon skin mucus were visualised in x, y and z planes using fluorescence mode of confocal laser scanning microscopy and activating channel 2. This was the basis of our experiment for determining mucus thickness of fish skin sample.

The image in figure 5.2 presents the distribution of Carboxylate modified nanoparticles in x, y and z direction in mucus. There was difference in distribution pattern of nanoparticles in various samples. Aggregates of nanoparticles can be seen along with the single particles in the image. These aggregates of nanoparticles were found in almost all different samples. Since the nanoparticles are charged particles they do not aggregate by themselves. So, they might have interacted with the mucus matrix components that induce cluster formation. The nanoparticles used here was of negatively charged and the mucins that provides the dense

network for mucus are also negatively charged. So, the above statement for cluster formation may not be always true. The nanoparticles of small size have higher surface area and higher number of surface atoms. Such surface atoms do not have complete coordination and each atom has vacant coordination sites. In this case more bonds need to be formed and such bonding formation occurs between adjacent particles that can cause aggregation. Black fields can also be seen in the images which is an expected observation. This might have observed because of the limited amount of nanoparticles added in the sample. At some instance it can also occur due to presence of debris other than mucus or air pockets that might prevent the nanoparticles diffusing in these areas of mucus.

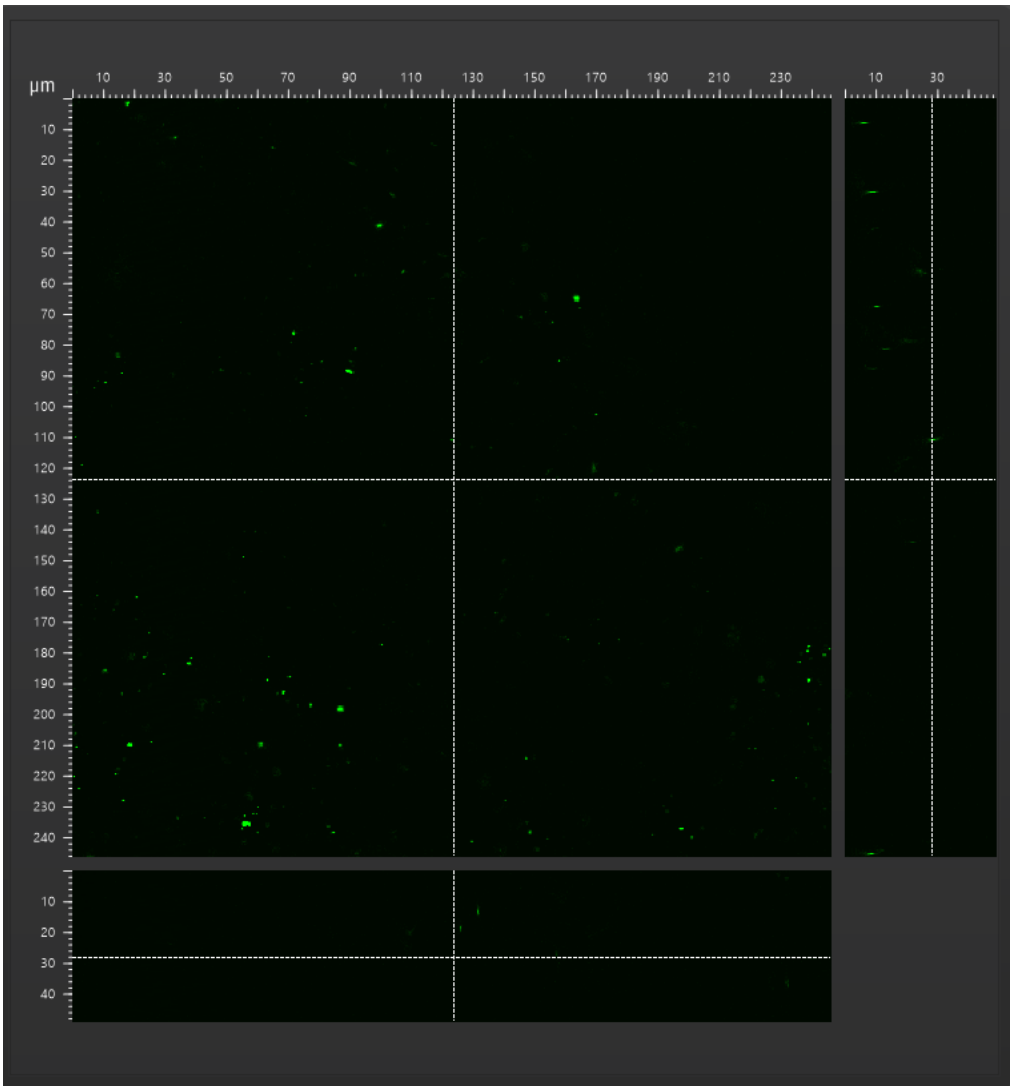

Figure 5.2: Fluorescence image of nanoparticles (both cluster and single particles) in mucus obtained in confocal laser scanning microscopy. The main panel shows the enface image at the z-depth. The bottom and side panels show the x-z and y-z cross-sectional image respectively.

5.1.3 Nanoparticles to scales distance

To measure the nanoparticles to scale distance both channels (channel 1 and channel 2) were activated. This activation enables the visualization of both nanoparticles and scales together so the distance between them could be measured as Z stacks. Z value for begin was set where nanoparticles starts to be visualised or simply after the reflectance signal from micro dishes and end value was set up to the scales. From this obtained Z stacks Z distance were measured using software Leica Application Suite. The Z distance was again multiplied with the step size to get nanoparticles to scale distance. The thickness of step size is determined by the numerical aperture of the objective, the wavelength of light and confocal pinhole size. However, the step size was set while taking images and the value can also be reported by image acquisition software.

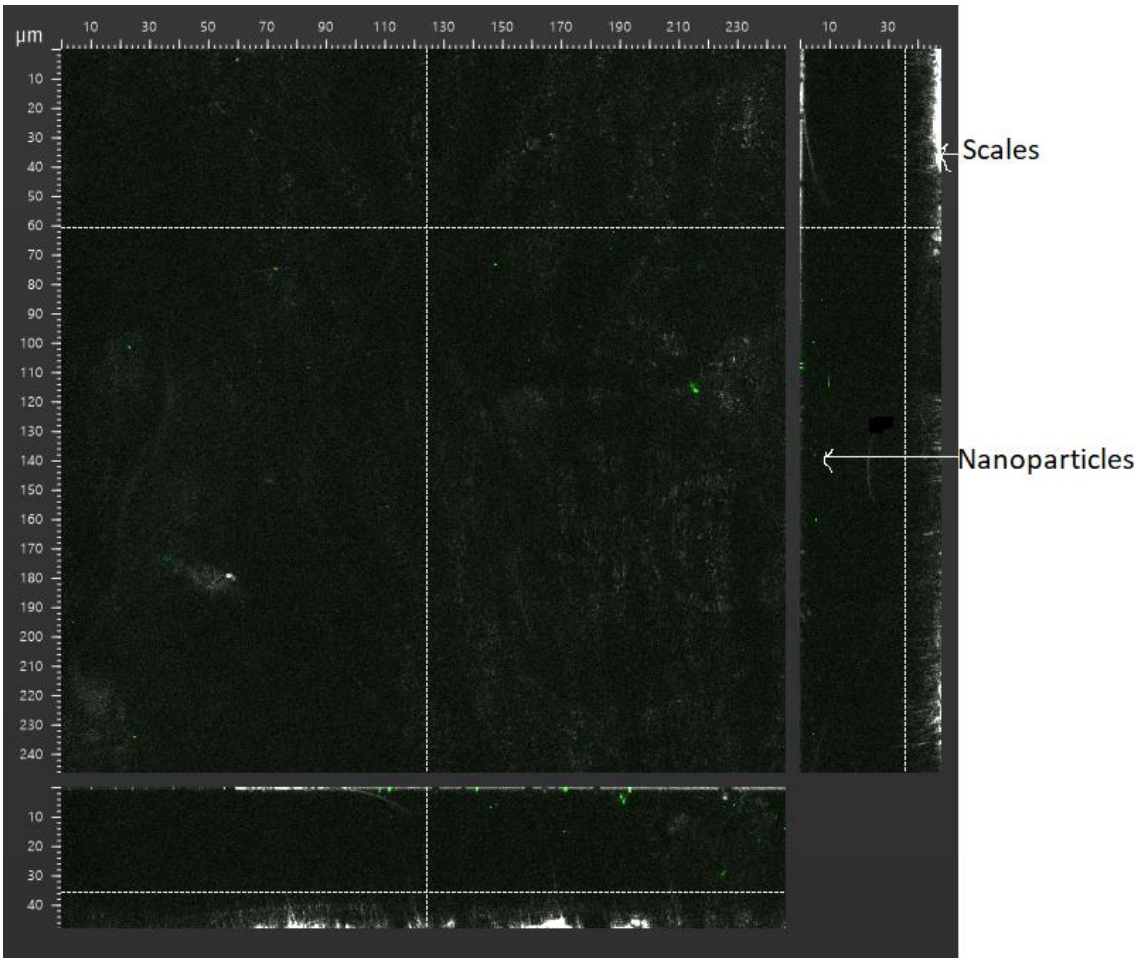

Figure 5.3: Fluorescence of nanoparticles and reflectance image of scales obtained in confocal laser scanning microscopy showing excluded distance between the nanoparticles to scales. The main panel shows the enface image at the z-depth. The bottom and side panels show the x-z and y-z cross-sectional image respectively. The dashed lines represent orthogonal sections of z-series.

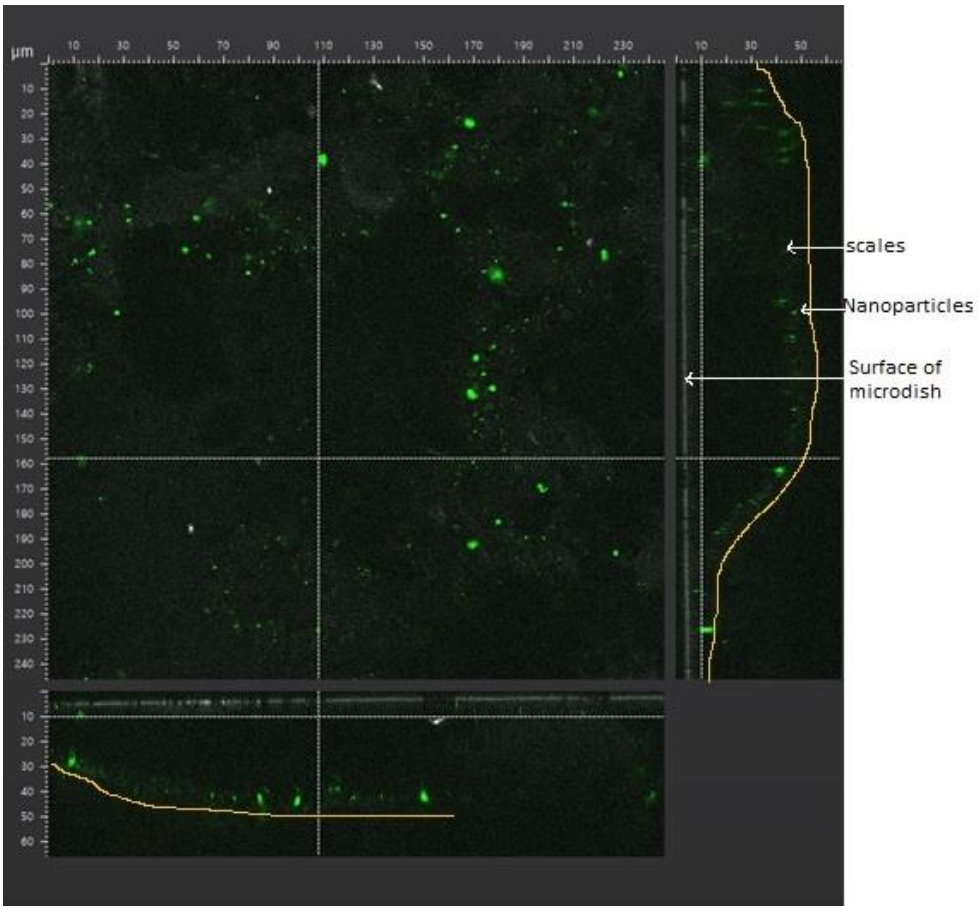

Figure 5.4: Fluorescence and reflectance image obtained in confocal laser scanning microscopy showing some nanoparticles diffusing up to the scales. The main panel shows the enface image at the z-depth. The bottom and side panels show the x-z and y-z cross-sectional image respectively. The dashed lines represent orthogonal sections of z-series.

Figure 5.3 shows there is excluded distance between the nanoparticles and scales whereas in figure 5.4 nanoparticles seems to be aggregated on mucus and diffused up to scales as well. This indicates the mucus barrier was not good enough to immobilize the nanoparticles. Most of the samples showed good separation and few showed no separation of scales. In the above image (figure 5.4) golden line is drawn just beneath the scales to clearly mark the position of scales.

The images presented in above figures are representative selection and might not reveal all the details regarding nanoparticles, scales and nanoparticles to scales distance.

5.2 Variance in the fish sample of the untreated groups

As shown in the images presented above there was considerable variability in the mucus and scale structures with in the same fish skin samples. For analysis purposes 3 punches were taken for each skin sample to increase the chance that the overall data was representative. Then 30 nanoparticles to scale distance measurement were made for each punch. X any Y position of stage was noted using mark and find function so that the same nanoparticles won't get measured again.

For sample 1 the average nanoparticles to scale distance for the individual punches ranged from 14.10µm (punch A) to 28.69µm (punch C). There was large variation within each punch. Standard deviation and range suggest that punch B has more consistent np-scale distance whereas punch C has less consistent np-scale distance. There was a significant difference between punch A, B and C ($p<0.05$).

Table 5.1: The table shows the average nanoparticles to scales distance of three punches of sample 1 with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	14.10	8.01	3.00-31.50
B	27.41	7.87	4.35-42.15
C	28.69	16.24	4.80-66.75
Combined (ABC)	23.40	13.06	3.00-66.75

For sample 2 the average nanoparticles to scale distance for the individual punches ranged from 12.68µm (punch B) to 16.20µm (punch A). In comparison with other samples it seems to have higher evenness in average mean within each punch. Punch A having higher standard deviation than other punches tell that punch A nanoparticle scale distance is more dispersed than punch B and C. The p value ($p=0.011$) indicates significant difference between punch A, B and C.

Table 5.2: The table shows the average nanoparticles to scales distance of three punches of sample 2 with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	16.20	5.02	8.10-29.55
B	12.68	2.85	7.50-19.35
C	14.03	3.7	9.15-26.55
Combined (ABC)	14.30	4.17	7.50-29.55

Similarly, for sample 3 the average nanoparticles to scale distance for the individual punches ranged from 23.55µm (punch A) to 34.50µm (punch C). Punch C has both a larger value for the range and larger standard deviation than other punches, it appears that the variation in nanoparticles scale distance in punch C are wider than those of other punches. There is significant difference between punch A, B and C ($p<0.05$).

Table 5.3: The table shows the average nanoparticles to scales distance of three punches of sample 3 with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	23.55	3.83	17.10-32.25
B	34.25	5.71	25.35-44.40
C	34.50	7.22	19.35-49.50
Combined (ABC)	30.76	7.66	17.10-49.50

For sample 4 the average of nanoparticles to scale distance for the individual punches ranged from 19.97µm (punch B) to 44.24µm (punch A). There seems to be large variation with in each punch. Punch A having higher standard deviation and larger range tells that nanoparticles scale distance are less consistent than punch B and C. The p value ($p<0.05$) indicates significant difference between punch A, B and C.

Table 5.4: The table shows the average nanoparticles to scales distance of three punches of sample 4 with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	44.24	7.33	29.10-56.10
B	19.97	3.17	14.55-26.70
C	26.27	6.30	17.85-44.55
Combined (ABC)	30.16	11.86	14.55-56.10

For each sample the data obtained from the separate punches was statistically significant different that is not unexpected as the multiple punch per sample approach was taken to account for variability in different regions of samples.

Comparing the four samples within the untreated groups, the average nanoparticles to scale distance ranged from 14.30µm (sample 2) to 30.76µm (sample 3).

Table 5.5: Comparison of average nanoparticles to scale distance among four different samples from untreated groups with S.D. and range. 1, 2, 3 and 4 are average of three punches of each samples respectively.

Sample	Mean	S.D.	Range
1	23.40	13.06	3.00-66.75
2	14.30	4.17	7.50-29.55
3	30.76	7.66	17.10-49.50
4	30.16	11.86	14.55-56.10

Samples 1, 3 and 4 all have means with 1 standard deviation of each other whilst sample 2 shows substantially lower nanoparticles to scale distances. Sample 1 has one punch (A) where the mean is within 1 standard deviation of the total mean for sample 2.

The same results from table 5.5 is presented in figure 5.5 below for more precise illustration. Statistical analysis showed sample 3 and 4 were not statistically different from each other but all other pairs were statistically different.

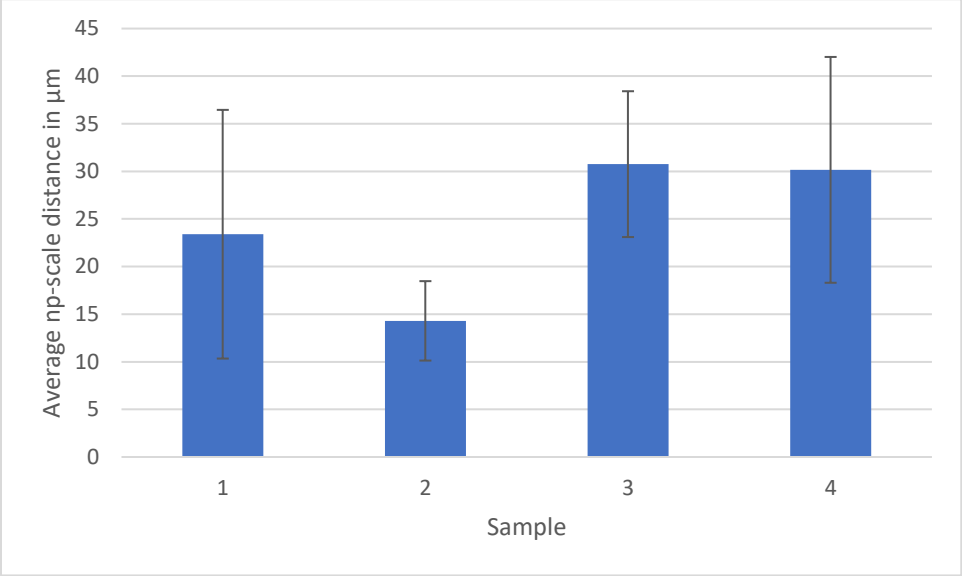

Figure 5.5: Comparison of nanoparticles to scales excluded distance among four different samples from untreated groups. 1, 2, 3 and 4 are the average of three punches of each samples respectively. There is significant difference between all pairs ($p<0.05$) except 3 and 4. The error bars specify standard deviation of the mean.

5.3 Variance in the fish sample of treated groups (Freshwater treated)

To investigate the variance in the fish sample of treated groups four samples were taken as in that of untreated groups and named as sample I, sample II, sample III and sample IV. For sample I the average nanoparticles to scale distance for the individual punches ranged from 18.83µm (punch A) to 25µm (punch B). There seems to be variation within each punch. Punch C having both larger value for standard deviation and the range, appears that the variation in nanoparticles scale distance at punch C are wider than those of punch A and B. There is a significant difference between punch A, B and C ($p=0.0001$).

Table 5.6: The table shows the average nanoparticles to scales distance of three punches of sample I with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	18.83	4.32	12.30-31.50
B	25.00	4.96	18.30-35.85
C	20.74	5.77	9.90-32.40
Combined (ABC)	21.52	5.63	9.90-35.85

Comparing the averages of nanoparticles to scale distance for the individual punches for sample II, it ranged from 18.36µm (punch A) to 60.85 µm (punch C). There was very large variation within each punch. Punch A having higher standard deviation and larger range, the variation in nanoparticles scale distance at punch A are wider than those of punch B and C. The p value ($p<0.05$) also indicates significant difference between punch A, B and C.

Table 5.7: The table shows the average nanoparticles to scales distance of three punches of sample II with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	18.36	6.07	9.00-27.75
B	32.24	4.55	22.80-42.90
C	60.85	4.73	43.80-67.95
Combined (ABC)	37.15	18.5	9.00-67.95

Similarly, for sample III the average nanoparticles to scale distance for the individual punches ranged from 23.86µm (punch A) to 62.27µm (punch B). There was also very large variation within each punch. Punch C having higher standard deviation and large range tells that punch C nanoparticles scale distance is more dispersed than that of punch A and B nanoparticles scale distance. There is significant difference between punch A, B and C.

Table 5.8: The table shows the average nanoparticles to scales distance of three punches of sample III with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	23.86	3.75	16.65-31.35
B	62.27	7.54	47.40-74.25
C	49.61	6.21	31.95-65.70
Combined (ABC)	45.24	17.14	16.65-74.25

For sample IV, the average nanoparticles to scales distance for the individual punches ranged from 35.73µm (punch B) to 77.19µm (punch A). The large variation within each punch remained to be continued for this as well. The standard deviation and range both suggest that punch B and C have more consistent nanoparticles scale distance than punch A. There is a significant difference between punch A, B and C ($p<0.05$)

Table 5.9: The table shows the average nanoparticles to scales distance of three punches of sample IV with S.D. and range. A, B and C are average of 30 measurements each and ABC is the average of A, B and C consisting 90 measurements.

Punch	Mean	S.D.	Range
A	77.19	7.25	65.25-90.45
B	35.73	5.95	20.55-48.45
C	42.63	5.96	31.80-56.70
Combined (ABC)	51.85	19.31	20.55-90.45

The data obtained from the separate punches for each sample from treated group was significantly different that is expected since the multiple punch per sample approach like that of untreated group was taken to account for variability in different regions of samples from treated groups.

Comparing four samples within treated groups the average nanoparticles to scales distance for individual samples ranged from 21.52µm (sample I) to 51.85µm (sample IV). There was also large variation within the samples from freshwater treated groups.

Table 5.10: Comparison of average nanoparticles to scale distance among four different samples from freshwater treated groups with S.D. and range. I, II, III and IV are average of three punches of each samples respectively.

Sample	Mean	S.D.	Range
I	21.52	5.63	9.90-35.85
II	37.15	18.5	9.00-67.95
III	45.25	17.14	16.65-74.25
IV	51.85	19.31	20.55-90.45

Samples II, III and IV all have means within 1 standard deviation of each other while sample I shows significantly lower nanoparticles to scale distance. Sample I have 2 punches (punch A and C) and sample II has 1 punch (punch A) where the mean is within 1 standard deviation of the total mean for sample I.

Again, the results obtained from table 5.10 are presented graphically in figure 5.6 for a more precise illustration. Statistical analysis showed all pairs were significantly different from each other.

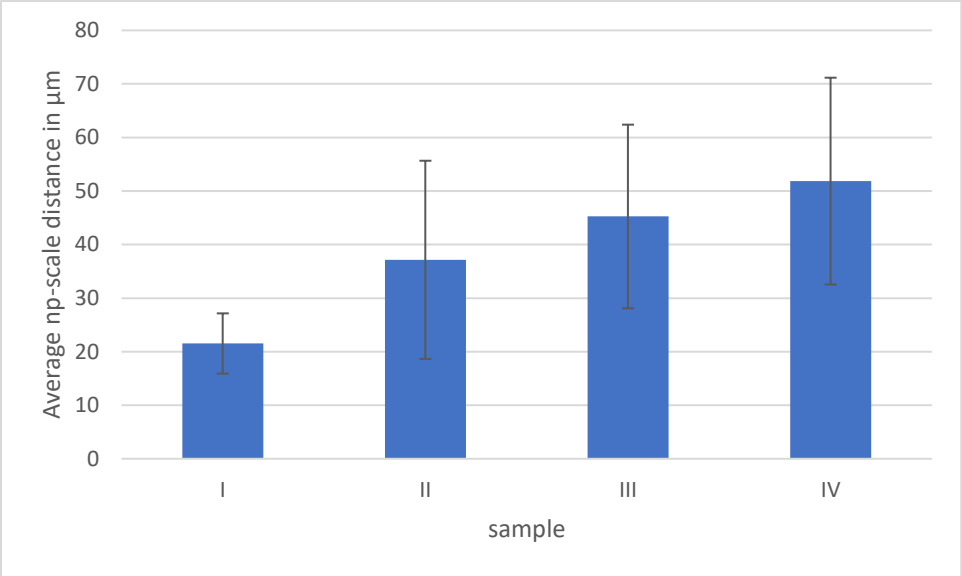

Figure 5.6: Comparison of nanoparticles to scale excluded distance among four different samples from treated groups. I, II, III and IV are the average of three punches of each samples respectively. There is significant difference between all pairs ($p<0.05$). The error bars specify standard deviation of the mean.

5.4 Variance in fish samples between treated and untreated groups

The average nanoparticles to scales distance in untreated samples is lower (24.65µm) than that of freshwater treated samples (38.94µm). Freshwater treated group has a large value for standard deviation (19.66) and large range (9.00-90.45) than that of untreated group which has standard deviation of 11.84 and range 3.0-66.75. It appears that the variation in nanoparticle scales distance at freshwater treated groups are wider than those of untreated groups. The average nanoparticles to scale distance shows significant difference between untreated and freshwater treated samples. From the graph below in figure 5.7 it can also be clearly observed that the mean of freshwater treated group is higher than mean plus standard deviation of untreated group.

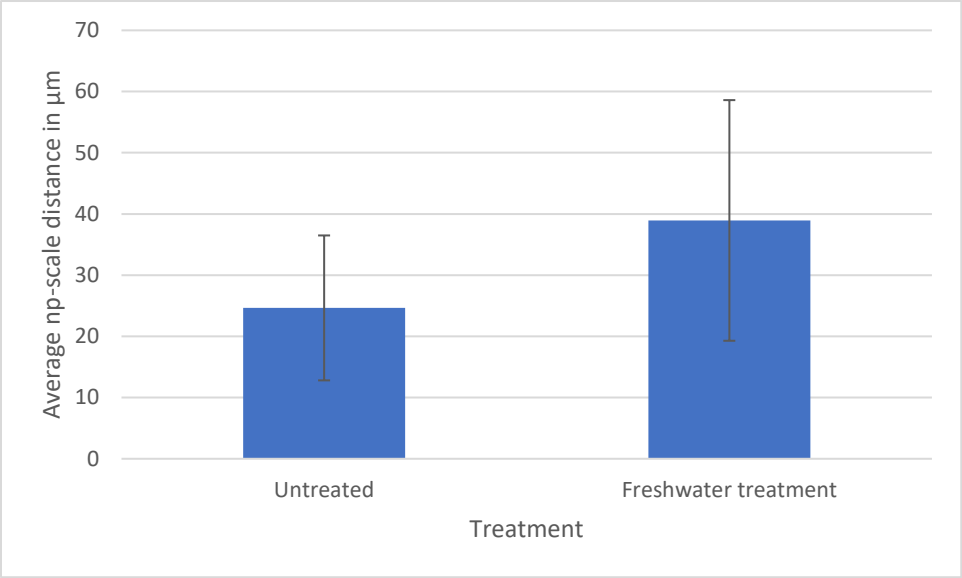

Figure 5.7: Comparison of average nanoparticles to scales distance of Atlantic salmon between two different treatment groups. There is significant difference between untreated and freshwater treated groups (p=0.0001). The error bar indicates standard deviation of the mean.

6 Discussion

This study set out to improve the knowledge about the barrier properties of Atlantic salmon skin mucus alteration due to lice treatment, one of the novel study to do so. It investigated the effect of salmon lice treatment on barrier properties of salmon skin mucus in two different group of samples: untreated and freshwater treated. The barrier property is studied by using fluorescent nanoparticles that are placed on the skin mucosal surface of salmon. These nanoparticles will be trapped in mucus or can diffuse up to the scales depending on the barrier properties of mucus.

6.1 Visualization of scales, nanoparticles and nanoparticles scale distance

The reflecting surface of fish scales can be visualized in x, y and z planes with the help of confocal laser scanning reflectance microscopy. Similarly, the carboxylate modified yellow green fluorescent nanoparticles that are used in our experiment can also be visualized in x, y and z planes with the help of fluorescent mode of confocal laser scanning microscopy. This was the basis to carry out this experiment where the average of nanoparticles to scale distance were measured and comparison of mucus thickness was done among treated and untreated group of samples. Through the use of fluorescent probes, the behaviour like interaction with the biological system, depth of penetration can be evaluated (Pygall, Whetstone, Timmins, & Melia, 2007). Similarly, the reflecting scales helped us in guiding where we are and to determine the end point (boundaries) of Z stacks as well. Z stack combines multiple images taken at different focal distance to provide a composite image with a greater depth of field (Larson & Banks, 2014). By the combination of these two modes (reflectance and fluorescence), a three-dimensional information from the salmon skin mucus can be viewed as a simple image by confocal laser scanning microscopy as shown in figure 5.3. A three-dimensional data was obtained by acquisition of several optical sections (x-y plane) taken at successive focal plane along the z axis. The depth information from salmon skin mucus was obtained by acquisition of x-z section. The images were obtained by scanning the specimen with one or more focus of beam of light. These images are called the optical sections. Optical sectioning is the method of collecting images noninvasively using light to section the sample instead of performing mechanical sections (Paddock, 2000). The reflectance signal of scales can be seen in figure 5.1 which were strong enough in almost all samples. As described earlier

the reflectance mode, an important feature of confocal microscopy is based on observation of own internal contrast by refractive indices of several cellular structure e.g. the fish scales here.

The barrier properties of mucus are due to its dense network of mucin fibres. These fibres contain highly glycosylated negatively charged segments that shows high affinity towards positively charged particles (Lai et al., 2009). Fluorescent Nanoparticles can be visualized and detected under confocal laser scanning microscopy. Distribution pattern of nanoparticles on mucus depends on size of the particles and the mucus mesh. The size of nanoparticles used in this experiment was of 0.2µm. Nanoparticles can form aggregate or distribute uniformly in the mucus. The cohesion between two or more particles results in aggregate structure of nanoparticles. Such aggregation of nanoparticles can also be seen in our study (figure: 5.2) though measurement of nanoparticles to scales distance was taken of single particles. The cluster formation can occur due to interaction of nanoparticles with mucus matrix constituent. As the Carboxylate modified nanoparticles used here are negatively charged and the mucin also possess the same charge there should be repulsion among them. Most likely, a layer of molecules on the surface of nanoparticles might have been created while interacting with mucus components. This layer thus formed might have shielded the repulsive force of nanoparticles and introduce Vander Waals forces, ionic interactions, electrostatic interactions or hydrogen bonds enabling the particles to aggregate (Chen, 2012). Also, the nanoparticles of this size (small) have higher number of surface atom that do not have complete coordination. More bonds need to be formed in this case and such bonding occurs between adjacent particles that can cause particles aggregation (Hilal, 2014).

We have assumed that nanoparticles that are placed on the surface of fish skin mucus will be trapped at the mucus surface if the mucus is of good quality acting as a perfect barrier. In this case the nanoparticles to scale distance will be higher. Similarly, the opposite will be true in case of damaged mucus or poor-quality mucus thus reducing the nanoparticles to scales excluded distance. In most of the measurements and or images obtained mucus seems to have acted as a barrier. However, in the image shown in figure 5.4, the nanoparticles are not immobilized by mucus and are in contact with fish scales. We can say the mucus here is not acting as a perfect barrier.

6.2 Variance in mucus thickness

The mucus thickness for each group of samples were determined, which are the measure of average nanoparticles to scales distance. All measurements were done in series of three for

each sample, and the given values are the average of these three. Very few/or no studies have been conducted related with nanobead penetration in fish skin mucus. Due to the use of different methods in our study, the findings are not directly comparable, but general conclusion can be drawn.

Mucosal immune system protects the body of vertebrates from first encounter of pathogens and have central role in protective immunity (Esteban, 2012). Mucosal barrier is more important in aquatic animals like salmon than those of in terrestrial animals as aquatic species are in constant contact with the microbiota in their environment (Rombout, Yang, & Kiron, 2014). In fish, mucus is considered to be mainly found on the surface of skin, gills and along with the gut lining. The fish skin is a mucosal organ comprising a number of mucus producing goblet cells (Rakers et al., 2010). The structure of goblet cells of fish that produces mucus are similar to that of mammalian goblet cells (Harris & Hunt, 1975). So, response in fish may be similar to mammals. Mucus being the first line of defence in Atlantic salmon, it is very important to study the barrier properties of salmon skin mucus.

A key to understanding the barrier properties of salmon skin mucus is to be able to measure them. In our experiment two samples (sample 2 of untreated group and sample I of treated groups) seems to be outlier in comparison with other samples. Outliers here include the sample minimum, so we can say average nanoparticles to scale distance is lower (thinner mucus) for this two samples. Thinner mucus layer seen in these two samples might be due to post mortem damage of the sample. Mucus may easily get removed during tissue preparation and is difficult to fix. The factors such as season, disease, stress, development stage, handling and environmental conditions influence the cellular makeup of the fish skin. It seems unavoidable that the composition and mucus thickness will also differ. Many species of fish exposed to adverse environmental condition like high level of UV lights (Roberts & Bullock, 1980), acid rain and aluminium ions at low PH (Muniz & Leivestad, 1980) and heavy metals in solution (Eddy & Fraser, 1982) develop thick layer of mucus on gill and non-gill surfaces. Goblet cell multiplication may also be induced by the factors like handling fish (Pickering & Macey, 1977) or change in salinity (P. Laurent, 1984). Infection causes increase in mucus secretion rates leading to development of thick layer of mucus for example in ‘bacterial gill disease’ and ‘cold water disease’ (Richards, 1978). It has been seen that many diseases show visible layers of mucus appearing on skin surfaces of fish (Shephard, 1994). Cone (2009) stated that the thickness of mucus is increased by toxic and irritating substances by

stimulating mucus secretion. So, the mucus thickness can increase in response to damage for example the infection caused by salmon lice or due to chemicals used to treat sea lice.

Variance in average nanoparticles to distance was observed among samples in both untreated and freshwater treated groups. The variance in mucus thickness within the different samples of same treatment group might be due to the samples from different anatomical location. Though no data found relating mucus thickness variability on different anatomical locations in fish skin, the mucus thickness in GI tract of human varies from 50-300µm (Ensign, Cone, & Hanes, 2012) where the thickest layer can be found in stomach and colon. Similarly, a study based on confocal laser scanning microscopy suggest that the airway mucus of human can range in thickness from 5-55µm (Clunes & Boucher, 2007). The thickness of epidermis and mucus can also be influenced by size, conditions, sex, and degree of sexual maturation of fish (Elliott, 2011). A layer of macromolecular gel over epidermal surface of fish referred to as cuticle may reach thickness up to 10µm (M Whitear & Mittal, 1984) (Mary Whitear, 1977). The term cuticle is now generally applied to any distinct layer of material found on fish skin containing integrate products of goblet cells, secretory cells, Malpighian cells and other cellular debris (Shephard, 1994).

The salmon skin mucus thickness in our study was significantly influenced by treatment. There is a marked difference in average nanoparticles to scale distance (mucus thickness) between freshwater treated sample (38.94µm) and untreated ones (24.60µm). The p value ($p=0.0001$) also suggest significant difference between treated and untreated groups. Though there are no comparative estimates available for the correlation between treatment and mucus thickness, it has been reported that the transfer of fish from hyperosmotic seawater to a hypoosmotic freshwater environment at high fish densities, crowding and handling procedure is likely to cause stress in the fish (Powell et al., 2015). Stress can cause change in status of mucus in fish surface, where more mucus is produced by stressed fish than that of unstressed. This claim is supported by the observations that the cortisol produced as stress response in teleost influence mucus release (Marshall, 1979) and catecholamines influence goblet cell development (Ojha & Munshi, 1974). Returning of *Salmo salar* to fresh water to spawn may develop thick layer of mucus on non-gill skin (Oosten, 1957). Opposing water qualities than that of sea lead to the production of thick layer of mucus along with proliferation of mucus cells in fish (Zuchelkowski, Lantz, & Hinton, 1981). A hypothesis is that the mucus that is built on the surface of gills and bodies of stressed fish in freshwater contributes the ability of fish retaining electrolytes (Handy, Eddy, & Romain, 1989). The stress like transfer to dilute

media have an immediate ion depleting effect which is corrected by stress mediated response of the fish (Handy et al., 1989). Surface mucus of fish provides a mechanism to cope with Heavy metal ions that occur naturally in freshwater by increasing mucus thickness (Simkiss, 1984). In contrary increase in temperature and stressors in trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) were found to decrease epidermis thickness whereas the epidermal area comprising mucus cells increased. The decrease of epidermal thickness was suggested to be due to rate of cell shedding surpassing the rate of cell replacement (Jensen et al., 2015).

The study done by Nolan, Hadderingh, Spanings, Jenner, and Wendelaar Bonga (2000) reported that after removal of acute stressors, epidermal parameters will be normalized. This was not true in our case where there is significant difference in mucus thickness between treated and untreated groups though sampling was done after more than a month of treatment date. In our case freshwater may not have act as acute stressors. But we cannot always conclude mucus thickness only as epidermal parameters. However, normalization of parameters after removal of chronic stressors has not been reported yet (Jensen et al., 2015).

7 Conclusion and future work

During this study the effect of lice treatment on salmon skin mucus of two different group of samples (freshwater treated and untreated) have been studied. The current work has demonstrated that salmon lice treatment can cause variance in salmon skin mucus thickness. Experimental results showed that there was considerable variability in mucus thickness with in the same sample, within the same treatment and between treatments. In conclusion mucus layer was significantly thicker in freshwater treated fish skin samples.

Microscopic assessment of nanobead (Carboxylate modified fluospheres) penetration in salmon fish skin mucus have proved to be a valuable tool in defining mucus barrier. Thus, the methodology used in this study have confirmed to be useful in accessing mucus thickness.

To ascertain the results found in this study, future work could include using samples from individuals prior to and after different lice treatment strategies that allow comparison. In addition, rheological methods and fluorescence recovery after photobleaching (FRAP) of fluorescent molecules can be utilised to investigate the mucus barrier properties. Study of the fresh samples could provide more accurate results. Moreover, single size and only one nanoparticle was studied here to access mucus thickness. Using nanoparticles of different types and various size could provide additional idea and information.

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Appendix

Appendix A: Confocal Laser Scanning Microscope Setup

Table A.1: Parameters used for assessment of fluospheres® Carboxylate modified fluospheres (0.2µm 2% solids yellow green fluorescent) with Confocal laser scanning microscope from Leica Microsystems.

Scanner settings parameters	**Value**
Visible shutter	1
Scan Mode	XYZ
Pinhole (m)	111.4
Pinhole (airy)	999.92
Step size	0.15µm
Zoom	1
Scan Direction	1
Sequential mode	0
Frame Accumulation	1
Frame Average	1
Line Average	1
Resolutions	8 bits
Channels	2

Table A.2: Hardware settings for asseement of fluospheres® Carboxylate modified fluospheres (0.2µm 2% solids yellow green fluorescent) with Confocal laser scanning microscope from Leica Microsystems.

AOTF (458)	10.00 %
AOTF (476)	10.00 %
AOTF (488)	0.00 %
AOTF (496)	20.00 %
AOTF (514)	0.00 %
AOTF (561)	0.00 %
AOTF (633)	0.0 %

HyD 2	Inactive
HyD 4	Inactive
PMT 1	Active
PMT 1 (Offs.)	-0.3 %
PMT 1 (HV)	630.0
PMT 1 (HV_Unit)	V
PMT 1 (Preamp)	Direct
PMT 3	Active
PMT 3 (Offs.)	0.0 %
PMT 3 (HV)	724.0
PMT 3 (HV_Unit)	V
PMT 3 (Preamp)	Direct
PMT 5	Inactive
PMT Trans	Inactive
System Number	5100000310
Laser (Argon, visible)	On
Laser (Argon, visible) (Power)	20 %
Laser (DPSS 561, visible)	Off
Laser (HeNe 633, visible)	Off
Scan Field Rotation	0 degrees
Z Scan Actuator (POS)	0.000 µm
Scan Speed	400 Hz
Objective	HCX PL APO CS 63.0x1.20 WATER UV
Numerical aperture (Obj.)	1.2
Refraction index	1.33
DMI6000 Stage Pos x	0.03851735332598
DMI6000 Stage Pos y	0.01690957630323
Emission bandwidth PMT 1: begin - end	451nm - 467nm
Emission bandwidth PMT 3: begin - end	505nm - 540nm