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Characterization of fish skin mucus using mass spectrometry methods

Investigation of biochemical differences in mucus of different fish species and sea lice

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Abstract

The epidermal mucus layer of fish covers the entire exterior, including gills and fins. This barrier plays a crucial role in protecting and maintaining the skin heath, while it also reflects the fish's welfare. The primary aims of this master's thesis were to develop an in-lab methodology for analyzing fish skin mucus using Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) and to differentiate between species based on the protein content levels. It was also aimed to investigate distinctive variations in low molecular weight compounds within the mucus of salmonid species using Liquid Chromatography Mass Spectrometry (LC-MS). Using a purification method involving using zip-tip (C18), a form of SPE (solid-phase extraction) together with 60% acetonitrile (ACN) and 0.1% trifluoracetic (TFA), enhanced spectra on MALDI-TOF MS. In contrast, the methods involving untreated mucus, as well as mucus treated with dichloromethane, and with chloroform, yielded poorer spectra and was therefore not continued with. The superior purification method enabled the analysis and differentiation of Atlantic salmon, Atlantic cod, and coalfish based on the protein content levels in their skin mucus. Salmonid species could also be differentiated, although their varying spectra complicated the distinction of certain specimens. Skin mucus from salmonid species, along with whole lice, was analyzed using Liquid Chromatography Mass Spectrometry (LC-MS). The components of the mucus were depicted in a Principal Component Analysis (PCA) plot, which demonstrated that species tended to cluster more closely with its own type, rather than with others. The PCA-plot did not reveal any significant similarities between the sea lice, sea lice-infected specimens, and the non-infected specimens. The metabolites from the skin mucus of the salmonid species were compared through differential analysis. Although no notable difference between the sea lice-infected specimens and the non-infected specimens were observed in terms of metabolite composition, several other identified metabolites were found – which could in further research be interesting to proceed with. Ultimately, skin mucus remains a relatively unexplored topic, offering exciting opportunities in the future.

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Abbreviations

ACN	Acetonitrile
AS–AQ	Atlantic salmon from aquaculture facility
AS–TARS	Atlantic salmon from Tromsø Aquaculture Research Station
AQ	Aquaculture
BTS	Bacterial Test Standard
FWHM	Full width at half maximum
GC-MS	Gas Chromatography Mass Spectrometry
HCCA	α-Cyano-4-hydroxycinnamic acid
hPa	Hectopascal
HSE	Health, Safety, and Environment
INT.	Intensity
LC-MS	Liquid Chromatography Mass Spectrometry
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
M/Z	Mass-to-charge
PCA	Principal component analysis
PS–ocean	Pink salmon ocean
PS–river	Pink salmon river
PPM	Parts per million
R.INT.	Relative intensity
RPM	Revolutions per minute
S/N	Signal-to-noise
TARS	Tromsø Aquaculture Research Station
TFA	Trifluoroacetic Acid

1 Introduction

1.1 Aquaculture

The world's population is growing at an unprecedented rate, projected to reach approximately 8.5 billion by the year 2030 (1). This rapid population growth has created a critical demand for novel and sustainable sources of food, especially as the consumption of animal protein continues to escalate. In alignment with the United Nations Sustainability Development goal number 14, there is a coordinated effort to enhance the utilization of marine resources, concurrently aiming to curtail overfishing and the sale of illegally caught and unregulated fishing by 2030 (2). Aquaculture emerges as a feasible solution in this context, facilitating the cultivation of fish, shellfish, and algae, thereby addressing several of these objectives. The expanding aquaculture sector offers a viable alternative to reduce dependence on terrestrial meat sources and thus transition towards more sustainable marine-based food sources (3). However, the expansion of this industry requires the adoption of sustainable practices that integrate biological and biotechnological methodologies. Ensuring good animal health and welfare is crucial for the biological success of aquaculture (4), while advancements in aquaculture technology and biotechnology are essential to facilitate the rapid growth of this sector (5). Aquaculture has undergone substantial growth in recent decades (6). Currently, salmonid aquaculture predominates along the Norwegian coast (7), with Norway producing approximately 1.5 million tons of Atlantic salmon (*Salmo salar*, Linnaeus, 1758) in 2022 (8). Nonetheless, the production of Atlantic salmon faces multiple challenges affecting the health and welfare of the fish (9).

1.2 Skin mucus: a protective barrier

Fish health and welfare in Norwegian aquaculture have since the 1990s become increasingly important (10). The industry faces numerous challenges related to parasites, bacteria, and viruses, and operational issues, highlighting the need for frequent monitoring and a robust immune system in the fish (10). The fish immune system can be divided into two main systems: the innate and the adaptive immune system. The innate immune system serves as the first line of defense, activating the adaptive immune system when necessary. Essential elements in the innate immune system are lymphocytes, natural killer cells (NK-cells), and the major histocompatibility (MHC) receptors (11). The adaptive immune system develops

immunological memory through exposure to infectious pathogens, allowing for a more efficient immune response to future infections caused by the same pathogen (11). The primary defense in the fish's skin is the mucus. This mucous layer covers the whole fish, from the anterior to posterior end, the fins, the gastro-intestinal tract, and the gills (12). This protective barrier plays an important part in many of the fish's physiological functions, including osmoregulation, gas exchange, communication, protection, and parental feeding (13). Importantly, mucus contains various host-defense components, such as immunity-related or antimicrobial molecules (14). The presence of these molecules emphasizes their key role as a primary defense mechanism against fish pathogens. Important components of mucus include mucin, various antimicrobial peptides (AMPs) and antibodies (15).

Pathogens have a benefit when the protective mucus layer of fish skin is compromised (16). Previous research highlight the importance of maintaining an intact skin mucus barrier as a defense mechanism (17). Alterations in the protein composition of fish skin mucus have been documented, with these changes ascribed to the negative effects of sea lice infestation (17). Mucus can immobilize and trap pathogens, preventing them from penetrating the fish's skin. Moreover, a healthy fish typically has a continual renewal of the mucus layer, effectively hindering adherences and colonization of microorganisms, as well as infestation by parasites (15).

1.3 Biology and ecology of sea lice

Sea lice, ectoparasitic copepods belonging to the family Caligidae, are prevalent in marine environments around the world and affect both wild and farmed fish populations (18). The parasite is a crustacean and belongs to the subclass of Copepoda (19). The parasites feed on blood, skin, and mucus (18), potentially resulting in stress, damage to skin and mucus, anemia, reduced osmoregulation, and mortality in cases of extensive infestations (20, 21). Species considered problematic in Norwegian aquaculture are *Lepeophtherius salmonis* (Krøyer, 1837), *Caligus elongatus* (Nordmann, 1832), and *Caligus curtus (*Müller, 1785*)* (22, 23). *L. salmonis*, commonly known as salmon lice, is a species-specific parasite only infesting salmonid species (24). In contrast, *Caligus* spp. demonstrates a broader host range, having the capability to infest more diverse species compared to salmon lice (25). *C. elongatus* parasitizes a range of fish species, such as salmonids, herring, cod, and lumpsuckers (26). Meanwhile, *C. curtus*,

commonly known as the cod louse, shares morphological similarities with salmon lice, only it exclusively parasitizes cod species (27).

L. salmonis is endemic to the marine environments in the northern hemisphere (18). The life cycle of the salmon louse includes five phases and ten stages (**Figure 1**), and each phase is separated by molting (26, 28). The life cycle of the lice begins with two naupliar stages (phase 1), during which they behave as plankton and are free-swimming (29), posing no threat to potential hosts (30). Following these initial stages, the lice transition into a sessile stage as copepodite (phase 2), at which point they attach to a host. The third phase involves four stages as an attached chalimus. The last three stages of development are motile, occurring while the lice are on the host, and are divided into two adult stages (phase 4) followed by the mature stage (phase 5) (19, 28).

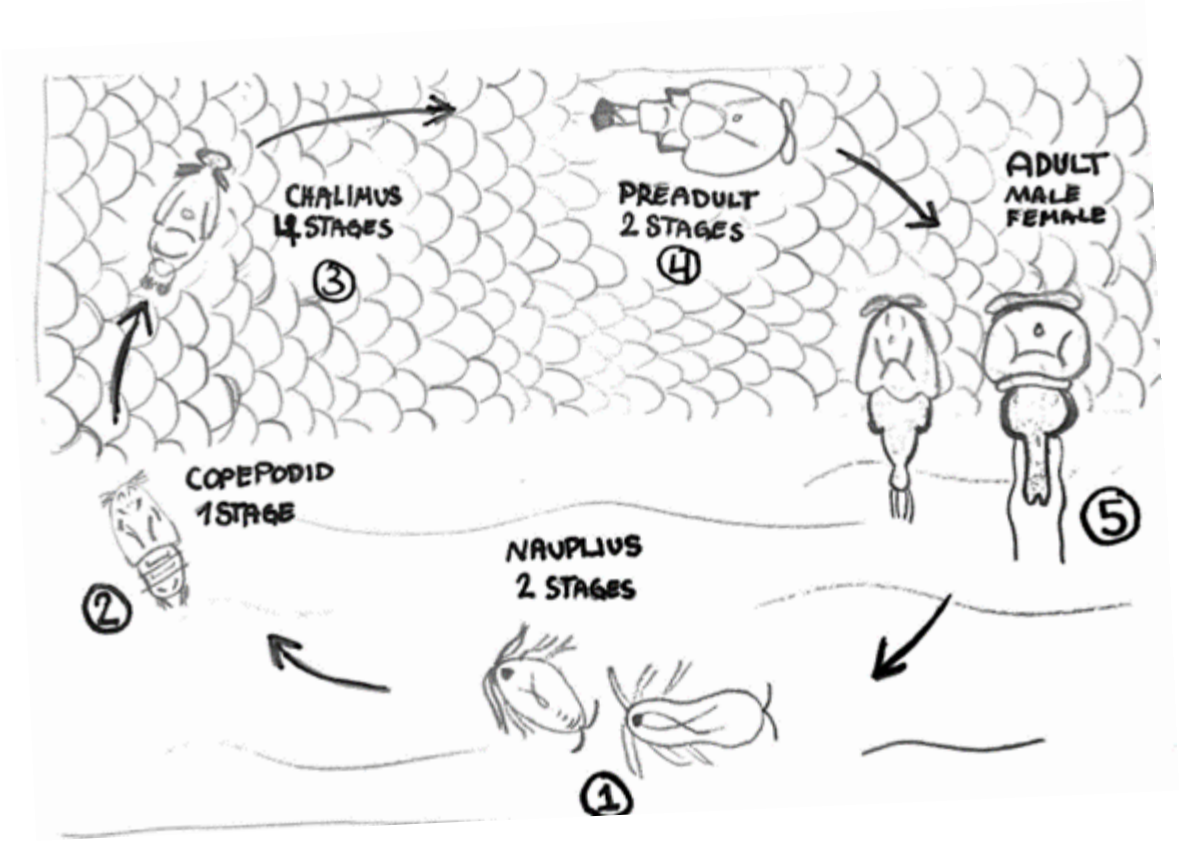

Figure 1. The life cycle of L. salmonis. The five main phases – from free-living Nauplius and Copepodid, to Chalimus stages, and finally the preadult and adult stage. Graphic design: Adele Knutson Dahl, based of Kari Sivertsen, NINA, in Thorstad et al. (31).

As female salmon lice reach maturity, they develop two egg strings, each louse capable of producing from 100 to 1000 eggs (18). These mature females are capable of producing eggs continuously throughout the year, with egg development and hatching able to proceed even at temperatures as low as 2 ˚C (32). The development of the eggs and the survival of the larvae are temperature-dependent, lower temperatures tend to result in reduced viability and survival, whereas higher temperatures enhance both egg development and larval survival rates of the larva (30). In controlled laboratory conditions, the life cycle duration of salmon lice exhibit sexual dimorphism. For females, the progression from egg to full maturity requires approximately 500-degree days, whereas for males, the cycle is approximately ten days shorter (18, 33). Salmon lice inhabit sub-Arctic and temperate areas, including Canada, Irland, Scotland, and Norway. The optimal temperature for salmon lice to complete their life cycle is at 4 ˚C or higher, which correlates with a higher prevalence of lice from around March to late summer in Norway (34).

The infestation of *L. salmonis* can be attributed to a coevolutionary relationship with their primary host, wherein a series of adaptations have led each species to evolve in response to the other (35, 36). The rapid growth of salmonid aquaculture along the Norwegian coast has resulted in an increase of susceptible hosts for the salmon lice, contributing to their proliferation (37). Farmed salmons (unless treated) have a higher abundance of salmon lice compared to their wild counterparts (30, 37). This is likely due to the dense concentrations of hosts within a confined area that enhance the lice's ability to detect and infest hosts, as opposed to the more dispersed wild population (38). Successful host recognition by the salmon lice involves a combination of locating, correctly identifying, and settling on the host. To accomplish these tasks, the lice utilize a diverse array of sensory modalities, including physical, chemical, and mechanical stimuli (39, 40). Physical cues, involving light levels in the water column and reflections from potential host fish, serve as navigation tools for the lice. Chemical cues, primarily olfactory, enhance the lice's ability to detect host odors, which guides them toward potential hosts (38, 41). Copepodites exhibit a 'sink and swim' strategy when recognizing host odors, engaging in burst swimming or 'circle attack' to approach the host (30). Once attached, the lice use antennae on their heads to confirm (mechanical cue) the suitability of the host and to ingest the host's mucus (42). If the host is deemed unsuitable, the lice reject the host's mucus and depart (30). In contrast to the species-specific salmon lice, *C. elongatus*, demonstrates a

broader dietary range, feeding on mucus from various fish species. Furthermore, *C. elongatus* exhibits behavioral changes in response to chemical cues from different hosts (38).

Semiochemicals represent substances released by an organism that elicit specific behaviors in target organisms (43). These chemicals are broadly categorized into allelochemicals and pheromones. Allelochemicals encompass substances that facilitate certain interspecific interactions, exemplified by kairomones (38). A study conducted by Difford et al. (44) suggests that volatile organic compounds (VOCs), particularly kairomones emitted by Atlantic salmon, facilitate host-seeking/locating behavior of salmon lice. However, there is no *in vivo* or any established link between salmon lice infestation in genetic trials and mucosal VOCs (44). It has been documented that kairomones primarily mediate interactions between fish and zooplankton, serving as a signaling channel from the fish to the plankton (transmitter – receiver) (38). For *L. salmonis* copepodites, preadults, and adults, to be able to determine whether it's a host or non-host, identifying kairomones released from the host is fundamental (38, 41). Pheromones facilitate intraspecific interactions, that is, where fish of the same species react to a cue for mutual advantage, which can lead to social aggregation (45).

1.4 Challenges in aquaculture related to salmon lice infestations

Fish in aquaculture face significant welfare challenges due to sea lice (46). Unfortunately, the strategies employed for removing sea lice also pose substantial challenges to fish welfare (47). The aquaculture industry uses various of techniques to eliminate this parasite, including mechanical, chemical, and biological methods. Mechanical methods encompass brushing, water jets (e.g., Hydrolicer, Optilicer), hot baths (e.g., Thermolicer), freshwater treatments, lice skirts, and snorkel cages. Chemical treatments involve chemotherapeutants administrated through baths or fish feed, while biological strategies include the use of cleaner fish, and selective breeding (48). However, these delousing treatments can result in increased stress, scale loss, lesions, and secondary infections in fish (49, 50, 51). Due to environmental concerns and the emergence of resistance among sea lice populations, the use of chemical methods has declined over recent decades (34, 49, 52). Furthermore, biological methods have shown limitations, resulting in poor welfare outcomes for cleaner fish due to predation, disease susceptibility, and harm during delousing operations (48, 53). Other research has also explored vaccine development against sea lice. Boxaspen (34) discusses various strategies, however,

developing a vaccine is challenging due to the molecular complexity of the parasite and brief duration of lice-host immune interactions (34).

1.5 Epidermal mucus

The mucosal layer of fish skin serves multiple essential functions. It not only lubricates the skin but also provides it with chemical and physical protection against pathogens (13). The mucus producing cells secrets a mucus which consists of various macro molecules. When released from the secreting cells, they take up water and expand. This way the mucus is distributed all over the body of the fish (54). The composition and abundance of mucus varies between fish species and where it is secreted from (55). Contributing factors to the variation are both endogenous and exogenous. Whereas the endogenous factors can be secreted lipids, glycoproteins, trefoil factors, sex, and development stage, and exogenous factors can be pH, stress, and infections (56, 57).

In environments with turbid and polluted waters, the mucosal layer has precipitative abilities, where it can clear toxic ions and other suspends from the gills and the skin. The continuing renewal and sloughing of the mucosal layer maintain the skin moist and prevents pathogen infestation (13). Hydration also controls the viscoelasticity of the mucus, which helps avoid invasion (56). Diverse types of mucus-producing cells secrete mucin, which is a family of high molecular weight glycosylated proteins with antimicrobial activities (58). Mucus production on the epithelial layer originates from mucosal cells in the skin, which comprises three primary layers in most fish species: the epidermis, the dermis, and the hypodermis (13). The epidermis is the outermost layer and is exposed to the external environment. It holds the scales, the mucus producing cells, and has an inner layer called *stratum spongiosum*. Within the dermis, blood vessels, pigment cells, nerves, and some adipose tissues are found. This layer is mostly fibrous tissue. The innermost part is called hypodermis, and is composed of adipose tissue (13, 59). The mucus production is carried out by various cell types, including goblet cells, sacciform cells, and club cells (60). When a wound is detected by the host defense, epithelial cells from the surrounding area migrate towards the wound's center. They migrate in groups and maintain contact between each cell, which forms into a mechanical protection from pathogens and other microorganisms (61). The time required for repair varies, ranging from only between a few hours to multiple days, depending on temperature of surrounding water and the severity of the wound (13, 62).

1.6 Skin mucus components and their role in host defense

Skin mucus is composed of glycoproteins, along with salts, lipids, and proteins, such as immunoglobulins and other important immunological factors (63). Major types of proteins found in fish skin mucus are proteases, lectins, lysozymes, and AMPs (57). It is shown that these proteins and peptides play a vital role in the innate immunity of fishes, where they take part in host defense (antibacterial, antiviral, antifungal activities) and wound healing (56).

Proteases have a major catalytic function where they hydrolyze peptide bonds of proteins. In the immune system, proteases are important when it comes to degrading invasive pathogens and harmful proteins or toxins (64, 65). Proteases are categorized into four classes: serine-, cysteine-, aspartic- and metalloproteases. The categorization depends on which chemical factor that is responsible for catalyzation (65). The proteases are also important in activating and supporting other factors in the innate immune system: such as AMP's, immunoglobulins and complement factors (57). In Atlantic salmon and rainbow trout mucus, proteases such as trypsin (serine protease), cathepsin B and L (cysteine proteases), cathepsin D (aspartic protease) and metalloproteases have been identified (64, 66). In the skin mucus of Atlantic salmon, an increase of proteases is seen as a response to infection with salmon lice (16).

Lectins are carbohydrate-binding proteins that work as receptors and are presented at the cell surface where they recognize and bind to polysaccharides located on the surface of microbes (67). When bound, they act as opsonins, where they promote phagocytosis of microbial cell by macrophages (64). Glycoproteins and mucin are heavily glycosylated proteins. These carbohydrates create the viscous consistency of mucus and protect the fish by trapping pathogens and particles (56). Mucins (like albumin) are glycoproteins, and are important for the viscosity of the mucus, to capture pathogens, and protect the epidermal surface (59). Within the goblet cells, the glycoproteins are N- or O- glycosylated (63), before being secreted into the epidermal layer of the skin (13).

There is scarce literature on lipid composition in fish skin mucus and its pivotal role, and needs further studies (63). It is mentioned that lipids in skin mucus have a role contributing to the barrier function, and possibly some antimicrobial properties (66, 68). In skin mucus, both saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty

acids (PUFA) have been observed. Such fatty acids could have a role in the protection against pathogenic microorganisms (57).

1.7 Norwegian coastal fish

Norwegian coastal waters are home to a variety of fish species, each playing a unique role in the marine ecosystems (69). In this master's project, skin mucus from four fish species is studied. Atlantic salmon (*S. salar*), Atlantic cod (*Gadus morhua*, Linnaeus 1758), and coalfish (*Pollachius virens*, Linnaeus 1758) are all commonly found in Norwegian coastal waters (70). Meanwhile, pink salmon (*Oncorhynchus gorbuscha*, Walbaum 1792), also known as 'Humpback salmon', is an introduced species originating from the northern parts of the Pacific Ocean and the Bering Sea (59, 71). The Atlantic salmon and the pink salmon both belong to the family of Salmonidae, both anadromous fish species, meaning they complete their life cycle in both fresh- and saltwater environments (71, 72). The Atlantic cod and the coalfish both belong to the family of Gadidae and are marine fish species (70, 73) .

1.8 MALDI-TOF MS and LC-MS

MALDI-TOF MS is a familiar technique in the analysis of macromolecules with a high molecular weight, and with a common application for the identification of bacteria (74, 75). Employing a laser to target crystalized macromolecules within an appropriate matrix on a MALDI-plate, this method generates ionized compounds. The determination of the molecules mass-to-charge (*m/z*) ratio is achieved by measuring the time used to travel the length of a flight tube, where lighter ions use shorter time traveling the tube than heavier ones (74, 76). The principle behind the TOF analyzer provides spectra very fast with high sensitivity and accuracy which allows determination of molecular formulas of smaller molecules (76, 77). Normally, bacterial samples are compared with known purified strains for the identification of their fingerprint mass spectra (78). With a similar concept, MALDI-TOF MS is also a promising method for the authentication of meat and fish products (79). LC-MS is another MS technique, more suitable for the analysis of compounds of low molecular weight, as well as the purity analysis of different samples (77). The LC-MS analyses aqueous samples through a system based on a High Performance Liquid Chromatography (HPLC) system, connected to a MS system (80). The LC-MS technique is commonly used for therapeutic drug monitoring and toxicology, biochemical screening for genetic disorders, as well as other important areas of biochemistry (77).

1.9 Aims

The main aim of this master's thesis is to develop an in-lab methodology for the analysis of fish skin mucus using MALDI-TOF MS.

The secondary objectives of this master's thesis are as follows:

1. Utilizing MALDI-TOF MS to differentiate the skin mucus protein profile of diverse marine fish species.
2. Apply the same analytical technique to distinguish between two related species within the Salmonidae family.
3. Detect distinctive variations in low molecular weight compounds within the mucus of salmonid species using LC-MS.

2 Material and method

2.1 List of chemicals

All chemicals utilized in this master's thesis were of analytical quality and are presented in **Table 1.**

Table 1. List of chemicals used throughout the whole master's thesis, including chemical name, supplier, purity, and serial number.

Chemical name	**Supplier**	**Purity**	**Serial number**
Acetone	Sigma-Aldrich, Missouri, USA	100%	20066.296
Acetonitrile	VWR International AS, Part of Avantor, Pennsylvania, USA	>99,95%	83642.290
Bruker Bacterial Test Standard	Bruker Daltonics GmbH, Massachusetts, USA	--	6030223023
Benzoak (Benzocain)	ACD Pharmaceuticals AS, Leknes, Norway	200 mg/mL	--
Chloroform	Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany	99,4%	32211
Dichloromethane	VWR International AS, Part of Avantor, Pennsylvania, USA	100%	23366.293
α-Cyano-4-hydroxycinnamic acid (HCCA)	Sigma-Aldrich, Missouri, USA	>99.0%	70990-1G-F
Methanol	VWR International AS, Part of Avantor, Pennsylvania, USA	99,9%	83638.290
Millipore milli-Q water	Millipore, MA, USA		F2NB92820
Trifluoroacetic acid	VWR International AS, Part of Avantor, Pennsylvania, USA	>99,9%	153112E

Zalo	Orkla, Oslo, Norway	--	--

2.2 Fish species utilized in this study

Mucus samples were obtained from Atlantic salmon, Atlantic cod, coalfish, and pink salmon. A total of eight attached whole salmon lice from Atlantic salmon were also sampled, where sex and maturity varied between the individual lice. The fish obtained in this study was collected from different locations and production systems. Atlantic salmon was obtained from 1) Tromsø Aquaculture Research Station **(**TARS) Land-based and Sea-based Research Facility, and 2) from a local aquaculture (AQ) facility in Troms region. Atlantic cod and coalfish were provided by a local fish shop, Eide Handel AS. Pink salmon, wild caught from the ocean and a river in Finnmark, and the Atlantic salmon from TARS was obtained through a collaboration with Nofima AS.

Key parameters, including the date of capture and source of the fish were carefully considered due to the variation among species, with some originating from wild catch and some from aquaculture. Additionally, the capture data was important, as certain samples were obtained during the summer of 2023 and others were obtained in the winter of 2024 (See **Table 2**). The methods of euthanization differed depending on the source of the fish. Atlantic salmon, Atlantic cod, and coalfish from TARS were euthanized with an overdose of Benzocaine, an anesthetic. Fresh caught Atlantic cod and coalfish, obtained from Eide Handel AS, were wild caught by professional fishermen, and as euthanization the fish was bled. Atlantic salmon from a local aquaculture facility were collected *post mortem* due to an undetermined cause, thus eliminating the need for anesthetics. Pink salmon, from the ocean and river, captured by Nofima AS were also wild-caught, and euthanized by bleeding the fish. In contrast, Atlantic salmon obtained from Nofima AS were euthanized with a blow to the skull.

For the rest of this master's thesis, to be able to differentiate between the different Atlantic salmon specimens, all Atlantic salmon originating from outdoors aquaculture will be labeled **AS-AQ**, and those from indoors aquaculture **AS-TARS**. Atlantic salmon from indoor aquaculture (not exposed to sea lice) was included for comparison to Atlantic salmon from outdoor, standard aquaculture where the fish had been exposed to sea lice. To differentiate

between the pink salmon specimens, all pink salmon from the ocean will be labeled **PS–ocean** and the pink salmon from river **PS–river**.

Table 2. Fish groups used in this study, structured into species, number of individuals used in method development, MALDI-TOF MS analysis, LC-MS analysis, and peak lists, time of skin mucus sampling, fish species origin, and habitat when sampling (saltwater/freshwater). MALDI-TOF MS = Matrix Assisted Laser/Desorption Time-Of-Flight Mass-Spectrometry, SW = saltwater, FW = freshwater, AQ = aquaculture, and TARS = Tromsø Aquaculture Research Station.

Species:	**Method development:**	**MALDI-TOF MS**	**LC-MS:**	**Peak lists:**	**Time of sampling:**	**Origin and region/area:**	**Saltwater/ freshwater:**
Atlantic cod	14	7	0	7	24.5.2023 25.9.2023	Wild caught, Troms	SW
Atlantic salmon AQ	21	13	5	13	27.9.2023	Outdoor aquaculture facility, Troms	SW
Atlantic salmon TARS	17	10	4	10	8.11.2023 8.1.2024	Indoor aquaculture research facility, Tromsø Aquaculture Research Station	SW
Coalfish	20	7	0	7	25.9.2023	Wild caught, Troms	SW
Pink salmon ocean	0	5	5	5	7.11.2023	Wild caught, Finnmark	SW
Pink salmon river	0	5	5	5	7.11.2023	Wild caught, Finnmark	FW
Salmon lice	0	5	5	0	27.9.2023	Outdoor aquaculture facility, Troms	SW

2.3 Sample collection

The following methods for sampling mucus was conducted during the first excursion: 1) scraping mucus with a knife or spatula (**Figure 2A**), 2) placing the fish in a plastic bag, and gently massaging the skin without contaminating the sample (**Figure 2B**), and 3) absorbing with paper tissue (81). The method that resulted as the least invasive and leading to minimal contamination, involved scraping with a spatula and collecting with a long spoon (**Figure 2A**). Thus, further sampling was carried out as follows: fish were placed on a plastic mat with their dorsal side facing upwards and their anterior side facing leftwards. Using a Wilton spatula, and a long and thin spoon, mucus was collected by dragging the spatula from the anterior to the posterior end. Mucus accumulating in front of the spatula was collected with the spoon. Mucus was then stored in Falcon tubes (50 mL) on ice. To remove contaminants (like scales), mucus samples were centrifuged (Centrifuge 5804 R, Eppendorf) at 5000 rpm for three minutes at 10 ˚C, before transferring the mucus to another tubes. The samples were then stored at -20 ˚C until analysis.

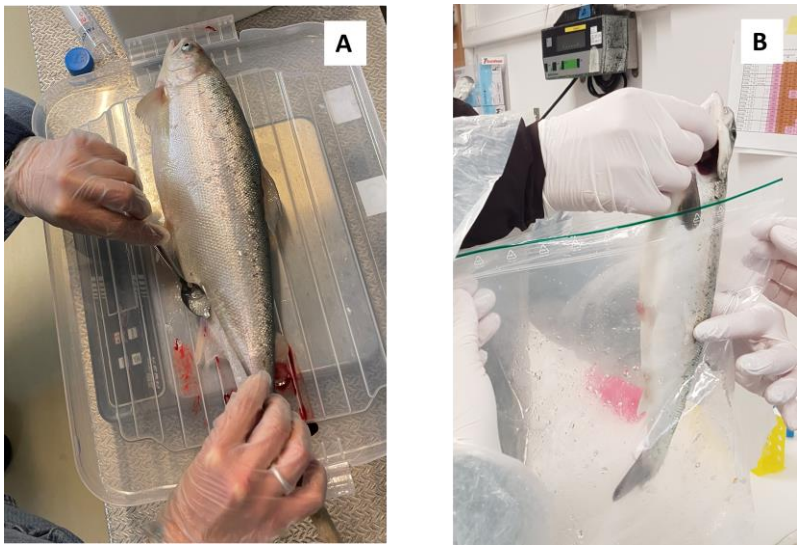

Figure 2. Sampling methods of skin mucus from Atlantic salmon using A) spatula and spoon, B) plastic bag and massaging of skin. Photo: Adele Knutson Dahl.

2.4 Sample preparation for MALDI-TOF MS analysis

Method No. 1

Initially, untreated skin mucus was subjected directly to MALDI-TOF MS analysis. This method was used as a starting point and basis for comparison for further method development.

Methods No.2 and No. 3

A volume of 400 µL methanol was added to each Eppendorf-tube holding 100 µL mucus sample and the tube was vortexed. A volume of 100 µL of dichloromethane (Method No.2) or chloroform (Method No.3) was added and vortexed, before 300 µL milli-Q-water was added and the sample was vortexed one final time. Each sample was centrifuged (Centrifuge 5424 R, Eppendorf) at 12 000 rpm for one minute, at 10 ˚C. When finished, each sample had separated into three layers. The top aqueous layer, containing mostly milli-Q-water and methanol was transferred to a new Eppendorf-tube. The middle layer and bottom organic layer was vortexed and stored at - 20 ˚C until further analysis (82).

Method No. 4

Each mucus sample was added 0.2% trifluoroacetic acid (TFA) until it constituted 50% of the sample. A C18 zip-tip pipette tip (Merck Millipore, MA, USA) was used to extract proteins from the salt-containing mucus. Firstly, the pipette tip (C-18 material) was activated with methanol, and subsequently rinsed in 0.1% TFA, both procedures by pipetting the solution up and down 10 times. The mucus sample was then pipetted 10 times, and on the last hold, 10 µL of 0.1% TFA was pipetted up and down, before extracted proteins were eluted with 10 µL of 60% acetonitrile (ACN) containing 0.1% TFA. The Eppendorf-tube containing the extracted sample was kept in -20 ˚C until further use (79).

2.5 Preparation before MALDI-TOF MS analysis

Cleaning the MALDI-TOF MS plate

The MALDI-TOF MS-plate was properly cleaned before use. Firstly, the plate was rinsed in a small container containing acetone. After two minutes, the plate was thoroughly rinsed with non-abrasive liquid soap (Zalo) and tap water. Soft paper was used to dry off any remaining

water. Subsequently, the plate was rinsed in running deionized water for two minutes and placed in a small plastic container filled with deionized water. The container was then submerged into an ultrasonic bath (filled with tap water) for five minutes to ensure thorough cleaning. After sonicating the plate, it was rinsed again with deionized running water for an additional two minutes. Finally, the plate was quickly rinsed with 90% methanol and left to dry (83).

Preparation of standard solvent solution

A mixture of 500 µL ACN, 475 µL deionized water and 25 µL of TFA was added to a tube (all reagents of MS quality). The 50% ACN solution mixture was vortexed properly (84).

Preparation of matrix solution mixture

The solution was made at a concentration of 10 mg/mL: 200 µL MALDI-grade standard solvent solution mixture was vortexed with 2 mg α-cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonik). The solution was afterwards vortexed again and centrifuged at 12 000 rpm for one minute at room temperature (84). This solution was used in MALDI-TOF MS analysis.

Preparation of Bruker Test Standard (BTS)

The cap at the BTS tube was removed, and the tube was allowed to equilibrate at room temperature for five minutes. Next, 50 µL of the standard solvent mixture was added to the BTS tube pellet. The solution was thoroughly mixed and incubated for five minutes. After incubation, the mixture was mixed again (by pipetting up and down twice) and the cap was securely screwed on. The tube was then centrifuged at 13 000 rpm for 10 seconds at room temperature. Subsequently, the solution was aliquoted in five µL portions into small 1.5 mL Eppendorf tubes (85). The mass range of proteins in the BTS extends from 3638 to 16952 Da (**Table S1**).

2.6 MALDI-TOF MS analysis

Skin mucus samples from AS–AQ, AS–TARS, Atlantic cod, coalfish, PS–ocean and PS–river, were analyzed using MALDI-TOF MS. Each sample was thoroughly vortexed before use. One µL of BTS was added to the first spot on the MALDI-TOF-plate and mixed 10 times with one

μL HCCA, without the pipette head touching the plate. Subsequently, one μL of the sample was added to another spot on the MALDI plate and pipetted up and down inside a droplet with one μL HCCA (10 mg/mL) solution mixture (86). Three technical replicates of each sample were run on MALDI-TOF MS. The samples were analyzed using an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). The program used for analysis was FlexControl – autoflex TOF in linear positive mode, covering a mass range from 2-21 kDa, between *m/z* 2000 to 18000. The sum of each laser shot was obtained using an auto execute mode.

2.7 Analysis of MALDI-TOF MS data

For analysis of MALDI-TOF MS data, the bioinformatics software package Identification Bacteria (IDBac, version 1.1.10), was used to analyze, visualize, and distinguish proteins and other metabolites in the samples. The software presented the diversity that existed within the samples, as well as the similarities of *m/z* peaks within different protein spectra. Peak (protein) lists for all samples/replicates, including their relative intensity in individual spectra, were analyzed manually to identify proteins present in different fish species.

The three replicates of each sample were combined into one replicate and the dendrograms depicting phylogenetic clustering of different mucus samples was generated using IDBac version 1.1.10. Peaks were retained with a *m/z* value between 3000 to 15000, and a signal-to-noise ratio (*s/n*) was set to 2.5 (83).

2.8 Sample preparation for LC-MS analysis

A total of 24 mucus samples from AS–AQ, AS–TARS, PS–ocean, PS–river, and whole body of salmon lice were prepared for LC-MS analysis. All samples were initially stored at -80 ˚C for two hours and subsequently freeze dried (Scanvac CoolSafe) until the vacuum pressure reached below 0.01 hPa. A solution containing 60% ACN and 0.1% TFA was added to the dried samples to achieve a final sample concentration of 10 mg/mL. The samples were then vortexed and placed on a shaker at 4 ˚C for 24 hours. The following day, the samples were centrifuged, and the supernatant was carefully transferred to glass tubes (87). These tubes were then dried in a vacuum centrifuge dryer (Scan Speed 40, Labogene ApS, Denmark).

The dried samples were dissolved in milli-Q water containing 0.05% TFA to a concentration of 10 mg/mL. A solid-phase extraction (SPE) procedure was subsequently performed on each

sample to remove salts and other highly polar compounds. The SPE columns, Sep-Pak C_{18} 1 cc Vac Cartridges, 100 mg (Waters, MA, USA) were connected to a SPE vacuum manifold (Supelco) and were first activated with 0.2 mL of ACN, before being equilibrated with 0.2 mL of 0.05% TFA. The samples were then loaded onto the columns, before washing the columns with 0.3 mL of 0.05% TFA. The retained compounds were eluted with 0.4 mL 80% ACN with 0.05% TFA. Throughout the extraction the flow was adjust to one drop (ca. 20 µL) per second. The SPE eluates were transferred to Eppendorf tubes and dried in a vacuum centrifuge. The dried samples were resuspended in milli-Q water to a concentration of 10 mg/mL (88) and stored at 4 ˚C before analyzed on LC-MS.

2.9 LC-MS analysis

Samples analyzed with LC-MS analysis was conducted by personnel at the Proteomics and Metabolomics Core Facility (PRiME) at UiT. The skin mucus samples were analyzed on a Thermo Scientific Vanquish Horizon UHPLC system connected to a Thermo Scientific Orbitrap ID-X Tribrid Mass Spectrometer (Waltham, MA, USA). The mobile phase consisted of two solvents: A) 5 mM ammonium formate, adjusted to a pH of 3.1, and B) ACN containing 0.1% formic acid. The injection volume was three µL. After injection, the system was equilibrated at 10% B for 0.5 min, continuing with a gradient going from 10-98% B for 16 min, then a washout with 98% B for 2 min before it returned to initial conditions at 10% over 0.5 min. The whole analysis took 18.5 min per sample. The analytical column used was an Aqcuity HSS T3 (150x2.1 mm, 1.8 Å) (Waters, Milford, USA), and the flow rate was 0.35 mL/min. All samples were analyzed in positive electrospray ionization (ESI+) mode with a scan range between 70-800 m/z. The data obtained were analyzed with Compound discoverer 3.3 (Thermo Scientific Inc., Waltham, Massachusetts, USA) (89).

3 Results

3.1 Method development

The main aim of this master's thesis was to develop a purification protocol for fish skin mucus, suitable for subsequent analysis using MALDI-TOF MS. Given the scarcity of established methodologies targeting mucus purification the primary objective was to develop a technique for species identification and examination of mucus protein composition. This section of the master's thesis presents an overview of methods explored and elucidates the rationale behind the selection of the optimal method. The method development section was conducted with three fish species common in Norwegian waters. The identification of the most efficacious purification method laid the foundation for the rest of the results presented in this master's thesis.

Overall, four different methods (named No.1-4) were tested, and the method development process is illustrated by a flow diagram in **Figure 3**. The first strategy for analyzing fish skin mucus involved a direct analysis of untreated mucus samples using MALDI-TOF MS**.** This approach resulted in bad spectra, not viable for data analysis, and method No. 1 was therefore discontinued. The second method (No. 2) was inspired by a study by Freitas et al. (82), which successfully differentiated the geographical origins of *Sparus aurata*, by analyzing their mucus using MALDI-TOF MS (82). In this modified method, organic solvents were used to remove salt and other contaminants from the mucus sample. To avoid using the hazardous solvent chloroform, used by Freitas et al., a mixture of dichloromethane and methanol was used as a substitute to purify the mucus samples. This treatment was followed by centrifugation which resulted in formation of three distinct phases: 1) an aqueous layer at the top containing methanol and water, 2) a precipitated layer in the middle with organic materials, and 3) an organic layer at the bottom containing dichloromethane and extracted lipids (**Figure 4**). However, when each of these layers were analyzed by MALDI-TOF MS, none of them produced usable protein spectra. This was observed as absence of any clear *m/z* peaks over the signal-to-noise threshold (**Figure 5a and 5b**). In method No. 3, the protocol described by Freitas et al. (82) was explored as original described. Here, the samples were extracted with a mixture of chloroform, methanol, and water – and like method No.2, this method also separated the samples into three layers. Each layer was individually analyzed using MALDI-TOF MS. The protein spectra obtained

from this method showed an improvement over those from method No. 2, with less disturbance and higher intensity of the observed peaks (**Figure 5c and 5d).**

However, the quality did not meet the standards necessary for this study, which demanded clear and well-defined mass spectra with *m/z* peaks with high intensity, surpassing the signal-to-noise threshold and with minimal interference. To resolve this problem, the organic, chloroform-containing bottom layer and the middle later were merged and collectively analyzed using MALDI-TOF MS. The top aqueous methanol layer was discarded. This modification was effective, resulting in high-quality spectra, and consequently, purification method No. 3 was initially chosen as method. Observation of spectra obtained from AS–AQ proved to be highly variable between each specimen without many similar peaks.

Due to revised health, safety, and environment (HSE)-requirement at UiT – The Arctic University of Norway in October 2023, which specified mandatory use of specialized gloves when handling chlorinated solvents such as chloroform, all lab work was paused until these gloves were ordered. Because of late shipping and therefore a long pause in the work, this delay led to the development of method No. 4. This method was inspired by research conducted by Bi et al. (79) on fish skin - and refined through knowledge gained from previous laboratory work with various marine materials, performed by the Bioprospecting research group at UiT. In method No. 4, mucus was extracted with a solution of 60% ACN and 0.1% TFA, and using zip-tip (C18), a form of SPE (solid-phase extraction), which subsequently was analyzed using MALDI-TOF MS **(Figure 6)**. The spectra obtained from this approach were superior to those produced by method No. 3, which utilized chloroform. Method No. 4 was therefore selected as the final uniform approach for mucus samples analysis in this study.

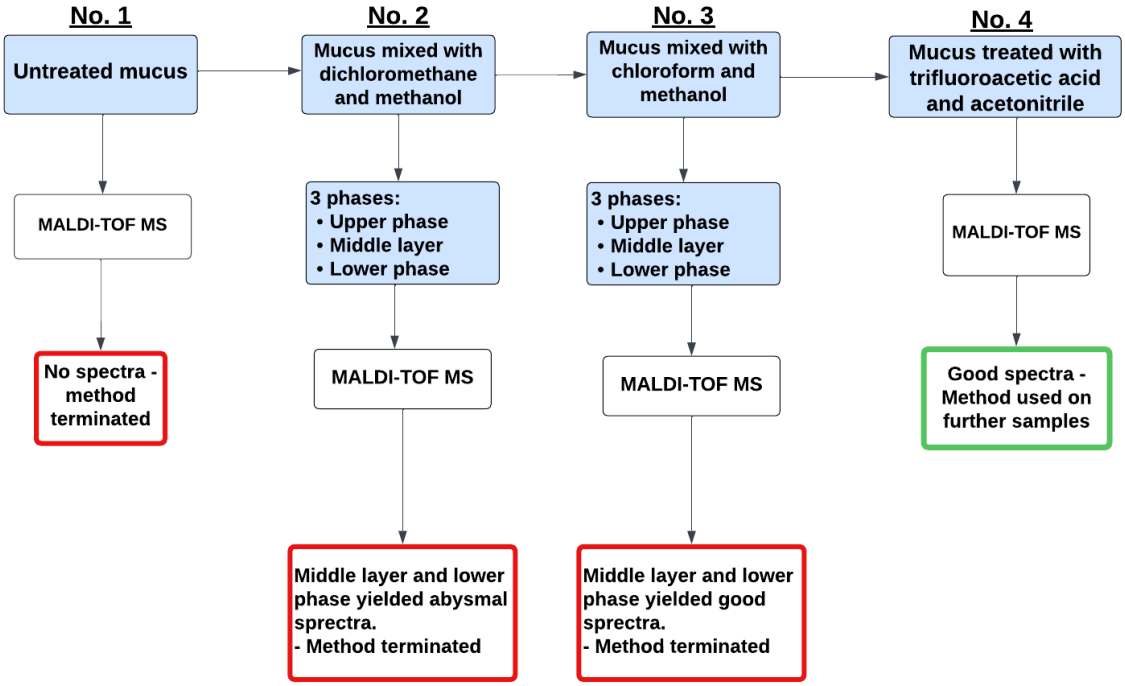

Figure 3. The flow diagram describing the different methods used in the pretreatment of skin mucus samples before MALDI-TOF MS. Method number 1 displayed no treatment of the mucus, in method number 2 and 3 the sample was prepared using a mixture of dichloromethane and methanol, or a mixture of chloroform and methanol, respectively. All these three methods were terminated, while method number 4, which used acetonitrile with trifluoroacetic acid as a pretreatment, was used in further analysis. The flow diagram was created in Lucid (Lucid.co). MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry analysis.

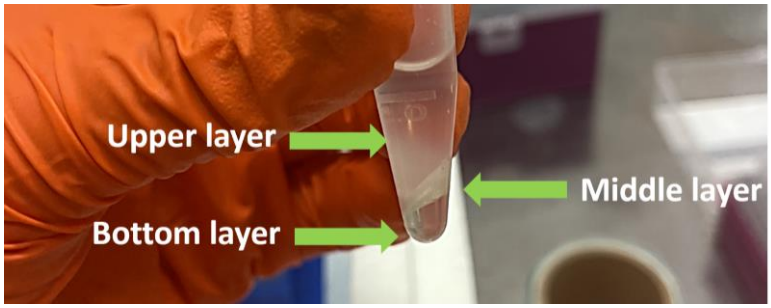

Figure 4. Skin mucus from Atlantic salmon–AQ separated into three distinct phases after purification with a mixture of dichloromethane, methanol, and milli-Q water (method No. 2) and subsequently centrifuged (photo: Adele Knutson Dahl). AQ = aquaculture.

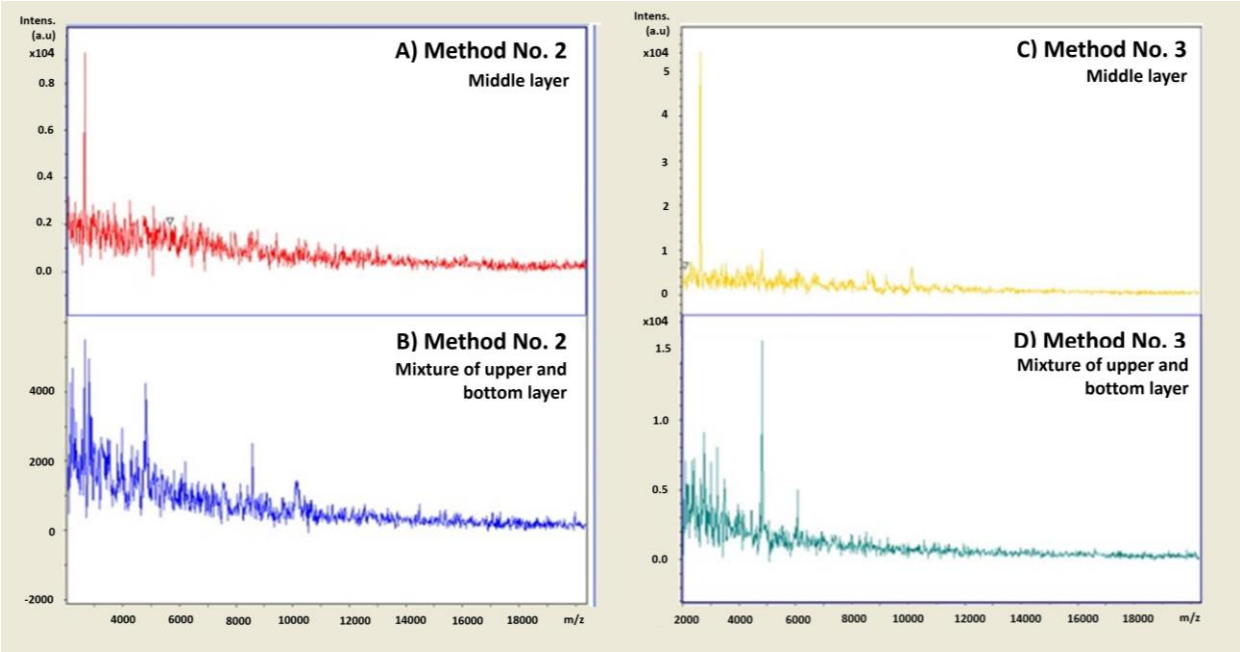

Figure 5. MALDI-TOF MS spectrum of skin mucus from Atlantic salmon–AQ purified with method No.2 and No.3: A) Middle layer (protein precipitation) B) mixture of the upper- (methanol and water) and bottom layer (dichloromethane). C) Middle layer (protein precipitation), D) mixture of the upper- (methanol and water) and bottom layer (chloroform). Photo from analysis program Bruker Daltonics FlexAnalysis. AQ = aquaculture, MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass.

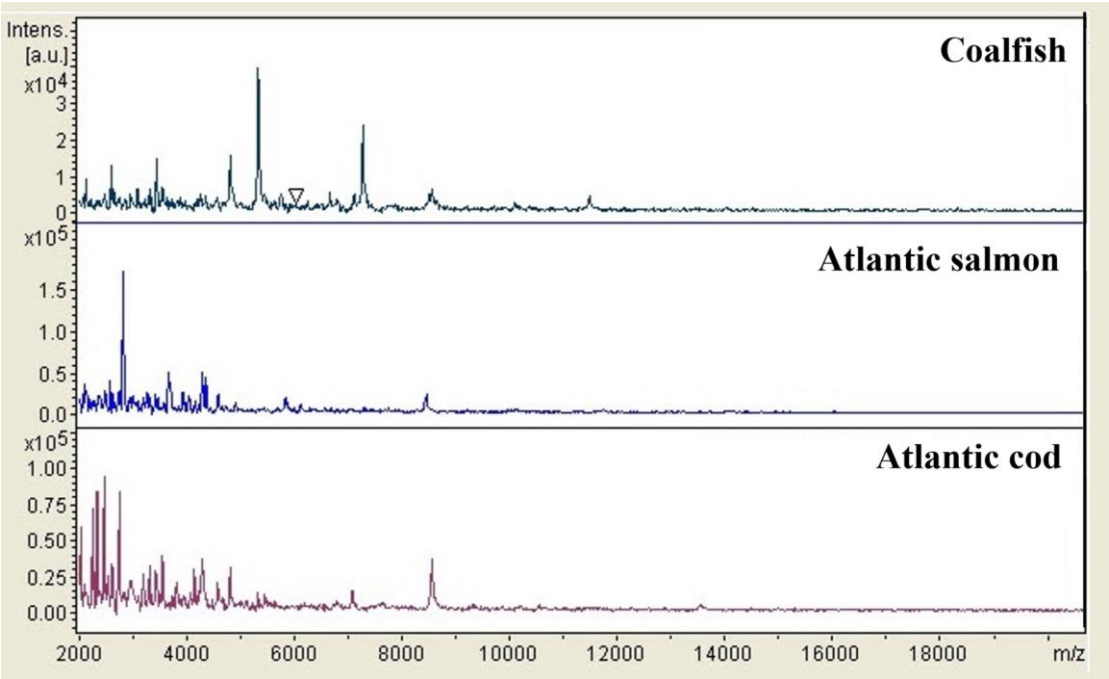

Figure 6. MALDI-TOF MS spectrum of purified fish skin mucus from Atlantic salmon–AQ, Atlantic cod, and coalfish compared using method No. 4. Photo derived from the Bruker Daltonics FlexAnalysis software. MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass. AQ = aquaculture.

3.2 Skin mucus variation

Appearance and texture of skin mucus varied widely between each fish species. AS–AQ exhibited a runny, thin skin mucus that was transparent and difficult to sample, requiring the use of a spoon. The skin mucus was mostly found above the lateral line, around the dorsal fin, and gills. In contrast, the Atlantic cod contained a thick skin mucus with high viscosity, and with coloration resembling the habitat from where it was sampled. Darker shades were observed above the lateral line, while lighter shades were seen around the abdomen and pelvic fins. Coalfish, on the other hand, possessed a thick skin mucus layer that closely resembled a paper layer, with an abundant distribution across the lateral line, dorsal fin, and abdomen. Notably, the coloration of the mucus mirrored that of the fish's skin. PS–ocean exhibited a minimal presence of skin mucus, with their skin almost seeming dry. The skin mucus that was present was primarily located around the gills and above the lateral line and was nearly white in appearance. In contrast, the PS–river had a dense layer of white skin mucus with a sticky consistency, predominantly found around the abdomen and above the lateral line (**Figure 7**).

Figure 7. Skin mucus from abdomen of pink salmon–river (Photo: Adele Knutson Dahl).

3.3 MALDI-TOF MS

Spectra of mucus samples from a set of individual specimens analyzed using MALDI-TOF MS was converted to lists of *m/z*-values (peaks) detected having s/n ≥ 2.5 and *m/z*-values between 3000 and 15000. The *m/z*-lists from every specimen (including three replicates) within each fish group was then combined in separate excel sheets. All *m/z*-values were sorted from smallest to largest value, and peak with similar *m/z*-values manually identified in ≥ 50% of the specimens were presented as "peak lists" for each fish group. Peak lists for AS–AQ, AS–TARS, PS–ocean and PS–river, Atlantic cod, and coalfish are presented in further results. The peak list of AS–AQ (13 specimens) contained 22 proteins raging from *m/z* 4222 to *m/z* 14168, where

two *m/z*-values were consistent in 10 specimens. The seven specimens with the overall best spectra had nine similar *m/z*-values (**Table S2**). The peak list of AS–TARS represented 10 specimens, where only one *m/z*-value (*m/z* 10119) was consistent in all specimens. The seven specimens with best overall spectra had 9 *m/z*-values that were similar (**Table S3**). For PS–ocean, 9 *m/z*-values occurred in all five specimens, while PS–river had only two *m/z*-values reoccurring in four out of five specimens (**Table S4**). The peak list for Atlantic cod represented seven specimens, where two *m/z*-values were identified in all seven specimens (**Table S5**). Of the seven coalfish specimens examined, three *m/z*-values consistent for all (**Table S6**).

3.3.1 Dendrogram of Atlantic salmon, Atlantic cod, and coalfish

The initial analysis of protein spectra derived from fish skin mucus using MALDI-TOF MS was done to determine if species-level phylogenetic differentiation could be performed among fish skin mucus samples. This analysis was conducted using a dendrogram generated on IDBac. The dendrogram comprised of seven selected samples of skin mucus from AS–AQ, Atlantic cod, and coalfish (**Figure 8**) (n=7). The dendrogram showed that AS–AQ split into two main clades, one diverting into Atlantic cod and coalfish, and the other separating the AS–AQ specimens. Among the AS–AQ clades, specimen 116 was separated into a single leaf while the rest of the species clustered more together (**Figure 8**). Within the Atlantic cod clade, two clades separated all samples, 205 branching out to a single leaf, and the rest of the specimens clustering more together. The coalfish was separated in six clades and specimen 175 branched out in a single leaf, separating from the rest of the specimens.

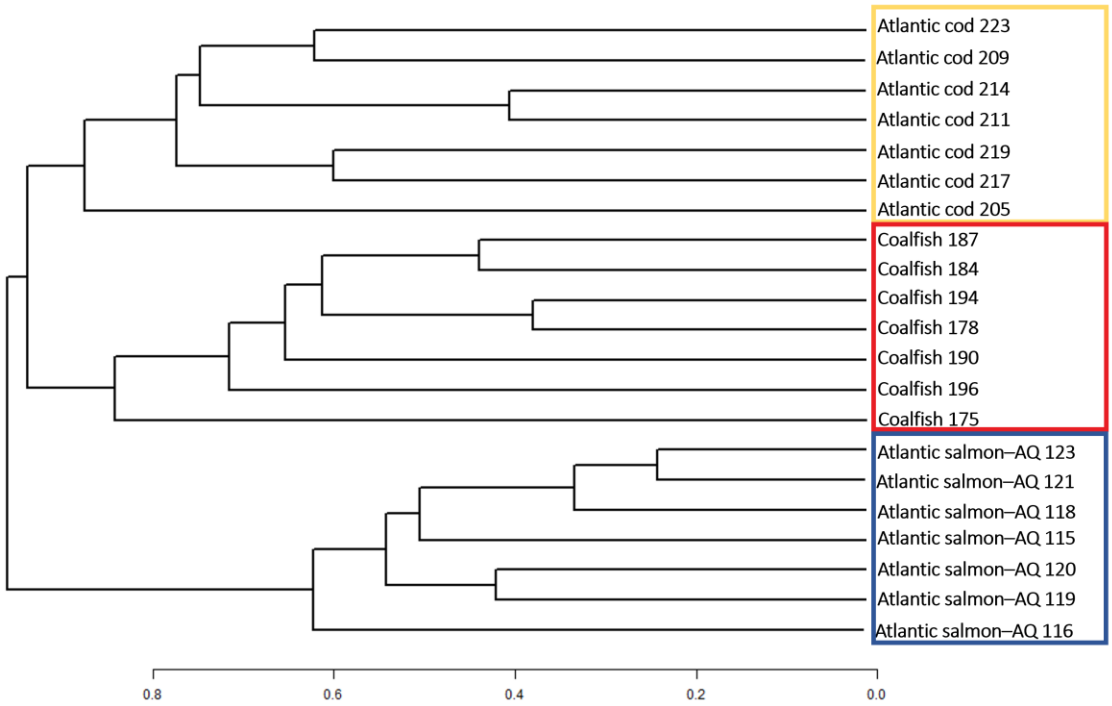

Figure 8. Dendrogram depicts the phylogenetic clustering of skin mucus samples from Atlantic salmon–AQ, Atlantic cod, and coalfish (n=7). Settings used on IDBac: *m/z* range 3000-15000, 2.5 signal-to-noise, 1000 parts-per-million tolerance. Protein spectra were made with three replicates for each sample. Figure created with IDBac version 1.1.10. AQ = aquaculture.

3.3.2 Dendrogram of high-quality spectra of salmonids

Five high quality MALDI-TOF MS spectra of AS–AQ, AS–TARS, PS–ocean, and PS–river (n=5), were analyzed using IDBac. The results, presented in dendrogram (**Figure 9**), showed that all AS–AQ and AS–TARS divided into two distinct clades: one comprising clustering of individuals of AS–AQ and the other from AS–TARS. PS–ocean clustered with specimens from PS–river, whereas only two PS–river specimens, 302 and 303, formed each a distinct single leaf, separate from all other specimens (**Figure 9**).

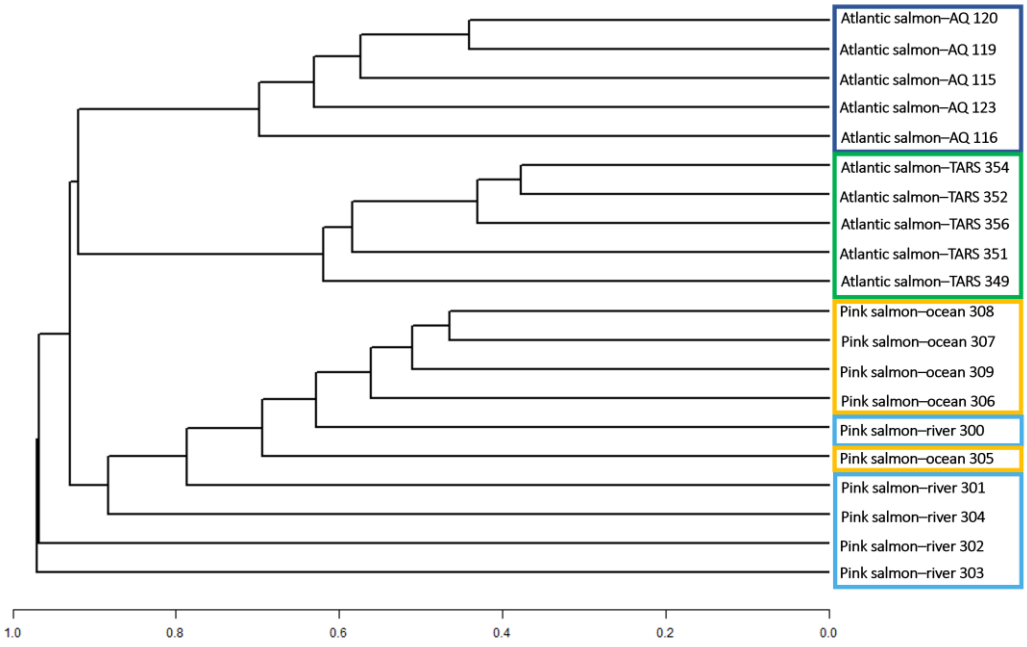

Figure 9. Dendrogram depicts the phylogenetic clustering of high-quality spectra of skin mucus samples from Atlantic salmon–AQ, Atlantic salmon–TARS, and pink salmon–ocean and pink salmon–river (n=5). Settings IDBac: *m/z* range 3000-15000, 2.5 signal-to-noise, 1000 parts-per-million tolerance. Protein spectra were made with three technical replicates for each individual fish. Figure created with IDBac version 1.1.10. AQ = aquaculture, TARS = Tromsø Aquaculture Research Station, IDBac = Identification Bacteria.

3.3.3 Dendrogram of low-quality spectra of salmonid skin mucus

Figure 10 represents five specimens of PS–ocean and PS–river, (as in **Figure 9)**, together with five randomly picked specimens of AS–AQ and AS–TARS, most of them with less *m/z* peaks (suboptimal spectra) compared to specimens in **Figure 9**. The dendrogram showed that one sample of AS–AQ was separated first into a single leaf, away from the rest of the specimens. PS–ocean and PS–river showed in general closer clustering to each other. However, two of the PS–river samples clustered together with two AS–AQ specimens. The AS–TARS species showed more clustering to each other than to the rest of the species (**Figure 10**).

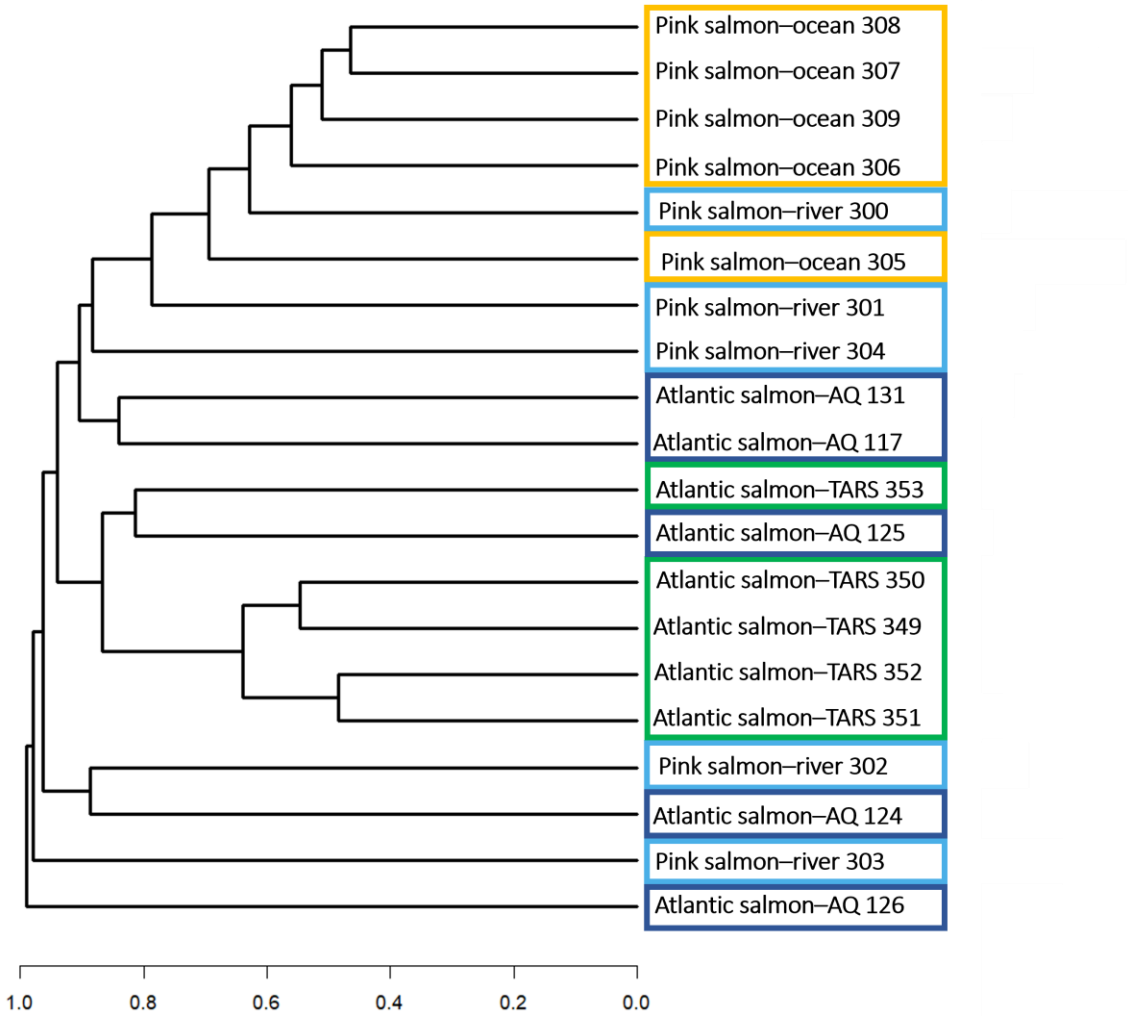

Figure 10. Dendrogram depicts the phylogenetic clustering of low-quality spectra of skin mucus samples from Atlantic salmon–AQ, and Atlantic salmon–TARS, pink salmon–ocean, and pink salmon–river (n=5). Settings IDBac: *m/z* range 3000-15000, 2.5 signal-to-noise, 1000 parts-per-million tolerance. Protein spectra were made with three technical replicates for each individual fish. Created with IDBac version 1.1.10. AQ = aquaculture, TARS = Tromsø Aquaculture Research Station, IDBac = Identification Bacteria.

3.4 LC-MS

3.4.1 PCA-plot of compounds from salmonid skin mucus and salmon lice

The same set of 20 salmonid skin mucus samples that underwent MALDI-TOF MS analysis (as detailed in section 3.3.3, **Figure 10**), were subjected to LC-MS analysis, together with five individuals of salmon lice. The analysis collectively identified a total of 3227 low molecular weight compounds across all specimens. However, one AS–TARS sample was not analyzed due to loss during transport, resulting in only four AS–TARS specimens being included in the LC-MS analysis.

A two-dimensional Principal Component Analysis (PCA) was used to illustrate the clustering and differentiation of the specimens (**Figure 11**). The first dimension (X-axis) represented 19.0% of variance within all the specimens, while the second dimension (Y-axis) represented 13.2% of the variance. The PCA-plot revealed to some extent separate clustering for each species. Specimens of AS–AQ were spread across both the negative and positive section of the PCA- plot. Two of these clustered together on the positive side of the X-axis, while the rest were scattered on the negative side. For the PS–ocean, a clustering between the individuals appeared on both the negative and positive side of the Y-axis. The specimens from PS–river clustered close together on the negative side of both the X- and Y-axis. The AS–TARS had a clustering of three specimens, and one was scattered closer to AS–AQ. The salmon lice specimens were clustered together, even though somewhere closer to AS–TARS (**Figure 11**).

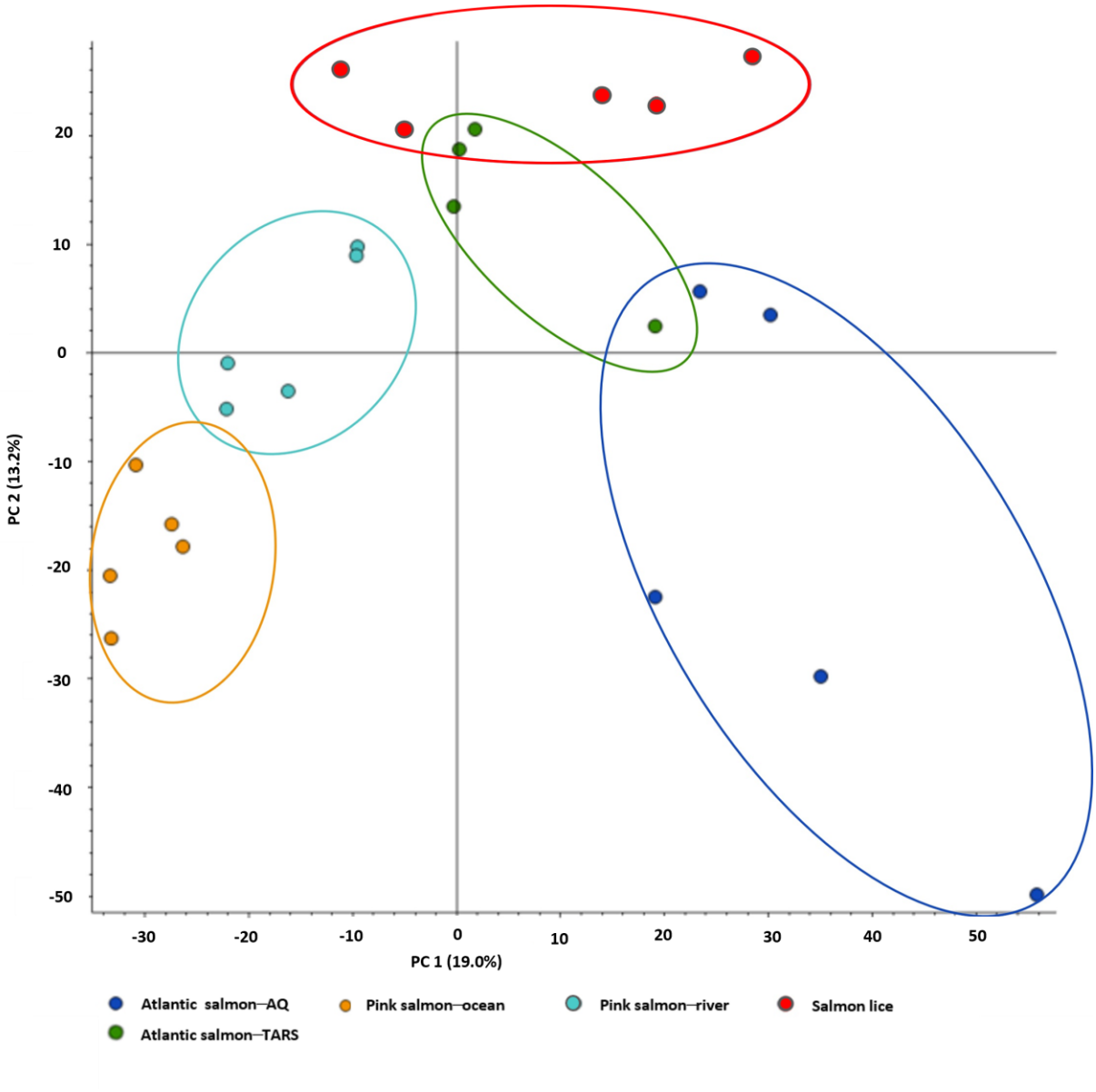

Figure 11. Two-dimensional Principal Component Analysis (PCA) plot of five species Atlantic salmon–AQ, Atlantic salmon–TARS, salmon lice, pink salmon–ocean, and pink salmon–river, each species containing five specimens (n=5), exept Atlantic salmon–TARS containing only four individuals, (n=4). The X-axes represent 19.0% variance off all the samples, while the Y-axis represent 13.2% of the variance of the specimens. PCA-plot created using Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). AQ = aquaculture, TARS = Tromsø Aquaculture Research Station.

The compounds (m/z-values) with highest intensity in salmonids species and salmon lice detected by LC-MS are listed in **Table 3**. Of note, two compounds, Tris(hydroxymethyl)aminomethane and 4-acetamidobutyrate were present in both AS–AQ and AS–TARS, as well as cinnamic acid in both PS–ocean and PS–river.

Table 3. Compounds with highest intensity in salmonids species and salmon lice detected by LC-MS. The analysis was preformed LC-MS/MS mode and software Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was used for compound identification. AQ = Aquaculture, TARS = Tromsø Aquaculture Research Station.

Compound	**Highest in species**	**Formula**	**m/z value**
Valine	Salmon lice	$C_2H_{11}NO_2$	118.08
Tryptophane	Salmon lice	$C_{11}H_{12}N_2O_2$	205.09
H-Gly(Ally)-OH	Salmon lice	$C_5H_9NO_2$	116.07
Guanidinobutyrate	Salmon lice	$C_5H_{11}N_3O_2$	145.98
DL-phenylalanine	Pink salmon–ocean	$C_9H_{11}NO_2$	166.08
Carnitine	Pink salmon–ocean	$C_7H_{15}NO_3$	162.11
DL-Histidine	Pink salmon–river	$C_6H_9N_3O_2$	156.07
Taurine	Pink salmon–river	$C_2H_7NO_3S$	126.02
Hypoxanthine	Pink salmon–river	$C_6H_9N_3O_2$	156.07
Cinnamic acid	Pink salmon–ocean Pink salmon–river	$C_9H_8O_2$	166.08
Hypoxanthine	Atlantic salmon–AQ	$C_6H_9N_3O_2$	156.07
2-aminobutanoic acid	Atlantic salmon–AQ	$C_4H_9NO_2$	104.07
Tris(hydroxymethyl)aminomethane	Atlantic salmon–AQ	$C_4H_{11}NO_3$	122.08
4-acetamidobutyrate	Atlantic salmon–AQ	$C_6H_{11}NO_3$	146.08
Tris(hydroxymethyl)aminomethane	Atlantic salmon–TARS	$C_4H_{11}NO_3$	122.08
4-acetamidobutyrate	Atlantic salmon–TARS	$C_6H_{11}NO_3$	146.08

3.4.2 Differential analysis of metabolites found in salmonid species

A differential analysis was conducted with Compound Discoverer to assess changes in metabolite levels in the skin mucus of AS–AQ, AS–TARS, PS–ocean, and PS–river. The analysis was able to compare the different groups. Both -log10 p-value and log2 fold change threshold were set at 0.05 and 1.00, respectively, for all analyses. For each differential analysis, an overview of compounds showing most deviation from these thresholds was provided, along with their respective chromatograms. **Table 4** presents the compound's formulas, RT-min, log2 fold change, and -log10 p-value. Five specimens from each salmonid species were subjected to and analyzed with differential analysis.

Differential analysis of skin mucus in AS–AQ vs. AS–TARS

Figure 12 presents a volcano plot used in differential analysis, comparing the skin mucus of AS–AQ (n=5) with that of AS –TARS (n=4). The plot showed the log2 fold change and the notable significance of all compounds detected in the skin mucus of the salmonids. The green square indicates 271 metabolites that have significantly decreased in concentration, exhibiting a log2 fold change of -1 or smaller in the AS –AQ when compared to AS –TARS. Conversely, the red square encompasses 532 metabolites that have significantly increased, each with a log2 fold change of one or greater in the AS –AQ relative to the AS –TARS. The marked dots labeled A through D denote selected metabolites with the most significant decrease (A and B) or increase (C and D).

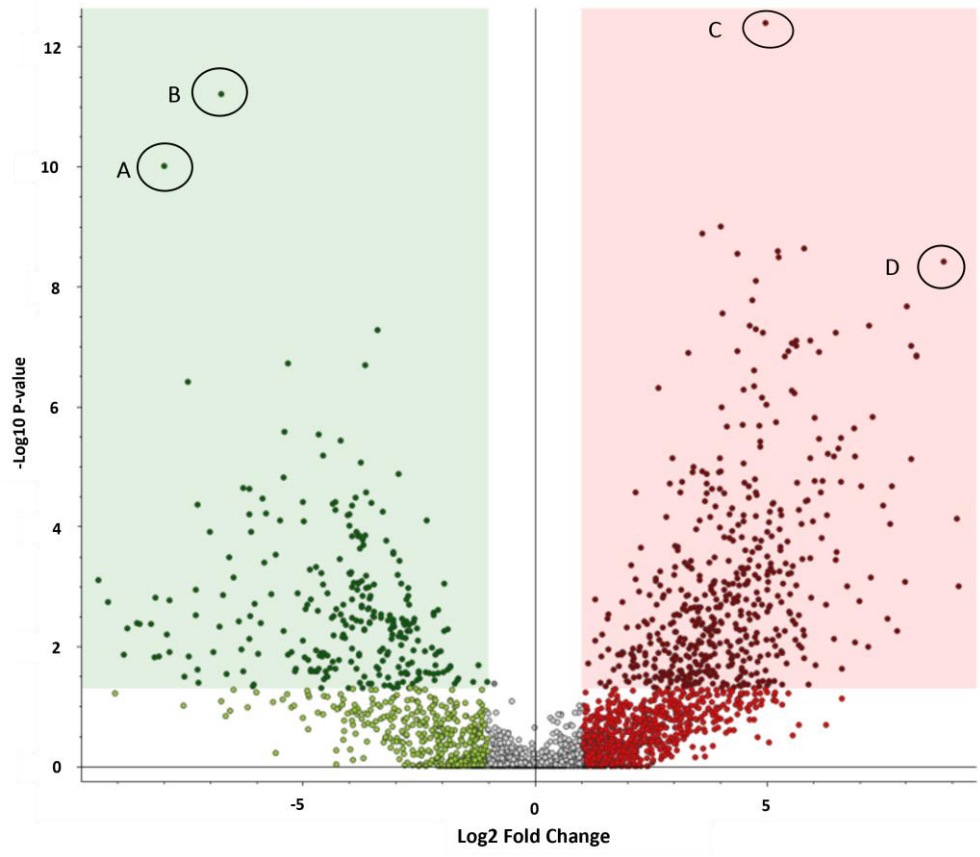

Figure 12. Volcano plot of differential analysis of Atlantic salmon–AQ vs. Atlantic salmon–TARS. The green square indicates 271 metabolites that have significantly decreased in concentration, exhibiting a log2 fold change of -1 or smaller in the Atlantic salmon–AQ when compared to Atlantic salmon–TARS. Conversely, the red square encompasses 532 metabolites that have significantly increased, each with a log2 fold change of one or greater in the Atlantic salmon–AQ relative to the Atlantic salmon–TARS. The marked dots labeled A through D denote the metabolites with the most significant decrease or increase. Volcano plot created with Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). AQ = aquaculture, TARS = Tromsø Aquaculture Research Station.

Differential analysis of PS–river vs. PS–ocean

Figure 13 presents a volcano plot, comparing skin mucus of PS–river against PS–ocean (n=5). The green square indicates 235 metabolites that have significantly decreased in concentration, exhibiting a log2 fold change of -2 or smaller in the PS–river when compared to PS–ocean. Whereas the red square encompasses six metabolites that have significantly increased, each with a log2 fold change of one or greater in the PS–river relative to the PS–ocean. The marked dots labeled E through H denote selected metabolites with the most significant decrease (E and F) or increase (G and H).

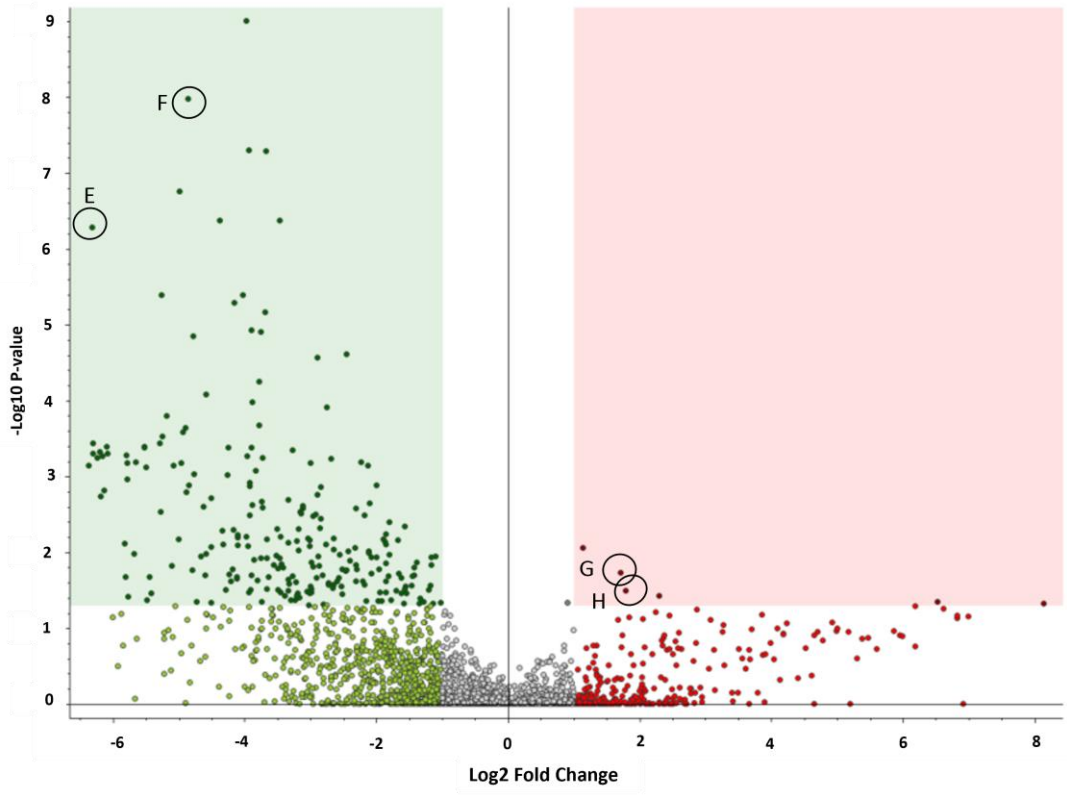

Figure 13. Volcano plot of differential analysis of pink salmon–river vs. pink salmon–ocean. The green square indicates 235 metabolites that have significantly decreased in concentration, exhibiting a log2 fold change of -2 or smaller in the pink salmon–river when compared to pink salmon–ocean. Whereas the red square encompasses six metabolites that have significantly increased, each with a log2 fold change of one or greater in the pink salmon–river relative to the pink salmon–ocean. The marked dots labeled E through H denote the metabolites with the most significant decrease or increase. Volcano plot created with Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Differential analysis of AS–AQ vs. PS–ocean

Figure 14 compares skin mucus of AS–AQ against PS–ocean (n=5). The green square indicates 497 metabolites that have significantly decreased in concentration, exhibiting a log2 fold change of -5 or smaller in the AS–AQ when comparing to PS–ocean. Conversely, the red square encompasses 1067 metabolites that have significantly increased, each with a log2 fold change of four or greater in AS–AQ relative to the PS–ocean. The marked dots labeled I through L denote selected metabolites with the most significant decrease (I and J) or increase (K and L).

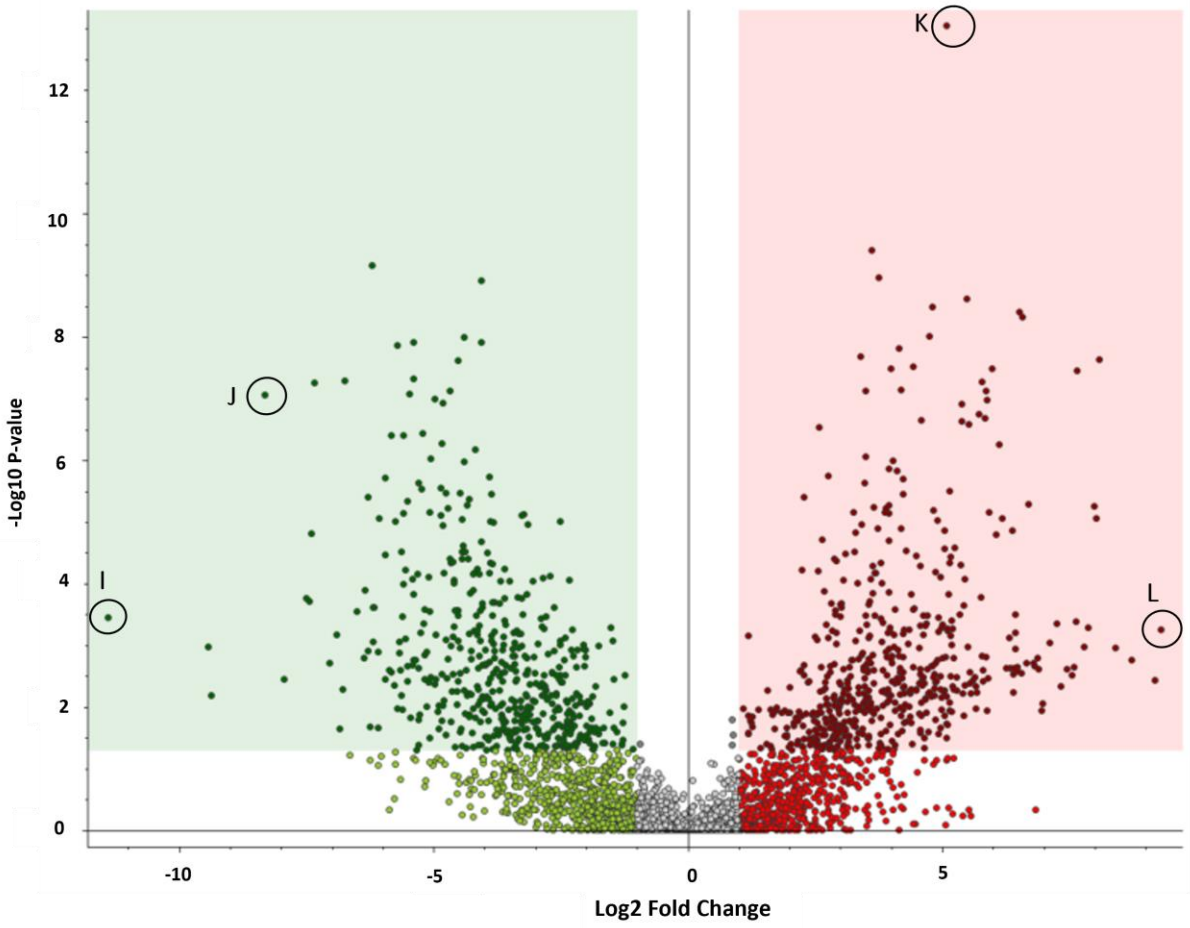

Figure 14. Differential analysis of Atlantic salmon–AQ vs. pink salmon–ocean. The green square indicates 497 metabolites that have significantly decreased in concentration, exhibiting a log2 fold change of -5 or smaller in the Atlantic salmon–AQ when compared to pink salmon–ocean. Conversely, the red square encompasses 1067 metabolites that have significantly increased, each with a log2 fold change of four or greater in Atlantic salmon–AQ relative to the pink salmon–ocean. The marked dots labeled I through L denote the metabolites with the most significant increase or decrease. Volcano plot created with Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). AQ = aquaculture.

Figure 15 and **16** presents the LC-MS chromatograms of the metabolites labeled A through L which denote the metabolites with the most significant decrease or increase in **Figure 12, 13, and 14**. The chromatograms visualize the intensity of the selected metabolites within the four groups of salmonids. For information about formula, *m/z*-value, and retention time (RT) for each compound, see **Table 4**.

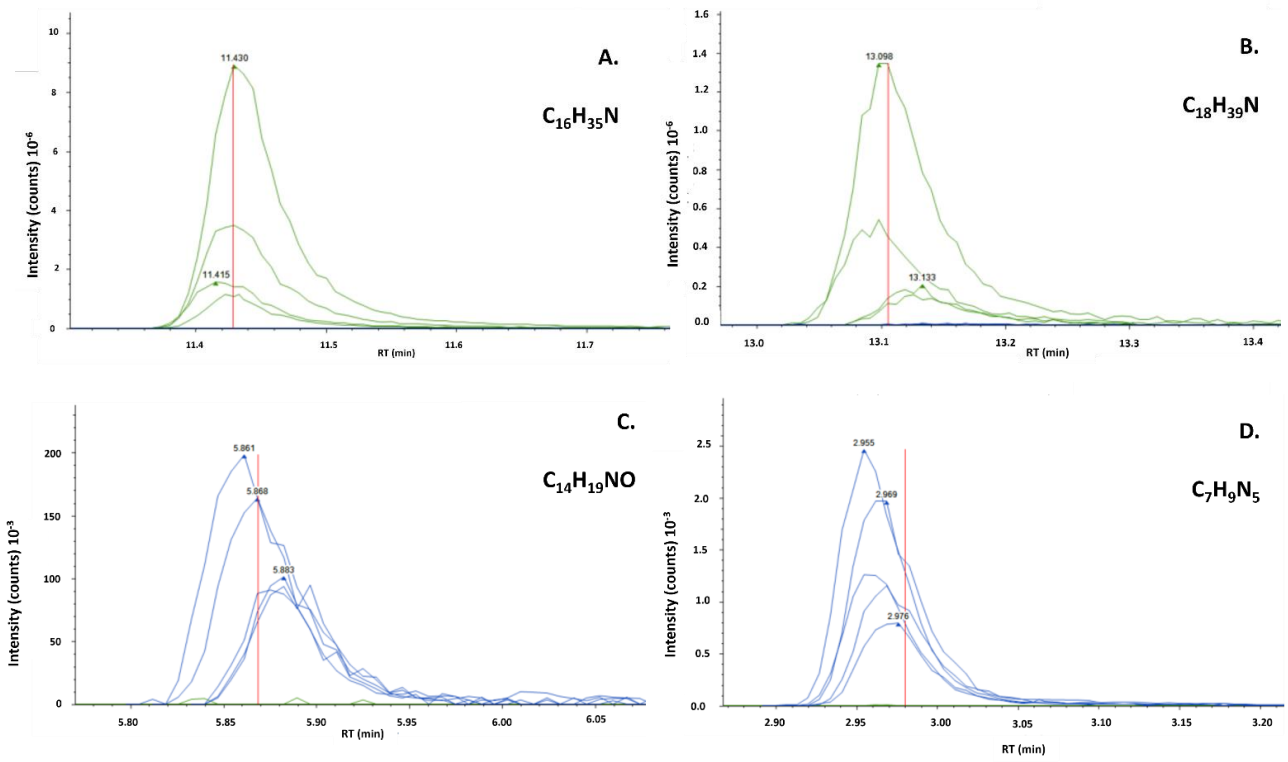

Figure 15. Chromatograms of compounds A, B, C, and D from volcano plot of Atlantic salmon–AQ and Atlantic salmon–TARS. Atlantic salmon–AQ is presented in dark blue, Atlantic salmon–TARS is presented in green. The x-axis represents retentional time in minutes (RT-min), and y-axis represents intensity (counts). Chromatograms created with Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). AQ = aquaculture, TARS = Tromsø Aquaculture Research Center.

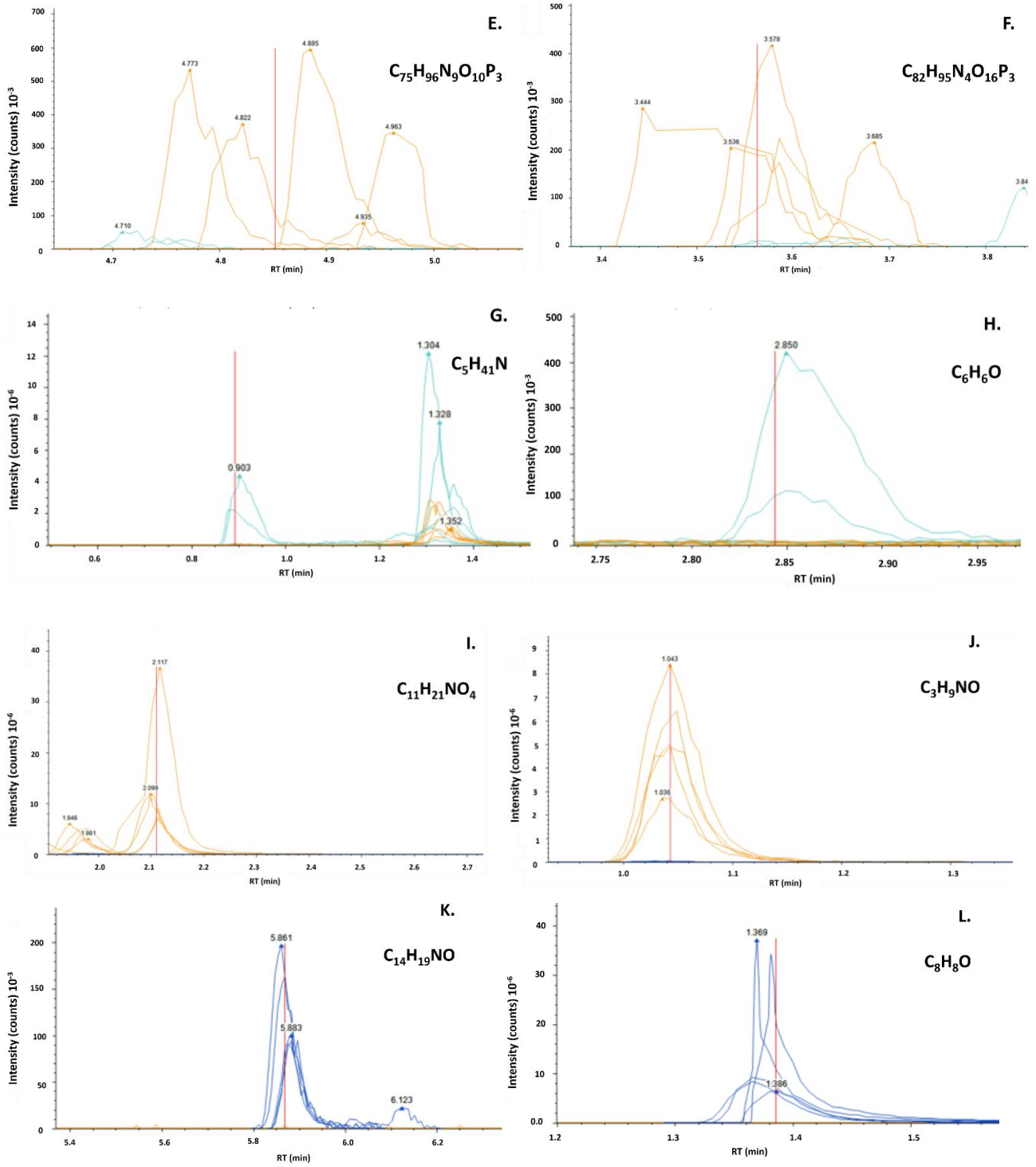

Figure 16. Chromatograms of compounds E, F, G, H, I, J, K, and L from volcano plot of pink salmon–river and pink salmon–ocean, and Atlantic salmon–AQ and pink salmon–ocean. Atlantic salmon–AQ is presented in dark blue, pink salmon–ocean is presented in orange, and pink salmon–river is presented in light blue. The x-axis represents retentional time in minutes (RT-min), and y-axis represents intensity (counts). Chromatograms created with Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). AQ = aquaculture, TARS = Tromsø Aquaculture Research Center.

Compounds detected in different salmonids using differential analysis (with formula, m/z value, retention time (RT) in minutes, log2 fold change, and -log10 p-value) are shown in **(Table 4**). The compounds identification and formula (elemental composition) generation were generated based on LC-MS/MS data, retention time, and matching against compound library databases using Compound discoverer (3.3).

Table 4. Compounds identified by differential analysis comparing LC-MS data of different salmonids. Threshold for log2 fold change and -log10 p-value: 0.05 and 1. Detected compounds were identified using Compound Discoverer 3.3 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). TARS = RT (min) = retention time in minutes, m/z = mass-to-charge ratio. AQ = aquaculture, TARS = Tromsø Aquaculture Research Center.

Species		**Formula**	**Compound**	***m/z***	**RT (min)**	**log2 fold change**	**-log10 p-value**
Atlantic salmon–TARS	**A**	$C_{16}H_{35}N$	Unknown compound	242.28	11.42	-7.9	10.0
Atlantic salmon–TARS	**B**	$C_{18}H_{39}N$	Octadecylamine	270.31	13.10	-6.7	11.2
Atlantic salmon–AQ	**C**	$C_{14}H_{19}NO$	Ethoxyquin	218.15	5.86	4.9	12.3
Atlantic salmon–AQ	**D**	$C_7H_9N_5$	N, N-dimethyl-9H-purin-6amine	164.09	2.98	8.8	8.4
Pink salmon–ocean	**E**	$C_{75}H_{96}N_9O_{10}P_3$	Unknown compound	688.83	4.85	-6.3	6.2
Pink salmon–ocean	**F**	$C_{82}H_{95}N_4O_{16}P_3$	Unknown compound	743.30	3.56	-4.8	7.9
Pink salmon–river	**G**	$C_5H_{41}N$	2-Methylpyrrolidine	86.09	0.89	1.7	1.4
Pink salmon–river	**H**	C_6H_6O	Phenol	95.04	2.84	2.2	1.4
Pink salmon–ocean	**I**	$C_{11}H_{21}NO_4$	N-(tert-Butoxycarbonyl)-L-leucine	232.15	2.11	-11.3	3.4
Pink salmon–ocean	**J**	C_3H_9NO	1-Amino-2-propanol	76.07	1.94	-8.3	7.0
Atlantic salmon–AQ	**K**	$C_{14}H_{19}NO$	Ethoxyquin	218.15	5.86	5.0	13.0
Atlantic salmon–AQ	**L**	C_8H_8O	Acetophenone	121.05	1.38	9.3	3.2

4 Discussion

The primary aim of this master's thesis was to develop a purification method tailored for the analysis of fish skin mucus utilizing MALDI-TOF MS. The optimized method, designated as method No. 4, which included precipitation with 60% ACN and 0.1% TFA, not only satisfied the stringent quality criteria for protein spectra but also complied with HSE regulation concerning chemical usage in the laboratory. Subsequently, MALDI-TOF MS was employed to distinguish between marine fish species, namely Atlantic salmon, Atlantic cod, and coalfish based on their respective fish skin mucus profiles. Similarly, differentiation among the more closely related salmonid fish species, such as AS–AQ, AS–TARS, PS–ocean, and PS–river, was achieved using MALDI-TOF MS, however, with less pronounced distinctions. Complementary analysis of fish skin mucus using LC-MS demonstrated that each species formed distinct clusters. Moreover, LC-MS could identify several thousand metabolites, which varied significantly across the species examined.

The following discussion delves deeper into these results, exploring their implications in a broader context. Additionally, it will address the limitations and possibilities for future work.

4.1 Method development

The existing body of literature on methods for analyzing fish skin mucus using MALDI-TOF MS, particularly concerning the fish species discussed in this study was notably limited (79, 82, 90). Consequently, the development of a more efficient and reliable method for protein analysis in fish skin mucus using MALDI-TOF MS, was important to facilitate broader species-level identification. The primary goal was to produce clear and reliable spectra by MALDI-TOF MS, thereby enabling more precise identification and differentiation of various species at the protein content level. To standardize the purification process of mucus obtained from various fish species prior to MALDI-TOF MS analysis, a methodological development was undertaken. Initial attempt using untreated mucus (method No.1) resulted in spectra that were deemed unusable, characterized by the absence of reliable peaks (i.e. *m/z*) values. This inefficacy can be attributed to the complexity and high abundance of components in biological samples, which are known to cause signal suppression of smaller components (91, 92). Therefore, it became essential to achieve clear spectra with high intensity peaks that accurately

reflect the mass pattern of proteins present in mucus (82). This necessitated detailed sample preparation prior to analysis (93).

The significance of establishing a well-validated method lies in obtaining purified samples (i.e. removal of interfering compounds like salts, as well as up-concentrated) that yield clear and reliable peaks on MALDI-TOF MS, thereby facilitating the detection of proteins. A subsequent method (No. 3) incorporated a protein precipitation process using methanol together with chloroform. However, due to stringent laboratory HSE-regulations, chloroform was substituted with dichloromethane (94, 95). The efficacy of this substitution was evaluated in method No.2. Despite the purification efforts, the analysis using MALDI-TOF MS revealed the presence of mainly background noise and poor-quality spectra. Consequently, the original method (No.3) was revisited, which resulted in the strongest and most consistent peaks. A similar result has been reported in a study exploring protein precipitation using methanol and chloroform, with and without deoxycholic acid, which aids in solubilizing proteins and thereby facilitates easier protein identification in proteomics research (96). The study showed higher protein identification rates with methanol and chloroform purification compared to other solvents such as acetone. Additionally, protein loss subsequent to precipitation were noted, which prompted the inclusion of deoxycholic acid to preserve and solubilize proteins after purification (96). This underscores the critical nature of selecting appropriate solvents and additives in protein purification protocols to optimize outcomes in proteomic analyses.

Furthermore, as a consequence of employing methanol and chloroform as a precipitation agent, the samples exhibited phase separation, resulting in two liquid layers and one solid layer in between. Given chloroform's higher density compared to methanol and water (97, 98, 99), it was presumed that the lower layer primarily consisted of chloroform, while the upper liquid layer was composed of water and methanol. The selection of methanol and chloroform, as components in the protein precipitating process was strategically aimed at eliminating salts, lipids, and other impurities from the sample (100). Protein precipitation is designed to transition proteins from their dissolved state to an insoluble form, concurrently altering the aqueous conditions surrounding them. This process is influenced by several factors, including pH, temperature, protein concentration, surface characteristics of proteins, and the choice of precipitation agents, all of which may alter the proteins within the sample (101).

Method No.4 was developed in response to updated HSE-regulations concerning the use of chlorinated solvents such as chloroform and dichloromethane, which are associated with significant health risk including carcinogenicity and potential organ damage through inhalation or skin contact (97). The transition from method No. 3 to method No. 4 emerged as a more efficacious purification technique, particularly demonstrated by the facilitated comparison between individuals of Atlantic cod and coalfish, owing to more consistent and pronounced peaks and *m/z* values (**Figure 6**). Like method No. 3, the MALDI-TOF MS spectra for different Atlantic salmon specimens exhibited limited similarities in peaks, which could be caused by variation of the specimens quality. Although a few recurring peaks were observed across the Atlantic salmon specimens, the variability in *m/z* values and intensity levels posed challenges in assessing their reliability.

A notable challenge encountered with method No. 4 was the difficulty in pipetting samples from fish species with a more viscous mucus, which frequently led to clogged filter pipettes and necessitated the restarting of the method. This issue frequently led to additional dilution of the samples. There is potential for optimization or refinement of this method to ensure a consistent purification process across all samples. Alternatively, exploring a different approach for purification might yield improved outcomes and enhance the overall efficacy of the analytical process.

4.2 Mucus variation

The mucus layer serves as a protective barrier against pathogens and other microorganisms (57, 102). Skin mucus specimens from both AS–AQ and AS–TARS were observed to be thin and runny. These observations may indicate that a thinner skin mucus barrier could be more susceptible to parasites and bacterial infections. Supporting this hypothesis, a study conducted by Fast et al. (103) compared various non-specific humoral parameters among different salmonids. It was found that Atlantic salmon possessed the thinnest epidermal layer, the least close distribution of mucosal cells, and the lowest mucous lysozyme activity among the salmonids examined (103). It has also been observed that skin mucus from farmed Atlantic salmon contains proteins that bacteria might exploit as a nutrients during colonization (104). The diverse results obtained from AS–AQ from this study using MALDI-TOF MS and LC-MS suggest that contamination by bacteria or other microorganisms could have contributed to the variability observed in the specimens. A paper by Ángeles Esteban (56) explained that when

hydration to skin mucus is lowered, a change in viscoelasticity occurs. The paper explain how a more hydrated skin mucus makes the viscoelasticity in the mucus lower, and could therefore be easier for bacteria to penetrate (56). The runny and thin mucus layer to AS–AQ and AS–TARS could therefore be considered more susceptible for bacteria to penetrate.

In a review by Boxaspen (34), it was suggested that Atlantic salmon might exhibit a higher susceptibility to *L. salmonis* compared to other salmonids (34). A variation in protein composition between AS–AQ and AS–TARS was somewhat anticipated, given the infestation of salmon lice in the AS–AQ specimens, but not in AS–TARS specimens. However, analysis using MALDI-TOF and LC-MS did not provide clear evidence that the mucus variation between the AS–AQ and AS–TARS was attributable to these factors. Research by Easy and Ross (17) investigated how skin and muscle tissue change following an infestation with salmon lice. They observed an alteration in identified proteins before and after an infestation (17), whereas Nolan et al. (105) noted a reduction in mucosal cells in salmonid skin (105). Nevertheless, in this master's project, only a few attached salmon lice were found on each AS–AQ specimen. For MS analysis in general, the low number of attached sea lice may have been insufficient for the analysis to detect any significant difference between the two species based on findings related to salmon lice infestations.

Collecting mucus samples from PS–ocean proved challenging due to minimal availability of skin mucus. In contrast, PS–river specimens were characterized by a considerable viscous mucus layer that covered most of the abdomen and above the lateral line. Previously research has indicated that a less hydrated mucus layer may alter its viscoelasticity properties, thereby appearing denser and more solid (56). Additionally, variations in salinity may have influenced the texture of the skin mucus in PS–river specimens. Ordóñez-Grande et al. (106) studied the secretion of skin mucus across different salinities, and discovered that fish produced less mucus in environments with lower salinity (106). For PS–river specimens, which exhibited a distinct mucus texture compared to PS–ocean specimens, the differences in saline conditions might not fully account for the observed differences. However, it is important to acknowledge that both the PS–ocean specimens and the PS–river specimens were frozen and thawed prior to the collection of mucus, which is expected to impact the samples (at least to some extent). This occurrence could explain the more viscous skin mucus observed in the PS–river specimens compared to that of the PS–ocean specimens.

4.3 MALDI-TOF MS

The MALDI-TOF MS technique, applied to the extracted mucus samples, successfully differentiated AS–AQ, Atlantic cod, and coalfish into three distinct clusters. Atlantic cod and coalfish exhibited closer clustering than AS–AQ, which aligns with their closer genetic relationship belonging to the Gadidae family (70). Although all three fish species originated from marine environments, only the AS–AQ was sourced from an aquaculture facility, while the Atlantic cod and coalfish were wild-caught. This distinction suggests that factors such as breeding, confinement in sea cages, stress, and exposure to handling operations could influence AS–AQ skin mucus barrier (47, 50, 51, 107). In contrast, the wild-caught Atlantic cod and coalfish were not subjected to controlled conditions, breeding, or dietary regimes. Another parameter to consider is the stress levels experienced by aquaculture fish, which may have contributed to differences in mucus composition. Research by Lanfermeijer et al. (109) on metabolites in the skin mucus of stressed aquaculture fish identified components such as cortisol and melatonin, which could serve as biomarkers of stress (108). These components were not detected in fish species in this study, possibly due to the analysis method not being designed for such detection.

Another critical aspect to consider is that the *m/z* cutoff used in IDBac differed from that used in the MALDI-TOF MS analysis. The MALDI-TOF MS analysis encompassed *m/z*-values ranging from 2000 to 18000, whereas IDBac used a range from 3000 to 15000. The signal-to-noise (s/n) ratio for all dendrograms were also set to 2.5. Consequently, a lower *m/z* cutoff would include a broader array of peaks, and allow the inclusion of low intensity peaks. The spectra for AS–AQ and AS–TARS exhibited few comparable peaks, suggesting that a lower cutoff might have included additional peaks for the AS–AQ. An article discussing the impact of chemical background noise in MALDI MS and MS/MS, elucidated how noise from desorption and ionization could influence the spectra, resulting in the emergence of peaks not originated from the sample. The article further noted that such noise could stem from impurities, which are challenging to eliminate, thus underscoring the necessity for an effective purification method. Additionally, the article addressed how the matrix could also generate chemical noise, presenting a more challenging problem to overcome (110). This highlights the importance of a robust purification, as developed in this study.

The dendrogram which elucidates the spectra from four groups of salmonid species (**Figure 9**), distinctly separates them into individual clusters. However, despite being the same species, AS–AQ and AS–TARS, subclustered into two separate groups. Similarly, PS–ocean and PS–river formed a separate cluster, although PS–river had a few specimens which showed greater divergence from PS–ocean. These findings suggest that differentiating salmonids species based in their protein spectra might be feasible. The subsequent dendrogram (**Figure 10**) encompasses the same four groups of salmonid species but with several individuals from AS–AQ and AS–TARS exhibiting lower quality spectra. This dendrogram presents a clustering different from the previous dendrogram. This variation highlights the critical importance of generating high quality spectra through robust purification processes to ensure reliable results, particularly if the method potentially is employed for species identification.

Similarities within the species could enhance the reliability for species identification based on protein content level in the mucus. Given the prevalence of food fraud cases, particularly fish fraud, using skin mucus to identify species based on "fingerprints'' could prove beneficial. According to peak lists (**Table S2 to S6)**, it was observed several consistent peaks in all the specimens. Further research should look closer at similar and consistent peak patterns in mucus samples from specific species - which could be used as a fingerprint for that certain species. Mass-fingerprint technique on fish skin mucus have already been tested and resulted in several biomarkers in the skin mucus (82).

4.4 LC-MS

The PCA-plot demonstrated how different salmonids and salmon lice specimens clustered based on the components identified in their skin mucus using LC-MS. Notable clustering was observed within each group of PS–ocean and PS–river specimens, though they did not cluster together. Their distinct origins - one at a freshwater phase and the other saltwater phase, may explain their separation in the PCA-plot. Among the salmonids, PS–river, collected exclusively from a freshwater environment, and was that species that clustered furthest away from the others on the PCA-plot. As noted in the literature, freshwater environments are highly influenced by sediments, terrestrial impact, and microorganisms (111). Such influence could have altered the mucus composition of the PS–river specimens, thereby explaining their distinct clustering from the marine environment.

The PCA-plot showed that the salmon lice specimens clustered with the AS–TARS specimens. However, there was no significant clustering between the salmon lice and AS–AQ, despite AS–AQ being the species primarily exposed to the lice during mucus collecting. A more systematic approach might be necessary to distinguish significant differences between AS–AQ and AS–TARS based on their skin mucus composition. In a study by Braden et al. (113), comparative defense mechanisms in salmonid skin against sea lice infestation were investigated. In their experiment, 10 individuals each of pink, chum, and Atlantic salmon were exposed to 50 sea lice for approximately 20 minutes (112). During this period, three to five sea lice attached to each fish, remaining attached post-experiment. Significant changes in immunological components in the skin were observed in all three species within 24 to 48 hours post-exposure. Notably, Atlantic salmon exhibited a greater alteration in expressed immunological components compared to the other species (112). Therefore, a similar experimental approach involving sea lice infestation could be incorporated into further research.

Before extraction and subsequent analysis using MALDI-TOF MS and LC-MS analysis, mucus specimens were stored at -20 ˚C. After extractions, mucus samples were stored at 4 ˚C until analyzed. For some samples, there was considerable gap between the extraction analysis date due to inadequate preparation and a limited number of mucus samples. This extended storage may have caused the degradation of important metabolites in the samples. A study on protein quality and degradation in saliva samples demonstrated that proteins remained stable at room temperature for 24 hours, but began to degrade shortly after (113). Although this research focused on human saliva, it is relevant to this study as it underscores the effects of prolonged storage on protein degradation. The storage conditions for PS–ocean and PS–river was distinct from those of the other fish groups. These two groups were frozen and then thawed prior to mucus sampling. A study on identifying artifacts in fish that had undergone freeze-thawed cycles observed changes in the myocytes of thawed fish (114). Consequently, artifacts may have been introduced in PS–ocean and PS–river due to their storage before sampling, potentially impacting the results of the MALDI-TOF MS and LC-MS analyses. However, given the limited number of specimens examined in this study, further research with more controlled parameters is essential.

Analysis using LC-MS provided insight into the compounds recognized in AS–AQ, AS–TARS, salmon lice, PS–ocean and PS–river (**Table 3**). Discussed further are some of the metabolites

presented in **Table 3**. Salmon lice exhibited the highest level of valine and tryptophane, which are essential amino acids crucial for protein synthesis (115, 116). This finding does not necessarily indicate anything beyond the fact that salmon lice are a distinct species compared to fish, and they utilize these essential amino acids throughout their bodies (117). PS–ocean samples showed the highest levels of phenylalanine and carnitine. A study by Rigault et al. (119) noted that salmon was the fish species with the highest carnitine levels (118), indicating that this amino acid plays an important role in fatty acid metabolism (119). The compounds found at the highest levels in PS–river were histidine, taurine, and hypoxanthine. Hypoxanthine has been observed during the parr-smolt transformation in salmon (120). Further research could explore whether this metabolite plays a role in de-smoltification when the salmon returns to the river, as observed in PS–river specimens. Both AS–AQ and AS–TARS exhibited high levels of tris(hydroxymethyl)aminomethane and 4-acetaminobutyrate, a similarity that may be common for Atlantic salmon species. However, no literature supports this theory.

The differential analysis depicted in volcano plots, compared two groups of salmonids and highlighted the metabolites in each group with highest significant differences. When comparing AS–AQ to AS–TARS, metabolite B and C exhibited a significant increase. Metabolite B, ($C_{18}H_{39}N$), was more abundant in AS–TARS and presented in chromatogram B (**Figure 15).** Based on the formula of metabolite B, it appears to be octadecylamine (ODA) (121). Metabolite C, ($C_{14}H_{19}NO$), most abundantly found in AS–AQ, was identified as the quinolone ethoxyquin, a food additive previously used in fish feed (122, 123). A study evaluating ethoxyquin in fish feed also observed *m/z*-values similar to those in this study (*m/z* 218.15 – **Table 4**) (124). This suggests that the metabolite identified in this study may originate from the fish feed in aquaculture. Its presence in fish skin mucus suggest accumulation from the digestive tract and throughout the tissues, ultimately manifesting in the skin mucus barrier.

When comparing metabolites found in PS–ocean and PS–river, metabolites E, ($C_{75}H_{96}N_9O_{10}P_3$), and F, ($C_{82}H_{95}N_4O_{16}P_3$), both presumably phospholipids, were notable and more abundant in PS–ocean (**Figure 16**). In the comparison between AS–AQ and PS–ocean, both of which are salmonids inhabiting marine environments, metabolites I, ($C_{11}H_{21}NO_4$), and L, (C_8H_8O), emerged as the most significant and substantial changes. Conversely, metabolite K, ($C_{14}H_{19}NO$), which was most abundant in AS–AQ, were as previously mentioned (metabolite C) identified to be ethoxyquin (**Figure 16**–C). This observation could suggest

differences in the nutritional content of fish feed between AS–AQ and the natural diet of PS–ocean specimens. Metabolite L, identified as acetophenone and most abundant in AS–AQ, is detailed in the chromatogram in **Figure 16** and **Table 4**. Acetophenone, a methyl ketone, serves various roles (125). Zubkov & Kouznetsov (127) discuss acetophenone's diverse and multi-purpose nature, characterized by its ketone structure containing aromatic groups, across a range of organisms. While the review article does not specifically address acetophenone's role in fish skin mucus, it highlights its significance in skin microbiota and its susceptibility to manipulation by vector-borne parasites (126, 127). Moreover, acetophenone is recognized as a natural metabolite and semiochemical (126).

In the context of fish, semiochemicals, which naturally occur in their skin mucus, have been briefly explored in relation to salmon lice (40, 41, 44, 128). Semiochemicals are predominantly volatile organic compounds (VOCs) (44, 129). In a study by Difford et al. (44), the skin mucus of Atlantic salmon was analyzed for VOCs in the context of sea lice infestation. The researchers noted a pronounced expression of VOCs in the skin mucus of fish that harbored a high density of lice, observed *in vivo* (44). This finding is interesting and suggests the need for additional analysis of the specimens used in the present study. VOCs, which are gaseous compounds (130), elude detection via LCMS, due to the requirement for the compounds to be soluble (131). Consequently, Gas Chromatography Mass Spectrometry (GC-MS) presents a viable alternative method for the detection of VOCs in these skin mucus samples.

5 Conclusion

Developing an in-lab methodology for analyzing fish skin mucus using MALDI-TOF MS proved to be successful, using zip-tip (C18), a form of SPE (solid-phase extraction) together with 60% ACN and 0.1% TFA. This approach made it possible to differentiate the three species Atlantic salmon, Atlantic cod, and coalfish based on the protein content level in the skin mucus. This method was also able to distinguish between different salmonid species, AS–AQ, AS–TARS, PS–ocean, and PS–river. By utilizing LC-MS, the composition of skin mucus from salmonid species and whole body of salmon lice were differentiated. LC-MS analysis did not distinguish any clear difference between the non-salmon lice infected salmonids and the infected species based on potential compounds related to an infestation. However, the analysis did find components in the mucus of Atlantic salmon (AS–AQ), such as acetophenone, that could be interesting to analyze further.

Future perspective

For further research, exploring semiochemicals, specifically VOCs, in the skin mucus of lice-infected fish could be beneficial for understanding more about the host-parasite connection. Analysis of VOCs in the skin mucus by utilizing GC-MS will provide more accurate results on the composition in the skin mucus compared to using LC-MS. Another future perspective would be to analyze and use the *m/z*-values of spectra from protein content level in the skin mucus, for mass fingerprinting. More specimens of freshly samlpled Atlantic salmon, pink salmon, Atlantic cod, and coalfish could give a more refined and accurate overview of the proteins and metabolites present in the mucus of each species.

References

1. United Nations DoEaSA, Population Division. Population 2030: demographic challenges and opportunities for sustainable development planning. 2015.
2. United Nations DoEaSAPD. Conserve and sustainably use the oceans, seas and marine resources for sustainable development: Department of Economic and Social Affairs: Sustainable Development; 2024 [updated 2024.05.06. Available from: https://sdgs.un.org/goals/goal14#overview.
3. Costello C, Cao L, Gelcich S, Cisneros-Mata MÁ, Free CM, Froehlich HE, et al. The future of food from the sea. Nature. 2020;588(7836):95-100.
4. Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, et al. Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish Physiology and Biochemistry. 2012;38(1):85-105.
5. Gui J-F. Fish biology and biotechnology is the source for sustainable aquaculture. Science China Life Sciences. 2015;58(2):121-3.
6. Naylor RL, Hardy RW, Buschmann AH, Bush SR, Cao L, Klinger DH, et al. A 20-year retrospective review of global aquaculture. Nature. 2021;591(7851):551-63.
7. Agnalt A-L, Diserud OH, Dunlop KM, Escobar R, Fiske P, Folkedal O, et al. Risikorapport norsk fiskeoppdrett 2024 - Produksjonsdødelighet hos oppdrettsfisk og miljøeffekter av norsk fiskeoppdrett. Havforskningsinstituttet; 2024.
8. Norwegian Directorate of Fisheries. Production of salmon and trout Barentswatch: Nofima, SINTEF Ocean and BarentsWatch; 2024 [updated 2024.05.01. Available from: https://www.barentswatch.no/havbruk/produksjonlaksefisk.
9. Santurtun E, Broom DM, Phillips CJC. A review of factors affecting the welfare of Atlantic salmon (Salmo salar). Animal Welfare. 2018;28(3):193-204.
10. Bøgwald J, Dalmo RA. Historisk blikk – og nåværende vaksiner i norsk fiskeoppdrett. Naturen. 2020;144(5):207-13.
11. Rauta PR, Nayak B, Das S. Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. Immunology Letters. 2012;148(1):23-33.
12. Beck BH, Peatman E. Mucosal Health in Aquaculture. San Diego, UNITED STATES: Elsevier Science & Technology; 2015.
13. Elliott D. Muscles, skeleton, skin, and movement. In: Farrell AP, editor. Encyclopedia of fish physiology: From genome to environment: Elsevier 2011. p. 506-19.
14. Saurabh S, Sahoo PK. Lysozyme: an important defence molecule of fish innate immune system. Aquaculture Research. 2008;39(3):223-39.
15. Benhamed S, Guardiola F, Mars M, Esteban M. Pathogen bacteria adhesion to skin mucus of fishes. Veterinary Microbiology. 2014;171(1-2):1-12.
16. Ross N, Firth K, Wang A, Burka J, Johnson S. Changes in hydrolytic enzyme activities of naïve Atlantic salmon Salmo salar skin mucus due to infection with the salmon louse Lepeophtheirus salmonis and cortisol implantation. Diseases of Aquatic Organisms. 2000;41:43-51.
17. Easy R, Ross NW. Changes in Atlantic salmon (Salmo salar) epidermal mucus protein composition profiles following infection with sea lice (Lepeophtheirus salmonis). Comparative Biochemistry and Physiology Part D, Genomics and Proteomics. 2009;4(3):159-67.
18. Costello MJ. Ecology of sea lice parasitic on farmed and wild fish. Trends in Parasitology. 2006;22(10):475-83.

19. Gjelland K, Serra-Llinares R, Hedger R, Arechavala-Lopez P, Nilsen R, Finstad B, et al. Effects of salmon lice infection on the behaviour of sea trout in the marine phase. Aquaculture Environment Interactions. 2014;5(3):221-33.
20. Coates A, Phillips BL, Bui S, Oppedal F, Robinson NA, Dempster T. Evolution of salmon lice in response to management strategies: a review. Reviews in Aquaculture. 2021;13(3):1397-422.
21. Grimnes A, Jakobsen PJ. The physiological effects of salmon lice infection on post‐smolt of Atlantic salmon. Journal of Fish Biology 1996;48:1179‐94.
22. Hamre LA, Lunestad BT, Hannisdal R, Samuelsen OB. An evaluation of the duration of efficacy of emamectin benzoate in the control of Caligus curtus Müller infestations in Atlantic cod, Gadus morhua L. Journal of Fish Diseases. 2011;34(6):453-7.
23. Fast MD. Fish immune responses to parasitic copepod (namely sea lice) infection. Developmental and Comparative Immunology. 2014;43(2):300-12.
24. Wagner GN, Fast MD, Johnson SC. Physiology and immunology of Lepeophtheirus salmonis infections of salmonids. Trends in Parasitology. 2008;24(4):176-83.
25. Costello MJ. How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. Proceedings of the Royal Society B: Biological Sciences. 2009;276(1672):3385-94.
26. Sommerset I, Wiik-Nielsen J, Oliveira VHSd, Moldal T, Bornø G, Haukaas A, et al. Fiskehelserapporten 2022. Veterinærinstituttet; 2023. Report No.: Veterinærinstituttets rapportserie nr. 5a/2023.
27. Kabata Z. Copepods parasitic on fishes : keys and notes for the identification of the British species. [Rev.]. ed. Shrewsbury: Field Studies Council; 2003.
28. Johnson SC, Albright LJ. The developmental stages of Lepeophtheirus salmonis (Krøyer, 1837) (Copepoda: Caligidae). Canadian Journal of Zoology. 1991;69(4):929-50.
29. Heuch PA, Knutsen JA, Knutsen H, Schram TA. Salinity and temperature effects on sea lice over-wintering on sea trout (*Salmo trutta*) in coastal areas of the Skagerrak. Journal of the Marine Biological Association of the United Kingdom. 2002;82(5):887-92.
30. Tully O, Nolan DT. A review of the population biology and host–parasite interactions of the sea louse Lepeophtheirus salmonis (Copepoda: Caligidae). Parasitology. 2002;124(7):165-82.
31. Thorstad E, Todd C, Uglem I, Bjørn P, Gargan P, Vollset K, et al. Effects of salmon lice Lepeophtheirus salmonis on wild sea trout Salmo trutta: a literature review. Aquaculture Environment Interactions. 2015;7(2):91-113.
32. Boxaspen K, Næss T. Development of eggs and planktonic stages of salmon lice (Leophtheirus salmonis) at low temperature. Contributions to Zoology. 2000;69:51-5.
33. Pike AW, Wadsworth SL. Sealice on Salmonids: Their Biology and Control. In: Baker JR, Muller R, Rollinson D, editors. Advances in Parasitology. 44: Academic Press; 1999. p. 233-337.
34. Boxaspen K. A review of the biology and genetics of sea lice. ICES Journal of Marine Science. 2006;63(7):1304-16.
35. Yazawa R, Yasuike M, Leong J, Von Schalburg KR, Cooper GA, Beetz-Sargent M, et al. EST and mitochondrial DNA sequences support a distinct pacific form of salmon louse, Lepeophtheirus salmonis. Marine Biotechnology. 2008;10(6):741-9.
36. Barrett LT, Oppedal F, Robinson N, Dempster T. Prevention not cure: a review of methods to avoid sea lice infestations in salmon aquaculture. Reviews in Aquaculture. 2020;12(4):2527-43.

37. Torrissen O, Jones S, Asche F, Guttormsen A, Skilbrei OT, Nilsen F, et al. Salmon lice - impact on wild salmonids and salmon aquaculture. Journal of Fish Diseases. 2013;36(3):171-94.
38. Mordue AJ, Birkett MA. A review of host finding behaviour in the parasitic sea louse, Lepeophtheirus salmonis (Caligidae: Copepoda). Journal of Fish Diseases. 2009;32(1):3-13.
39. Fields DM, Skiftesvik AB, Browman HI. Behavioural responses of infective‐stage copepodids of the salmon louse Lepeophtheirus salmonis, Copepoda: Caligidae to host‐related sensory cues. Journal of Fish Diseases. 2018;41(6):875-84.
40. Bailey RJE, Birkett MA, Ingvarsdóttir A, Mordue AJ, Mordue W, O'Shea B, et al. The role of semiochemicals in host location and non-host avoidance by salmon louse (Lepeophtheirus salmonis) copepodids. Canadian Journal of Fisheries and Aquatic Sciences. 2006;63:448-56.
41. Ingvarsdóttir A, Birkett MA, Duce I, Genna RL, Mordue W, Pickett JA, et al. Semiochemical strategies for sea louse control: host location cues. Pest Management Science. 2002;58(6):537-45.
42. Brooker AJ, Skern-Mauritzen R, Bron JE. Production, mortality, and infectivity of planktonic larval sea lice, Lepeophtheirus salmonis (Krøyer, 1837): current knowledge and implications for epidemiological modelling. ICES Journal of Marine Science. 2018;75(4):1214-34.
43. Sorensen PW, Johnson NS. Theory and application of semiochemicals in nuisance fish control. Journal of Chemical Ecology. 2016;42(7):698-715.
44. Difford GF, Haugen JE, Aslam ML, Johansen LH, Breiland MW, Hillestad B, et al. Variation in volatile organic compounds in Atlantic salmon mucus is associated with resistance to salmon lice infection. Scientific Reports. 2022;12(1).
45. Li K, Buchinger TJ, Li W. Discovery and characterization of natural products that act as pheromones in fish. Natural Product Reports. 2018;35(6):501-13.
46. Bornø G, Hansen H. Parasittsykdommer hos laksefisk i oppdrett. Veterinærinstituttet 2024. Report No.: Veterinærinstituttet rapportserie nr 8a/2024.
47. Gismervik K, Nielsen KV, Bjørklund K, Osnes MN, Gåsnes S, Stige LC, et al. Fiskevelferd. Veterinærinstituttet 2024. Report No.: Veterinærinstituttet rapportserie nr 8a/2024.
48. Brooker AJ, Papadopoulou A, Gutierrez C, Rey S, Davie A, Migaud H. Sustainable production and use of cleaner fish for the biological control of sea lice: recent advances and current challenges. Veterinary Record. 2018;183(12):383-.
49. Overton K, Dempster T, Oppedal F, Kristiansen TS, Gismervik K, Stien LH. Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. Reviews in Aquaculture. 2019;11(4):1398-417.
50. Gismervik K, Gåsnes SK, Gu J, Stien LH, Madaro A, Nilsson J. Thermal injuries in Atlantic salmon in a pilot laboratory trial. Veterinary and Animal Science. 2019;8:1-8.
51. Gismervik K, Nielsen KV, Lind MB, Viljugrein H. Mekanisk avlusing med FLS avlusersystem - dokumentasjon av fiskevelferd og effekt mot lus. Veterinærinstituttets rapportserie 2017(6-2017).
52. Aaen SM, Helgesen KO, Bakke MJ, Kaur K, Horsberg TE. Drug resistance in sea lice: a threat to salmonid aquaculture. Trends in Parasitology. 2015;31(2):72-81.
53. Hvas M, Folkedal O, Oppedal F. Fish welfare in offshore salmon aquaculture. Reviews in Aquaculture. 2021;13(2):836-52.
54. Kryvi H, Poppe T. Fiskeanatomi. 2 ed: Fagbokforlaget 2021.

55. Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology. 2001;25(8):827-39.
56. Ángeles Esteban M. An overview of the immunological defenses in fish skin. ISRN Immunology. 2012;2012:1-29.
57. Dash S, Das S, Samal J, Thatoi H. Epidermal mucus, a major determinant in fish health: a review. Iranian Journal of Veterinary Research. 2018;19(2):72-81.
58. Rose MC. Mucins: structure, function, and role in pulmonary diseases. American Journal of Physiology. 1992;263(4):413-329.
59. Helfman GS, Collette BB, Facey DE, Bowen BW. The Diversity of fishes : biology, evolution, and ecology. Second edition. ed. Chichester: Wiley-Blackwell; 2009.
60. Reverter M, Tapissier-Bontemps N, Lecchini D, Banaigs B, Sasal P. Biological and ecological roles of external fish mucus: a review. Fishes. 2018;3(4):41.
61. Fontenot D, Neiffer D. Wound management in teleost fish: Biology of the healing process, evaluation, and treatment. The Veterinary clinics of North America: Exotic animal practice. 2004;7:57-86.
62. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult Zebrafish as a Model System for Cutaneous Wound-Healing Research. Journal of Investigative Dermatology. 2013;133(6):1655-65.
63. Brinchmann MF. Immune relevant molecules identified in the skin mucus of fish using -omics technologies. Molecular BioSystems. 2016;12(7):2056-63.
64. Abbas AK, Lichtman AH, Pillai S. Basic immunology: functions and disorders of the immune system. Fifth ed: Elsevier 2016.
65. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology Reviews. 1998;62(3):597-635.
66. Díaz-Puertas R, Adamek M, Mallavia R, Falco A. Fish skin mucus extracts: An underexplored source of antimicrobial agents. Marine Drugs. 2023;21(6):350.
67. Elumalai P, Rubeena AS, Arockiaraj J, Wongpanya R, Cammarata M, Ringø E, et al. The role of lectins in finfish: a review. Reviews in Fisheries Science and Aquaculture. 2019;27(2):152-69.
68. Lewis RW. Fish cutaneous mucus: A new source of skin surface lipid. Lipids. 1970;5(11):947-9.
69. Hamre J. Biodiversity and exploitation of the main fish stocks in the Norwegian - Barents Sea ecosystem. Biodiversity and Conservation. 1994;3(6):473-92.
70. Froese R, Pauly D. FishBase [World Wide Web electronic publication]. 2024 [Available from: www.fishbase.org.
71. Schiewe MH. Salmon. In: Levin SA, editor. Encyclopedia of Biodiversity (Second Edition). Waltham: Academic Press; 2013. p. 522-31.
72. McCormick SD. Ontogeny and Evolution of Salinity Tolerance in Anadromous Salmonids: Hormones and Heterochrony. Estuaries. 1994;17(1):26.
73. Cargnelli LM, Griesbach SJ, Packer DB, Berrien PL, Johnson DL, Morse WW. Essential fish habitat source document. Pollock, Pollachius virens, life history and habitat characteristics. NOAA technical memorandum NMFS-NE. 1999(131):1-30.
74. Singhal N, Kumar M, Kanaujia P, Virdi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Frontiers in Microbiology. 2015;6(791):1-16.

75. Mortier T, Wieme AD, Vandamme P, Waegeman W. Bacterial species identification using MALDI-TOF mass spectrometry and machine learning techniques: A large-scale benchmarking study. Computational and Structural Biotechnology Journal. 2021;19:6157-68.
76. Gross JH. Mass Spectrometry : A Textbook. Berlin, Heidelberg: Springer Berlin Heidelberg : Imprint: Springer; 2011.
77. Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. The Clinical Biochemist Reviews. 2009;30(1):19-34.
78. Yi J, Qin Q, Wang Y, Zhang R, Bi H, Yu S, et al. Identification of pathogenic bacteria in human blood using IgG-modified Fe3O4 magnetic beads as a sorbent and MALDI-TOF MS for profiling. Microchimica Acta. 2018;185(12).
79. Bi H, Zhong C, Shao M, Wang C, Yi J, Qiao L, et al. Differentiation and authentication of fishes at the species level through analysis of fish skin by matrix assisted laser desorption/ionization time of flight mass spectrometry. Rapid Communications in Mass Spectrometry. 2019;33(16):1336-43.
80. McMaster MC. LC/MS: a practical user's guide: John Wiley 2005.
81. Fæste CK, Tartor H, Moen A, Kristoffersen AB, Dhanasiri AKS, Anonsen JH, et al. Proteomic profiling of salmon skin mucus for the comparison of sampling methods. Journal of Chromatography B. 2020;1138.
82. Freitas J, Silva P, Perestrelo R, Vaz-Pires P, Câmara JS. Improved approach based on MALDI-TOF MS for establishment of the fish mucus protein pattern for geographic discrimination of Sparus aurata. Food Chemistry. 2022;372:131237.
83. Clark CM, Costa MS, Conley E, Li E, Sanchez LM, Murphy BT. Using the open-source MALDI TOF-MS IDBac pipeline for analysis of microbial protein and specialized metabolite data. Journal of Visualized Experiments. 2019(147).
84. Bruker Daltonik GmbH. Bruker guide to MALDI sample preparation Bremen: Bruker Daltonik GmbH; 2014.
85. Bruker Daltonik GmbH. Instructions for Use MALDI Biotarget 48. Bremen: Bruker Daltonik GmbH; 2011.
86. Stahl A, Schröder U. Development of a MALDI–TOF MS-based protein fingerprint database of common food fish allowing fast and reliable identification of fraud and substitution. Journal of Agricultural and Food Chemistry. 2017;65(34):7519-27.
87. Çelik EE, Cömert ED, Gökmen V. The power of the QUENCHER method in measuring total antioxidant capacity of foods: Importance of interactions between different forms of antioxidants. Talanta. 2024;269:1-9.
88. Haug T, Kjuul AK, Stensvåg K, Sandsdalen E, Styrvold OB. Antibacterial activity in four marine crustacean decapods. Fish & Shellfish Immunology. 2002;12(5):371-85.
89. Grijseels S, Vasskog T, Heinsvig PJ, Myhre T, Hansen T, Mardal M. Validation of an LC-HRMS-based untargeted metabolomics analytical platform for a long-term clinical metabolomics study. ChemRxiv. 2024.
90. Hong Y, Brise N, Quinn B, Li Y, Jia W, Ruth Sv, et al. MALDI-ToF MS and chemometric analysis as a tool for identifying wild and farmed salmon Food Chemistry. 2023;432.
91. Hosseini S, Martinez-Chapa SO. Fundamentals of MALDI-ToF-MS Analysis : Applications in Bio-Diagnosis, Tissue Engineering and Drug Delivery. Singapore, SINGAPORE: Springer Singapore Pte. Limited; 2016.
92. Krause E, Wenschuh H, Jungblut PR. The Dominance of Arginine-Containing Peptides in MALDI-Derived Tryptic Mass Fingerprints of Proteins. Analytical Chemistry. 1999;71(19):4160-5.

93. Thermo Fisher Scientific Inc. Protein sample preparation and quantitation for mass spectrometry: Thermo Fisher Scientific Inc,; 2007.
94. Chaves AL, Vergara CE, Mayer JE. Dichloromethane as an economic alternative to chloroform in the extraction of DNA from plant tissues. Plant Molecular Biology Reporter. 1995;13(1):18-25.
95. Sigma-Aldrich. Chloroform Safety data sheet. 2023 2024.01.10.
96. Shahinuzzaman ADA, Chakrabarty JK, Fang Z, Smith D, Kamal AHM, Chowdhury SM. Improved in‐solution trypsin digestion method for methanol‐chloroform precipitated cellular proteomics sample. Journal of Separation Science. 2020;43(11):2125-32.
97. National Center for Biotechnology Information. PubChem Compound Summary for CID 6212, Chloroform. 2024 [Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Chloroform.
98. National Center for Biotechnology Information. PubChem Compound Summary for CID 887, Methanol 2024 [Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Methanol.
99. National Center for Biotechnology Information. PubChem Compound Summary for CID 962, Water 2024 [Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Water.
100. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Analytical Biochemistry. 1984;138(1):141-3.
101. Martinez M, Spitali M, Norrant EL, Bracewell DG. Precipitation as an Enabling Technology for the Intensification of Biopharmaceutical Manufacture. Trends in Biotechnology. 2019;37(3):237-41.
102. Subramanian S, Ross NW, MacKinnon SL. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2008;150(1).
103. Fast MD, Ross NW, Mustafa A, Sims DE, Johnson SC, Conboy GA, et al. Susceptibility of rainbow trout Oncorhynchus mykiss, Atlantic salmon Salmo salar and coho salmon Oncorhynchus kisutch to experimental infection with sea lice Lepeophtheirus salmonis. Diseases of Aquatic Organisms. 2002;52:57-68.
104. Minniti G, Rød Sandve S, Padra JT, Heldal Hagen L, Lindén S, Pope PB, et al. The farmed Atlantic salmon (Salmo salar) skin–mucus proteome and its nutrient potential for the resident bacterial community. Genes. 2019;10(7):515.
105. Nolan DT, Ruane NM, Van Der Heijden Y, Quabius ES, Costelloe J, Bonga SEW. Juvenile Lepeophtheirus salmonis (Krøyer) affect the skin and gills of rainbow trout Oncorhynchus mykiss (Walbaum) and the host response to a handling procedure. Aquaculture Research. 2000;31(11):823-33.
106. Ordóñez-Grande B, Guerreiro PM, Sanahuja I, Fernández-Alacid L, Ibarz A. Environmental Salinity Modifies Mucus Exudation and Energy Use in European Sea Bass Juveniles. Animals. 2021;11(6):1580.
107. Vera LM, Migaud H. Hydrogen peroxide treatment in Atlantic salmon induces stress and detoxification response in a daily manner. Chronobiology International. 2016;33(5):530-42.
108. Lanfermeijer M, van Winden LJ, Starreveld DEJ, Razab-Sekh S, Faassen Mv, Bleiker EMA, et al. An LC-MS/MS-based method for the simultaneous quantification of melatonin, cortisol and cortisone in saliva. Analytical Biochemistry. 2024;689:1-6.
109. Kulczykowska E. Stress Response System in the Fish Skin—Welfare Measures Revisited. Frontiers in Physiology. 2019;10:1-3.

110. Krutchinsky AN, Chait BT. On the nature of the chemical noise in MALDI mass spectra. Journal of the American society for mass spectrometry. 2002;13(2):129-34.
111. Staley C, Gould TJ, Wang P, Phillips J, Cotner JB, Sadowsky MJ. Sediments and soils act as reservoirs for taxonomic and functional bacterial diversity in the Upper Mississippi river. Microbial Ecology. 2016;71(4):814-24.
112. Braden LM, Barker DE, Koop BF, Jones SRM. Comparative defense-associated responses in salmon skin elicited by the ectoparasite Lepeophtheirus salmonis. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics. 2012;7(2):100-9.
113. Rosa N, Marques J, Esteves E, Fernandes M, Mendes VM, Afonso Â, et al. Protein Quality Assessment on Saliva Samples for Biobanking Purposes. Biopreservation and Biobanking. 2016;14(4):289-97.
114. Kagan R, Viner TC. Identification of freeze-thaw artifact in fresh and decomposed black rockfish (Sebastes melanops) and steelhead trout (Oncorhynchus mykiss). Veterinary Pathology. 2022;59(6):1022-30.
115. Jamshed L, Debnath A, Jamshed S, Wish JV, Raine JC, Tomy GT, et al. An Emerging Cross-Species Marker for Organismal Health: Tryptophan-Kynurenine Pathway. International Journal of Molecular Sciences. 2022;23(11):1-26.
116. Hod Y, Hershko A. Relationship of the pool of intracellular valine to protein synthesis and degradation in cultured cells. Journal of Biological Chemistry. 1976;251(14):4458-67.
117. van der Meeren T, Olsen R, Hamre K, Fyhn H. Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. Aquaculture. 2008;274:375-97.
118. Rigault C, Mazué F, Bernard A, Demarquoy J, Le Borgne F. Changes in l-carnitine content of fish and meat during domestic cooking. Meat Science. 2008;78(3):331-5.
119. Reda E, D'Iddio S, Nicolai R, Benatti P, Calvani M. The carnitine system and body composition. Acta Diabetologica. 2003;40(S1):s106-s13.
120. Staley KB, Ewing RD. Purine levels in the skin of juvenile coho salmon (Oncorhynchus kisutch) during Parr-smolt transformation and adaptation to seawater. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 1992;101(3):447-52.
121. National Center for Biotechnology Information. PubChem Compound Summary for CID 15793, Octadecylamine. 2024.
122. Błaszczyk A, Augustyniak A, Skolimowski J. Ethoxyquin: An Antioxidant Used in Animal Feed. International Journal of Food Science. 2013;2013:1-12.
123. Bampidis V, Azimonti G, Bastos MDL, Christensen H, Dusemund B, Fašmon Durjava M, et al. Safety and efficacy of a feed additive consisting of ethoxyquin (6‐ethoxy‐1,2‐dihydro‐2,2,4‐trimethylquinoline) for all animal species (FEFANA asbl). ESFA Journal. 2022;20(3).
124. Negreira N, Regueiro J, Valdersnes S, Berntssen MHG, Ørnsrud R. Comprehensive characterization of ethoxyquin transformation products in fish feed by traveling-wave ion mobility spectrometry coupled to quadrupole time-of-flight mass spectrometry. Analytica Chimica Acta. 2017;965:72-82.
125. National Center for Biotechnology Information. PubChem Compound Summary for CID 7410, Acetophenone. 2024.
126. Zubkov FI, Kouznetsov VV. Traveling across Life Sciences with Acetophenone—A Simple Ketone That Has Special Multipurpose Missions. Molecules. 2023;28(1):370.

127. Zhang H, Zhu Y, Liu Z, Peng Y, Peng W, Tong L, et al. A volatile from the skin microbiota of flavivirus-infected hosts promotes mosquito attractiveness. Cell. 2022;185(14):2510-22.e16.
128. Delfosse C, Pageat P, Lafont‐Lecuelle C, Asproni P, Chabaud C, Cozzi A, et al. Effect of handling and crowding on the susceptibility of Atlantic salmon (Salmo salar L.) to Lepeophtheirus salmonis (Krøyer) copepodids. Journal of Fish Diseases. 2021;44(3):327-36.
129. McBride CS, Baier F, Omondi AB, Spitzer SA, Lutomiah J, Sang R, et al. Evolution of mosquito preference for humans linked to an odorant receptor. Nature. 2014;515(7526):222-7.
130. David E, Niculescu V-C. Volatile Organic Compounds (VOCs) as Environmental Pollutants: Occurrence and Mitigation Using Nanomaterials. International Journal of Environmental Research and Public Health. 2021;18(24):13147.
131. Yashin YI, Yashin AY. Chapter 10 - Liquid Chromatography. In: Picó Y, editor. Chemical Analysis of Food: Techniques and Applications. Boston: Academic Press; 2012. p. 285-310.
132. Bruker Daltonik GmbH. Bruker bacterial test standard Bremen: Bruker Daltonik GmbH; 2012.

Supplement

Reference calibration points for BTS

Table S1: Bruker test standard calibration proteins with a mass tolerance error limit of +/- 300 ppm. Data collected from: Bruker Daltonik GmbH (2012).

Protein	***m/z* ion detected**	**Reference mass (average mass)**	**+/- 300 error ppm range**
RL29	$(M+2H)^{2+}$	3637.8 Da	3636.7 Da – 3638.8 Da
RS32	$(M+H)^{+}$	5096.8 Da	5095.3 Da – 5098.3 Da
RS34	$(M+H)^{+}$	5381.4 Da	5379.8 Da – 5383.0 Da
RS33meth	$(M+H)^{+}$	6255.4 Da	6253.5 Da – 6257.3 Da
RL29	$(M+H)^{+}$	7274.5 Da	7272.3 Da – 7276.7 Da
RS19	$(M+H)^{+}$	10300.1 Da	10297.0 Da – 10303.2 Da
RNAse A	$(M+H)^{+}$	13683.2 Da	13679.1 Da – 13687.3 Da
Myoglobin	$(M+H)^{+}$	16952.3 Da	16947.2 Da – 16957.4 Da

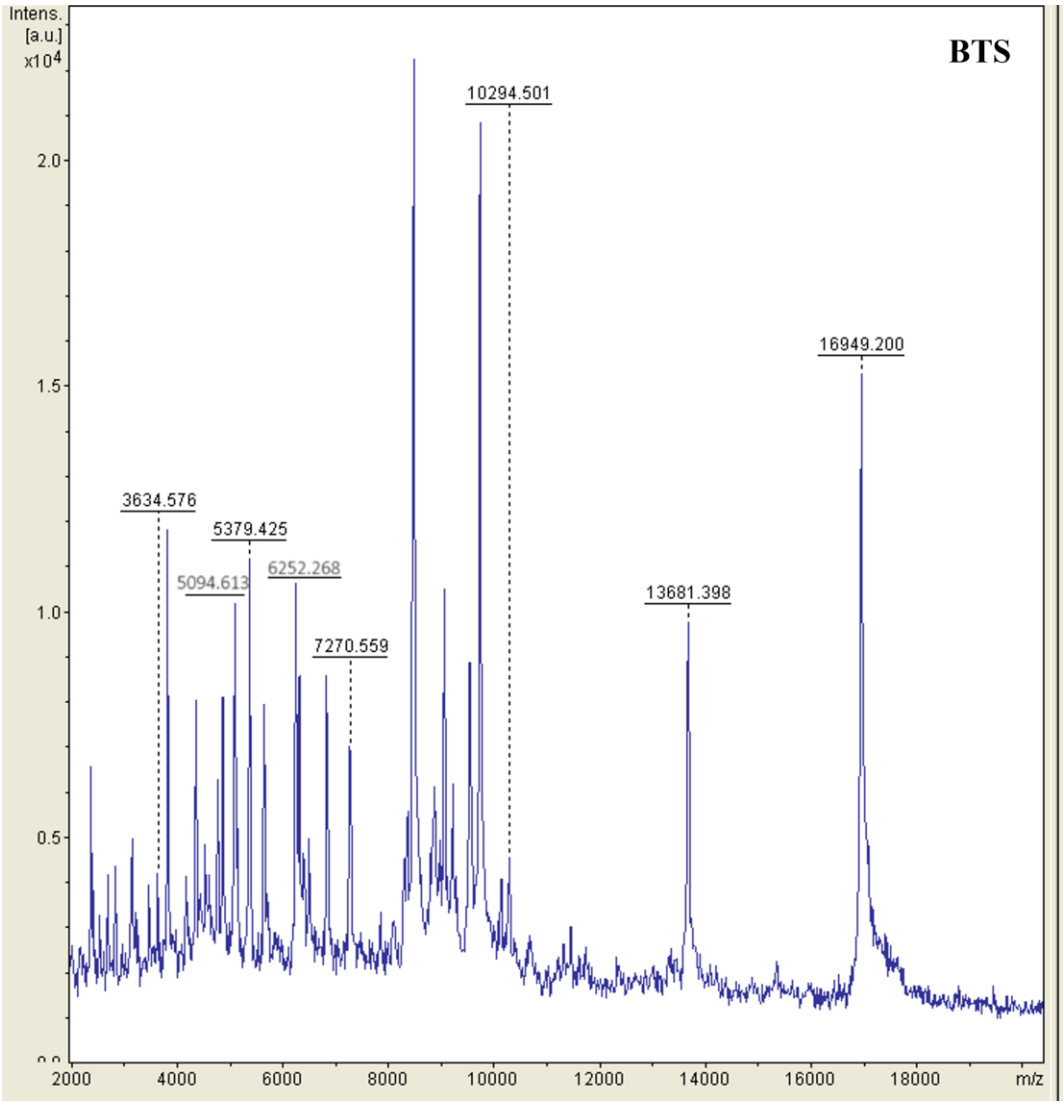

Figure S1. Calibration mass of BTS on MALDI-TOF MS. BTS = bacterial test standard, MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry.

Reference MS spectrum for HCCA and HCCA and chloroform

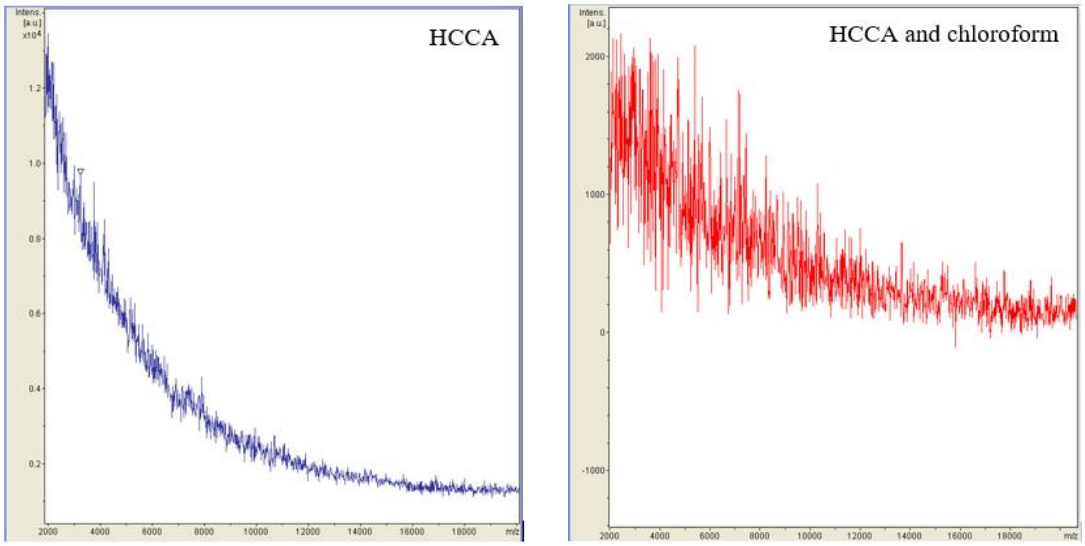

Figure S2. Reference spectrum of HCCA and of HCCA mixed with chloroform obtained after MALDI-TOF MS. HCCA = α-Cyano-4-hydroxycinnamic acid, MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry.

Peak list of characteristic proteins in AS–AQ from MALDI-TOF MS

Table S2. Characteristic proteins detected by MALDI-TOF MS in seven or more individuals (>50%) of Atlantic salmon obtained from an outdoor aquaculture (AQ) facility. The proteins are listed by their average m/z-values ([M+H]+) and these are calculated based on all individuals where the respective protein is detected. Peaks retained with a signal-to-noise at 2.5, and m/z-values between 3000 and 15000. MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry, AQ = aquaculture

Average m/z ± SD	Relative intensity (%) ± SD	Detected in # individuals	
		Of total individuals (13)	Of best individuals (7)
4222.2 ± 1.8	50.0 ± 17.0	9	7
4230.2 ± 2.0	37.7 ± 17.8	7	3
5050.4 ± 2.1	48.9 ± 15.6	7	6
5061.1 ± 3.3	57.3 ± 20.2	7	7
5076.7 ± 4.4	38.6 ± 8.1	7	6
5881.0 ± 3.4	38.3 ± 17.7	10	7
6571.1 ± 1.8	59.4 ± 30.6	8	7
7069.3 ± 3.4	33.1 ± 22.9	8	5
7078.7 ± 2.1	44.3 ± 26.2	7	7
8392.9 ± 2.1	35.9 ± 16.1	9	7
8450.3 ± 1.4	68.5 ± 32.9	10	7
8486.5 ± 3.9	36.1 ± 17.8	7	3
10036.5 ± 3.0	34.3 ± 17.3	7	6
10103.6 ± 2.5	67.2 ± 32.9	8	6
10114.5 ± 3.5	56.4 ± 37.6	9	5
10125.9 ± 3.0	34.2 ± 29.8	7	4
10139.2 ± 2.4	29.6 ± 24.4	7	4
10156.9 ± 3.3	32.2 ± 16.0	8	6
10176.6 ± 4.1	28.2 ± 15.6	8	5
10268.9 ± 1.9	37.3 ± 16.4	7	6
11762.1 ± 3.4	30.8 ± 15.1	9	7
14168.2 ± 4.0	28.4 ± 15.1	8	7

Peak list of characteristic proteins in AS–TARS from MALDI-TOF MS

Table S2. Characteristic proteins detected by MALDI-TOF MS in five or more individuals (≥50%) of Atlantic salmon obtained from an indoor aquaculture (TARS) facility. The proteins are listed by their average m/z-values ($[M+H]^+$) and these are calculated based on all individuals where the respective protein is detected. Peaks retained with a signal-to-noise at 2.5, and m/z-values between 3000 and 15000. MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry, TARS = Tromsø Aquaculture Research Station

Average m/z ± SD	Relative intensity (%) ± SD	Detected in # individuals	
		Of total (10)	Of best individuals (7)
3247.2 ± 1.6	57.3 ± 22.4	7	6
3313.8 ± 1.7	62.5 ±27.2	8	6
3338.5 ± 1.3	43.4 ± 14.1	9	7
3383.8 ± 1.7	63.7 ± 21.3	6	7
3927.0 ± 1.3	63.5 ± 28.0	7	5
4283.6 ± 1.3	46.6 ± 16.3	8	7
4355.4 ± 1.3	80.3 ± 19.0	8	7
5062.1 ± 2.7	32.3 ± 20.9	8	6
6071.5 ± 2.3	25.2 ± 11.3	9	7
6624.8 ± 2.0	29.7 ± 13.0	8	6

6648.8 ± 2.3	21.8 ± 12.0	7	5
7631.9 ± 1.8	19.3 ± 12.5	5	4
8568.1 ± 1.6	32.4 ± 7.7	7	7
10119.5 ± 2.6	35.2 ± 22.7	10	7
10131.8 ± 3.4	17.7 ± 7.9	6	4
10161.9 ± 3.2	20.8 ± 13.1	8	7
10181.3 ± 1.8	16.4 ± 8.1	7	6
10192.4 ± 4.0	15.2 ± 8.2	8	7

Peak list of characteristic proteins in PS–river and PS–ocean from MALDI-TOF MS

Table S4. Characteristic proteins detected by MALDI-TOF MS in five or more individuals (≥50%) of pink salmon caught from the ocean (saltwater, SW) and river (freshwater, FW). The proteins are listed by their average m/z-values ([M+H]+) and are calculated based on all individuals where the respective protein is detected. Peaks retained with a signal-to-noise at 2.5, and m/z-values between 3000 and 15000. MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry.

Average m/z ± SD	**Relative intensity (%) ± SD**	**Detected in # individuals**		
		Ocean (5 in total)	**River (5 in total)**	**Ocean+river (10 in total)**
3116.5 ± 0.9	76.5 ± 18.4	5	1	6
3140.6 ± 1.5	45.2 ± 17.1	4	1	5
3155.6 ± 2.1	30.6 ± 14.2	4	4	8
3227.7 ± 0.8	66.3 ± 22.8	5	1	6
3241.0 ± 2.3	32.2 ± 8.2	4	1	5
3680.2 ± 1.7	40.0 ± 18.4	4	3	7
4225.0 ± 1.9	32.6 ± 15.3	3	1	4
4735.1 ± 2.4	38.2 ± 7.0	4	0	4
4806.5 ± 0.9	60.5 ± 15.8	5	0	5
4822.2 ± 1.1	52.3 ± 8.6	5	0	5
4849.5 ± 3.2	50.0 ± 7.6	4	0	4
4858.2 ± 2.5	53.0 ± 22.0	4	1	5
4898.1 ± 1.8	31.2 ± 5.2	3	2	5
4935.2 ± 1.8	46.0 ± 15.6	3	2	5
5159.3 ± 1.3	48.4 ± 19.3	5	1	6
5423.6 ± 0.6	58.6 ± 21.9	4	0	4
5611.5 ± 1.3	45.7 ± 23.3	5	2	7
6143.3 ± 2.5	52.3 ± 17.0	4	0	6
6153.6 ± 1.4	48.8 ± 21.4	5	1	6
8456.0 ± 1.7	25.2 ± 8.5	5	2	7
8570.1 ± 2.6	24.5 ± 13.3	5	2	7
9719.5 ± 2.2	32.5 ± 31.7	4	2	6
12168.2 ± 1.6	25.0 ± 19.5	3	1	4
13445.8 ± 2.6	21.5 ± 19.5	3	1	4
13468.5 ± 3.2	17.3 ± 18.8	2	4	6

Peak list of characteristic proteins in Atlantic cod from MALDI-TOF MS

Table S5. Characteristic proteins detected by MALDI-TOF MS in four or more individuals (>50%) of wild caught Atlantic cod. The proteins are listed by their average m/z-values ($[M+H]^+$) and these are calculated based on all individuals where the respective protein is detected. Peaks retained with a signal-to-noise at 2.5, and m/z-values between 3000 and 15000. MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry.

Average m/z ± SD	Relative intensity (%) ± SD	Detected in # individuals of total (7)
3425.7 ± 2.9	24.6 ± 8.1	5
3772.4 ± 2.0	31.1 ± 7.4	4
4134.6 ± 1.0	77.4 ± 28.0	5
5155.2 ± 1.4	52.4 ± 20.1	4
5254.6 ± 1.9	51.5 ± 27.5	5
5420.3 ± 2.9	42.6 ± 14.6	4
5439.8 ± 1.6	26.7 ± 8.1	4
5616.4 ± 1.8	53.4 ± 28.4	4
5731.1 ± 1.7	64.4 ± 22.5	6
6875.9 ± 3.2	49.7 ± 31.0	6
6928.6 ± 3.1	39.0 ± 15.6	7
6990.0 ± 2.5	35.9 ± 15.5	4
7076.5 ± 1.8	46.6 ± 25.4	7
8564.7 ± 2.4	33.6 ± 23.4	5
11417.2 ± 2.7	28.8 ± 9.2	4
11460.4 ± 2.3	51.6 ± 36.3	7

Peak list of characteristic proteins in coalfish from MALDI-TOF MS.

Table S6. Characteristic proteins detected by MALDI-TOF MS in four or more individuals (>50%) of wild caught coalfish. The proteins are listed by their average m/z-values ($[M+H]^+$) and these are calculated based on all individuals where the respective proteins is detected. Peaks retained with a signal-to-noise at 2.5, and m/z-values between 3000 and 15000. MALDI-TOF MS = Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry.

Average m/z ± SD	Relative intensity (%) ± SD	Detected in # individuals of total (7)
3501.1 ± 2.0	26.9 ±10.1	4
3621.9 ± 2.0	18.2 ± 7.3	4
3743.1 ± 1.3	45.1 ± 24.5	4
3843.9 ± 1.5	26.0 ± 12.6	5
4008.5 ± 1.1	18.9 ± 6.6	4
4220.2 ± 2.1	40.7 ± 27.2	4
4351.1 ± 0.5	25.2 ± 6.6	4
4653.5 ±1.2	28.1 ± 8.0	5
4695.7 ± 2.1	64.5 ± 34.7	5
4808.4 ± 2.7	48.1 ± 22.9	7
5151.7 ± 1.7	28.2 ± 10.9	5
5187.1 ± 1.5	48.5 ± 27.7	5
5261.7 ± 2.6	51.5 ± 39.2	4
5315.9 ± 2.3	77.8 ± 28.3	6
5623.3 ± 4.3	27.1 ± 13.4	5
5745.7 ± 2.9	37.0 ± 24.5	6

6608.0 ± 3.2	42.4 ± 26.6	6
6655.6 ± 2.4	23.3 ± 12.5	6
7116.1 ± 1.3	40.7 ± 22.5	6
7152.2 ± 3.4	23.4 ± 13.8	4
7262.9 ± 2.5	51.4 ± 34.4	7
7297.9 ± 2.4	18.9 ± 5.7	4
8449.9 ± 1.9	37.1 ± 33.4	4
8507.3 ± 2.6	19.4 ± 14.6	5
8564.0 ± 1.8	16.2 ± 5.1	4
10108.4 ± 3.4	14.8 ± 9.6	7
11459.1 ± 3.6	10.7 ± 5.0	4
11486.0 ± 2.6	31.2 ± 30.4	7
13222.5 ± 2.1	27.8 ± 21.7	6