Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Emma Vangen

The quality of thawed Atlantic salmon (*Salmo salar*) fillets as affected by sub-chilling prior to freezing

Master's thesis in Biotechnology
Supervisor: Jørgen Lerfall
Co-supervisor: Bjørn Tore Rotabakk
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Abstract

The export of fish, especially Atlantic salmon (*Salmo salar*), is one of Norway's most important industries. When exported to Asia and the US, the fish is usually chilled with ice and shipped in expandable polystyrene (EPS) boxes by air freight. In 2019, more than 75% of the exported salmon was unprocessed, which profoundly increases the edible product's emission. However, by freezing and shipping by boat, transportation emissions can be reduced by up to 90%. Although better emission-wise, freezing can alter the quality of the product, creating a product that is inherently different from the fresh product.

This master's thesis aimed to examine how the internal temperature of Atlantic salmon before freezing affected the quality of the fish after it had been thawed. Sub-chilling is a form of super-chilling in which the temperature of the fish is lowered to about -1.5 °C. This method of chilling was compared with traditional chilling on ice. The sub-chilling used was refrigerated seawater (RSW)- a setup created using a 7% NaCl brine and ice, creating an environment of around -1.0 °C. Ice-chilled fish remained in EPS boxes with ice. A storage trial was also introduced, comparing sub-chilled fish and ice-chilled fish stored fresh, 1 month frozen, and 4 months frozen. The fillets were thawed and placed in a cooling room at the end of the freezing time. Analyses were performed after 1, 5, 12, and 16 days in the cooling room. The focus was on water content, drip loss, texture, color, protein denaturation, microbial growth, and degradation of adenosine triphosphate.

Sub-chilled fish had higher drip loss than ice-chilled fish, less growth of mesophilic bacteria, and higher concentrations of inosine monophosphate. In combination with frozen storage, sub-chilling provided a better product in several of the measured parameters. Significant differences were found when looking at the storage method, where frozen fish had higher drip loss than fresh fish; fresh fish had more growth of psychrotrophic bacteria, required more force regarding breaking force and firmness, and had a higher chroma and lower hue. A conclusion was made that sub-chilling in combination with frozen storage provides a better-quality product than chilling with ice; there was not a great difference between the frozen samples regarding the duration of freezing, and as storage days increased in a cooling room, the samples deteriorated accordingly.

Sammendrag

Eksport av fisk, spesielt atlantisk laks (*Salmo salar*), er en av Norges viktigste næringer. Når fisken eksporteres til Asia og USA, blir fisken vanligvis nedkjølt med is og sendt i ekspanderbare polystyrenbokser (EPS) med fly. I 2019 var mer enn 75 % av den eksporterte laksen ubearbeidet, noe som øker det spiselige produktets utslipp betydelig. Ved frysing og frakt med båt kan imidlertid transportutslippet reduseres med opptil 90 %. Selv om det er bedre utslippsmessig, kan frysing endre kvaliteten på produktet, og skape et produkt som er forskjellig fra det ferske produktet.

Denne masteroppgaven hadde som hensikt å undersøke hvordan den indre temperaturen til Atlantisk laks før frysing påvirket kvaliteten på fisken etter at den var tint. Subkjøling er en form for superkjøling der temperaturen på fisken senkes til ca. -1,5 °C. Denne metoden for nedkjøling ble sammenlignet med tradisjonell kjøling på is. Et nedkjølt sjøvann (eng. refrigerated seawater) (RSW)-oppsett ble lagd ved å bruke 7 % saltlake og is. Temperaturen på løsningen var rundt -1,0 °C og fisken ble deretter tilført oppsettet. Isavkjølt fisk ble liggende i EPS-bokser med is. Et lagringsforsøk ble også introdusert hvor subkjølt fisk og iskjølt fisk lagret fersk, 1 måned frossen og 4 måneder frossen ble sammenliknet. På slutten av frysetiden ble filetene tint og plassert i et kjølerom. Analyser ble utført etter 1, 5, 12 og 16 dager i kjølerommet. Det ble fokusert på vanninnhold, drypptap, tekstur, farge, proteindenaturering, mikrobiell vekst og nedbrytning av adenosintrifosfat.

Resultatene viste at subkjølt fisk hadde høyere drypptap enn iskjølt fisk, mindre vekst av mesofile bakterier, og høyere konsentrasjoner av inosinmonofosfat. I kombinasjon med fryselagring ga subkjøling et bedre produkt i flere av forsøksparameterne. Signifikante forskjeller ble funnet når man så på lagringsmetoden hvor frossen fisk hadde høyere drypptap enn fersk fisk, fersk fisk hadde mer vekst av psykrotrofe bakterier, krevde mer kraft mht. bruddkraft og fasthet, hadde høyere kroma og lavere fargetone. Det ble konkludert med at subkjøling i kombinasjon med fryselagring gir et produkt av bedre kvalitet enn kjøling med is, det var ikke stor forskjell mellom de frosne prøvene mht. varigheten av frysetid og ettersom lagringsdagene økte i kjølerommet, forringet prøvene deretter.

Preface

This master's thesis has been completed as a final thesis in the 5-year master's program of Biotechnology at the Norwegian University of Science and Technology (NTNU) Trondheim, counting for 60 credits. The thesis was carried out in collaboration with NTNU and Nofima AS and is a part of the research project nr. 901635 «*Nye metoder for bedre holdbarhet og miljøvennlig transport av lakseprodukte*r», founded by FHF – Norwegian Seafood Research Fund. The FHF project is divided into three parts, where part 1 involves finding new conservation methods for salmon that can reduce the environmental impact while maintaining quality. Part 2 examines consumers and the market's attitude towards these changes. Part 3 quantifies the environmental impact and makes economic calculations of the conservation methods. The thesis is mainly within part 1.

I want to thank my main supervisor Professor Jørgen Lerfall at NTNU, for introducing me to this project, for the help I got processing results, and completion of the thesis. I appreciate the time and effort you have put into supporting me. A big thank you to my co-supervisor, Dr. Bjørn Tore Rotabakk at Nofima, for welcoming me to Stavanger, helping me settle, and being available whenever I had any questions. You have been there for the breakthroughs and the breakdowns, you have been patient, and you have exceeded my expectations for a supervisor. I appreciate the time and effort you have put into helping me, and last but not least, thank you for eating lunch with me almost every day. I want to thank Nofima for giving me this opportunity and everyone working at Nofima for being so welcoming. I would also like to thank laboratory technician Karin Tranøy for all the help in the laboratory and thanks to staff engineer John-Kristian Jameson for the help I received when performing HPLC.

To my classmates, thank you for these memorable five years, for the support during the ups and downs, for the countless hours studying together, for the parties and the after-parties, and for the long-lasting friendship I have developed. I just want to say: We did it! I would also like to thank my family, friends, and boyfriend for the never-ending support. Without you, I could not have done this.

Emma Rakvåg Vangen

Emma Vangen

Trondheim,
May 16th 2022

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Abbreviations

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
APC	Aerobic Plate Count
ATP	Adenosine Triphosphate
CAF	Calcium-Activated Factors-
Cu	Copper
DHA	Docosahexaenoic Acid
DMA	Dimethylamine
DSC	Differential Scanning Calorimetry
EPA	Eicosapentaenoic Acid
EPS	Expanded Polystyrene
FA	Formaldehyde
GLM	General Linear Model
Hx	Hypoxanthine
HPLC	High-Pressure Liquid Chromatography
H_2S	Hydrogen Sulfide
IMP	Inosine Monophosphate
Ino	Inosine
LC-PUFA	Long Chain Polyunsaturated Fatty Acids
L&H	Long & Hammer
L*	Lightness
Mn	Manganese
MUFA	Mono-Unsaturated Fatty Acid
NQC	Norwegian Quality Cut
PUFA	Polyunsaturated Fatty Acid
RSW	Refrigerated Sea Water
Se	Selenium
SFA	Saturated Fatty Acid
SSOs	Specific Spoilage Organisms
T_m	Peak Maximum Temperatur
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TVC	Total Psychrotrophic Viable Count
UA	Uric Acid
UN	United Nations
WHC	Water Holding Capacity
Zn	Zinc

1 Introduction

In 2015, the United Nations (UN) general assembly provided a plan for the world to develop in a more sustainable matter: The 2030 Agenda for Sustainable Development. A blueprint for "peace and prosperity, for the people and the planet, now and into the future" was provided with 17 sustainable development goals as the framework and 169 targets as a secondary structure (UN, 2015). The agenda has become important regarding the socio-economic and environmental issues within a country, both developed and developing (Abualtaher et al., 2021; Regost et al., 2004). For Norway, which has been a member since the UNs foundation in 1945, it has been important to actively partake in and implement the goals in the country's industry.

One of Norway's most important industries is the production of Atlantic salmon (*Salmo salar*). In fact, Norway contributes 55.3% of the total production share globally, and the fish export created a value of NOK 81.4 billion in 2021 (Iversen et al., 2020; Sørli and Aandahl, 2020). Even though the industry has experienced massive growth from the first farmed salmon was slaughtered in 1971 until now; the goal is to reach an export value of NOK 240 billion in 2050 (Midsund, 2021; Olafsen et al., 2012). Together with the aim of growth within the industry, there is also a goal that Norway's seafood industry will be responsible for the world's most environmentally friendly production of healthy food (Alsos, 2018). Here, the UN's sustainable development goals play an important role. Especially number 2- zero hunger, 3- good health and well-being, 8- decent work and economic growth, 12- responsible consumption and production, 13- climate action, and 14- life below water are important goals relevant to Norway's wish for sustainable aquaculture.

With increased production and export, a natural increase in emission is expected, especially since fresh produce is the most sought-after. In 2019, more than 75% of the exported salmon was unprocessed, meaning a lot of by-products like head and tail, ice, and polystyrene boxes were being transported across the world, increasing the emission of the actual edible product profoundly (Winther et al., 2020). When looking at Figure 1.1, fresh, gutted salmon exported by air freight to Asia emits the absolute most greenhouse gasses. This is also the dominating transportation method today when exporting to Asia. By solely super-chilling the fish, the

emission due to transport can be reduced by 20%; by both filleting and super-chilling, the greenhouse gas emission can be reduced up to 50% (column 6) (Iversen et al., 2021). It is, however, worth noting that when looking solely at emissions from transportation, the most improvement lies in substituting air freight with boat transportation. As this type of transportation is more time-consuming when shipping to Asia, solely sub-chilling the fillets will not be sufficient, and freezing will be necessary. By freezing the fillets, transportation emissions can be reduced by up to 90% (column 4) (Rotabakk et al., 2020).

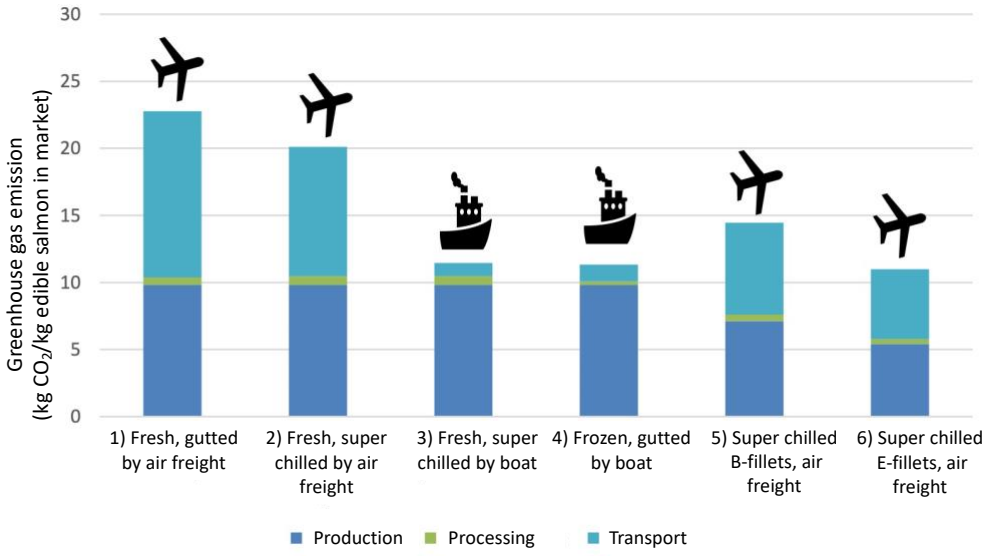

Figure 1.1: Amount of greenhouse gas emission in kg CO_2/kg edible product from six different production, processing- and transportation chains regarding export to Asia (Shanghai). Modified from Iversen et al. (2021).

Super-chilling has proven effective in maintaining the quality and improving the shelf-life of tuna and poultry (Kang et al., 2020; Kaewthong et al., 2019). Several research projects have also implemented this technology to understand its effect on salmon, with promising results (Erikson et al., 2011; Beaufort et al., 2009; Chan et al., 2020b). Super-chilling is the practice of reducing the internal temperature of the fish to 1-2 °C below the initial freezing point, while sub-chilling is a form of super-chilling, reducing the temperature of the fish to around -1.5 °C (Duun and Rustad, 2007). By keeping the fish at this temperature, both bacterial and enzymatic activity is slowed down, and the formation of damaging ice crystals is stalled, resulting in a product that has better quality and longer shelf-life (Koutsoumanis et al., 2021). To look further into this kind of research, Norwegian Seafood Research Fund (FHF) has granted the project «*Nye metoder for bedre holdbarhet og miljøvennlig transport av lakseprodukte*r»

(FHF project nr. 901635). The project's main objective was to find alternatives to the current conservation methods by evaluating existing methods, assessing new methods, and a combination of these, with emphasis on quality, durability, environmental impact, consumer acceptance, and economy. This master's thesis is a part of that project, and the goal is to enhance understanding regarding the conservation of the quality of thawed Atlantic salmon by sub-chilling before freezing.

1.1 Objectives

The specific objective of this master project consists of several parts and focuses on the following topics:

1. Examine if the initial temperature before freezing affects the quality of the fish after thawing.
2. Comparing fresh and frozen fish regarding quality.
3. Examine how storage time, both with regards to days stored in a cooling room and days stored as frozen, affects the fish's quality.

The project's objectives were evaluated by following the evaluation of microbial activity, drip loss, water content, color, texture, protein denaturation, and adenosine triphosphate (ATP)-metabolites throughout the experiment.

2 Theory

Looking into the dietary benefits of consuming salmon, it becomes clear why it is an excellent source of nutrients. It has a high content of proteins, low content of saturated fat and cholesterol, and a decent amount of minerals and vitamins. However, it is the omega-3, long-chain polyunsaturated fatty acids (LC-PUFA) that people mostly associate salmon with. The LC-PUFA, eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) are extremely important in human health as they play critical roles in cardiovascular health but also in neurological development (Colombo and Mazal, 2020). Nonetheless, salmon is not just salmon. There are several different species of salmon in the world that differ in everything from the range of age, size, location, and biological-and chemical composition. However, this project focuses only on Atlantic salmon.

2.1 Chemical Composition of Salmon Muscle

The muscle of Atlantic salmon is composed of mostly water (60-65%), a large portion of proteins (18-20%), and a large amount of lipids (16-19%) (Einen et al., 1998; Shearer, 1994). Other important molecules found in smaller quanta are vitamins, minerals, pigment, and non-protein-nitrogen (Lynum and Rustad, 1997).

2.1.1 Water

Around 85% of the water in a muscle cell is found within the myofibrils, some water is located between the myofibrils and the cell membrane, and some is found between the myofibrils (Huff-Lonergan and Sosnicki, 2002; Huff-Lonergan and Lonergan, 2005). Since water is a dipolar molecule, it can attract charged molecules, like proteins. In fact, one-tenth part of the total water in muscle cells is bound to proteins, and this water is relatively resistant to freezing. Another fraction is referred to as entrapped water. This is water kept in place by steric effects or by the attraction to the water bound to proteins. This water is not resistant to freezing. The last fraction is called free water. Free water is held by weak surface forces, and its flow within the tissue is unhindered (Huff-Lonergan and Lonergan, 2005).

2.1.2 Fat

Salmon is considered a fatty fish, meaning it stores its lipids in the flesh, contrary to lean fish storing its lipids in the liver (Calder, 2021). Lipids can be classified as fats, phospholipids, sphingomyelins, waxes, and sterols, and they each have different roles within the body and the cells (Halver, 1978). Oxidation of fatty acids and triacylglycerols is a primary provider of metabolic energy, and fatty acids are generally the preferred energy source for growth, reproduction, and swimming (Tocher, 2003). Phospholipids are found in the membranes of the cellular structures creating a hydrophilic-hydrophobic environment, sphingomyelins are mostly found within the brain tissue, sterols are found in the endocrine system, and waxes can be oxidized and used for energy (Halver, 1978).

Fatty acids are named based on the number of carbons and double bonds. A saturated fatty acid (SFA) has no double bonds, mono-unsaturated has one double bond (MUFA), and polyunsaturated fatty acids (PUFA) contain more than one double bond (Moghadasian and Shahidi, 2017). Salmon needs, like any other vertebrate, LC-PUFAS in their diet to maintain cellular function and integration, growth, reproduction, and synthesis of other molecules like hormones. Especially DHA (22:6*n*−3) and EPA (20:5*n*−3) are of importance, as these are fatty acids that the salmon cannot synthesize *in vivo* (Sargent et al., 1999). Wild salmon obtain their dietary fatty acids from consuming smaller fish and algae, while farmed salmon is dependent on the feed containing the fatty acids (Strobel et al., 2012).

2.1.3 Proteins

Proteins found in the muscle tissue are classified as myofibrillar, stromal, or sarcoplasmic proteins (Strasburg et al., 2008). As the name implies, myofibrillar proteins are the proteins within the myofibrillar and constitute the largest fraction with approximately 50-60% of total muscle protein. Common proteins in this group are actin, myosin, tropomyosin, and troponin. Stromal proteins constitute 10-20% of total protein content in the muscles, and these proteins form the connective tissue layers such as epimysium, perimysium, and endomysium. Collagen is the most abundant stromal protein. The sarcoplasmic proteins are found in the sarcoplasm and constitute about 30% of total muscle protein content and are primarily enzymes like glycolytic enzymes and myoglobin (Strasburg et al., 2008).

Myosin is the most abundant protein in the muscle and is the operational unit for muscle contraction (Strasburg et al., 2008). The protein is composed of a heavy chain consisting of an α-helix of amino acids and a head that contains the ATP binding site seen in Figure 2.1 (Jena, 2020).

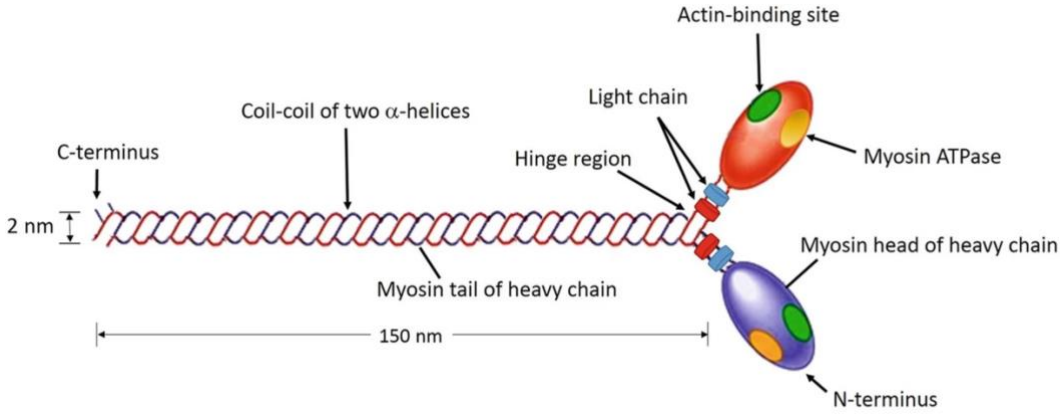

Figure 2.1: Illustration of myosin showing the head with actin binding site and ATPase activity, and the α-helix tail (Jena, 2020).

Actin is the second most abundant protein in the muscle and makes up around 20% of the myofibrillar protein content (Strasburg et al., 2008). The protein can transition between a monomeric form (G-actin) and a filamentous form (F-actin) (Dominguez and Holmes, 2011). When actin binds to myosin, it activates myosin's ATPase activity, allowing myosin to act as a molecular motor that pulls the actin past the myosin, eventually resulting in a shortened sarcomere. Actin also binds troponin and tropomyosin, which are regulatory proteins that regulate the interaction between actin and myosin as a response to changes in the concentration of calcium (Strasburg et al., 2008). Myofibrillar proteins are salt-soluble, meaning that the proteins are soluble if a solution has an ionic strength > 0.3M (Widyastuti et al., 2020).

Sarcoplasmic proteins consist mainly of enzymes involved in glycolysis, glycogen synthesis, and glycogenolysis. However, other proteins like myoglobin, albumins, creatine kinase, and calpains are also classified as sarcoplasmic proteins. Their enzymatic activity contributes to the quality deterioration prior to bacterial degradation (Strasburg et al., 2008). These proteins are the water-soluble proteins in a fish (Lin et al., 2021).

2.1.4 Pigment

The characteristic red-pink color of salmonoids results from the carotenoids found in the muscles. Several factors determine the degree of pigmentation, like sexual maturation, size and growth rate, age, and genetic background (Torrissen and Naevdal, 1988). It is essential to know about the pigmentation in salmon as it is one of the most important quality parameters (Anderson, 2012).

Carotenoids are the pigment group responsible for the yellow to red colors in the cells. Over 600 carotenoids have been identified, and most of them are hydrocarbons containing 40 carbo-atoms. The fish itself cannot synthesize this specific group, and it is crucial for the fish to obtain the compound via the consumption of food (Anderson, 2012). For salmon, astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) and canthaxanthin (β,β-carotene-4,4'-dione) are the most important carotenoids (Matthews et al., 2006). The process by which the carotenoid is absorbed, cleaved, and deposited in the muscle is poorly studied in vertebrates; however, researchers have tried to create models that mimic the retention of pigment in order to understand the process (Rajasingh et al., 2006).

2.1.5 Vitamins and Minerals

Vitamins and minerals are essential micronutrients that do not work as energy sources but are rather crucial for cellular mechanisms and pathways. Vitamins are categorized as water-soluble (vitamin B and C) or fat-soluble (vitamin A, E, D, and K) and cannot be synthesized endogenously. Salmon is, therefore, dependent on obtaining the necessary vitamins through food consumption (Kangsen et al., 2022; Hamre et al., 2004). Minerals are essential elements needed by an organism to function appropriately (Zoroddu et al., 2019).

The tissue of salmon is, as mentioned, rich in fatty acids. Fatty acids are highly susceptible to peroxidation, especially *in vivo*, simply by normal metabolism, which can cause inflammation and other cellular damage. Therefore, it is essential that the fish has a defense against this oxidation, and a part of this defense are different vitamins and minerals acting as antioxidants (Hamre et al., 2004). Studies have concluded that vitamin E and C are the primary antioxidants in animal tissue. Vitamin E hinders lipid peroxide radicals from reacting with PUFAs, while vitamin C donates electrons to radicals formed in the water phase, eventually terminating

their reactivity (Martínez-Alvarez et al., 2005). Vitamin C can also regenerate the antioxidative state of vitamin E by reducing tocopheroxyl radicals (Pehlivan, 2017). Some dietary minerals like manganese (Mn), copper (Cu), selenium (Se), and Zinc (Zn) have also been proven effective when it comes to the prevention of lipid peroxidation (Martínez-Alvarez et al., 2005).

2.1.6 Nonprotein-Nitrogen

Nonprotein-nitrogenous compounds are water-soluble compounds that do not contain amino acids (Simidu, 1962). The compounds include miscellaneous substances like urea, purines, pyrimidines, guanidine and imidazole derivatives, and ammonia and trimethylammonium bases, which all contribute to different processes in the fish. The compounds constitute only a tiny portion of the total nitrogen content in the fish; however, they are of great importance when processing the fish and preserving fish products (Velankar and Govindan, 1958).

Salmon is rich in the imidazole derivative anserine (β-alanyl-N-methylhistidine). This compound is important as it has a strong buffering capacity and regulates the muscle pH when anaerobic burst swimming is necessary (Ogata et al., 1998). The derivative is also thought to govern the postmortem pH, an important factor for the textural properties (Puolanne and Kivikari, 2000). Trimethylamine N-oxide (TMAO) is an important osmolyte found in all teleost fish (Shumilina et al., 2016). TMAO can be degraded to trimethylamine (TMA), dimethylamine (DMA), and formaldehyde (FA) by endogenous enzymes or by bacterial breakdown contributed by the fish's microflora (Summers et al., 2017). Although found in small amounts in salmon, the breakdown of the compound is one of the main reasons fish acquires the characteristic "fishy" smell of spoiled fish (Fidalgo et al., 2019).

2.1.7 Energy

Like any other living species, the Atlantic salmon is dependent on ATP production for several cellular mechanisms. ATP is a nucleoside triphosphate acting as a coenzyme that transports chemical energy within cells. In muscle cells, the energy is used to do mechanical work and synthesize proteins (Hong et al., 2017). The precursor for cellular metabolism is glucose, which can be catabolized to ATP in three processes subsequently: glycolysis, Krebs cycle, and oxidative phosphorylation (Bonora et al., 2012). Glycolysis breaks glucose into two pyruvate molecules, a three-carbon compound that is further catabolized in the Krebs cycle (Kumari,

2018). Prior to entering the Krebs cycle, the pyruvate molecules must be further converted to Acetyl-CoA, which happens when the molecules are transported from the cytosol and into the mitochondria (Bonora et al., 2012). In the matrix of the mitochondria, Acetyl-CoA is converted to oxaloacetate through a series of steps, eventually forming three NADH molecules, one $FADH_2$ molecule, and two ATP (Fernie et al., 2004). Electrons from NADH and $FADH_2$ are further utilized in oxidative phosphorylation, where the net gain of ATP is 36. Oxidative phosphorylation is aptly named after the dependence of oxygen as the last electron acceptor (Cardol et al., 2009; Melkonian and Schury, 2022). When the cells are oxygen-deprived, the pyruvate does not enter the mitochondria and is instead converted to lactate, and the primary ATP production is glycolysis. The glycolysis produces, as mentioned, two ATP molecules. However, the glycolysis is approximately 100 times faster than oxidative phosphorylation, making it a suitable energy production for muscle tissue rapidly contracting (Melkonian and Schury, 2022).

2.2 Muscle Composition of Salmon

65% of the body mass in salmonoids is muscle tissue, making it the most abundant type of tissue (Johnston et al., 1999). Contrary to terrestrial animals, fish does not have muscles that connect to the skeleton, meaning there is little to no skeleton support. Instead, the muscle cells are structured in parallel bundles, called myotomes, which are attached to connective tissue called myocommata, which again are attached to both the skin and skeleton (Huss, 1995). The muscles consist of around 90% muscle fibers and 10% connective tissue and fat (Listrat et al., 2016). The connective tissue consists mainly of collagen, elastin, and reticular fibers and provides the transmission of contractile forces between muscle segments, the skeleton, and the skin (Lynum and Rustad, 1997). Muscle segments and connective tissue are structured in a W-shape, as shown in Figure 2.2. The connective tissue that encloses an entire muscle is called the epimysium. Endomysium encloses the individual muscle fibers, while perimysium encloses the fascicles, as seen in Figure 2.3 (Listrat et al., 2016). The two types of muscle fibers found in salmon are red and white, whereas red is mainly used for low-speed swimming using aerobic respiration, and white is for short periods of maximum speed using glycolysis (Kiessling et al., 2006; Syme and Shadwick, 2011).

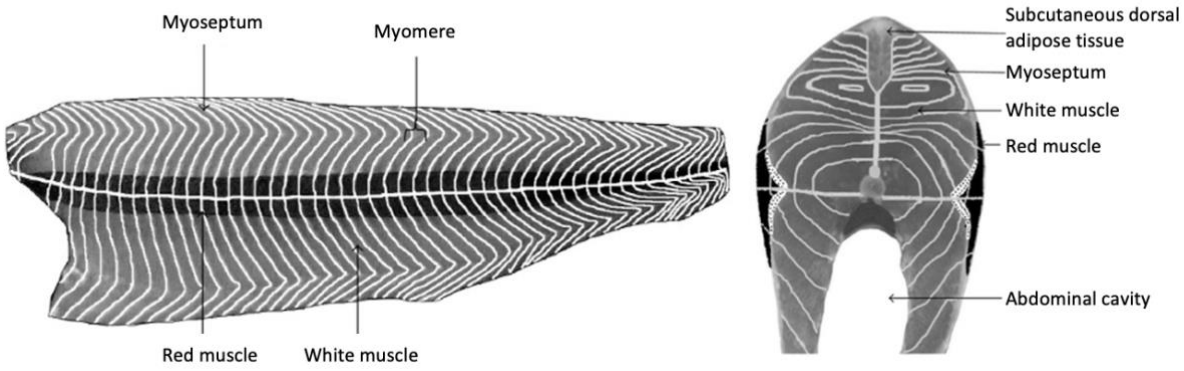

Figure 2.2: Sagittal cross-section of a salmon showing the W-shape of the muscle segments and the connective tissue, and a transverse cross-section of the muscle showing the red and white muscle. Modified from Listrat et al. (2016).

The muscle cells are commonly referred to as muscle fibers or myofibers as they appear long and threadlike. A bundle of muscle fibers creates the secondary structure known as a fascicle, and several fascicles create a muscle (Huff-Lonergan and Lonergan, 2005). The muscle fibers are constructed of myofibrils consisting of myofilaments, consisting of the proteins actin, troponins T, I, and C, tropomyosin, and myosin (Huss, 1995). When dissecting a myofibril longitudinal, alternating dark A-bands and light I-bands can be observed, whereas Z-lines separate the I-bands. Between the Z-lines, as a repeating unit, the sarcomere is found, which is responsible for the myofibril's contractive forces (Listrat et al., 2016). These structures can be seen in Figure 2.3. Surrounding the muscle fibers is a plasma membrane called the sarcolemma, which acts as a barrier between the extracellular and intracellular parts of the muscle cell. A sub-sarcolemma protein assembly known as costamere connects the sarcomeres to the sarcolemma. This assembly consists of two major protein complexes: the dystrophin-glycoprotein complex and the integrin–vinculin–talin complex (Peter et al., 2011). The complexes regulate the interactions between the extracellular matrix and the cytoskeleton, whereas dystrophin is thought to have additional structural properties (Peter et al., 2011; Jaka et al., 2015).

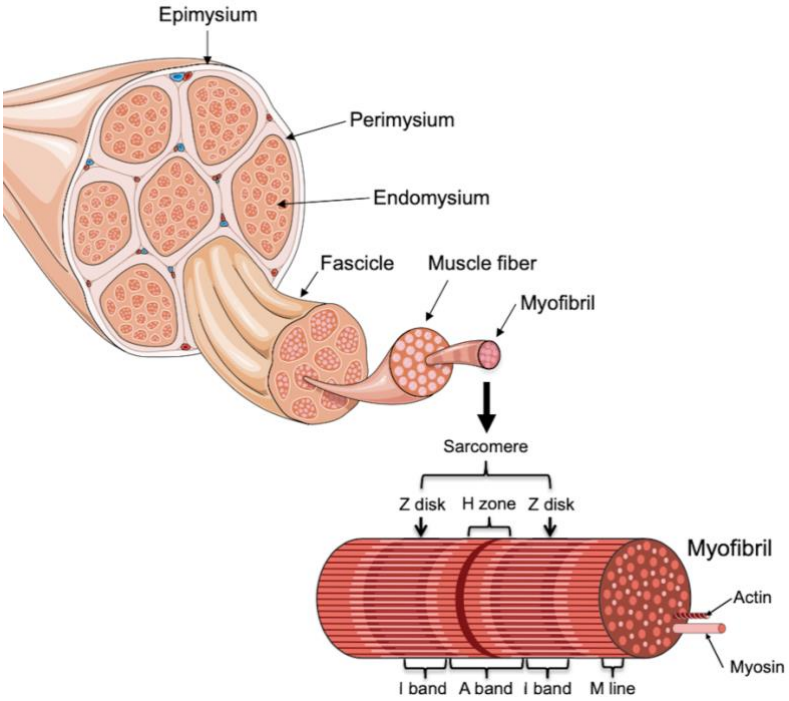

Figure 2.3: Illustration of the muscle organization from epimysium to individual proteins like actin and myosin. The sarcomere is shown with the Z-disks, H-zone, I-bands, and the A-band. The sarcolemma is the plasma membrane surrounding the muscle fiber (Bonetto and Bonewald, 2019).

2.2.1 Muscle contraction

A muscle contraction starts when a nerve impulse changes the permeability of the muscle cell membrane, and Ca^{2+} ions enter the myofibril, which activates myosin. Myosin is the dominant protein of the A-band. Activated myosin cleaves ATP, and the released energy is transferred to a chemical binding between actin and myosin, shortening the functional unit between the Z-lines as seen in Figure 2.4 (Lynum and Rustad, 1997). The shortening happens as the myosin has multiple hinged segments on the end near the actin, which can bend and create a "walking"-like movement on the actin. This grab- and release of the actin filament is known as myosin-actin cycling (Krans, 2010).

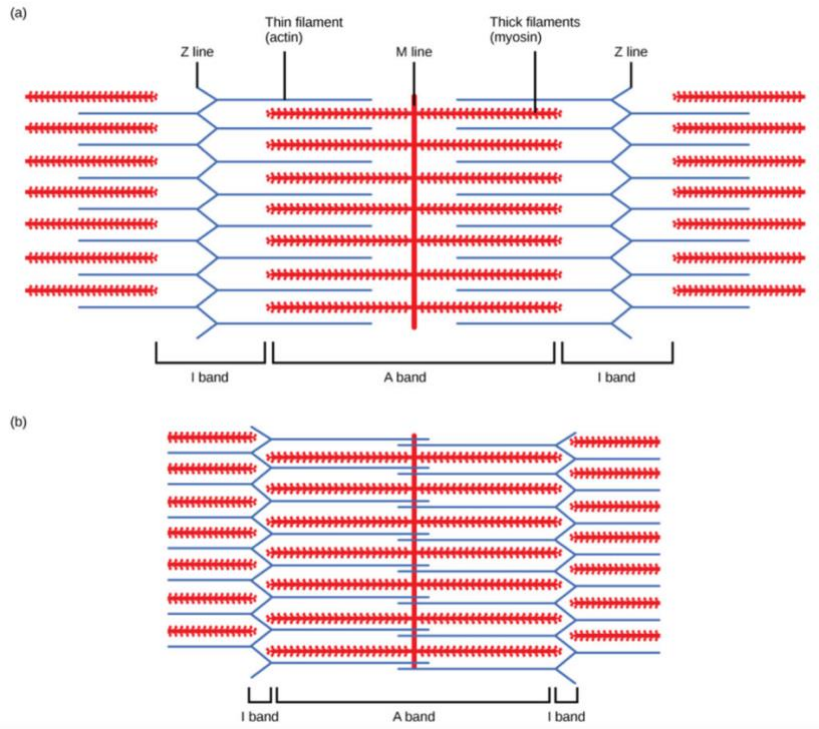

Figure 2.4: Illustration that shows how the functional unit of a muscle works during a muscle contraction. The distance between the Z-lines is shortened as myosin "walks" on actin (Rye et al., 2016).

It is, however, important for living fish that a muscle does not remain contracted. One way to control the contractive forces is to regulate the Ca^{2+}-permeability of the cell membrane and remove the ions from the myosin. This can be achieved by inhibiting the receptor-operated and voltage-operated Ca^{2+} channels. Another way the cells control the Ca^{2+} uptake is to activate the sarcoplasmic reticular Ca,Mg-ATPase, which binds two Ca^{2+} ions and translocate them to the luminal side of the sarcoplasmic reticulum (Webb, 2003).

2.3 Quality

Quality can be defined as consumer acceptance; however, this is a vague description as this will differ from consumer to consumer. Each consumer also has their own quality barometer, where what is perceived as high quality greatly depends on where that barometer starts (Moskowitz, 1995). When considering the quality of fresh fish, it usually spans from sensory to biochemical to microbiological to nutritional characteristics (Chan et al., 2021c). Depending on who is being asked, different aspects will be more important. The nutritional-physical value and hygienic-toxicological parameters might be considered the most important for the industry. For consumers, quality is based chiefly on organoleptic parameters like odor, appearance, and taste. Coloration is also an important aspect for consumers (Komolka et al., 2020). However, both parties consider freshness as significant when evaluating if a product has high quality or not (Cheng et al., 2014).

A highly accepted graph to show changes regarding quality in slaughtered fish was provided by Hans Henrik Huss (Huss, 1976). Figure 2.5 presents how Huss divides the deterioration of the muscle and degrading of quality into four phases. In phases 1 and 2, quality degradation is primarily due to autolysis, while in phases 3 and 4, bacterial activity influences the quality the most. When discussing the freshness of a fish, it generally refers to the autolysis part of the degradation, while spoilage is mainly associated with bacterial activity.

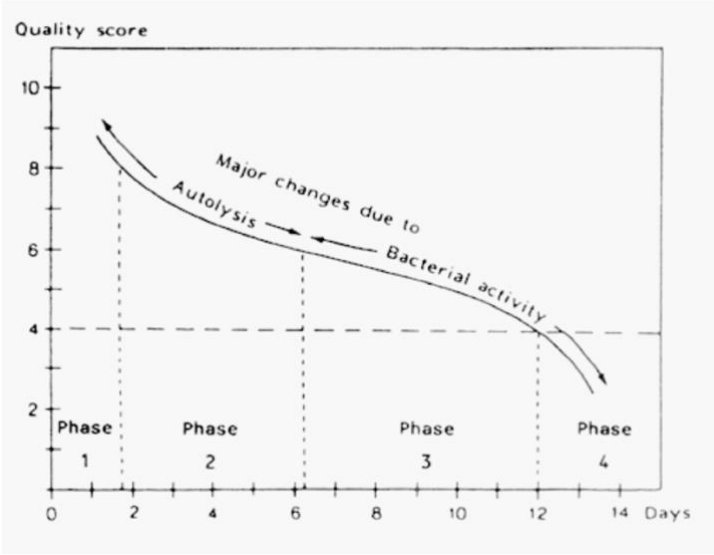

Figure 2.5: Graph provided by Huss in 1976. A quality score of 10 indicates absolute freshness, 8 indicates good quality, 6 indicates neutral quality, and 4 is the rejection level for consumption. From day 0 to around day 6, the fish experiences autolysis, while from day 6 and onward bacterial activity is the main cause of spoilage (Huss, 1976).

2.3.1 Rigor mortis

Rigor mortis is the process in which muscle stiffens soon after death. This process causes sensory changes, which affect the quality of the meat. These changes include changes in odor, texture, and taste (Huss, 1995). The stiffening is a product of a biochemical chain reaction that starts with the oxygen circulation being stopped after the fish deceases. This forces the muscle cells to produce ATP anaerobically via glycolysis, where glycogen is broken down, and lactate is the primary end-product (Camacho et al., 2020). Accumulation of lactic acid will eventually lead to the inhibition of glycolytic enzymes as the pH is altered, and as the glycogen reserves are depleted, the ATP production will eventually be terminated (Hultin, 1984). The muscle enters a full-*rigor mortis* when the ATP concentration is decreased to about 1 µmol/g (Wang et al., 1998).

For muscle contraction to occur, a certain concentration of Ca^{2+} must release into the sarcoplasm. In alive organisms, there are ATP-gated ion-pumps that always control the concentration of Ca^{2+} ions in the sarcoplasm. However, when the organism dies, these pumps no longer receive ATP energy, and the control of Ca^{2+} stops. No control of the ion-pumps makes the sarcoplasm fill up with Ca^{2+} ions resulting in a continuous contracted muscle (Daskalova, 2019).

2.3.2 Autolysis

Autolysis is the process of self-digestion and endogenous enzymes breaking down ATP to its catabolites adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino), hypoxanthine (Hx), and uric acid (UA), as seen in Figure 2.6 (Huss, 1995). This occurs when the glycogen storages are depleted as glycolysis requires glycogen, and ATP production via this catabolic pathway stops. However, enzymes breaking down ATP continue to work, resulting in the loss of ATP over time (Hultin, 1984). Prior to this process, the glycolysis has altered the pH of the muscle, affecting the physical properties of the muscle significantly (Huss, 1995). The adenosine phosphate molecules (ATP, ADP, AMP) are rapidly deaminated to IMP following postmortem, while dephosphorylation of IMP to Ino and eventually Hx is a slower process (Fogarty et al., 2019a).

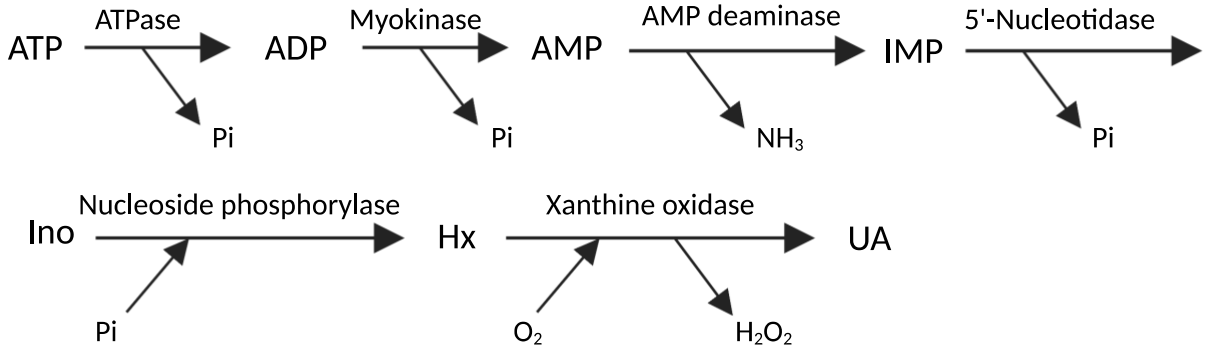

Figure 2.6: Illustration of the breakdown of ATP to UA and the enzymes needed for the process to proceed. The figure is made at www.biorender.com and adapted from Okuma and Watanabe (2002).

The breakdown products of ATP contribute to different flavors of the flesh. IMP contributes to the pleasant fresh flavor of the fish meat known as umami; meanwhile, Ino and Hx contribute to an unpleasant bitter flavor (Kawai et al., 2002; Karim et al., 2019). ATP degradation to IMP is mainly due to endogenous autolytic enzyme activity. In contrast, both enzymatic and microbial activity is responsible for the breakdown from IMP to Ino and Hx (Gram and Huss, 1996).

Autolysis is also responsible for the softening of the muscles post-*rigor mortis*. The degradation of ATP to its catabolites proceeds simultaneously as this process. The degree of autolysis can readily be examined by measuring the force needed to puncture the fillets or by calorimetry to determine the degree of protein denaturation. It is thought that the enzymes digesting essential components of the *rigor mortis* complex are the crucial factors for this softening (Huss, 1995). Two things happen as the *rigor mortis* complex is degraded. First, the Z-disk becomes weaker and eventually degraded, and second, essential muscle proteins (nebulin, desmin, troponin T, vinculin) become degraded. These proteins are important to keep the myofibrils stable (Daskalova, 2019). These two processes are catalyzed by proteolytic enzymes that become activated as the Ca^{2+} concentration rises (Hultin, 1984). Calcium-activated factors (CAF) or calpains are especially important when it comes to the hydrolyzation of troponin and tropomyosin but do not hydrolyze myosin or actin (Daskalova, 2019).

Cathepsins are another group of proteolytic enzymes whose work results in meat tenderness. They are often found in the lysosomes and are thought to be inactivated in the presence of ATP. This explains why cathepsins are only active in postmortem fish muscles (Hultin, 1984). There are 13 known cathepsins; however, only cathepsins B, D, H, and L are significant for myofibrils' proteolysis and the softening of the muscles post-*rigor*. Cathepsin B breaks down myosin's heavy chain when pH is around 6.0; cathepsin D has optimum pH below 6.0 and must be in the presence of other cathepsins to be activated. Cathepsin D can alter Z-disk myofibrils by hydrolyzing myosin, both heavy and light chains, and stimulate alteration in the troponin-tropomyosin complex. Cathepsin H and L both generate peptide fragments that need further degradation (Ahmed et al., 2015). Deterioration of fatty acids has a major impact on the quality of the fish meat. Hydrolysis and lipid oxidation are the main processes that are associated with odor and off-taste, and because fish lipids are highly unsaturated and low in endogenous antioxidants, they are more susceptible to oxidation (Sae-leaw and Benjakul, 2014).

2.3.3 Freshness indicators

The concentration of ATP and its corresponding breakdown products are highly acknowledged indicators of fish freshness prior to microbial spoilage (Hong et al., 2017). The K-value, first proposed by Saito, Arai, and Matsuyoshi in 1959, utilizes the concentration of all the ATP-breakdown products and is one of the most used indicators of freshness. The equation for the K-value is presented in Equation 1.

Equation 1:

$$K\ (\%) = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [Ino] + [Hx]} \times 100$$

(Saito et al., 1959).

Each compound in the equation is expressed as a molar concentration, meaning that the lower the concentration of ATP and ADP, the higher the concentration of Ino and Hx, creating a higher K-value (Wang et al., 1998). Saito et al. (1959) also created some limits, whereas if K>40%, the fish is not edible, while K<20% indicates that the fish is fresh enough for raw

consumption. However, newer research conducted by Erikson and colleagues suggests that a K-value of 40-50% represents a fish of excellent quality, while fish with a K-value of 70-80% is of good quality and still edible (Erikson et al., 2006).

The concentration of ATP, ADP, and AMP drastically decrease during the first 24 hours postmortem, so an alternative K-value that excludes these metabolites has been developed, as seen in Equation 2. However, researchers have concluded that this modified K-value is only suitable for monitoring the early stages of storage, and other non-invasive methods might even be better at these stages (Hong et al., 2017).

Equation 2:

$$K_1(\%) = \frac{[Ino] + [Hx]}{[IMP] + [Ino] + [Hx]} \times 100$$

(Hong et al., 2017).

2.3.4 Bacterial Activity

Spoilage is defined as changes in the quality rendering it unfit for consumption either due to odor and/or changes in texture and appearance (Odeyemi et al., 2020). Fresh fish is highly perishable due to extrinsic and intrinsic factors like high water content and pH and will start to deteriorate quickly after capture and during storage, if not stored at appropriate temperatures (Fogarty et al., 2019b). Like any other fresh produce, fish inhabit various microorganisms in their natural microflora. Some contribute directly to the spoilage process, some might be pathogenic, and some are neutral (Gram and Dalgaard, 2002). The microorganisms directly attributed to spoilage are specific spoilage organisms (SSO). The flesh of the fish remains sterile while the fish is alive or is newly caught, while the microorganisms mainly inhabit the skin, gills, and intestinal tract (Huss, 1995). The most common spoilage bacteria in salmon stored under aerobic and refrigerated conditions are *Pseudomonas spp*., *Carnobacterium spp*., and *Shewanella spp*. (Xie et al., 2018). However, it is worth noting that high levels of microorganisms do not equal spoilage, and only the microorganisms with a high spoilage potential able to produce off-flavors and off-odors should be considered SSOs, as shown in Figure 2.7 (Gram and Huss, 1996). The bacteria present when the fish spoils are known as spoilage flora and do not necessarily partake in the spoilage (Huss, 1995).

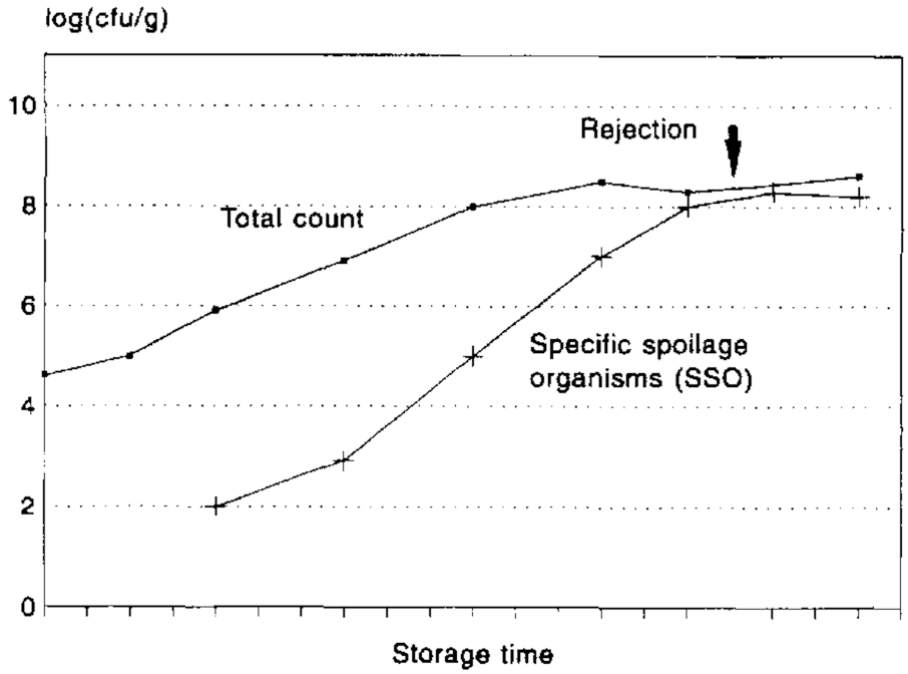

Figure 2.7: Illustration showing the relationship of total counts of bacteria and SSO in fish during storage (Huss, 1995).

Microbial growth on fish after deceased occurs because of several processes. First, the fish's immune system is no longer active, increasing the chance of the skin's bacterial flora, gills, and intestine becoming intruders. The flesh of a fish is sterile as long as the skin barrier does not get broken; however, if the skin barrier is broken, bacteria are free to move between the muscle fibers (Huss, 1995). Second, catabolites from the ATP degradation can also be metabolized by microbes resulting in ammonia, biogenic amines (putrescine, histamine, and cadaverine), and sulfur compounds being produced, affecting the quality (Fogarty et al., 2019b). The production of these spoilage products might also increase enzymatic activity, further denaturate the muscle proteins, and cause structural damage to the cell membranes, which reduce the water-holding capacity, increase weight loss, and causes textural changes (Xie et al., 2018).

The most common pathogenic microorganisms found on salmon are *Escherichia coli, Staphylococcus aureus, Aeromonas* sp., and *Listeria monocytogenes*, most likely reaching the fish through processing (Nespolo et al., 2012). These microorganisms are more important concerning food safety and do not attribute directly to spoilage *per se*. However, from the point of view of public health, these microorganisms are important to have knowledge about (Novoslavskij et al., 2016).

2.3.5 Water Holding Capacity and Drip Loss

Water holding capacity (WHC) is the ability the muscle proteins have to hinder water from being released from the three-dimensional structure when exposed to stress from external forces (Chan et al., 2021b). Drip loss is the release of water during thawing or storage and is associated with nutrient loss (Kaale et al., 2014). The drip loss consists mainly of water, proteins, and lipids (Rotabakk et al., 2018). Both these properties are important quality measures for salmon.

As mentioned, postmortem glycolysis is responsible for a drop in the pH (Hultin, 1984). When the pH has reached the isoelectric point of the major muscle proteins, the net charge becomes zero, meaning an equal part of positive and negative charges are found on the protein. This causes the protein to lose its ability to hold water as the water attracts only charged proteins (Huss, 1995). Also, the charge between the muscle proteins repels, which creates space within the myofibril. When the isoelectric point is zero, this repulsion is diminished, resulting in a reduced area within the myofibril, pressing the water out (Huff-Lonergan and Lonergan, 2005).

Furthermore, during *rigor mortis*, cross-bridges will form between the actin and myosin, further reducing the space for water. Research has also confirmed that water is mostly held in the I-band (Huff-Lonergan and Lonergan, 2005). As Figure 2.4 shows, the length of the I-band drastically decreases when a muscle is contracted, which happens during *rigor mortis*. This decrease in length and volume of the I-band, in conjunction with pH-induced shrinkage of the myofibrils, can expel the water from within the myofibril structure to the extramyofibrillar space, eventually leading to drip loss (Bendall and Swatland, 1988).

2.4 Preservation

There are several ways in which Atlantic salmon can be processed in Norway. Slaughterhouses specifically for Atlantic salmon are found all over Norway's coastline, typically within proximity to the salmon farms. The salmon is reared for about 12-18 months until it reaches a weight of 3-6 kg. When the fish has reached market-ready weight, they are transported alive, from the feeding pens in well-boats to waiting pens where they are allowed to de-stress. The fish is transported from the waiting pens into the processing plant, where they are stunned and

slaughtered. The fish is left in cold freshwater to bleed out before being gutted and sorted according to quality (Salmon-from-Norway, 2022). Another way is to anesthetize and euthanize the fish onboard a stun-and-bleed boat right by the pens. The fish is left to bleed out inside the boat before being placed in a chilled tank before being transported to the processing plant, where it gets gutted and packaged (Optimar, 2022).

2.4.1 Traditional chilling on ice

From Norway, farmed Atlantic salmon is usually exported as bled and gutted whole fish stored on ice (Skare et al., 2021). After the fish is sorted according to quality, the fish is placed with belly cavity down in polystyrene boxes with enough ice to keep the core temperature around 0 °C (Margeirsson et al., 2017). Depending on the season and the temperature of the seawater, up to 25% of the weight of the fish in ice might be necessary to obtain the desired core temperature (Jessen et al., 2014). Normally, the Expanded Polystyrene (EPS) boxes contain 22 kg of fish and around 3-5 kg of ice, and transportation time varies from 2 to 7 days, depending on the market (Sivertsvik et al., 1999). Traditional chilling of a product relies on the fact that no freezing should occur on the surface, resulting in ineffective heat removal. This process becomes even more extensive for larger products due to low thermal conduction and low thermal conductivity, making this process highly energy consuming and, last but not least, time-consuming (Magnussen et al., 2008).

2.4.2 Chilling

Super-chilling is the process by which the temperature of the product is lowered to 1-2 °C below the initial freezing point (Duun and Rustad, 2007). Sub-chilling is the process by which the temperature of the product is lowered to around -1.5 °C (Skaginn3X, n.d). These definitions tend to be used interchangeably, but super-chilled products normally obtain lower temperatures than sub-chilled products. For both procedures, ice formation is found on the surface of the fish, which will conduct heat from the interior part of the fish to the exterior, eventually reaching an equilibrium during storage (Kaale et al., 2011). This surface ice also acts as a cold reservoir and the need for external ice sojourns. The ice will also absorb heat from the surroundings keeping the fish at a stable temperature (Magnussen et al., 2008). When super-chilling and sub-chilling the fish, the enzymatic and bacterial activity ceases, and stopping microbial growth is one of the most critical factors in increasing the shelf-life of food

products (Kaale et al., 2011). It is reported that the shelf life of food that has been super-chilled can be extended by 1.5-4 times compared to the same food that was merely chilled (Duun and Rustad, 2007).

There are several methods of sub-chilling, such as refrigerated seawater (RSW) slurry, partial ice formation, chilled seawater, impingement, and nitrogen freezing (Magnussen et al., 2008). RSW is an ice-water suspension kept at subzero temperature with ice particles surrounded by seawater. The advantages include faster chilling due to faster heat exchange and less physical damage to the fish compared to flake ice since the ice particles are not directly in contact. The seawater must be salty enough to obtain a low enough temperature. Therefore, excess salt is often added to the RSW system (Piñeiro et al., 2004). A more recent technique is the "SuperChiller" cooling technique, which surface cools fillets by conveying the fillets through a freezing tunnel. The skin-side of the fillets are placed on a Teflon-coated aluminum belt which holds a temperature of -8 to -6 °C, and while being transported through the tunnel cold air is blasting (Wu et al., 2014).

Another common super-chilling method is freezing with impingement. This freezer is divided into zones ranging from -30 °C to -40 °C, where the zone closest to the inlet is the warmest, and the zone closest to the outlet is the coldest. This allows for maximum thermodynamic usage of the refrigerant. The zones also have independent impingement jets, which enable maximum velocity air to produce maximum heat transfer (Kaale et al., 2011). Salvadori and Mascheroni (2002) reported that processing times in an impingement freezer were lower than the time needed in a conventional belt tunnel freezer, making impingement freezing a promising way of preserving food products. According to Stevik and Claussen (2011), impingement freezing only takes about 45 seconds.

2.4.3 Freezing

Freezing is the process by which the water is converted to ice. In foodstuff like salmon, this process slows endogenous enzyme activity that otherwise would have degraded proteins and inhibits microbial growth, eventually extending the shelf life (Tan et al., 2021). As mentioned, salmon contains around 65% water, and when temperatures inside the fish reach around -1 °C, the water starts to change from liquid to solid (Jessen et al., 2014). The process can be

divided into four phases, as shown in Figure 2.8. 1) sensible heat from the product is removed until the product reaches its initial freezing temperature, 2) cooling continues until the temperature passes the phase changing temperature, and nucleation of ice crystals starts (the super-cooling phase), 3) latent heat is removed, and ice crystals start to form simultaneously, and last 4) ice crystal recrystallization occurs (Tan et al., 2021). Freezing is an exothermic process, and as most of the water turns into ice crystals, the excess heat must be removed from the fish and depending on what method of freezing is utilized, this process might be slow or fast (Jessen et al., 2014).

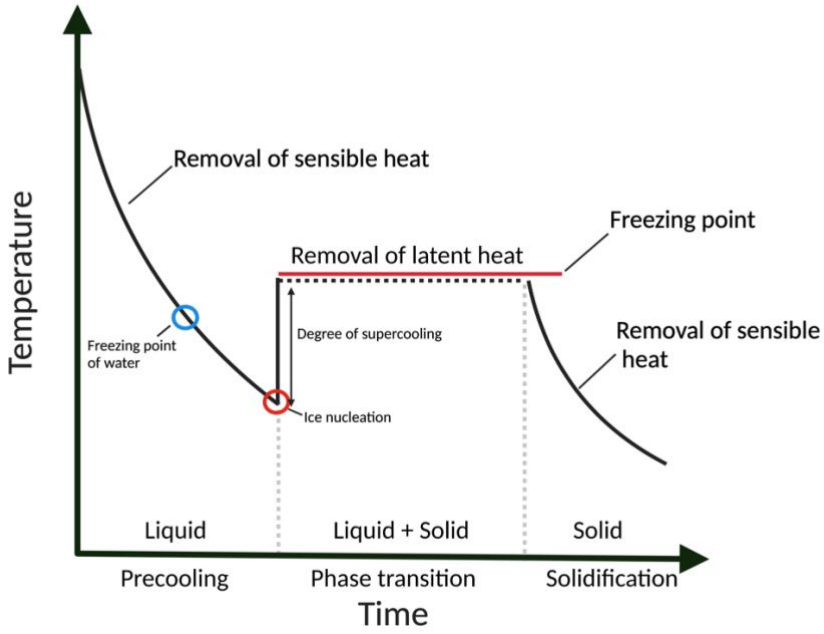

Figure 2.8: Time-temperature graph of water freezing, made at www.biorender.com and adapted from Tan et al. (2021).

The freezing rate is critical to the nucleation and formation of ice crystals (Alizadeh et al., 2007). A rapid decrease in temperature and fast removal of latent heat supports the growth of numerous small ice crystals (Gokoglu and Yerlýkaya, 2015). When these criteria are met, small ice crystals form both in the sarcoplasm and the extracellular space, creating opposite forces that result in uniform spacing. This does not cause extensive damage to the muscle cells (Bello et al., 1981). On the other side, slow freezing creates more significant structural damage to the muscle structure resulting in drip loss, protein denaturation, and color changes (Sigurgisladottir et al., 2000; Tan et al., 2021). The ice crystals in the slow freezing form in an irregular pattern. This distinctive feature can be observed in the myofibers and endomysium,

disrupting random matters. Here, a few large ice crystals can be observed inside and outside the cell, creating pressure on the myofibers and eventually distorting them (Bello et al., 1981). Recrystallization is when already formed ice crystals change shape, size, and amount, and when the average size of ice crystals increases, the amount of ice crystals decreases (Tan et al., 2021). The driving force is the tendency for all systems to lower the amount of free energy (Gokoglu and Yerlýkaya, 2015). Recrystallization results from temperature fluctuations, which are often inevitable during storage and transportation, and as the smaller ice crystals fuse, the larger ice crystals are created (Zhang et al., 2020). As mentioned, larger ice crystals do more damage to the cellular structures than smaller ice crystals, which is undesirable.

2.4.4 Thawing

Thawing is essentially the opposite of freezing; heat is introduced instead of being extracted (Haugland, 2002). When the product is ready to be thawed, the ice crystals formed during freezing melts. If the tissue has experienced minimal destruction, the proteins will reabsorb the water. However, if the muscle proteins have been denatured as a result of the freezing, the ice crystals will melt into the empty space creating softer texture, drip loss, and changes in flavor (Nakazawa and Okazaki, 2020). Drip loss implies nutrient loss as water-soluble proteins remain in the water released during thawing, which results in lower quality of the meat (Kaale et al., 2014).

The required thawing time depends mainly on the temperature difference between the thawing medium and the product, and the characteristics of ice and water. As mentioned, when salmon is frozen, a layer of ice forms on the surface of the fish, efficiently transferring heat due to the thermal conductivity of ice. When this layer melts, it works as an insulator instead of acting as a conductor, slowing the thawing process immensely (Gokoglu and Yerlýkaya, 2015). This melted layer will make the thawing process slower and slower as more water is melted. Because of this, thawing is a more complex process to control and predict than freezing (Haugland, 2002).

3 Material and Methods

3.1 Experimental Setup

Analyses for this experiment were conducted in Stavanger at Nofima and in Trondheim at NTNU. However, all the samples were obtained in Stavanger from Grieg Seafood Rogaland, a two-hour drive Nofima's facilities. The RSW-setup was created with two 400-liter polyethylene fish chilling tanks containing a mix of 7% NaCl brine and ice, creating an environment of ∼-1.0 °C. The salmon were electrically stunned and bled on board the stun-and-bleed boat MS Seibas ultimo September. The fish was unloaded to the processing facility 9-12 hours postmortem. 35 fish (weight: 4.2 ± 0.6 kg) of superior quality were chosen from the processing line and packed five and five into EPS boxes with ice. 45 fish (weight: 4.6 ± 0.3 kg) of superior quality came prepacked from the Grieg Seafoods' cold room, though still from the same stun-and-bleed boat. The fish were divided equally and randomized when designated to RSW-cooling and traditional cooling on ice, which is the first design variable aptly named chilling method. For each group (RSW, ice), five TrackSense Pro temperature loggers (Ellab A/S, Denmark) were inserted in five random fish mid-abdomen. The temperature was monitored, and the solution was stirred upon arrival at Nofima.

At Nofimas lab, all the fish were filleted by hand and marked with ear tags. Left fillets were designated to odd numbers while the right fillets acquired the even numbers. When marked, the fillets were vacuum-packed at 99% vacuum. Once again, the fillets were randomized when dived into three groups with 20 fillets from both RSW and ice-chilling in each group: fresh, one-month freezing, and four-months freezing. The fresh fillets were used as a control group. The fillets in the fresh group were placed in a refrigerated room (0.6 ± 0.5 °C), while the fillets to be frozen were flash-frozen for 30 minutes with dry ice (∼-78.5 °C) before being placed in a freezer (-28.5 ± 1.4 °C) for the designated time (one month, four months). Fresh and frozen storage is the second design variable, which is referred to as the storage method. The experimental setup is summarized in Figure 3.1.

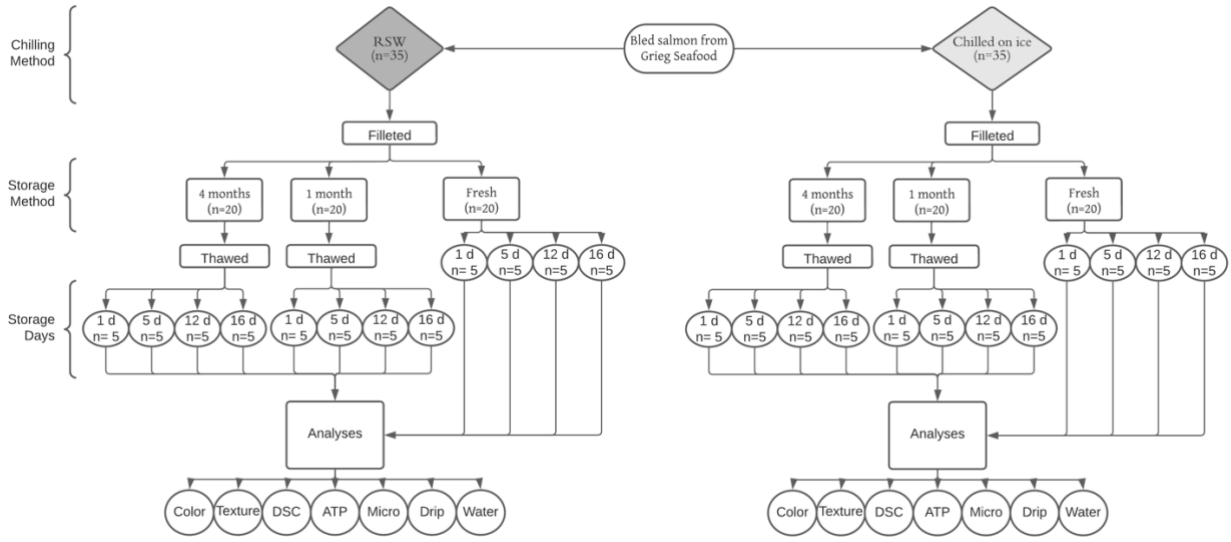

Figure 3.1: Schematic illustration of the experimental setup where the fish from Grieg was divided into two groups: RSW-chilling and traditional chilling on ice. The fish was further filleted and vacuum-packed and randomized into three groups: fresh, one month, and four months freezing. The analyses include color analyses, texture, protein denaturation, analysis for ATP metabolites, microbiological analyses, drip loss, and water content, and were conducted after 1, 5, 12, and 16 days in a cooling room.

When the time for analyses, the frozen fillets were thawed rapidly in a water bath (4 °C) for 4 hours before being placed in a refrigerated room (0.6 ± 0.5 °C) for the remaining days. Thawing was considered day 0, while analyses were conducted on days 1, 5, 12, and 16. For each day, five fillets were selected randomly. Storage days are the last design variable. The fillets were weighted with the vacuum bag, removed from the bag, patted dry, and weighted again without the bag to determine drip loss. The Norwegian Quality Cut (NQC) was then cut out and divided into four (as shown in Figure 3.2) for protein denaturation analysis, microbiological analyses, determining water content, and examining ATP degradation. Color and- texture analyses were conducted at the top loin on the day of sampling, together with microbial analysis. The three remaining pieces of the NQC were vacuum-packed and stored at -80 °C until time for further analyses.

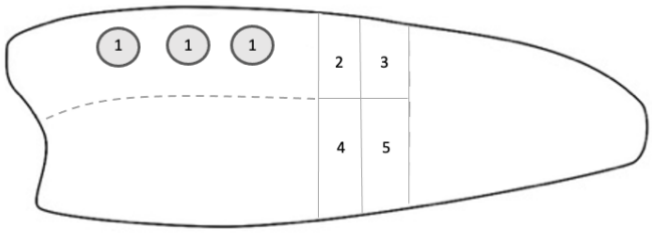

Figure 3.2: Illustration showing sampling location. 1) for texture analysis, 2) for ATP metabolites, 3) protein denaturation, 4) for microbiological analyses, 5) for water content, and the top loin for color analysis.

3.2 Water Content

The belly part of the NQC was used to determine the water percentage (Nr. 5, Figure 3.2). Each sample was frozen at -80 °C for at least 24 hours. The samples were thawed for 22 hours at 0.6 ± 0.5 °C. The analysis for water content was conducted according to NMKL method No. 23 (NORDISK METODIKKOMITE FOR NÆRINGSMIDLER c/o National Veterinary Institute, 1991). An aluminum bowl was marked and weighed. A uniform sample of both fat and muscle tissue was extracted with a scalpel and minced. 10.0 ± 0.5 g was then transferred over to the aluminum bowl. The samples were placed in a heating cabinet (Termaks, Norway) at 102-105 °C for 18 hours to dry. The samples were afterwards placed in a desiccator (SICCO Star-Vitrum Desiccator, borosilicate glass 3.3, Germany) for 30 minutes and weighed again. Water percent was calculated using Equation 3.

Equation 3:

$$W\% = \frac{W_1 - W_2}{W_1} \times 100$$

where W_1 = weight of sample before drying and W_2 = weight of sample after drying.

3.3 Drip loss

Ten empty bags were weighed on the day of filleting to find the average weight. After thawing, the fillets were patted dry with tissue paper and weighted again without the bag. Drip loss (%) was calculated using Equation 4.

Equation 4:

$$DL\% = \frac{D_0 - D_t}{D_0} \times 100$$

D_0 = original weight (g) without bag and D_t = weight (g) without bag after patted dry.

3.4 Microbiology

The microbial analyses were conducted according to NMKL method No. 184 (NORDISK METODIKKOMITE FOR NÆRINGSMIDLER c/o National Veterinary Institute, 2006). Iron agar-pour plates were used to determine total viable counts, measured as aerobic plate count of mesophilic bacteria (APC), while Long & Hammer (L&H) plates were used for total psychrotrophic viable counts (TVC). A part of the hypaxial muscle of the NQC (~20g) was excised from the skin aseptically (Figure 3.2), transferred to a stomacher bag, and weighed. A 1:10 dilution was prepared with sterile buffered peptone water and salmon that was homogenized using a Smasher © Stomacher (AES Laboratorie, bioMériux Industry, USA) for 120 seconds. The homogenate was transferred to sterile Eppendorf tubes, and dilution series was prepared (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} for iron agar, and 10^{-1}, 10^{-2} for L&H) by using 150 µL sample to 1350 µL sterile buffered peptone water. 49.2 µL of each dilution was plated on L&H plates by Eddy Jet 2 Spiral Plater (IUL micro, Spain). 1 mL of each dilution was transferred to sterile petri dishes, and 10-12 mL of sterile iron agar containing L-cysteine (0.04%, v/v), pre-cooled to 45.0 ± 1.0 °C, was added. The inoculum was carefully mixed, and a thin layer of iron agar was applied after the medium had solidified. The L&H plates were incubated at 15 °C for 7 days, and the iron agar plates were incubated at 25 °C for 72 ± 6 hours. Microbial concentrations are presented as log CFU/g sample.

3.5 Texture Analysis

The texture was analyzed by the TA.TXplus Texture Analyzer (Stable Micro Systems Ltd, UK) equipped with a 25mm DIA stainless steel flat-ended probe. A 50 kg load cell was used to measure the force (N) needed to compress 80% of the sample's height at 2 mm/s. Three puncture holes starting at the anterior end towards the posterior end of the top loin were consecutively conducted on each sample (Nr. 1, Figure 3.2). The force (N) was logged as a force-time graph in the Texture Exponent software (Stable Micro Systems). A pre-made macro-program was utilized to obtain data like fillet height, breaking force, and 60% compression (firmness).

3.6 Color Analysis

For color analysis of the fillets, a digital color measurement system was implemented (DigiEye full system, VeriVide Ltd, UK) and connected to a camera (Nikon D90, 35 mm lens, Nikon Corp., Japan). Each fillet contained the top loin, loin, and belly; however, the area of analysis was from above the pin bones and up until the first ridge of fat back to where the NQC was cut out. The fillets were placed on a green cutting board and positioned inside a lightbox (Verivide daylight, 6400 K, UK). The pictures were analyzed with DigiPix software (VeriVide Ltd.). The data obtained was quantified as L*a*b* values, where L* represents lightness (L* = 100 = white, L* = 0 = black), a* describes the color intensity in a red-green scale (a* > 0 = red, a* < 0 = green), and b* describe the color intensity in a yellow-blue scale (b* > 0 = yellow, b* < 0 = blue). Hue and chroma can be derived from the a* and b* values. Hue is normally expressed as degrees within the color space (°H = 0 red hue, °H = 60 yellow hue), whereas chroma, which is the distance from the achromatic center, expresses the intensity of a color (greyness) (Dawson et al., 2018). Chroma was calculated using Equation 5.

Equation 5:

$$Chroma = \sqrt{a^2 + b^2}$$

(CIE, 1987).

Hue angle was calculated using Equation 6.

Equation 6:

$$Hue\ angle = tan^{-1}\left(\frac{b}{a}\right)$$

(CIE, 1987).

3.7 ATP-metabolism

ATP compounds were determined by High-Pressure Liquid Chromatography (HPLC). Each sample was frozen at -80 °C before being transported to Trondheim from Stavanger. Samples from days 1, 5, 12, and 16 were utilized to determine the degree of ATP degradation. The samples were prepared by grating the muscle part without the skin into a fine mixture. 1.5 ± 0.1 grams were then transferred to centrifuge tubes (50 mL), and for each sample, three parallels were created. 7.5 mL of 7% trichloroacetic acid ($C_2HCl_3O_2$, Sigma-Aldrich, CAS: 76-03-9) was added to each tube before the mixture was homogenized (T25 Digital ULTRA TURRAX®, IKA®, Germany) for 120 seconds. Post homogenization, 3.25 mL, 1 M potassium hydroxide (KOH, Merck, CAS: 1310-58-3) was added, and the mixture was mixed by turning the tubes upside down a few times. The tubes were then centrifuged at 4 °C with 7000 RPM for 10 minutes (ROTINA 420R, Hettich®, Germany). The supernatant (7mL) was carefully pipetted over to new centrifuge tubes (10 mL) before being put back into the freezer (-80 °C).

Before the HPLC analysis, pre-extracted samples were thawed for approximately one hour at room temperature. Approximately 1.5 mL of the sample was filtrated (25mm, 0.45m Polyethersulphone membrane, VWR, USA) into HPLC tubes (1.5 mL vial ND9 amber P.+cap SIL/PTFE, VWR, USA). The system consisted of Agilent 1290 Infinity connected to a Diode Array Detector (Agilent Technologies, USA) with InfinityLab Proshell 120 EC-C18 column (3.0 * 5 mm, 2.7 m, Agilent, prod. nr.: 823750-911 + 3.0*100mm 2.7 m, Agilent, prod. nr.: 695976-302, USA). The mobile phase was a buffer containing 1.5% acetonitrile (C_2H_3N, Sigma-Aldrich, CAS: 75-05-8), 0.25 M potassium dihydrogen phosphate (KH_2PO_4, Merck, CAS: 7778-77-0), and 0.0023 M tetrabutylammonium hydrogen sulfate ($[CH_3(CH_2)_3]_4N(HSO_4)$, Sigma-Aldrich, CAS: 32503-27-8). The pH of the buffer was adjusted to 6.26 by 5M NaOH. The flow of the buffer was set up as a gradient with 0.2 mL/min from 0-9 minutes, 0.8 mL/min from 9-11 minutes, and 0.2 mL/min from 12-14 minutes. The temperature in the column was set to 20 °C with a maximum pressure of 600 bar. The metabolites were detected at 210 nm (ATP, ADP, and AMP) and 260 nm (IMP, Ino, and Hx).

3.8 Protein Denaturation

Analyses for protein denaturation were performed on the top part of the NQC using the Mettler Differential Scanning Calorimetry (DSC) 1 (Mettler Toledo AG, Schwerzenbach, Switzerland) calibrated with indium. When the time for the analysis, the samples were thawed in a cooling room (0.6 ± 0.5 °C) for approximately 22 hours. Only day 1 and day 16 were of interest for this analysis, and four samples from each group were selected with two parallels. Aluminum pans with pins (40 mg) and aluminum lids were used for the measurement. The pan and lid were weighed, and 20 mg of muscle fibers were placed into the pan. The fibers were extracted using a scalpel cutting between the myosepta in the muscle. A reference pan based on the water percentage of the fish (68% of 20 mg = 13.6 mg) was prepared in a second pan with distilled water. After sealing with a crucible sealing press (Mettler Toledo, Switzerland), the sample pans and the reference pan were placed carefully into the DSC with tweezers. The samples were kept on ice prior to loading to avoid unintended thermal denaturation. The samples were heated 5 °C/min until a maximum temperature of 88 °C.

Thermograms were presented by the StarE software version 9.10 (StarE System, Schwerzenbach, Switzerland). The peaks were identified by the peak maximum temperature by comparison to literature data (Bircan and Barringer, 2002). Each sample's denaturation enthalpy was defined using a spline baseline outlining the denaturation peak. The total enthalpy of denaturation was found by integrating the denaturation peak. The onset and endset of the denaturation peaks were standardized. The results are presented as Joules per gram of sample.

3.9 Statistical Analyses

The data were analyzed using Minitab Version 19 (www.minitab.com, Minitab Inc., USA). Data are presented as average values with associated standard deviations unless otherwise stated. All data were subjected to analysis of normality by Levene's test and analysis of the equality of variance by testing the homoscedasticity. Analysis of variance (ANOVA) was performed using general linear modeling (GLM) to detect significant differences between groups with a significance level set to $p < 0.05$. Tukey's pairwise comparisons test with a confident level of 95% was performed to analyze if a significant difference existed between the parameters.

Pearson’s correlation coefficient was utilized to determine the correlation between the parameters in the discussion. When analyzing texture, fillet height was added as an additional covariate. Microsoft Excel 2022 (Microsoft Office, Norway) was utilized for graphing.

4 Results

4.1 Temperature

Temperature loggers monitored the temperature inside the fish and the environment around. The logging started when the fish was placed in the RSW tank or the EPS boxes with ice. Fish stored in the RSW tank obtained a temperature of ~-1.1 °C, while fish stored in the boxes with ice acquired a temperature of ~0.6 °C. Figure 4.1 shows the temperature differences between the fish at different hours.

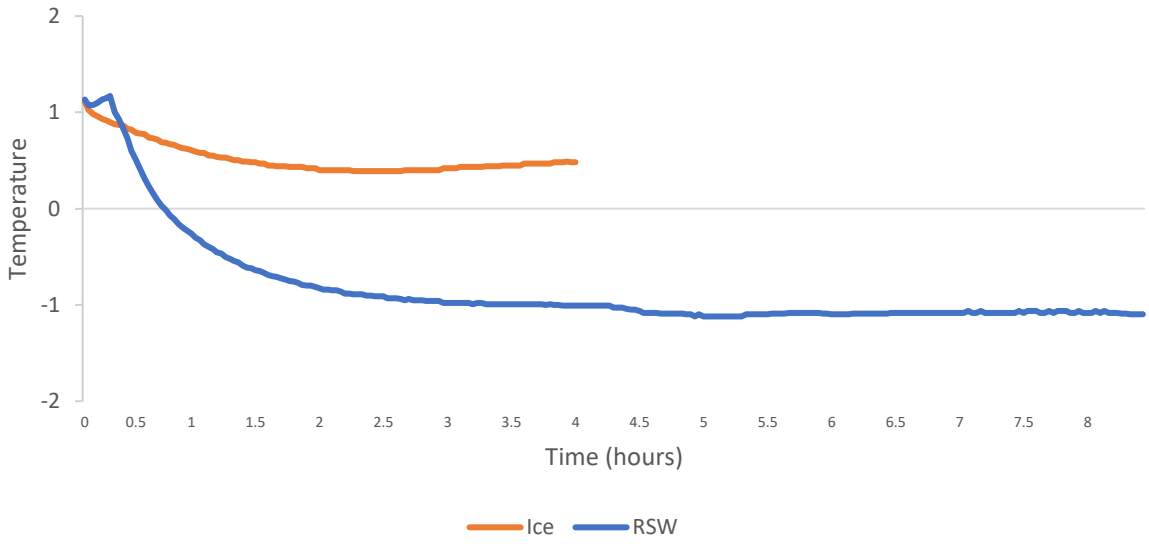

Figure 4.1: Graph showing the temperature differences between fish stored in RSW tanks and fish stored in EPS boxes with ice. Time is hours from the fish being placed in the RSW tanks or the boxes with ice.

4.2 Water Content and Drip Loss

Water content was measured for each sample on each sampling day. The average water content for all samples was 68.1 ± 3.1% (n=112). Table 4.1 displays the effect of the storage method ($p<0.001$), with the frozen samples having the highest water content. Storage days as a design variable also significantly differed ($p=0.023$), with day 12 having the lowest amount. No other significant differences between the design variables were observed ($p>0.05$).

The drip loss was measured for each sample on each sampling day. All the design variables had a significant impact ($p<0.05$) on the drip loss and are summarized in Table 4.1. Fish chilled with RSW had significantly higher drip loss ($p=0.004$) compared to fish chilled with ice, and a gradual increase in drip loss as storage days increased ($p<0.001$) was observed. Frozen samples had higher drip loss than the fresh samples ($p<0.001$). No significant interactions between the design variables were observed ($p>0.05$).

Table 4.1: Effect of chilling method (RSW, Ice), method of storage (fresh, 1-month, and 4-months frozen), and storage days (1, 5, 12, 16) on water content and drip loss in Atlantic salmon fillets. A-c indicates a significant difference (p<0.05) within the design variables based on Turkey's pairwise comparisons test. The p-value of the experimental variables is shown in cursive.

	Amount (%)		
	Water content	Drip loss	n
Chilling Method			
RSW	67.7 ± 2.1	1.7 ± 0.3	57
Ice	68.5 ± 2.4	1.5 ± 0.2	55
Effect of chilling method (p-value)[1]	*0.155*	*0.004*	
Method of Storage			
Fresh	67.1 ± 2.3[a]	0.9 ± 0.2[a]	38
1 month frozen	67.0 ± 1.8[a]	1.9 ± 0.3[b]	37
4 months frozen	70.0 ± 2.6[b]	2.0 ± 0.3[b]	37
Effect of Storage Method (p-value)[1]	*<0.001*	*<0.001*	
Storage Days			
1	69.3 ± 1.8[a]	0.8 ± 0.1[a]	27
5	67.4 ± 2.8[ab]	1.5 ± 0.2[b]	26
12	67.0 ± 2.1[b]	2.0 ± 0.4[c]	29
16	68.5 ± 2.2[ab]	2.2 ± 0.3[c]	30
Effect of Storage Days (p-value)[1]	*0.023*	*<0.001*	

[1]Significance level p<0.05, GLM

4.3 Microbiology

APC of mesophilic bacteria was determined by incubation on iron agar. A significant difference was found as a function of the design variables storage days ($p<0.001$) and the method of chilling ($p<0.001$), and this is shown in Figure 4.2A. An interaction between the design variables method of storage and the method of chilling ($p<0.001$) was also observed. Samples chilled with ice had more mesophilic growth on all sampling days than samples chilled with RSW. The method of storage as a design variable was insignificant ($p>0.06$), although shown in Figure 4.2B as a function of storage days.

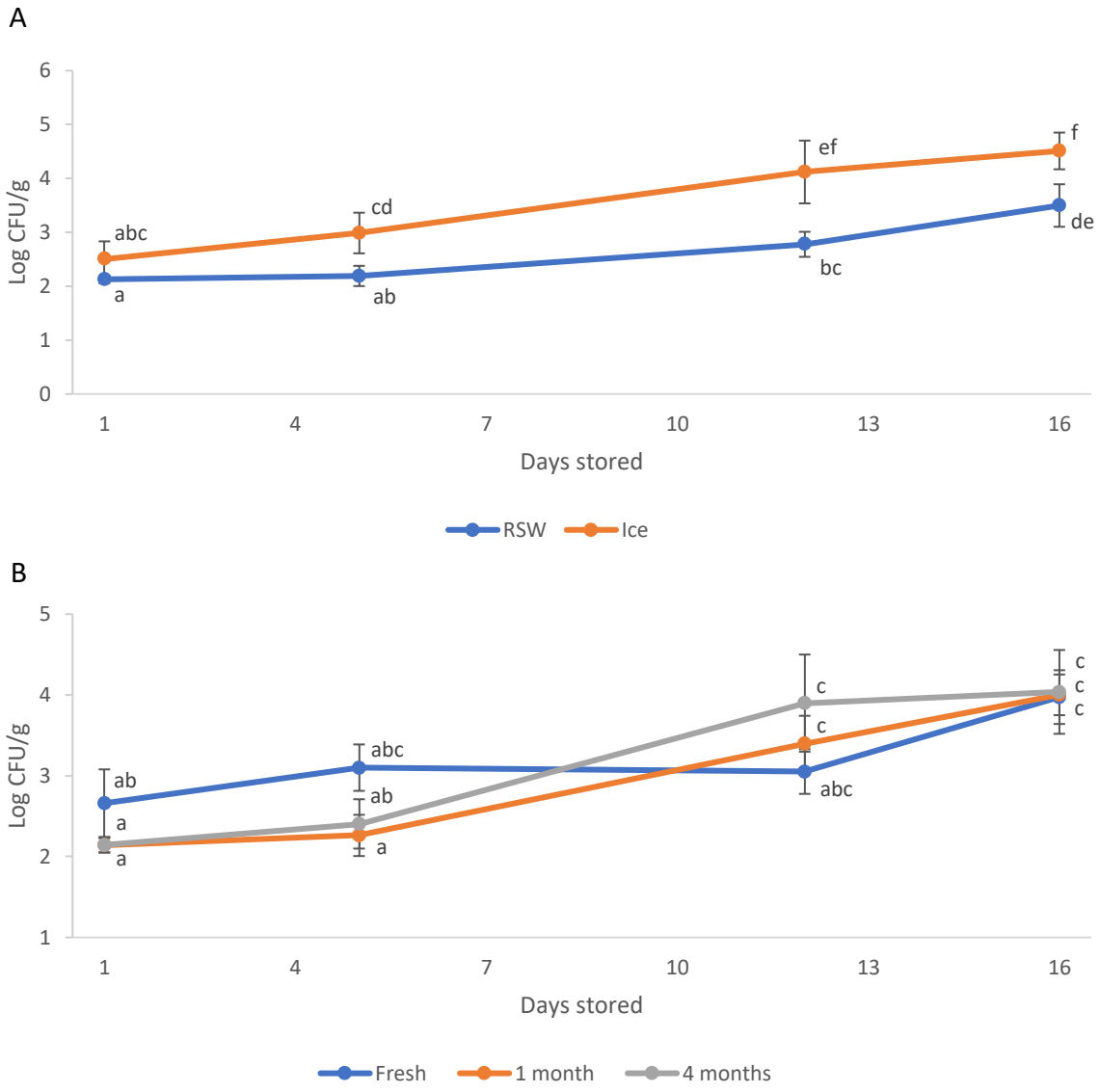

Figure 4.2: A) APC of mesophilic bacteria in RSW chilled and ice-chilled samples throughout storage (significance level $p<0.05$, GLM). The quantification limit was set to 25-250. Samples with colonies under 25 were assigned the value 12.5, translating to the respective value in log CFU/g. Letters a-f indicates significant variance ($p<0.05$) between the storage days and the chilling method. B) APC of fresh, 1-month, and 4-months frozen samples shown as log CFU/g. A-c indicates a significant difference between storage days and method of storage. A significant difference was not detected regarding the storage method.

TVC of psychrotrophic bacteria in the samples was determined by incubation on L&H agar. A significant difference between the design variables storage days ($p<0.001$) and method of storage ($p<0.001$) was observed and is shown in Figure 4.3B. A significant interaction between the storage method and the chilling method was also observed ($p<0.001$). The fresh samples had more growth than the 4-months frozen samples on all sampling days and more growth than 1-month frozen samples on days 1, 12, and 16. For 1-month frozen samples, a rapid increase of growth from day 1 to day 5 can be observed. The growth declined slightly from day 5 to 12 before increasing growth occurred again. Tukey's pairwise comparison test determined a significant difference ($p<0.05$) between the sampling days and between the 4-month frozen samples and fresh- and 1-month frozen samples. No significant difference was observed for the method of chilling ($p>0.643$); however, the growth trend for both chilling methods is shown in Figure 4.3A as a function of storage days.

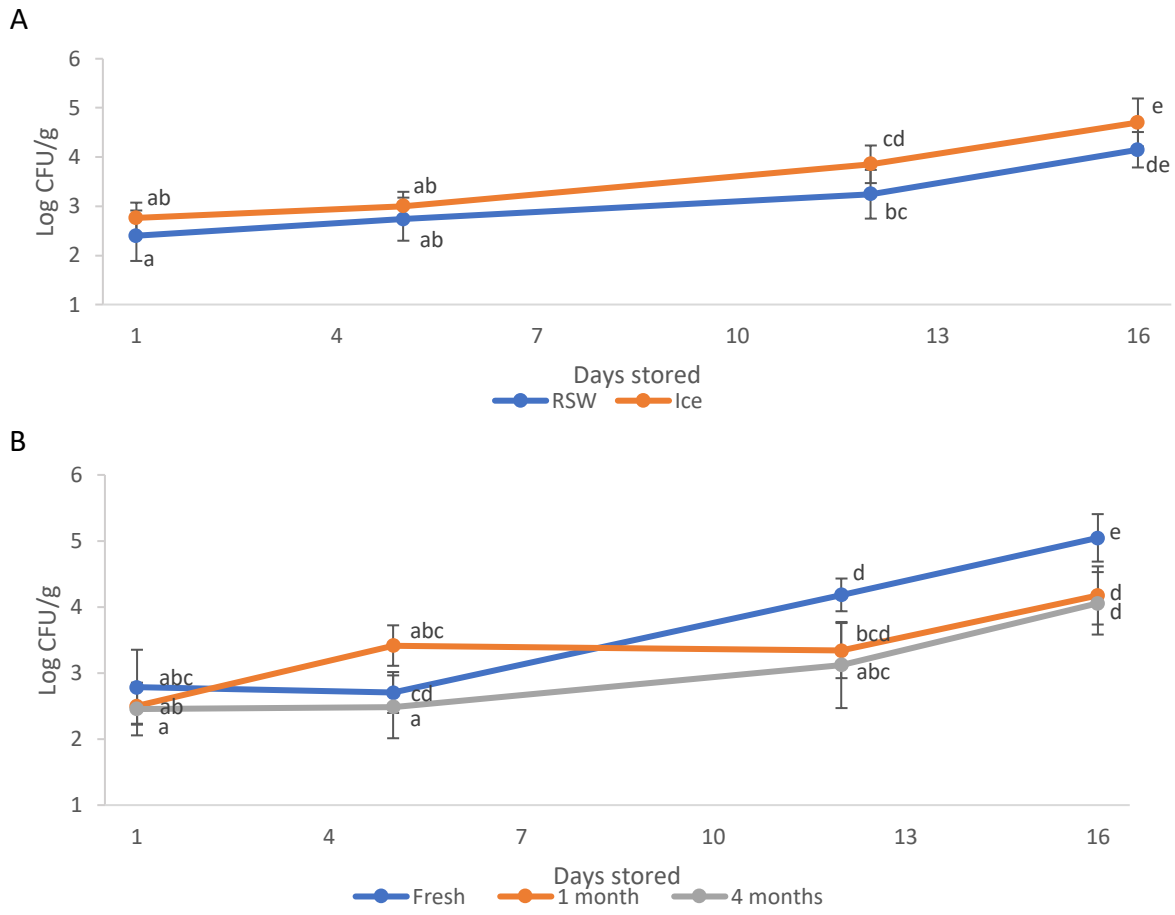

Figure 4.3: A) TVC of psychrotrophic bacteria in RSW-chilled and ice-chilled samples. Lowercase letters a-e imply a significant difference within the storage day of the chilling methods. No significant difference was observed in the chilling method. B) TVC of psychrotrophic bacteria in fresh, 1-month, and 4-months frozen samples during storage. The quantification limit was set to 1, where no growth was detected. Samples with colonies under 1 were assigned the value 0.5, translating to the respective value in log CFU/g. Lower case letters a-e indicate significant variance ($p<0.05$) between storage days and the storage method.

4.4 Texture

Texture analyses were conducted on the top loin of all the samples. Primary data is found in APPENDIX A. Firmness of the fish is described with 60% compression force. As shown in Table 4.2, the storage method had a significant impact on both the parameters breaking force and firmness ($p<0.001$ and $p<0.001$, respectively). For firmness, a significant decrease was observed as a function of storage days ($p<0.001$). For breaking force, the height of the fillet was significant ($p=0.011$). No other significant differences or interactions between the design variables were observed ($p>0.05$).

Table 4.2: Effect of the chilling method, method of storage, and storage days on the textural properties breaking force and firmness on Atlantic salmon fillets. A-b indicates a significant difference (p<0.05) within the variables based on Tukey's pairwise comparison test. The p-values for the experimental variables are shown in cursive.

	Force (N)		
	Breaking Force	Firmness[2]	n
Chilling Method			
RSW	35.6 ± 5.4	32.6 ± 5.0	57
Ice	34.5 ± 6.7	32.9 ± 5.3	55
Effect of chilling method (p-value)[1]	*0.302*	*0.572*	
Method of Storage			
Fresh	38.5 ± 7.3[a]	36.0 ± 4.6[a]	38
1 month frozen	32.4 ± 4.7[b]	30.4 ± 4.3[b]	37
4 months frozen	34.2 ± 5.8[b]	33.1 ± 6.4[ab]	37
Effect of Storage Method (p-value)[1]	*<0.001*	*<0.001*	
Storage Days			
1	38.0 ± 7.4	37.5 ± 5.1[a]	27
5	34.7 ± 4.7	32.7 ± 4.2[b]	26
12	33.8 ± 6.1	31.7 ± 4.9[b]	29
16	33.6 ± 5.9	30.7 ± 6.2[b]	30
Effect of Storage Days (p-value)[1]	*0.103*	*<0.001*	
Effect of Fillet Height (p-value)[1]	*0.011*	*0.443*	

[1]Significance level p<0.05, GLM.

[2]Firmness is described by 60% compression force.

4.5 Color Measurements

Primary data for the colorimetric analyses are found in APPENDIX B. As shown in Table 4.3, the design variable storage method contributed a significant difference (p=0.005) regarding the parameter chroma. For hue, both the variable's storage method and storage days significantly differed (p<0.001 and p=0.002, respectively). An interaction between the design variables chilling and storage method was observed for L* with p=0.026, as shown in Figure 4.4. An interaction between the method of storage and the chilling method was observed for chroma (p=0.004). No other design variables were significant (p>0.05), and no other interactions between the design variables were observed (p>0.05).

Table 4.3: Effect of the chilling method, method of storage, and storage days on L, chroma, and hue on Atlantic salmon fillets. A-b implies a significant difference (p<0.05) within the variables, and the p-values are shown in cursive.*

	L*	Chroma	Hue	n
Chilling Method				
RSW	58.2 ± 1.5	49.1 ± 2.3	47.0 ± 0.7	57
Ice	57.4 ± 1.9	49.1 ± 2.0	46.9 ± 0.5	55
Effect of chilling method (p-value)[1]	*0.432*	*0.390*	*0.756*	
Method of Storage				
Fresh	58.0 ± 1.7	48.3 ± 1.8[a]	46.1 ± 0.6[a]	38
1 month frozen	57.5 ± 1.8	49.4 ± 2.3[b]	47.5 ± 0.7[b]	37
4 months frozen	57.8 ± 1.5	49.6 ± 2.3[b]	47.3 ± 0.5[b]	37
Effect of Storage Method (p-value)[1]	*0.300*	*0.005*	*<0.001*	
Storage Days				
1	58.0 ± 1.8	50.0 ± 2.8	47.2 ± 0.8[a]	27
5	57.3 ± 1.8	48.8 ± 1.7	47.1 ± 0.5[a]	26
12	57.9 ± 1.7	48.6 ± 2.2	47.1 ± 0.5[a]	29
16	58.1 ± 1.4	49.0 ± 1.8	46.5 ± 0.6[b]	30
Effect of Storage Days (p-value)1	*0.192*	*0.311*	*0.002*	

[1]Significance level p<0.05, GLM

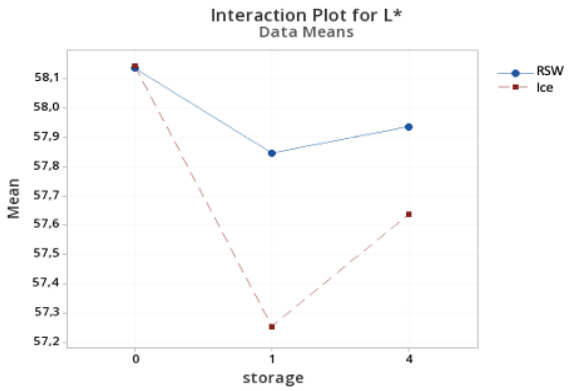

Figure 4.4: Interaction plot for L regarding the method of storage and chilling (p=0.026).*

4.6 ATP Degradation and K-value

ATP degradation was analyzed in all the samples with three parallels on each sample by HPLC. Only one out of the 360 samples contained ATP. This was an RSW-sample from the fresh group stored one day in the cooling room that contained 0.564 µmol/g ATP. Concentrations detected of IMP, Ino, and Hx are presented in Figure 4.5, Figure 4.6, and Figure 4.7, respectively. The K-value is presented in Figure 4.8

As Figure 4.5 shows, the amount of IMP detected decreased as the storage days increasd. A significant difference was observed for the design variables storage days ($p<0.001$) and storage method ($p<0.001$). Tukey's pairwise comparison test determined a significant difference ($p<0.05$) between all sampling days and between the fresh and frozen samples. The fresh samples had a higher content of IMP than the frozen samples on all sampling days. No other design variables were significant ($p>0.05$), and no other interactions between the design variables were observed ($p>0.05$).

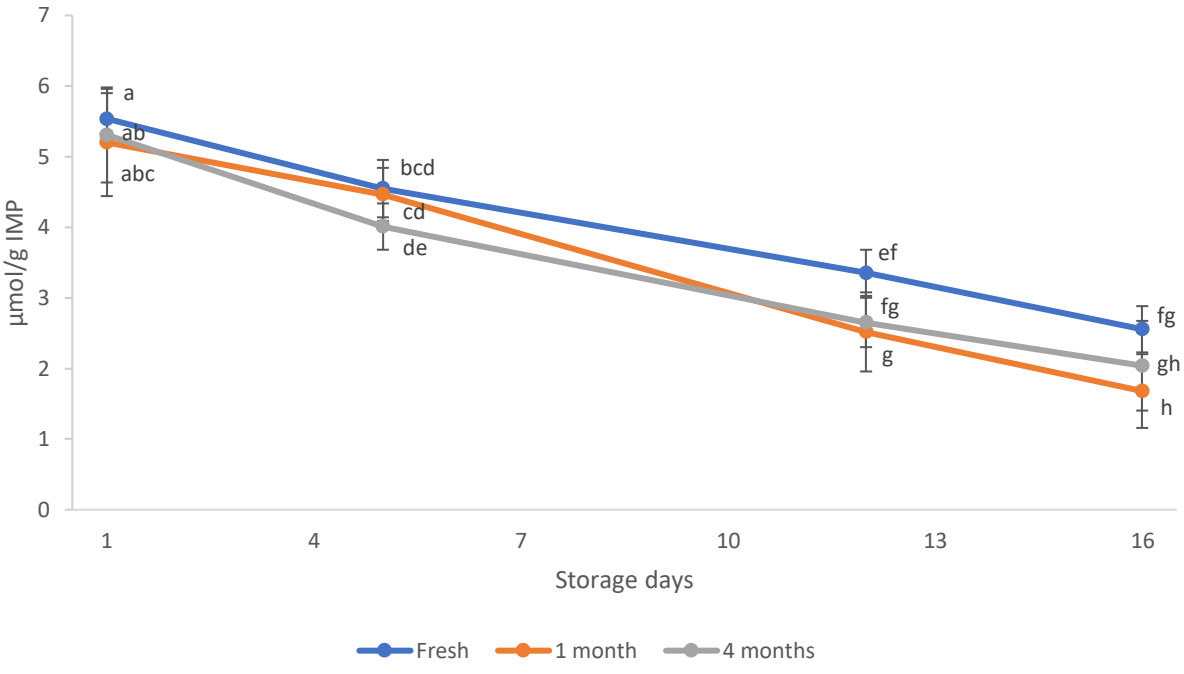

Figure 4.5: A) IMP content of fresh, 1-month, and 4-months frozen samples as a function of storage days. A significant difference ($p<0.05$) regarding storage method and storage days was observed. Lowercase letters a-h indicate a significant difference regarding storage days and storage method, and a significant difference was found between fresh and 1-and 4-months frozen samples based on Tukey's pairwise comparisons test.

The amounts of Ino detected in the samples increased as storage time increased, as Figure 4.6 shows. Days stored and storage method were the only design variables providing significant differences between the samples ($p<0.001$ and $p=0.018$, respectively). The chilling method was insignificant ($p>0.579$). Day 16 of the 4-month frozen fish had, on average, the highest amount of Ino with 3.75 ± 0.52 µmol/g. The lowest amount detected was 1.25 ± 0.23 µmol/g on day 1, 1-month frozen samples. Tukey's pairwise comparison test showed a significant difference between the sampling days and between 1-month and 4-months freezing time ($p<0.05$). An interaction between the design variables storage days and the chilling method was observed ($p=0.007$).

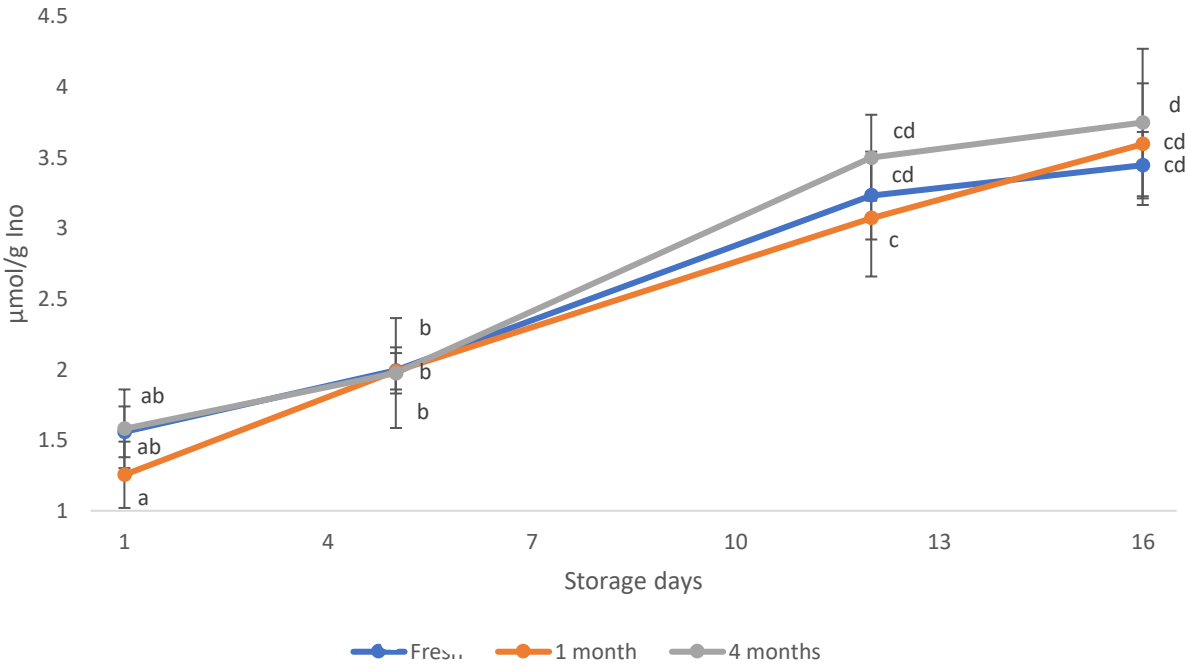

Figure 4.6: The concentration of Ino (µmol/g) of the samples stored fresh, 1 month frozen, and 4 months frozen. Both these design variables were significant ($p<0.05$). Lowercase letters a-d imply a significant difference between the storage days and the storage method ($p<0.05$) based on Tukey's pairwise comparison test.

The amount of Hx detected in the samples increased as storage time increased. A steady increase from day 1 to day 16 is demonstrated in Figure 4.7. Day 1 had the lowest amount of Hx detected (0.07 ± 0.09 µmol/g, average across the chilling methods), while day 16 had the highest amount (1.70 ± 0.34 µmol/g, the averages across the chilling methods). Days stored were the only design variable providing a significant difference ($p<0.001$); however, the concentrations of Hx of the different chilling methods are shown in the same figure. No significant interactions between the other design variables and no interactions were observed ($p>0.05$).

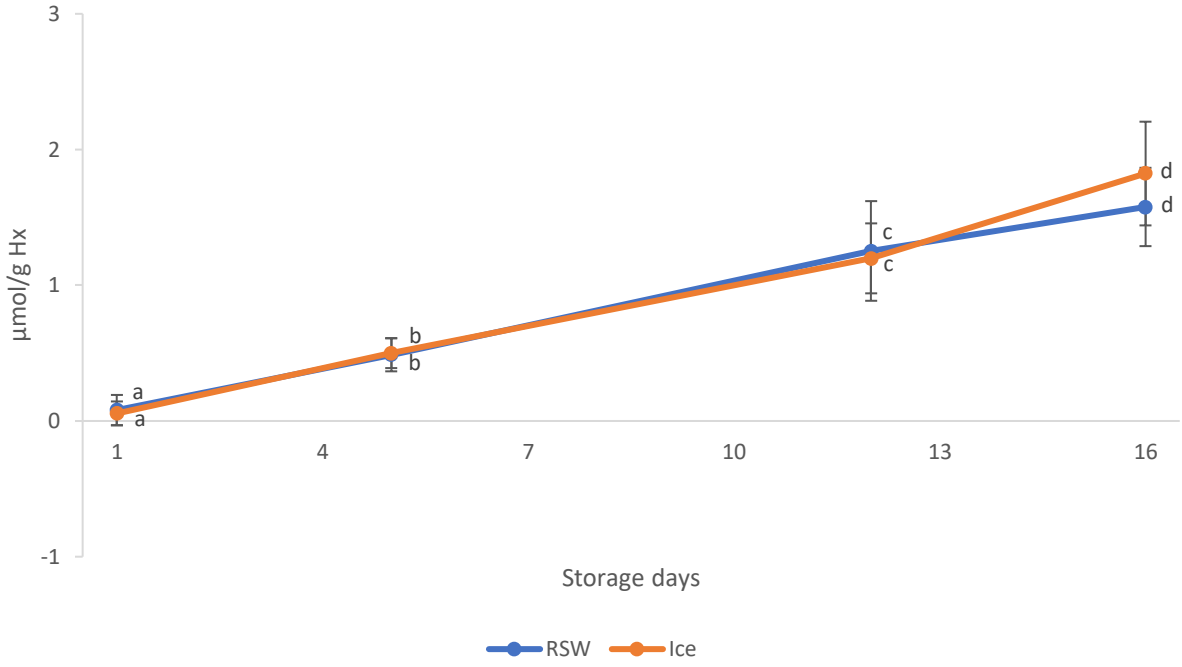

Figure 4.7: Content of Hx (µmol/g) during the different sampling days. The letters a-d indicates a significant difference ($p<0.05$) based on Tukey's pairwise comparisons test between the storage days and chilling method. Storage days were the only design variable significant; however, the concentrations of Hx detected in samples chilled with RSW and ice are also shown. The chilling method was insignificant ($p>0.388$).

The K-value increased as storage time increased. The K-value was only found to be significantly affected by the design variable storage days ($p<0.001$). The storage and chilling methods were insignificant ($p>0.05$), though chilling method as a function of storage days is shown in Figure 4.8A. An interaction between the variables storage days and storage method was detected ($p=0.011$) (Figure 4.8B).

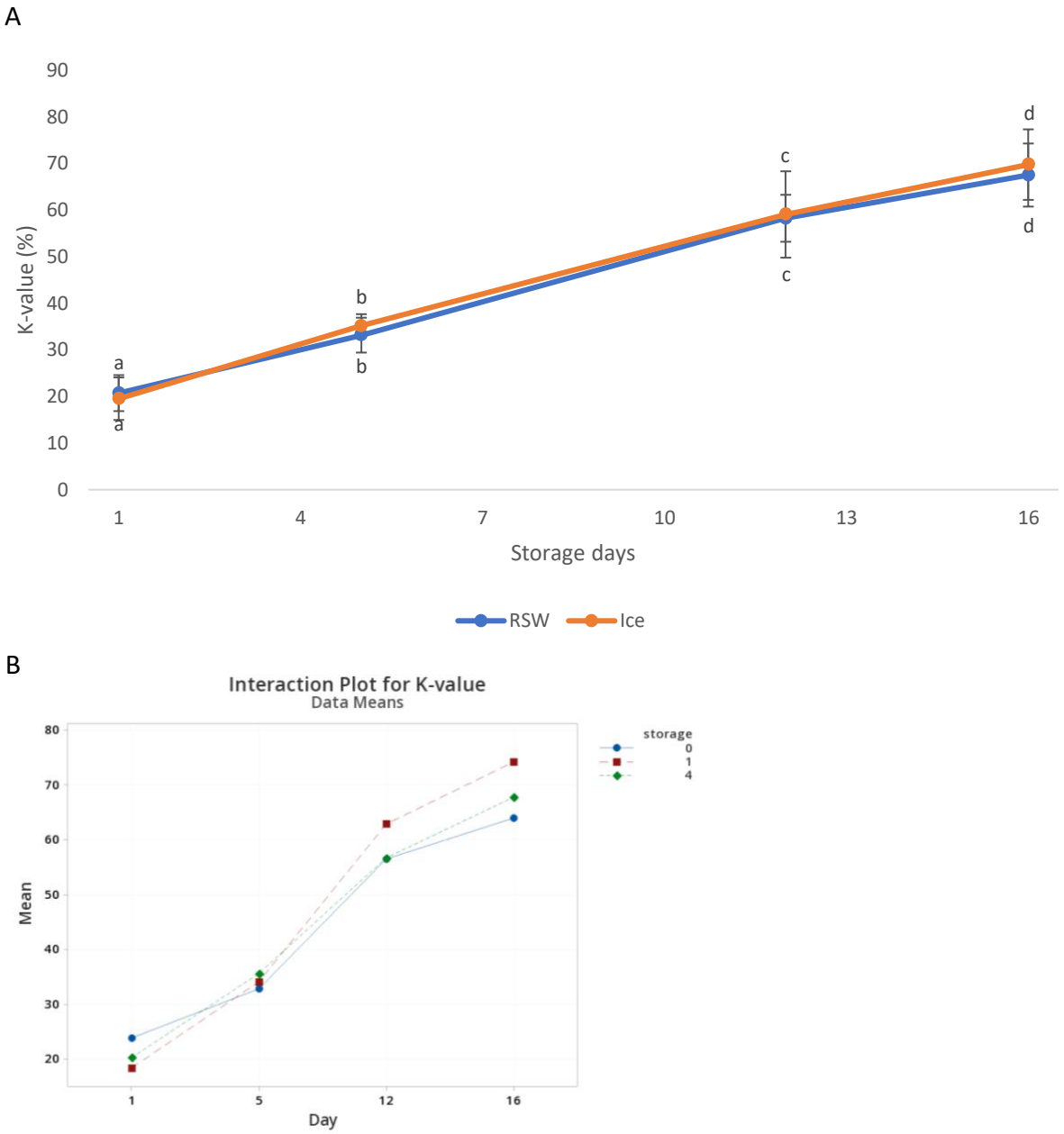

Figure 4.8: A) The increase of K-value throughout storage, with day 16 having the highest. Storage days were the only design variable significant. However, the chilling method is shown as a function of storage days. No significant difference was found between the chilling methods on the different days based on Tukey's comparison test, shown as lowercase letters a-d. However, a significant difference between each storage day was observed. B) Interaction plot for storage days and storage method, where 0= fresh, 1= 1 month frozen, and 4= 4 months frozen.

4.7 Protein Denaturation

Four samples with two parallels from both RSW and ice-chilled samples on day 1 and day 16 were used to determine the degree of protein denaturation. DSC was applied to find the peak maximum temperature (T_m), and the enthalpy needed to denaturate the different types of proteins in the muscle. Figure 4.9 demonstrates a thermogram for one of the samples where the peaks were integrated using a spline baseline. According to literature, peak 1 is myosin, peaks 2 and 3 are denaturation of sarcoplasmic proteins, while peak 4 is actin (Ofstad et al., 1996).

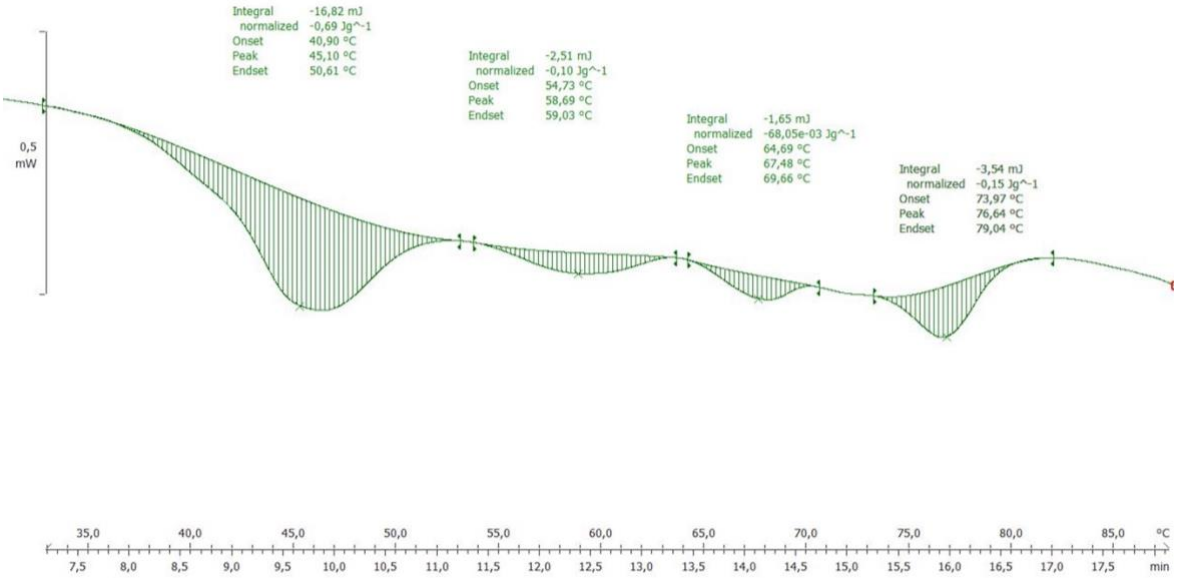

Figure 4.9: Example of thermogram for one of the samples where the peaks have been integrated using a spline baseline.

T_m was standardized and compared to literature. Table 4.4 presents the onset- and endset temperature for the peaks and T_m alongside Ofstad et al. (1996) results for T_m.

Table 4.4: Standardized peak temperatures for onset, endset, and T_m from present work, and Ofstad et al. (1996) T_m for salmon kept on ice.

	Temperature °C				
	Peak 1	Peak 2	Peak 3	Peak 4	
Onset	40.1 ± 0.8	54.7 ± 0.8	64.5 ± 0.4	73.6 ± 0.3	Present work
T_m	44.4 ± 0.9	58.4 ± 0.5	67.1 ± 0.3	76.4 ± 0.4	
Endset	50.0 ± 0.6	61.2 ± 1.4	69.4 ± 0.3	78.7 ± 0.3	
T_m	44	56-57	65	73-74	Ofstad et al. (1996)

The design variables method of storage (p=0.015) and storage days (p=0.06) contributed to a significant difference regarding peak 1. For peak 2, a significant difference in the method of storage (p=0.038) was observed. Interactions between the variables storage days and method of storage (p=0.025) and chilling method and method of storage (p=0.010) were also observed for peak 2, as shown in Figure 4.10A and B. Peak 3 was found to be significantly affected by storage days (p<0.001), and an interaction between the chilling method and method of storage was observed (p=0.008) (Figure 4.10C). For peak 4 an interaction between storage days and the chilling method was observed (p=0.023) (Figure 4.10D). No other significant differences between the design variables were observed (p>0.05). The results are summarized in Table 4.5.

Table 4.5: Effect of the chilling method, method of storage, and storage days on enthalpy (J/g) utilized to denaturate proteins. The letters a-b indicates a significant difference (p<0.05) within the design variable, and the p-values are shown in cursive.

	Enthalpy (J/g)				
	Peak 1	Peak 2	Peak 3	Peak 4	n
Chilling Method					
RSW	0.51 ± 0.10	0.08 ± 0.02	0.04 ± 0.01	0.15 ± 0.02	46
Ice	0.61 ± 0.07	0.08 ± 0.01	0.04 ± 0.01	0.16 ± 0.02	44
Effect of chilling method (p-value)[1]	*0.071*	*0.285*	*0.334*	*0.670*	
Method of Storage					
Fresh	0.62 ± 0.07[a]	0.09 ± 0.02[a]	0.05 ± 0.01	0.16 ± 0.02	32
1 month frozen	0.59 ± 0.08[ab]	0.07 ± 0.02[b]	0.04 ± 0.01	0.16 ± 0.03	30
4 months frozen	0.51 ± 0.08[b]	0.08 ± 0.01[ab]	0.04 ± 0.01	0.15 ± 0.02	30
Effect of Storage Method (p-value)[1]	*0.015*	*0.038*	*0.193*	*0.413*	
Storage Days					
1	0.60 ± 0.09	0.08 ± 0.02	0.05 ± 0.01	0.13 ± 0.03	48
16	0.52 ± 0.08	0.09 ± 0.02	0.03 ± 0.01	0.16 ± 0.02	44
Effect of Storage Days (p-value)[1]	*0.009*	*0.074*	*<0.001*	*0.281*	

[1]Significance level p<0.05, GLM

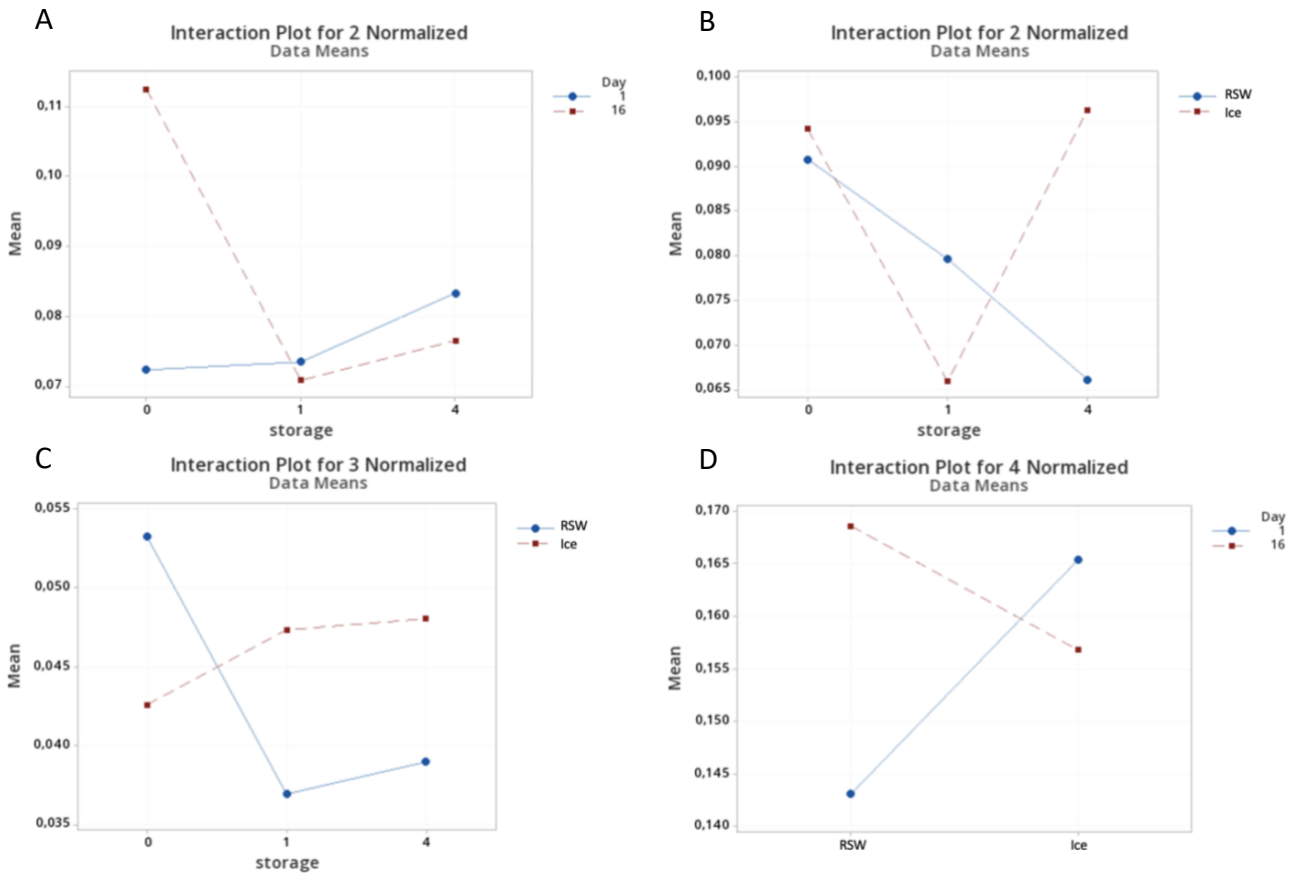

Figure 4.10: Interaction plots for peaks 2, 3, and 4. A) For peak 2, both method of storage and storage days, and B) method of chilling and method of storage were significant ($p<0.05$). C) For peak 3, only the method of chilling and storage days was significant ($p<0.05$), and for D) peak 4, only the method of chilling and storage days was significant ($p<0.05$).

5 Discussion

The present thesis aimed to investigate how the temperature of Atlantic salmon prior to freezing, and frozen-storage time affected the quality of fillets post thawing. A group of fresh fillets was analyzed as controls. Analyses were conducted on days 1, 5, 12, and 16 after storage in a cooling room for all three groups (fresh, 1 month, and 4 months frozen). The results were thereafter discussed and compared to the literature. A mistake was made early in the experiments regarding the microbiology results, resulting in hydrogen sulfide (H_2S) producing bacteria not being counted individually. APC and TVC are, therefore, the only results presented and discussed. Some fillets lost their vacuum during storage, rendering the fillets unfit for sampling and making the number of samples vary, as seen in the tables in chapter 4.

Pearson's correlation coefficient was utilized to determine the relationship between the design variables. The coefficient with the corresponding relationship is presented in Table 5.1.

Table 5.1: Pearson's correlation coefficient and the corresponding relationship (Evans, 1996).

r value	
+ 0.70 →	Very strong positive relationship
+ 0.40 to + 0.69	Strong positive relationship
+ 0.30 to + 0.39	Moderate positive relationship
+ 0.20 to + 0.29	Weak positive relationship
+ 0.01 to + 0.19	Negligible relationship
0	No relationship
- 0.01 to - 0.19	Negligible relationship
- 0.20 to - 0.29	Weak negative relationship
- 0.30 to - 0.39	Moderate negative relationship
- 0.40 to - 0.69	Strong negative relationship
- 0.70 →	Very strong negative relationship

5.1 Method of Chilling

The water content of Atlantic salmon is around 60-70%, and it is highly undesired that the fish loses large amounts of water during storage (Dawson et al., 2018). The fish used for these experiments were slaughtered in September and had an average water content of 68.1%. It was not expected that the water content would fluctuate between the samples as it is a relatively constant parameter when fish is captured in the same season (Aksnes et al., 1986). Berg and Bremset (2005) could present riverine Atlantic salmon having the highest water content during spring. The high water content was correlated to the protein composition and fat content. However, only minor fluctuations were found within the season. Therefore, it is assumable that fish from any other season would have a slightly different water content.

Sub-chilled samples (chilled in RSW) had, on average, a higher drip loss than the samples chilled with ice. This is not in line with Kaale et al. (2014), where drip loss was significantly lower in super-chilled samples compared to chilled and frozen ones. There are, however, contradicting results regarding whether super-chilling is an optimal way to chill fish when looking at drip loss. Bahuaud et al. (2008) could present -1.5 °C short super-chilled Atlantic salmon *pre-rigor* fillets having the same damage as Atlantic salmon frozen at -20 °C due to large intra- and extracellular ice crystals. On the other side, Chan et al. (2020a) did not find any significant differences regarding drip loss in fish chilled on ice and fish chilled with RSW.

Kaale et al. (2013) demonstrated that a high super-chilling rate is preferred, resulting in the formation of smaller ice crystals. As mentioned in chapter 2.4.3, smaller ice crystals result in more uniform freezing, allowing the cells to maintain their integrity and reducing the drip loss during thawing. When cell integrity is maintained, the water from the ice crystals is reabsorbed by the proteins, who, when thawed, obtain their natural isoelectric point and eventually bind the water. If the proteins have been denaturated during freezing, the proteins will not bind to the water, the water is lost, and the quality of the fish meat deteriorates significantly (Nakazawa and Okazaki, 2020).

It is essential that the temperature variations when sub-chilling are kept minimal. When looking at Figure 4.1, the temperature fluctuates slightly, potentially causing the RSW samples to have the degree of drip loss observed. Even minor temperature changes might result in the

ice crystals melting and recrystallizing, resulting in the ice crystals changing size and location, increasing the degree of destruction, and increasing drip loss (Wu et al., 2014).

One of the main limiting factors of shelf life is the growth of microorganisms. Samples that were sub-chilled had a lower amount of bacterial growth regarding APC of mesophilic bacteria, and the ice-chilled samples had almost one log unit more bacterial growth across all the storage days. For TVC of psychrotrophic bacteria, sub-chilled samples also had less growth; however, differences between ice-chilled and sub-chilled samples were not statistically significant. Sub-chilling seems to inhibit the growth of mesophilic microorganisms, but not psychrotrophic microorganisms. This is not in accordance with Erikson et al. (2011), who found that RSW-chilling was very effective in inhibiting the growth of microorganisms contributing to TVC. However, Bono et al. (2017) found that super-chilling in combination with ozone did not affect the growth of psychrotrophic bacteria in European anchovy (*Eugraulis encrasicolus*) and sardine (*Sardina pilchardus*). Sarmast et al. (2019), on the other hand, found that ice-glazing in combination with super-chilling storage delayed the growth of both mesophilic and psychrotrophic bacteria. There are contradicting results from previous studies, and sub-chilling might therefore be a limiting factor regarding the growth of microorganisms. However, since H_2S-producing bacteria were not counted, it is hard to determine whether it is a suitable method for limiting the growth of the SSOs. The growth of microorganisms does not necessarily equal spoilage, and without the characterization of the organisms, a conclusion cannot be made regarding the inhibition of spoilage.

The reason sub-chilled samples have less bacterial growth can be explained by the difference in temperature. According to Bréand et al. (1997), the most important environmental factor influencing the growth of bacteria is the temperature. Psychrotrophic bacteria are able to grow at temperatures below 0 °C but have an optimum temperature of around 15 °C, while mesophilic bacteria have an optimum temperature between 25-40 °C (Thomas et al., 2008). When the microorganisms are exposed to temperatures outside their growth range, known as stress-induced temperatures, the organisms redirect their energy to adapt rather than grow (Park et al., 2021). This might explain why sub-chilling haltered the growth of mesophilic bacteria as sub-chilled samples reached a lower temperature, ultimately inducing more stress on the microorganisms.

There are variable results regarding the indication of spoilage and end of shelf-life, with Robson et al. (2007) reporting end of shelf-life as 5-6 log CFU/g and Dalgaard et al. (1997) reporting end of shelf-life as 8-9 log CFU/g. All the ice-chilled and RSW-chilled samples were far from the reported limits after 16 days of being stored in a chilling room, indicating that the samples were of high microbial quality.

Whether the fish was chilled using ice or RSW had no significant effect on textural or colorimetric properties. This might indicate that sub-chilling provides a product of the same quality as the traditional practice.

IMP, Ino, and Hx are the ATP-catabolites of interest regarding food quality (Hong et al., 2017). This is because ATP is rarely found in foodstuff unless the animal is slaughtered, and analyses are conducted immediately. As mentioned in chapter 2.3.2, ATP is readily degraded when the glycogen storages are depleted. The enzyme breaking down ATP, ATPase, is highly active when the temperature is around -0.8 °C to -5.0 °C (Gill, 2000). All the samples obtained temperatures within or close to the temperature range, resulting in the ATP being degraded rapidly.

The chilling method did not significantly affect the concentration of IMP, Ino, or Hx, and neither was the K-value affected. Both RSW-chilled fish and ice-chilled fish had approximately the same levels of the three catabolites. The K-value not being affected by the chilling method is not in line with Mozuraityte et al. (2021), who found that samples of Atlantic mackerel (*Scomber scombrus*) stored in super-chilled conditions were of good quality 9 days longer than samples chilled on ice. However, the samples in this study were all stored at the same temperatures, which explains the contradicting results compared to Mozuraityte et al. (2021) and indicates that sub-chilling treatments prior to freezing might be extraneous.

Protein denaturation can be measured with DSC. For both RSW-chilled samples and ice-chilled samples, four peaks were observed. This is in line with Ofstad et al. (1996), who reported four peaks at 44 °C, 57 °C, 65 °C, and 74 °C. The first peak is attributed to myosin, the two peaks following are sarcoplasmic proteins, and the last peak is attributed to actin (Liu et al., 2013). It is the endothermic energy required to unfold the protein from its original structure

compared to the linear plot of the reference pan that is registered by the DSC machine (Hastings et al., 2006). The results fit according to Ofstad et al. (1996) temperatures, indicating that it is, in fact, myosin, sarcoplasmic proteins, and actin that have become denaturated. RSW-chilled fish required, on average, less energy to denaturate myosin, which might indicate that the myosin had been denaturated during the chilling process. Another explanation might be that the myosin in the samples was denaturated during the waiting time prior to being inserted into the DSC machine. The chilling method was insignificant ($p>0.05$) for the other proteins, and there was almost no difference in denaturation energy. The sarcoplasmic proteins and actin are more stable than myosin, withstanding the chilling temperature better, according to Konno (2017).

5.2 Storage

Quality deterioration over time is inevitable for almost everything—especially organic materials like fresh-and frozen foodstuff. By sub-chilling and freezing, enzymatic and bacterial activity are haltered, prolonging the shelf-life immensely (Elíasson et al., 2018; Dawson et al., 2018). There are, however, other consequences when foodstuff is frozen. As mentioned in chapter 2.4.3, freezing can be destructive for the cellular structures resulting in dry and tough meat, higher drip loss, and more extensive protein denaturation. Storing thawed or fresh foodstuff for a more extended period typically increases the spoilage potential.

When comparing the fresh and frozen samples, the frozen samples have approximately double the drip loss percentage as the fresh samples. This is as expected since freezing disrupts the myofibrillar space and, depending on freezing time, disrupts other important cellular components like denaturating proteins. When these structures have been disrupted and denaturated, the water cannot be absorbed when thawed and thereby end up as drip loss (Kaale et al., 2014). All the frozen samples were flash-frozen with dry ice for 30 minutes before being placed in a freezer at -30 °C. Therefore, it is safe to assume that all the samples experienced a rapid freezing time where smaller ice crystals were formed. However, when the samples were placed in the freezer, the temperature difference might have induced recrystallization resulting in more extensive and more destructive ice crystals (Roos, 2021). Zhao and Takhar (2017) state that temperature fluctuations during storage are inevitable, and Syamaladevi et al. (2012) could report that the size of the ice crystals in Atlantic salmon

increases by 22.1% when frozen for 4 weeks at -35 °C with no provoked temperature fluctuations. The study observed that the longer the storage time in the freezer, the more extensive the drip loss became. This is in line with the results from this experiment, as samples frozen for 4 months have the highest drip loss for all the sampling days. Claussen et al. (2017) also reported super-chilled salmon having a more considerable drip loss than regular chilled salmon, stating that freeze damage during partial freezing prior to entering the super-chilled state causes extensive drip loss in addition to the drip loss caused by freezing.

Drip loss also increased as storage days increased, with day 16 having almost three times the amount of drip loss as day 1. However, the difference in percentage lost as drip loss was overall higher when looking solely at the amount at the first two sampling days, and the amount ceased towards the end of the storage trial. Day 12 and 16 were significantly different from day 5 and day 1, which is in line with Claussen et al. (2017), who also reported an increase in drip loss at the beginning and a plateauing towards the end of the storage. Feng et al. (2020) found that the pH of salmon fillets stored in cooling rooms had a sudden drop during the first three days before the pH increased. This might explain why the first days of storage have such a high amount of drip loss as pH alters the isoelectric points of the proteins imposing that the proteins might not reabsorb the water to the extent possible. This is just a supposed resonance as the pH was not measured at any point during the experiments.

Seafood is highly perishable, and an increased bacterial growth was expected as storage days increased. This is shown in Figure 4.2, where for APC, the samples had the lowest count on day 1 and the highest count on day 16. RSW-chilled samples have a significantly lower amount of bacterial growth than ice-chilled samples on all sampling days, similar to what Hansen et al. (2009) found when comparing super-chilled fillets to chilled fillets after 21 and 28 days. Interestingly, Tukey's comparison test registered that day 16 of RSW-chilled samples were compatible with day 5 ice-chilled samples, implying that RSW-chilled samples were of higher microbial quality throughout storage. After a more extended storage period, analyses would have to be conducted to confirm if this is the case following 16 days of storage.

Figure 4.3 shows the TVC for fresh, 1-month, and 4-months frozen samples. It was expected that the fresh samples would have more growth than the frozen ones, which is the case for

days 1, 12, and 16 (Ge et al., 2012). Although both the fresh and 4-months frozen samples follow approximately the same growth curve, the sudden increase in bacterial growth on day 5, 1-month frozen samples might imply that the 1-month frozen fillets were of poorer microbial quality when initially frozen. The increase might also be explained by a combination of systematic and human errors. Ge et al. (2012) reported less bacterial diversity in frozen fish compared to fresh fish, which might explain why frozen samples, in general, had less growth than the fresh samples.

Even though the storage method was insignificant alone as a design variable for APC, it was significant as an interaction with the chilling method. Remarkably, sub-chilled samples have less microbial growth stored as fresh and 4-month frozen compared to samples chilled with ice. When frozen for 1 month, however, sub-chilled samples have more growth. The same interaction was observed for TVC. It is hard to pinpoint the cause, as many factors play an essential role. It would mostly be speculations trying to explain why 1-month frozen sub-chilled samples suddenly had an increase in microbial growth. New microbial analyses with the same experimental design would need to be conducted to determine whether 1 month of freezing is not optimal when sub-chilling.

The method of storage was significant for breaking force and firmness. Fresh samples required more force to break the surface than the frozen samples, and the 1-month frozen samples required the least amount of force. It was expected that the frozen samples would require less force to break the surface as freezing damages some of the cellular structures, causing softening of the tissue. Rehbein and çaklı (2000) could report lysosomal enzyme activity as higher in samples of various fish that had been frozen compared to fresh samples, which corresponds with the results of this experiment. Sigurgisladottir et al. (2000), on the other hand, report that freezing contributes to a loss in juiciness and increased toughness. This might explain why 4-month frozen samples require more force to break the surface than 1-month frozen samples. The 4-months frozen samples might have been more freeze damaged than the 1-month frozen samples, and less water has been obtained by the proteins when thawed, resulting in dryer fillets. This explanation might apply to the same trend observed for firmness as well. When looking at drip loss, 4-month frozen samples have the highest amount, supporting Sigurgisladottir et al. (2000) findings that freezing contributes to a loss in juiciness.

Storage days were not significant for breaking force, although a decrease in force as storage days increase can be observed. Storage days were significant for firmness, with the firmness decreasing as the storage days increased. Chan et al. (2021c) could also report a significant decrease in firmness as storage days increased, but not in breaking force. This indicates that softening within the flesh occurs during storage, but the surface is not affected.

Taylor et al. (2006) report that changes in the textural properties of Atlantic salmon mainly happened from day 0 to day 5, and the samples from day 5 to day 14 were very similar. This is in line with the current results, as day 1 is significantly different ($p<0.05$) from the rest of the sampling days. Fiber-to-fiber attachment is lost within 24 hours postmortem, while after 1 day of storage, the fibers are detached from the myofibers. After 5 days of storage, the fibers are separated from the myocommata (Taylor et al., 2006). A sudden decrease in force needed to compress 60% of the fillet is observed from day 1 to day 5, indicating that the fibers are detached from the myocommata. However, this would have to be confirmed by microscopy. As mentioned in chapter 2.1, dystrophin acts as a structural component in the costamere. Taylor et al. (2006) could also report that within 24 hours postmortem, the dystrophin complex had been degraded, resulting in a rapid loss of textural quality. This corresponds with the results for firmness. The longer the fish is stored, the more enzymatic and bacterial activity is anticipated. Enzymes are active in the cells even after death has occurred and are especially important in the quality changes that occur during the first few days of storage.

The drip loss had a strong negative correlation ($r= -0.461$, $p<0.001$) to the firmness and a moderate negative correlation ($r= -0.363$, $p<0.001$) to breaking force, according to Table 5.1. These relationships indicate that higher drip loss results in less force needed to break the surface and compress 60% of the fillet. This was anticipated as high amounts of drip loss are usually indicators of cellular destruction and protein denaturation (Chan et al., 2020a). However, Pearson's correlation coefficient only reflects the linear correlation of the variables, meaning other relationships could be ignored (Kotu and Deshpande, 2019). Although there is a negative linear relationship between drip loss and textural properties, a correlation between increased drip loss and increased toughness can still be there.

L* was affected by neither method of storage nor storage days. Therefore, a fillet frozen for 4 months and stored for 12 days is presumably precepted equally as a fresh fillet stored for 1 day, regarding L*. An interaction, however, between the method of chilling and the method of storage was observed, as seen in Figure 4.4. There was little to no difference between fresh RSW-samples and ice samples, indicating that sub-chilling is negligible regarding the lightness of fillets when stored fresh. The ice-chilled samples had a decrease in L* when frozen for 1 month compared to fresh samples and a slight increase when frozen for 4 months compared to 1-month samples, and the same trend could be observed for RSW-chilled samples as well, but to a lesser degree. Chilling with ice prior to freezing indicates that the fillets become a little darker than sub-chilling prior to freezing. The results for both the chilling methods are not in line with Rørå and Einen (2003), who could present L* increasing in frozen cold-smoked salmon fillets stored at -30 °C for 6 days compared to fresh cold-smoked salmon fillets. The astaxanthin content was analyzed, and they found that the pigment was broken down during freezing. Regost et al. (2004) could also report astaxanthin concentration of raw Atlantic salmon fillets decreasing with freeze storage at -20 °C for 3 months and L* increasing.

Changes in lightness have previously been correlated with increased drip loss and protein denaturation. Robb et al. (2000) reported increased L* in rainbow trout throughout storage due to proteins denaturating causing water to be lost as drip loss which eventually altered the surface reflection of light. In this study, L* had a negligible relationship to drip loss ($r= 0.053$, $p>0.583$) and to protein denaturation (all proteins: $r< 0.19$, $p>0.438$). In fact, L* had a negligible relationship to all experimental variables, except chroma and hue, making it reasonable to suggest that there might be other factors affecting L*, such as lipid oxidation which Stien et al. (2005) could present happening in cod (*Gadhus mohua*).

Chroma was only significantly affected by the method of storage, where an increase in the chroma was observed as the samples were frozen. An increase in chroma suggests that the color intensity became stronger during the freezing of the samples. This is not in accordance with Santos-Yap (1996), who reported a fading of the characteristic color of salmon during freezing. The storage days not affecting the color intensity are also not in accordance with either Erikson et al. (2011) or Chan et al. (2021a), who reported a decrease in chroma as storage days increased. Contrarily, Rørå and Einen (2003) could report that the color intensity

of the fillets increased as astaxanthin content decreased. They also reported that color intensity correlated with a harder texture, which is not the instance for the results in the present study ($r= 0.113$, negligible relationship, $p<0.236$).

However, an interaction between the chilling method and the method of storage was observed for chroma. Sub-chilled fresh samples had a higher color intensity than ice-chilled fresh samples. When 1 month frozen, however, the ice-chilled samples had a vast increase in color intensity while the sub-chilled samples had a slight decrease. When 4 months frozen, the samples had an equal color intensity, with ice-chilled samples having a slight decrease compared to the samples frozen for 1 month, and sub-chilled samples having a slight increase. Therefore, choosing the most suitable chilling method seems to depend on how the salmon is stored and how long the salmon is supposed to stay frozen.

Contrary to the chroma, the hue was significantly affected both by the method of storage and storage days. Fresh samples were significantly different from the frozen samples, with hue increasing 1° when samples were frozen. An increase in hue indicates an increase in yellowness as 0° = red, while 60° = yellow. This is in line with Regost et al. (2004), who reported an increased hue in Atlantic salmon with frozen storage. The pigment astaxanthin has been reported to have antioxidation properties, and according to Jensen et al. (1998), the pigment partakes to protect lipid oxidation in the early stages. In Atlantic herring (*Clupea harengus)*, the increase in yellowness was found to be caused by lipid oxidation, according to Hamre et al. (2003). As Atlantic salmon is a particularly fatty fish, the observed increase in yellowness might be due to a combination of lipids oxidating and astaxanthin being used as an antioxidating agent.

Hue decreased as storage days increased, with day 16 being significantly different from the rest of the storage days. Decreasing hue means the color of the samples is becoming redder. This might indicate that a color change visible to the human eye occurs only after 12 days of storage in a cooling room. This is in accordance with Chan et al. (2021a), who also found a decrease in hue during storage. Chan et al. (2021a) explained the decreasing hue with an increasing chroma throughout the storage time as the fillets did not exhibit an increased

redness (a*). The results in this study show a similar trend, and the decreasing hue might therefore be explained by the increasing color intensity.

The nucleotide degradation commences postmortem in fish muscle and progresses throughout storage. ATP is rapidly deaminated to ADP and further to AMP and IMP, while IMP dephosphorylation to Ino and Hx is a slower process (Fogarty et al., 2019a). The presence of IMP is desired as this catabolite contributes to the umami flavor (Kawai et al., 2002). Both the storage method and storage days were significant regarding the amount of IMP detected in the samples, and the amount of IMP decreased as storage days increased. This is expected and in line with Karim et al. (2019), who found that in both haddock (*Melanogrammus aeglefinus)* and Atlantic herring, the concentration of IMP decreased significantly throughout storage of 14 days. The dephosphorylation of IMP will proceed either by enzymes or by bacterial activity as long as the nucleotide is present. Fresh samples were significantly different from the frozen samples and had higher concentrations of IMP on all sampling days. Rehbein and çaklı (2000) could present that lysosomal enzyme activity was much higher in frozen and thawed fish compared to fresh fish in a variety of species. This might explain why the levels of IMP are lower in the frozen samples and why the difference between the fresh and frozen samples increases as storage days increase.

As the concentration of IMP decreased throughout storage, the concentration of Ino and Hx increased. The concentration of IMP was inversely related to Ino ($r= -0.825$, $p<0.001$) and Hx ($r= -0.858$, $p<0.001$). This is as expected as IMP is the precursor to Ino and Hx, and a decrease in IMP will lead to an increase in Ino and Hx. Endogenous enzymes and bacterial enzymes are responsible for the dephosphorylation of IMP. The rate at which IMP is dephosphorylated is highly dependent on temperature (Gill, 2000). It has also been proved that the rate of IMP dephosphorylation follows the Arrhenius function for some species at low temperatures (Yang et al., 1998). Meaning, that when temperature increases, the activation energy for the enzymes decreases, and the rate of the reaction increases. Higher concentrations of Ino were observed on all storage days compared to Hx. This is in accordance with Creelman and Tomlinson (1960), who reported a higher concentration of Ino in Pacific salmon (*Oncorhynchus tshawytscha*), and Kyrana and Lougovois (2002), who reported the same in European sea bass (*Dicentrarchus labrax*). Species like rainbow trout (*Oncorhynchus mykiss*)

and rockfish (*Helicolenus dactylopterus*), however, have been found to have higher concentrations of Hx at the end of storage (Mendes et al., 2001; Howgate, 2005).

Microbial growth is an important factor regarding nucleotide degradation. APC had a very strong positive relationship regarding amount of Hx ($r= 0.707$, $p<0.001$) and Ino ($r= 0.713$, $p<0.001$), while TVC had a strong positive relationship ($r= 0.683$, $p<0.001$ and $r= 0.631$, $p<0.001$, respectively). This is expected as the degradation degree of IMP is highly dependent on the growth of microorganisms, and the more growth of microorganisms, the more IMP is degraded by bacterial enzymes into Ino and Hx. On the other hand, IMP had a very strong negative relationship with APC ($r= -0.716$, $p<0.001$) and a strong negative relationship with TVC ($r= -0.631$, $p<0.001$). This is also expected as more bacterial growth equals less IMP in the samples. Boyle et al. (1991) confirmed this by packaging whitefish (*Coregonus clupeaformis*) and rainbow trout in a modified atmosphere, limiting microorganisms' growth and eventually decreasing the Hx concentrations.

The K-value has been well documented as a good indicator of freshness in fish as it assesses the concentration of the spoilage nucleotides (Howgate, 2005; Ashie and Simpson, 1996; Hong et al., 2017). For Atlantic salmon, the limits of acceptability have been reported to be 70-80% by Erikson et al. (2006). Therefore, the fish in this experiment was acceptable for consumption after 16 days of storage. There was no significant difference between RSW-chilled fish and ice-chilled fish or if the fish had been frozen. However, an interaction between the method of storage and storage days was observed. For day 1, samples stored for 1-month in the freezer had the lowest K-value. Interestingly, the K-values on day 12 and day 16 had an opposite trend, where samples frozen for 1 month had the highest values. This is not in accordance with Fernández-Segovia et al. (2012), who reported a steady increase in the K_1-value in Atlantic salmon samples as days stored in the freezer increased.

Storage in a cooling room after 1-month freezing results in a considerably higher increase in K-value than fresh or 4-months frozen fish. 1-month frozen samples have a higher count regarding TVC on day 5, as seen in Figure 4.3; however, this does not explain the high K-value of the last storage days of 1-month frozen samples. Miki and Nishimoto (1984) investigated the K-value for skipjack (*Katsuwonus pelamis*), Atlantic mackerel, and red seabream (*Pagrus*

major) at different temperatures. They reported that the K-value changes even at freezing temperatures. However, it would be expected that the 4-months frozen samples would have similar K-values on the different sampling days as the 1-month frozen samples. A steep increase in the K-value can be observed from day 5 to day 12, and this can be explained by the fact that there were 7 days between the analyses and not 4 as there were between the other sampling days.

Regarding protein denaturation, only myosin was significantly affected by freezing. There was a difference between the fresh samples and the samples frozen for 4 months, and the energy required to denaturate the proteins decreased as the storage time in the freezer increased. It has been reported that myosin is less temperature stable than actin. According to Chan et al. (2006), certain regions of the protein causes it to be less thermostable. For the other proteins, freezing did not affect the denaturation. On the other hand, storage days were significant for both sarcoplasmic proteins and myosin. A decrease in energy required to unfold the proteins was observed as the storage days increased. This indicates that the proteins are denaturated while stored in the cooling room. This is also in accordance with Jensen and Jørgensen (2003), who found that storage in a freezer and storage in a cooling room had independent effects on the denaturation of proteins in cod mince.

As mentioned in chapter 2.1.3, sarcoplasmic proteins make up a good portion of the water-soluble proteins in a fish, while salt-soluble proteins consist of mostly myofibrillar proteins (Lin et al., 2021). For peak 2- sarcoplasmic proteins, two interactions were observed. One was between storage days and method of storage, where fresh samples had an increase in denaturation energy from day 1 to day 16. In contrast, the frozen samples had a decrease in denaturation energy. The difference in denaturation energy required for the different days was minor for both 1-month and 4-months frozen samples. This can be explained by freeze damage due to recrystallization, which denaturates proteins and disrupts cellular structures. It is well documented that an increase in sarcoplasmic proteins during storage occurs. Lin et al. (2021) could report an increase in sarcoplasmic proteins in Atlantic mackerel stored at 0 °C and 4 °C after five days, while Lu et al. (2017) reported the same in Bighead carp (*Aristichthys Nobilis*). One explanation for why the fresh samples had an increase in denaturation energy throughout storage is that the content of salt-soluble proteins decreases throughout storage,

leading to increased hydrophobicity. Increased hydrophobicity eventually leads to the formation of non-covalently and covalently linked aggregates between the water-soluble proteins, which increases the energy required to denaturate these proteins (Badii and Howell, 2002).

The other interaction for peak 2 was between the chilling and storage methods. There was no great difference between sub-chilled and ice-chilled samples when the samples were stored fresh. The difference increased as storage time in the freezer increased, and at 1-month frozen, sub-chilled samples required more energy to denaturate proteins which is the opposite of fresh and 4-month frozen samples. This might imply that the sarcoplasmic proteins in the sub-chilled samples do not aggregate throughout storage in a freezer, but the proteins in the ice-chilled samples do after 1 month of storage. This is not in accordance with Gallart-Jornet et al. (2007), who could report that freezing resulted in a decrease in sarcoplasmic proteins.

An interaction for peak 3, sarcoplasmic proteins, was observed between the storage and method of chilling. Fresh and sub-chilled samples required more energy to denaturate the proteins. While for the frozen samples, ice-chilled samples required more energy. This might also be due to the aggregation of sarcoplasmic proteins. For actin, peak 4, none of the parameters were significant; however, an interaction between the method of chilling and storage days was observed. Actin is a reasonably stable protein due to bound nucleotides and divalent cations; it was therefore expected that actin was unaffected by the design variable (Rosin et al., 2014). Ice-chilled samples required less energy to denaturate the proteins on day 16 than on day 1, which is expected as proteins become denatured throughout storage by various processes. On the other hand, sub-chilled samples required less energy to denaturate the proteins on day 1, indicating that the proteins aggregate throughout storage. Kaale and Eikevik (2016) could report that salt-soluble proteins increased significantly in super-chilled salmon, while Sankar and Fathiraja (2015) reported that tilapia (*Oreochromis mossambicus*) stored on ice experienced a decrease in salt-soluble proteins. Even though salt-soluble proteins include both myosin and actin, these findings moderately support the results of this experiment.

The denaturation of proteins did not have a strong correlation to the texture. In fact, denaturation of myosin and peak 2 (sarcoplasmic proteins) had a negligible relationship both for the breaking force ($r= 0.177$, $p>0.239$ and $r= 0.064$, $p>0.673$ respectively) and firmness ($r= 0.145$, $p>0.335$ and $r= 0.010$, $p>948$, respectively). In comparison, peak 3 (sarcoplasmic proteins) had a moderate positive relationship regarding the firmness ($r= 0.311$, $p=0.035$) of the fillets and a negligible relationship with the breaking force ($r= 0.147$, $p>0.329$). Actin had negligible relationships with both the breaking force ($r= 0.024$, $p>0.872$) and firmness ($r=-0.024$, $p>0.874$). This indicates that for this experiment, the denaturation of the proteins does not excessively affect the fillet's texture.

6 Conclusion

In this study, the goal was to examine whether the internal temperature of Atlantic salmon by chilling with RSW and ice before freezing affected the quality when thawed. A group of fresh fillets was analyzed as a control group. Additionally, storage time was included as a design variable regarding months frozen and days stored in a cooling room as fresh or thawed.

The chilling method interacting with the storage method provided significant differences. For fresh storage, RSW-chilling was better regarding microbiology, considerably better for texture, and somewhat better for protein degradation. For 1 month of freezing, RSW chilling was better regarding color, texture, ATP degradation, and protein denaturation. For 4 months of freezing, RSW chilling was better regarding microbiology, color, ATP degradation, and protein denaturation. Therefore, sub-chilling can be concluded to be a somewhat better chilling method than ice-chilling. As expected, the quality of the salmon samples deteriorated as storage time in the cooling room increased. Regarding fresh and frozen samples, significant differences were observed. Fresh samples had more bacterial growth, while frozen samples had more drip loss and Ino and decreasing breaking force, firmness, hue, and IMP. 4-month frozen samples had more Ino and less psychrotrophic growth than 1-month frozen samples. The rest of the parameters showed no difference regarding storage time in the freezer. The difference between a sample stored 1 month and 4 months in a freezer was minor and did not profoundly affect the quality of the product. Based on this thesis's results, a frozen product might therefore compete with a fresh product, especially when looking at the environmental benefits, when sub-chilled prior to being frozen.

Regarding the UN's sustainable development goals, the world is far from achieving the goals mentioned, and measures need to be taken relatively soon. As mentioned in chapter 1, freezing and shipping fish by boat can reduce emissions by 90%. This is crucial as the ocean becomes even more acidic as years pass and the planet's temperature rises due to emissions of greenhouse gases. At least in this study, sub-chilling before freezing seems to provide a better-quality product when thawed. This is important information for when future preservation- and export methods will be evaluated.

7 Future Perspective

It would have been advantageous to test the different samples' WHC if the experiment was repeated. It would have been interesting to compare the textural properties against the WHC since these properties are closely related. Another variable that should have been included in this thesis was the enzymatic activity of calpains and cathepsins. These enzymes are essential for the tenderization of the muscle, and it would have been valuable to see if there was a correlation between the textural properties, the coloration, protein denaturation, and the enzyme activity. The pH should have been measured in all the fish prior to entering the respective chilling method and for each sample on the day of sampling, as this is a factor that highly affects the drip loss. It would also have been beneficial to log the thawing process with temperature loggers. Thawing is important regarding drip loss, which greatly influences the textural properties.

Concerning the application of sub-chilling industrially, it seems that the industry is getting on board. Skaginn 3X could already report in 2019 that several modern fish processing plants had been launched in both Iceland and Russia (Skaginn3X, 2019). As mentioned in chapter 2.4, there are several steps to handling farmed Atlantic salmon today. By introducing sub-chilling onboard boats, the traditional slaughter and handling on land can be shifted to be at sea introducing advantages like quicker cooling (Chan, 2021). This logistical shift would be advantageous as the quality of the fish would be preserved faster, and export by boat could proceed at a faster rate with fewer steps in the handling process. With a sub-chiller from Skaginn 3X on board, the fish will come graded, pre-cooled, frozen, and packaged to land. This limits the need for sorting on land, and the product emerges ready for further transportation. Fewer steps in the handling process are advantageous regarding the conservation of quality.

The UN's responsible consumption and production goal is still far from being reached. 14% of the food produced is lost due to spoilage before expiration (UN, 2015). As there were not detected large differences in the fish frozen for 1 month and 4 months, this makes it clear that freezing is a great way to preserve some of the food resources that are currently being lost. However, a shift in consumer acceptance would need to happen as most of us currently want fresh food, but producing a better-quality product when thawed is an excellent place to start.

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Appendices

APPENDIX A: Primary data from textural analyses

APPENDIX B: Primary data from colorimetric analyses

APPENDIX A

Primary data from textural analyses showed as mean ± SD.

Table A.1: Results from texture analyses contducted on fish chilled with ice and fish chilled with sub-chilling, storage (fresh, frozen), and number of days stored.

Group		Day	BF (N)	80% (N)	60% (N)	40% (N)	20% (N)	n
Fresh	Ice	1	38.1 ± 15.8	68.0 ± 9.6	39.6 ± 3.7	31.4 ± 9.5	2.9 ± 0.5	5
		5	34.9 ± 5.7	67.2 ± 10.7	34.2 ± 4.1	34.0 ± 5.9	4.5 ± 1.1	4
		12	35.2 ± 8.5	59.6 ± 9.6	33.8 ± 4.5	32.5 ± 6.0	4.5 ± 1.0	5
		16	42.0 ± 6.6	62.4 ± 7.9	33.3 ± 4.1	38.6 ± 5.6	4.8 ± 1.0	5
	RSW	1	43.6 ± 4.7	60.1 ± 6.9	37.1 ± 2.6	32.2 ± 3.2	3.1 ± 0.5	5
		5	41.4 ± 10.9	85.8 ± 8.1	38.3 ± 5.1	37.8 ± 5.0	4.6 ± 0.6	4
		12	38.3 ± 4.8	69.1 ± 11.1	36.2 ± 5.4	32.3 ± 3.0	4.2 ± 0.6	5
		16	34.6 ± 4.0	71.7 ± 13.9	35.3 ± 7.1	34.8 ± 3.1	5.7 ± 1.7	5
1 month	Ice	1	34.0 ± 3.5	66.6 ± 8.0	36.8 ± 3.8	32.1 ± 6.6	4.8 ± 1.8	4
		5	32.0 ± 4.8	54.4 ± 10.1	28.2 ± 3.2	30.4 ± 5.8	5.0 ± 1.3	5
		12	29.1 ± 7.8	50.4 ± 9.9	25.8 ± 4.5	26.1 ± 7.9	4.2 ± 1.5	5
		16	32.7 ± 6.6	63.5 ± 12.4	29.6 ± 7.1	31.1 ± 5.4	5.0 ± 0.6	5
	RSW	1	41.1 ± 4.3	77.0 ± 11.4	38.5 ± 4.2	39.8 ± 3.8	7.5 ± 2.0	4
		5	30.0 ± 1.0	57.8 ± 11.2	28.2 ± 2.6	28.2 ± 3.3.	4.9 ± 1.6	4
		12	29.6 ± 3.5	56.7 ± 2.9	28.5 ± 2.7	26.7 ± 4.5	4.8 ± 0.9	5
		16	30.5 ± 5.8	59.2 ± 10.5	27.9 ± 6.5	30.9 ± 6.1	6.8 ± 2.4	5
4 months	Ice	1	36.8 ± 7.0	59.6 ± 10.5	38.5 ± 9.0	27.3 ± 6.9	1.9 ± 0.3	4
		5	33.9 ± 3.2	60.7 ± 6.5	32.9 ± 6.7	31.7 ± 2.3	6.3 ± 1.7	4
		12	34.8 ± 4.2	62.9 ± 13.1	33.3 ± 6.2	33.1 ± 6.4	5.1 ± 1.1	4
		16	30.1 ± 6.5	53.8 ± 10.4	28.6 ± 6.2	29.3 ± 5.8	5.7 ± 0.9	5
	RSW	1	34.4 ± 9.0	61.0 ± 10.4	34.2 ± 7.4	28.8 ± 7.6	2.1 ± 0.4	5
		5	35.9 ± 2.7	64.5 ± 15.3	34.6 ± 3.7	35.0 ± 4.0	5.5 ± 1.2	5
		12	35.4 ± 7.9	67.8 ± 10.1	32.8 ± 6.3	34.2 ± 8.2	6.9 ± 1.1	5
		16	31.9 ± 5.8	58.0 ± 10.4	29.7 ± 6.0	29.7 ± 6.0	6.0 ± 2.0	5

APPENDIX B

Primary data from colorimetric analyses showed as mean ± SD.

Table B.1: Results from colorimetric analyses on fish stored fresh, 1 month frozen, and 4 months frozen, chilled on ice or in an RSW-tank, and days stored in a cooling room.

Group		Day	L*	a*	b*	C	H	n
Fresh	Ice	1	57.7 ± 1.6	34.4 ± 1.9	36.2 ± 1.9	50.0 ± 2.7	46.5 ± 0.5	5
		5	59.1 ± 2.4	31.9 ± 1.0	33.5 ± 0.9	46.3 ± 1.3	46.4 ± 0.4	4
		12	57.3 ± 2.1	32.3 ± 1.6	34.0 ± 1.6	46.9 ± 2.2	46.5 ± 0.1	5
		16	57.7 ± 1.4	34.2 ± 1.0	34.7 ± 1.5	48.7 ± 1.8	45.4 ± 0.4	5
	RSW	1	58.4 ± 2.1	33.6 ± 2.3	35.1 ± 1.4	48.6 ± 2.4	46.3 ± 1.6	5
		5	57.5 ± 1.2	34.6 ± 0.9	35.7 ± 1.1	49.7 ± 1.4	45.9 ± 0.3	4
		12	57.6 ± 1.9	33.6 ± 1.4	35.2 ± 1.0	48.6 ± 1.7	46.3 ± 0.6	5
		16	58.9 ± 0.9	33.3 ± 1.2	34.2 ± 0.8	47.7 ± 1.3	45.8 ± 0.8	5
1 month	Ice	1	56.4 ± 2.0	35.4 ± 1.9	38.6 ± 1.7	52.4 ± 2.5	47.5 ± 0.4	4
		5	54.7 ± 2.3	33.6 ± 2.0	36.2 ± 1.3	49.4 ± 2.3	47.1 ± 0.8	5
		12	56.7 ± 1.7	32.8 ± 1.9	35.4 ± 1.2	48.3 ± 1.9	47.3 ± 0.6	5
		16	58.5 ± 2.0	33.5 ± 1.1	35.9 ± 1.1	49.1 ± 1.5	47.0 ± 0.6	5
	RSW	1	59.0 ± 2.6	32.0 ± 2.8	35.8 ± 2.0	48.1 ± 3.4	48.2 ± 1.0	4
		5	57.3 ± 1.4	32.0 ± 1.8	35.3 ± 1.2	47.7 ± 2.1	47.8 ± 0.8	4
		12	58.7 ± 1.2	33.0 ± 2.1	36.5 ± 1.7	49.3 ± 2.6	47.9 ± 0.7	5
		16	59.1 ± 0.9	34.9 ± 1.6	37.5 ± 1.1	51.2 ± 1.9	47.1 ± 0.6	5
4 months	Ice	1	57.1 ± 1.6	34.8 ± 3.0	37.9 ± 2.1	51.5 ± 3.6	47.5 ± 0.9	4
		5	58.0 ± 1.6	33.3 ± 0.7	36.6 ± 0.8	49.4 ± 1.1	47.7 ± 0.2	4
		12	58.8 ± 1.9	33.2 ± 1.6	36.3 ± 1.1	49.2 ± 1.9	47.5 ± 0.6	4
		16	56.8 ± 1.6	33.0 ± 1.2	35.6 ± 1.9	48.4 ± 1.3	47.0 ± 0.5	5
	RSW	1	59.3 ± 0.8	33.8 ± 1.9	36.5 ± 1.7	49.8 ± 2.5	47.3 ± 0.3	5
		5	56.9 ± 1.6	34.1 ± 1.6	37.1 ± 1.3	50.4 ± 2.0	47.4 ± 0.2	5
		12	58.2 ± 1.1	33.6 ± 1.8	36.1 ± 2.1	49.3 ± 2.8	47.0 ± 0.4	5
		16	57.2 ± 1.7	33.3 ± 2.3	35.4 ± 0.7	48.7 ± 2.9	47.0 ± 0.6	5

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