Research Article

Received: 14 August 2023 Revised: 19 March 2024 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.13643

The quality of frozen–thawed Atlantic salmon (*Salmo salar* L.) fillets as affected by sub-chilling before freezing

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Abstract

Background: To maintain the quality of frozen Atlantic salmon after thawing and highlight the potential for moving from air fright to boat for long-distance export, a study was designed to investigate the effects of sub-chilling before rapid freezing on the quality of thawed fillets. Atlantic salmon chilled on wet ice before filleting and freezing was used as a control for the experimental factor chilling, whereas fresh fillets were used for the frozen–thawed samples.

Results: The pre-freezing chilling method interacted with the storage protocol and significantly affected the product. For fresh stored fillets, sub-chilling improved the microbiological and textural stability and degradation of proteins. After 1 month of frozen storage, sub-chilled fillets gave better color and textural properties, less adenosine triphosphate degradation and protein denaturation. In addition, sub-chilled 4-month-frozen fillets also showed improved microbial stability compared to those initially chilled with ice before frozen storage. Quality was lost as a function of storage. Fresh fillets generally had higher bacterial counts, surface breaking force, firmness, hue and contents of inosine monophosphate, and lower drip loss and inosine (HxR) levels than those stored frozen–thawed. Moreover, 4-month-frozen fillets had higher HxR levels and lower psychrotrophic viable count growth than those that were frozen for 1 month. The time fillets were stored frozen did not profoundly affect their quality.

Conclusion: It is concluded that a frozen product might be competitive with a fresh product when sub-chilling is performed before freezing, especially when including the environmental benefits of frozen export by boat rather than air freight.

Keywords: Atlantic salmon quality; cold chain; sub-chilling; sustainable food systems

INTRODUCTION

Norway plays a pivotal role in the global production of Atlantic salmon (*Salmo salar* L.), contributing over 55% to the total output. In 2022, salmon exports from Norway reached a value of NOK105.3 billion.[1,2] Despite substantial growth since the first farmed salmon was slaughtered in 1971, the industry aspires to achieve an export value of NOK240 billion in 2050.[3] In addition to the expansion of the industry, Norway's seafood sector aims to lead in environmentally conscious practices. The goal is to establish the world's most eco-friendly production of food.[4]

With the rise in production and exports, a natural increase in emissions is anticipated, particularly given the high demand for fresh products. Notably, in 2019, over 75% of exported salmon remained unprocessed, resulting in the transportation of significant amounts of byproducts such as heads and tail, ice and polystyrene boxes across the globe. This practice significantly amplified the emissions associated with the actual edible product.[5] Among transportation methods, gutted salmon exported by air freight emits the highest levels of greenhouse gases and remains the dominant choice for transporting salmon to Asia. However, there are effective strategies to mitigate emissions. By exclusively super-chilling the fish, it has the potential to reduce the emissions due to transport by 20%. Alternatively, filleting and super-chilling can achieve an even more substantial reduction of up to 50% in greenhouse gas emissions.[6,7] Super-chilling has demonstrated its efficacy in preserving salmon quality and extending the shelf-life of salmon.[8-10] Nevertheless, when focusing solely on emissions from transportation, the greatest gains come from replacing air freight with boat transportation. Although boat transportation to Asia is more time-consuming, solely sub-chilling the fillets will not be sufficient, and freezing will

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be necessary. Freezing the fillets before export can reduce transport emissions by up to 90%.[6]

To maintain the quality of frozen products after thawing and highlight the potential for moving from air fright to boat when transporting salmon over long distances, a rapid freezing rate[11] and proper thawing are critical.[12-14] One way to increase the freezing rate is by sub-chilling the fish first. Sub-chilling is a form of super-chilling, reducing the temperature of the salmon to around −1.5 °C but still above the freezing point.[6] When freezing the fish subsequently, crystallization of water will, in theory, occur almost instantly. However, the combined effect of sub-chilling before freezing on the quality of thawed salmon fillets is not well studied. Therefore, the study presented here investigated the impact of sub-chilling before rapid freezing on the quality of thawed Atlantic salmon fillets. Atlantic salmon chilled on wet ice before filleting and freezing was used as controls for the experimental factor chilling, whereas fresh fillets (both sub-chilled and ice-chilled) were used as controls for the frozen–thawed samples. The objectives were evaluated following microbial growth, fillet drip loss, water content, color, texture, protein denaturation and flesh freshness expressed as adenosine triphosphate (ATP) denaturation[15] through storage.

MATERIAL AND METHODS

Raw material and experimental design

Atlantic salmon were obtained from Grieg Seafood Rogaland. A chilled seawater (CSW) setup was created with two 400 L polyethylene fish chilling tanks containing a mix of 7% NaCl brine and ice, creating an environment of approximately −1.0 °C.[16] The salmon were electrically stunned and bled on board the stun-and-bleed boat MS Seibas in late September 2021 and were transported to the processing facility within 9–12 h post-mortem. Eighty salmon (average of 4.4 ± 0.25 kg) were randomized and divided equally between CSW chilling (CSW) and traditional chilling on wet ice (Ice), which was the first design variable hereby designated as *chilling method*. The temperature was monitored using five TrackSense Pro temperature loggers (Ellab A/S, Denmark) inserted in the mid-abdomen of five random fish from each group (CSW and Ice). The solution was stirred upon arrival at the laboratory. The temperature profile during pre-chilling of the experimental Atlantic salmon is shown in Fig. 1.

After the pre-chilling, all the fish were immediately filleted by hand before being vacuum packaged at 99% vacuum. Thereafter, the fillets were randomized again, and divided into three groups with 20 fillets from both CSW and ice-chilling in each group: fresh, one-month freezing and four-month freezing. The fresh fillets were used as a control group. The fresh fillets were then placed in a refrigerated room (0.6 ± 0.5 °C), while the fillets of other groups were flash-frozen for 30 min with dry ice (−78.5 °C) before being placed in a freezer (−28.5 ± 1.4 °C) for the designated time (one and four months). Fresh and frozen storage was the second design variable, referred to as the *storage method*. The experimental design, as modified after Vangen,[16] is summarized in Fig. 2(A).

After one and four months of frozen storage, frozen fillets were thawed in a pre-cooled water bath (4 °C) for 4 h before being placed in a refrigerated room (0.6 ± 0.5 °C) for conducting the storage experiments. The day of thawing was considered day 0, while analyses were performed on days 1, 5, 12 and 16. For each day, five fillets of each group were sampled randomly. *Storage days* (as fresh or thawed) was the last design variable. At each sampling point, fillets (including the vacuum bag) were weighted, removed from the pack, patted dry and weighted again without the bag to calculate drip loss. The Norwegian Quality Cut (NQC) was then divided into four (Fig. 2(B)) for protein denaturation analysis, microbiological analyses and water content determination, and to examine ATP degradation products. Microbiological analysis was conducted on the sampling day, while the remaining NQC part were vacuum-packaged and stored at −80 °C until further analyses. Colorimetric and textural analyses were conducted on a fillet's back loin on the stated sampling days.

Analytical parameters

Water content and drip loss

The water content was analyzed gravimetrically in the abdomen part of the NQC (Fig. 2(B)) according to NMKL method no. 23.[17] Samples (10.0 ± 0.5 g) were placed in a heating cabinet (Termaks, Norway) at 102–105 °C for 18 h. The dried samples were placed in a desiccator (SICCO Star-Vitrum Desiccator, borosilicate glass 3.3, Germany) for 30 min before being weighed. The water percentages were calculated using Eqn (1):

$$W\,(\%) = \frac{W_1 - W_2}{W_1} \times 100 \qquad (1)$$

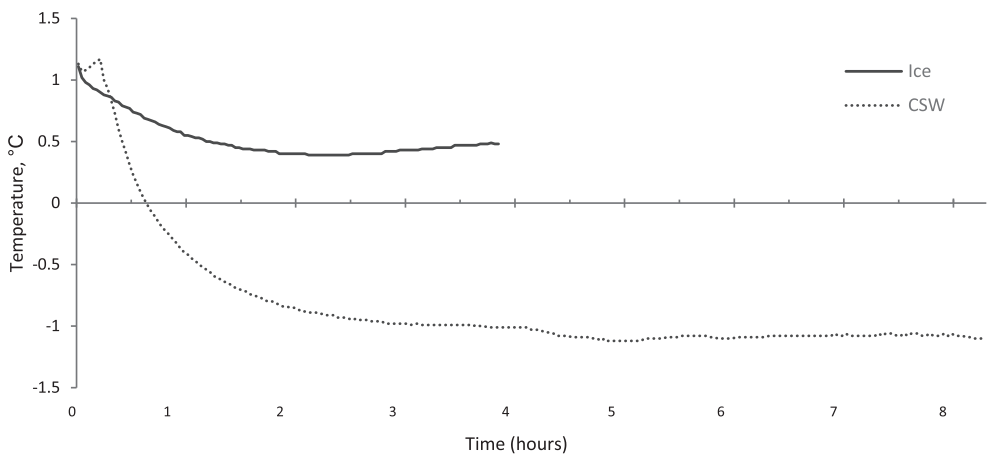

Figure 1. Temperature evaluation of Atlantic salmon stored in a CSW tank and fish stored in EPS boxes with ice. Time is hours from the fish being placed in the CSW tank or in the boxes with ice.

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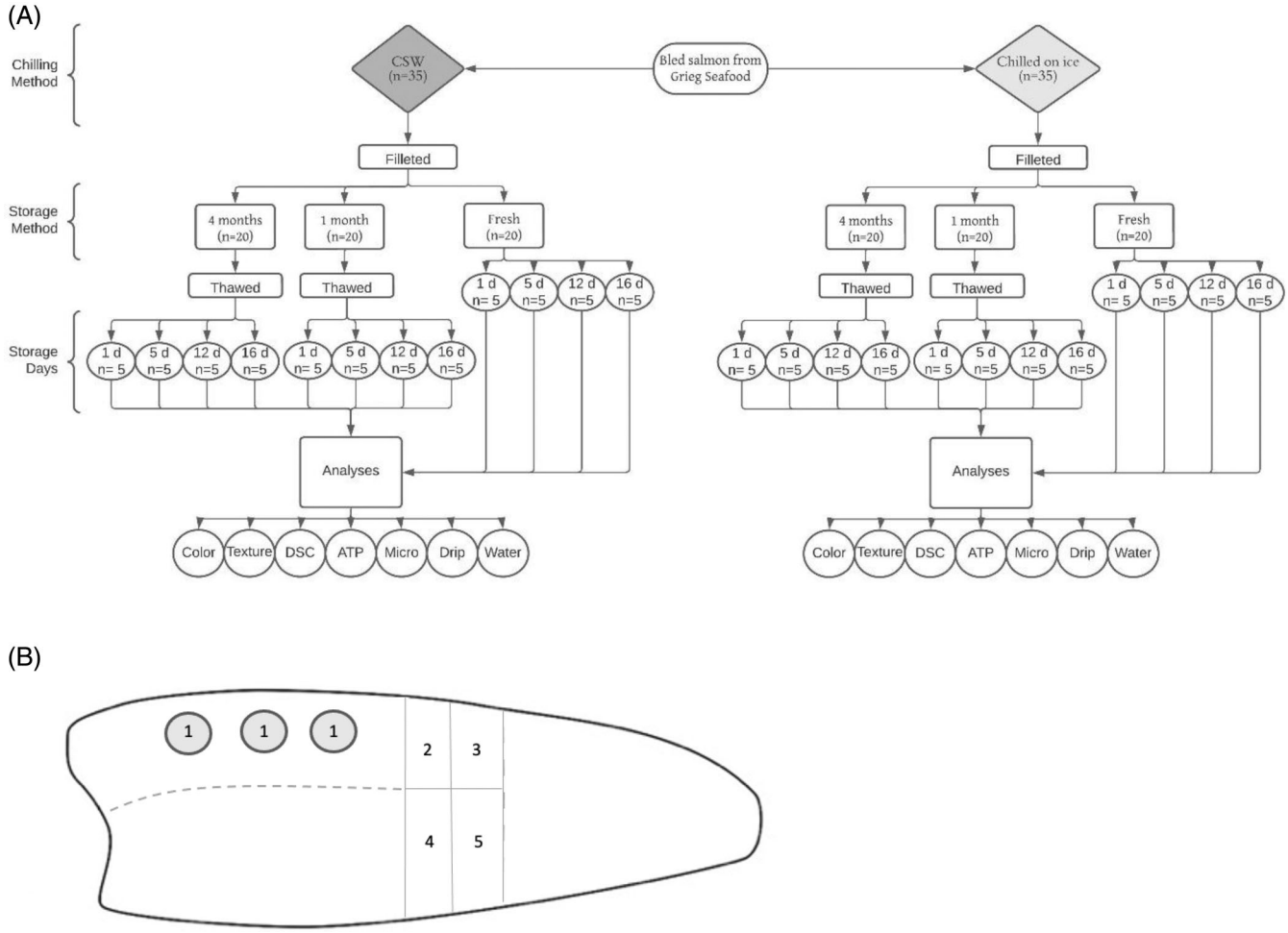

Figure 2. (A) Schematic of experimental design where the fish from Grieg Seafood was divided into two groups: CSW chilling and traditional chilling on ice. The fish was further filleted and vacuum-packed and randomized into three groups: fresh, 1 month and 4 months freezing. The analyses include color 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 cold room. (B) Illustration showing sampling locations (1) for texture analysis, (2) for ATP metabolites, (3) for protein denaturation, (4) for microbiological analyses, (5) for water content, and the top loin for color analysis.

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

The drip loss was measured as the difference between the initial fillet weight (g) and the weight on the sampling day (g). The drip loss (%) was calculated using Eqn (2):

$$\mathrm{DL}\,(\%) = \frac{D_0 - D_t}{D_0} \times 100 \qquad (2)$$

where D_0 = initial weight (g) without bag and D_t = weight (g) without bag after patting dry.

Textural and colorimetric properties

Textural properties were assessed in triplicate for each sample (as shown in Fig. 2(B)) using a TA.TXplus texture analyzer (Stable Micro Systems Ltd, UK). The analyzer was equipped with a 25 mm DIA stainless steel flat-ended probe. A 50 kg load cell measured the force (N) needed to compress 80% of the sample's height at 2 mm s^{-1}. The resulting force data were logged as a force–time graph using Texture Exponent software (Stable Micro Systems). The key parameters assessed were the point of surface rupture (breaking force) and the force (N) at 60% compression (indicating firmness).

Colorimetric properties were measured on the salmon fillet back loin in front of the NQC using a DigiEye full system (VeriVide Ltd, UK) connected to a Nikon D90 camera (35 mm lens, Nikon Corp., Japan). The samples were placed on a green background inside a lightbox (Verivide daylight, 6400 K, UK). Pictures were analyzed using DigiPix software version 2.8 (VeriVide Ltd). The data obtained were quantified as L^*, hue (°H) and chroma (C^*) values, where L^* represents lightness (L^* = 100 = white; L^* = 0 = black), °H (0° = red hue; 60° yellow hue), whereas C^*, which is the distance from the achromatic center, expresses the intensity of a color (greyness).[18,19]

Freshness measured as degradation of ATP

The content of ATP degradation compounds was measured in all samples using high-performance liquid chromatography (HPLC) according to a modified method by Sellevold *et al.*[20] Sample extracts were prepared by grating the muscle (without the skin) into a homogeneous mixture, followed by adding 7.5 mL of 7% trichloroacetic acid ($C_2HCl_3O_2$; Sigma-Aldrich) into samples of

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1.5 ± 0.1 g. Three parallels from each sample were homogenized using a T25 Digital ULTRA TURRAX® (IKA®, Germany) for 120 s. Then, 3.25 mL of 1 mol L^{-1} potassium hydroxide (KOH; Merck) was added, and the mixture was mixed gently before being centrifuged at 4 °C and 2950 × *g* for 10 min (ROTINA 420R, Hettich®, Germany). The supernatant (7 mL) was carefully transferred by pipetting into new centrifuge tubes (10 mL) before being frozen at −80 °C until further analysis.

Prior to HPLC analysis, pre-extracted samples were thawed for approximately 1 h at room temperature before being filtered into HPLC tubes (1.5 mL vial ND9 amber P. + cap SIL/PTFE, VWR, USA) by using a polyethersulfone membrane (0.45 µm; VWR, USA). The HPLC system consisted of an Agilent 1290 Infinity connected to a diode array detector (Agilent Technologies, USA) with an InfinityLab Proshell 120 EC-C18 column (3.0 × 5 mm, 2.7 m (Agilent, no. 823750-911) + 3.0 × 100 mm 2.7 m (Agilent, no. 695976-302, USA)). The mobile phase consisted of a buffer containing 1.5% acetonitrile (C_2H_3N; Sigma-Aldrich), 0.25 mol L^{-1}M potassium dihydrogen phosphate (KH_2PO_4; Merck) and 0.0023 mol L^{-1} tetrabutylammonium hydrogen sulfate ($[CH_3(CH_2)_3]_4N(HSO_4)$; Sigma-Aldrich). The buffer's pH was adjusted to 6.26 using 5 mol L^{-1} NaOH. The flow of the buffer was set up as a gradient with 0.2 mL min^{-1} from 0 to 9 min, 0.8 mL min^{-1} from 9 to 11 min and 0.2 mL min^{-1} from 12 to 14 min. The column temperature was fixed to 20 °C with a maximum pressure of 600 bar. The metabolites were detected at 210 nm (ATP, adenosine diphosphate (ADP) and adenosine monophosphate (AMP)) and 260 nm (inosine monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx)). Quantification was performed following the methods described in Lerfall *et al.*[21] and Abel *et al.*[22]

Protein denaturation

The protein denaturation was analyzed in the dorsal part of the NQC (Fig. 2(B)) using a differential scanning calorimetry (DSC) instrument (Mettler Toledo AG, Schwerzenbach, Switzerland) calibrated with indium. Before analysis, the samples were thawed in a cold room (0.6 ± 0.5 °C) for approximately 22 h. Protein denaturation was measured in fresh and thawed samples stored for 1 and 16 days. Four samples from each group were selected randomly, and two parallels of each sample were analyzed. Aluminium pans with pins (40 mg) and aluminium lids were used for holding a sample during the measurement. Muscle fibers (20 mg) were extracted using a scalpel cutting between the muscle myosepta. As a reference, a pan consisting of 13.6 mg of distilled water was used (the amount of water was calculated based on the fish water percentage, on average 68%). After sealing with a crucible sealing press (Mettler Toledo, Switzerland), the sample pans and the reference were kept on ice before loading to avoid unintended thermal denaturation. The samples were analyzed by a heating gradient of 5 °C min^{-1} until a maximum temperature of 88 °C.

Data were analyzed using StarE software version 9.10 (StarE System, Schwerzenbach, Switzerland). The peaks were identified by comparing the peak maximum temperature to literature data.[23] The specific sample's denaturation enthalpy was obtained using a spline baseline defining the denaturation peak, whereas the total denaturation enthalpy was found by integrating the specific peak. The onset and end set of the denaturation peaks were standardized. The results are presented as joules per gram of sample.

Microbial growth

Microbial analyses were conducted as described by Vangen[16] using NMKL method no. 184.[24] Iron agar-pour plates were employed to assess total viable counts, measured as aerobic plate count of mesophilic bacteria (APC). Meanwhile, Long & Hammer (L&H) plates were used for total psychrotrophic viable counts (PC). A part of the hypaxial muscle of the NQC (approximately 20 g) was excised from the skin aseptically (Fig. 2(B)), transferred to a stomacher bag and weighed. A 1:10 dilution was prepared with sterile buffered peptone water and homogenized salmon using a Smasher© stomacher (AES Laboratorie, bioMériux Industry, USA) for 120 s. 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 plates) by using 150 µL samples to 1350 µL of sterile buffered peptone water. An amount of 49.2 µL of each dilution was plated on L&H plates using an Eddy Jet 2 spiral plater (IUL micro, Spain). Moreover, 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), precooled 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 h. Microbial counts are presented as log CFU g^{-1} sample.

Statistics

The data were analyzed according to Vangen,[16] using Minitab Version 19 (www.minitab.com, Minitab Inc., USA). Data are presented as average values with their associated standard deviations unless otherwise specified. All data were subjected to normality analysis by Levene's test and analysis of the equality of variance by testing the homoscedasticity. Analysis of variance 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 confidence level of 95% was performed to analyze if a significant difference existed between the parameters. Pearson's correlation coefficient was used to determine the correlation between connected parameters. When analyzing texture, fillet height was added as an additional covariate.

RESULTS

Water content and drip loss

The water content was affected by the storage method ($P < 0.05$), with the frozen–thawed samples frozen for 4 months showing the highest value (70.0 ± 2.6%; Table 1). An effect of the design variable storage days was also observed ($P < 0.05$), with day 12 having a lower water content than day one.

All the design variables were found to affect the drip loss ($P < 0.05$) that ended at 2.2 ± 0.3% after 16 days of storage (Table 1). Salmon sub-chilled with CSW showed a slightly higher drip loss (on average 0.2% higher; $P < 0.05$) than those samples initially chilled with ice. Moreover, on average, frozen samples had approximately 1% higher drip loss than those stored fresh ($P < 0.05$).

Textural and colorimetric properties

The design variable storage method affected the fillet breaking force and firmness ($P < 0.05$; Table 2). Regarding firmness, a significant decrease (on average, 6.8 N) was observed as a function of storage days ($P < 0.05$). Regarding colorimetric properties, the design variable storage method affected the parameter chroma,

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Table 1. Effect of the design variables chilling method (CSW, Ice), storage method (fresh, one-month frozen, four-month frozen) and storage days (1, 5, 12, 16) on water content and drip loss in Atlantic salmon fillets

	Amount ± SD (%)		
	Water content	Drip loss	*n*
Chilling method			
CSW	67.7 ± 2.1	1.7 ± 0.3	57
Ice	68.5 ± 2.4	1.5 ± 0.2	55
Effect of chilling method (*P*)[1]	*0.155*	*0.004*	
Storage method			
Fresh	67.1 ± 2.3^{a}	0.9 ± 0.2^{a}	38
One-month frozen	67.0 ± 1.8^{a}	1.9 ± 0.3^{b}	37
Four-month frozen	70.0 ± 2.6^{b}	2.0 ± 0.3^{b}	37
Effect of storage method (*P*)[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*)[1]	*0.023*	*<0.001*	

[1] Significance level $P < 0.05$, GLM. Different lowercase letters a–c indicate a significant difference ($P < 0.05$) within the design variables based on Tukey's pairwise comparisons test. The *P* value of the experimental variables is shown in italics.

Table 2. Effect of the design variables chilling method (CSW, Ice), storage method (fresh, one-month frozen, 4 month-frozen) and storage days (1, 5, 12, 16) on the textural (breaking force and firmness) and colorimetric properties (*L**, chroma, hue) of Atlantic salmon fillets. Values are expressed as the mean ± SD

	Textural properties (N)		Colorimetric properties			
	Breaking force	Firmness[2]	*L**	Chroma	Hue	*n*
Chilling method						
CSW	35.6 ± 5.4	32.6 ± 5.0	58.2 ± 1.5	49.1 ± 2.3	47.0 ± 0.7	57
Ice	34.5 ± 6.7	32.9 ± 5.3	57.4 ± 1.9	49.1 ± 2.0	46.9 ± 0.5	55
Effect of chilling method (*P*)[1]	*0.302*	*0.572*	*0.432*	*0.390*	*0.756*	
Storage method						
Fresh	38.5 ± 7.3^{a}	36.0 ± 4.6^{a}	58.0 ± 1.7	48.3 ± 1.8^{a}	46.1 ± 0.6^{a}	38
One-month frozen	32.4 ± 4.7^{b}	30.4 ± 4.3^{b}	57.5 ± 1.8	49.4 ± 2.3^{b}	47.5 ± 0.7^{b}	37
Four-month frozen	34.2 ± 5.8^{b}	33.1 ± 6.4^{ab}	57.8 ± 1.5	49.6 ± 2.3^{b}	47.3 ± 0.5^{b}	37
Effect of storage method (*P*)[1]	*<0.001*	*<0.001*	*0.300*	*0.005*	*<0.001*	
Storage days						
1	38.0 ± 7.4	37.5 ± 5.1^{a}	58.0 ± 1.8	50.0 ± 2.8	47.2 ± 0.8^{a}	27
5	34.7 ± 4.7	32.7 ± 4.2^{b}	57.3 ± 1.8	48.8 ± 1.7	47.1 ± 0.5^{a}	26
12	33.8 ± 6.1	31.7 ± 4.9^{b}	57.9 ± 1.7	48.6 ± 2.2	47.1 ± 0.5^{a}	29
16	33.6 ± 5.9	30.7 ± 6.2^{b}	58.1 ± 1.4	49.0 ± 1.8	46.5 ± 0.6^{b}	30
Effect of storage days (*P*)[1]	*0.103*	*<0.001*	*0.192*	*0.311*	*0.002*	
Effect of fillet height (*P*)[1]	*0.011*	*0.443*				

[1] Significance level $P < 0.05$, GLM.
[2] Firmness is described by 60% compression force. Different lowercase letters a, b indicate a significant difference ($P < 0.05$) within the design variables based on Tukey's pairwise comparisons test. The *P* value of the experimental variables is shown in italics.

showing higher values (on average 1.2 units) for frozen–thawed fillets than for those stored fresh ($P < 0.05$; Table 2). For hue, an effect of the variables storage method and storage days was observed ($P < 0.05$). Moreover, an interaction between the design variables chilling and storage method was found ($P < 0.05$; data not shown).

Freshness measured as degradation of ATP

The fillet IMP content decreased during storage (Fig. 3(A)), where significant effects of the design variables storage days ($P < 0.05$) and storage method ($p < 0.05$) were observed. Tukey's pairwise comparison test revealed a significant difference ($P < 0.05$) between all sampling days and both fresh and frozen stored

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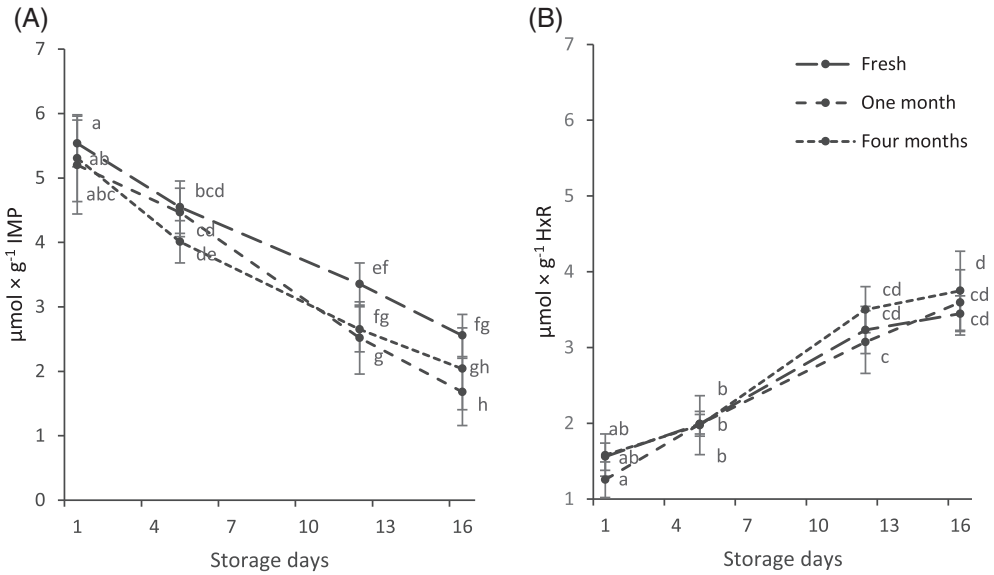

Figure 3. (A) IMP content of fresh, one-month- and four-month-frozen samples as a function of storage days. A significant difference ($P < 0.05$) regarding storage method and storage days was observed. (B) Concentration of HxR of samples stored fresh, one and four months frozen. Both these design variables were significant ($P < 0.05$). Lowercase letters a–h indicate a significant difference regarding storage days and storage method for each parameter based on Tukey's pairwise comparisons test.

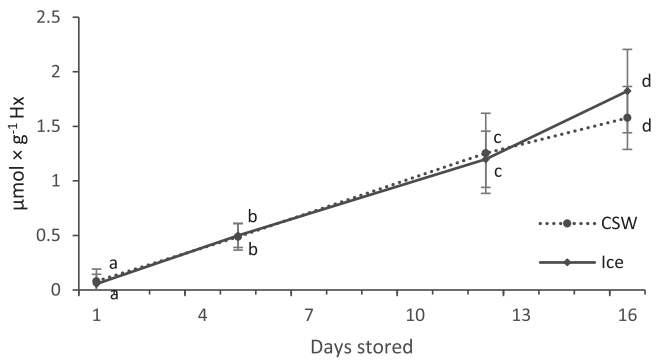

Figure 4. Development of Hx as affected by storage. The letters a–d indicate a significant difference ($P < 0.05$) based on Tukey's pairwise comparisons test between storage days and chilling method.

samples. The fresh samples had a slightly higher content of IMP than the frozen–thawed samples indicating a slower conversion of IMP to HxR.

The fillet HxR content increased as a function of storage (Fig. 3 (B)). Days stored and the storage method were the discriminants explaining the observed differences ($P < 0.05$), whereas the design variable chilling method did not affect the fillet HxR content ($P > 0.05$). The highest amount of HxR was measured in the 4-month-frozen fillets stored for 16 days after thawing (3.75 ± 0.52 µmol g^{-1}). Tukey's pairwise comparison test showed a significant difference between the sampling days and one-month and four-month freezing time ($P < 0.05$). Moreover, an interaction was found between the design variables storage days and chilling method ($P < 0.05$; data not shown).

The fillet Hx concentration increased as a function of storage (Fig. 4). Samples from day 1 had the lowest Hx concentration (0.07 ± 0.09 µmol g^{-1}, average across the chilling methods), while day 16 showed the highest (1.70 ± 0.34 µmol g^{-1}). Days stored was the only design variable affecting the Hx content ($P < 0.05$).

The *K*-value representing fillet freshness was only found to be significantly affected by the design variable storage days ($P < 0.05$; Fig. 5(A)). Moreover, an interaction between the variables storage days and storage method was found (Fig. 5(B); $P < 0.05$).

Protein denaturation

The design variables storage method and storage days contributed to a significant difference ($P < 0.05$) regarding peak 1 (myosin) (Table 3). For peak 2 (sarcoplasmic proteins), a significant difference between storage methods ($P < 0.05$) was observed. Interactions between the variables storage days and storage method ($P < 0.05$) and chilling method and storage method ($P < 0.05$) were also found for peak 2 (Fig. 6(A),(B)). The third peak (sarcoplasmic proteins) was found to be significantly affected by the factor storage days ($P < 0.05$; Table 3). An interaction between the chilling and storage methods was found ($P < 0.05$; Fig. 6(C)). For peak 4 (actin), an interaction between storage days and chilling method was found ($P < 0.05$; Fig. 6(D)). The results are summarized in Table 3. According to the literature, the first peak is myosin, the second and third are the denaturation of sarcoplasmic proteins and peak 4 is actin.[25]

Microbial growth

APC was determined by incubation on iron agar. A significant effect was found in the design variables storage days ($P < 0.05$) and chilling method ($P < 0.05$; Fig. 7(A)). APC increased as a function of storage days, ending at an average of 4.0 ± 0.03 log CFU g^{-1} after 16 days of storage, where the averages for the CSW and ice-chilled salmon were 3.5 ± 0.4 and 4.5 ± 0.3 log CFU g^{-1}, respectively ($P < 0.05$). An interaction between the design variables storage days and chilling method ($P < 0.05$) was also found. The storage method as a design variable was insignificant ($P > 0.05$), shown in Fig. 7(B) as a function of storage days.

PC was determined by incubation on L&H agar. A significant effect was found in the design variables storage days ($P < 0.05$) and storage method ($P < 0.05$), whereas the chilling method

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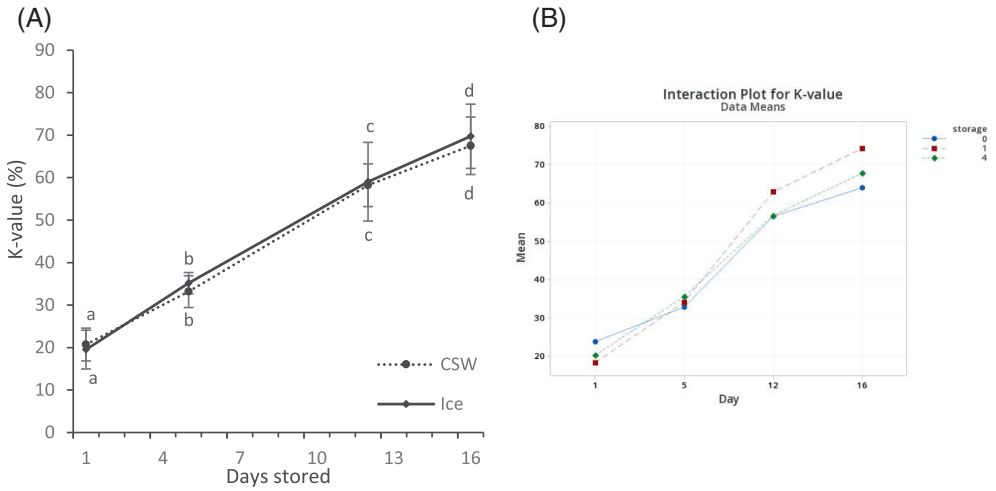

Figure 5. (A) Increase of *K*-value throughout storage, with day 16 having the highest value. Storage days was the only significant design variable based on Tukey's comparison test, shown as lowercase letters a–d. (B) Interaction plot for storage days and storage method, where 0 = fresh, 1 = one month frozen and 4 = four months frozen.

Table 3. Effect of the chilling method (CSW, Ice), storage method (fresh, one-month frozen, four-month frozen) and storage days (1, 16) on the enthalpy utilized to denature proteins

	Enthalpy (J g^{-1})				
	Peak 1	Peak 2	Peak 3	Peak 4	*n*
Chilling method					
CSW	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*)[1]	*0.071*	*0.285*	*0.334*	*0.670*	
Storage method					
Fresh	0.62 ± 0.07^{a}	0.09 ± 0.02^{a}	0.05 ± 0.01	0.16 ± 0.02	32
One-month frozen	0.59 ± 0.08^{ab}	0.07 ± 0.02^{b}	0.04 ± 0.01	0.16 ± 0.03	30
Four-month 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*)[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*)[1]	*0.009*	*0.074*	*<0.001*	*0.281*	

[1] Significance level $P < 0.05$, GLM. Different lowercase letters a, b indicate a significant difference ($P < 0.05$) within the design variables based on Tukey's pairwise comparisons test. The *P* value of the experimental variables is shown in italics.

was insignificant (Fig. 8). However, a significant interaction was found between storage days and chilling method ($P < 0.05$). The fresh samples had higher PC than the four-month-frozen stored samples on all sampling days and the one-month-frozen samples on days 1, 12 and 16. Tukey's pairwise comparison test determined a significant difference ($P < 0.05$) between the sampling days and the four-month-frozen and fresh and one-month-frozen samples. The chilling method as a design variable was insignificant ($P > 0.05$), shown in Fig. 8(A) as a function of storage days.

DISCUSSION

Quality deterioration over time is an inevitable process for nearly everything, particularly organic materials like fresh or frozen foodstuffs. By employing sub-chilling and freezing, enzymatic and bacterial activity can be halted, prolonging shelf-life immensely.[18,26] However, there are other repercussions associated with frozen foodstuffs. The freezing process can negatively impact cellular structures resulting in dry and tough meat, higher drip loss and more pronounced protein denaturation. Additionally, storing thawed or fresh foodstuffs for an extended period often increases the risk of spoilage.

Effects of chilling method on quality of fresh or frozen–thawed salmon fillets

On average, CSW samples had a higher drip loss when stored fresh or after thawing than ice-chilled samples. Contradicting results have previously been reported on whether super-chilling is an optimal way to chill fish, considering the drip loss. Bahuaud *et al.*[27] reported short-time super-chilled (−1.5 °C) *pre-rigor* fillets having the same structural damage as Atlantic salmon frozen at −20 °C, where large intra- and extracellular ice crystal formation

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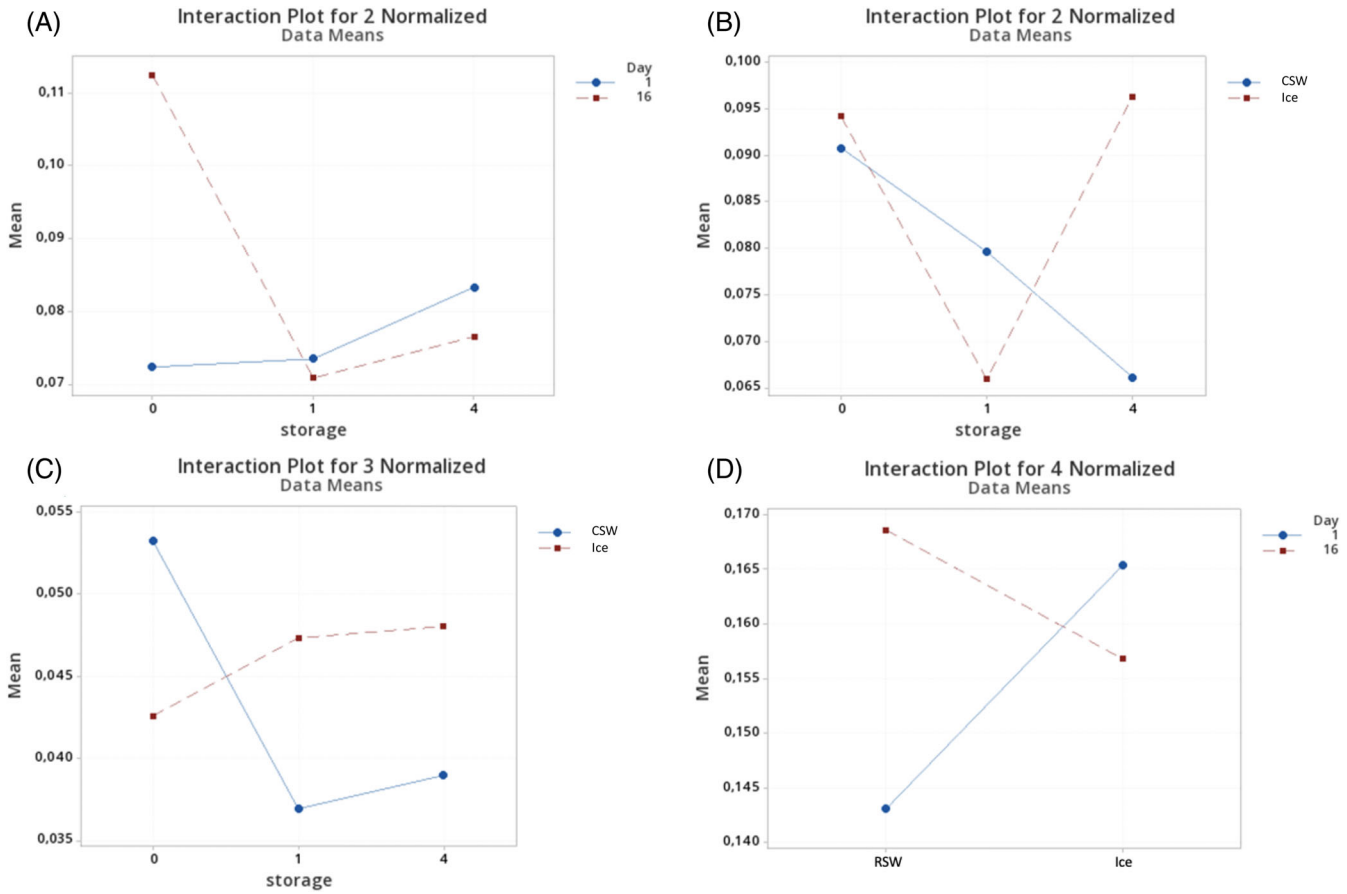

Figure 6. Interaction plots for peaks 2, 3 and 4. (A) For peak 2, both storage method and storage days and (B) chilling method and storage method were significant ($P < 0.05$). (C) For peak 3, only chilling method and storage days were significant ($P < 0.05$). (D) For peak 4, only chilling method and storage days were significant ($P < 0.05$).

caused structural changes. Conversely, Chan *et al.*[28] found no significant differences in drip loss between ice-chilled and sub-chilled Atlantic salmon.

Whether the fish was chilled using CSW or ice in the present study did not affect the fillet textural or colorimetric properties indicating that sub-chilling provides a product of the same quality as today's traditional practice. Protein denaturation is commonly measured with DSC. Four peaks were observed for the CSW and ice-chilled samples (Table 3). The first peak (at 44 °C) is attributed to myosin, the two peaks (at 57 and 65 °C) following are sarcoplasmic proteins, whereas the last peak (at 74 °C) is attributed to actin.[25,29] It is the endothermic energy required to unfold the protein from its original structure compared to the linear plot of the reference pan registered by DSC.[30] The results of the present study were comparable to those reported by Ofstad *et al.*,[25] indicating that it is, in fact, myosin, sarcoplasmic proteins and actin that have become denatured. The sub-chilled salmon investigated in the present study needed, on average, less energy to denature myosin than ice-chilled salmon, indicating myosin is denatured during the chilling process. For other proteins, the effect of the chilling method was insignificant ($P > 0.05$). Sarcoplasmic proteins and actin are known to be more stable than myosin, with the potential to withstand chilling temperatures better.[31]

A significant factor limiting a product's shelf-life is the growth of spoiling microorganisms. Sub-chilled samples showed lower APC and the ice-chilled samples had almost one log unit higher counts for all storage days. For PC, no significant differences were seen as affected by the chilling method. Sub-chilling seems to inhibit mesophilic microorganisms' growth but not that of psychrotrophic ones. This observation did not follow Erikson *et al.*,[8] who found that CSW chilling effectively inhibited the growth of microorganisms contributing to PC. However, Bono *et al.*[32] found that super-chilling in combination with ozone did not affect the development of psychrotrophic bacteria in European anchovy (*Eugraulis encrasicolus*) and sardine (*Sardina pilchardus*). There are contradictory results in previous studies. However, based on the presented results, sub-chilling might be a limiting factor regarding the growth of microorganisms, i.e. an improved product shelf-life.

Effects of storage condition on quality of fresh or frozen–thawed salmon fillets

When comparing fresh and frozen samples, the frozen samples had approximately double the drip loss percentage compared with the fresh ones, which was expected since freezing disrupts the myofibrillar space and, depending on freezing time, disrupts other important cellular components such as proteins. When these structures have been disrupted and denatured, water cannot be absorbed when thawed, resulting in drip loss.[33] All the frozen samples were flash-frozen with dry ice for 30 min before being placed in a freezer at −30 °C. Therefore, it is safe to assume that most formed ice crystals were small. Nevertheless, when the

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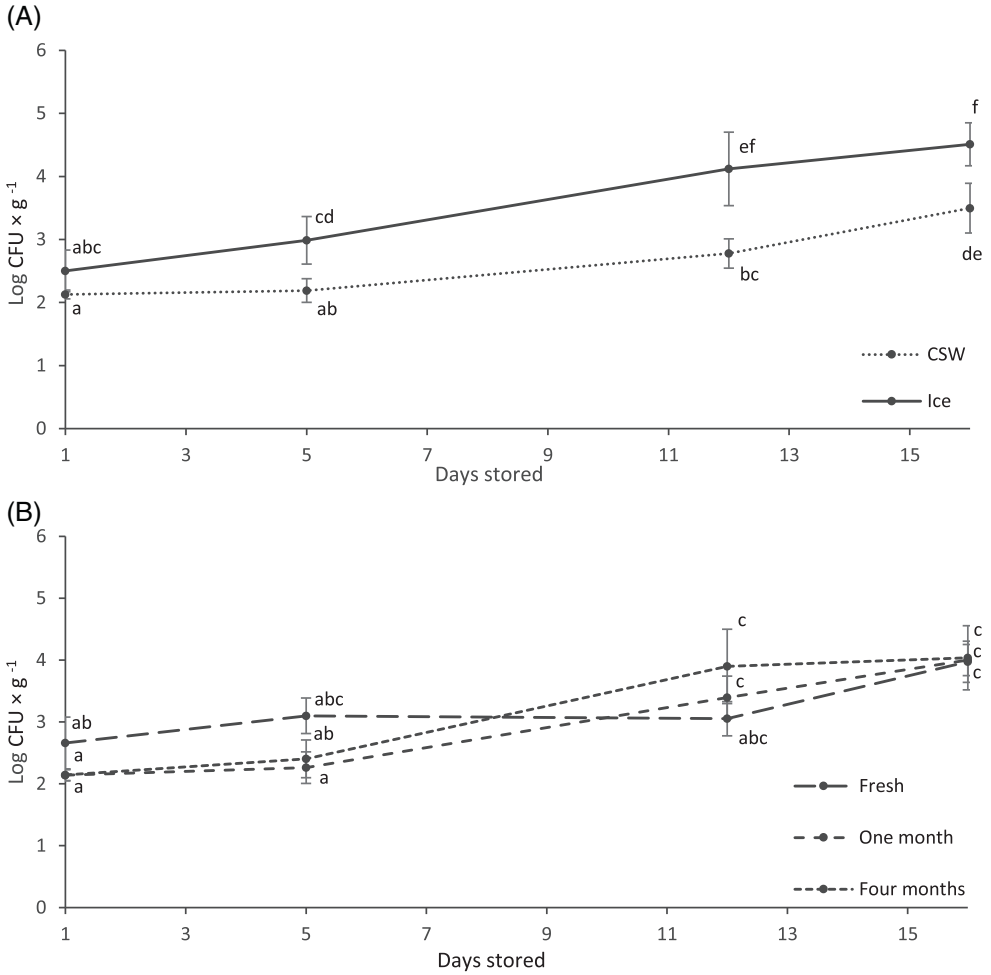

Figure 7. (A) APC of mesophilic bacteria in CSW-chilled and ice-chilled fresh and frozen–thawed samples throughout 16 days of cold storage (significance level $P < 0.05$, GLM). The quantification limit was set to 25–250. Samples with colonies under 25 were assigned the value 12.5, translating to the respective value in log CFU g^{-1}. Letters a–f indicate significant variance ($P < 0.05$) between the storage days and the chilling method. (B) APC of fresh, one-month- and four-month-frozen samples shown as log CFU g^{-1}. Letters a–c indicate a significant difference between storage days and storage methods. A significant difference was not detected regarding the storage method.

samples were placed in the freezer, the temperature variations could have triggered recrystallization, leading to the formation of larger and more damaging ice crystals.[34] According to Zhao and Takhar,[35] temperature fluctuations during storage are inevitable. Additionally, Syamaladevi *et al.*[36] observed a 22.1% increase in the size of ice crystals in Atlantic salmon after 4 weeks of freezing at −35 °C, even in the absence of provoked temperature fluctuations. Moreover, increased drip loss was observed as a function of storage time, which was in line with the results from the present study, as samples frozen for 4 months had the highest drip loss. Claussen *et al.*[37] reported that super-chilled salmon had a higher drip loss than those chilled regularly. They attributed this observation to the freeze damage occurring during partial freezing before the salmon enters the super-chilled state. In addition to the inherent drip loss caused by the freezing itself, this freeze damage contributes to the overall liquid loss in super-chilled salmon.

The storage method did significantly affect the fillet breaking force and firmness. Fresh samples required more energy to damage the surface than the frozen samples, and the one-month-frozen samples required the least. The frozen samples were expected to require less force to break the surface as freezing damages some cellular structures, causing tissue softening. Rehbein and Çaklı[38] reported lysosomal enzyme activity as higher in samples of various fish that had been frozen compared to fresh ones, which supports the results of the present experiment. On the other hand, Sigurgisladottir *et al.*[39] reported that freezing contributes to a loss in juiciness and increased toughness. This might explain why four-month-frozen samples had the most considerable drip loss, were firmer and required more force to break the fillet surface than those frozen for one month before thawing and fresh storage.

Although no effects of storage were found on the surface breaking force, the fillet firmness decreased during storage, for both fresh and thawed fillets. These results confirm the observations by Chan *et al.*,[40] who reported a significant reduction in firmness as a function of storage. However, they did not find any corresponding decrease in breaking force. Moreover, Taylor *et al.*[41] reported changes in the textural properties of Atlantic salmon to mainly happen from slaughtering until day 5 and that the change from day 5 to day 14 is minor.

The drip loss showed a medium negative correlation to fillet firmness ($r = -0.461$, $P < 0.05$) and breaking force ($r = -0.363$,

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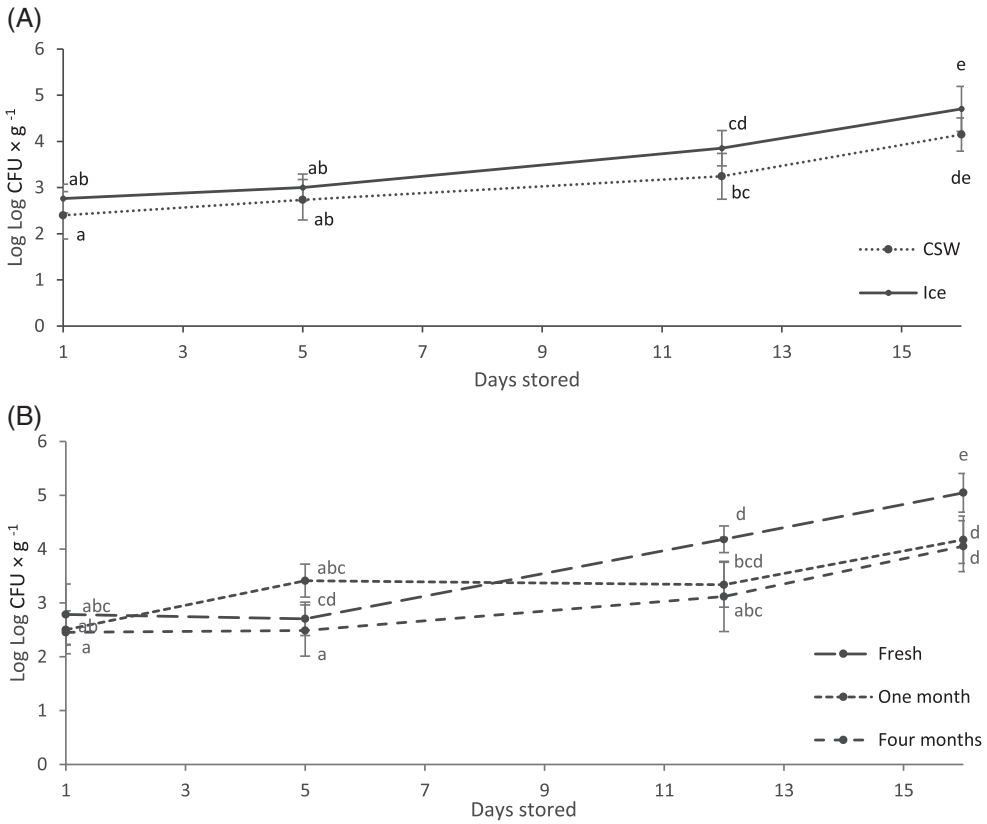

Figure 8. (A) PC of psychrotrophic bacteria in CSW-chilled and ice-chilled fresh and frozen–thawed samples throughout 16 days of cold storage. Lowercase letters a–e imply a significant difference within the storage day of the chilling methods. (B) PC of psychrotrophic bacteria in fresh, one-month- and four-month-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^{-1}. Lowercase letters a–e indicate significant variance ($P < 0.05$) between storage days and the storage method.

$P < 0.05$). These relationships indicate that a higher drip loss results in less force to break the fillet surface, which was anticipated as high drip loss usually indicates cellular destruction and protein denaturation.[28]

The fillet lightness (L^*) remained unaffected by storage method and storage duration. Consequently, a fillet that has been frozen for 4 months and stored for 12 days is presumably perceived equally as a fresh fillet regarding lightness. However, an interesting interaction emerged between chilling method and storage conditions ($P < 0.05$). There was little to no difference between fresh CSW-chilled and ice-chilled samples, indicating that sub-chilling is negligible regarding the fillet lightness when stored fresh. Chilling with ice before freezing leads to the fillets becoming slightly darker after thawing compared with sub-chilled samples. It is worth noting that changes in fillet lightness have previously been linked to increased drip loss and protein denaturation.[42]

Chroma was the only colorimetric variable affected by the storage method, where an increase in chroma was observed after frozen storage and thawing. Increased chroma indicates that the color intensity became stronger. Santos-Yap[43] reported the opposite: a fading salmon color during frozen storage. Moreover, the frozen storage duration did not affect the color intensity in the present study, showing non-compliance with results reported by Erikson *et al.*[8] and Chan *et al.*,[44] who reported a decrease in chroma as a function of storage days. However, an interaction between chilling and storage method was observed for chroma. Sub-chilled samples had a higher color intensity than ice-chilled samples when stored fresh. When frozen for 1 month, the ice-chilled samples significantly increased their color intensity when stored as thawed, while the sub-chilled samples slightly decreased. After 4 months of frozen storage, the samples had an equal color intensity, with ice-chilled samples having a slight decrease compared to those frozen for 1 month and sub-chilled samples showing a slight increase. Therefore, the chilling method seems to influence color stability when stored frozen.

Contrary to the chroma, the hue was significantly affected by the storage method and days stored whether fresh or thawed. Fresh samples differed significantly from those frozen–thawed, with a 1° higher hue. An increase in hue in the range 0° to 90° indicates an increase in yellowness as 0° represents a reddish hue, while 90° is yellowish. Regost *et al.*[45] reported an increased hue in Atlantic salmon fillets after frozen storage. The pigment astaxanthin, known for its antioxidation properties, plays a role in protecting lipid oxidation, as previously shown by Jensen *et al.*[46] As Atlantic salmon is a particularly fatty fish, the observed increase in yellowness might be due to a combination of lipid oxidation and astaxanthin being used as an antioxidative agent. Moreover, the present study showed a change towards a more reddish hue as a function of storage, with the fillets sampled after 16 days being significantly different from the others. These results correspond to those of Chan *et al.*,[44] who reported reduced hue during storage and increased chroma.

Nucleotide degradation commences post-mortem in fish muscle and progresses throughout storage. ATP is rapidly deaminated

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to ADP and further to AMP and IMP, while IMP dephosphorylation to HxR and Hx is a slower process.[47] IMP is desired as this metabolite contributes to the umami flavor.[48] Both storage methods and days affected samples' IMP level, which decreased as storage days increased. The dephosphorylation of IMP will proceed by either enzymes or bacterial activity if nucleotides are present. Fresh samples significantly differed from the frozen–thawed fillets and had higher concentrations of IMP on all sampling days. Rehbein and Çaklı[38] attributed this phenomenon to the increase in lysosomal enzyme activity often observed in frozen and thawed fish compared to fresh fish of various species.

As the IMP concentration decreased throughout the storage of fresh and frozen–thawed fillets, the HxR and Hx concentrations increased. The concentration of IMP was inversely related to HxR ($r = -0.825$, $P < 0.05$) and Hx ($r = -0.858$, $P < 0.05$). IMP is the precursor to HxR and Hx, and a decrease in IMP will increase HxR and Hx. Both endogenous and bacterial enzymes play a role in the dephosphorylation of IMP. However, the reaction rate highly depends on the temperature.[49] In the present study, higher concentrations of HxR were observed on all storage days compared to Hx. Microbial growth is an essential factor regarding nucleotide degradation. APC had a strong positive relationship to the amount of Hx ($r = 0.707$, $P < 0.05$) and HxR ($r = 0.713$, $P < 0.05$), while PC had a strong positive relationship ($r = 0.683$, $P < 0.05$ and $r = 0.631$, $P < 0.05$, respectively). The extent of IMP degradation is highly dependent on microbial growth. As microorganisms proliferate, bacterial enzymes break down IMP to HxR and Hx. Conversely, IMP had a strong negative correlation with APC ($r = -0.716$, $P < 0.05$) and PC ($r = -0.631$, $P < 0.05$).

The *K*-value has been well documented as a good freshness indicator as it assesses the concentration of spoilage nucleotides.[15,50,51] For Atlantic salmon, the limits of acceptability have been reported to be a *K*-value of 70–80%.[52] Therefore, the fresh and frozen–thawed fillets of the present study were acceptable for consumption after 16 days of storage. On the first sampling day, samples stored frozen for 1 month in the freezer had the lowest *K*-value. Interestingly, the *K*-values exhibited contrasting trends on day 12 and day 16. Notably, samples frozen for 1 month had the highest values. This does not follow the results of Fernández-Segovia *et al*.,[53] who reported a steady increase in the *K*-value in Atlantic salmon samples as a function of frozen days.

In the context of protein denaturation, only myosin was significantly affected by frozen storage. There was a difference in enthalpy between the fresh samples and those stored frozen for 4 months. Myosin has been reported to be less temperature stable than actin due to specific thermo-unstable regions.[54] Moreover, a decrease in energy required to unfold the proteins was observed as a function of storage. This indicates that the proteins denature as a function of cold storage.[55] Two interactions were observed for sarcoplasmic proteins (Fig. 6, peak 2). Firstly, between storage days and storage methods, fresh samples increased their denaturation energy from days 1 to 16. In contrast, the frozen samples had a decrease in denaturation energy. However, the difference in denaturation energy between days was minor between one- and four-month-frozen samples, probably because of freeze damage due to recrystallization, which denatures proteins and disrupts cellular structures. It is well documented that a release of sarcoplasmic proteins occurs during storage. Lin *et al*.[56] reported an increase in sarcoplasmic proteins in Atlantic mackerel stored at 0 and 4 °C after 5 days, while Lu *et al*.[57] reported the same in bighead carp (*Aristichthys nobilis*). Secondly, an interaction between the factors chilling and storage was noted. Notably, there was no considerable difference between sub-chilled and ice-chilled samples when the samples were stored fresh. However, the difference increased as a function of frozen storage, and the one-month-frozen, sub-chilled samples required more energy to denature proteins which was the opposite of those stored fresh and frozen for four months before further storage as thawed. This might imply that the sarcoplasmic proteins in the sub-chilled samples do not aggregate during frozen storage, but those in the ice-chilled fillets do.

Moreover, an interaction for peak 3 (Fig. 6) was observed between the factors chilling and storage method. Fresh and sub-chilled samples required more energy to denature the proteins. While for the frozen samples, ice-chilled fillets required the most. For actin, peak 4, none of the different factors were significant; however, an interaction was observed between the chilling method and storage days. Actin is a reasonably stable protein due to bound nucleotides and divalent cations.[58]

The denaturation of proteins did not have a strong correlation to the texture. Denaturation of myosin and peak 2 (associated with sarcoplasmic proteins) had a negligible relationship for both the breaking force ($r = 0.177$, $P > 0.05$ and $r = 0.064$, $P > 0.05$, respectively) and firmness ($r = 0.145$, $P > 0.05$ and $r = 0.010$, $P > 0.05$, respectively). In contrast, peak 3 (also associated with sarcoplasmic proteins) had a moderate positive relationship regarding fillet firmness ($r = 0.311$, $P < 0.05$) and a negligible association with breaking force ($r = 0.147$, $P > 0.05$). Actin had negligible relationships with both breaking force ($r = 0.024$, $P > 0.05$) and firmness ($r = -0.024$, $P > 0.05$).

Seafood is highly perishable, and an increased bacterial growth was expected as storage days increased. This is shown in Fig. 7, where the lowest APC was observed on day 1 and the highest on day 16. CSW-chilled samples have a significantly lower amount of bacterial growth than ice-chilled samples on all sampling days, similar to what Hansen *et al*.[59] found when comparing super-chilled fillets to chilled fillets after 21 and 28 days. Interestingly, CSW-chilled fillets stored for 16 days had comparable APC values to those ice-chilled on day 5, implying that CSW-chilled fillets were of high microbial quality throughout storage. Moreover, frozen–thawed samples had lower PC in general compared to those stored fresh after 12 and 16 days of chilled storage, indicating that the freezing process inhibits growth after thawing. Ge *et al*.[60] reported less bacterial diversity effect was found in frozen fish than in fresh fish, which might explain why frozen samples had less growth than those stored fresh.

Although the storage method was insignificant alone as a design variable for APC, a significant interaction with the applied chilling method was observed. Notably, sub-chilled samples had less microbial growth stored as fresh and four-month frozen than those ice-chilled. The same interaction pattern was observed for PC. It is hard to pinpoint the cause, as many factors might be at play.

CONCLUSION

The objective of this study was to investigate the impact of internal temperature on Atlantic salmon quality when chilled using CSW and ice prior to freezing. Fresh-stored fillets were analyzed as a control group. Additionally, storage time was included as a design variable regarding months frozen and days stored in a cold room as fresh or thawed.

The chilling method was found to interact with the storage method providing significant quality differences. Among fresh

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stored fillets, sub-chilling improved quality characteristics such as microbial stability, texture and protein degradation. Moreover, compared to ice-chilled fillets, it is concluded that sub-chilling before frozen storage and further storage as thawed improves quality parameters such as microbial stability, fillet color, texture (only fillets stored frozen for one month), ATP degradation and protein denaturation.

As expected, quality is lost as a function of storage, and differences were observed between fresh stored salmon fillets and those stored frozen. Fresh fillets generally had higher bacterial counts, surface breaking force, firmness, hue and contents of IMP, and lower drip loss and HxR levels than those stored frozen–thawed. Moreover, four-month-frozen fillets had higher HxR levels and lower PC growth than those frozen for one month. The difference between fillets frozen for one and four months was minor and did not profoundly affect the quality. Based on the presented results, it is concluded that a frozen product might be competitive with a fresh product when sub-chilling is performed before freezing, especially when including the environmental benefits of frozen export.

ACKNOWLEDGEMENT

The FHF funded this project – Norwegian Seafood Research Fund project number 901635. The authors would like to thank the technical staff at Nofima AS, Stavanger, Norway, who contributed to the project with technical guidance at the laboratory. The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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