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Effects of −1.5 °C Super-chilling on quality of Atlantic salmon (*Salmo salar*) *pre-rigor* Fillets: Cathepsin activity, muscle histology, texture and liquid leakage

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ABSTRACT

The aim of this study was to evaluate the impact of super-chilling on the quality of Atlantic salmon (*Salmo salar*) *pre-rigor* fillets. The fillets were kept for 45 min in a super-chilling tunnel at −25 °C with an air speed in the tunnel at 2.5 m/s, to reach a fillet core temperature of −1.5 °C, prior to ice storage in a cold room for 4 weeks. Super-chilling seemed to form intra- and extracellular ice crystals in the upper layer of the fillets and prevent myofibre contraction. Lysosome breakages followed by release of cathepsin B and L during storage and myofibre–myofibre detachments were accelerated in the super-chilled fillets. Super-chilling resulted in higher liquid leakage and increased myofibre breakages in the fillets, while texture values of fillets measured instrumentally were not affected by super-chilling one week after treatment. Optimisation of the super-chilling technique is needed to avoid the formation of ice crystals, which may cause irreversible destruction of the myofibres, in order to obtain high quality products.

1. Introduction

Refrigeration of gutted fish or fish fillets is an important parameter and has to be controlled, in order to deliver high quality, fresh food products to consumers. Today, ice is the most commonly used means of maintaining a chilling temperature in farmed salmon during transportation. When transporting gutted salmon in an average-sized truck, 21% of the space is taken by external ice (Haugland et al., 2006). Reducing the amount of ice during transportation from producers to customers will directly reduce transport weight and costs, and also have a positive impact on the environment. Super-chilling is one of the techniques that can be used to fulfil these objectives.

Super-chilling consists in freezing or partly freezing a food product, lowering the product temperature 1–2 °C below its initial freezing point. Instead of adding external ice to the product to ensure its refrigeration during distribution, part of the internal water is frozen and acts as a refrigeration reservoir, reducing the overall transported weight. Super-chilling has already been studied on different food products, most of them emphasising the many advantages of the technique, especially when it comes to freshness and extension of shelf-life (Dion, 1997; Duun & Rustad, 2007, 2008; Fik, Surowka, & Leszczynskafik, 1988; Gallart-Jornet, Rustad, Barat, Fito, & Escriche, 2007; Lee & Toledo, 1984; Nowlan, Dyer, & Keith, 1974; Sivertsvik, Rosnes, & Kleiberg, 2003). Trained consumer panels also found better quality attributes in super-chilled fish, when scored on visual aspects and taste (Jensen, 2007). However, most of these studies dealt with several days super-chilling storage of gutted fish or fillets processed *post-rigor*. To our knowledge, this paper reports the first study focusing on a short-term super-chilling process of Atlantic salmon *pre-rigor* fillets, prior to a storage period on ice in a cooling room.

Formation of ice within tissues during freezing can cause irreversible damage. Ice crystallisation occurs in two steps: first, nuclei are formed and second, they grow to a specific crystal size. It is known that freezing rate and final temperature of the process determine the size and location of ice crystals formed during freezing. Slow freezing results in a low number of large ice crystals, while more rapid freezing gives a large number of small ice crystals (Bello, Luft, & Pigott, 1982; Martino, Otero, Sanz, & Zaritzky, 1998).

Today, the salmon industry is interested in increasing the production of *pre-rigor* fillets, instead of fish filleting after the onset of *rigor mortis*. *Pre-rigor* filleting allows the fish to be processed

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directly after slaughter; therefore no storage period before filleting is necessary. *Pre-rigor* fillets reach the market earlier, compared to *post-rigor* fillets and, as a matter of quality, show a reduction in severity of gaping, a firmer flesh texture, a positive effect on colour and an increased thickness of the fillet (Skjervold, Fjæra et al., 2001, Skjervold, Rørå et al., 2001). This early processing also increases the fresh fillet value and reduces waste product transport by 20%, decreasing considerably transportation costs and energy wastage.

Post-mortem softening of the fillet is an important parameter when dealing with fish quality. Ando, Toyohara, Shimizu, and Sakaguchi (1991) and Ofstad, Olsen, Taylor, and Hannesson (2006) suggested that gradual degradation of the extra-cellular matrix may be one of the causes of *post-mortem* tenderisation of fish muscle. Taylor, Fjæra, and Skjervold (2002) have also shown that breaks in muscle cell cytoskeleton and connective tissue (detachments between myofibres and between myofibres and myocommata) determined fish flesh texture. They suggested that fish muscle tenderisation paralleled loss of attachments between myofibres.

Lysosomal and cytosolic enzymes have been associated with *post-mortem* tenderisation of fish muscle (Ando et al., 2001; Chéret, Delbarre-Ladrat, de Lamballerie-Anton, & Verrez-Bagnis, 2007; Yamashita & Konagaya, 1991). Degradation of the fine structure of muscle myofibrils and intramuscular connective tissue is probably caused by proteases such as cathepsins, as well as calcium-dependent proteases (Okitani, Matsukura, Kato, & Fujimaki, 1980). In several fish species, cathepsins B, D and L are considered as the enzymes playing the most important role in *post-mortem* muscle softening, with a particularly high participation of cathepsin L (Ando et al., 2001; Ladrat, Verrez-Bagnis, Noel, & Fleurence, 2003; Yamashita & Konagaya, 1990, 1991).

Modified atmosphere packaging (MAP) is used to reduce microbial growth and delay the production of amine compounds (Sivertsvik, Jeksrud, & Rosnes, 2002) of packed food products, even on frozen and thawed cod (Bøknæs, Osterberg, Nielsen, & Dalgaard, 2000). MAP of super-chilled stored Atlantic salmon *post-rigor* fillets has been studied by Sivertsvik et al. (2003). The combination of super-chilling storage with MAP increased the sensory and microbial shelf-life of the fillets by 17 days, when compared to air-stored fillets at chilled temperature.

The aim of this study was to investigate the consequences of −1.5 °C short super-chilling technology on *pre-rigor* farmed Atlantic salmon fillets. Physical and chemical degradation of the muscle was followed during storage time and studied by light microscopy, lysosomal enzymatic analyses, and instrumental texture and liquid leakage measurements.

2. Materials and methods

2.1. Fish

Twenty-four Atlantic salmon (*Salmo salar*) were raised at Akvaforsk Research Station, Averøy, on the west coast of Norway. They were fed a standard commercial extruded dry feed (“Optiline”, 12 mm, Skretting, Stavanger, Norway). In December 2006 (sea temperature: 8 °C, salinity: 33‰, average body weight of the fish: 5 kg), the fish were starved for five days before being killed by percussive stunning, followed by gill cutting and subsequent bleeding in a tank with running water. The gutted fish were filleted immediately *post mortem* by experienced workers (*pre-rigor* fillets) and subjected to two different treatments. The right fillets of each fish were placed in a mobile super-chilling tunnel, in a freezing room at a temperature of −25 °C, with an air velocity of approximately 2.5 m/s above the fish. The fillets were super-chilled for 45 min, after which the temperature of the fillet cores had reached −1.5 °C. The super-chilled fillets were immediately packed on ice in standard Styrofoam boxes and stored in a cold room (+5 °C) for further analyses (ice-stored fillets), until 145 h after slaughter. The left fillets served as a control and were directly packed in the same way and placed in the cold room without a previous super-chilling treatment.

Besides the ice-stored fillets, pieces of *pre-rigor* fillets (15 × 10 cm) from 5 other salmon, were packed in modified atmosphere (MAP) and stored in the cold room (+5 °C) for long term analyses, until 4 weeks after slaughter. Before being MAP packed, five right fillets were also super-chilled. The other five left fillets were immediately MAP packed without super-chilling and served as control for the rest of the experiment with MAP. In this present study, MAP was mainly used in order to extend shelf-life of the fillets to be able to run long term analyses with low bacterial disturbance. The effect of MAP was not measured as a priority, but some observations were reported, comparing 144 h ice-stored samples with 1 week MAP samples.

Super-chilling formed an ice layer on the top of the fillets of approximately 4 mm. After previous testing, it was estimated that about 25% of the free water contained in the fish was frozen at the end of the super-chilling treatment.

2.2. Microscopy

Six super-chilled and six corresponding control *pre-rigor* fillets were used for light microscopy analyses at each time point. Using a total of 24 fish for this experiment allowed us to make a maximum of three measurements per fillet (positions A, B and C, close to each other), always in the same cut (Norwegian Quality Cut), avoiding any effect of the measurement location during the different analyses.

Muscle blocks (2 × 2 × 3 mm) were taken from the upper layer of the front part of randomised super-chilled and control fillets, in the dorsal section, above the mid-line, and fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.3. Sampling was performed 0 h, 12 h, 24 h, 48 h, 144 h, 1 week and 4 weeks after treatment (time at slaughter +10 min filleting +45 min super-chilling). The fixed samples were transported by truck in refrigerated boxes to a laboratory and kept there at cool temperature (+4 °C) until they were embedded for histological analyses.

After being rinsed in cacodylate buffer for 2 × 15 min, the muscle blocks were dehydrated in different gradients of ethanol (70% and 96% for 2 × 20 min, 100% for 1 × 20 min) and embedded in a cold plastic resin based on hydroxyethylmethacrylate (Technovit 7100, Heraeus Kulzer GmbH, Hanau, Germany). The direction of the myofibres in the embedded samples was oriented so as to obtain both cross- (for every time point) and longitudinal- (for the 144 h samples) sections. Light microscopy observations at 10 times magnification were made on thin sections (3 μm), stained (using the method of Ofstad et al. (2006), slightly modified), in 0.05 g/100 ml toluidine blue dissolved in 0.1 M aqueous sodium acetate buffer.

In the cross-section samples, muscle degradation was evaluated according to the method of Taylor et al. (2002), slightly modified. Briefly, we counted the percentage of degraded endomysium between myofibres (myofibre–myofibre detachments), as well as detachments between myofibres and connective tissue (perimysium and epimysium) (myofibre–myocommata detachments). Two samples per fish and per time point were analysed. For each sample, detachments were counted based on 250 junctions between myofibres, and 50 junctions between myofibres and myocommata.

In the longitudinal-oriented 144 h samples, the quality of the *pre-rigor* fillets was determined by counting myofibre breakages for both super-chilled and control fillets. Two to four samples per fish were analysed. For each sample, the percentage of myofibre

breakages was evaluated, based on a total of 30 myofibres per sample.

The percentage of contracted myofibres was also evaluated on the same samples as for the myofibre breakages, using the same amount of myofibres.

2.3. Subcellular fractionation

Extraction and subcellular fractionation was performed, according to Ertbjerg, Larsen, and Moller (1999), with slight modifications. Briefly, tissue samples (1.5 g) from the same 6 fillets/group/time point as for the microscopic observations, were taken randomly next to the part used for histology, above the mid-line of the fillet, 0 h, 6 h, 12 h, 24 h, 48 h, 144 h, 1 week and 4 weeks after treatment. They were first homogenised in a buffer (100 mM sucrose, 100 mM KCl, 50 mM Tris, 10 mM sodium pyrophosphate, 1 mM Na_2EDTA, pH 7.2), with a Potter-Elvehjem homogeniser making 10 complete passes at 1500 rpm. The homogenate was filtered using a cheesecloth and fractionated by serial centrifugations at 1100*g* for 10 min (myofibrillar fraction; subsequently discarded), 3000*g* for 10 min (heavy mitochondrial fraction) and 16,000*g* for 30 min (lysosomal fraction). The mitochondrial and lysosomal fractions were resuspended in a storage buffer (85 mM Na acetate, 15 mM acetic acid, 1 mM Na_2EDTA, pH 5.5). Together with the filtrated homogenate and the remaining supernatant after lysosomal fractionation (lysosomal supernatant), these samples were frozen at −80 °C until further analyses.

2.4. Cathepsin B and L analyses

Cathepsin B and L activity was measured fluorimetrically, according to the method of Kirschke, Wood, Roisen, and Bird (1983). The procedure used *N*-CBZ-L-phenylalanyl-L-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-Nmec) as a substrate. It was performed on the different subcellular fractions of both super-chilled and control fillets. Special emphasis was given to the lysosomal fraction at all time points. Cathepsin B and L activity was also measured on the filtered homogenate (for total activity), mitochondrial fraction and lysosomal supernatant 0 h and 24 h after treatment. In all cases, the assays were run twice in triplicates.

2.5. Texture analyses

Instrumental texture analyses were performed on the same 6 fillets/group/time point as for histology and enzyme analyses, above the mid-line of the fillets, 0 h, 2 h, 4 h, 6 h, 12 h, 24 h, 40 h, 144 h and 1 week after treatment, using a texture analyser TA-XT2 (Stable Micro Systems Ltd., Godalming, UK) equipped with a 5 kg load cell. A flat-ended cylindrical probe (12.5 mm diameter; type P/0.5) was used to analyse the firmness of the fillets, by measuring the force required to penetrate the fillet surface (breaking force) (Mørkøre, Netteberg, Johnsson, & Pickova, 2007). Only the results obtained 1 week after treatment of MAP fillets are directly reported here.

2.6. Liquid leakage

Liquid leakage during storage of super-chilled and control MAP fillets was measured 1 week after treatment using the method of Mørkøre et al. (2007) on 6 fillets/group. Briefly, we placed 15 g of muscle on a thin-bedded, honeycombed pad. The pad and muscle were sealed together in a polyethylene bag and placed in a refrigerated room (+3 °C) for three days. After three days, liquid loss was calculated as

$$100 \times \frac{\text{weight increase of the mat}}{\text{initial muscle weight}}.$$

2.7. Statistical analyses

Most of the data were evaluated statistically, according to the analysis-of-variance approach of Langsrud, Jørgensen, Ofstad, and Næs (2007), and the corresponding software (www.matforsk.no/ola) was utilised. Myofibre–myofibre detachments, myofibre–myocommata detachments, myofibre breakages and myofibre contraction were analysed as percentages. Analysis of those observations was based on a model with the model terms "fish identity", "time after treatment", "treatment" and "time after treatment" × "treatment". In addition, separate analyses with "treatment" as the model term were conducted within each time point. For the analysis of texture (breaking force) and cathepsin B and L activity, the parameters "location of the measurement on the fillet" and "treatment" × "location" were added to these models. In the models mentioned above, "time after treatment" was considered as a categorical variable. Results from modelling "time after treatment" as a continuous variable were also checked. In that case, the logarithmic transformation of the "time after treatment" (hours), $t = \log(\text{time} + 4)$, was performed. Then, polynomial terms such as t, t^2 and t^3 were included in the model. Interactions between such terms and "treatment" were also included. The chosen analysis approach ensured that maximal polynomial complexity could be used. Cathepsin B and L activity, myofibre–myofibre detachments and myofibre–myocommata detachment results of the MAP fillets were analysed according to a two-way model with the terms "time after treatment", "treatment" and "time after treatment" × "treatment". For those three response variables, the MAP and the ice-stored samples data were also compared together: 1 week MAP fillets data were combined with 144 h ice-stored fillets data in a two way model ("treatment", "packaging method" and "treatment" × "packaging method").

One-way analyses of variance, using Minitab®Release 14.20, were also carried out on 1 week liquid leakage and texture, as well as cathepsin B and L activity, in the different isolated muscle fractions (homogenate, mitochondrial fraction and lysosomal supernatant) data, to analyse the effect of treatment (super-chilling *vs.* control).

3. Results

3.1. Microscopy

Light microscopy observations were performed, in order to detect eventual physical damage due to super-chilling. Observations of the upper layer of the fillets showed several freezing imperfections in all super-chilled fillets, most probably consequences of the development of large intra- and extra-cellular ice crystals during super-chilling (Figs. 1 and 2). Intra-cellular ice crystals seemed to be formed mostly at the border of the myofibres, while extracellular crystals affected the endomysium. On the contrary, no traces of intra- or extra-cellular ice crystals formation were noticed in the control fillets.

3.1.1. Myofibre–myofibre detachments

Microscopic observations of the cross-section muscle samples showed an increase in myofibre–myofibre detachments with storage time, up to 144 h after treatment (Figs. 1 and 3) for both super-chilled and control fillets ($p < 0.0001$). Statistical analysis showed that super-chilling caused more myofibre–myofibre detachments than in the control during storage time ($p < 0.0001$). Super-chilling accelerated myofibre–myofibre detachments

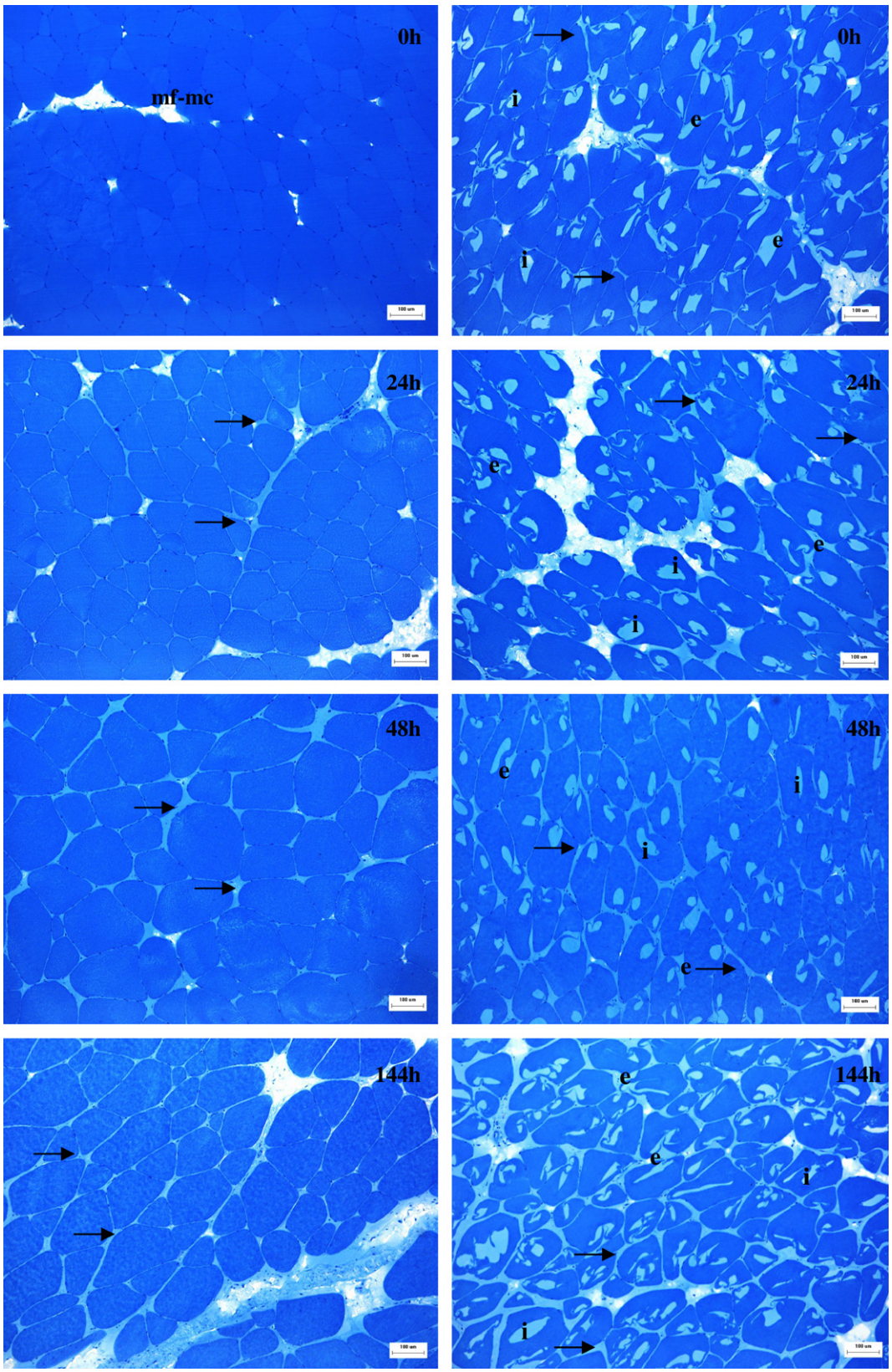

Fig. 1. Light microscopy observations 10 × (cross-sections) of ice-stored muscle samples, control (left column) and super-chilled (right column) at corresponding time after treatment. Arrows: myofibre–myofibre detachments; mf-mc: myofibre–myocommata detachments; i: trace of intracellular ice crystals; e: trace of extracellular ice crystals.

(Figs. 1 and 3), showing already a significant difference between the two groups immediately after treatment (0 h samples), with 27.1% of detachments in the super-chilled fillets and 8.8% in the control fillets. The difference was still significant 12 h and 24 h after treatment. However, from 48 h, no significant differences between the two groups were noticed.

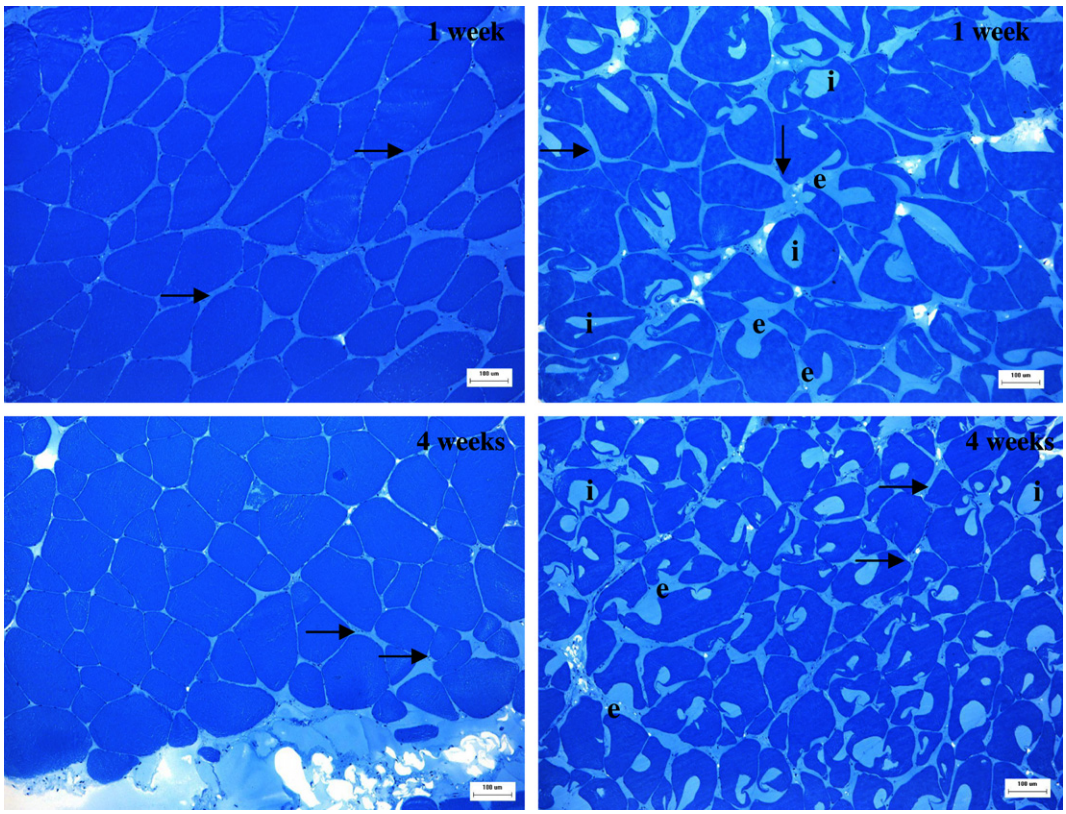

Fig. 2. Light microscopy observations 10 × (cross sections) of MAP muscle samples, control (left column) and super-chilled (right column) 1 and 4 weeks after treatment. Arrows: myofibre–myofibre detachments; i: trace of intracellular ice crystals; e: trace of extracellular ice crystals.

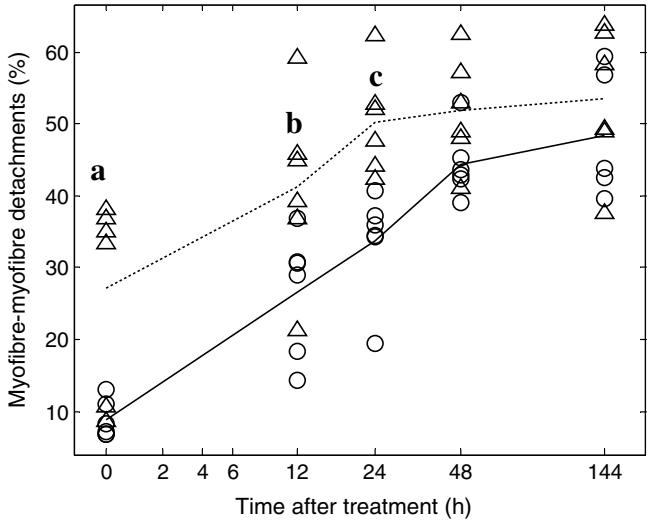

Fig. 3. Myofibre–myofibre detachments in ice-stored fillets (%): dotted line and triangles = super-chilled; full-line and circles = control. a, b, c: results showing significant difference comparing to control (a: $p = 0.009$; b: $p = 0.040$; c: $p = 0.003$).

In the MAP fillets, the super-chilled fillets showed significantly more myofibre–myofibre detachments than the control fillets both 1 and 4 weeks after treatment (Fig. 2 and Table 1). While the level of detachments in the control fillets seemed to stabilise 144 h after treatment, a trend towards further increase of myofibre–myofibre detachments could be seen in the super-chilled fillets up to 4 weeks of storage.

Table 1
Myofibre–myofibre detachments in MAP fillets (%)

	1 week MAP		4 weeks MAP	
	Mean	Standard deviation	Mean	Standard deviation
Super-chilled	66.88	15.17	73.64	7.46
Ice-chilled	45.40	6.4	44.04	2.05
p-Value	0.019		<0.0001	

3.1.2. Myofibre–myocommata detachments

In contrast to myofibre–myofibre detachments, analyses of myofibre–myocommata detachments of super-chilled and control fillets did not show any significant differences between treatment (super-chilling and control) or storage time (from 0 h to 4 weeks after treatment) (Fig. 4 and Table 2).

3.1.3. Myofibre breakages and contraction

Microscopic observations of longitudinal sections of the super-chilled fillets 144 h after treatment showed a significantly higher amount of myofibre breakages, compared to the control fillets (Figs. 5 and 6). Contracted muscle fibres observed microscopically were found almost twice as often in the control fillets as in the super-chilled fillets (Figs. 5 and 6).

3.2. Cathepsin B and L analyses

3.2.1. Lysosomal activities

Cathepsin B and L activity significantly decreased during storage time in the lysosomal fraction ($p < 0.0001$) of both super-chilled and control fillets, as would be expected during storage degradation (Fig. 7). Statistical analysis showed that in general,

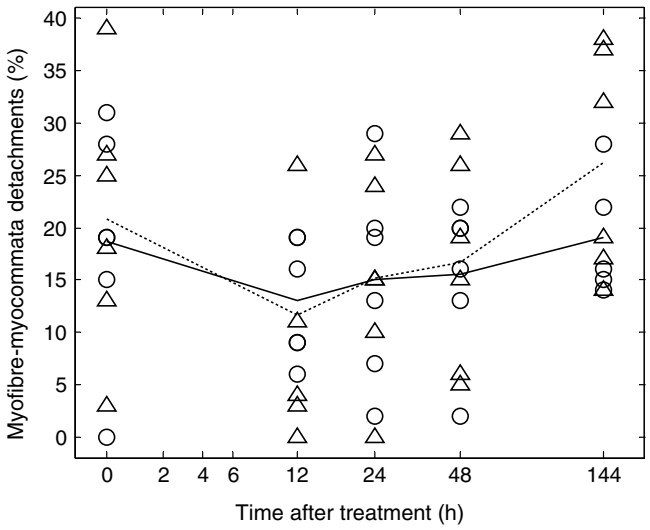

Fig. 4. Myofibre–myocommata detachments in ice-stored fillets (%): dotted line and triangles = super-chilled; full-line and circles = control.

Table 2
Myofibre–myocommata detachments in MAP fillets (%)

	1 week MAP		4 weeks MAP	
	Mean	Standard deviation	Mean	Standard deviation
Super-chilled	16.20	6.98	27.00	7.48
Ice-chilled	21.50	13.99	22.20	8.38
p-Value	0.527		0.367	

super-chilling caused a higher release of cathepsin B and L from the lysosomes ($p < 0.000001$).

Super-chilling accelerated the release of these enzymes from the lysosomes of the ice-stored fillets (Fig. 7). Already 6 h after treatment, almost half of the activity had disappeared from the lysosomes of the super-chilled fillets, while in the control fillets, the activity remained stable. Cathepsin B and L activity in the lysosomes of the super-chilled fillets stayed significantly lower than in the control fillets at every time point, up to 144 h after treatment, where the cathepsin B and L activity in the lysosomes of the control fillets finally reached the level of the super-chilled fillets. The activity in the lysosomes of the control fillets did not start to decrease until between 24 h and 48 h after treatment. Statistical analysis showed a negative correlation between the level of myofibre–myofibre detachments and the level of cathepsins B and L in the lysosomal fraction (−0.711).

Cathepsin B and L activity in the lysosomes of the MAP fillets significantly decreased between 1 and 4 weeks after treatment ($p < 0.000001$) (Table 3). One week after treatment, the super-chilled fillets showed a much lower cathepsin B+L activity than the control fillets. The same significant difference could still be observed 4 weeks after treatment, although the remaining activity was very low in both groups.

3.2.2. Cathepsin B and L activity in the other fractions

Cathepsin B and L activity was also measured in the other fractions of the fish muscle (homogenate (for total activity), mitochondrial fraction and lysosomal supernatant) 0 h and 24 h after treatment (Fig. 8). The total activity of cathepsin B and L remained stable from 0 h to 24 h after treatment and no differences were found due to super-chilling treatment. In the mitochondrial fraction, cathepsin B and L activity showed the same trend as for the

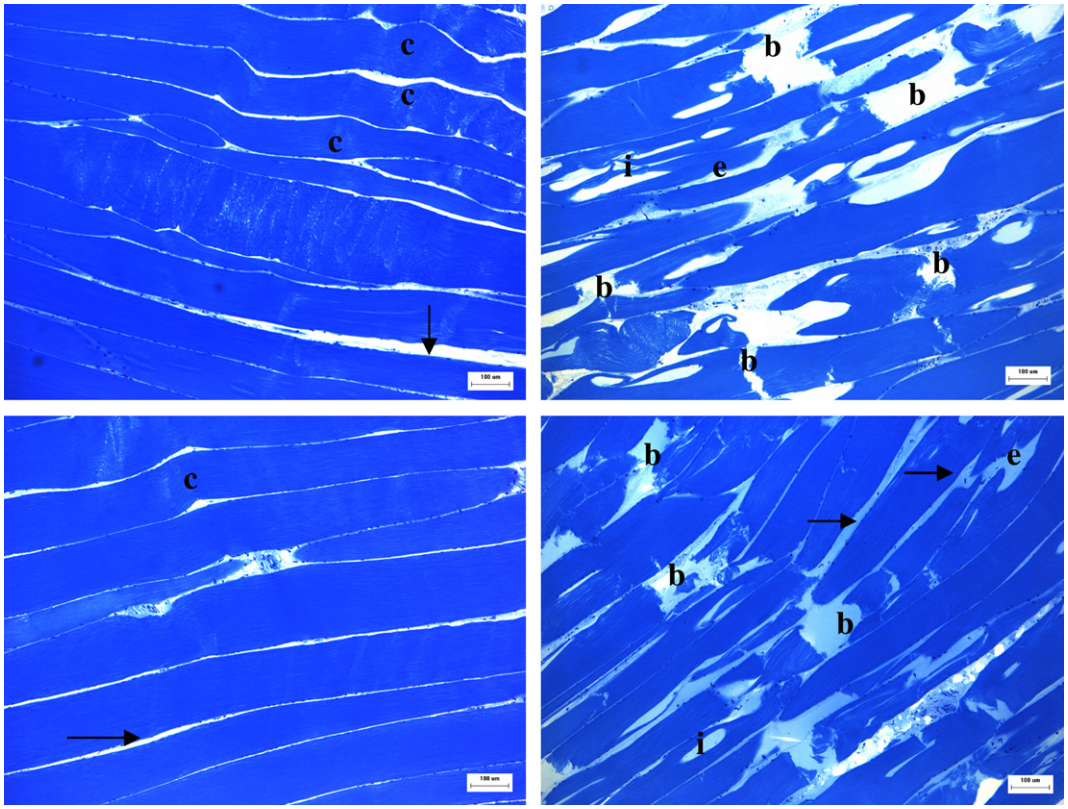

Fig. 5. Light microscopy observations 10 × (longitudinal sections) of two ice-stored control fillets (left column) and the corresponding super-chilled fillets (right column). c: contracted myofibres; b: myofibre breakages; i: trace of intracellular ice crystals; e: trace of extracellular ice crystals; arrows: myofibre–myofibre detachments.

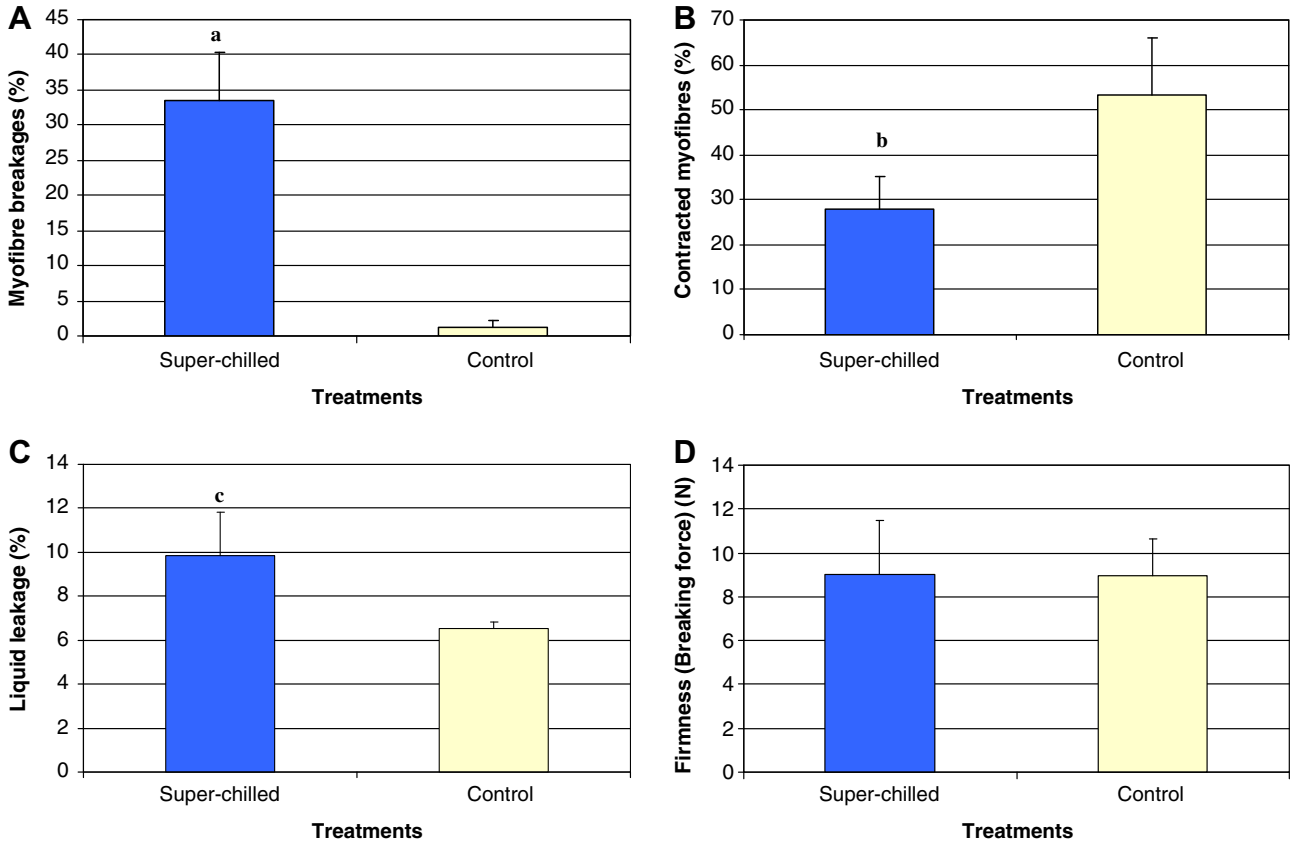

Fig. 6. (A) Myofibre breakages (%) in super-chilled and control ice-stored fillets observed by light microscopy, 144 h after treatment. a: results showing a significant difference compared to control ($p = 0.005$). (B) Contracted myofibres (%) in super-chilled and control ice-stored fillets observed by light microscopy, 144 h after treatment. b: results showing a significant difference compared to control ($p = 0.04$). (C) Liquid leakage (% body weight) in super-chilled and control MAP fillets, 1 week after treatment. c: results showing a significant difference compared to control ($p = 0.004$). (D) Firmness (breaking force) (N) of super-chilled and control MAP fillets measured instrumentally, 1 week after treatment.

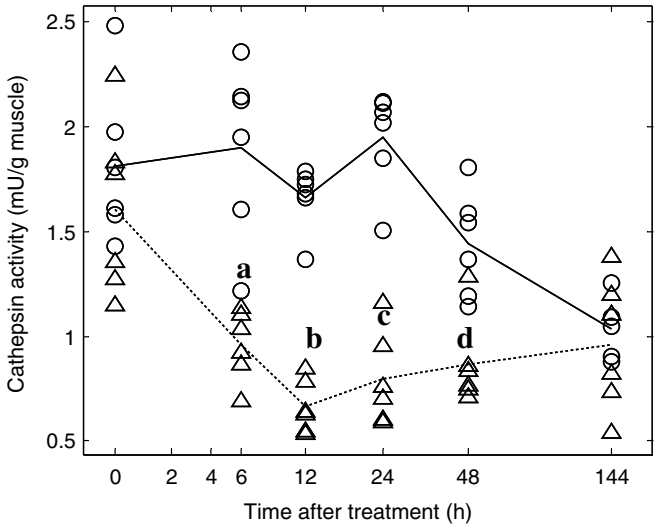

Fig. 7. Cathepsin B and L activity in the lysosomes of ice-stored fillets (mU/g muscle): dotted line and triangles = super-chilled; full-line and circles = control. a, b, c, d: results showing a significant difference compared to control ((a) $p = 0.004$; (b) $p = 0.00004$; (c) $p = 0.0003$ and (d) $p = 0.0018$).

lysosomal fraction, but in a lower amount; there was no significant difference of cathepsin B and L activity between super-chilled and control fillets 0 h after treatment, but the activity was significantly lower in the super-chilled fillets 24 h after treatment. Part of the cathepsin B and L seemed to have been released upon storage from the broken lysosomes in the lysosomal supernatant. Cathepsin B and L activity increased significantly in the lysosomal supernatant from 0 h to 24 h after treatment ($p = 0.003$) in the control fillets, but not in the super-chilled fillets ($p = 0.118$). No significant difference between the super-chilled and the control groups was noticed either 0 h or 24 h after treatment.

Table 3
Cathepsin B and L activity in the lysosomes of MAP fillets (mU/g muscle)

	1 week MAP		4 weeks MAP	
	Mean	Standard deviation	Mean	Standard deviation
Super-chilled	1.53	0.18	0.09	0.09
Ice-chilled	2.39	0.31	0.53	0.33
p-Value	<0.001		0.021	

3.3. Quality parameters

Liquid leakage 1 week after treatment was significantly higher in the super-chilled fillets than in the control fillets (Fig. 6). There was no difference in texture measured instrumentally as breaking force, between super-chilled and control fillets 1 week after treatment (Fig. 6). No correlation over time between firmness of the fillets and level of myofibre–myofibre detachments (−0.180) on one hand, nor level of cathepsin B and L activity in the lysosomes (+0.294) on the other hand, was observed.

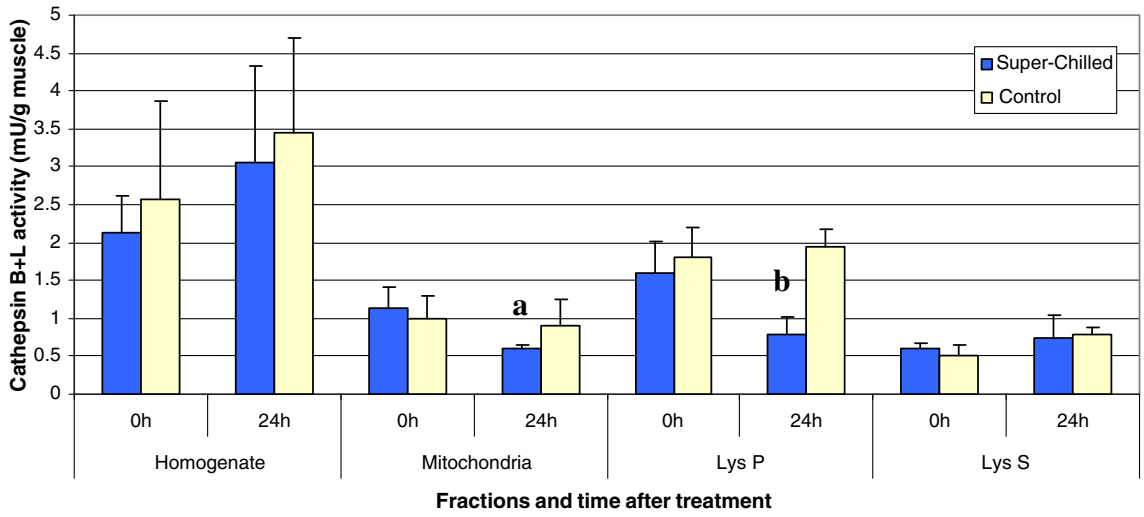

Fig. 8. Cathepsin B and L activity in 4 different fractions of super-chilled and control ice-stored fillets, 0 h and 24 h after treatment. a and b: results showing a significant difference compared to control at the same time (a: $p < 0.0001$; b: $p = 0.0003$).

3.4. Effect of MAP

3.4.1. Myofibre–myofibre detachments

There was no significant difference in myofibre–myofibre detachments between 144 h ice-stored fillets and 1 week MAP fillets (super-chilled and control fillets taken together) (Figs. 1–3 and Table 1). Therefore, MAP did not have any effect on myofibre–myofibre detachments during storage of *pre-rigor* Atlantic salmon fillets 1 week after treatment. MAP control fillets showed the same myofibre–myofibre detachments level 1 week after treatment as the ice-stored control fillets 144 h after treatment. For the super-chilled fillets, the level of myofibre–myofibre detachments in the ice-stored fillets 144 h after treatment was significantly lower than in the MAP fillets 1 week after treatment, but this difference was due to the treatment (super-chilling) ($p = 0.013$), and not to the packaging method (MAP) ($p = 0.250$).

3.4.2. Myofibre–myocommata detachments

As for myofibre–myofibre detachments, MAP did not seem to influence the loss of attachments between myofibres and myocommata, since no significant difference could be seen between 144 h ice-stored fillets and 1 week MAP fillets.

3.4.3. Cathepsin B and L activity in the lysosomes

When compared to the ice-stored fillets 144 h after treatment, 1 week MAP fillets presented a higher cathepsin B and L activity in the lysosomes of both super-chilled and control fillets ($p < 0.000001$) (Fig. 7 and Table 3). This level was close to the one obtained immediately after treatment (0 h samples) in both fillet groups. Therefore, MAP may have had an impact, decreasing lysosomal breakages with time, independently from treatment.

4. Discussion

In the present study, super-chilling seemed to result in the irreversible formation of large intra- and extra-cellular ice crystals. The optimum freezing rate for a food product depends a lot on the freezing system. Slow classical freezing will form extracellular ice crystals, generally decreasing the product quality through extensive mechanical damage, accelerated enzyme activity and increased oxidation rates, while ultra-rapid freezing can reduce ice crystal size, but still may result in intracellular ice crystallisation and/or mechanical cracking in the tissues. Intracellular ice crystals can affect the internal structure of the muscle (Molina-Garcia et al., 2004) and cause protein denaturation (Gomez & Calvelo, 1982; Martino & Zaritzky, 1988). The size and location of the formed ice crystals have been shown to affect the textural quality of a frozen food, as well as drip loss after thawing (Bello et al., 1982; Martino et al., 1998).

As found here in −1.5 °C super-chilled Atlantic salmon *pre-rigor* fillets, extracellular ice crystal formation was also previously noticed by Choi and Park (1991) in −3 °C super-chilled yellowtail fish. Lee and Toledo (1984) found that ice crystals formed in subzero temperature stored (−2 °C) mullet caused less structural changes than in −20 °C frozen mullet, which possessed destructive ice crystals in the muscle. However, those results were obtained on *post-rigor* gutted fish, not on *pre-rigor* fillets, as in our study. In any case, it seems that −1.5 °C short super-chilling observed on Atlantic salmon *pre-rigor* fillets in this present study, with formation of large intra- and extracellular ice crystals, results in similar damage to that observed in both −20 °C classical freezing and subzero super-chilling of fish and/or meat.

Molina-Garcia et al. (2004) found that classical freezing has, as consequences, disruption and separation of muscle fibre bundles in pork meat, due to the occurrence of numerous extracellular and intracellular ice crystals. On the other hand, they found that super-cooled meat did not show any freezing damages (the samples lacked intra-cellular ice crystals and the muscle fibre bundles were undisturbed) and even preserved the quality of the muscle. They explained these results by the difference in freezing volume between classical freezing and super-cooling, classical freezing inducing bigger ice crystals than super-cooling. In this present study, the ice crystals formed during super-chilling, as indicated by the microscopy observations, were large in size and big enough to damage the integrity of the fish muscle.

The increase of myofibre–myofibre detachments with storage time found in our study in both super-chilled and control fillets was also observed by Ofstad et al. (2006), and Taylor et al. (2002). It illustrated the normal degradation process of fish muscle during storage. However, the acceleration of myofibre–myofibre detachments in our super-chilled fillets seemed to be a direct consequence of freezing damage, as discussed previously, and confirmed by the large intra- and extracellular ice crystals formed during super-chilling.

No significant difference or clear trend was noticed in myofibre–myocommata detachment level between super-chilled and

control fillets. This agreed with Ofstad et al. (2006) and Taylor et al. (2002), who did not notice any changes in myofibre–myocommata attachments before five days of storage in Atlantic salmon and seven days in wolffish. This confirms that myofibre–myocommata detachments occur later than myofibre–myofibre detachments in those fish species.

The higher amount of contracted myofibres in the control fillets observed in our experiment agreed with the results of Mørkøre et al. (2007), who measured only 5% of *rigor* contraction in super-chilled *pre-rigor* Atlantic salmon fillets and 18% in control fillets. This is also what Einen, Guerin, Fjæra, and Skjervold (2002) showed in nitrogen-frozen *pre-rigor* Atlantic salmon fillets, which did not develop any thaw *rigor* muscle contraction while unfrozen *pre-rigor* fillets contracted about 14% in length during *rigor*. They also observed that this absence of contraction had a negative effect on the final quality of the fillet (such as decrease in the firmness of the fillet).

The higher amount of myofibre breakages observed in the super-chilled fillets agreed with our results on myofibre–myofibre detachments, whose level was accelerated by super-chilling. According to Sanz et al. (1999), formation of ice crystals in muscle dehydrate the fibres, resulting in a higher concentration of salt in the tissue. This dehydration could lead to denaturation of the muscle proteins and structural damage of the membranes (Huss, 1988). This damage could explain the high amount of breakages detected almost exclusively in our super-chilled fillets.

Cathepsins belong to a large family of lysosomal cysteine proteases playing a major role in *post-mortem* degradation of mammalian and fish muscle. Measuring cathepsin activity level in the lysosomal fraction of fish muscle has been previously used and is a precise and efficient mean to evaluate fish flesh degradation (Aoki & Ueno, 1997). A decrease of cathepsin activity in the lysosomal fraction indicates the breakage of the lysosomal membrane, releasing proteolytic enzymes, and eventually, the start of fish muscle proteolysis. In our study, a decrease of cathepsin B and L in the lysosomal fraction of both super-chilled and control fillets during storage time was found. Between 1 and 4 weeks of storage, our results suggested an even stronger lysosomal degradation, as the cathepsin B and L activity decreased considerably in both groups almost until disappearance. Decrease of cathepsin activity during storage time was also observed by Aoki and Ueno (1997) in mackerel muscle. This corresponds to the normal degradation process of the muscle during storage, cathepsins being released from the lysosomes to start proteolysis.

In this present study, cathepsin B and L were released faster from the lysosomes of the super-chilled fillets. These results suggested that super-chilling deteriorated the muscle structure and affected the integrity of the muscle lysosomes more rapidly than the control. This agreed well with our results of myofibre–myofibre detachments, which already showed that super-chilling accelerated muscle deterioration. This was a consequence of freezing damage and formation of ice crystals in the upper layer of the fillets. Muscle autolysis of mature chum salmon due to the activity of cathepsins B and L has been shown to be enhanced, if the muscle proteins were previously denaturised (Yamashita & Konagaya, 1991). The ice crystals damaging myofibres in the upper layer of our super-chilled fillets could thus be one of the causes of the accelerating release of proteolytic enzymes in the super-chilled group.

Our results on cathepsin B and L activity in the lysosomal fraction could be correlated with those found on yellowtail fish by Choi and Park (1991). As we found that the integrity of the lysosomes was degraded by super-chilling, they showed that −3 °C partial freezing resulted in deleterious changes of the mitochondrial inner membrane. However, Duun and Rustad (2008) concluded that super-chilling did not influence cathepsin B and L activity in the muscle (at −1.4 °C and −3.6 °C), this activity being measured as total activity in the muscle homogenate. In our study, we also observed stable total cathepsin B and L activity during storage, with no significant difference between control and super-chilled fillets. Thus, measuring cathepsin B and L total activity would not give any indication about the impact of super-chilling on fillet quality, as the total enzyme activity should not vary from one treatment to the other, if the treatment is done *post-mortem*.

In the lysosomal supernatant, we would have expected to observe an opposite activity to that of the lysosomal fraction for both super-chilled and control fillets, suggesting that the enzyme released from the lysosomes would be found in the lysosomal supernatant, as Aoki and Ueno (1997) noticed in mackerel muscle. However, in our study, no significant differences were seen between control and super-chilled samples in this fraction. Regardless, our results indicate that the lysosomal fraction (pellet) is appropriate for analysing muscle degradation.

The results on cathepsin B and L activity in the lysosomal fraction observed in the present study are not totally in line with the results of Gallart-Jornet et al. (2007). Their measurements indicated that −1 °C super-chilling and storage of Atlantic salmon for 9 and 16 days did not cause any lysosomal membrane damage. This damage was evaluated by measurement, in the centrifugal cell tissue fluid, of lysosomal α-glucosidase activity, efficient in detection of freeze-thaw treatment of fish. However, in our study, cathepsin B and L activity in the lysosomal fraction seemed to be more appropriate to directly evaluate muscle softening, as cathepsins have been shown to be directly responsible for fish muscle degradation. Losada, Piñeiro, Barros-Velazquez, and Aubourg (2005) also observed that −1.5 °C super-chilling storage of gutted *post-rigor* horse mackerel using slurry ice reduced nucleotide autolytic degradation during storage time. However, the super-chilling technique they used in their experiment did not appear to cause any freezing damage or formation of ice crystals, which would explain the absence or reduction of autolytic degradation in their super-chilled fish.

In the present study, liquid leakage after one week of storage was found to be higher in the super-chilled than in the control fillets. Sivertsvik et al. (2003) observed less drip loss in −2 °C super-chilled stored MAP fillets than in control MAP fillets, probably due to the fact that, in their study, no ice crystals were formed in the upper layer of the super-chilled fillets. Our results agreed more with the ones from Einen et al. (2002), who found a higher drip loss in −25 °C frozen Atlantic salmon *pre-rigor* fillets, compared to fresh fillets; classical freezing in this case probably forming ice crystals. Gallart-Jornet et al. (2007) explained a decrease in water-holding capacity of −1 °C super-chilled Atlantic salmon *post-rigor* fillets by possible structural changes in the muscle during super-chilled storage. Einen et al. (2002), Mackie (1993), and Sikorski and Kolakowska (1994) were more precise, by suggesting that a lower water-binding capacity in fish muscle can be due to cell damage, lower protein solubility, and protein denaturation and aggregation during freezing and thawing. These explanations agree with our results on liquid leakage and myofibre breakages being observed in a higher amount in the super-chilled fillets.

The instrumental texture measurement of the fillets that we used showed no significant difference in firmness between super-chilled and control fillets 1 week after treatment. Taylor et al. (2002) suggested that texture was determined by several distinct structures and events, especially myofibre–myofibre and myofibre–myocommata attachments/detachments. We found more myofibre–myofibre detachments in the super-chilled group at this time point. This was also supported by a higher level of lysosomal breakages (and lower cathepsin B and L activity in the lysosomal fraction) 1 week after treatment, as well as more myofibre breakages 144 h after treatment in the super-chilled fillets. According to these results, we expected a softer texture in the super-chilled fillets

1 week after treatment. On the other hand, we observed the same myofibre–myocommata detachments level in both groups and a large variation between fillets' texture results (breaking force), especially in the super-chilled group. Besides, at this time point, both fillet groups contained more than 45% of myofibre–myofibre detachments. At this level of deterioration, it is possible that a threshold has been reached, above which no differences in texture could be measured instrumentally. However, it was interesting to notice that at this time point, two of the super-chilled fillets (out of six in the group) showed a very soft texture, which reached the limit of acceptance (5.31 and 6.93 N) for fish quality (Mørkøre & Rørå, 1999). No extreme results like those were detected in the control fillets.

Therefore, instrumental texture measurement in this present study was difficult to correlate directly with myofibre–myofibre detachments, myofibre breakages and/or level of cathepsin B and L activity in the muscle. Guillerm-Regost et al. (2006) showed that a sensory panel could detect texture differences, due to a higher/lower liquid leakage in a product, better than a texture instrument. Thus, when talking about fish texture, a sensory method seems to be more sensitive than instrumental texture measurements. As liquid loss was found higher in the super-chilled than in the control fillets 1 week after treatment in our study, we may have detected texture differences between the two treatments, if a sensory analysis had been performed.

MAP seemed to have prevented or reduced the release of cathepsins B and L from the lysosomes, eventually decreasing muscle proteolysis in both super-chilled and control fillets 1 week after treatment. Sivertsvik et al. (2003) showed that combining MAP and super-chilling extended the shelf-life of Atlantic salmon *post-rigor* fillets, maybe because of a higher dissolution of CO_2 at the super-chilled temperature. One explanation to our results could then be that the CO_2 contained in the MAP, while dissolving into the fillets, could prevent, besides bacterial development, muscle lysosomal breakages. This would slow down the release of cathepsin B and L into the cytosol. However, more work is needed to evaluate this hypothesis. Sivertsvik et al. (2003) found that CO_2 level decreased linearly from day 7 to day 24 of storage in the MAP headspace of super-chilled stored and control Atlantic salmon *post-rigor* fillets. The CO_2 had gone after 4 weeks, this could explain why at this time point, MAP had no positive effect anymore on the release of cathepsin B and L in our fillets.

5. Conclusions

According to these results, −1.5 °C super-chilling of Atlantic salmon (*S. salar*) *pre-rigor* fillets for 45 min, followed by chilled storage on ice in a cold room at +5 °C, affected the quality of the fish fillets. Super-chilling prevented the fillets from *rigor* contraction, having an impact on the final fish quality. The upper layer of the super-chilled fillets showed consequent freezing damage characterised by the formation of large intra- and extracellular ice crystals during super-chilling. Freezing damage due to super-chilling accelerated the amount of detachments between myofibres and increased the amount of myofibre breakages during storage time. These microscopic observations were confirmed and correlated with cathepsin B and L activity measurement in the muscle lysosomes, which indicated that super-chilling accelerated the release of these proteolytic enzymes from the lysosomes, causing an acceleration of fish muscle degradation. These lysosomal breakages seemed to be due to freezing damage, confirmed by the formation of ice crystals during super-chilling.

MAP did not influence the effects of super-chilling (presence of ice crystals and level of detachments between myofibres), but seemed to slow down the release of cathepsins B and L from the lysosomes, independently of the treatment, at least until one week after treatment.

It seems that the effects of super-chilling on fish fillet quality vary dramatically, according to fish species, super-chilling and storage temperature and time, super-chilling technique itself, fish packaging, size of the studied samples, and time between slaughter and filleting (*post-* or *pre-rigor* filleting).

We concluded that super-chilling in the conditions presented in this study had almost the same consequences on Atlantic salmon fillet quality (studied as integrity of the muscle) as classical slow and rapid freezing. Consequently, super-chilling of Atlantic salmon *pre-rigor* fillets needs to be optimised, in order to avoid the formation of irreversibly destructive ice crystals. This would hopefully prevent a fast muscle proteolysis, while maintaining the advantages that super-chilling has offered so far.

Super-chilling of *pre-rigor* Atlantic salmon is still a very promising technique. Combined with MAP, the technique could offer even more quality advantages to the fish industry for long-term storage. However, optimisation of super-chilling, in terms of temperature and air-speed, is necessary in order to obtain high quality Atlantic salmon *pre-rigor* fillets.

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