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Effect of temperature control on the efficiency of modified atmosphere packaging of cod loins in bulk

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Report summary

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Titill / Title	**Effect of temperature control on the efficiency of modified atmosphere packaging of cod loins in bulk**		
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Ágrip á íslensku:	Markmið tilraunarinnar var að bera saman ferskleika, gæði og geymsluþol undirkældra (CBC) þorskhnakka við geymslu í lofti og í loftskiptum pakkningum (MAP) við stýrt hitastig til að líkja eftir hitasveiflum við flutninga og dreifingu á Evrópumarkaði. Fylgst var með breytingum á samsetningu gassins í pakkningunum og gert skynmat og örveru- og efnamælingar. Fiskurinn var veiddur í botnvörpu að vorlagi og unninn þremur dögum frá veiði. Tveggja daga lenging varð á ferskleikatímabili og eins dags á geymsluþoli fisks í loftskiptum pakkningum (2,7 kg í bakka) miðað við loft (3,1 kg) í frauðplasti þrátt fyrir að 0.5 °C munur hafi verið á meðalhitastigi hópanna og var lofthópurinn geymdur við lægra hitastig (-0.3 ± 0.9 °C). Mestu hitasveiflurnar leiddu til mestrar styttingar á ferskleika-tíma í loftskiptum pakkningum. Þorskhnakkar sem geymdir voru undirkældir við -1.1 ± 0.1 °C höfðu 13 daga geymsluþol. Niðurstöður örverutalninga og efnamælinga sýndu hversu mikilvæg *Photobacterium phosphoreum* er við TMA-myndun í skemmdarferli þorskhnakka við geymslu bæði í lofti og loftskiptum pakkningum. MAP og undirkæling hægðu á og breyttu skemmdarferlinu. MAP jók drip um 2% á seinni stigum geymslunnar.		
Lykilorð á íslensku:	*Loftskiptar umbúðir – Undirkæling - Þorskur – Ferskleiki - Geymsluþol –Útflutningur*		

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Summary in English:

The aim of this study was to compare freshness, quality deterioration and shelf life of CBC (combined blast and contact)-treated cod loins packaged in bulk under different atmospheres (air or modified atmosphere, MA) and stored under different temperature profiles to mimic temperature changes during transport and distribution to European markets. Sensory, chemical, microbial and headspace gas composition analyses were performed regularly. The fish was caught by trawler in the spring and processed 3 days post catch. Following simulation of current sea freight conditions and distribution to European markets, a 2-day and 1-day increase in freshness period and shelf life of MA-packaged fish (2.7 kg in trays), respectively, was observed compared to air-stored loins (3.1 kg in EPS boxes). This is despite a mean product temperature difference of 0.5 °C between the products, being lower (-0.3 ± 0.9 °C) for air-stored fish. Abusive conditions had the greatest impact on the reduction of the freshness period for MAP fish. Superchilled storage of MAP loins (-1.1 ± 0.1 °C) resulted in a 13-day shelf life. Evaluation of microbial and chemical indicators emphasised the importance of *Photobacterium phosphoreum* and TMA formation in the deterioration of cod loins stored in air or MA, while superchilled MAP storage delayed as well as modified the spoilage pattern. MAP increased drip loss by about 2% at late storage.

English keywords: *Modified atmosphere packaging –Superchilling - Cod – Freshness – Shelf life – Quality deterioration - Export*

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1. AIM OF STORAGE TRIAL

The aim of the storage trial was to compare freshness, quality deterioration and shelf life of CBC (combined blast and contact)-treated cod loins packaged in bulk under different atmospheres (air or modified atmosphere, MA) and stored under different temperature profiles to mimic temperature changes during transport and distribution to foreign markets. Sensory and headspace gas composition analyses were performed regularly and changes in chemical and microbial indicators were also evaluated.

2. EXPERIMENTAL DESIGN

2.1 Fish processing and packaging

Atlantic cod (*Gadus morhua*) was caught in April 2010 on the east coast of Iceland (fishing area #412) by the trawler Bjartur. The fish was bled, gutted, rinsed, iced in tubs and stored chilled until processed at Samherji (Dalvík, Iceland) three days later. At the processing plant, activity had already started with a 4-day old raw material, followed by de-heading, filleting, liquid cooling in brine, CBC cooling, skinning, trimming and cutting of the fillets into loins for this packaging trial. The gas mixture applied during MA-packaging contained % $CO_2/O_2/N_2$: 50/5/45. The plastic trays were made of polypropylene (2325-100, Mo-PP; FÆRCH, Denmark), had a volume of 5.85 L (32.8 x 26.8 cm at tray top and 10 cm depth) and was sealed with an appropriate film from FÆRCH. A Sealpack packaging unit was provided by Plastco hf. (Reykjavík, Iceland). All packages used were lined with absorbent pads (3 pads of 17.7 x 10.4 cm). MA-packed fish contained 2.67 ± 0.05 kg of cod loins (Mc, Mab, Ms), except for one group (Mcc) which weighed 2.89 ± 0.02 kg similarly to air-stored fish (3.10 ± 0.03 kg) packaged in expanded polystyrene (EPS) boxes. See Table 1 for details on experimental groups.

To monitor the product temperature from packaging, temperature loggers (iButton DS1922L, Maxim Integrated Products Inc, USA) were inserted aseptically at four positions (bottom corner inside package, underneath the fish bulk, at its centre and top fillet) in two packages for each treatment. The ambient temperature was similarly recorded with two loggers positioned on two sides of the selected packages. The logger accuracy was of ±0.5 °C and its resolution of 0.0625 °C. All packages were identified and assembled on a pallet shipped to Matís in Reykjavík the following morning.

2.2 Storage at Matís

This study included six test groups (Table 1), two of them stored aerobically (Ec and Eab) in EPS boxes and four others packaged under MA (Mc, Mcc, Mab, Ms). The packages were stacked per test group in the storage room as shown in Figure 1, with each stack built on an empty EPS box to avoid the fish packages to touch directly the floor. MA-packages had a paperboard between them to protect the plastic film sealing the trays. The package on top of each stack identified with an asterisk (*) was temperature-logged throughout storage. The storage room temperature was set to -1 °C for 6 days, then control groups (Ec, Mc and Mcc) stored for 4 h at 5 °C followed by storage at 2 °C for the rest of the trial. Abused groups (Eab and Mab) were kept at 5 °C for 8 h on day 6, then at 2°C until day 7 where they were stored at 5 °C for the rest of the trial. The superchilled group (Ms) was stored at -1 °C during the whole storage period.

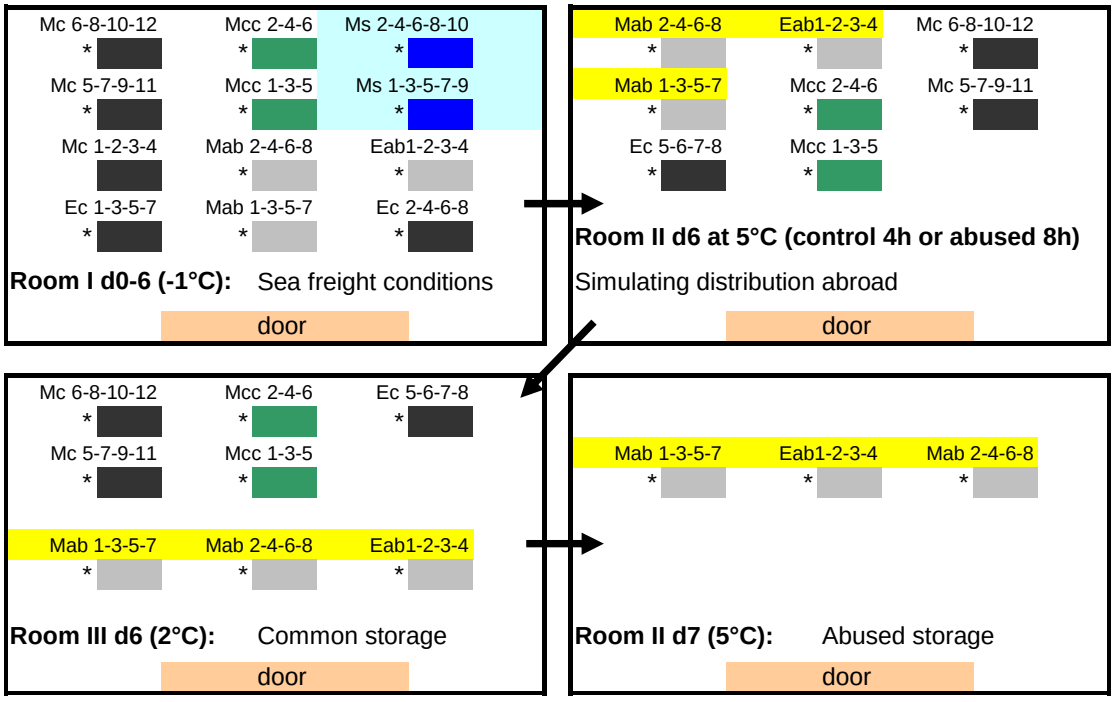

Figure 1. Setup of packages and temperature conditions in the cooling rooms during storage at Matís

An asterisk denotes that the top package of the stack is being temperature-logged. Ec, control air-stored fish (3 kg); Eab, abused air-stored fish (3 kg); Mc, control MAP fish (2.75 kg); Mcc, control MAP fish (3 kg); Mab, abused MAP fish (2.75 kg); Ms, superchilled MAP fish (2.75 kg).

3. ANALYSIS OF SENSORY, MICROBIOLOGICAL AND CHEMICAL PARAMETERS

3.1 Materials and Methods

3.1.1 *Sampling*

Samples were obtained in duplicate using two packages per group, selecting six loins from the upper fish layer from one package as one sample replicate for microbiological and chemical analyses. The rest of loins was used for sensory analysis. Regular sampling was performed as described in Table 1.

Table 1. Definition of test groups evaluated

Sample name	Description (fish bulk in kg)	Sampling days
Raw material	Loins from the lot processed	0
Ec	Air-stored loins, control (3 kg)	2, 6, 9, 12
Eab	Air-stored loins, abused (3 kg)	2, 6, 9, 12
Mc	MAP loins, control (2.75 kg)	2, 6, 9, 12
Mcc*	MAP loins, control (3 kg)	9, 13
Mab	MAP loins, abused (2.75 kg)	2, 6, 9, 12
Ms	MAP loins, superchilled (2.75 kg)	2, 6, 13, 15

* Mcc loins not evaluated by sensory analysis. Lipid-related analysis was not performed on day 9.

3.1.2 *Sensory evaluation*

Five of the six groups of cod loins were evaluated with sensory evaluation (Ec, Eab, Mc, Mab, Ms). The main purpose was to evaluate differences in quality deterioration and shelf life according to sensory evaluation by a trained panel. Quantitative Descriptive Analysis (QDA), as introduced by Stone and Sidel (2004), and Torry freshness score sheet (Shewan *et al*., 1953) were used to assess cooked samples. Twelve panellists participated in the sensory evaluation. They had all been trained according to international standards (ISO 8586, 1993); including detection and recognition of tastes and odours, use of scales and in the development, and use of descriptors. The members of the panel were familiar and experienced in using the QDA method and Torry freshness score sheet for cod. The panel was trained in recognition of sensory characteristics of the samples and describing the intensity of each attribute for a given sample using an unstructured scale (from 0 to 100%). Most of the attributes were defined and described by the sensory panel during other projects (Sveinsdottir *et al.* 2009). The sensory attributes were 30 and are described in Table 2.

Portions weighing about 40 g were cut from the loins and placed in aluminium boxes coded with three-digit random numbers. The samples were cooked for 6 minutes in a pre-warmed oven (Convotherm Elektrogeräte GmbH, Eglfing, Germany) at 95-100 °C with air circulation and steam, and then served to the panel. Each panellist evaluated duplicates of each test group in a random order in seven sessions (maximum four samples per session). A computerised system (FIZZ, Version 2.0, 1994-2000, Biosystèmes) was used for data recording.

Table 2. Sensory attributes for cooked cod and their description

Sensory attribute	Short name	Description of attribute
Odour		
sweet	o-sweet	Sweet odour
shellfish, algae	o-shellfish	Shellfish, algae, characteristic fresh odour
meaty	o-meat	Meaty odour, reminds of boiled meat or halibut
vanilla/warm milk	o-vanilla	Vanilla, sweet heated milk
boiled potatoes	o-potatoes	Reminds of whole, warm, boiled potatoes
frozen storage	o-frozen	Refrigerator, freezer storage odour
table cloth	o-cloth	Reminds of a table cloth (unclean, damp cloth to clean kitchen table, left for 36 h)
TMA	o-TMA	TMA odour, reminds of dried salted fish, amine
sour	o-sour	Sour odour, sour milk, spoilage sour, acetic acid
sulphur	o-sulphur	Sulphur, matchstick, boiled kale
Appearance		
colour	a-dark	Left end: light, white colour. Right end: dark, yellowish, brownish, grey
appearance	a-disc	Left end: homogeneous, even colour. Right end: discoloured, heterogeneous, stains
white precipitation	a-prec	White precipitation in the broth or on the fish surface
Flavour		
salt	f-salt	Salty taste
metallic	f-metallic	Characteristic metallic flavour of fresh cod
sweet	f-sweet	Characteristic sweet flavour of fresh boiled cod
meaty	f-meaty	Meaty flavour, reminds of boiled meat
frozen storage	f-frozen	Reminds of wood which has soaked in refrigerator/freezing flavour
pungent	f-pungent	Pungent flavour, bitter
sour	f-sour	Sour taste, spoilage sour
TMA	f-TMA	TMA flavour, reminds of dried salted fish, amine
off-flavour	f-off	Strength of off-flavour (spoilage flavour/off-flavour)
Texture		
flakiness	t-flakes	The fish portion slides into flakes when pressed with the fork
soft	t-soft	Left end: firm. Right end: soft. Evaluate how firm or soft the fish is during the first bite
juicy	t-juicy	Left end: dry. Right end: juicy. Evaluated after chewing several times: dry - draws juice from the mouth
tender	t-tender	Left end: tough. Right end: tender. Evaluated after chewing several times
mushy	t-mushy	Mushy texture
meaty mouthfeel	t-meaty	Meaty texture, meaty mouthfeel, crude muscle fibers
clammy	t-clammy	Clammy texture, tannin (dry red wine)
rubbery	t-rubbery	Rubbery texture, springy

3.1.3 *Microbiological analysis*

Loins were aseptically minced, assessing two pooled loins for each sample. Two replicate samples (packages) were evaluated for each group. Minced flesh (20 g) was mixed with 180 g of cooled Maximum Recovery Diluent (MRD, Oxoid, UK) in a stomacher for 1 minute. Successive 10-fold dilutions were done as required. Total viable psychrotrophic counts (TVC) were performed on modified Long and Hammer´s agar (mLH) according to van Spreekens (1974) with 1% NaCl and on iron agar (IA) as described by Gram *et al*. (1987) with the exception that 1% NaCl was used instead of 0.5% with no overlay. Counts of H_2S-producing bacteria were evaluated on IA. Plates were spread-plated and incubated at 17 °C for 5 days.

Counts of all colonies (both white and black) on IA gave the number of total count and counts of black colonies gave the number of H_2S-producing bacteria. Enumeration of presumptive pseudomonads was performed using modified Cephaloridine Fucidin Cetrimide (mCFC) agar as described by Stanbridge and Board (1994). *Pseudomonas* Agar Base (Oxoid, UK) with CFC selective Agar Supplement (Oxoid) was used and the plates were incubated at 22 °C for 3 days. Mean bacterial numbers are presented as $\log_{10}$ numbers of colony-forming units (cfu) g^{-1} fish.

Estimation of *Photobacterium phosphoreum* (Pp) counts was achieved by a quantitative Polymerase Chain Reaction (qPCR) method developed at Matís (E. Reynisson, unpublished). Briefly, 10 ml of the 10-fold diluted fish sample in MRD buffer was frozen at -20 °C for later DNA extraction. For the DNA extraction, the diluted samples were centrifuged at 11.000 x g for 7 min to form a pellet. The supernatant was discarded and DNA was recovered from the pellet using the promeganesil KF, Genomic system (MD1460) DNA isolation kit (Promega Corporation, Madison, USA) in combination with King Fisher magnetic beads automatic DNA isolation instrument (Thermo Lab systems, Waltham, USA) according to the manufacturers' recommendations. All PCR reactions were done using the MX 3005p instrument. The PCR was done using Brilliant QPCR master mix (Stratagene, La Jolla, CA, USA). Primers were synthesised and purified with HPLC (MWG, Ebersberg, Germany). The DNA standard used for quantification of *P. phosphoreum* was previously calibrated against the PPDM-Malthus conductance method (Dalgaard *et al*., 1996; Lauzon, 2003) using fish samples from storage trials.

3.1.4 *Chemical analysis: Total Volatile Base Nitrogen (TVB-N), trimethylamine (TMA), pH, water holding capacity, moisture and salt content*

All chemical analyses were performed in duplicate. The method of Malle and Tao (1987) was used for total volatile bases (TVB-N) and trimethylamine (TMA) measurements in the previously prepared mince. TVB-N was measured by steam distillation (Struer TVN distillatory, STRUERS, Copenhagen) and titration, after extracting the fish muscle with 7.5% aqueous trichloroacetic acid (TCA) solution. The distilled TVB-N was collected in boric acid solution and titrated with sulphuric acid solution. TMA was measured in TCA extract by adding 20 ml of 35% formaldehyde, an alkaline binding mono- and diamine, TMA being the only volatile and measurable amine. The pH was measured in 5 g of minced loins mixed with 5 ml of deionised water using the Radiometer PHM 80. Measurements were done within 30

min from sampling for MAP fish. The pH meter was calibrated using the buffer solutions of pH 7.00 ± 0.01 and 4.01 ± 0.01 (25 °C).

Water holding capacity, moisture and salt content were evaluated from two loins for each sample. Moisture content was measured by drying 5 g of the sample, mixed with sand, in a ceramic bowl at 103 ± 2 °C for 4 h (ISO 6496, 1999). Salt content was measured with the Volhard titration method (AOAC 976.18, 2000). Water holding capacity (WHC) was measured with the centrifugal method described by Eide and others (1982). Approximately 2 g of the samples were weighed precisely into a vial and centrifuged (Sorvall RC-5B, Dupoint Company, USA) at 210 g (1300 rotations min^{-1}) at 2-5 °C for 5 min. WHC (%) was calculated as the ratio of water in the sample after centrifugation to water in the sample before centrifugation.

3.1.5 *Analysis of total lipids, lipid hydrolysis and oxidation*

Lipid analysis was performed using two loins for each sample, analysing two packages (duplicate samples) per group. Mcc fish group was not analysed. Total lipids (TL) were extracted from 25 g samples (moisture content=80±1%) with methanol/chloroform/0.88% KCL (1/1/0.5, v/v/v) according to the Bligh and Dyer (1959) method. The lipid content was determined gravimetrically and the results were expressed as grams lipids per 100 g wet muscle. Free fatty acids (FFA) were determined on the TL extract according to Lowry and Tinsley (1976), with modifications from Bernardez *et al.* (2005). FFA concentration was calculated as micromolar oleic acid based on a standard curve spanning a 2-22 µmol range. Results are expressed as grams FFA per 100 g of lipids.

Assessment of primary lipid oxidation by the determination of the lipid hydroperoxide value (PV) with a modified version of the ferric thiocyanate method (Santha and Decker Eric, 1994) was intended to be performed in light and dark muscle separately. However, several of the PV samples lost their identity while being stored in the freezer and could not be measured. The few data obtained are therefore not presented here.

A modified method of Lemon (1975) was used for measuring thiobarbituric acid reactive substances (TBARS representing secondary lipid oxidation compounds). A sample (5.0 g) was homogenised with 5.0 ml of TCA extraction solution (7.5% TCA, 0.1% propyl gallate and 0.1% ethylene diamine tetraacetic acid (EDTA) mixture prepared in ultra pure water) using a homogeniser (Ultra-Turrax T-10 basic, IKA, Germany) at maximum speed for 10 s. The homogenised samples were then completed with 5.0 ml TCA extraction solution and centrifuged at 9400 *x g* for 15 min (Model Z323K, Hermle laboratories, Germany). The

supernatant (0.5 ml) was collected and mixed with the same volume (0.5 ml) of thiobarbituric acid (0.02 M) and heated in a water bath at 95 °C for 40 min. The samples were cooled down on ice and immediately loaded into 96-wells microplates (NUNC A/S Thermo Fisher Scientific, Roskilde, Denmark) for reading at 530 nm (POLARstar OPTIMA, BMG Labtech, Offenburg, Germany). A standard curve was prepared using tetraethoxypropane. Dark and light muscle was analysed separately. The results are expressed as µmol of malonaldehyde diethylacetal (MDA) per kg of samples.

Tertiary oxidation compounds were investigated by measuring the formation of interaction compounds between primary and secondary lipid oxidation products and nucleophilic molecules (protein-like) present in the fish muscle. The formation of interaction compounds was assessed by the fluorescence ratio. Fluorescence measurements (Perkin Elmer LS 50B) were made at 393/463 and 327/415 nm excitation/emission maxima, according to other researchers (Aubourg *et al*., 1997; Aubourg *et al*., 1998; Aubourg, 1999a,b; Aubourg, 2001). The excitation and emission slit was set at 2.5 nm. The relative fluorescence (RF) was calculated as $RF=F/F_{st}$, where F is the sample fluorescence intensity at each excitation/emission maximum and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 µg/ml in 0.05M H_2SO_4) at the corresponding wavelength. The fluorescence shift (δF) was calculated as the ratio between the two RF values, i.e. $\delta F = RF_{393/463nm} / RF_{327/415nm}$, and was analysed on the organic phase (δF_{or}) resulting from the lipid extraction.

3.1.6 *Headspace gas analysis and drip loss*

Gas composition was evaluated using a PBI Dansensor (CheckMate 9900, Denmark) gas measuring device. Septums were put on the film to enable measurements, gas collected twice via the sampling needle and the latter measurement recorded for each pack. Two packs were evaluated for each group at each sampling day, while four packs were measured during packaging (day 0). Drip was evaluated throughout storage. The % drip was calculated as the ratio of the water lost during storage to the original weight of the fish x 100.

3.1.7 *Data analysis*

Principal Component Analysis (PCA) on significant mean values of QDA sensory attributes was performed, using full cross validation. Analysis of variance (ANOVA) was carried out in the statistical program NCSS 2000 (NCSS, Utah, USA) on sensory data (see Appendix II) as well as for the microbial and chemical data (see Appendix III). Comparison of data with respect to treatments was performed using the Tukey-Kramer multiple comparison test. The significance level was set at 5%.

3.2 Results and Discussion

3.2.1 *Temperature monitoring*

The ambient and product temperature of the six groups was monitored by temperature loggers during the storage trial. Table 3 summarises the mean product and ambient temperature recorded during the 12- or 13-day period, whereas Figure 2 illustrates the temperature profiles that took place. The temperature profiles of Mcc fish is not shown since they were similar to those measured in the Mc group. Ambient temperature was adjusted to mimic sea freight shipping under superchilled conditions, followed by chilled storage after distribution (Ec, Mc, Mcc) or abusive conditions (Eab, Mab). One group (Ms) was maintained under isothermal condition at -1 °C for the whole storage in order to determine the maximal shelf life of the product. Table 3 shows the mean temperature differences between control and abused groups, the latter having a fish temperature of at least 1 °C higher than their control counterpart. Further mean product temperature was influenced by the packaging method applied, being higher in MAP than air-stored groups. A difference of 0.5 °C and 0.7 °C was observed between control and abused groups, respectively. A mean product temperature gradient of 0.3 °C was observed in air-stored fish (Ec, Eab) between bottom corner and center positions in the packages in contrast to 0.1 °C for MAP fish.

Table 3. Mean temperature and standard deviation (± SD °C) of the different fish groups as influenced by the loins' position in the package and their environment during the 12-day storage period

Group	$T_{ambient}$ (°C) n=4	$T_{product}$ (°C) n=6	$T_{bottom\ corner}$ (°C) n=2	T_{center} (°C) n=2
Ec	0.0 ± 2.8	-0.3 ± 0.9	-0.2 ± 1.5	-0.5 ± 0.8
Eab	1.2 ± 3.4	0.7 ± 2.4	0.8 ± 2.4	0.5 ± 2.3
Mc	0.4 ± 1.9	0.2 ± 1.5	0.3 ± 1.6	0.2 ± 1.5
Mcc	0.4 ± 2.1	0.3 ± 1.4	0.2 ± 1.6	0.3 ± 1.4
Mab	1.5 ± 2.9	1.4 ± 2.8	1.5 ± 2.9	1.4 ± 2.8
Ms*	-1.2 ± 0.4	-1.1 ± 0.1	-1.1 ± 0.2	-1.1 ± 0.0

* Temperature calculated for the storage period of 13 days for Ms fish.

The generally colder product temperature of air-stored fish compared to MAP fish may be explained by greater insulation properties of EPS boxes compared to PP trays, leading to a better temperature maintenance of the superchilled fish. In spite of the colder storage temperature during the 8-h shipping period of the air-stored products (-20 to -10 °C) compared to MAP fish (-5 to -2 °C), as illustrated in Figure 2, the mean product temperature among the groups was similar (-1.0 to -0.9 °C) at reception.

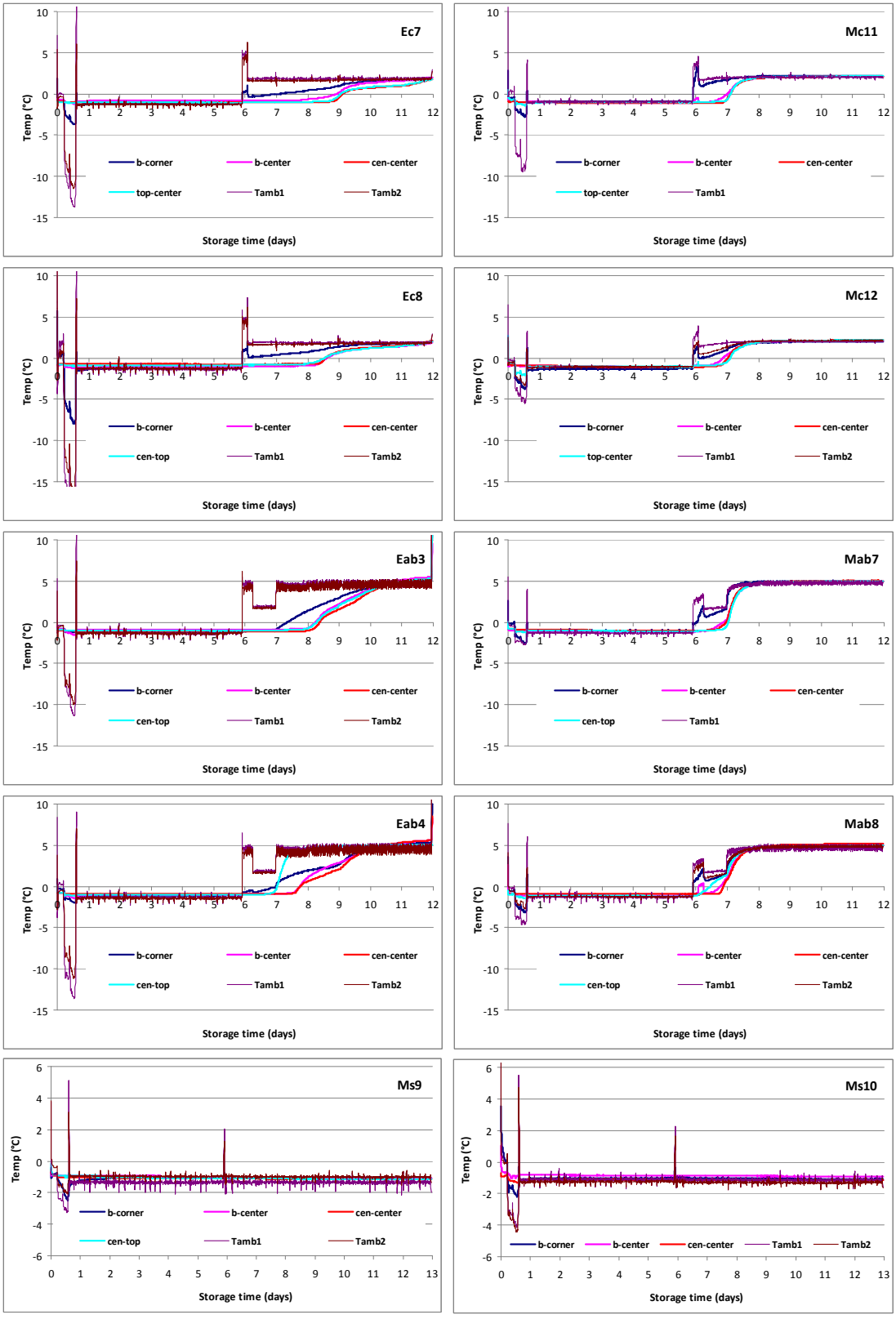

Figure 2. Product and ambient temperature of five of the groups evaluated, showing the recordings for duplicate samples stored in air (E-groups) or under MA (M-groups). b-corner, bottom corner in package; b-centre, bottom centre under fish bulk; cen-centre, core of fish bulk; top-centre, under top loin; Tamb1 or 2, ambient temperature measured on package walls by logger 1 or 2.

The main advantage of EPS boxes is visualised after 6 days of superchilled storage followed by chilling at 2 °C, as it took almost 6 days to reach a temperature otherwise attained in MAP fish within 2 days (Ec vs Mc). Under abusive conditions, MAP fish reached 5 °C within 2 days (Mab) in contrast to 4 days for EPS (Eab).

3.2.2 *Headspace gas analysis*

On the packaging day, the gas mixture flushed aimed to contain % $CO_2/O_2/N_2$: 50/5/45. Figure 3 shows the changes in headspace gas composition from packaging ($CO_2/O_2/N_2$: 51.6/4.1/44.3) to the end of the storage trial. Gas equilibrium in the package generally occurs between 12 (Jakobsen and Bertelsen, 2004) and 48 h (Sivertsvik *et al*., 2004) after packaging. Six to nine days post-packaging, headspace CO_2 level had dropped by 25-30% as it dissolved into the muscle water phase, while the headspace O_2 concentration reached 5%.

Mcc trays had the lowest headspace CO_2 concentration on day 9 as they contained in average 2.89 ± 0.02 kg fish which corresponded to a gas to fish ratio of 1.11 to 1.14 in comparison to 1.26 to 1.34 for Mc and Mab groups (2.67 ± 0.05 kg fish), assuming a fish density of 1.05 kg m^{-3} (Lowndes, 1955). This could be expected since increasing degree of filling (DF) has been shown to have a negative impact on the concentration of CO_2. This is because increasing DF results in less CO_2 to dissolve in an increasing mass of product (Rotabakk *et al*., 2007). Lesser CO_2 dissolved in the fish water phase implies an inferior antibacterial action. This should be kept in mind during the selection of the gas composition and DF (volume of fish to volume of package).

Following microbial growth, O_2 concentration was slowly reduced as storage time progressed, leading to the formation of additional CO_2 in the headspace of the package as observed from day 12. Overall, it is observed that the tightness of the MA package was appropriate since the balance between O_2 and CO_2 was apparently maintained, i.e. a reduction of O_2 generally corresponded to the CO_2 formed during late storage.

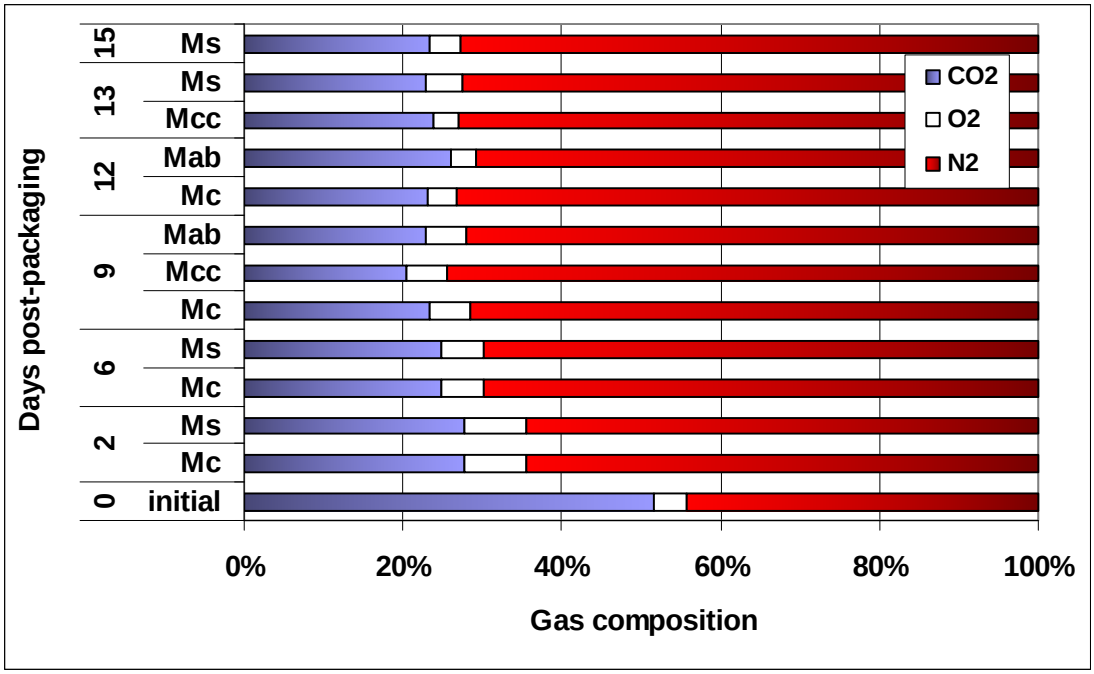

Figure 3. Mean headspace gas composition (% $CO_2/O_2/N_2$) in trays sampled throughout storage (n=2, but on day 0 n=4)

3.2.3 *Sensory evaluation*

Sensory evaluation of cooked cod was performed by two methods; Torry freshness assessment (Figure 4) and QDA method describing several attributes relating to odour, appearance, flavour and texture (Figure 5 and Figure 6).

Figure 5 shows how the different groups were characterised by sensory attributes during the shelf life study. Minor changes were observed between groups until day 12-13, where Ms fish received higher scores of boiled potato odour than other groups. At the same time scores for TMA and sour odours and flavour were low, but high for other groups, especially Eab and Mab. Figure 6 shows how the sensory profile of the different groups evolves with storage time. Altogether 96% of the sensory variance is accounted for in PC1 and PC2. At the beginning of storage, both Ec and Mc are mainly characterised by sweet and shellfish like odours, sweet and metallic flavours. As the storage time progresses, these attributes become less evident, but odour of vanilla more prominent after 6-9 days and odour of potatoes of the Ms groups stored 13 and 15 days. After 12 days (the end of storage for all other groups) attributes like off flavour, sulphur odour, sour odour and flavour, and especially for Mab and Eab TMA odour and flavour were dominating the sensory profile.

The fish was 3 days post catch at processing which means that the freshness period could be expected to reach at least 6 days considering the superchilled storage conditions applied

(Lauzon *et al.*, 2010). Indeed, some freshness characteristics were still detected 6 days post packaging but no significant difference was observed in Torry scores (Figure 4) and QDA profiles (Figure 5 and Figure 6) among the groups investigated (p>0.05). Freshness loss is generally characterised by a Torry score of 7 (out of 10, see Appendix I) and QDA freshness attributes approaching values less than 25-30% (sweet and shellfish odour; sweet and metallic flavour). Further, comparison of QDA scores for air-stored and MAP fish products at each sampling point indicates that there was no significant difference in any of the QDA attributes evaluated during the 12 days of storage (see Appendix II for average data and statistical analysis). On the other hand slightly better scores were seen for Mc than Ec and abused groups (Mab, Eab) from day 9, reflecting a slightly slower deterioration rate. This agrees with the Torry scores obtained (Figure 4).

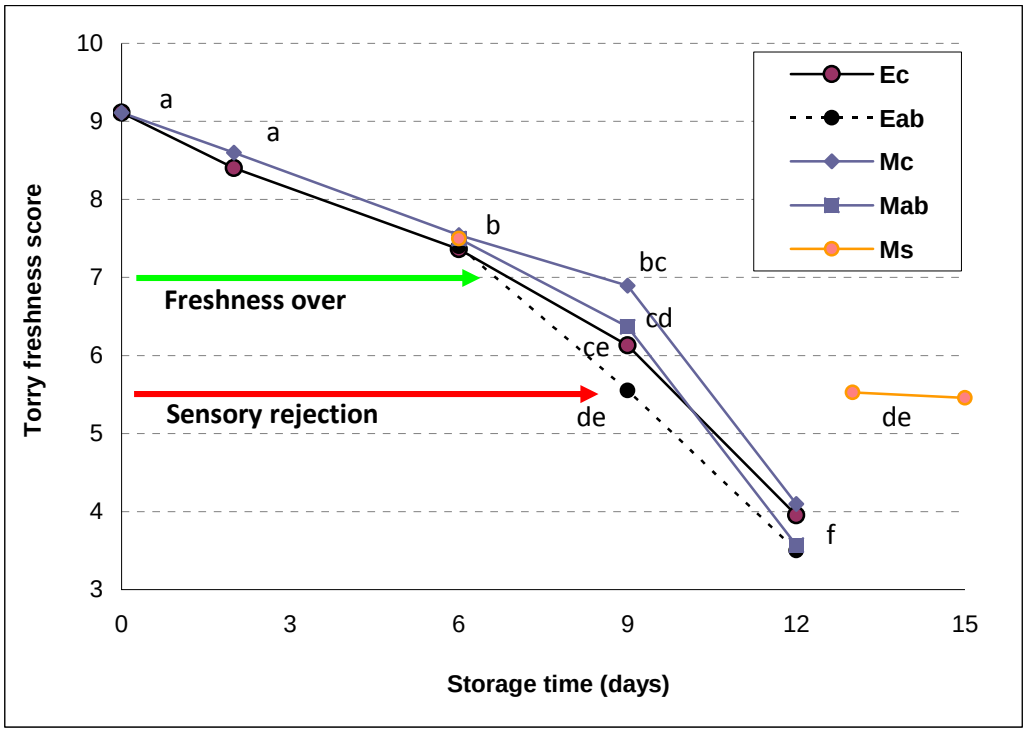

Figure 4. Mean Torry freshness scores for cooked cod loins during storage

Table 4 summarises the freshness and shelf life data intrapolated from the Torry curve for each group. Torry score of 5.5 is used to determine the end of shelf life, while a score of 7 indicates the loss of freshness characteristics. The abusive treatment generally led to a 1-day shelf life reduction for both air-stored (Eab) and MAP (Mab) fish, while a reduction in freshness period was greater for abused MAP fish than air-stored loins. This finding emphasises the importance of good temperature control of MAP products to enhance their freshness. Further the mean temperature difference (0.5 °C) between air-stored and MAP

fish is undoubtedly concealing the real gain in quality extension generally provided by MA-packaging and low temperature storage (Lauzon *et al*., 2010). Based on the Seafood Spoilage Predictor software (SSSP at http://www.dfu.min.dk/micro/ssp), it can be calculated that a 1-day shelf life reduction for air-stored fish could be expected if the mean product temperature would have been like that of MAP fish. This implies a reduction of the freshness period as well. It is therefore predicted that a gain in freshness period and shelf life of MAP products approached 3 and 2 days, respectively, compared to air-stored fish at a similar temperature.

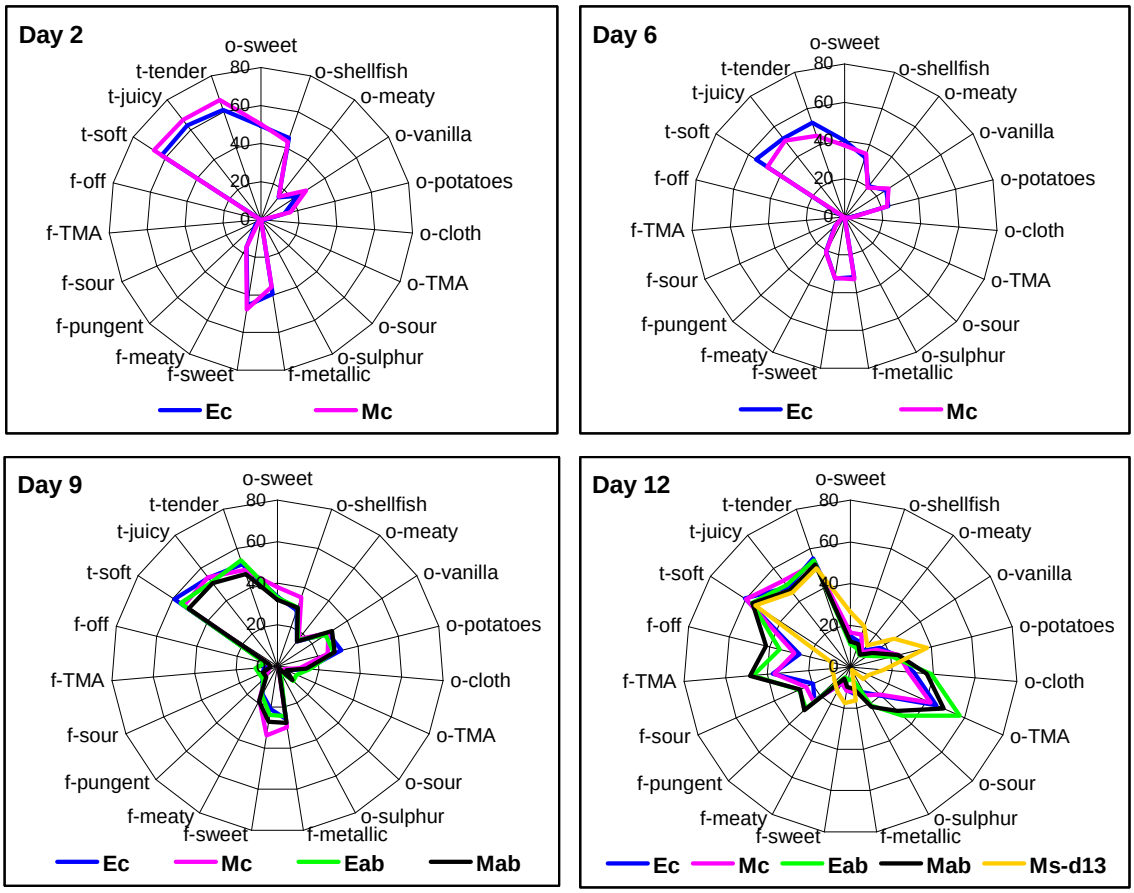

Figure 5. Spiderplots of QDA data (scale 0-80) for cod loins evaluated on days 2 to 12. Only attributes of significance among samples are shown (see Appendix II).

Superchilled cod loins stored under MA (Ms) was not evaluated between days 6 and 13 of storage. It is therefore not possible to estimate the freshness period, but it is expected to be at least more than that observed for Mc fish (>9 days). Ms fish was judged unfit for consumption 13 days post packaging. This optimal storage only slightly increased the shelf

life of cod loins, which in fact was shorter than expected based on earlier trials (Lauzon *et al*., 2010).

Table 4. Estimation of the freshness period and shelf life (in days) of cod loins based on Torry scores

Product group	Product temperature (± SD °C)	Freshness period (days)	Shelf life (days)
Ec	-0.3 ± 0.9	7	10
Eab	0.7 ± 2.4	6.5	9
Mc	0.2 ± 1.5	9	10.5-11
Mab	1.4 ± 2.8	7.5	10
Ms	-1.1 ± 0.1	(9+)	13

Value in parenthesis is only an expectation since it was not evaluated between days 6 and 13, and it is expected to be of better quality than Mc.

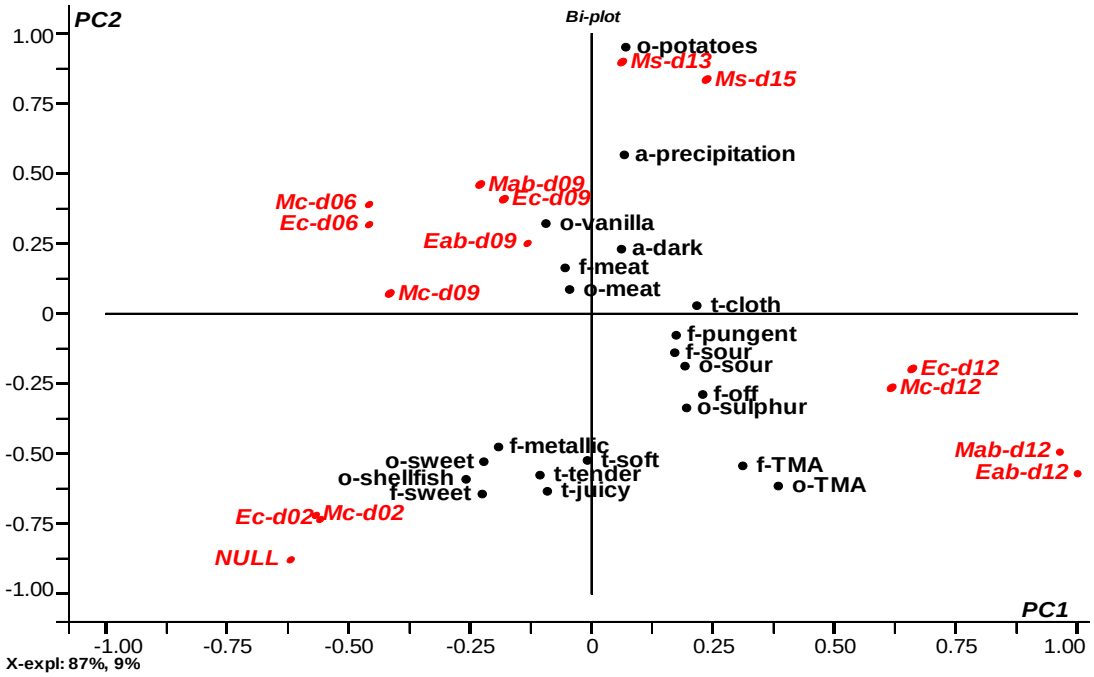

Figure 6. PCA describing sensory quality, odour (o-), appearance (a-), flavour (f-) and texture (t-) of the sample groups with storage time in days (d). PC1 VS PC2 (X-expl.: 87% and 9%). Bi-plot of scores (samples) and loadings (sensory attributes). *NULL* means newly processed loins at day 0.

3.2.4 *Microbiological analysis*

Microbial contamination of fresh fish during processing is difficult to avoid as the fish skin generally harbour at least 1000-fold higher levels of bacteria than what is measured in the flesh, if aseptically handled. The processing plant involved in the study performs a 2-step cooling of skin-on fillets, first using brine as the pre-cooling medium and then CBC-processing to superchill the fillets to about -1 °C followed by skinning, trimming and

packaging. The microbial load of the brine and fillets prior to and after liquid cooling is shown in Figure 7. On that processing day, a 4-day old fish batch had just been introduced to the cooling tank when initial sampling of the brine was performed at 7:30. The brine was already contaminated by low levels of H_2S-producing bacteria and pseudomonads, while 3 h later *P. phosphoreum* (Pp) was dominating among the culturable microbiota.

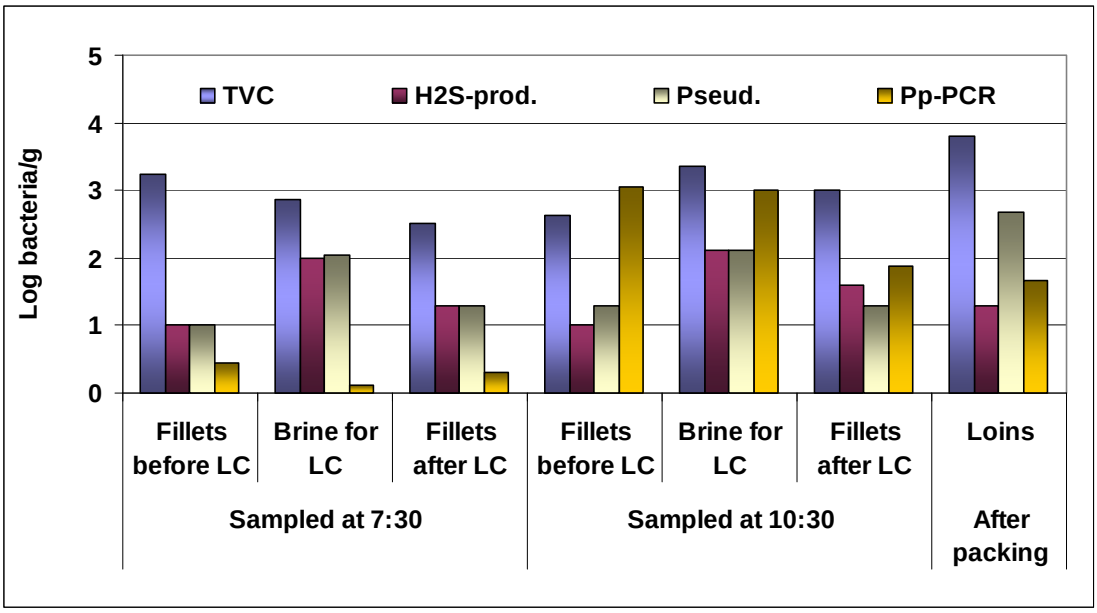

Figure 7. Microbial load in cooling brine and cod fillets prior to and after liquid cooling (LC). TVC, total viable psychrotrophic counts; H2S-prod., counts of H_2S-producing bacteria; Pseud., pseudomonad counts; Pp-PCR, estimated counts of *P. phosphoreum*.

These three bacterial groups are considered as important spoilage bacteria in fresh, coldwater marine fish stored aerobically (Olafsdottir *et al*., 2006a,b), while the latter species (Pp) is the main spoilage bacterium in MAP fish products (Dalgaard *et al.,* 1997). Growth of H_2S-producing bacteria and pseudomonads in the brine was not evidenced during the time elapsed between sampling. The higher Pp levels on fillets processed towards the end of the packaging trial (10:30) compared to those processed early (7:30) could be explained by the faster growth rate of Pp, especially as temperature increases (Lauzon *et al*., 2010). It is therefore possible that Pp became established and grew on the processing line and then in the brine as the processing time progressed. Brine temperature was recorded, starting at 0.8 °C but reaching 1.2 °C at final sampling. Further the fact that older raw material was processed earlier, inevitably contaminating the processing line and brine, may have enhanced fillet contamination of the newer raw material. Microbial load of newly packed loins shown on Figure 7 indicates that 10-fold higher TVC and pseudomonad counts were

detected in the final product than after liquid cooling (10:30). This could be explained by the tolerance of pseudomonads to superchilling (CBC-process) and their ability to form biofilms on processing equipment (Guðbjörnsdóttir, 2004; Lauzon *et al*., 2009). This suggests the possible proliferation of pseudomonads as well as other background microbiota on the processing line after CBC-process, leading to further fillet contamination.

Figure 8 presents the microbial growth in the differently treated cod groups as storage time progressed. TVC obtained using mLH medium is only shown since IA gave lower counts, independently of the packaging method used (see Appendix III). The initial microbiological quality of the raw material was satisfactory (6600 ± 2700 cfu g^{-1}), with about 10-fold lower levels of pseudomonads but 100-fold lower H_2S-producing bacteria and Pp counts compared to the overall culturable microbiota. It should be kept in mind that the mean ambient temperature during the first 6 days post-packaging was -1.3 ± 1.1 °C for MAP fish and -1.9 ± 2.5 °C for air-stored fish, after which three different temperature profiles were applied.

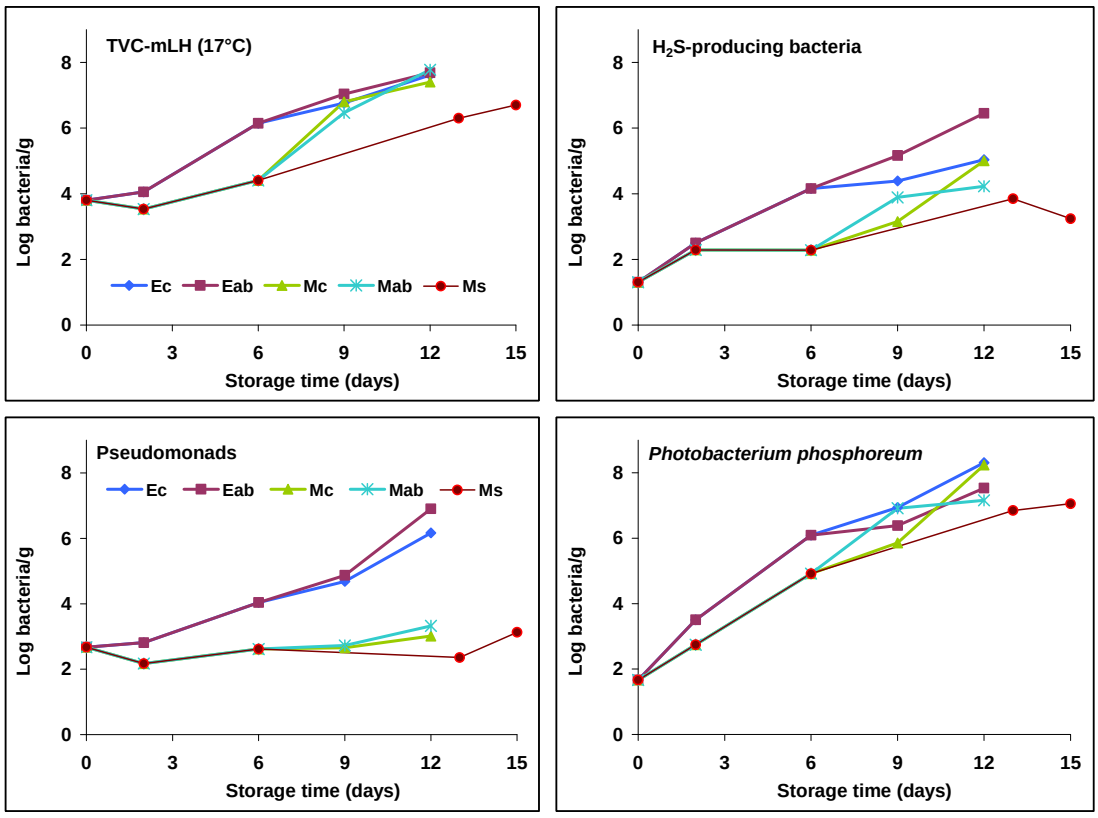

Figure 8. Total viable psychrotrophic counts (TVC-mLH) and counts of H_2S-producing bacteria, pseudomonads and *P. phosphoreum* (Pp) in differently treated cod groups (mean ± SD, n=2).

The combined use of modified atmosphere and superchilling significantly reduced the growth rate of the overall culturable microbiota, especially H_2S-producing bacteria and

pseudomonads as demonstrated by the lower slope of the curves at day 6. From that day, ambient temperature was raised to mimic distribution and storage in a traditional chilling room (2 °C) or under abused conditions (5°C). Enhanced growth was generally seen in abused groups compared to controls which proved to be insignificant in most cases. The microbiota of Ms-cod loins superchilled during the whole storage period developed at a slower rate, though the least difference was observed for Pp growth. In fact, the data clearly show the synergistic inhibitory action of MAP and low temperature storage towards Pp evolution which was readily eliminated upon poor temperature control at later storage. Average microbial data and statistical analysis are provided in Appendix III.

3.2.5 *Chemical analysis: TVB-N, TMA, pH, water holding capacity, moisture and salt content*

Changes in TVB-N and TMA content as well as pH in minced fish were evaluated throughout storage as shown in Figure 9 and Figure 10, respectively. Formation of TVB-N and TMA was influenced by the packaging method and superchilled storage. TVB-N content of air-stored fish was approaching the EU limit of acceptability (35 mg N/100 g fish) about 10 days post-packaging while a slower formation was seen in MAP fish. Formation of TMA followed a similar trend, with 10-15 mg N/100 g flesh generally corresponding to unfitness for consumption (Connell, 1995). TVB-N and TMA content in superchilled fish was approaching these limits about 15 days post packaging. These findings reflect the proliferation of *P. phosphoreum,* an important TMA producer (Dalgaard, 1995), in the differently treated cod groups.

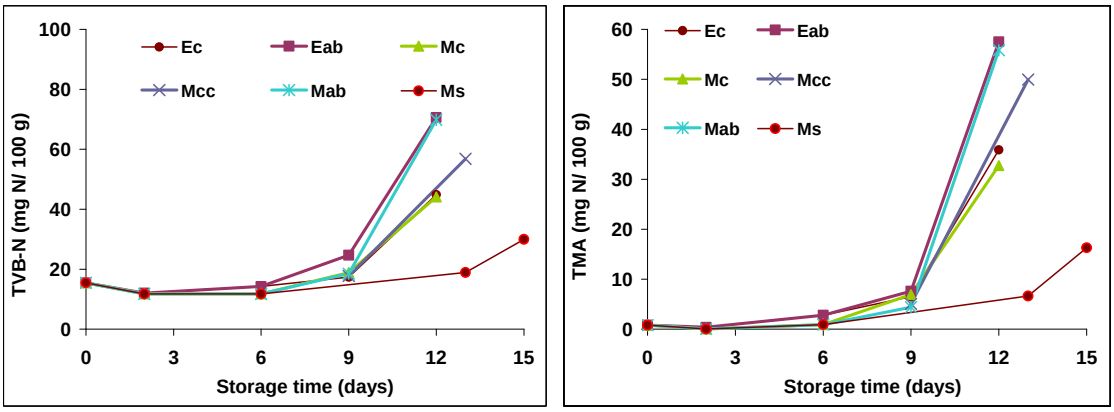

Figure 9. Total Volatile Base Nitrogen (TVB-N) and trimethylamine (TMA) content in differently treated cod groups (mean ± SD, n=2)

Measurements of pH revealed only slightly lower values for MAP fish compared to air-stored loins (Figure 10). Dissolved CO_2 is easily lost from the fish muscle as time elapses from opening of the package until pH analysis of the mince prepared aseptically is performed. Overall, all groups that received a thermal simulation for distribution and chilled or abused storage showed a pH rise as time progressed, corresponding to the amine formation. On the other hand, pH of superchilled fish (Ms) was observed to decrease during the first 13 days of storage, suggesting the production of acidic products, after which a pH rise was seen in agreement with amine formation.

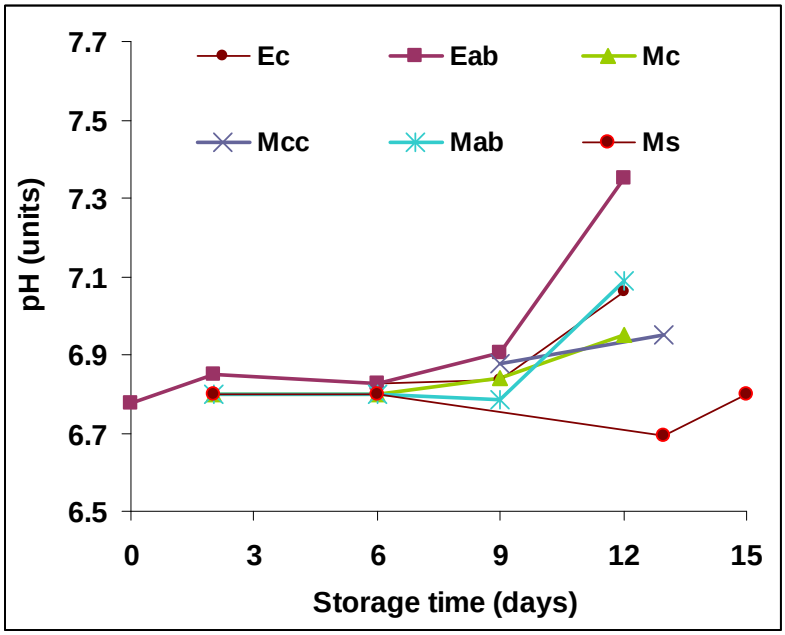

Figure 10. Measurements of pH in differently treated cod groups (mean ± SD, n=2)

Table 5. Moisture content and WHC of differently treated cod groups: Minimum and maximum mean values (n=2) measured during the storage study on days 0, 6, 9 and 12

Parameters	Ec	Eab	Mc	Mab
Moisture content (%)	81.3-81.7	81.2-81.7	81.0-81.7	81.1-81.7
WHC (%)	89.0-90.1	88.2-92.9	89.4-91.0	89.4-93.6

Table 5 demonstrates that little difference was observed in moisture content and water holding capacity of the differently treated loins during storage (p>0.05). The salt content of newly processed and liquid cooled fillets was 0.2 ± 0.0% and 0.4 ± 0.0%, respectively, similarly to newly packaged cod loins (0.4 ± 0.1%). Average chemical data and statistical analysis are provided in Appendix III.

3.2.6 *Lipid analyses*

Figure 11 to Figure 13 present the data obtained by lipid analyses. Total lipids in cod loins amounted to 0.5 ± 0.1 g per 100 g fish (n=24). FFA values ranged between 2 and 28 g FFA/100 g lipids over the storage period, where the values had increased significantly for most groups (Ec, Eab, Mab) on day 12 in comparison to day 6 (Figure 11). The general trend observed is that abusive conditions contributed to a faster FFA formation, with MA-packaging slightly delaying the process but insignificantly. Superchilling combined with MA-packaging (Ms loins) promoted a slow degradation of lipids. Accumulation of FFA has been related to some extent to lack of acceptability, because FFA are known to have detrimental effects on ATPase activity, protein solubility and relative viscosity (Careche and Tejada, 1994), to cause texture deterioration by interacting with proteins (Mackie, 1993) and to be interrelated with lipid oxidation development (Han and Liston, 1987). Being relatively small-size molecules, FFA have shown to undergo a faster oxidation rate than higher molecular weight lipid classes (triglycerides and phospholipids) by providing a greater accessibility to oxygen and pro-oxidant molecules (Labuza and Dugan, 1971; Miyashita and Takagi, 1986). FFA content has been successfully used to assess fish deterioration during frozen storage (de Koning and Mol, 1991) and chilled storage (Barassi *et al*., 1987). FFA are important not only because of their susceptibility to oxidation, but also because by themselves they cause taste deterioration (Refsgaard *et al*., 2000).

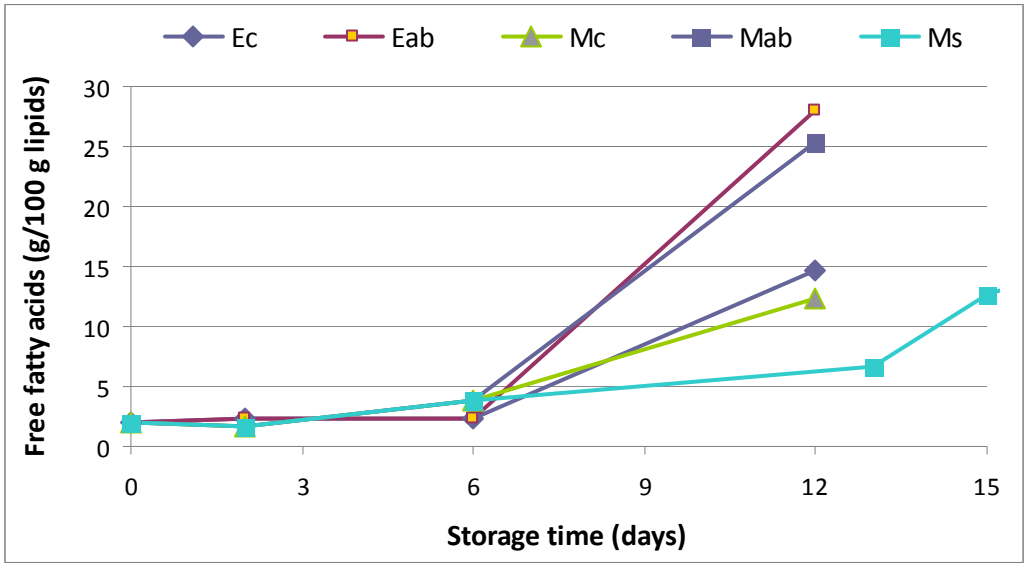

Figure 11. Evolution of FFA content in cod loins as influenced by packaging method and temperature profile during storage

Formation of secondary lipid oxidation compounds, hydroperoxides given by TBARS values (µmol MDA kg^{-1}) in light and dark muscle of cod loins, is presented in Figure 12. TBARS values ranged from 1.5 to 6.4 (light muscle) or 35.2 (dark muscle) µmol MDA kg^{-1} sample. The results showed higher concentration of TBARS in the dark muscle compared with the light muscle. This difference can be related to the fatty acid composition of these two muscle types, but studies have indicated that the polyunsaturated fatty acids in the dark muscle are more easily destroyed (Dulavik *et al*., 1998). Thus, it can be expected that fatty acid oxidation occurs mainly in the dark muscle. A value of 20 µmol MDA kg^{-1} sample has been suggested to correspond to noticeably rancid fish. Our results show that there was a trend for higher TBARS levels in MAP fish compared to air-stored loins. This was specifically detected on day 6 in the dark muscle, indicating a peak concentration after six days of storage under superchilled conditions. This was followed by a reduction as storage time progressed and temperature was raised. It is likely that this reduction was caused by further reactions involving these compounds. The results therefore suggest that the packaging method influenced the formation of secondary lipid oxidation compounds, while temperature had little effect.

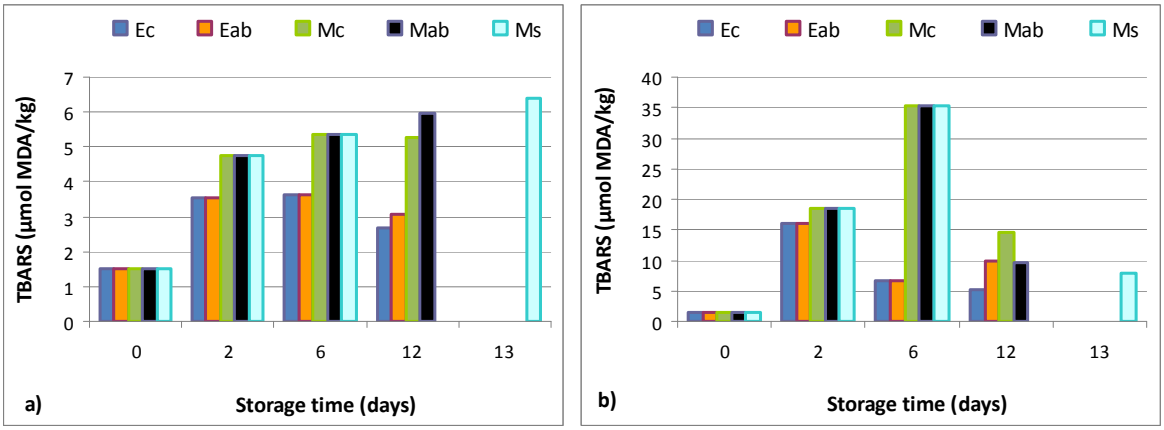

Figure 12. TBARS values (µmol MDA/kg) in a) light and b) dark muscle of cod loins as influenced by the packaging method and temperature profile during storage

The formation of interaction compounds was assessed by the fluorescence ratio. Studies have shown that fluorescence detection (δF value) is a valid method to assess lipid oxidation (Aubourg *et al*., 1995; Aubourg *et al*., 2007; Rodrígez *et al*., 2009). According to the mean values of the organic phase, an increasing trend was detected for all the samples (Figure 13). The formation of these tertiary oxidation compounds is the result of the interaction between lipid oxidation products (primary and secondary) and protein-like molecules present in the

fish muscle (Aubourg, 1999a). The electrophilic character of most lipid oxidation compounds leads them to interact with food constituents possessing nucleophilic functions. Such interactions are highly favoured by a temperature increase of oxidised lipids, particularly in protein-rich foodstuffs such as marine source, which have high portion of essential and reactive amino acids such as lysine and methionine. As expected, a low ratio of tertiary oxidation compounds was measured in the newly processed loins while a significant increase was seen on day 6. Similar values were obtained on days 12-15. The values obtained in the present study were rather high compared with other studies of lean fish species (Aubourg, 1999b; Aubourg and Medina, 1999; Aubourg *et al*., 2007). Based on the data similarity among the cod groups, it can be concluded that the treatments applied did not apparently influence the formation of these interaction compounds. Average lipid data and statistical analysis are provided in Appendix III.

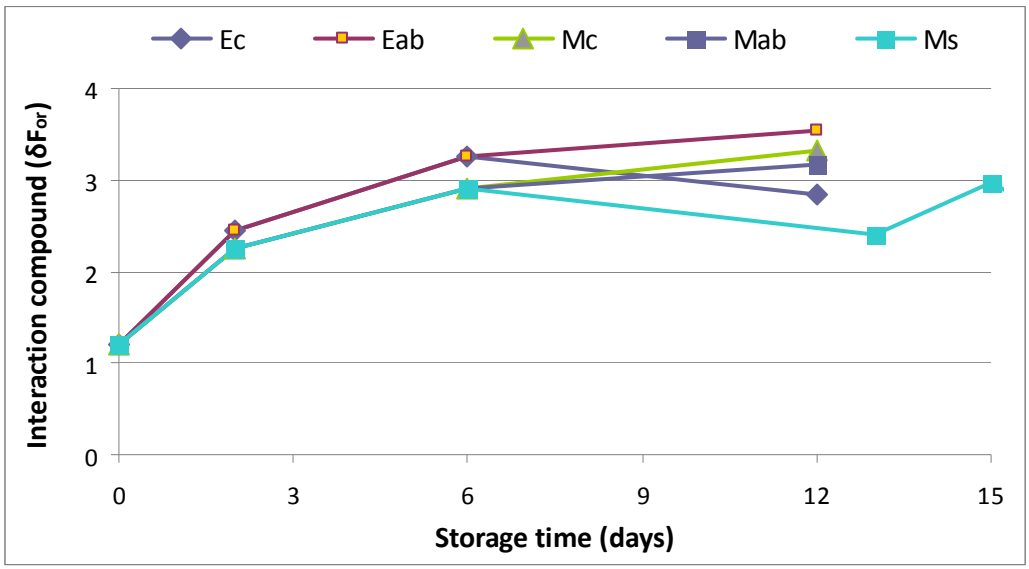

Figure 13. Fluorescence shift ratio of the organic phase resulting from Bligh and Dyer lipid extraction of cod loins as influenced by packaging method and temperature profile during storage

3.2.7 *Drip loss*

Water loss in fish muscle was evaluated throughout storage (Figure 14). Upon sampling on day 12, it was observed that EPS boxes containing air-stored fish were leaking from the bottom. The drip loss calculated on day 12 therefore underestimates the real water loss that occurred in air-stored fish. Otherwise the data indicate that MA-packaging significantly increased drip loss after 9 days of storage, but insignificant increase in drip of MAP fish was observed onwards. Little difference in drip was observed for MAP fish weighing 2.67 ± 0.05

kg (Mc) and 2.89 ± 0.02 kg (Mcc). Drip loss in superchilled fish (Ms) was evaluated shortly after removing the boxes from the cooler. It follows that underestimation of drip in that group cannot be precluded. Overall, it can be concluded that close to the end of shelf life, air-stored fish had lost about 3% of their water in comparison to 5% for MAP fish.

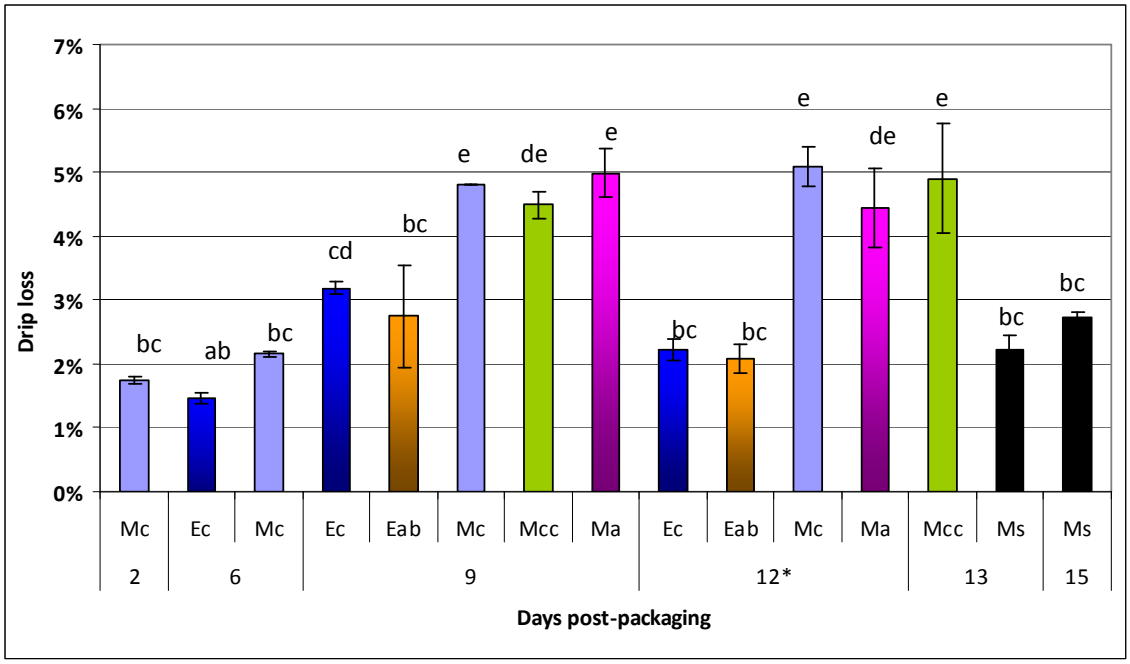

Figure 14. Drip measurements in differently treated cod groups (mean ± SD, n=2). Different letters indicate significant difference (*p*<0.05) among sample groups (assuming day 0 = 0% drip); *, Ec and Eab boxes were leaking on day 12 (underestimated drip).

3.3 Overview of the study and conclusions

Table 6 lists values or estimates of potential microbial and chemical spoilage indicators at sensory rejection of the differently treated cod products. The dominance of *P.phosphoreum* in all groups was generally related to TMA production, while the lower oxygen tension establishing in MAP fish as time progressed apparently enhanced TMAO reduction to TMA. The bacterial reduction of TMAO to TMA is known to proceed at a faster rate at conditions of low oxygen tension (Huss, 1972). This is also clearly shown by the P ratio indicating the higher contribution of TMA in MAP fish over other amine compounds. However, superchilled MAP storage conditions delayed TMA formation which indicates that other spoilage compounds were influencing the spoilage profile. Oxidation products are suspected. This is suggested by the QDA data showing low TMA odour and flavour but a high level of "boiled potato" odour from Ms fish (day 13) compared to other fish groups (day 12). This "boiled potato" odour is related to heptanal, an aldehyde corresponding to secondary lipid oxidation

compounds and reported to increase during cod deterioration (Olafsdottir *et al*., 2005). Aldehydes have been suggested as spoilage indicators in fatty fish species (Aro *et al.,* 2003).

Table 6. Values or estimates of spoilage indicators at sensory rejection for the different cod groups

Parameters evaluated (at sensory rejection)	**Ec (10 d)**	**Eab (9 d)**	**Mc (10.5 d)**	**Mab (10 d)**	**Ms (13 d)**
TVC (log cfu g^{-1})	7.1	7.0	7.1	7.0	6.3
H_2S-producing bacteria counts (log cfu g^{-1})	4.6	5.2	4.1	4.0	3.8
Pseudomonad counts (log cfu g^{-1})	5.2	4.9	2.9	2.9	2.4
P. phosphoreum counts (log cfu g^{-1})	7.4	6.4	6.6	7.0	6.8
pH (units)	6.9	6.9	6.9	6.9	6.7
TVB-N (mg N/100 g fish)	27	25	31	36	19
TMA (mg N/100 g fish)	16	13	20	23	7
P ratio (TMA/TVB-N)	0.59	0.52	0.65	0.64	0.37
FFA (g/100 g lipids)*	14.6	28.0	12.3	25.3	6.7
TBARS values in light muscle (µmol MDA kg^{-1})*	3.0	3.1	5.3	6.0	6.4
TBARS values in dark muscle (µmol MDA kg^{-1})*	5.1	9.8	14.5	9.6	8.0
Tertiary oxidation compounds (δF_{or})*	2.8	3.5	3.3	3.2	2.4

* Data obtained on day 12 (Ec, Eab, Mc, Mab) or day 13 (Ms).

Lipid hydrolysis in cod loins occurred at a faster rate with increasing temperature, with MA-packaging slightly delaying the process. On the other hand, the oxidation pattern that developed in fish products was apparently influenced by the packaging method, with MAP fish reaching higher levels of secondary lipid oxidation products than air-stored fish. Whether this is directly related to the condition of the fish at processing and/or MAP is uncertain. Insufficient bleeding, i.e. residual haemoglobin in fish muscle, has been shown to induce lipid oxidation (Wang *et al*., 2010). However, secondary and tertiary oxidation products were not influenced by the temperature range (-1 to 5 °C) evaluated. These findings may reflect the greater importance of these oxidation products in superchilled MAP fish where TMA level was low at sensory rejection. Further, protein denaturation may result from slow freezing, and may be enhanced under MAP due to CO_2 dissolution and its pH lowering effect. It has been demonstrated that the temperature of maximum activity is in the region of -2 to -1 °C (Johnston *et al*., 1994). Other quality changes at subzero conditions

include flavour and odour deterioration, pigment degradation, enzymatic browning and lipid oxidation.

Under the experimental conditions presented, MA-packaging slightly extended the freshness period and shelf life of fish when compared to air-stored products. The freshness period of iced, gutted cod has been reported to last 8-9 days post catch, while superchilling generally delays freshness loss in cod products (Lauzon *et al.,* 2010). Temperature rise in MAP fish during storage decreases CO_2 dissolution hence counteracting the antibacterial action otherwise provided. The importance of good temperature control of MAP products to enhance their freshness was emphasised.

Overall, the quality data presented indicate that the fish used in this experiment spoiled faster than observed in a similar trial conducted with cod caught during the fall season (Martinsdóttir *et al*., 2005). In that experiment, the freshness period and shelf life of similarly superchilled (-1 °C) cod fillets processed 3 days post catch was 10 and 16 days under air storage (EPS) and 15 and 19 days under MA-packaging, respectively. It is therefore possible to achieve a longer freshness period and shelf life, especially if fresher raw material is processed. However, the gas to fish ratio was higher in that experiment (1.6 to 2) and a gas mixture of 55% CO_2/ 5% O_2/40% N_2 was used. Higher CO_2 concentration and gas to fish ratio have been suggested to enhance antibacterial action. The main factors influencing the effectiveness of MA packaging are temperature, the amount of available CO_2 that can dissolve into the food, as given by the partial pressure of CO_2 inside the package, and the degree of filling (DF), i.e. food volume to package volume. Indeed, the solubility of CO_2 has been shown to increase with increasing initial CO_2 in headspace as well as with decreasing temperature and DF (Rotabakk *et al*., 2007). Taking into account the lower gas to fish ratio (higher DF) in the present study, it can be proposed that slightly increasing CO_2 concentration in the gas mixture could enhance the antibacterial action in the fish muscle resulting in higher quality fish products. However, textural defects must be avoided. Further, alternative gas mixtures (and components) could also lead to high quality products.

4. ACKNOWLEDGEMENTS

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6. APPENDIX I: SCHEME FOR TORRY FRESHNESS EVALUATION OF COOKED COD

Odour	Flavour	score
Initially weak odour of sweet, boiled milk, starchy followed by strenghthening of these odours	Watery, metallic, starchy. Initially no sweetness but meaty flavours with slight sweetness may develop	**10**
Shellfish, seaweed, boiled meat	Sweet, meaty, characteristic	**9**
Loss of odour, neutral odour	Sweet and characteristic flavours but reduced in intensity	**8**
Woodshavings, woodsap, vanillin	Neutral	**7**
Condensed milk, boiled potato	Insipid	**6**
Milk jug odours, boiled clothes- like	Slight sourness, trace of off-flavours	**5**
Lactic acid, sour milk, TMA	Slight bitterness, sour, off-flavours, TMA	**4**
Lower fatty acids (eg acetic or butyric acids) composed grass, soapy, turnipy, tallowy	Strong bitter, rubber, slight sulphide	**3**

7. APPENDIX II: STATISTICAL ANALYSIS OF SENSORY DATA

Appearance	colour	appearance	precipitate	flakes
p-value	***0.037***	0.072	***0.004***	0.514
D0	19	24	22 b	44
Ec-D02	15	20	30	47
Ec-D06	20	24	41 a	45
Ec-D09	25	24	30	51
Ec-D12	25	28	35	52
Eab-D09	21	23	30	53
Eab-D12	29	29	35	56
Mc-D02	17	19	31	47
Mc-D06	23	27	39	47
Mc-D09	21	22	30	45
Mc-D12	26	27	37	52
Mab-D09	21	26	32	42
Mab-D12	30	30	36	53
Ms-D13	28	32	42 a	46
Ms-D15	27	33	46 a	52

Texture	soft	juicy	tender	mushy	meaty	clammy	rubbery
p-value	***0.020***	***0.000***	***0.000***	0.144	0.207	0.571	0.327
D0	64	62 ac	65 ab	30	27	13	16
Ec-D02	62	63 ab	61 ac	26	25	12	10
Ec-D06	55	52 bd	52	29	32	17	21
Ec-D09	59	54	52	36	27	17	15
Ec-D12	60	49 cd	55	38	25	17	18
Eab-D09	56	51 bd	54	31	25	15	14
Eab-D12	56	50 bd	54	36	23	15	13
Mc-D02	67 a	67 a	67 a	30	25	9	11
Mc-D06	48 b	51 bd	45 d	22	36	17	23
Mc-D09	54	54	49 bd	28	34	19	16
Mc-D12	60	52 bd	52	38	23	15	15
Mab-D09	51	51 bd	47 cd	28	37	21	19
Mab-D12	56	47 d	52	37	22	15	14
Ms-D13	54	45 d	50 bd	30	25	16	17
Ms-D15	55	49 cd	50 bd	28	34	25	18

Odour	sweet	shellfish	meaty	vanilla	potatoes	frozen storage	cloth	TMA	sour	sulphur
p-value	***0.000***	***0.000***	***0.003***	***0.000***	***0.000***	0.960	***0.000***	***0.000***	***0.000***	***0.000***
D0	54 a	49 a	19	27	14 bc	1	2 d	0 b	0 c	0 d
Ec-D02	49 ab	45 ab	15	23	13 c	1	2 d	0 b	1 c	0 d
Ec-D06	40 ac	33 ad	20 a	26	24	1	3 d	0 b	1 c	0 d
Ec-D09	34 bd	29 bf	15	29 ab	32 ab	1	14 bd	5 b	8 bc	3 cd
Ec-D12	15 ef	13 eg	10	17	22	1	31 ab	45 a	20 ab	14 ac
Eab-D09	33 bd	30 be	16	28	28	1	16 bd	10 b	10 bc	3 cd
Eab-D12	11 f	9 g	7 b	10 c	21	1	39 a	57 a	34 a	21 a
Mc-D02	50 ab	43 ab	15	28	16 bc	1	1 d	0 b	1 c	0 d
Mc-D06	37 ac	35 ac	19	28	23	1	3 cd	1 b	1 c	0 d
Mc-D09	38 ac	35 ac	17	29 ab	25	1	11 cd	4 b	6 bc	1 d
Mc-D12	17 df	16 dg	9	15	24	1	28 ab	42 a	19 ab	15 ab
Mab-D09	32 bd	30 be	15	31 a	28	1	14 bd	5 b	10 bc	1 d
Mab-D12	12 f	12 fg	7	13 bc	24	1	37 a	49 a	31 a	22 a
Ms-D13	26 cf	21 cg	13	25	38 a	2	16 bd	10 b	8 bc	2 cd
Ms-D15	17 df	15 eg	12	21	36 a	2	20 bc	18 b	12 bc	5 bd

Flavour	salt	metallic	sweet	meaty	pungent	frozen storage	sour	TMA	off-flavour
p-value	0.472	***0.000***	***0.000***	***0.001***	***0.000***	0.461	***0.000***	***0.000***	***0.000***
D0	4	51 a	42 ab	19	3 de	1	1 d	0 b	0 d
Ec-D02	6	39 ab	46 a	16	4 de	0	1 d	0 b	0 d
Ec-D06	6	32 ae	33 ac	20 ab	7 de	1	1 d	2 b	1 d
Ec-D09	6	26 bg	21 ce	15	10 ce	2	8 cd	6 b	5 d
Ec-D12	5	13 eg	11 de	9	24 ac	1	20 ac	37 a	25 ac
Eab-D09	6	24 bg	24 ce	16	9 ce	2	9 cd	11 b	6 d
Eab-D12	4	6 g	6 e	5 c	29 a	2	27 a	47 a	35 a
Mc-D02	7	36 ac	48 a	17	2 e	0	1 d	0 b	0 d
Mc-D06	4	33 ad	32 ac	21 a	6 de	1	2 d	1 b	0 d
Mc-D09	7	30 bf	34 ac	19	9 de	2	5 d	4 b	5 d
Mc-D12	5	12 fg	12 de	9	24 ab	2	23 ab	37 a	28 ab
Mab-D09	7	28 bf	27 bd	19	7 de	2	8 cd	3 b	4 d
Mab-D12	4	11 fg	10 de	6 bc	30 a	2	27 a	48 a	42 a
Ms-D13	4	16 cg	18 ce	13	10 be	3	9 cd	9 b	9 cd
Ms-D15	5	15 dg	15 de	13	16 ad	3	11 bc	15 b	16 bd

Different letters within a column indicate significant difference among samples (p<0.05).

8. APPENDIX III: STATISTICAL ANALYSIS OF MICROBIAL AND CHEMICAL DATA

Groups	TVC-mLH (log cfu g^{-1})	TVC-IA (log cfu g^{-1})	H_2S-prod. (log cfu g^{-1})	PCR-Pp (log cfu g^{-1})	*Pseud.* (log cfu g^{-1})	pH (units)	TVB-N (mg N $100g^{-1}$)	TMA (mg N $100g^{-1}$)
p value	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*	*0.000*
Loins d0	3.8 ± 0.2 a	3.6 ± 0.1 a	1.3 ± 0.4 a	1.3 ± 0.5 a	2.7 ± 0.2 ab	6.78 ± 0.11 ab	15.5 ± 4.8 a	0.8 ± 0.4 a
Ec-d2	4.1 ± 0.2 a	3.8 ± 0.4 a	2.5 ± 0.7 ac	3.5 ± 0.9 bc	2.8 ± 0.0 ab	6.85 ± 0.07 ac	12.0 ± 0.5 a	0.4 ± 0.0 a
Mc-d2	3.5 ± 0.0 a	3.5 ± 0.1 a	2.3 ± 0.7 ab	2.7 ± 0.2 ab	2.2 ± 0.2 a	6.80 ± 0.00 ab	11.8 ± 0.7 a	0.0 ± 0.0 a
Ec-d6	6.1 ± 0.1 b	5.6 ± 0.3 bc	4.2 ± 0.4 ce	6.1 ± 0.2 df	4.0 ± 0.1 cd	6.83 ± 0.04 ab	14.3 ± 1.6 a	2.8 ± 0.8 a
Mc-d6	4.4 ± 0.7 a	3.7 ± 0.5 a	2.3 ± 0.4 ab	4.9 ± 1.0 cd	2.6 ± 0.4 ab	6.80 ± 0.00 ab	11.8 ± 0.4 a	0.9 ± 0.2 a
Ec-d9	6.8 ± 0.3 bd	5.8 ± 0.1 bc	4.4 ± 0.1 de	6.9 ± 0.2 dg	4.7 ± 0.4 d	6.84 ± 0.09 ab	17.4 ± 0.4 a	6.5 ± 3.0 a
Mc-d9	6.8 ± 0.4 be	5.9 ± 0.7 bc	3.2 ± 0.2 bd	5.9 ± 0.6 de	2.7 ± 0.3 ab	6.84 ± 0.06 ab	18.8 ± 3.2 a	6.9 ± 3.9 a
Mcc-d9	6.7 ± 0.1 bd	6.1 ± 0.7 bd	4.0 ± 0.5 be	6.5 ± 0.1 dg	2.3 ± 0.0 ab	6.88 ± 0.01 ac	17.8 ± 0.1 a	5.1 ± 0.6 a
Eab-d9	7.0 ± 0.2 be	6.4 ± 0.3 be	5.2 ± 0.4 ef	6.4 ± 0.6 dg	4.9 ± 0.3 d	6.91 ± 0.02 ad	24.7 ± 5.6 a	12.6 ± 2.7 a
Mab-d9	6.5 ± 0.2 bc	6.1 ± 0.0 bc	3.9 ± 0.6 be	6.9 ± 0.0 dg	2.7 ± 0.2 ab	6.79 ± 0.02 ab	18.5 ± 0.9 a	5.7 ± 1.8 a
Ec-d12	7.6 ± 0.0 de	6.7 ± 0.2 cf	5.0 ± 0.6 ef	8.3 ± 0.1 g	6.2 ± 0.1 e	7.06 ± 0.04 cd	44.9 ± 1.9 bc	35.9 ± 3.1 bd
Mc-d12	7.4 ± 0.0 ce	6.8 ± 0.1 cf	5.0 ± 0.0 ef	8.2 ± 0.6 g	3.0 ± 0.2 ac	6.95 ± 0.07 bd	44.1 ± 5.5 bc	32.8 ± 5.1 bc
Eab-d12	7.7 ± 0.0 de	7.6 ± 0.1 ef	6.5 ± 0.2 f	7.5 ± 0.1 eg	6.9 ± 0.1 e	7.35 ± 0.07 e	70.6 ± 13.8 d	57.5 ± 17.2 e
Mab-d12	7.8 ± 0.0 e	7.7 ± 0.1 f	4.2 ± 0.4 ce	7.2 ± 0.4 eg	3.3 ± 0.1 bc	7.09 ± 0.01 d	69.8 ± 2.0 d	55.8 ± 4.6 de
Mcc-d13	7.6 ± 0.2 de	7.4 ± 0.1 df	3.8 ± 0.3 be	7.9 ± 1.0 fg	3.2 ± 0.7 ac	6.95 ± 0.07 bd	56.8 ± 2.0 cd	50.0 ± 1.5 ce
Ms-d13	6.3 ± 0.1 b	5.2 ± 0.3 b	3.8 ± 0.2 be	6.8 ± 0.3 dg	2.4 ± 0.1 ab	6.70 ± 0.04 a	18.9 ± 5.5 a	6.7 ± 3.5 a
Ms-d15	6.7 ± 0.2 bd	5.7 ± 0.3 bc	3.2 ± 0.3 bd	7.1 ± 0.0 eg	3.1 ± 0.2 ac	6.80 ± 0.00 ab	30.0 ± 5.5 ab	16.3 ± 4.2 ab

Different letters within a column indicate significant difference among samples (p<0.05).

Groups	Moisture (%)	WHC (%)	Total lipids (g/100 g fish)	FFA (g/100 g TL)	TBARS-light (MDA/kg fish)	TBARS-dark (MDA/kg fish)	Tertiary prod. $\delta F_{organic}$
p value	0.190	0.338	0.098	*0.000*	*0.000*	*0.000*	*0.002*
Loins d0	81.7 ± 0.1	90.1 ± 2.5	0.6 ± 0.1	2.0 ± 0.1 ab	1.5 ± 0.6 a	1.5 ± 0.7 a	1.1 ± 0.3 a
Ec-d2			0.6 ± 0.1	2.4 ± 0.1 ab	3.8 ± 1.8 ac	16.1 ± 0.7 e	2.5 ± 0.6 ab
Mc-d2			0.5 ± 0.0	1.6 ± 0.2 a	4.8 ± 1.4 bc	18.5 ± 3.1 e	2.2 ± 0.6 ab
Ec-d6	81.3 ± 0.2	89.7 ± 1.0	0.4 ± 0.1	2.3 ± 0.4 ab	4.1 ± 2.2 ac	6.7 ± 2.7 ac	3.3 ± 0.3 b
Mc-d6	81.3 ± 0.5	89.4 ± 0.9	0.6 ± 0.0	3.8 ± 1.3 ac	5.4 ± 1.0 bc	35.2 ± 3.7 f	2.9 ± 0.1 b
Ec-d9	81.4 ± 0.1	89.0 ± 2.3					
Mc-d9	81.0 ± 0.4	90.1 ± 0.9					
Mcc-d9							
Eab-d9	81.3 ± 0.1	89.4 ± 2.6					
Mab-d9	81.3 ± 0.3	88.1 ± 3.9					
Ec-d12	81.6 ± 0.7	90.0 ± 2.6	0.5 ± 0.1	14.6 ± 2.9 d	3.0 ± 1.0 ab	5.1 ± 2.0 ab	2.8 ± 0.3 b
Mc-d12	80.7 ± 0.1	91.0 ± 1.5	0.5 ± 0.0	12.3 ± 1.6 cd	5.3 ± 0.6 bc	14.5 ± 1.8 de	3.3 ± 0.6 b
Eab-d12	81.4 ± 0.2	92.9 ± 0.8	0.4 ± 0.3	28.0 ± 1.6 e	3.1 ± 0.5 ab	9.8 ± 3.5 bd	3.5 ± 0.8 b
Mab-d12	81.7 ± 0.0	93.6 ± 0.6	0.6 ± 0.0	25.3 ± 5.9 e	6.0 ± 1.5 cd	9.6 ± 1.4 bd	3.2 ± 0.6 b
Mcc-d13			0.6 ± 0.1	11.0 ± 0.2 bd	5.2 ± 1.8 bc	11.4 ± 3.9 ce	2.4 ± 0.5 ab
Ms-d13			0.5 ± 0.1	6.7 ± 0.6 ad	6.4 ± 1.4 cd	8.0 ± 1.7 bc	2.4 ± 1.7 ab
Ms-d15			0.6 ± 0.0	12.7 ± 3.9 cd			3.0 ± 0.4 b

Different letters within a column indicate significant difference among samples (p<0.05).