MICROBIAL GROWTH IN MODIFIED ATMOSPHERE PACKAGED BLUE MUSSELS (*MYTILUS EDULIS)*

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Abstract
Microbial growth was investigated in live blue mussels (*Mytilus edulis*) packaged in modified atmosphere (MA) and stored at -1 and 4 ^{o}C for 19 days. The mortality of mussels was 0.8 % after 19 days of storage with lowest mortality at -1 and in CO_2 atmosphere. The increase in bacterial numbers in the mussel flesh was low, 1- 2 log units, measured as Aerobic Plate Counts, H_2S-producing bacteria, and psychrotrophic bacteria. There was significant lower bacterial growth ($p<0.05$) in an atmosphere with CO_2:O_2 compared air and N_2:O_2 and at -1 ^{o}C. An initial level of < 3 mg Tri-Methylamine Oxid-N (TMAO-N)/ 100g was found in the mussels, detectable levels of trimethylamine-N (TMA) were observed after 11 days of storage and TMA-N increased to 1 – 3 mg/100g in 19 days. Total Volatile Nitrogen (TVN) increased from 3.4 to 18 mg/100g in 19 days and may be an important chemical indicator to be used in defining the shelf life. Low temperature (-1 ^{o}C) did not significantly increase the shelf life, but the superchilled temperature will control pathogenic growth. MA packaging of live blue mussels is a promising packaging technology for retail distribution, with a shelf life of 11-12 days based on sensory scores.

Introduction
Modified atmosphere packaging (MAP) has been used to enhance shelf life of different seafood products and has been reviewed by several authors in the last decade (Brody 1989, Ogrydziak and Brown 1982, Parkin and Brown 1982, Pedrosa-Menabrito and Regenstein 1990, Skura 1991, Stammen *et al*. 1990, Reddy *et al*. 1992, Sivertsvik *et al*. *2002*). Crustacean shellfish keep up to 30 % longer at 0°C in a modified atmosphere than in other types of packaging, and the onset of blackspot in shell-on products is delayed (Cann 1988). A MA of 80 % CO_2 and 20 % air was found to be the preferred atmosphere for storage of freshwater crawfish (*Procambaris clarkii*) tail meat compared to 100 % CO_2 or air (Gerdes *et al*. 1989). CO_2-enriched atmosphere was found to increase the shelf-life of whole cooked shrimp by 200 %, compared with shrimp stored on ice and exposed to air (Sivertsvik *et al*. 1997). CO_2-atmospheres have also been found beneficial for storage of fish-cakes (Yokoseki *et al*. 1956), and also for chilled storage of raw squid and white octopus (Morales *et al*. 1997).

MA storage of live mussels differs from other processed MA packaged seafood in that they have a functioning immune system and that they have an active respiration during storage. The immune system protects the mussels from microbial attack and degradation. The active metabolism, aerobic and anaerobic, may on the other side produce metabolites adding unpleasant taste and flavour to the mussels in a closed packaging system. The replacement of air in the package with elevated concentration of oxygen may, however, support an extended survival time. One method with the potential to extend the period of prime quality in fish is “superchilling” or “partial freezing” (Haard 1992). In this technique, the temperature in the fish is reduced to 1 ^{o}C to 2 ^{o}C below the initial freezing point

and some ice is formed inside the product (Haard 1992, Sikorski and Sun 1994). Storage at superchilled conditions may enhance phospholipids hydrolysis and protein denaturation (Ashie *et al*. 1996), but superchilling will inhibit most autolytic and microbial reactions, and thereby in many instances increase shelf life (Chang *et al*. 1998, Huss 1995). An important advantage is that superchilled temperatures effectively will inhibit growth of pathogenic microorganisms at temperatures at 0 °C or below. The aim of this work was to examine MA packaging of blue mussels and examine effects of processing, packaging and storage temperature conditions on microbial growth, chemical spoilage products and survival rate.

Materials and Methods

Raw material and sample preparation

Farmed blue mussels (*Mytilus edulis*) were obtained from a local mussel farmer (FruMar/Mytilus AS) near Stavanger, and packaged at the packaging facility Norshell in Rogaland, Norway. Mussels were 40-70 mm long and farmed during a 3 year period in fjords with a mean salt concentration of 30 ‰, varying over a year from 20‰ to 32‰. The mussels were harvested and clots of mussels were placed in large tubs (500 L) in 3 days for conditioning in circulating seawater to imitate normal tidal waters, and to reduce stress. One sample group was exposed to changing periods with dry storage and under water storage to simulate tidal water changes. The mussels were harvested and clots of mussels were placed in large tubs (500 L) for 3 days for conditioning in circulating seawater to reduce stress. All samples were stabilised at 4.0°C before packed in plastic trays. The same day as packaging the mussels were transported on crushed ice to storage rooms with temperatures of +4 °C and ÷1 °C respectively, variables according to Table 1.

Table 1. The following packaging and storage conditions were used

No.	Gas mixture	Storage temp. (°C)	Tidal conditioning
1	O_2:N_2 = 40 %:60 %	4	Without
2	O_2:N_2 = 40 %:60 %	4	With
3	Air (sealed package)	4	Without
4	O_2:N_2 = 40 %:60 %	-1	Without
5	CO_2:O_2 = 50 %:50 %	4	Without

Temperature measurements

Sample's core temperatures were measured every 5 min during storage using electronic temperature loggers (Ebro Electronic, Ingolstadt, Germany) at both temperatures and in both packaging methods to ensure stable storage temperatures.

Packaging and packaging materials

Portions of 850 to 1050 g mussels were packaged in blue Dynopack trays No 551, PE-HD with PA/PE top film (Polimoon, Kristiansand, Norway). The gas mixture were added in gas flushing cycles from vacuum (-1 bar) to overpressure (0.1 bar) in a Dynopack packaging machine (Polimoon ibid.) before heat-sealing.

Gas mixtures and gas measurements

The gas composition (O_2, CO_2 and N_2, %) in the packages was measured in triplicate using an oxygen and carbon dioxide analyser (M.A.P. Test 4000, Hitech Instruments, Luton, UK). A 30 ml aliquot of the gas was collected through a syringe from the head space after intrusion of the top foil and analysed. Before intrusion of the syringe, a foam rubber septum (Nordic Supply, Skodje, Norway) was added to the top foil to avoid introduction of false atmosphere into the gas analyser. The

analyser was calibrated against a certified gas mixture (O_2:CO_2:N_2 1.1:44.1:54.8) and air before each sampling

Microbiological analysis
Samples of 25 g mussel tissue were taken at random and homogenised in 225 ml of 0.9 % NaCl (w/v) and 0.1 % peptone (w/v) for 120 sec in a Stomacher 400 Laboratory Blender (Seward Medical, London, U.K.). Total viable counts/ aerobic plate counts (APC) were measured after an aliquot a suitable dilution had been added to melted and temperated (44 °C) iron agar (Agar Lyngby, IA, Oxoid CM 867, Basingstoke, Hampshire, U.K.) supplemented with L-cysteine, and incubated at 20±1 °C for 3 days. Black colonies were counted as H_2S-producing bacteria, APC were counted as the total of black and white colonies. The content of psychrotrophic bacteria was determined by a spread plate count method with plate count agar (PCA, Merck, Darnstadt, Germany) with 1 % NaCl, and incubated at 8 °C for 7-10 days. Average results of duplicate measurements are presented as log colony-forming units (cfu) per gram mussel.
Chemical analysis
Tri-methylamine oxide (TMAO), tri-methylamine (TMA) and total volatile basic nitrogen (TVN) were determined in homogenised mussel meat (25 g) in duplicate using a modified Conway microdiffusion method (Conway and Byrne 1933) and expressed as mg TMAO/TMA/TVN- N/100 g product.

Determination of live mussels
Mussels were determined as live when the two half shells closed after a light knocking on the shell surface or after a gentle touch on the closing muscle with a knife. Only live mussels were subjected to microbial and chemical analysis. Number of live mussels was determined within 3 packages from each variant at each sampling.

Shellfish tissue pH
The pH of the mussel tissue was determined in triplicate using a pH meter (Beckman 72, Dan Mezansky, Oslo, Norway) on 25 g of homogenate of mussel muscle with 25 ml of 0.1 M KCl in distilled water.

Formation of exudate and exudate pH
The exudate formation was measured gravimetrically and reported as percentage of initial net weight of mussels in the package. pH in exudate was measured directly in the exudate using same instrument as above.

Sensorial off-odour determination
Samples from the storage experiment were analysed by a sensory panel of 3 panelists. The packages were allowed to acclimitise to room temperature and an off-odour formation was scored in a 10 point scale where 10 was no off-odour/seafresh odour, 1 a putrid/rotten odour and very strong and unpleasant off-odours. A score of 5-5.5 was the lower acceptance level.

Statistical analysis
Univariate analysis of variance were performed with Minitab 13.3 (Minitab, Coventry, UK) using Tukey's HSD test at level $p<0.05$ (95 %), to obtain confidence intervals for all pair wise differences between level means of temperature and packaging type on each sampling time (days of storage).

Results and discussion

Mortality

After 19 days of storage 41 of 5095 mussels (0.8 %) died during the storage period (Table 2). About 170-200 mussels were tested for mortality at each sampling day from each packaging variant. Dead mussels were found in packaging variant 1, 2 and 3 and mortality increased with storage time.

The best survival rate was observed for O_2-N_2 at -1 °C and with CO_2. Low mortality may be due to a low respiration rate in the mussels at low temperatures (-1 °C) or higher microbial inhibition (CO_2-atmosphere).

Table 2. Mortality of mussels in packages with different atmospheres and temperatures

		Packaging variants				
Days		1 O_2:N_2	2 O_2:N_2-T	3 Air	4 O_2:N_2-1	5 CO_2:O_2
4	Live	198	179	171	180	194
	No.dead (%)	0	0	0	0	0
7	Live	203	179	174	188	190
	No.dead (%)	0	0	0	0	0
11	Live	190	183	121	199	188
	No.dead (%)	0	1 (0.5)	2 (1.6)	0	0
14	Live	198	193	172	179	196
	No.dead (%)	2 (1.0)	1 (0.5)	0	0	0
19	Live	65	192	44	188	200
	No.dead (%)	1 (1.5)	6 (3.0)	18 (29.0)	0	0

Microbiological analysis

The microbiological analysis showed that there were only minor differences in bacterial growth caused by different atmospheres and temperatures (Table 3). One day after packaging the number of psychrotrophic bacteria was log 4.9 cfu/g. This was 1.7 log-units higher than the APC numbers and 2.1 log-units higher than H_2S producing bacteria. The psychrotrophic numbers were higher than the others during the whole storage period. A general trend for APC, H_2S-producing bacteria and psychrotrophic bacteria was a slow growth during the storage period. Comparison of the effects of gas mixtures on microbial growth showed that for APC there were no differences between packages with O_2:N_2, O_2:N_2-T, air, or even O_2:N_2 at -1 °C, Table 3. However, there were significant ($p<0.05$) lower growth in CO_2 atmosphere after 4 and 7 days of storage, compared to O_2:N_2 atmosphere at 4 °C. In the last part of the storage period (day 11 and 19) no differences were observed independent of temperature or package atmosphere. The mean of all samples showed that CO_2:O_2 and O_2:N_2 – 1 °C were significant lower than O_2:N_2 and air at 4°C.

The psychrotrophic counts also were significantly lower for O_2:N_2 at – 1°C and for CO_2:O_2 at 4 °C, and except for variant 1 the same result was found for H_2S-producing bacteria (Table 2). A general trend for all microbiological results was that there were some differences between the variants in the beginning of the storage time, after 4 and 11 days, but not after 14 and 19 days.

Packaging of mussels constitutes special challenges for microbial spoilage and safety compared to other seafood products. Mussels are packaged live without removing

the parts of the organisms that contain high levels of bacteria e.g. gills, intestine, surfaces as for fish products. Due to the filter feeding system, the micro flora of molluscan shellfish therefore directly reflects the environments from where they are harvested. It comprises the natural commensal microorganisms and the microorganisms accumulated from the water during feeding.

Photobacterium phosphoreum have previously been found to be the main spoilage organism of MA packaged salmon (Emborg *et al*. 2002) and on cod at 0 ºC (Dalgaard *et al*. 1993, Dalgaard 1995). *P. phosphoreum* tolerates high CO_2 concentrations and can grow on PCA with salt and are enumerated unspecified as psychrotrophic bacteria. It produces 10-100 fold more TMA per cell than *S. putrefaciens* due to the large size of the former (5 µm) (Dalgaard 1995, Dalgaard *et al*. 1996). As a consequence a lower level of *P. phosphoreum* cell per gram is necessary for the spoilage of fresh fish. The spoilage of chilled, CO_2 packaged fish is seen at a level of 10^7 *P. phosphoreum* per gram (Dalgaard *et al*. 1993). This level was not reached for any of the packages in 19 days of storage.

Chemical analysis
Measurements of TVN –N (Total volatile base nitrogen), TMAO-N (trimethylamine oxide nitrogen), TMA-N (trimethylamine nitrogen, and DMA-N (dimethylamine nitrogen) have been used to determine fish quality. Öelenschläger (1992) concludes that for ice-stored cod the best indicator for spoilage is TMA, produced from the precursor TMAO, and/or TVN-N.
Low levels of TMAO were found in the mussels, never exceeding 3 mg TMAO/100g, and the majority of the samples had levels below 1.5 mg/100g (Table 3). TMA was not detectable during the first 11 days of storage, and then the concentration increased after 14 and 19 days to levels of 1.0 – 2.8 mg/100g. There were no significant differences ($p < 0.05$) in the levels of TMAO and TMA in packages with O_2-N_2 gas mixtures, CO_2 levels or air, and also independent of tidal water conditioning and storage temperature.

Table 3. Individual differences between gas atmospheres and storage temperatures

	Storage time (days)						
	1	4	7	11	14	19	Mean all
APC (log cfu/g)							samples
1-O_2:N_2	3,2	3,8 ab	4,6 a	4,7	4,2	4,8	4,4 ab
2- O_2:N_2-T	3,2	4,3 a	4,6 a	5,4	4,8	4,9	4,8 a
3-Air	3,2	4,2 a	4,0 ab	5,0	4,3	4,8	4,4 ab
4- O_2:N_2-1	3,2	3,9 ab	4,1 ab	4,5	4,5	4,5	4,3 b
5-CO_2:O_2	3,2	3,1 b	3,5 b	4,5	4,1	4,6	3,9 c
Psychrothrophic (log cfu/g)							
1-O_2:N_2	4,9	5,6 a	5,5 a	5,6	5,3	5,9	5,6 a
2- O_2:N_2-T	4,9	5,6 a	5,9 a	5,7	5,7	5,3	5,6 a
3-Air	4,9	5,6 a	5,3 a	5,2	5,0	5,7	5,4 a
4- O_2:N_2-1	4,9	4,4 b	4,7 ab	5,0	5,1	5,4	4,9 b
5-CO_2:O_2	4,9	4,4 b	4,4 b	5,0	4,5	5,4	4,7 b
H2S-producing (log cfu/g)							
1-O_2:N_2	2,8	3,2	3,4 ab	3,9	3,3	4,0 ab	3,6 ab
2- O_2:N_2-T	2,8	3,5	4,0 a	4,3	3,6	3,9 ab	3,9 a
3-Air	2,8	3,5	3,1 ab	4,5	3,9	4,4 a	3,9 a

4- O_2:N_2-1	2,8	3,3	2,9 ab	3,9	3,1	3,4 b	3,3 b
5-CO_2:O_2	2,8	2,7	2,5 b	3,3	3,0	3,9 ab	3,0 b
pH muscle							
1-O_2:N_2	6,5	7,0	6,5	6,5	6,5 a	6,5 a	6,6 a
2- O_2:N_2-T	6,5	7,0	6,6	6,7	6,4 ab	6,2 bc	6,6 a
3-Air	6,5	6,9	6,5	6,5	6,0 c	6,1 c	6,4 b
4- O_2:N_2-1	6,5	6,8	6,5	6,5	6,2 bc	6,4 ab	6,5 ab
5-CO_2:O_2	6,5	6,9	6,7	6,7	6,0 c	6,4 ab	6,6 a
TMA (mg –N/100g)							
1-O_2:N_2	0,0	0,0	0,0	0,0	1,0	1,6	0,5
2- O_2:N_2-T	0,0	0,0	0,0	0,0	1,2	2,0	0,6
3-Air	0,0	0,0	0,0	0,0	2,2	2,6	1,0
4- O_2:N_2-1	0,0	0,0	0,0	0,0	2,0	2,8	1,0
5-CO_2:O_2	0,0	0,0	0,0	0,0	1,6	1,0	0,5
TVN (mg –N/100g)							
1-O_2:N_2	3,5	5,6	8,0	7,2	7,0 a	7,8 a	7,1
2- O_2:N_2-T	3,5	5,2	6,6	6,6	6,8 a	10,0 ab	7,0
3-Air	3,5	5,4	7,2	7,2	15,8 b	13,0 b	9,7
4- O_2:N_2-1	3,5	5,6	6,2	6,2	15,8 b	8,8 a	8,5
5-CO_2:O_2	3,5	5,4	6,0	7,0	17,6 b	12,8 b	9,8
TMAO (mg –N/100g)							
1-O_2:N_2	1,3	0,0	2,8 a	0,4	0,2	1,4	1,0
2- O_2:N_2-T	1,3	1,6	1,4 ab	0,6	0,4	0,6	0,9
3-Air	1,3	0,6	2,8 a	0,0	0,2	0,2	0,8
4- O_2:N_2-1	1,3	0,8	0,2 b	0,2	0,6	1,0	0,6
5-CO_2:O_2	1,3	0,6	0,4 b	0,2	0,0	0,0	0,2

(Table 3 Continued)

Development of total volatile bases increased from 3.5 mg/100g after 1 day of storage to levels of 8- 12.8 mg/100g, with some high spot measurements after 14 days. There were no significant differences ($p < 0.05$) in the levels of TVN in packages with N_2-O_2 gas mixtures, CO_2 levels or air during the first 11 days of storage, but some differences was observed at the end of the storage period. The low levels of TMAO and TMA render these analyses useless in detecting end of shelf, but TVN may be a very promising chemical marker.

pH increased for 4 days then slightly decreased during the rest of the storage period. Mussel pH in live mussels ranged from 7.0 to 6.0 and the pH decreased during the period from 7 to 19 days of storage. There were no significant differences ($p < 0.05$) in the levels of pH in packages with O_2-N_2 gas mixtures, CO_2 levels or air during the first 11 days, but differences were observed after 14 days of storage.

Gas measurements

CO_2 levels in the headspace of chilled MA packages were 1.6-7.6 % the day after packaging. Solubility of CO_2 increases with decreasing temperature (Carroll *et al.* 1991). The level of CO_2 increased in the headspace during storage to levels between 8 and 22 % at day 19. The level of oxygen was from 17.4 (air) to 33.6 % after one day of storage. The level of oxygen decreased during storage, and there were low levels

(<5 %) O_2 left in all packages except in the package with air (0.0 %) after 19 days of storage.

The oxygen levels after 1 day was 33 % for packages with added oxygen and 17.4 % for packages with air. Oxygen is used by the mussels' respiration and no oxygen was left in packages with air after 11 days. Oxygen level decreased in the other packages to 5.6 – 6.7 % in O_2:N_2 packages.

Exudate formation and pH in exudate
The results from formation of exudate in the packages and from measurements of the pH in the exudate are shown in Table 4. The exudate formation was high in all variants and stabilised around 20 % from day 7 of storage. The exudates in the conditioned tidal shell (variant 2) were not lower than the other non-trimmed shells. Reduction of the exudate formation must be a goal for further trials, in order to reduce the amount of over-weight the producers need in the package and in order to meet the declared net-weight towards the end of storage. The pH in the exudate did not differ between the variants. At day 19 a drop in pH was observed for all variants except no. 4 stored at superchilled conditions.

Table 4. Individual differences between exudate and exudate pH, and raw odour in blue mussels stored under modified gas atmospheres and storage temperatures.

	Storage time (days)				
	4	7	11	14	19
Exudate (%)					
1-O_2:N_2	13.4	18.2	17.8	15.8	18
2- O_2:N_2-T	17.7	21.2	20.0	19.5	22.3
3-Air	19.2	19.3	22.6	16.3	29.7
4- O_2:N_2-1	18.6	19.2	18.6	23.4	17.2
5-CO_2:O_2	16.2	19.2	14.6	19.1	17.4
pH in exudate					
1-O_2:N_2		6.7	6.7	6.8	6.3
2- O_2:N_2-T		6.6	68	6.7	6.5
3-Air		6.8	6.9	6.8	6.0
4- O_2:N_2-1		6.7	6.8	6.7	6.7
5-CO_2:O_2		6.5	6.6	6.8	6.4
raw odour					
1-O_2:N_2	9.0	7.0	7.0	5.3	4.0
2- O_2:N_2-T	7.7	6.7	7.0	5.7	4.7
3-Air	8.0	7.0	6.7	4.3	2.0
4- O_2:N_2-1	10.0	8.3	7.0	6.0	6.0
5-CO_2:O_2	7.7	8.0	7.0	5.7	4.3

Raw odour
Raw odour scores are shown in Table 4. High quality (>7.5) was maintained in all variants at day 4 of storage, but only in O_2:N_2 atmosphere at superchilled conditions. CO_2:O_2 at chilled conditions held high quality after 7 days of storage. All

variants had acceptable odours after 11 days of storage, the lowest being in the air packaged mussels. Off-odour in all packages was strong from day 11 of storage directly after package opening; however 15 minutes later most of these strong off-odours were lost. At day 14 of storage the odour-quality was only marginal in MA-packages and the air samples were spoiled, and only the superchilled samples (variant 4) were not totally spoiled after 19 days of storage.

Conclusions

In the measurements carried out in this experiments the superchilled mussels packaged in 60 % O_2 and 40 % N_2, and the chilled mussels packages in 50 % CO_2 and 50 % O_2 attained the lowest microbial numbers, lowest TMA/TVN formations and highest odour scores. Using MA for live mussels and keeping the storage temperature low a long shelf-life is obtainable. Further studies should include the combination of superchilled storage together with e.g 50-50 CO_2-O_2 mixtures. Methods to reduce off-odour upon package opening and to reduce the formation of exudate should also be sought in future studies.

Acknowledgement

This work was a part of a project "Mild preservation of new farmed seafood species" supported by the Norwegian Research Council and "Marinepack" supported by Norwegian Research Council and industrial partners.

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