QUALITY CHARACTERISTICS OF NEWFOUNDLAND CULTURED BLUE MUSSELS (Mytilus edulis) AT PRE- AND POST-HARVEST STAGES

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Quality characteristics of Newfoundland cultured blue mussels (*Mytilus edulis*) at pre- and post- harvest stages

By

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Abstract

Despite the rapid increase in production of Newfoundland cultured blue mussels in the last few years and publication of various studies addressing aspects of culturing and production, information with regard to the quality of cultured blue mussels at pre- and post-harvest stages is scarce and fragmented in the literature. In order to produce, process, package, transport, distribute, market, store and consume blue mussels with superior quality as well as to fill an important gap in the scientific literature regarding the quality of Newfoundland blue mussels, this research was designed to: (1) enumerate bacteria from cultured blue mussels from Newfoundland using different forms of agar; (2) evaluate changes in shell size, meat content, microbial quality, fatty acids, sterols and lipid composition of blue mussels during their growth at two aquaculture sites; (3) evaluate the effect of ice storage on proximate composition and quality of mussels; (4) assess the use of bacterial fatty acids as microbial quality indicators; and (5) examine the effects of mechanical handling and ascorbic acid treatment on lipid oxidation and quality of blue mussels.

Results indicate that marine agar is better than plate count agar to evaluate microbial quality of bivalves. Ocean water temperatures at the aquaculture site affected chlorophyll *a* content or algae forms and their fatty acid compositions, as well as microbial quality of water, which in turn affected microbial quality, lipid and fatty acid composition, meat content and shell size of cultivated mussels. The measurement of n-3 polyunsaturated fatty acids (PUFA) and phytosterols in Newfoundland cultured mussels may add to there market value. Cultured mussels can reach their commercial size within a year following cultivation. Their optimum harvest time would be either spring or fall of each year.

Meanwhile, microbial growth and lipid oxidation of ice stored mussels leads to their spoilage and an increase in flavor deterioration index. Conventional agar was the best substrate to monitor microbial changes during ice storage. Other potential indicators would be the bacterial fatty acids *ai*15:0, *i*17:0 or total bacterial fatty acids as determined by H_2SO_4-MeOH transmethylation reagent. Ice stored mussels were more susceptible to lipid oxidation than the fresh mussels as reflected in their high 2-thiobarbituric acid reactive substances (TBARS) values. However, the current rapid mechanized handling practice in the blue mussel industry, which includes washing, sorting, washing again and packaging, should not affect their oxidative status. Furthermore, exposing live blue mussels to specific concentrations of ascorbic acid may control lipid oxidation during their ice storage for only 5 days. Thereafter, the remaining ascorbic acid may become a pro-oxidant in ice stored mussels.

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Table of Contents

List of abbreviation

ACAsc	Antioxidant capacity of ascorbic acid
ACL	Antioxidant capacity of lipid soluble antioxidant
ACS	American Chemical Society
ACT	Antioxidant capacity of tocopherol
ACW	Antioxidant capacity of water soluble antioxidant
ai	Anetiso
APC	Aerobic plate count
Asc	Ascorbic acid
BF_3 - MeOH	Boron trifluoride methanol
FAME	Fatty acid methyl esters
BHA	Butylated hydroxylanisole
BHT	Butylated hydroxyltoluene
BSA	N, O- bis-trimethylsilyl acetamide
BSTFA	Bis (trimethyl silyltrifluroacetamide)
CA	Charles Arm
CFU	Colony forming unit
DHA	Docosahexanoenoic acid (22:6 n-3)
EPA	Eicosapentaenoic acid (20:5 n-3)
FH	Fortune Harbor
FSW	Filtered sea water
GC	Gas chromatography
GC - MS	Gas chromatography - Mass spectrometry

H_2SO_4 - MeOH	Sulfuric acid – methanol
i	Iso
MA	Marine agar
MUFA	Monounsaturated fatty acids
NaAs	Sodium ascorbate
PCA	Plate count agar
PG	Propyl gallate
PL	Phospholipids
PPC	Psychrotrophic plate count
PSU	Particle salinity units
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
NaAs	Sodium Ascorbate
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substances
TBHQ	tert-Butylhydroxyquinone
TCA	Trichloroacetic acid
THB	Total heterotrophic bacteria
TL	Total lipids
TMS	Trimethyl silyl

List of tables

List of figures

List of appendices

Chapter I

General Introduction and Literature Review

1.1 Seafoods

Seafoods are consumed for their nutritional and potential health benefits as well as distinct flavor and taste. They represent up to 25% of the animal protein supply world-wide (Shahidi, 1998). Furthermore, n-3 polyunsaturated fatty acids (PUFA), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), derived from α-linolenic acid by chain elongation and desaturation, are prevalent in seafoods (Newton, 2000). Epidemiological studies, from Greenland and Japanese populations, have indicated that consumption of seafood has beneficial effects in the prevention of various clinical disorders, including cardiovascular risk factors (Senanyake and Shahidi, 2000). In addition, diets enriched in n-3 PUFA delay the growth of experimentally induced tumours (Gill and Valivety, 1997).

Polyunsaturated fatty acids (n-3 and n-6) are critical components of cell membranes, and are therefore important in the structure, dynamics and control of membrane-associated biochemical functions (Newton, 2000). These observations have been supported by recent animal and human clinical studies (Uauy *et al.*, 2000). The use of DHA in the treatment of inflammatory diseases such as arthritis as well as asthma and diabetes has shown satisfactory results (Gill and Valivety, 1997). The most significant effects of DHA relate to the normal growth of brain and retina, and thus it is approved for incorporation into infant formula (Newton, 2000).

1.2 Blue mussels (*Mytilus edulis*)

1.2.1 Biology

Blue mussels are bivalve (two valves joined at the top) molluscs, which also include oysters, scallops and clams. They are filter feeders which retain microscopic phytoplankton cells as the main source of their food (Shumway *et al.*, 1985). Mussels usually spawn in late spring or early summer by releasing their eggs and sperm into the water. The larvae shell appears within 48 h of external fertilization, After another 3-4 weeks, the larvae grow to juveniles with a foot and a gill. These juvenile mussels anchor themselves to hard surfaces by secreting a byssus. After one year, these mussels (spat) are used as starting materials (seed) in the culture of mussels (Sutterlin *et al.*, 1981).

1.2.2 Distribution and production

Wild blue mussels are distributed in cool temperature regions around the world, from the Kara Sea to the Mediterranean Sea, East Asia, and from North Carolina to Canada. They live on coasts that have hard substrates such as rocks, ropes, wood and boats, inter-tidally to 40 m deep, attached by threads to rocks and piers within the harbors, estuaries and rocky shores of the open coast (Anonymous, 2001a). Wild populations are supplemented by aquaculture to accommodate the high demand and protect the natural mussel beds. Cultured mussels generally have a higher meat yield (20-25%) than wild mussels. The meat is of high quality and free of grit and sand (Sutterlin *et al.*, 1980; Anonymous, 2001a; Anonymous, 2001c).

World production of cultured mussels in the year 2000 was 1.3 million tons, of which 80% were blue mussels (*Mytilus edulis*). The main producers are China, Spain, France,

Netherlands, Britain, Ireland, Greece, Australia, Chile and New Zealand (Mcleaod, 2000; Anonymous, 2003a). Canadian production of blue mussels in 2000 was 21,287 tons, of which Newfoundland produced only 1,045-1,051 tons (Anonymous, 2000; MacDonald *et al.*, 2002). Nonetheless, blue mussels are the single largest cultured shellfish product of Newfoundland. More than 95% of the blue mussels cultivated in Newfoundland are grown in the Notre Dame Bay area. Cultivation began in 1984, but commercial production started in the early 1990s (Anonymous, 2003b).

1.2.3 Cultivation

Mussels are usually cultured in sheltered areas by three methods, plastic rope with mesh tubes hanging from rafts, post culture and bottom culture (Anonymous, 2003a). Blue mussels are cultured throughout Newfoundland with the rope technique. The ropes are anchored at both ends and supported by buoys at certain intervals. Spat (approximately one year old mussels) are loaded into the mesh tubing or socks and tied at intervals on the long ropes and suspended approximately 4 m below the surface of the water (Anonymous, 1988). The mussels reach market size (shell length of 5 – 6 cm) after another one or two years with a bluish-black shell color and white, pink or dark blue meat color (Anonymous 2001b; Anonymous, 2001c).

1.2.4 Harvesting and processing

Cultured mussels (2-3 years old) are harvested by pulling the socks out of water using powerful winches, loading them into small boats and transporting them to the processing plant. Upon arrival, the mussels are stripped from the socks and washed

thoroughly (Anonymous, 2001b; Anonymous, 2001c). Filtered sea water (5-15°C) is used in all washing steps and the temperature in the processing plant is usually 2-6°C. Clumps of mussels are separated, sorted and washed again before final packaging in bags with a wide mesh. The bags are placed in insulated containers or special boxes and kept at 2-4°C (Anonymous, 2003b). Mussels are mainly marketed live and must be kept as such until consumption. However, frozen and vacuum packed, raw or cooked mussels, are also marketed (Anonymous, 2001c).

1.2.5 Nutritional value

The nutritional quality of blue mussels varies depending on season of harvest, place of origin, method of cooking and techniques used for analysis (Choi, 1970; Suryanarayanam and Alexander, 1972; Slabyj *et al.*, 1977; Slabyj *et al.*, 1978, Anonymous, 2002 a; Anonymous 2002 b). Table 1.1 summarizes the proximate and main nutritional composition of cultured blue mussels. Three ounces (84.9 g) of cooked mussels provide about 1/3 (20 g) of the daily-recommended protein requirement. Furthermore, mussel proteins are easily digested because they contain less connective tissue than red and poultry meats (Anonymous, 2002b; Anonymous, 2002c). Blue mussels are also a rich source of iron, selenium and vitamin B_{12} (providing 20 % or more of the daily recommended intake) and a good source of zinc (providing 10-20% of recommended daily intake) (Anonymous, 2002a; Anonymous, 2002c).

Cultured blue mussels are rich in healthful n-3 PUFA. The n-3 PUFA content of blue mussels (0.5 g/100 g) is greater than that of many popular seafoods, including Atlantic cod, halibut, yellowfin tuna, skipjack tuna, blue crab, shrimp, snow crab, spiny

lobster and northern lobster (Anonymous, 2000b). Meanwhile, cultured blue mussels contain less cholesterol than 100 g beef steak or other types of seafoods (Anonymous, 2001b; Anonymous, 2002d).

Blue mussels contain various sterols, including phytosterols (Appendix 1.1) with cholesterol being at relatively low concentrations compared with other types of seafoods and meat (Krzynowek, 1985; King *et al.*, 1990; Napolitano *et al.*, 1993). The major phytosterols (plant sterols) are sitosterol, stigmasterol and campesterol which are not only present in vegetable oils, cereals, nuts and vegetables, but also in mussels and scallops (King *et al.*, 1990; Napolitan *et al.*, 1993; Piironen *et al.*, 2000). The daily recommended intake of cholesterol is 100 mg, which is mainly obtained from animal sources. However, high intakes of cholesterol may lead to serious cardiovascular problems (King *et al.*, 1990). Various nutritional and clinical studies have indicated that phytosterols can reduce plasma cholesterol concentration, and thus lower or eliminate cardiovascular clinical disorder risks (Miettinen *et al.*, 1995; Pelletier *et al.*, 1995; Jones *et al.*, 1999). Other clinical studies indicated that consuming mussels is beneficial in reducing triacyglycerols in blood, and therefore reduces cardiovascular risks (Anonymous, 2002c). Meanwhile, a clinical study showed that consuming mussels instead of meat, eggs and cheese increased the amounts of "good" cholesterol (high density lipoprotein (HDL) and thus decreased the ratio of "bad" to "good" cholesterol (low density lipoprotein (LDL) : HDL) (Anonymous, 2000b; Anonymous, 2002b).

Table 1.1 Nutritional and proximate composition values of cultured blue mussels as specified.*

Nutritional or Proximate composition	Units (per100g)
Ash	1.4 - 2.1 g
Moisture	77.0 - 81.8 g
Carbohydrates	0.95 - 5.8 g
Proteins	9.0 – 24 g
Fat	1.3 - 3.5 g
Cholesterol	28 - 63 mg
Omega-3 fatty acids	0.4 - 0.7 g
Sodium	270 - 286 mg
Iron	2.46 – 3.40 mg
Selenium	0.2 mg
Vitamin B_{12}	25 μg

*Results were summarized from Choi, 1970; Suryanarayanam and Alexander, 1972; Slabyj *et al.*, 1977; Slabyj *et al.*, 1978; Anonymous, 2002a; Anonymous, 2002b).

1.3 Quality of seafoods

1.3.1 Definition

Consumer perception of seafood safety and quality differs from that used by food scientists and nutritionists. Safety of seafoods is compromised by contamination with micro-organisms or chemicals, while quality is related to the appearance, odor, flavor and texture (Anderson *et al.*, 1991; Shahidi, 1998). One study (Ackman and Zhou, 2003) reported that the ratio of two common isoprenoid hydrocarbons pristane and phytane, which may affect the quality of blue mussels, are important in distinguishing their origin from algal source or environmental oil contamination.

Surveys are conducted regularly to examine the ability of consumers to discriminate between purchased seafoods of differing quality. One survey showed that 84% of consumers believed farm-raised mussels to be better than wild mussels (Anderson *et al.*, 1994). In this thesis, the term quality will be used in the context of how good are cultured Newfoundland blue mussels for human consumption based on their microbial, nutritional and flavor status.

1.3.2 Measurement techniques

Several microbial, chemical, biochemical and sensory techniques have been used to examine the quality of seafoods (Bayne and Thompson, 1970; Slabyj, 1978; Krzynowek, 1985; Sigurgisladottir *et al.*, 1993; Orban *et al.*, 2001). There is no universal method or instrument that can determine the quality of seafoods. However, sensory methods are perhaps the most accurate for predicting the quality, but these are not used for routine analysis due to the need for highly trained panels and the time consuming nature of

the procedures involved (Shahidi, 1998). Nonetheless, microbial methods are used in conjunction with sensory analysis to accurately predict the shelf life of seafoods (Koutsoumans and Nychas, 1999). The type of analysis, accuracy and the availability of instruments will determine the selection of a technique or an instrument to examine the quality of blue mussels.

1.3.2.1 Microbial content

Microbial content of seafoods affects their quality. Change in quality due to change in microbial content is generally evaluated by conventional plate count methods (Hunt *et al.*, 1984; Neufeld, 1984), which includes pour plate, spread plate and agar droplet (Busta *et al.*, 1984; Harrigan and Park, 1991a). Total plate count represents the total number of bacteria that are capable of forming visible colonies at a given temperature. Specific chemical agents may be incorporated into the agar in order to differentiate between bacterial species and enumerate target or indicator micro-organisms (Harrigan, 1998a). Other methods include membrane filtration and most probable number (Harrigan and Park, 1991a). Several rapid methods have been developed to handle large amounts of sample within reasonable time limits (Slabyj and Bolduc, 1987; Harrigan and Park, 1991b; De Boer and Beumer, 1999), including direct microscopic counts, bioluminescence measurement of ATP, direct epifluorescense (Vanne *et al.*, 1996; Duffy and Sheridan, 1998), flow cytometry, impedimetry and microcalorimetry (Harrigan and Park, 1991b; Shelef and Firstenberg-Eden, 1997; Harrigan, 1998b), fluorescence *in situ* hybridization using an array scanner (Stender *et al.*, 2001), using protease indicator (Jabbar and Joishy, 1999), or turbidimetric, dye reduction, electrometric (Harrigan and Park, 1991b),

capacitance (Russel, 1998), rapid CO_2 evolution (Chew and Hsieh, 1997), immunological and gene technology methods (Jay, 1996; Vanne *et al.*, 1996).

Despite the development of rapid microbiological methods, most of the provincial, national and international microbiological specifications for foods are still based on various types of colony counts from agar plates (Anonymous, 1992; Chung *et al.*, 1997; Bolton, 1998). Furthermore, standard plate count techniques are used to validate newly developed microbiological techniques and methods (Shelef and Firstenberg-Eden, 1997; Bolton, 1998; Townsend and Naqui, 1998; Salo *et al.*, 2000) and as indicators of the presence of pathogenic bacteria in foods (Bremer and Osborne, 1998; Siragusa *et al.*, 1998). Therefore, there is a constant search for an agar medium that gives the highest and most consistent recoveries of micro-organisms. Many of the newly developed methods may still take between 12 and 24 h (1-2 working days) for testing because of pre-enrichment steps or the need to prepare pure bacterial cultures (Harrigan and Park, 1991b). The microbial content of seafoods must be known before the product leaves the production area. Therefore, rapid identification methods of micro-organisms in seafoods are important for the aquaculture industry and public safety. These methods should not require the use of conventional agar, pre-enrichment steps or pure bacterial cultures and must be finished within 4-5 h. Fatty acids may be used to develop rapid methods that reflect the microbial content of seafoods.

Some specific fatty acids (Appendix 1.2) can be identified in a given micro-organism, and thus may serve as lipid biomarkers (Findly and Dobbs, 1993; Annous *et al.*, 1997). Iso- and anteiso-branched chain fatty acids have been detected in the well-known food-borne pathogen *Listeria monocytogens* (Mastronicolis *et al.*, 1996). Cyclopropanoic

acid has been observed in Gram-positive and Gram-negative bacteria, but not in fungi or animal biomarkers. Furthermore, hydroxy fatty acids (3-OH) such as 3-hydroxymyristic have been detected in Gram-negative bacteria such as *Haemophylus* (Osipov and Turova, 1997). Recently, 16:4n-1 has been suggested as a marker for the toxic alga *Pseudo-nitzchia* (Budge and Parrish, 1999). Therefore, lipid markers have been used to investigate microbial communities in clinical (Ahtonen, 1997; Osipov and Turova, 1997) and environmental samples (Smith *et al.*, 2000; Rutters *et al.*, 2002). However, the use of lipid markers to examine the microbial content and predict the storage of seafoods has not yet been reported. Because fatty acid determination may require only 2-4 h, the lipid marker method may be the most practical method to examine the microbial content of seafoods, including blue mussels.

1.3.2.2 Nutritional quality

Several standard and modified chemical and instrumental methods have been used to examine the nutritional qualities of seafoods, including blue mussels. These include determination of proteins by micro-Kjeldahl, the extraction of fat with a mixture of chloroform and methanol, fatty acid composition by gas chromatography, lipid classes by thin layer chromatography-flame ionisation detection (Iatroscan), sterol concentrations by gas chromatography or gas chromatography coupled with mass spectroscopy, vitamins and antioxidants by high performance liquid chromatography, carbohydrates by spectrophotometric or enzymatic methods, and moisture content determined by weight difference before and after oven drying (Bayne and Thompson, 1970; Slabyj, 1978;

Krzynowek, 1985; King *et al.*, 1990; Nalepa *et al.*, 1993; Sigurgisladottir *et al.*, 1993; Orban *et al.*, 2001; Siddaiah *et al.*, 2001).

1.3.2.3 Flavor quality loss due to lipid oxidation

Oxidation of PUFA and pigments (carotenoids) in seafoods contributes to the loss of *flavor*, *color*, and texture. Furthermore, oxidation may reduce the content of nutritional n-3 PUFA (Shahidi, 1998; Subagio and Morita, 2001). In the presence of oxygen and catalysts, autoxidation proceeds via self-generating destruction of PUFA to produce free radicals and peroxides. The primary oxidation products, usually hydroperoxides, are further degraded to secondary oxidation products such as aldehydes, hydrocarbons, ketones and alcohols (King *et al.*, 1995).

There is no universally accepted technique that can be employed to monitor the progression of lipid oxidation. Therefore, several chemical, instrumental and sensory techniques are used to monitor the oxidation of seafoods (St. Angelo and Spainer, 1993). While some techniques measure the loss of reactants such as oxygen or substrates such as fatty acids, others measure primary oxidation products such as free radicals, hydroperoxides or conjugated dienes and secondary oxidation products, namely alcohols, aldehydes, hydrocarbons and ketones (Shahidi, 1998; Shahidi and Wanasundara, 1998). Since some factors such as pH, temperature and content of antioxidants affect the oxidation of seafood lipids, these variables may also be measured and related to the progression of oxidation (Melton, 1983; King *et al.*, 1995).

The TBA (2-thiobarbituric acid) test is frequently used as a measure of lipid oxidation in meats, including seafoods (Table 1.2). One molecule of malonaldehyde (MA),

which is a secondary product of lipid oxidation, reacts with two molecules of TBA to form the TBA-MA complex, which has a maximum absorbance at 532 nm (Appendix 1.3) (Guzman-Chozas *et al.*, 1998). The accuracy of the TBA test has been questioned due to the reaction of TBA with other molecules such as sugars and oxidized proteins (Hoyland and Taylor, 1991). Moreover, TBA might also react with other oxidation products such as 2-alkenals and 2,4-alkadienals, and thus TBARS (TBA reactive substances) reflect the total amount of aldehydes rather than MA alone. Nonetheless, TBA values correlate well with sensory data (Melton, 1983; Shahidi, 1998).

1.4 Control of quality loss due to lipid oxidation and applications of antioxidant in seafoods

Seafoods have natural antioxidant systems which include enzymes as well as lipids and water-soluble compounds that can control lipid oxidation (Gieseg *et al.*, 2000; Bell *et al.*, 2000; Undeland *et al.*, 2003). However, the antioxidant capacity of seafoods may be affected during stress such as from harvesting, processing, packaging and distribution. Therefore, proper application of synthetic antioxidants, natural antioxidants and/or use of inert gas or vacuum packaging have been frequently used to control the oxidation of seafood lipids (Colby *et al.*, 1995; Young *et al.*, 2000; Abdel-Aal, 2001; Tang *et al.*, 2001; Medina *et al.*, 2003; Harpez *et al.*, 2003).

Table 1.2 Application of the 2-thiobarbituric acid reactive substances (TBARS) technique to monitor oxidation of muscle foods, including seafoods.

Application	Reference
Retail meat and fish	Siu and Draper, 1978
Muscle foods	Melton , 1983
Channel catfish	Gaitlin III *et al.*, 1992
Fresh and heat processed mackerel	Shahidi and Spurvey, 1996
Frozen and fresh meat	Wang and Brewer, 1997
Carp fillet	Khalil and Mansour, 1997
Fish	Dulvik *et al.*, 1998
Beef patties	Lee *et al.*, 1999
Fish	Sant' Ana and Mancini-Filho, 1999
Freeze dried meat	Wilkinson *et al.*, 2001
Meat, poultry and fish	Tang *et al.*, 2001
Atlantic mackerel	Saeed and Howell, 2000

Ideal synthetic and natural antioxidants for food applications should meet certain criteria such as safety, ease of incorporation, effectiveness at low concentration and absence of undesirable odor, *flavor* and *color* (Colby *et al.*, 1995). The most common lipid-soluble natural antioxidants are tocopherols. The main biochemical function of tocopherols is believed to be protection of PUFA against peroxidation. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are examples of commonly used phenolic antioxidants (King *et al.*, 1995; Halliwell *et al.*, 1997).

Antioxidants together with vacuum packaging are more effective in retarding oxidation of carp fillets than antioxidants alone without vacuum packaging (Khalil and Mansour, 1998). Lipid oxidation of refrigerated or frozen rainbow trout muscle has been controlled using dietary α-tocopherol supplement and surface application of the oleoresin rosemary (Akhtar *et al.*, 1998). Ascorbic acid (Asc) can control lipid oxidation in thornfish (Chien and Hwang, 2001), catfish (Erickson, 1997), antarctic fish (Gieseg *et al.*, 2000) and rainbow trout (Ciereszko *et al.*, 1999) by natural occurrence, dietary supplement or physical treatment.

1.4.1 Ascorbic acid

Ascorbic acid (Asc) has several functions in biological systems (Halliwell and Whiteman, 1997). Quantitative data on Asc for several fish, crustaceans and some molluscs indicate that Asc is vital for growth, behavior, immune response, wound healing, reproduction and response to chemical, biological or physical stress (Moreau *et al.*, 1999). Most seafood species lack the enzyme L-gluonolactone oxidase which catalyses the last

step in the conversion of glucose to Asc, and therefore it must be obtained from dietary sources (Moreau and Dabrowski, 2001).

Deficiency of Asc in some fish seafood species may increase mortality and infection rates (Gabaudan and Verlhac, 2001). L-Ascorbic acid is very labile and may be destroyed during feed preparation or extraction and analysis, therefore other more stable forms of Asc such its sodium salt, 2-glycoside, 2-phosphate or sulfate derivatives may be used for fish nutrition (Halver and Felton, 2001). L-Ascorbic acid (Asc) (Appendix 1.4) is an effective antioxidant which can help maintain iron- and copper-containing enzymes in their required reduced form. It can scavenge free radicals in biological systems, including lipids, thus preventing their damage by oxidation. It acts synergistically with tocopherol to reduce lipid peroxide radicals and regenerate tocopherols for further antioxidant reactions. Furthermore, Asc can also exhibit pro-oxidant activities depending on concentrations and the presence of metal ions in the system (Halliwell and Whiteman, 1997; Taso, 1997).

1.4.2 Measurement of antioxidants in biological systems

Antioxidants in biological systems, including seafoods, can be measured by various methods. Tocopherols in lipid extracts have been measured using spectrophometric, chemiluminescence and HPLC methods while Asc content has been detected by spectrophotometric, flow injection, capillary electrophoresis, chemiluminescence or HPLC procedures (Dabrowski and Hinterleitner, 1989; Lewin and Popov, 1994; Popov and Lewin, 1996; Eitenmiller and Landen, 1999 a and b). Asc is usually extracted from biological samples at low temperatures using 5% trichloroacetic acid (5% TCA) prior to antioxidant measurement (Eitenmiller and Landen, 1999).

The luminol-enhanced chemiluminescence method to detect antioxidants in biological systems, used in this work, is based on oxidation of luminol (3-aminophthalihydrazide) with a peroxy radical in the presence of dimethylsulfoxide, dimethylformamide, water or alcohol (Wheatley, 2000). This reaction yields a luminol radical or luminol dianion (Appendix 1.5) that emits light, which can then be detected by a luminometer. Antioxidants extracted from biological samples inhibit this chmiluminescence in proportion to their concentrations. The antioxidant capacity can be calibrated with standard antioxidants such as ascorbic acid or Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid), (Lewin and Popov, 1994; Popov and Lewin, 1996; Amarowicz *et al.*, 1999; Amarowicz, *et al.*, 2002).

1.5 Objectives and rationale

The blue mussel (*Mytilus edulis*) has been a favourite shellfish in Europe and the United States, and its cultivation is a fast-growing industry in Newfoundland. Experimental trials started in the 1970s and were followed by large-scale commercial production in the early 1990s (Anonymous, 2003b). In 1995 the production of blue mussels was 411 tonnes ($CAN 295,000), increasing rapidly to reach 1,700 tonnes in 1999 ($CAN 3,800,000) (Anonymous, 2000a). The blue mussel industry is expected to expand and grow further to meet the high demand in Europe, United States and Canada.

The economic importance of cultured Newfoundland blue mussels has lend to much research in this area. Sutterlin *et al.* (1981) compiled a report summarising mussel culture in Newfoundland waters, which includes a comparison between cultured and wild blue mussels in addition to other information related to mussel aquaculture sites. By 1988,

a guide to long line mussel culture in Newfoundland had been prepared (Anonymous, 1988). This culture method has since become the most popular in Newfoundland. In 1990, Mills assessed the culture of blue mussels in western Newfoundland and found that all sites examined were suitable for mussel culture. However, the author recommended further scientific evaluation of the sites examined. Dabinett and Clements (1994) investigated mussel productivity and spatfall in Notre Dame Bay and recommended that the mussel growers lower their mussel socks to a deeper area where food and temperature was more suitable for mussel growth. More recent studies (Budge and Parrish, 1999; Budge *et al.*, 2003) have examined the lipid composition of Newfoundland plankton and cultured mussels and found that up to 50 % of the lipids were PUFA with DHA and EPA as the main components of n-3 PUFA in mussels. Ibarra *et al.* (2000) developed a method to calculate meat yield of Newfoundland mussels. This method was based on two simple formulas, in the first formula the sum of steamed meats and shells was used, and in the second formula live weight was used. Furthermore, Struthers *et al.* (2002) found seasonal and geographic differences in blue mussel shell mechanical properties and breakage patterns.

Despite the rapid increase in production of Newfoundland cultured blue mussels in the last few years and various studies addressing different aspects of culturing and production, information on the quality of cultured blue mussels is scarce and fragmented in the literature. In order to produce, process, package, transport, distribute, market, store and consume blue mussels with superior quality, as well as to fill an important gap in the scientific literature regarding the quality of Newfoundland blue mussels at pre- and post-harvest stages, this research was designed to: (1) enumerate bacteria from cultured blue

mussels from Newfoundland using different types of agar; (2) evaluate changes of shell length, meat content, microbial status, fatty acids, sterols and lipid composition of blue mussels during their growth at two aquaculture sites (Charles Arm and Fortune Harbor ; (3) evaluate the effect of storage on ice on proximate composition and quality of mussels; (4) assess the use of bacterial fatty acids as microbial content indicators; and (5) examine the effects of mechanical handling and ascorbic acid treatment on lipid oxidation and quality of blue mussels.

Chapter II

Materials and Methods

2.1 Materials

Blue mussels and scallops were obtained respectively from natural populations, aquaculture sites and local seafood distributors. Details of sample origin are mentioned in the experimental design section. Compressed air, hydrogen and UHP helium were obtained from Canadian Liquid Air Ltd. (St. John's, NL). Marine and plate count agars were purchased from Becton Dickinson Microbiology Systems (Sparks, MD). Trichloroacetic acid was obtained from Fisher Scientific (Nepean, ON). PUFA 1, PUFA 3, Supleco 37 component FAME mixture, and bacterial fatty acid methyl ester mixture, N,O-bis(trimethylsilyl) acetamide (BSA) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Supelco, Canada Ltd. (Oakville, ON), GLC-461 standard fatty acid methyl ester preparation was obtained from Nu-Check-Prep, (Elyasin, MN). Silicic acid powder (100-200 mesh), 2-thiobarbituric acid (TBA), 1,1,3,3-teteramethoxypropane (TMP), L-ascorbic acid (ACS grade), L-ascorbic acid (SigmaUltra), sodium ascorbate, 14% BF_3-methanol, were from Sigma Chemical Co. (St. Louis, MO). Reagents, standards and buffers required for photochemiluminescence measurements of ascorbic acid and tocopherol were obtained from Analytica Jena USA (Delaware, OH). All other chemicals were ACS grade or better.

2.2 Sample collection and transportation

Appropriate numbers of mussels (3-30) or scallops (3-4) were used depending on their size, the experiment and the amounts required for various analyses. Plankton were collected with a SEA-GEAR model 900 plankton net (SEA-GEAR Corp., Melbourne, FL). The net length was 3 m with a 1 m mouth opening and a mesh size of 5 μm. Ten to fifteen millilitres of the suspended sample material were filtered onto a pre-combusted 47 mm GF/C Whatman glass fiber filter using a Fisherbrand filtration apparatus. The glass fibre filter, which contained the plankton material, was used in subsequent analyses. Details of sampling location and intervals are described in the experimental design section. The samples were transported to the laboratory in an insulated styrofoam container filled with crushed ice.

Mussels (Fortune Harbor and Charles Arm) were harvested by pulling the socks out of water, loading in a small boat and transporting to the processing plant. Upon arrival, the mussels were stripped from the socks and washed with filtered seawater. Mussels were separated from clumps, sorted and washed again before final packaging in socks with a wide mesh. Filtered seawater (10°C) was used in all washing steps and the temperature in the processing plant was 10-15°C.

2.3 Physical measurement of the samples

Mussel shell length (± 0.1mm) was measured using a Traceable Digital Caliper (Fisher scientific, Nippon, ON). The wet weight (± 0.1mg) of mussels was determined with a Mettler AE 100 analytical balance (Mettler Instruments AG, Zurich, Switzerland). Ocean water temperature and salinity were measured with a Sea-Bird SBE25 Sea logger CTD

(Sea-Bird electronics, Inc. Bellevue, WA) fitted with a WETStar fluorometer (WET Labs, Philomath, OR) for chlorophyll determination. The pH of sea water and homogenized mussel samples was determined by immersing a pH electrode into water or the homogenate for 60 s and observing the pH on a pre-calibrated Fisher Accumate pH meter model 805 MP (Fisher Scientific, Fair Lawn, NJ).

2.4 Sample preparation for analysis

Bivalve shells were opened and the shell liquor and meats collected in a beaker and subsequently homogenized for 60s period with a commercial Waring blender (Dynamic Corporation of America, New Hartford, CT). All subsequent analyses were performed on this homogenate. Ten grams of the homogenate were mixed with 90 mL 0.1% peptone water (10 g peptone and 5 g of NaCl in 1 L distilled water, pH: 7.2 - 7.3) for a further 30 s. Serial decimal dilutions from this mixture were carried out in 9 mL 0.1% peptone water. Water samples were collected with Niskin bottles.

2.5 Microbial analysis of prepared samples

Diluted samples (0.1 to 0.5 mL) of the prepared samples were spread with a sterile glass spreader on pre-poured marine and/or plate count agars (Becton Dickinson Microbiology Systems; Sparks, MD). Inoculated plates were incubated at 4°C for 10 days to determine psychrotrophic counts (PPC), and at 25 or 30°C for 48 h to determine aerobic plate counts (APC). Bacterial colonies on the agar were counted with a Quebec counter and recorded as colony forming unit CFU per mL ocean water or g mussel meat.

2.6 Determination of 2-thiobarbituric acid reactive substances (TBARS) as an index of off-flavor of mussel meat

Changes in oxidative state and hence off flavor development of mussel meat were evaluated with the direct 2-thiobarbituric acid-reactive substances (TBARS) test as described by Siu and Draper (1978) with minor modifications. Five grams of the homogenized sample were placed in a 50 mL centrifuge tube to which 20 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) and 20 mL distilled water were added and vortexed (Fisher Vortex Genie 2, Fisher Scientific, Nepean, ON) at high speed for 2 min. The mixture was then centrifuged at 4000*g* for 5 min and the supernatants filtered through a Whatman No 3 filter paper. One milliliter of a 0.01 M aqueous solution of 2-thiobarbituric acid and 4 mL of the filtrate were mixed. The mixture was heated in a boiling water bath for 25 min, cooled to room temperature, and the absorbence of the resultant colored solution was read at 532 nm with a Hewlett-Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Pals Alto, CA). TBARS values (expressed as mg malonaldehyde equivalents/kg mussel meat) were calculated by multiplying the absorbance readings by a factor of 10.2, which was obtained from a standard line prepared using 1,1,3,3-tetramethoxypropane as a precursor of malonaldehyde.

2.7 Proximate composition

2.7.1 Moisture and Ash content

Approximately 2 g of blue mussel homogenate were dried in a pre-weighed aluminum pan (Fisher Scientific, Nepean, ON) in a forced-air oven (Fisher Isotemp 300, Fair Lawn, NJ) at 105°C for 24 hours. The samples were cooled to room temperature in a

desiccator. Moisture content was calculated as percentage weight loss of sample during drying. Approximately 5-7 g of homogenized blue mussel sample were placed in pre-weighed porcelain crucibles with covers and charred using a Corning hot plate (Model PC-351, Corning, NY). The charred samples were heated in a muffle furnace (Thermolyne, F 62700, Dubuque, IA) at 550 °C overnight or until the entire sample turned white. The samples were cooled to room temperature in a desiccator and the weight of the residue was used to determine the ash content of each sample (AOAC, 1990).

2.7.2 Crude protein content

Crude protein content of homogenized blue mussels was determined by the Kjeldahl method (AOAC, 1990). Approximately 200 mg homogenized blue mussel sample was placed in a pre-labeled digestion tube with 20 mL concentrated sulfuric acid (Fisher Scientific Co., Fair Lawn, NJ) and two (5.0 g potassium sulfate and 0.25 g mercuric oxide) catalyst tablets (Kjeltabs, Profamo Analytical Service Inc., Dorval, PQ). The sample was digested in a Buchi 430 digester (Switzerland) for 50 to 60 min until the digest was clear. Fifty milliliters of distilled water and 150 mL 25% (w/v) sodium hydroxide were added to the digests, which was steam distilled (Buchi 321 distillation unit, Switzerland) and the distillate was collected in 50 mL 4% (w/v) boric acid containing 10 drops of methyl red/methylene blue indicator (EM SCIENCE, Gibbstown, NJ). Distillation was continued until 150 mL of condensate were collected. The condensate was then titrated with a 0.1 N standardized sulfuric acid solution to a pink color end point. A similar treatment of digestion and titration was performed with the blank, but without the sample. The nitrogen

content was calculated as a percentage using the equation below and reported as crude protein content (N% X 6.25).

$$N\ \% = \frac{(V_{sample} - V_{blank}) \times N \times 14.0067 \times 100}{W}$$

where, V_{sample} = volume of titrant for sample (mL), V_{blank} = volume of titrant for blank, N= normality of H_2SO_4 solution used in the titration and W = weight of sample (mg) .

2.7.3 Total lipid content

Total lipids of blue mussel homogenate were extracted and quantified by the Bligh and Dyer (1959) procedure as described by Budge and Parrish (2003). Approximately 4 g homogenate were mixed with 4 mL chloroform and 8 mL methanol in a 50 mL tube, flushed with nitrogen and vortexed (Fisher Vortex Genie 2, Fisher Scientific, Nepean, ON) at high speed for 2 min. The mixture was filtered through a Whatman No.1 filter paper in a Büchner funnel and the filtrate collected. The tissue and the filter were vortexed with another 4 mL chloroform and the mixture filtered again. Filtrates were combined and transferred to a separatory funnel, mixed with 4 mL water and flushed with nitrogen. The separatory funnel was kept in a Foster refrigerator at 2-4 °C (Drummondville, QC). The bottom chloroform layer was then separated and evaporated at 40°C under vacuum in a Büchi RE 111 rotoevaporator (Büchi Laboratories, Flawil, Switzerland). Lipid content was determined gravimetrically. Samples were stored under nitrogen at –20 °C for fatty acid analysis.

2.7.4 Carbohydrate content

Carbohydrate content in each sample was determined by difference as follows: Carbohydrate % = 100 – (% moisture + % ash + % protein + % lipid)

2.8 Fatty acid composition

2.8.1 Preparation of fatty acid methyl esters (FAME) using 6% (v/v) sulfuric acid in methanol

Blue mussel lipid extract (10 mg) was weighed into 6 mL chromerge-cleaned (Fisher Scientific, Fair Lawn, NJ), screw capped, teflon-lined, conical vials. Subsequently, 2 mL of transmethylation reagent were added to each vial and its contents mixed by vortexing (Fisher Vortex Genie 2, Fisher Scientific, Nepean, ON) at high speed for 1 min. This reagent was prepared freshly each day by adding 6 mL concentrated sulfuric acid to a 100 mL volumetric flask, then making up to volume with spectral-grade methanol and adding 15 mg t-butylhydroquinone (TBHQ) antioxidant. The mixture of fatty acids and reagent was incubated at 60 °C for 17 h. The vials were then, cooled to room temperature and 1 mL distilled water added. The fatty acid methyl esters (FAME) were extracted 3 times with 1.5 mL n-hexane. A few crystals of TBHQ were added to each vial before the extraction step to prevent oxidation of unsaturated fatty acids. The hexane layers were separated, combined, transferred to a clean dry tube and washed twice with distilled water. The hexane layer was separated and evaporated under a stream of nitrogen at room temperature (Khan and Shahidi, 2000).

2.8.2 Preparation of fatty acid methyl esters (FAME) using boron trifluoride methanol (14% BF_3-methanol) solution

In this method, FAME of lipid extracts of blue mussels and/or plankton were prepared using 14% BF_3-methanol mixture as described by Budge and Parrish (2003). Lipid extracts were dissolved in 0.5 mL n-hexane and 1.5 mL 14% BF_3-methanol mixture (Supelco, Oakville, ON). The samples were flushed with nitrogen and heated for 90 min at 85 °C. After cooling to room temperature, FAME were extracted three times with a mixture of 2 mL hexane and 2 mL water, followed by centrifugation for 2 min at 2000 *g*. Hexane layers, which contained FAME were separated, combined, and evaporated under a stream of nitrogen at room temperature.

2.9 Analysis of fatty acid methyl esters by gas chromatography

2.9.1 Analysis of fatty acid methyl esters with a Hewlett Packard 5890 II gas chromatograph

Fatty acid methyl esters, prepared as described previously, were analyzed with a Hewlett Packard 5890 II gas chromatograph (Hewlett Packard, Toronto, ON) equipped with a 30 m x 0.25 mm Supelcowax-10 column (SP 2330, Supelco Canada Ltd., Oakville, ON). The injector and flame ionization detector temperatures were both 270 °C. The oven temperature was initially 220 °C for 10.25 min and then increased to 240 °C at 30 °C/min and held there for 9 min. Helium (UHP) was used as the carrier gas. The FAME were identified by comparing their retention times with those of authentic standard mixtures (PUFA 1, Supleco 37 component FAME mixture and bacterial acid methyl ester mixture; Supelco, Canada Ltd. (Oakville, ON) and GLC-461 Nu-Check-Prep. (Elyasin, MN), quantified using area normalization and each fatty acid reported as % of the total fatty acids.

2.9.2 Analysis of fatty acid methyl esters with a Varian Model 3400 gas chromatograph

Fatty acid methyl esters were analyzed using a Varian Model 3400 gas chromatograph (Varian Canada Inc., St. Laurent, QC) fitted with split / splitless injector (Budge, 1999; Budge and Parrish, 1998) and a 0.25 μm film thickness Omegawax-coated fused silica column of 30-m length and 0.23 mm i.d. (Omegawax 320; Supelco, Bellfonte, PA). The flow rate of hydrogen (carrier gas) was 2 mL/ min and of air and helium 300 and 30 mL / min, respectively. The oven was programmed to hold at 65°C for 5 min followed by a ramp at 40°C / min and hold for 15 min at 195 °C, then ramp at 2°C min and hold for 0.75 min at 220°C. The flame ionization detector (FID) was isothermal at 260 °C and the injector temperature was raised from 150°C (hold for 0.5 min) to 250°C at a rate of 200°C / min. The injector was kept at 250°C for 10 min. The FAME were identified by comparing their retention times with those of authentic standard mixtures of PUFA1 (marine source), PUFA 3, Supleco 37 component FAME mixture and bacterial acid methyl ester mixture; Supelco, Canada Ltd. (Oakville, ON), quantified using area normalization and each fatty acid reported as % of total fatty acids.

2.10 Determination of lipid classes in mussel and plankton samples

2.10.1 Extraction of lipids from plankton

Lipids were extracted from plankton using a modified Folch method as described by Parrish (1987). Three millilitres of chloroform - methanol (2:1, v/v) were added to a plankton loaded filter (Section 2.2) in a 15 mL tube, and ground with a stainless steel rod. Then 1 mL of chloroform : methanol (2:1, v/v) and 0.5 mL of water were added. The mixture was sonicated for 4 min and centrifuged at 2000 *g* for 2 min. The mixture was

washed 3 times with 3 mL chloroform with repeated sonication and centrifugation. Chloroform layers, which contained the extracted lipids, were pooled and the volume reduced to 0.5 mL under a gentle stream of nitrogen. The samples were stored at –20 °C for determination of lipid classes, fatty acids and sterols.

2.10.2 Extraction of lipids from mussels

Lipids were extracted by the modified Folch method (Parrish 1987). Each mussel sample was placed in a 40 mL tubes with 10 mL chloroform and 5 mL methanol and homogenized with a Polytron homogenizer (Brinkman Instruments, Rexdale, ON). Between samples, the rod of the homogenizer was rinsed with 6 mL chloroform : methanol (2:1, v/v) and 5 mL chloroform-extracted water. Homogenates were vortexed, sonicated for 4 min and centrifuged at 2000 g for 2 min. The bottom chloroform layer containing the lipids layer was then removed using a double pipetting technique (one long Pasteur pipette inserted into a short one). This extraction procedure was repeated three times or until no colour was observed in the chloroform layer. Chloroform layers were pooled and evaporated under a gentle stream of nitrogen. The samples were stored at –20 °C for lipid class, fatty acid and sterol determinations.

2.10.3 Determination of lipid classes in blue mussel and phytoplankton lipid extracts using the MK-5 Chromarod Iatroscan system

Determinations of lipid classes in mussel and plankton lipid extracts were carried out with a MK-5 Chromarod Iatroscan system (Parrish, 1987). Briefly, lipid extracts were spotted onto silica gel coated quartz rods (Chromarods) and developed using various solvent systems. Hexane-diethyl ether-formic acid (99:1:0.5, v/v/v) was used twice to

obtain the first scan until after the ketone peak. Next, hexane-diethyl ether-formic acid (80:20:10, v/v/v) was used to resolve all classes up to the diacylglycerol peak. Finally, the rods were developed with two solvent systems, first with acetone, then with chloroform-methanol-water (50:40:10, v/v/v). Chromatograms from each extract were combined and lipid classes were quantified by comparing responses with those of reference standards.

2.11 Determination of sterols in mussels and plankton by gas chromatography-mass spectroscopy (GC-MS)

A Pasteur pipette was filled with 0.5 g of silicic acid and activated by heating at 150 °C for 1 hr. The silicic acid was washed with 1 mL diethyl ether followed by 2 mL hexane. Lipids were extracted from mussels and plankton and FAME were prepared as described above. The FAME dissolved in 0.5 mL hexane were applied to the top of the activated silicic acid column. Ten millilitres of hexane - diethyl ether (93:7, v/v) were used to elute the FAME through the column. Sterol and alkanol fractions were recovered by passing 10 mL of hexane - diethyl ether (50:50, v/v). Through a gentle stream of nitrogen, the solvents (hexane and diethyl ether) were removed. Two drops of N,O-bis(trimethylsilyl) acetamide (BSA) and four drops of bis(trimethylsilyl-trifluoroacetamide (BSTFA) were added to the sterol fraction. The mixture was vortexed and heated at 85°C for 15 min. Traces of BSA and BSFA were removed under nitrogen and the resultant TMS derivatives of the sterols re-suspended in hexane (Copeman et al., 2003, Halket, 1994).

2.11.1 Analysis of sterol composition

The trimethylsilyl (TMS) derivatives of sterols were analysed with GC-MS (Varian 3800 GC coupled with a Varian Saturn 2000R MS). One microlitre of the derivatized sample in hexane was injected in a splitless mode into a fused silica capillary column (CP-SIL 8 CB, 30m X 0.25 mm i.d., 0.25 μm film thickness, Varian Canada Inc. St. Laurent, QC). The initial column temperature (60°C) was increased from 60 to 100 °C at a rate of 25°C/min. The temperature was further increased from 100 to 150°C at a rate of 15°C/min. Finally, the temperature of the column was raised from 150 to 315°C at a rate of 3 °C/min and maintained for 4.07 min. Carrier gas (helium) flow rate was 20 psi. The mass analyser scanned over a mass range 60 to 520 m/z at an inoization potential of 70 ev. Sterol TMS derivatives were identified by comparing their spectral data with published spectral data (Heupel, 1989).

2.12 Photochemiluminescence (PCL) detection of water - soluble (ascorbic acid) and lipid - soluble (α-tocopherol) antioxidants in mussels

2.12.1 Extraction of ascorbic acid (Asc) from blue mussels

Approximately 2 g of the homogenate were mixed with 5 mL of ice – cold 10% TCA and the mixture vortexed using a Fisher Vortex Genie 2 (Fisher Scientific, Nepean, ON) at high speed for 2 min. The mixture was centrifuged at 4000*g* for 5 min and the supernatant filtered through a Whatman No.3 filter paper. The antioxidant capacity of the Asc was determined in the filtrate.

2.12.2 Determination of ascorbic acid and α-tocopherol in blue mussels

Antioxidant capacity of Ascorbic acid (ACAsc) in seawater and blue mussels (expressed as Asc equivalent/ L of seawater or g meat) and antioxidant capacity of tocopherol (ACT) in mussel lipid extract (expressed as μ mol Trolox equivalent / g meat) were assayed by a photochemiluminescence (PCL) method with a PHOTOCHEM® instrument (Analytica Jena USA, Delaware, OH).

The assay mixture to determine ACAsc consisted of 1.5 mL of reagent 1 (distilled water), 1 mL of reagent 2 (Na_2CO_3 buffer 0.1 mol / L + 0.1 mmol Na_2 EDTA, pH 10.5), 0.025 mL of photosensitizer (luminol) and 0.05, 0.01, 0.015, 0.02 and 0.025 mL reagent 4 (Asc standard solution) or sample solution (0.01 or 0.015 mL of an appropriate dilution of the seawater or blues mussels filtrate (2.12.1), (Lewin and Popov, 1994; Amarowicz et al., 1999). The lag phase generated was used to calculate Asc content using PCLsoft software v 3.1 (Analytica Jena USA, Delaware, OH).

The assay mixture to determine ACT consisted of 2.3 mL reagent 1 (methanol), 0.2 mL reagent 2 (Na_2CO_3 buffer 0.1 mol/L + 0.1 mmol Na_2 EDTA, pH 10.5) 0.025 mL of photosensitizer (luminol), and 0.05, 0.015, 0.02, 0.025, and 0.03 mL reagent 4 (Trolox standard solution) or mussel lipid extract (0.1 mL of an appropriate dilution of the extract). The presence of tocopherol in mussel samples decreased ACL intensity (Popov and Lewin, 1996; Amarowicz et al., 2002). The percentage inhibition was compared with the standard and expressed as Trolox equivalents using PCLsoft® v 3.1 (Analytica Jena USA, Delaware, OH).

2.13 Experimental designs

2.13.1 Chapter III experiments

Mussels (3 years old) were obtained from two aquaculture sites, Fortune Harbor and Charles Arm. Both sites are located in Notre Dame Bay, Newfoundland, Canada. Fortune Harbor mussels were obtained in the middle of June 2000, Charles Arm mussels a month later. Cultured mussels were obtained from Notre Dame Bay in June 2003 for the final experiment. Wild blue mussels, wild and cultured scallops (*Placopecten magellanicus*) were obtained at the beginning of August 2000 from Gilbert Bay, Labrador, Canada. The cultured scallops were kept inside lantern nets suspended at a depth of 8 m. for 48 months prior to use in this study. Mussels and scallops were separated and washed using filtered seawater.

Fortune Harbor samples from three batches (5-6 mussels x 3 batches) were stored for 10 days at three different temperatures (-12 ± 2°C), (3 ± 2°C) and (9 ± 2°C) in plastic bags with holes to drain the discharges. In another experiment, Charles Arm mussels, 5-6 in each of three bags, cultured and wild scallops, 3-4 in one bag for each, were stored at 2°C. Samples at 2°C were stored in a Foster refrigerator (Drummondville, QC), while samples at 9 and –12°C were stored in a household Cold Spot refrigerator (9°C) with an upper freezer compartment (-12°C). The samples were analyzed on days 0, 3, 7 and 10 as described in Section 2.4. Microbial examination of Fortune Harbor samples stored at 9°C and Charles Arm mussels stored at 2°C was terminated after 7 days due to visible spoilage of samples. In a final experiment, cultured blue mussels, harvested in June 2003, were stored on ice and analyzed for microbial changes after 0, 3, 7, 10, and 14 days.

2.13.2 Chapter IV experiments

Blue mussels used in this study were cultivated in June 2000 at two aquaculture sites. The two sites are located in Notre Dame Bay, north of St. John's, Newfoundland, Canada. The locations of the stations in each site were 49°21.617′ N 055° 17.0106′ W (Charles Arm station I), 49° 20.849′ N 055° 16.722′W (Charles Arm station II), 49° 30.942′ N 055° 15.186 W (Fortune Harbor station I) and 49° 31.693′ N 055° 16.545 W (Fortune Harbor station II). Mussel spat (12 months) were loaded into the mesh tubing or socks (three per station) and grown until October 2001. Samples of seawater, blue mussels (about 30-50) and net-tows were collected in June, August, September and October 2000 as well as in April, June, August, September and October 2001 (Table 2.1) and analysed as described above. Scheduled sampling intervals were not possible due to poor logistics or unsafe weather conditions. Analysis and measurements of samples were carried out as described in appropriate sections.

2.13.3 Chapter V experiments

Newfoundland cultured blue mussels (12-18 h from harvest) obtained from a local seafood supplier were used in this study. The mussels were immediately placed in 3 styrofoam containers filled with crushed ice. The mussels were separated from ice with a plastic sheet to protect the mussels from melting ice. The ice was replaced on a daily basis. Each container held between 90 and 100 mussels. Mussels (9-10) with closed valves, which mean they were alive, were removed on days 0, 3, 7, 10 and 14 and homogenized. The homogenate was used for the determination of pH, proximate composition, microbial

content, fatty acid composition and mussel meat off flavor as described in appropriate sections above.

2.13.4 Chapter VI experiments

2.13.4.1 Effect of mechanical handling

Mussels (3 years old) were obtained from two processing plants in Charles Arm and Fortune Harbor, Newfoundland. Three different batches (80-100 mussels per batch) of mussels were collected from each plant during processing (separation, sorting, washing and packaging), one at the initial stage, the second at the middle of processing and the third at the end of processing. TBARS analysis were performed on the homogenized samples as described in sections 2.4 and 2.6.

2.13.4.2 Effect of storage of mussels on ice

Mussels (3 years old) obtained from two different aquaculture sites, Fortune Harbor (FH) and Charles Arm (CA), in Notre Dame Bay, Newfoundland. Mussels were distributed in small bags (about 35 mussels per bag) with a wide mesh size. The bags were stored in a plastic container (70 cm length x 35 cm width x 25 cm height) filled with crushed ice (10 cm height). Mussels were separated from the ice by a plastic sheet (70 cm length x 35 cm width). TBARS analysis was carried out on days 0, 3, 7, 10 and 14.

Table 2.1 Details of sampling days for mussels and plankton obtained from two aquaculture sites (two stations per site), Charles Arm and Fortune Harbor

Sampling intervals		Sampling site			
Month	Year	Charles Arm		Fortune Harbor	
		Station I	Station II	Station I	Station II
June	2000	6	6	29	29
August		15	15	10	10
September		20	20	13	13
October		20	20	13	13
April	2001	7	ND	6	ND
May / June		May 30	May 30	June 3	June 3
August		10	10	8	8
September		ND	ND	19	19
October		3	3	19	19

ND; not determined. Numbers listed are actual sampling days.

2.13.4.3 Effect of ascorbic acid treatment

Commercial size blue mussels were obtained from a Charles Arm processing plant (12 hours after harvest). Twenty-five mussels were placed in a plastic container filled with 10 L filtered seawater (FSW) and held at 2°C for 24 hours for acclimation. The water was replaced with 9 L FSW and the mussels left for another 2 hours. Specific amounts of sodium ascorbate (NaAs), ACS grade ascorbic acid and ultra pure ascorbic acid were then dissolved in 1 L of FSW. The prepared solutions were poured into the containers (one solution per container). The final concentration for each type of Asc was 0.01 M. Mussels were exposed to various types of Asc for 24 hours. The mussels were then removed from water and stored on ice for 10 days as described above (Section 2.13.4.2). Controls were run with mussels but no Asc treatment. The mussels were homogenised and analysed on days 0, 5 and 10 for pH, fatty acid composition, TBARS, ACAsc and ACT as described above.

2.14 Statistical analysis

All experiments were replicated three times, except for scallop samples (limited numbers avialable). Mean ± standard deviation was reported for each case. Prior to statistical analysis, results of microbiological analysis were transformed to $\log_{10}$ values. After testing the normality of the data, analyses of variance (ANOVA) and Tukey's studentized test were performed at a level of $p < 0.05$ to evaluate the significance of differences between mean values. Regression and correlation analyses were performed to establish the relationships among various variables tested (Edward, 1984; Freund *et al.*, 1988).

Chapter III

Enumeration of total heterotrophic and psychrotrophic bacteria in blue mussels (*Mytilus edulis*) and sea scallops (*Placopecten magellanicus*) using different agars

3.1 Introduction

Various microbiological methods and media are used to evaluate the microbial content of seafoods (Atlas, 1995a; 1995b). The selection of a certain medium or method depends upon several factors, such as availability and sensitivity of the medium and technique, as well as the cost and time required to complete the analysis (Harrigan *et al.*, 1991; Harrigan, 1998b). Despite the development of some rapid microbiological methods (Chew *et al.*, 1997; Shelef and firstenberg-Eden, 1997; De Boer and Beumer, 1999), most of the provincial, national and international microbiological specifications for seafoods are still based on different types of colony count from agar plates (Busta *et al.*, 1984; Anonymous, 1992; Bolton, 1998). Furthermore, standard plate count techniques are used to validate newly developed microbiological methods (Townsend and Naqui, 1998; Salo *et al.*, 2000; Stender *et al.*, 2001) and as indicators of the presence of pathogenic bacteria in foods (Bremer and Osborne, 1998; Siragusa *et al.*, 1998; Besnard *et al.*, 2000). Therefore, there is a constant search for an agar medium that gives the highest and most consistent recoveries of micro-organisms.

Aerobic plate count (APC) or total heterotrophic bacteria (THB) in filter feeders such as mussels and scallops indicate the microbial content of the seafood and surrounding water as well as hygiene during washing and processing (Hunt *et al.*, 1988). Therefore, high APC ($>10^5$ (or $\log_{10}$ 5) CFU/g) may indicate a potential health hazard for consumers

(Anonymous, 1992Colby *et al.*, 1995;; Jay, 1996). Furthermore, psychrotrophic bacteria are the main contributors to the spoilage of seafoods at refrigeration temperatures (Gilliland *et al.*, 1984; Ray, 1996). Therefore, APC and psychrotrophic plate count (PPC) can be used to estimate the shelf life of seafoods.

Heterotrophic bacteria have specific nutritional requirements, and thus it is not possible to use a general-purpose agar medium. However, any medium must include water, peptides, amino acids, carbohydrates as an energy source, and accessory growth factors such as vitamins (Harrigan, 1998a). Furthermore, many marine bacteria are unable to grow unless critical amounts of certain salts and minerals are present in the growth medium (Bross and Matches, 1984; Sukoso *et al.*, 1998; Reed *et al.*, 1999). Plate count agar (PCA) has frequently been used for determination of APC in various seafoods (Hunt *et al.*, 1984; Neufeld, 1984; Nickelson II and Finne, 1984; McCarthy, 1997; Boskou and Debevere, 2000), although it lacks the salts and minerals necessary for the growth of marine micro-organisms. Because marine agar (MA) contains the essential elements required for the growth of marine bacteria, it has been used to enumerate both APC and PPC from marine samples (Bruni *et al.*, 1998; Lizarraga-Partida *et al.*, 1998; Giuliano *et al.*, 1999). Nonetheless, MA has rarely been used to evaluate the microbial content of seafoods (Grisez *et al.*, 1997), and to the best of our knowledge, no study has evaluated the use of MA to examine the microbial status of bivalves.

The objectives of this study, therefore, were 1) to enumerate bacteria in mussels and scallop stored at -12, 2 and 9°C with PCA and MA; 2) to establish a relationship between bacterial counts (Log_{10} CFU/g) on PCA and MA using linear regression analysis

and 3) to estimate the shelf life of cultured blue mussels stored on ice using APC on MA, PCA and PCA+1% NaCl.

3.2 Materials and Methods

Mussel and scallop samples were collected as described in subsection 2.13.1. Microbial analyses of samples were carried out as described in sections 2.4 and 2.5. Statistical analyses of the collected data were done as described in section 2.14.

3.3 Results

3.3.1 Changes in bacterial counts in Fortune Harbor mussels on PCA and MA and effect of storage time and temperature

Bacterial counts on MA were 1-3 log CFU/g higher than corresponding counts on PCA throughout the storage period at –12, 2 and 9°C (Figures 3.1 and 3.2). After 10 days of storage at 2 °C, APC counts of cultured mussels on MA were log 8.9 ± 0.13 CFU/g, while only 5.40 ± 0.53 CFU/g on PCA. Similarly, PPC counts on MA at 2°C were log 7.17 ± 0.32 CFU/g, but were only log 5.73 ± 0.23 CFU/g on PCA.

Bacterial counts of mussels were affected by storage temperature. Thus, APC and PPC counted from PCA and MA of samples stored at 9°C were significantly higher ($p < 0.05$) than samples stored at 2 and –12°C. For example, after 7 days of

Figure 3.1 Experimental aerobic plate count (APC) on plate count agar (PCA) and marine agar (MA) (Log CFU/g) in cultured blue mussels from Fortune Harbor stored at -12, 2 and 9°C for 10 days. (Mean ± SD, n=3). ND, no bacteria were detected, and thus bacterial counts were less than 10 CFU/g.

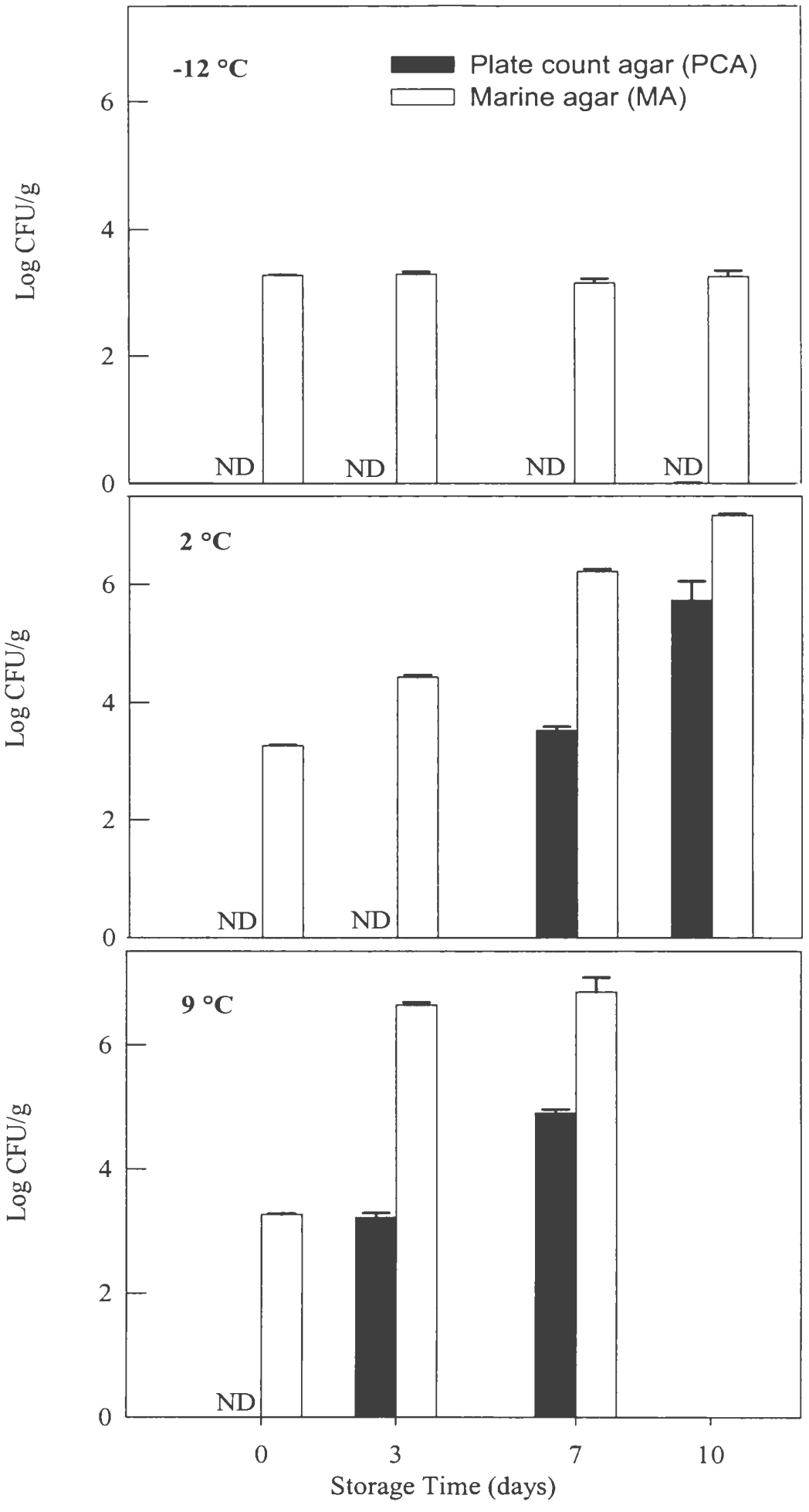
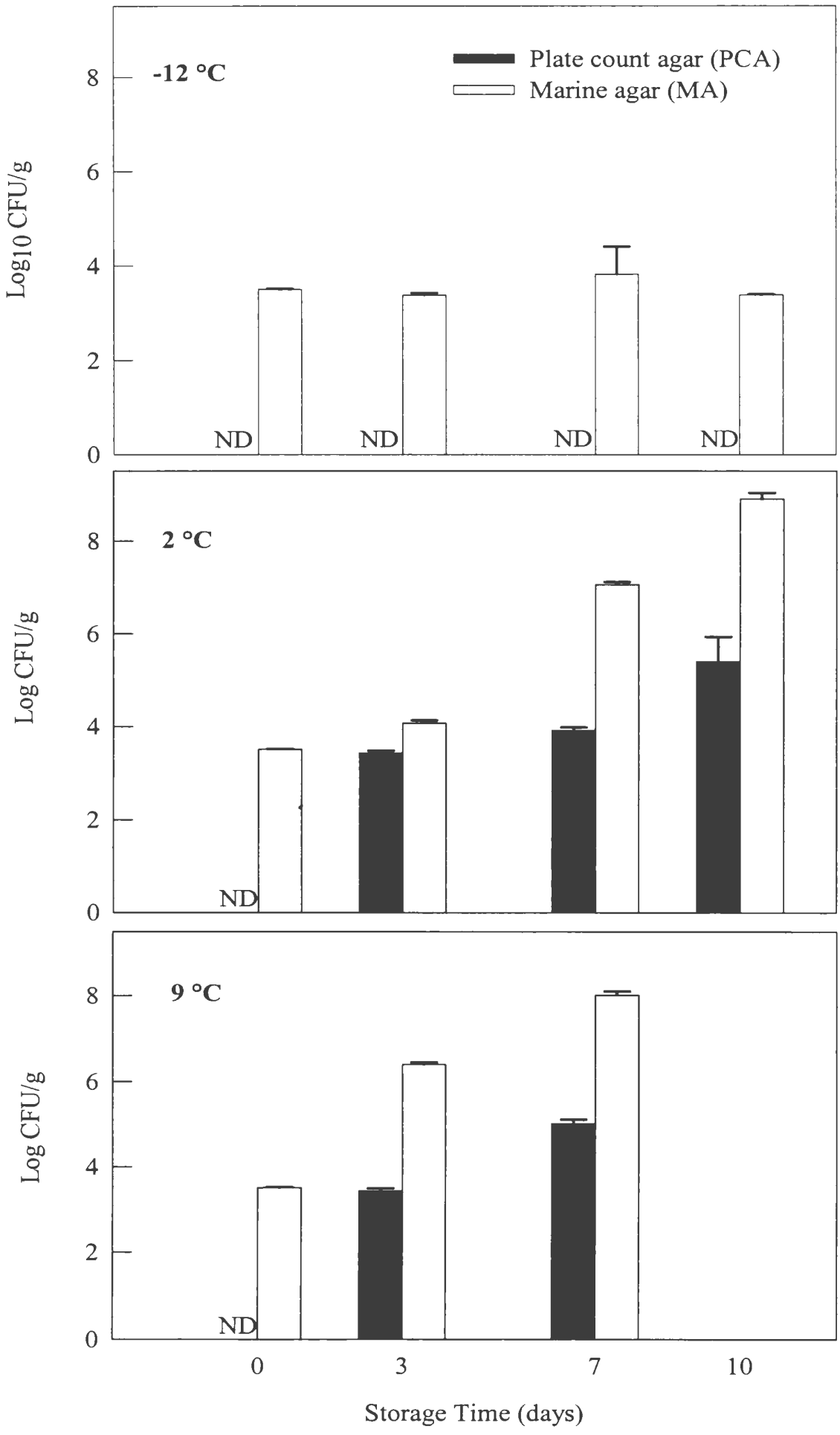

-12 °C
Plate count agar (PCA)
Marine agar (MA)
Log CFU/g
6
4
2
0
ND
ND
ND
ND
2 °C
Log CFU/g
6
4
2
0
ND
ND
9 °C
Log CFU/g
6
4
2
0
ND
0
3
7
10
Storage Time (days)

Figure 3.2 Experimental psychrotrophic plate count (PPC) on plate count agar (PCA) and marine agar (MA), (Log CFU/g). cultured blue mussels from Fortune Harbor stored at -12, 2 and 9°C for 10 days. (Mean ± SD, n=3). ND, no bacteria were detected, and thus bacterial counts were less than 10 CFU/g.


-12 °C
Plate count agar (PCA)
Marine agar (MA)
Log10 CFU/g
ND
ND
ND
ND
2 °C
Log CFU/g
ND
9 °C
Log CFU/g
ND
0
3
7
10
Storage Time (days)

storage at 9°C, APC counts on PCA were log 5.02 ± 0.09 CFU/g, but only log 3.92 ± 0.06 CFU/g for samples stored at 2°C and less than 10 log CFU/g for samples stored at –12 °C. A similar trend was observed for counts on marine agar. No significant differences ($p > 0.05$) were observed between APC and PPC of stored mussels on the same type of agar. After 10 days of storage, APC counts on MA of samples stored at -12°C were log 3.39 ± 0.09 CFU/g, while PPC counts were log 3.25 ± 0.09 CFU/g. A similar trend was observed when APC and PPC on PCA were considered.

3.3.2 Relationship between bacterial counts of Fortune Harbour mussels on MA and PCA

Equation 1c (Table 3.1) indicates a strong correlation (r= 0.990) between bacterial counts obtained from PCA and MA (Figure 3.3). The equation also had the lowest standard deviation values for the intercept (±0.16) and slope (±0.05). This linear equation was applied to experimental APC on PCA of Charles Arm mussels as well as wild mussels, cultured and wild scallops stored at 2°C to obtain predicted APC on MA and to compare values with the experimental APC on MA. Meanwhile, equation 1d was applied to experimental PPC of wild and cultured blue mussels and scallops stored at 2°C to predict PPC on MA. These predicted values were also compared with the experimental PPC on MA.

3.3.3 Prediction of APC and PPC of wild and cultured mussels on MA using bacterial counts on PCA and the accuracy of prediction

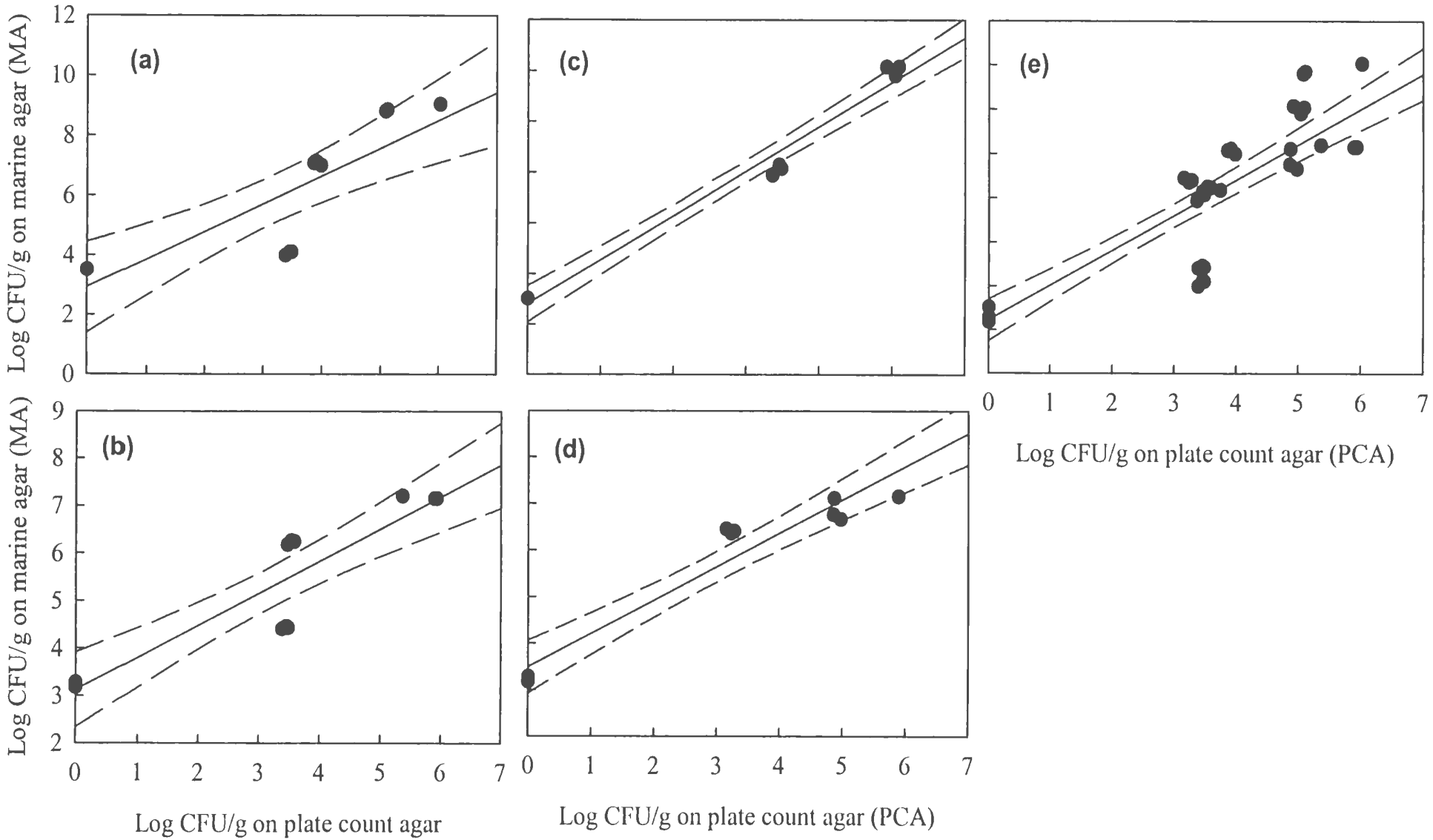
APC and PPC on PCA were incorporated into appropriate linear predictive equations to obtain the predicted bacterial counts on MA. The accuracy of predicting

Table 3.1 Linear regression analysis of aerobic plate count (APC)[a] and psychrotrophic plate count (PPC)[a] values for plate count agar (PCA) and marine agar (MA) in mussels cultured at Fortune Harbor stored at 2 and 9 °C.

Temperature	Type of bacteria	r	n	P	Equation	Linear relationship equation
2 °C	APC	0.838	12	0.0007	1.a	$y = 2.93 (\pm 0.72) + 0.92(\pm 0.19) * x$
2 °C	PPC	0.908	12	<0.0001	1.b	$y = 3.1 (\pm 0.37) + 0.67 (\pm 0.10) * x$
9 °C	APC	0.990	9	<0.0001	1.c	$y = 3.40 (\pm 0.16) + 0.87(\pm 0.05) * x$
9 °C	PPC	0.963	9	<0.0001	1.d	$y = 3.49 (\pm 0.26) + 0.72 (\pm 0.07) * x$
2 ° and 9 °C	APC and PPC	0.887	42	<0.0001	1.e	$y = 3.22 (\pm 0.23) + 0.79 (\pm 0.07) * x$

[a] Log CFU/g.; y, Log CFU/g on marine agar; x, Log CFU/g on plate count agar; r, coefficient correlation; n, number of observations, p, level of significance.

Figure 3.3 Linear regression lines of experimental aerobic plate count (APC) and psychrotrophic plate count (PPC) on plate count agar (PCA) and marine agar (MA) for cultured blue mussels from Fortune Harbor stored at 2 and 9°C (see Table 3.1 for abbreviation and regression parameters). Dotted lines are 95 % confidence intervals.


(a)
(b)
(c)
(d)
(e)
Log CFU/g on marine agar (MA)
Log CFU/g on marine agar (MA)
Log CFU/g on plate count agar
Log CFU/g on plate count agar (PCA)
Log CFU/g on plate count agar (PCA)

bacterial counts on MA using bacterial counts on PCA was calculated using the following formula: (experimental APC or PPC on MA/ predicted APC or PPC on MA)*100. The linear equations applied were suitable to predict the APC and PPC on MA from bacterial counts on PCA at relatively lower accuracy (60-80%) at initial stages of storage but with very high accuracy at the end of storage (90-99%) (Table 3.2). There are one or two exceptions to this general trend. For example, on day 0, the prediction accuracy of APC of cultured mussels was about 68%, but increased to 93% after 7 days of storage. Similarly, the accuracy of predicting PPC counts on MA using PPC counts on PCA of cultured mussels, on day 0, was 69% but more than 90% after 7 days of storage. The linear model used to predict APC counts on MA using APC on PCA of cultured scallops stored for 3 days at 2 °C showed accuracy of 72%; this value was up to 99% after 10 days of storage. The accuracy of predicting PPC counts of cultured scallops on MA using counts on PCA was only 72% after 3 days of storage, but more than 95% after 10 days of storage. Similar trends were observed when wild mussels and scallops were considered (Table 3.3).

3.3.4 Shelf life estimation of blue mussels and scallops

Both temperature and agar type greatly influenced shelf life estimation while the type of bacteria (APC and PPC) had a lesser effect. Therefore, shelf life based on APC and PPC on MA was 3-4 days less than that estimated using APC and PPC on PCA. The shelf life of frozen Fortune Harbor mussels based on APC and PPC on PCA and MA was more than 10 days, whereas the shelf life of mussels at 2°C was between 3 and 10 days.

Table 3.2 Experimental aerobic plate count (APC)[a] and psychrotrophic plate count (PPC)[a] on plate count agar (PCA), marine agar (MA) and predicted bacterial counts on MA of cultured Charles Arm mussels and wild blue mussels from Gilbert Bay stored at 2°C.

	Cultured mussels				Wild mussels			
	APC (Log CFU/g)							
Storage time (days)	PCA	MAexpr.	MApred.	Accuracy	PCA	Maexpr.	Mapred.	Accuracy
0	3.33±0.06	4.32±0.08	6.30±0.06	68.52±0.06	3.21±0.16	3.53±0.00	6.19±0.14	57.05±1.33
3	5.56±0.03	6.71±0.02	8.24±0.02	81.43 ±0.13	3.57±0.02	4.82±0.03	6.51±0.02	74.04±0.45
7	6.57±0.03	8.47±0.05	9.12±0.03	92.89±0.64	4.50±0.01	6.46±0.02	7.32±0.01	88.27±0.31
10	ND	ND	ND	ND	5.76±0.01	7.38±0.01	8.41±0.01	87.70±0.15
	PPC (Log CFU/g)							
0	2.87±0.05	3.38±0.05	5.55±0.03	69.02±0.59	3.11±0.07	3.36±0.02	5.73±0.05	58.73±0.41
3	3.49±0.00	4.51±0.033	6.00±0.00	75.13±0.44	3.56±0.01	4.40±0.03	6.05±0.01	72.63±0.48
7	5.69±0.11	6.87±0.01	7.58±0.08	90.59±0.95	4.26±0.34	5.97±0.02	6.56±0.24	91.08±3.51
10	ND	ND	ND	ND	5.41±0.02	6.90±0.52	7.39±0.01	93.41±7.16

[a] Log CFU/g.; ND, not determined (sampled spoiled completely); MApred. for APC and PPC were calculated using equation 1.c and 1.d in Table 1. Accuracy of prediction = (MAexpr. / MApre.) * 100., MAexp.; experimental counts on MA, MApre.; predicted counts on MA. (No ANOVA test was performed as the accuracy of prediction was used in the discussion).

Table 3.3 Experimental aerobic plate count (APC)[a] and psychrotrophic plate count (PPC)[a] on plate count agar (PCA), marine agar (MA) and predicted bacterial counts on MA of cultured and wild scallops stored at 2°C.

	Cultured scallops				Wild scallops			
	APC (Log CFU/g)							
Storage time (days)	PCA	MAexpr.	Mapred.	Accuracy	PCA	MAexpr.	MApred.	Accuracy
0	0	2.79	3.40	82.06	0	2.62	3.40	77.06
3	3.43	4.62	6.38	72.37	3.26	4.13	6.24	66.23
7	4.73	6.87	7.52	91.42	4.24	6.41	7.09	90.42
10	5.92	8.52	8.55	99.64	6.09	8.87	8.70	101.97
	PPC (Log CFU/g)							
0	0	2.83	3.49	81.09	0	2.93	3.49	83.95
3	3.28	4.22	5.85	72.12	3.19	4.13	5.79	71.37
7	4.58	5.46	6.79	80.44	4.21	6.23	6.52	95.53
10	5.28	6.97	7.29	95.59	6.66	7.21	8.29	87.02

[a] Log CFU/g.; ND, not determined; MApred. for APC and PPC were calculated using the equation 1.c and 1.d in Table 1. Accuracy of prediction = (MAexp. / Mapre.) * 100, MAexp.; experimental counts on MA, Mapre.; predicted counts on MA. (No ANOVA test was performed due to lack of larger sample size for at least n=3).

Similarly, wild mussels stored at 2°C were fit for consumption for 7 to10 days based on bacterial counts on PCA and only 3 to 7 days if bacterial counts on MA were considered. Shelf life of Charles Arm mussels at 2°C was shorter than for all other samples. The shelf life of wild and cultured scallops was estimated to be between 3 and 7 days, based on bacterial counts on MA and 7 to 10 days based on counts on PCA (Table 3.4).

3.3.5 Shelf life of blue mussels stored on ice based on bacterial counts from three types of agars

APC on MA and PCA + 1% NaCl were 1-3 log CFU/g units higher than their corresponding counts on PCA throughout the storage period (Figure 3.4). Therefore, shelf life was less than 3 days based on APC on MA, and up to 3 and 7 days based on bacterial counts on PCA +1% NaCl and PCA, respectively.

3.4 Discussion

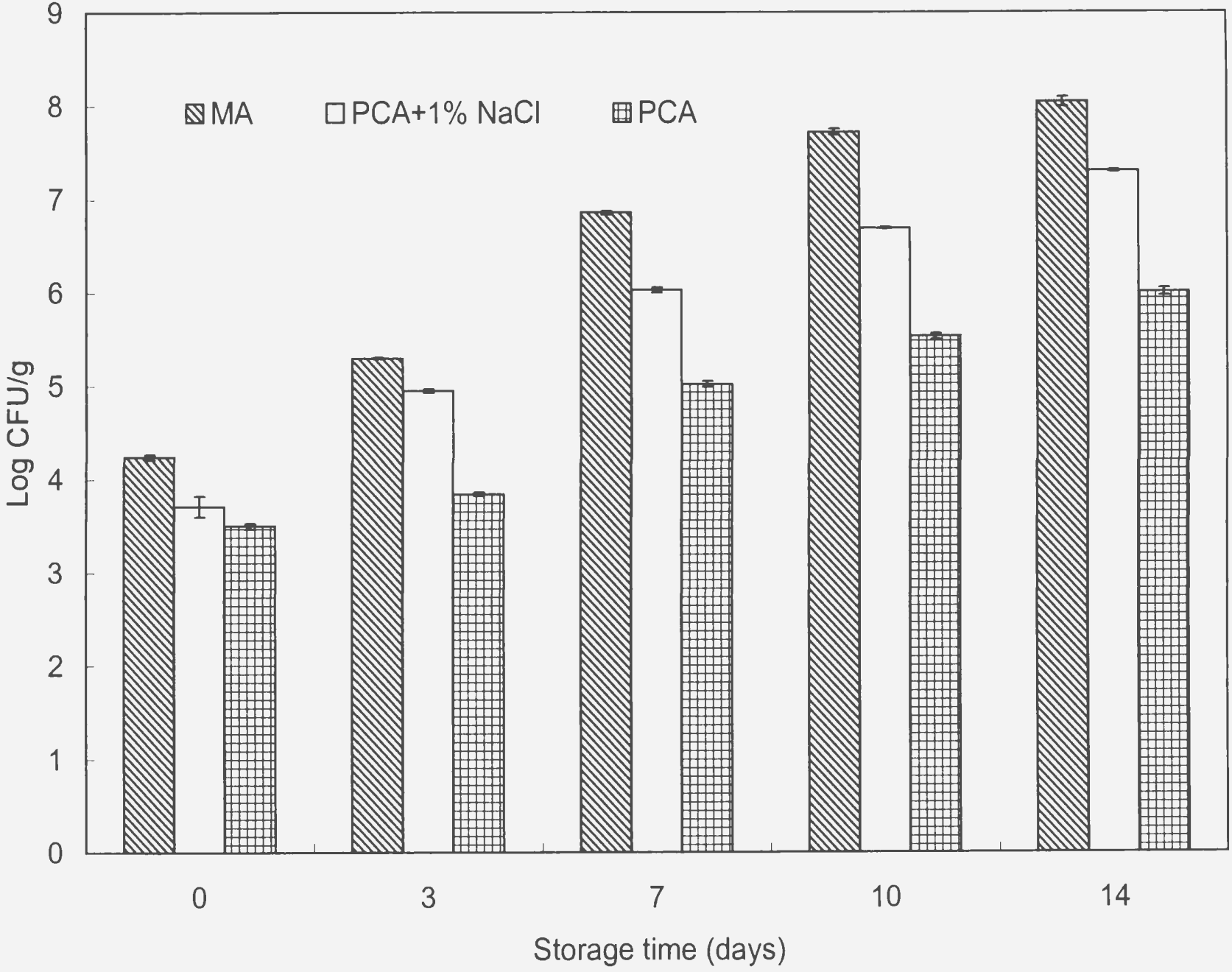
Seawater is composed of about 80 elements at concentrations, which can be tolerated by marine bacteria. Some elements and ions, such as calcium, magnesium, nitrogen, potassium, silicon, sodium, strontium, bicarbonate, boron, bromide, chloride, fluoride and sulfate support the growth of marine bacteria. However, the dominant components of seawater are sodium and chloride ions (Austin, 1988b). Therefore, all marine bacteria are either able to tolerate the high salinity of sea water (≈ 3 %) or salt is required for their growth and viability (Baross and Matches, 1984).

Table 3.4 Microbial content and estimated shelf life (days to reach Log 5 CFU/g) of cultured and wild blue mussels and scallops based on experimental aerobic plate count (APC) and psychrotrophic plate count (PPC), (CFU/g) on plate count (PCA) and marine (MA) agars.

Sample	Temperature (°C)	Aerobic plate count		Psychrotrophic plate count	
		PCA	MA	PCA	MA
Fortune Harbor mussels	-12	>10	>10	>10	>10
	2	≈10	3-7	≈10	3-7
	9	≈7	<3	≈7	<3
Charles Arm mussels	2	<3	<3	3-7	3-7
Wild mussels	2	7-10	3-7	7-10	3-7
Cultured mussels	2	7-10	3-7	7-10	3-7
Wild mussels	2	7-10	3-7	7-10	3-7

<3, less than three days; ≈ 7, about 7 days, ≈ 10, about 10 days, and >10, more than 10 days

Figure 3.4 Experimental aerobic plate count (APC) on plate count agar (PCA), (PCA) + 1% NaCl and marine agar (MA) (Log CFU/g), from cultured blue mussels (2003 harvest) stored on ice for 14 days. (Mean ± SD, n=3).


MA
PCA+1% NaCl
PCA
Log CFU/g
0
1
2
3
4
5
6
7
8
9
0
3
7
10
14
Storage time (days)

The aim of selecting the proper agar medium in this study was to obtain the highest consistent growth of APC and PPC from bivalve mollusks. Although the bivalve may be exposed to non-marine bacteria during transportation, distribution and storage, this exposure is limited because of the normal closure of the valves. Furthermore, numerous studies have indicated that various marine bacteria are the main contributors to microbial status of bivalve shellfish products due to accumulation of these bacteria within the bivalve tissues during the feeding and filtration processes. (Lee and Pfeifer, 1974; Cook, 1997; McCarthy, 1997; Hoi *et al.*, 1998).

There are few types of commercially available agar, such as MA and seawater agar, that contain all the necessary elements for the growth of marine bacteria (Atlas, 1995a; Austin, 1998a). MA has been used frequently to examine the microbial content of sea and ocean water samples (Lizarraga-Partida *et al.*, 1998; Bruni *et al.*, 1999; Giuliano *et al.*, 1999). Although seawater agar is recommended for isolation of *Vibrio* and *Pseudomonas* species from fish (Atlas, 1995a), its application in the food industry is limited or non-existent. Furthermore, many of the elements that required for the growth of marine bacteria are not present in plate count agar. This may explain, in part, the lower APC and PPC on PCA compared with MA counts throughout the storage period of Fortune Harbor mussels at –12, 2 and 9°C. Examination of additional wild and cultured mussels and scallops (year 2000) showed that MA recovery of APC and PPC from bivalves was three orders of magnitude higher than the recovery of these bacteria using PCA.

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommends that PCA be supplemented with 1% NaCl for better enumeration of marine bacteria which contribute to the spoilage of seafoods (Anonymous, 1992). The

results of the current research indicate that MA and not PCA + 1% NaCl or PCA alone gave the highest recovery of micro-organisms from bivalves, although PCA is still being used to obtain the total aerobic plate count of seafoods (McCarthy, 1997; Boskou and Debevere, 2000). However, even with PCA supplemented with 1% NaCl the bacterial counts enumerated from seafoods increase by only 1 order of magnitude (Cook, 1997). Meanwhile, bacterial counts on MA, in the current research, were up to two to three orders of magnitude higher than counts on PCA.

Although differences were observed between APC and PPC on the same type of agar for some samples, they did not influence the shelf life estimate. The incubation temperature of 30°C used in this study to obtain APC may have stimulated the growth of psychotropic bacteria (Gilliland *et al.*, 1984). In one study, PPC of refrigerated and frozen clams, oysters, crabmeat, shrimp and raw lobster tails were higher on agar incubated at 30°C than agar incubated at 35°C indicating the ability of seafood psychotropic bacteria to grow at up to 30°C (Swartzentruber *et al.*, 1980). Furthermore, when Solo *et al.* (2000) compared bacterial growth of different samples at various incubation times and temperatures, they did not observe any significant ($p > 0.05$) differences in bacterial counts at 25 or 30°C or after 48 or 72 hours of incubation. The NACMCF recommends the use of incubation temperature of 25°C to obtain the highest recovery of bacteria from molluscs. However, it appears from our results and a previous study (Solo *et al.*, 2000) that the use of an incubation temperatures of either 25 or 30°C will not adversely affect the recovery of total bacteria of blue mussels on MA or other similar types of agar.

Microbial spoilage of refrigerated seafoods is primarily due to the presence of psychrotrophic Gram-negative bacteria such as *Pseudomonas*, *Vibrio*, *Falvobacterium* and

Moraxella (Hunt *et al.*, 1984; Jay, 1996; Ray, 1996; Sakata, 1989). Nonetheless, the microflora of many spoiled seafoods, including bivalves, at refrigeration temperatures are composed mainly (90-100%) of genus *Pseudomonas* (Jay, 1996; Ray, 1996). Therefore, it is expected that many of the bacteria enumerated on MA belong to the *Pseudomonas* species. It is also possible that growth of some species of *Vibrio* on MA had occurred (Hariharan *et al.*, 1995; Hoi *et al.*, 1998). Clearly it is difficult to reach firm conclusions regarding the bacteria that contributed to the spoilage of blue mussels and scallops in this study without further identification. At very high bacterial counts (> log 7 CFU/g), microbial activity may produce off-odors because of production of undesirable primary metabolites such as trimethylamine from trimethylamine oxide, other amines and ammonia, hydrogen sulphide and carbonyl compounds from degradation of lipids (Colby *et al.*, 1995; Antoine *et al.*, 2002). Therefore, microbial examination of Charles Arm mussels stored at 9°C and Fortune Harbor mussels stored at 2°C was terminated after 7 days of storage.

Linear regression analysis was used to find the association between APC and PPC on PCA and MA of cultured mussel samples stored at 2 and 9°C Various parameters were used to gauge the accuracy of linear model systems; such as the correlation coefficient (r), level of significance (p) and standard deviation (SD) of the intercept and slope. High r values ($r > 0.7$) and low p ($p < 0.05$) and SD values for the intercept and slope indicate the suitability of a linear model for application in prediction of APC and PPC on MA using counts of PCA of wild and cultured bivalves (Edward, 1984; Chew, 1997; Freund, 1988). Regression analysis parameters and results indicate the applicability of the established linear models, described in the results section (Table 3.1), to predict APC and PPC counts

on MA using counts on PCA of various samples of wild and cultured blue mussels and scallops stored at 2°C. The samples were collected from different locations at various times, and thus had different initial bacterial counts. This may explain, in part, the power of prediction at the initial stages. It is possible that at the end of the storage period one type of bacteria had become predominant and was able to grow on MA and PCA, and thus the accuracy of prediction was high.

Chemical, biochemical and sensory analyses should be used in conjunction with microbial analysis to determine seafood shelf life (Colby *et al.*, 1995; Jay, 1996; Koutsoumans and Nychas, 1999; Boskou and Dbevere, 2000). Microbial analysis of cultured seafood, however, can be used to examine hygiene during processing, packaging, transportation, storage, distribution and estimation of their microbial shelf life as well as to comply with the regulations of food safety and protection agencies (Hunt, 1984; Anonymous, 1992; Harrigan, 1998a). To simulate the household and commercial storage of mussels, various temperatures and forms of storage were used. In the first experiment mussels were stored at three temperatures: 1) -12 ± 2°C, which is considered to be frozen storage temperature of seafood. 2) 2 ± 2°C which simulates normal temperature in household refrigerators. 3) 9± 2°C considered to be abuse temperature. In the second experiment all samples were stored at 2 ± 2°C. In the final experiment mussels were placed on crushed ice which is a normal practice during transportation and display in seafood stores.

Various maximum acceptable levels of APC (10^5 to 10^7) in fish and shellfish have been set in different countries (Busta, 1984; Hunt *et al.*, 1984; Colby *et al*, 1995; Valle *et al.*, 1998). However, it is generally recommended that APC on PCA supplemented with

1% NaCl should not exceed 10^5 or log 5 CFU/g (Anonymous, 1992). Because there are no data for the maximum acceptable levels of microbial counts on MA, log 5 was considered to be the maximum acceptable level in this study. Consequently, shelf life of Fortune Harbor mussels at 2°C was 3 to 4 days longer than for those stored at the "abuse" temperature (9°C). Similar trends were observed when other wild mussels' as well as cultured and wild scallops' were considered. Meanwhile, the short shelf life (<3 days) of Charles Arm mussels stored at 2°C (year 2000) can be attributed to the relatively higher initial counts. Low cold temperature storage can maintain the initial microbial status of the product, but can not improve it. Therefore, for improved shelf life it is important to obtain blue mussels with low initial bacterial counts. Storing blue mussels at –12°C increased the shelf life to more than 10 days. No further microbial analysis was carried out beyond 10 days, but bivalves may have a shelf life of several months when frozen (Jay, 1996).

Based on the results of this study, the use of MA instead of PCA and PCA + 1% NaCl to evaluate the general microbial content of bivalves should be considered. Furthermore, additional studies are required to obtain maximum acceptable bacterial levels on MA. Meanwhile, if PCA is to be used for microbial analysis of mussels, then regression equations established in the current study may be useful to obtain more accurate estimation of the microbial content.

Chapter IV

Changes in meat content, shell size, microbial quality and lipid composition during growth of Newfoundland blue mussels (*Mytilus edulis*) cultured at two sites (Charles Arm and Fortune Harbour)

4.1 Introduction

Various studies have examined chemical and biochemical seasonal changes in blue mussels (Choi, 1970). Slabyj *et al.* (1978) found seasonal effects on proximate composition and quality of wild and cultured blue mussels meats. Krzynowek and Wiggin (1979) found that seasonal variations in the proximate composition in cultured blue mussels affects the quality of cooked product during storage at –20 °C. The authors suggested that mussels in Northeast United States should be harvested in late spring or early summer for optimum quality retention in mussels stored at –20 °C. Krzynowek (1985) reported seasonal variations in cholesterol levels in blue mussels. Dolmer (1998) observed that growth of cultured blue mussels was affected by season, salinity and current speed. More recently, Orban *et al.* (2002) evaluated seasonal changes in chemical composition in Italian cultured blue mussels and found that fatty acid composition of mussels changes depending on harvest season. However, information with regard to seasonal variation in physical and environmental parameters of cultured Newfoundland blue mussels is either outdated (Sutterlin *et al.*, 1984; Dabinett and Clements, 1994; Mills, 1994;) or fragmented in the literature. It appears that there is no comprehensive up-to-date study in the literature examining the

variations in physical, chemical and microbial parameters of growing Newfoundland blue mussels and their aquaculture sites. Therefore, the objectives of this study were to: (1) measure seasonal variations in salinity, chlorophyll *a* content, temperature and microbial content of the oceanic water at two Newfoundland blue mussel aquaculture sites (two stations per site in various seasons and years); (2) determine seasonal variations in fatty acid composition of plankton from the aquaculture sites; (3) examine variations in meat content, shell size, microbial content, fatty acid profiles, and lipid classes during the growth of blue mussels within the four stations of the two aquaculture sites and during various seasons and years and 4) determine sterol composition of cultured mussels and their plankton food.

4.2 Materials and Methods

The experimental set up is described in subsection 2.13.2. Samples of mussels and plankton were collected as described in section 2.2. Physical measurements of mussels and aquaculture sites were taken as described in section 2.3. Sections 2.4 and 2.5 describe the microbial analysis of samples, subsections 2.8.2 and 2.9.2 the fatty acid determination, section 2.10 the lipid determination, section 2.11 the sterol determination and section 2.14 the statistical analysis.

4.3 Results

4.3.1 Seasonal and annual variations in salinity, temperature and chlorophyll *a*

Water salinity fluctuated within the four stations from a minimum of 28.1 psu in August 2001 (Fortune Harbour II) to a maximum of 32.2 psu in April 2001 (Fortune Harbour I). Water temperature raised from a minimum of -1.3°C in April 2001 (Fortune Harbour I) to a maximum of 16.6°C in August 2001 (Charles Arm II). Chlorophyll *a* ranged from 0.40 µg / L in June 2000 (Charles Arm I) to 1.84 µg / L in May / June 2001 (Fortune Harbour I).

Salinity (psu) was high in June 2000 then decreased in September and October 2000 followed by a sharp increase in April 2001, then decreased again from August to October 2001. The reverse trend was observed for seasonal variation in temperature. Initial values in June 2000 were low, thereafter they increased in September 2000 and then decreased to the lowest values in April 2001 followed by a gradual increase to reach high values in August 2001. There were no significant ($p > 0.05$) differences in Chlorophyll *a* throughout the sampling period from June 2000 to October 2001 (Figure 4.1).

4.3.2 Seasonal and annual variations in microbial content of mussels and water samples

There were no significant differences ($p > 0.05$) in APC and PPC of blue mussels and water samples within the four stations throughout the sampling period in years 2000 and 2001, with some exceptions. The highest APC of mussels was in August 2000 (3.41 ± 0.05 CFU/g) and August 2001 (3.61 ± 0.06 CFU/g) (Table 4.1).

Figure 4.1 Seasonal and annual variation in salinity (psu), temperature (°C) and chlorophyll *a* (μg/L) at Newfoundland blue mussel (*Mytilus edulis*) aquaculture sites in 2000 and 2001. Values are means of the four stations ± SD, values within each variable with different letters are different ($p < 0.05$) from one another.

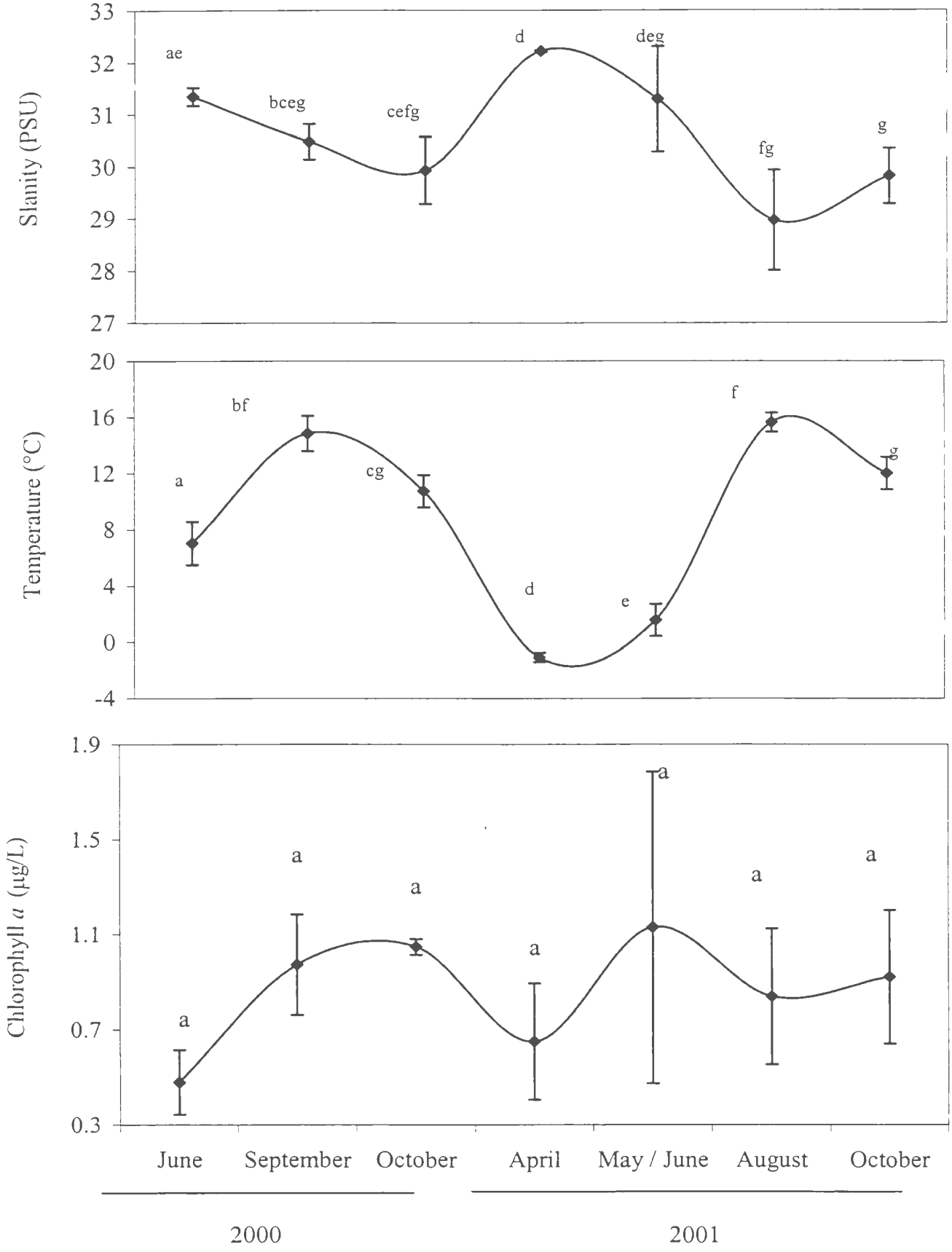

Salinity (PSU)
33
32
31
30
29
28
27
ae
bceg
cefg
d
deg
fg
g
Temperature (°C)
20
16
12
8
4
0
-4
a
bf
cg
d
e
f
g
Chlorophyll a (µg/L)
1.9
1.5
1.1
0.7
0.3
a
a
a
a
a
a
a
June
September
October
April
May / June
August
October
2000
2001

Table 4.1 Seasonal and annual variations in aerobic bacterial count (APC) and psychrotrophic plate count (PPC) in cultured Newfoundland blue mussels and water in aquaculture sites in 2000 and 2001

Month	Year	Mussels		Ocean water	
		APC Log (CFU/g)	PPC Log (CFU/g)	APC Log (CFU/g)	APC Log (CFU/g)
June	2000	3.35 ± 0.04^{abcefh}	3.14 ± 0.02^{adfh}	3.45 ± 0.05^{abch}	3.26 ± 0.10^{aeh}
August	2000	3.41 ± 0.05^{bcfh}	2.86 ± 0.06^{b}	3.49 ± 0.07^{bh}	2.92 ± 0.10^{b}
September	2000	3.35 ± 0.03^{cefh}	3.31 ± 0.19^{ceh}	3.46 ± 0.10^{cdh}	3.15 ± 0.21^{cadeh}
October	2000	3.26 ± 0.02^{def}	3.12 ± 0.06^{dfhi}	3.35 ± 0.06^{defh}	3.19 ± 0.07^{dfg}
April	2001	3.31 ± 0.04^{ef}	3.23 ± 0.02^{ef}	3.31 ± 0.01^{ef}	3.32 ± 0.01^{e}
May / June	2001	3.32 ± 0.17^{fh}	3.14 ± 0.07^{fh}	3.27 ± 0.09^{fh}	3.22 ± 0.07^{fg}
August	2001	3.61 ± 0.06^{g}	3.07 ± 0.34^{gi}	3.53 ± 0.07^{g}	3.20 ± 0.29^{gh}
September	2001	3.41 ± 0.10^{h}	3.32 ± 0.21^{h}	3.27 ± 0.09^{h}	3.37 ± 0.02^{h}
October	2001	3.18 ± 0.01^{i}	3.06 ± 0.05^{i}	ND	ND

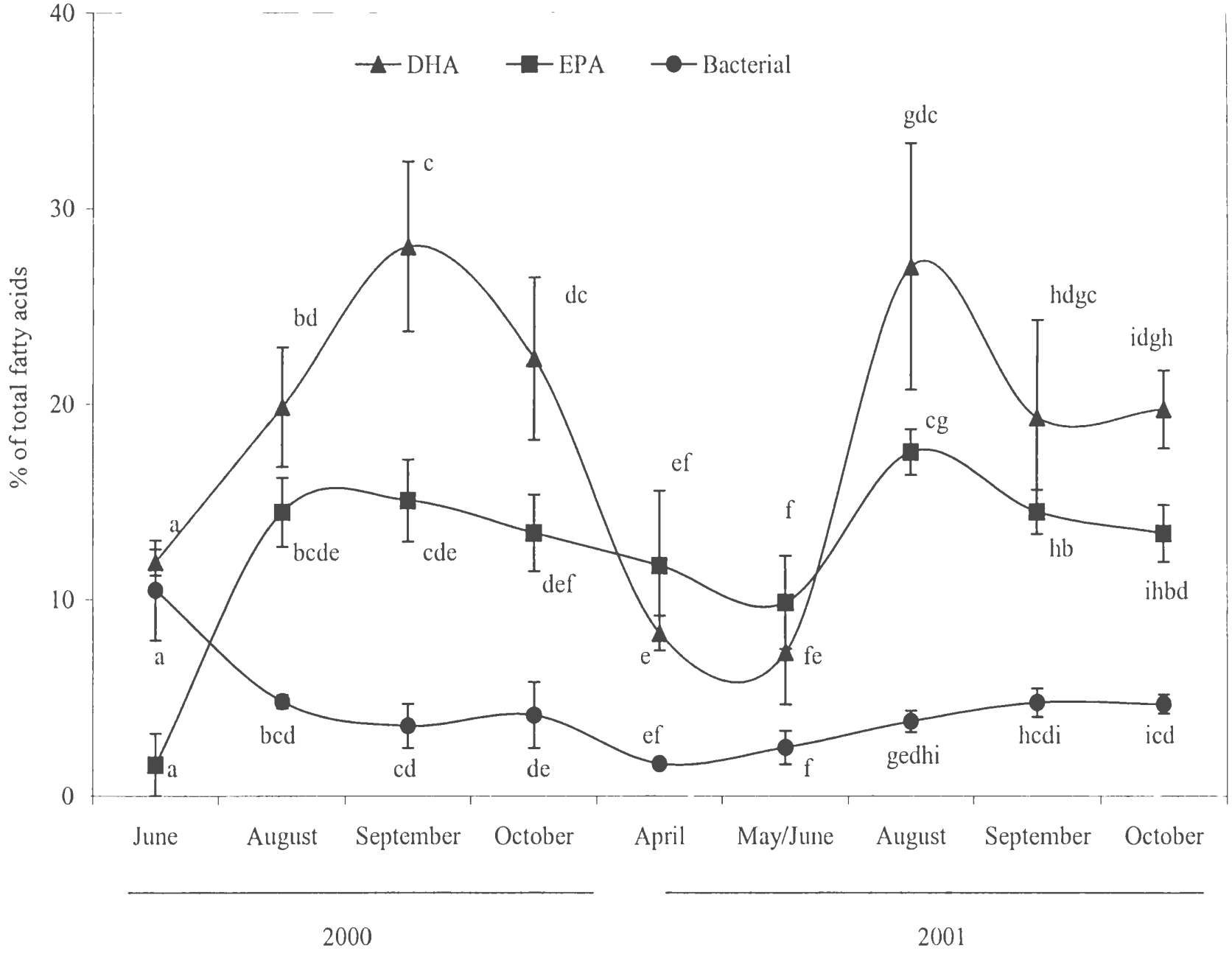
ND; not determined, values are means of the four stations ± SD, values within each column with different superscript are different ($p<0.05$) from one another.

These values corresponded with the lowest PPC (2.86 ± 0.06 CFU/g) and (3.07 ± 0.34 CFU/g) in August 2000 and 2001 respectively. A similar trend was observed when water samples were considered. The highest APC of water samples was in August 2000 (3.49 ± 0.07 CFU/g) and August 2001 (3.53 ± 0.07 CFU/g) which corresponded with low PPC values of 2.92 ± 0.10 CFU/g in August 2000 and 3.20 ± 0.07 CFU/g in August 2001.

4.3.3 Seasonal and annual variations in selected fatty acid profiles in plankton samples

No significant ($p > 0.05$) differences in DHA, EPA, or bacterial fatty acid composition (% of total fatty acids) of plankton samples among the four stations were observed throughout the sampling period in 2000 and 2001 (Appendices 4.1 and 4.2). Therefore, the fatty acid composition in the four stations was averaged and plotted against the sampling months to give the seasonal variations in fatty acid compositions at mussel culture sites (Figure 4.2). The DHA content was initially low (11.9 ± 0.68%) in June 2000, but increased gradually to reach 22.3 ± 4.15% in October 2000. A similar trend was observed in 2001. DHA content of plankton in May / June 2001 was 7.3 ± 2.6%, increasing gradually to 19.73 ± 2.00% in October 2001. EPA contents in June 2000 were low at 1.6 ± 1.5% in June 2000 and May/June 2001 at 9.86 ± 2.36%, but increased in August and September 2000 and 2001 followed by a decrease in October of both years.

Figure 4.2 Seasonal and annual variations in docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and total bacterial fatty acids (*i*15:0, *ai*15:0 15:0, *i*16:0, *ai*16:0, *i*17:0, *ai*17:0 and 17:0) content (% of total fatty acids) of plankton samples obtained from blue mussels (*Mytilus edulis*) aquaculture sites in 2000 and 2001. Value in each month is mean of the four stations ± SD, values within each variable with different letters are different ($p < 0.05$) from one another.


40
30
20
10
0
% of total fatty acids
DHA
EPA
Bacterial
a
bd
c
dc
a
bcde
cde
def
a
bcd
cd
de
ef
e
ef
f
fe
gdc
cg
f
gedhi
hdgc
idgh
hb
ihbd
hcdi
icd
June
August
September
October
April
May/June
August
September
October
2000
2001

The lowest value of bacterial fatty acids of plankton were detected in April 2000 (1.66 ± 0.01%), while the highest was obtained in June 2000 (10.4 ± 2.56%). There were no significant differences ($p>0.05$) in bacterial fatty acids in plankton samples from August to October 2000 or August to October 2001.

4.3.4 Seasonal and annual variations in shell length and meat content of cultured mussels

There was no significant difference ($p > 0.05$) in the meat content among the mussels obtained from the four stations throughout the sampling period in 2000 and 2001. Therefore, the weights were averaged for all sites and stations and plotted against the sampling intervals (Figure 4.3). There was a gradual linear increase in the meat content of mussels from 0.48 ± 0.28 g in June 2000 to 3.69 ± 0.96 g in October 2001. However, the liner growth reached a plateau in May / June 2001, after which there were no significant ($p > 0.05$) differences in average weights of harvested blue mussels.

There were no significant ($p > 0.05$) differences in shell length among mussels from the four stations throughout the sampling period. Therefore, length values were averaged for all sites and stations and plotted against the sampling months (Figure 4.4). There was a gradual linear increase in the average shell length of mussels from June 2000 (23.50 ± 0.23 mm) until a plateau was reached in April 2001 (50.85 ± 4.46 mm). Thereafter, there were no significant ($p > 0.05$) differences in average shell length of mussels obtained in April to October 2001, with one exception (August 2001).

Figure 4.3 Meat content (g) of cultured Newfoundland blue mussels (*Mytilus edulis*) during their growth in 2000 and 2001. Value in each month is mean of the four stations ± SD, values with different letters are different ($p < 0.05$) from one another.

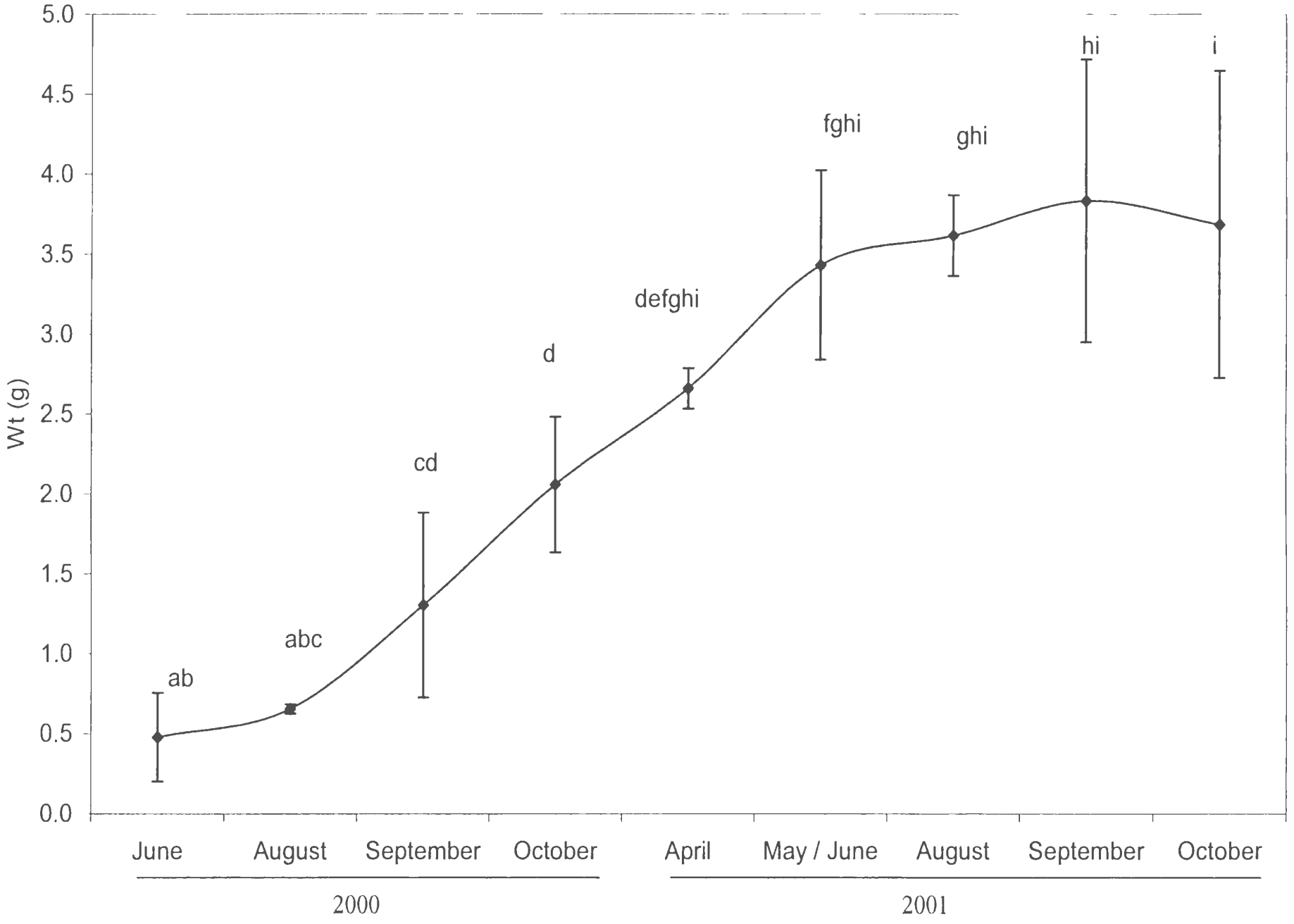
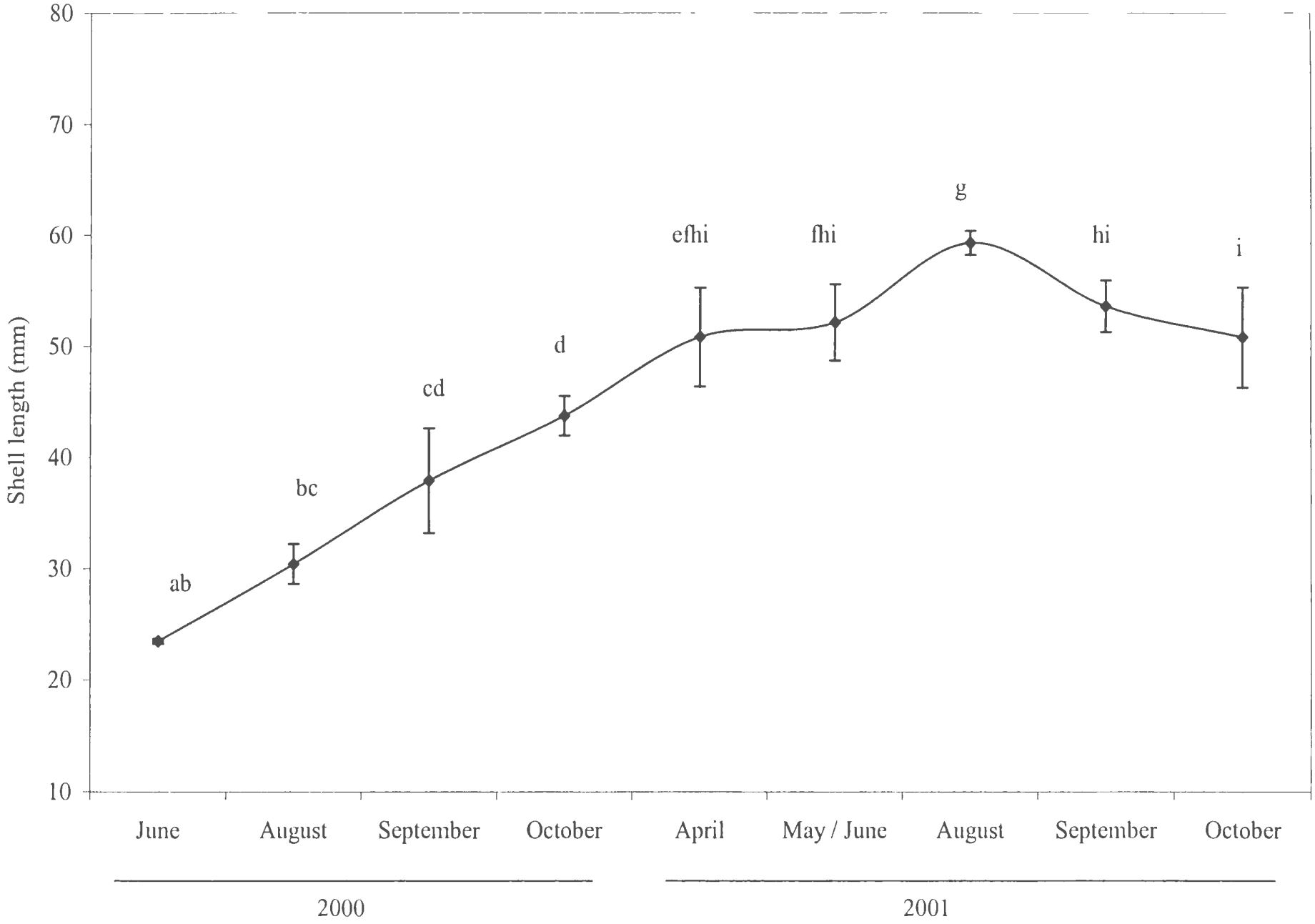

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Wt (g)
ab
abc
cd
d
defghi
fghi
ghi
hi
i
June
August
September
October
April
May / June
August
September
October
2000
2001

Figure 4.4 Shell length (mm) of cultured Newfoundland blue mussels (*Mytilus edulis*) during their growth in 2000 and 2001. Values are means of the four stations ± SD, values with different letters are different ($p < 0.05$) from one another.


80
70
60
50
40
30
20
10
Shell length (mm)
ab
bc
cd
d
efhi
fhi
g
hi
i
June
August
September
October
April
May / June
August
September
October
2000
2001

The average shell length of mussels collected in August 2001 was significantly ($p < 0.05$) higher than of other sampling times.

4.3.5 Seasonal variations in lipid composition of cultured mussels

There were no significant ($p > 0.05$) differences in the lipid classes of mussels among stations with the following exceptions; TAG from Charles Arm stations I and II were significantly ($p<0.05$) higher than TAG from Fortune Harbour stations I and II in June 2000 (Figure 4.5).

The TAG content of mussels from Charles Arm station I in October 2000 was significantly ($p < 0.05$) higher than other stations in Charles Arm and Fortune Harbour. Furthermore, sterol content of mussels from station II in Charles Arm in September 2000 contained significantly ($p < 0.05$) lower amounts of sterols, while in October 2000 mussels from the same station contained higher ($p < 0.05$) amounts of sterols. Phospholipid content in mussels from Fortune Harbour stations was higher ($p < 0.05$) than Charles Arm stations in August 2000. Mussels from station II in Fortune Harbour in September 2000 contained higher ($p > 0.05$) amounts of PL compared with other stations. Figure 4.6 presents the average values of each lipid class from mussels obtained from all stations versus sampling months. Total lipids, TAG, sterols and PL of mussels in June 2000 were significantly ($p > 0.05$) higher than in August to October 2000. Although total lipids and other lipid composition values increased gradually from August 2000, there was no significant ($p > 0.05$) difference in lipid composition of mussels from August to October 2000.

Figure 4.5 Triacylglycerol (TAG), sterols, phospholipids (PL), and total lipids (TL) concentrations (mg/g dry weight) in cultured Newfoundland blue mussels (*Mytilus edulis*) during their growth at various stations in Charles Arm and Fortune Harbour aquaculture sites. The value in each month is the mean of the four stations ± SD, values within each variable with different letters are different ($p < 0.05$) from one another.

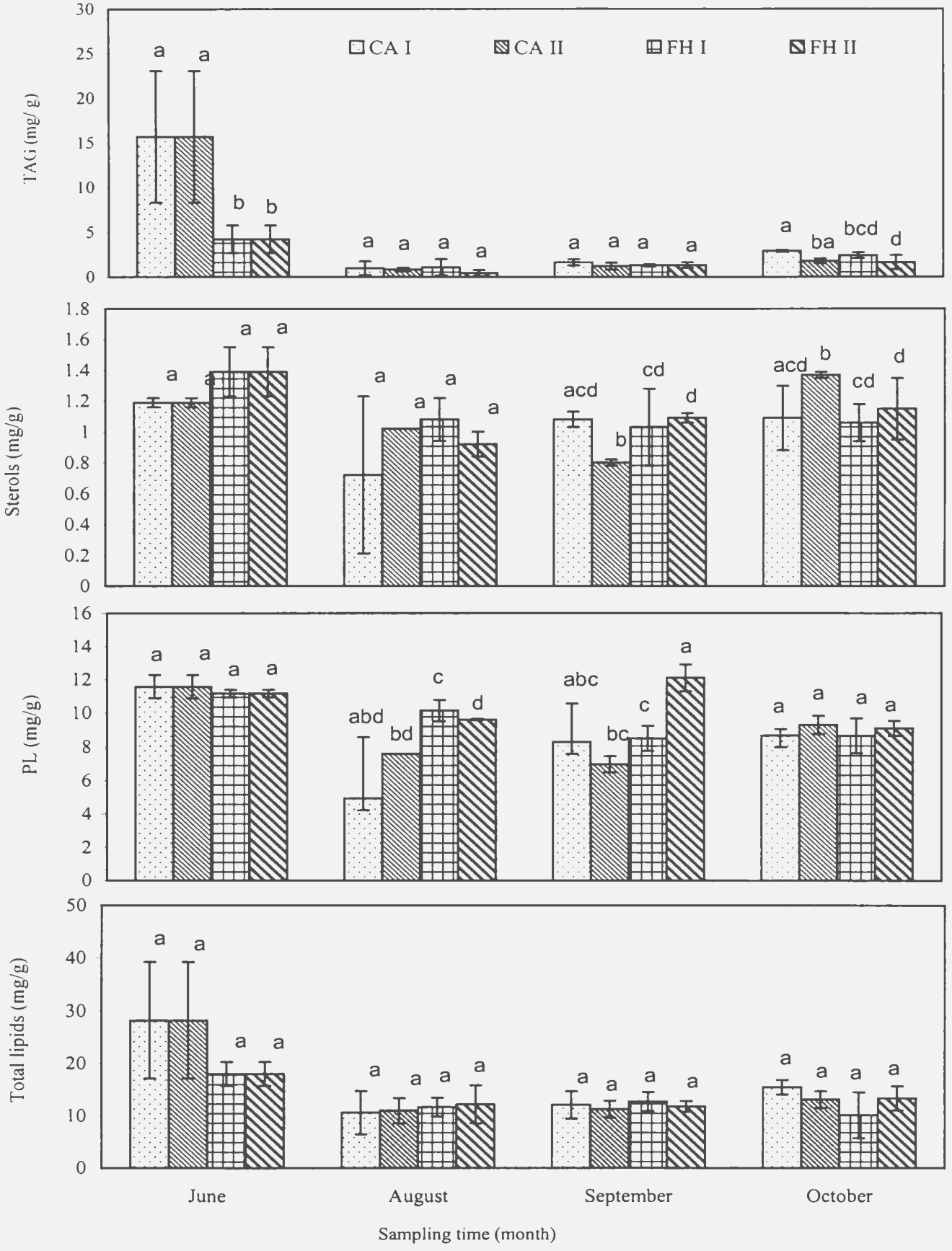
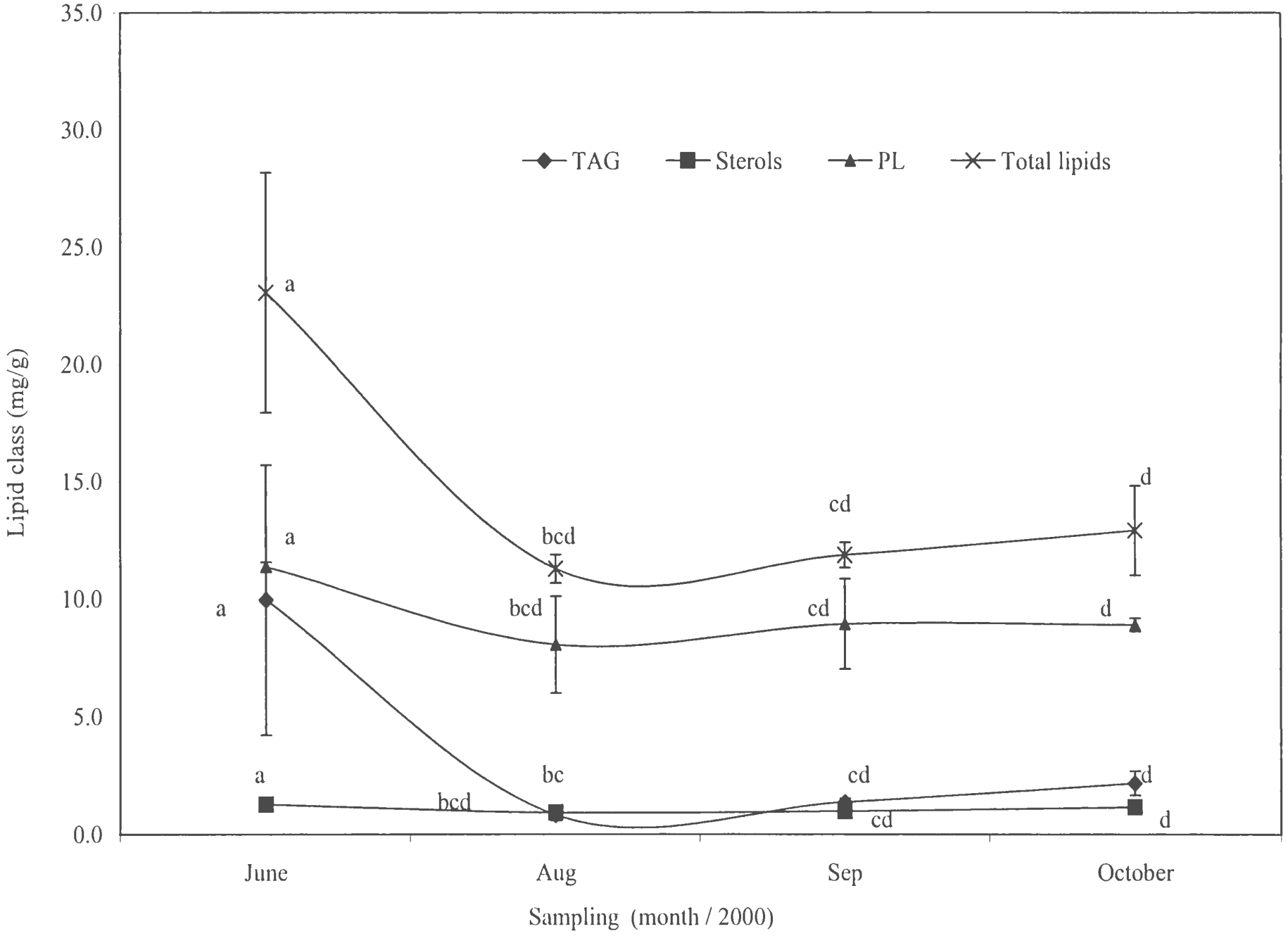

CA I
CA II
FH I
FH II
TAG (mg/g)
Sterols (mg/g)
PL (mg/g)
Total lipids (mg/g)
June
August
September
October
Sampling time (month)

Figure 4.6 Triacylglycerols (TAG), sterols, phospholipids (PL), and total lipids (TL) concentrations (mg/g dry weight) of cultured Newfoundland blue mussels (*Mytilus edulis*) during their growth in 2000. Value in each month is mean of the four stations ± SD. Each variable with different letters are different ($p < 0.05$) from one another.


35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Lipid class (mg/g)
TAG
Sterols
PL
Total lipids
a
a
a
bcd
bcd
bc
cd
cd
cd
d
d
d
June
Aug
Sep
October
Sampling (month / 2000)

4.3.6 Seasonal and annual variations in fatty acid profiles of cultured mussels

No significant ($p > 0.05$) differences were observed in DHA, EPA, or total bacterial fatty acids (% of total fatty acids) in mussels from the four stations throughout the sampling period in 2000 and 2001. To obtain seasonal variations in EPA, DHA, and total bacterial fatty acids during the growth of mussels in 2000 and 2001, values for the four stations were averaged and plotted against sampling months (Figure 4.7). EPA content was initially high at 19.5 ± 3.2 % in June 2000, then decreased to 13.2 ± 1.42% in October 2000. This trend was repeated in 2001 with mussels having higher ($p < 0.05$) amounts in April and May / June than in August to October. Meanwhile, DHA was initially low at 17.03 ± 3.71 % in June 2000, thereafter increased to 25.01 ± 1.53 % in October 2000. A similar trend was observed in 2001, where the DHA content was lowest in April and May / June (14.35 ± 3.2 %) and increased to 23.69 ± 3.56% in October. The initial total bacterial fatty acids were 4.04 ± 1.61% in June 2000, after which increased from August to October 2000. Similarly, the average bacterial fatty acids detected in blue mussels in May / June 2001 were 2.35 ± 0.52 %, followed by an increase from August to October 2001.

4.3.7 Sterol composition of mussel and plankton

Cholesterol was the main sterol in both plankton and mussel samples with relative ion counts (RIC) of 76.15 % and 45.08 %, respectively (Table 4.3). The main ion fragments from the mass spectrum of cholesterol were 458, 443, 368, 353, 329, 255, 147 and 129 (Appendix 4.5).

Figure 4.7 Seasonal and annual variations in docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and total bacterial fatty acids (*i*15:0, *ai*15:0 15:0, *i*16:0, *ai*16:0, *i*17:0, *ai*17:0 and 17:0) content (% of total fatty acids) of cultured Newfoundland blue mussels (*Mytilus edulis*) during their growth in 2000 and 2001. Values are means of the four stations ± SD. Each variable with different superscript are different ($p < 0.05$) from one another.

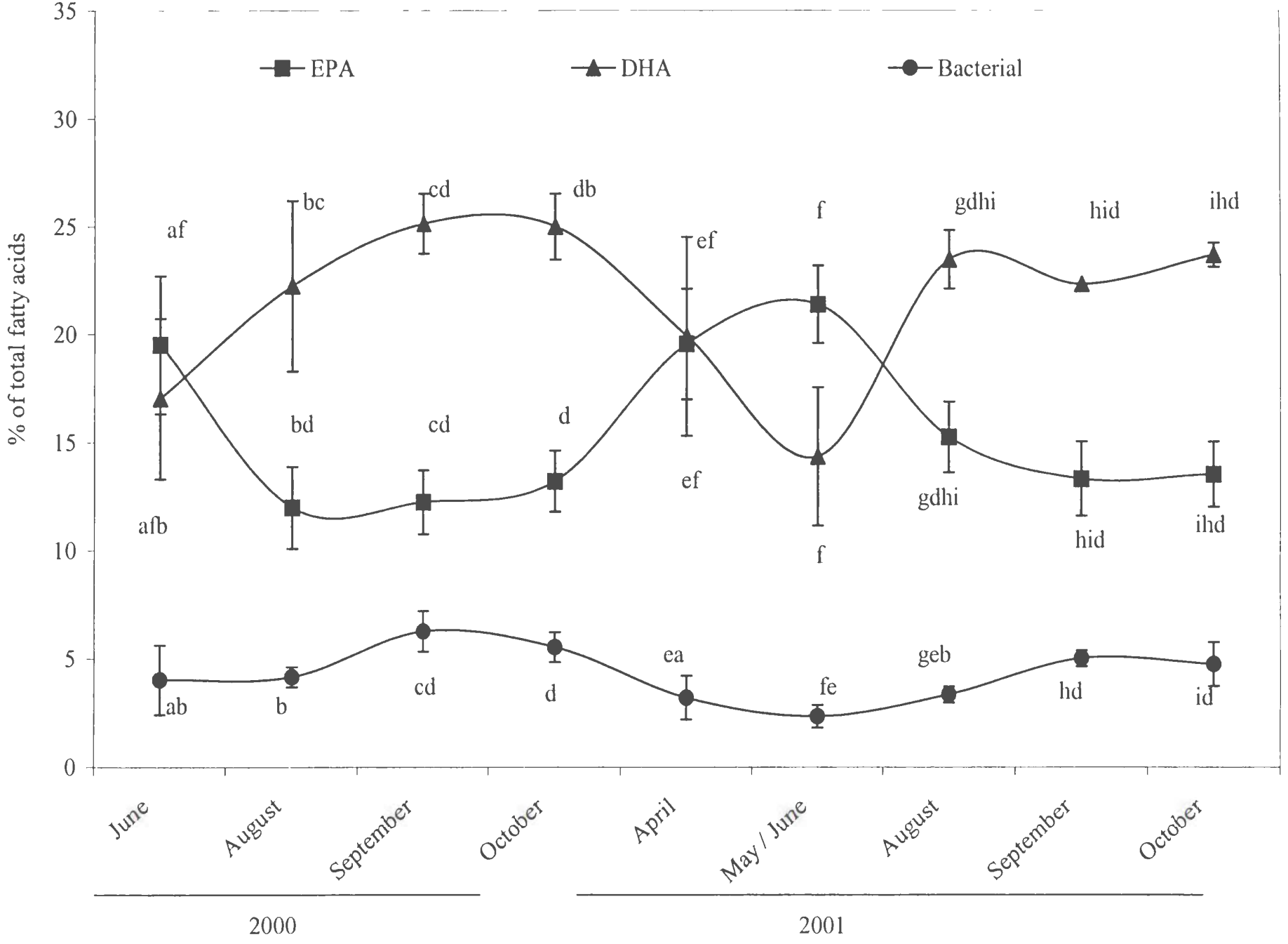

EPA
DHA
Bacterial
% of total fatty acids
35
30
25
20
15
10
5
0
af
bc
cd
db
f
ef
gdhi
hid
ihd
afb
bd
cd
d
f
ef
gdhi
hid
ihd
ab
b
cd
d
ea
fe
geb
hd
id
June
August
September
October
April
May / June
August
September
October
2000
2001

Table 4.2 Major sterols forms detected in mussel and plankton samples* and their relative ion counts

			% Relative ion counts	
M+1	Chemical name	Trivial name	Mussels	plankton
442	24-norcholesta-5, 22E-dien-3β-ol	24-nordehydrocholesterol	7.65	4.33
456	cholesta-5,22 Z-dien-3β-ol	cis-22-dehydrocholesterol	5.6	2.28
456	cholesta-5,22 E-dien-3β-ol	trans-22-dehydrocholesterol	16.69	9.96
458	Cholesta-5-en-3β-ol	cholesterol	45.08	76.15
456	cholesta-5,24-dien-3β-ol	desmosterol	0.61	ND
456	steradienol C_{27} with two double bonds	-	10.69	3.0
470	24-methylcholesta-5, 22E-dien-3β-ol	Brassicasterol / crinosterol	11.18	4.28
484	24-ethylcholesta-5, 22E-dien-3β-ol	stigmasterol / poriferasterol	0.42	ND
468	24-methylcholesta-5,7, 22E-trien-3β-ol	ergosterol	0.56	ND
470	24-methylcholesta-5, 24 (28)-dien-3β-ol	24-methylene cholesterol	0.54	ND

ND, not detected, *samples were obtained in October 2000 from station II in Charles Arm.

The second most abundant sterol in mussels and plankton was trans-22-dehydrocholesterol followed by brassicasterol, 24-nordehydrocholestrol, a C_{27} sterol with two double bonds and cis-22-dehydrocholesterol. Other sterols were detected in small quantities (less than 1 %) in mussels or not detected in the plankton samples. The main ion fragments in the phytosterol brassicasterol were 470, 380, 341, 282, 255, 213, 159, 129 and 69 (Appendix 4.6). The major ion fragments in the mass spectrum of sterols comes from TMS-sterols complex, the parent ion (M+) and the fragmentation of various functional groups (Jones *et al* ., 1994).

Discussion

Commercial production of cultured Newfoundland blue mussels normally takes 18 months from one year old mussels (spat) to market size mussels (2.5 years old). The mussels are cultivated in sheltered marine environments, which are known to be a good habitat for wild mussels. The selection of aquaculture sites, cultivation period and harvest time has generally been based on practical experience, market demands and outdated research studies. This study intended to find out: 1) if the two aquaculture sites, currently used for commercial production of blue mussels in Newfoundland, can render significant differences in the quality of the produced mussels; 2) which environmental, chemical and biological variables are most important for future selection of new mussel aquaculture sites and 3) what is the optimum harvest month during the year.

The general trend for all environmental variables was that there were no major differences among the four stations throughout the sampling period, with a few exceptions. Even when some differences were observed among the four stations, it cannot be directly related to the biochemical composition of the cultivated mussels because mussel composition would be determined during exposure to various environmental variables prior to the sampling day. Furthermore, these differences are not a major contributor to the marketing of the product nor do they adversely affect consumer perception of cultivated mussels. Therefore, the values of all variables examined were averaged and plotted against sampling months to obtain the seasonal and annual variations in these variables at Newfoundland mussel aquaculture sites.

The higher salinity values in April 2001 can be explained by low temperatures, which lead to the formation of ice and reduced runoffs because of water being trapped in ice and snow. Once the ice started to melt, run-offs of rain and river waters started to dilute the aquaculture sites during June to August, and salinity decreased. This fluctuation in salinity may not adversely affect the growth or biochemical composition of mussels as they can tolerate salinities from 25 to 33 psu (Sutterlin *et al.*, 1980). Years 2000 and 2001 have been noted as the initiation of major climate changes in North America (Richardson and Schoeman, 2004), therefore seasonal and annual variation in the temperature of ocean water should be considered as one of the criteria for blue mussel aquaculture site selection. Temperature fluctuation in the aquaculture sites affect chlorophyll *a* content or algal types and their fatty acid compositions, and the microbial composition of water, which in turn can affect microbial status, lipid and

fatty acid composition, meat content and shell size of cultivated mussels (Stirling and Okumus, 1995; McKenzie *et al.*, 2002; Orban *et al.*, 2002). No seasonal or annual variation in chlorophyll *a* was observed, although two bell shaped peaks were evident in September and October 2000 and May / June 2001. This can be attributed to the large standard deviation, which indicates that some stations may have had more chlorophyll at the time of sampling. Therefore, in order to obtain meaningful data for chlorophyll *a* content at each aquaculture site, more frequent measurements at different tidal cycles may be required (Ogilvie *et al.*, 2001).

High temperatures in August of 2000 and 2001 may explain the relatively high APC (bacteria which can grow between 15 and 30°C) but low PPC (bacteria which can grow between 0 and 10°C) in mussels and water (Hunt *et al.*, 1984; Gilliland *et al.*, 1984; Ray, 1996). This indicates that the microbial content of water is directly affecting the mussel microbial content. Furthermore, high APC counts in the water may indicate the presence of harmful bacteria, but this would have to be confirmed by further identification to species level (Anonymous, 1992; Colby *et al.*, 1995; Jay, 1996). Various maximum acceptable levels of APC (10^5 to 10^7) in bivalves have been set in different countries (Busta, 1984; Hunt *et al.*, 1984; Colby *et al.*, 1995), but it is generally recommended that APC should not exceed 10^5 or log 5 CFU/g (Anonymous, 1992). Based microbial counts, it is acceptable to harvest the mussels throughout the year as the levels of APC were far less than the maximum levels permitted. Furthermore, cultivated mussels have a shorter microbial shelf life (days on ice to reach 10^5 or log 5 CFU/g) in summer than in winter (Chapter I, Harding *et al* ., 2002).

Moreover, the bacterial counts at the examined aquaculture sites were lower than published values (up to 10^7 Log CFU/ ml) for marine environments elsewhere (Austin, 1988a; Lizarraga-Partida, 1998) and this perhaps can be explained by the relatively low temperatures observed in this study with a maximum of 16°C in the summer of 2000 .

The dominance of algal fatty acids by DHA at the aquaculture sites from August to October in 2000 and 2001 can be explained by the presence of dinoflagellates in which DHA is the major fatty acid (Budge *et al.*, 2001; McKenzie *et al* , 2002; Copeman and Parrish, 2003). Furthermore, the higher amounts of EPA in August 2000 and 2001 can be attributed to the presence of diatoms, in which EPA is the dominance fatty acid (Budge *et al.*, 2001; Ramos *et al.*, 2003). Both DHA and EPA were low in April 2001 indicating a lower algae population due to subzero temperatures (Ogilvie *et al.*, 2000) or inappropriate light conditions due to long nights and snow and ice coverage of the aquaculture site. The fatty acid composition of algae directly affected the fatty acid profiles of cultivated mussels in various seasons and years. This can be explained by the filter feeding behaviour of bivalves (Riisgard, 1991; Parrish *et al.*, 1995; Parrish *et al.*, 1998). Furthermore, EPA and DHA contents in cultured mussels showed opposite trends in the spring and summer indicating that one is increasing and the other is decreasing. This may be explained by temperature changes in the spring to subfreezing values which lead to an increase in the production of EPA in bivalves (Hall *et al.*, 2000; Hall *et al* ., 2002). The relatively higher values for total bacterial fatty acids in plankton and mussel samples in summer and fall 2000 and 2001 compared with spring 2001 may be attributed to contamination with runoff waters,

higher aerobic bacterial plate counts and spawning of mussels. It is also possible that mussels are consuming bacteria associated with algae (Sutterlin *et al.*, 1980; Rheinheimer, 1982).

The optimum growth temperatures of mussels are between 10 and 20 °C, explaining the rapid growth (in shell length and meat weight) of mussels from June to October 2000, after which growth slowed down from October to April 2001. Furthermore, mussels reached market size by May / June 2001 with no further significant growth in shell length or meat weight. The high value of shell size in August 2001 is considered an anomaly, perhaps due to the collection of relatively larger shells.

A raise of water temperature from sub zeros to 8-10 °C, and thus a decreases in salinity by the end of June in 2000 and 2001 may have induced spawning in mussels (Bayne and Thompson, 1970) which affected their lipid composition. The most marked effect was a reduction in TAG content of mussels. This can be explained by the fact that gametes, which are released into the water for external fertilization, are composed mainly of TAG. Furthermore, lack of recovery in TAG from August to September may indicate that spawning was occurring throughout this period. It is possible that mussels started to recover from spawning by the beginning of October or November. Spawning can reduce the quality of mussels and thus their market value and therefore proper harvest time should exclude the spawning period. One interesting observation was the significantly ($p < 0.05$) higher amount of TAG in mussels from Charles Arm than in those from Fortune Harbour in June 2000 (the starting time). This can be explained by the actual sampling schedule. Charles Arm was sampled on June 6, while Fortune

Harbour on June 29. It is possible that spawning may have started in Charles Arm but not in Fortune Harbour. It is important to try to sample sites as closely in time as possible if results are to be used for comparative purposes.

Cholesterol is the main sterol detected in blue mussels (Krzynowek, 1985; King *et al.*, 1990; Copeman *et al.*, 2002). However, high values of cholesterol in plankton samples are unusual, and may be explained by the presence of zooplankton in the net tows used to collect algae samples (Copeman and Parrish, 2003). Furthermore, some taxa of algae have cholesterol as their dominant sterol (Napolitano *et al.*, 1993; Parrish, 1998; Copeman *et al.*, 2002).

Sterol composition of algae affected the sterol composition of mussels, which is consistent with previous studies and is attributable to the filter feeding ability of bivlaves (Napolitano *et al.*, 1993; Hall *et al.*, 2000). The presence of additional sterols which are not present in algae may either indicate anomalies due to contamination (as they were less than 1 %) or metabolic transformation by mussels (Napolitano *et al.*, 1993; Copeman *et al.*, 2002; Orban *et al.*, 2002).

Various nutritional and clinical studies in humans have indicated that n-3 PUFA and phytosterols can reduce the risk of many clinical disorders, including cardiovascular diseases (Ling, *et al* , 1995; Miettinen *et al.*, 1995; Pelletier *et al.*, 1995; Shahidi, 1998; Jones *et al* ., 1999; Newton, 2000). Therefore, occurrence of n-3 PUFA and phytosterols in Newfoundland cultured mussels may add to their marketing value (Ackman, 2002; Copeman and Parrish, 2003).

Chapter V

Effect of storing cultured Newfoundland blue mussels (*Mytilus edulis*) on ice on their microbial content, lipid oxidation, proximate composition and fatty acid profiles

5.1 Introduction

Blue mussels are a favorite shellfish that is consumed in North America, Europe, Japan and China (Smaal, 1991; Kaiser *et al.*, 1998; Mcleaod, 2002). Although frozen, vacuum packed, pickled, smoked and canned mussels are available in the market, most of the cultured mussels in Newfoundland or elsewhere are kept alive on ice or refrigerated (2-4°C) until consumed (Anonymous, 2001b; Anonymous, 2001c; Anonymous, 2003b). During refrigeration of fresh fish and shellfish, some microbiological, chemical, physical, biochemical and sensory changes may occur depending on duration and condition of storage as well as the initial quality of the seafood (Flick *et al.*, 1992; Balsundaris *et al.*, 1997; Aubourg and Madina, 1999; Lauzon, 2000). There is no universal method or instrument that can determine the quality of seafoods. Sensory methods, perhaps, are the most accurate for predicting the quality, but these are not used for routine analysis due to the need for highly trained personnel and the time consuming procedures involved (Melton, 1983; Shahidi, 1998; Koutsoumans and Nyches, 1999). Nonetheless, microbial, chemical and biochemical methods are used in conjunction with sensory analysis to evaluate the quality of seafoods (Chang *et al.*, 1998; Suvanich *et al.*, 1998; Taliadourou *et al.*, 2003). The type of analysis required by food regulations, accuracy and the availability

of instruments will determine the selection of a technique or an instrument to examine the quality of blue mussels.

Microbial content of blue mussels is generally evaluated by the conventional total plate count method, which may take up to three days before results are available (Neufeld, 1984; Harrigan and Park, 1991a; Harrigan, 1998b). Total plate count represents the number of bacteria that are capable of forming visible colonies at specified temperature, and thus high total bacterial counts (10^6 – 10^7 CFU/g) may indicate poor handling, inappropriate storage and transportation temperature, as well as old or expired seafood products (Hunt *et al.*, 1984; Jay, 1996; Bremer, 1998). The microbial content of seafoods must be known before the product leaves the production area. Therefore, rapid identification methods for micro-organisms in seafoods are important for the aquaculture industry and public safety (Harrigan, 1998b; Jabbar and Joishy, 1999; Koutsoumans and Nyches, 2000). Several rapid methods have been developed to handle large amounts of sample within reasonable time limits (Slabyj and Bolduce, 1987; Harrigan and Park, 1991b; De Boer and Beumer, 1999; Stender *et al.*, 2001). Many of the newly developed testing methods may still take between 12 and 24 h (1-2 working days) because of pre-enrichment steps or the need to prepare pure bacterial cultures (Russel, 1998; De Boer and Beumer, 1999). Specific fatty acids (odd carbon number and branched chain) are found in micro-organism, and thus may serve as lipid bacterial markers (Findly and Dobbs, 1993; Brown and Leff, 1996; Annous *et al.*, 1997; Budge *et al.*, 2001). Fatty acid profiles have been used not only to investigate the microbial communities in clinical and environmental samples, but also to examine the occurrence of healthful fatty acids in seafoods (King *et al.*, 1995; Osipov and Turova, 1997; Kirkegaard and White 2000; Rutters *et al.*, 2002; Budge and Parrish 2003).

However, the use of lipid bacterial markers to examine the microbial content of blue mussels has not been reported. Because fatty acid determination may require only 2 - 4 h, the lipid marker method may serve as the most practical to examine the microbial content of seafoods, including blue mussels.

Proximate composition (protein, fat, carbohydrate, moisture and ash) and lipid oxidation of mussels and other seafoods reflect the nutritional and flavor qualities of the stored product (Slabyj, 1977; Nalepa *et al.*, 1993; Orban *et al.*, 2001). No universal technique is available to monitor the progression of lipid oxidation, and several chemical, instrumental and sensory techniques are therefore used for seafoods (St. Angelo and Spainer, 1993; Shahidi, 1998; Shahidi and Wanasundara, 1998). While some techniques measure the loss of reactants such as oxygen or substrates such as fatty acids, others measure primary oxidation products such as hydroperoxides or conjugated dienes and secondary oxidation products, namely alcohols, aldehydes, hydrocarbons and ketones (Shahidi and Wanasundara, 1998). The TBA (2-thiobarbituric acid) test is frequently used to measure lipid oxidation in seafoods (Khalil and Mansour, 1997; Dulvik *et al.*, 1998; Lee, 1999; Sant' Ana and Mancini-Filho, 1999; Tang *et al.*, 2001; Saeed and Howell, 2002). One molecule of malonaldehyde (MA), a secondary product of lipid oxidation, reacts with two molecules of TBA to form the TBA-MA complex, which has a maximum absorbance at 532 nm. TBA may also react with other oxidation products such as 2-alkenals and 2,4-alkadienals, and thus TBARS (TBA reactive substances) reflect the total amount of aldehydes rather than MA alone (Hoyland and Taylor, 1991). Nonetheless, TBA values correlate well with sensory data (Shahidi and Wabasundara, 1998).

Despite the rapid increase in the production of blue mussels, information about the quality changes during storage of cultured mussels on ice is scarce and fragmented in the literature. In order to devise proper strategies to market premium quality cultured mussels and fill an important gap in the scientific literature, the current study was designed to: (1) examine the effect of storing cultured Newfoundland blue mussels *(Mytilus edulis)* on ice on their microbial content, lipid oxidation, proximate composition and fatty acid profiles; and (2) evaluate the use of fatty acid bacterial markers as rapid technique to examine microbial status of blue mussels compared to standard plate count techniques.

5.2 Materials and Methods

The experimental set up is described in subsection 2.13.3. Section 2.3 describes the pH measurement, sections 2.4 and 2.5 the microbial analysis of samples, section 2.6 TBARS measurement, section 2.6 proximate composition analyses, section 2.8 and subsection 2.9.1 fatty acid composition using two different methods, and section 2.14 statistical analysis.

5.3 Results

5.3.1 Microbial counts and pH changes

There was a rapid increase in total bacterial counts for 10 days in mussels stored on ice, after which bacterial growth reached a plateau (Figure 5.1). Initial bacterial counts varied from log 4.22 to log 4.28 (CFU/g) at 0 day, while after 14 days of storage on ice the bacterial counts varied from log 7.98 to log 8.08 (CFU/g). Initial pH values were around

6.4, thereafter increasing to about 6.5 and remaining there throughout the storage period (Figure 5.2).

5.3.2 Lipid oxidation and proximate composition changes

TBARS values increased gradually and reached the peak after 10 days of storage on ice to 5.53 ± 0.5 mg malonaldehyde equivalent / kg mussel meat, thereafter declining to 4.19 ± 0.11 mg malonaldehyde equivalent / kg mussel meat (Figure 5.3). There were no significant ($p > 0.05$) differences in the moisture, protein and lipid content throughout the storage period (Table 5.1). The moisture content was about 80 % and lipid content was about 2.5 %, while protein content ranged from 12.02 to 12.88 %. Ash content of mussels stored on ice was significantly ($p < 0.05$) higher for mussels stored for 14 days compared to those stored for 0 or 3 days. There was no significant ($p > 0.05$) difference in ash content among mussels stored for 7, 10 and 14 days. Ash content ranged from 1.41 to 2.53 %. Carbohydrate content was significantly ($p < 0.05$) lower ice mussels stored on ice after 14 days compared to mussels stored for 3 days. However, there was no significant difference ($p > 0.05$) in carbohydrate content among mussels at days 0, 7, 10 and 14 of storage.

5.3.3 Fatty acid profiles

Appendix 5.1 presents the fatty acid composition of mussels stored on ice as determined by the 14% boron trifluoride - methanol (14% BF_3 - MeOH) method, while Appendix 5.2 presents the fatty acid composition as determined by the sulfuric acid – methanol (H_2SO_4 - MeOH) method. Only selected (16:0, 16:1n-7, 22:6 n-3 and *ai*15:0) fatty acids and total saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and bacterial fatty acids (BFA) were statistically analysed to evaluate the storage on ice.

Figure 5.1 Total bacterial counts (CFU/g) in blue mussels stored on ice for 14 days. (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

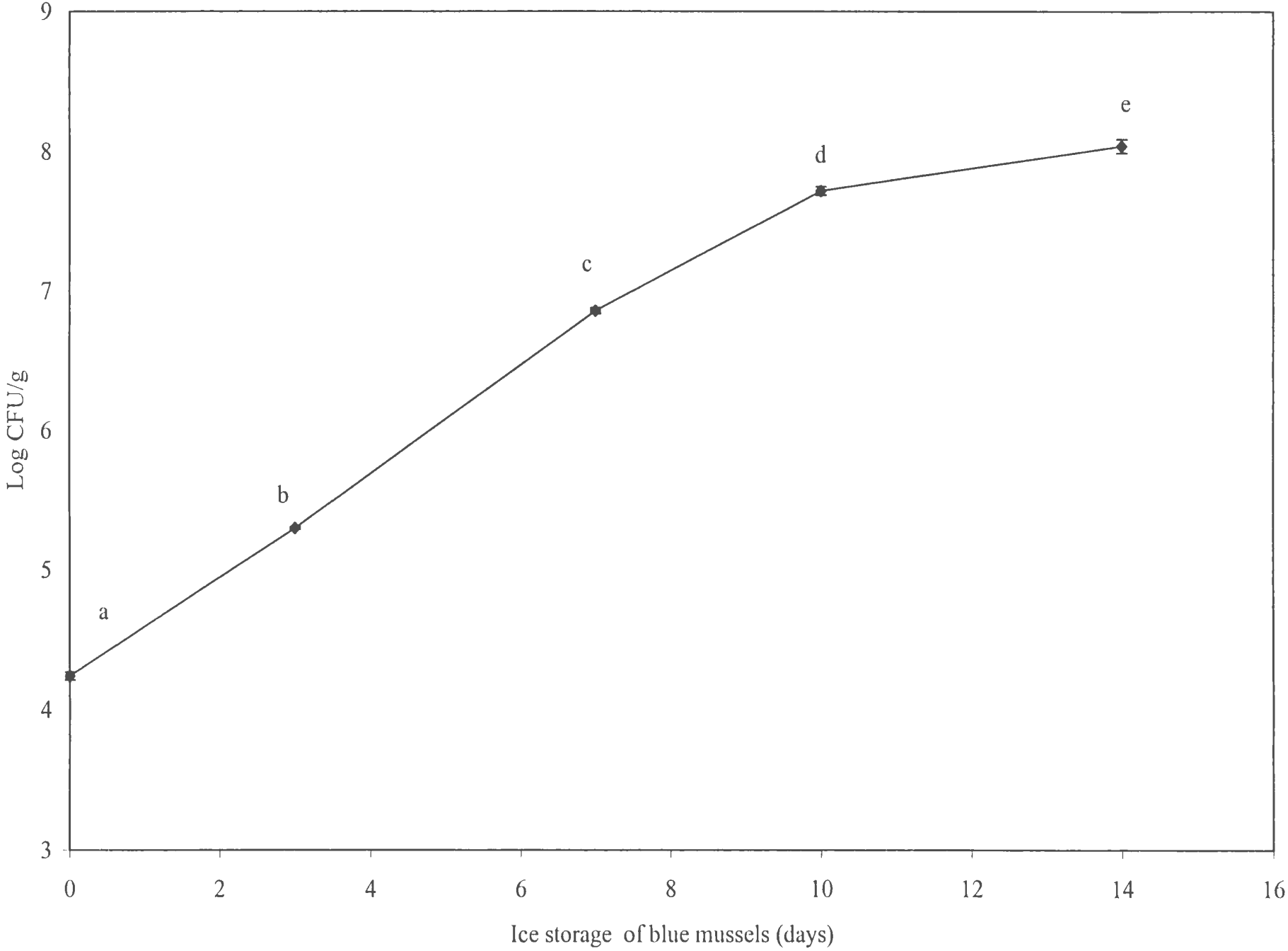

9
8
7
6
5
4
3
Log CFU/g
a
b
c
d
e
0
2
4
6
8
10
12
14
16
Ice storage of blue mussels (days)

Figure 5.2 pH of blue mussels stored on ice for 14 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

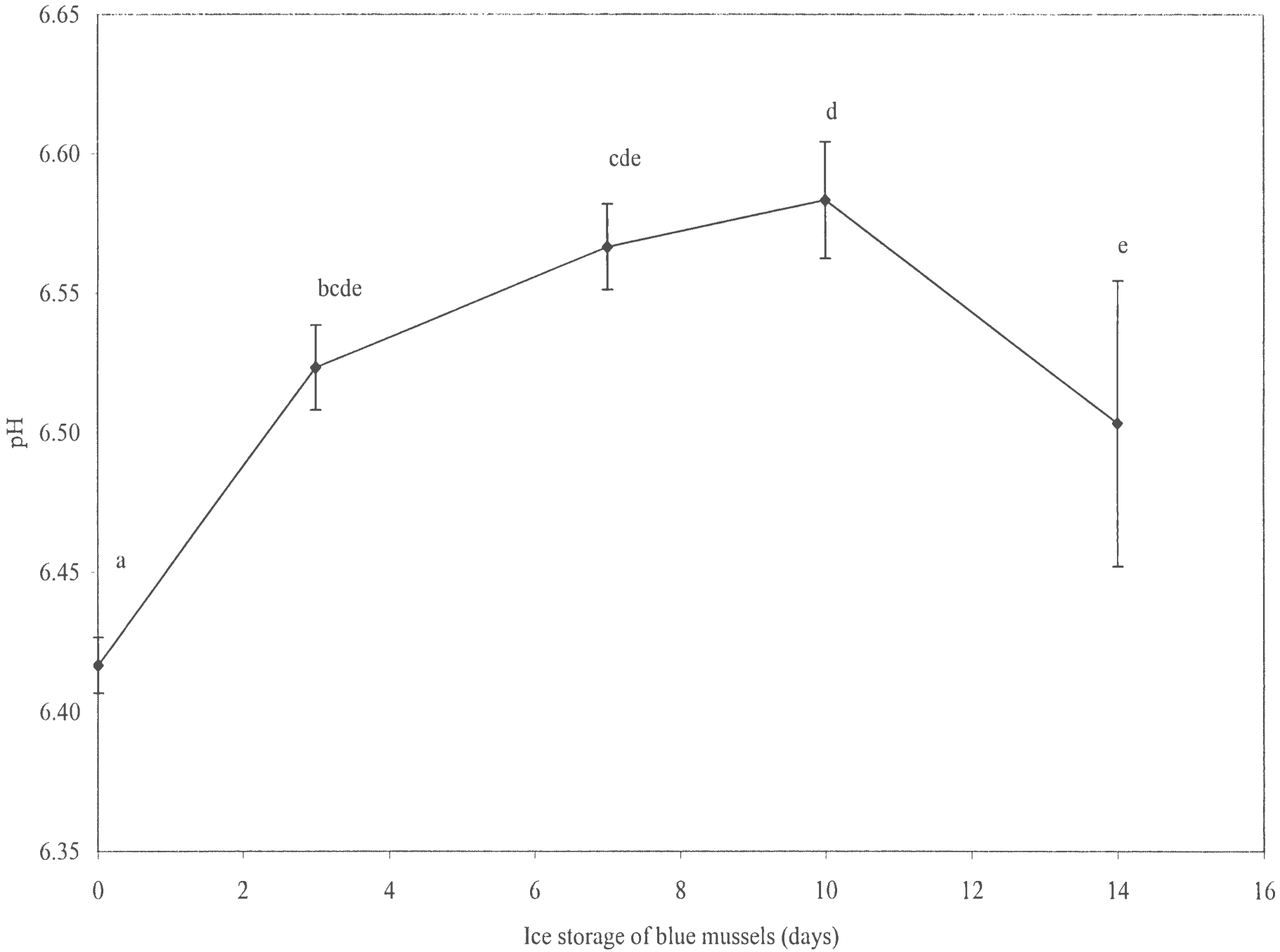

6.65
6.60
6.55
6.50
6.45
6.40
6.35
pH
a
bcde
cde
d
e
0
2
4
6
8
10
12
14
16
Ice storage of blue mussels (days)

Figure 5.3 Thiobarbituric acid reactive substances (TBARS) (mg malonaldehyde equivalents / kg meat) of blue mussels stored on ice for 14 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

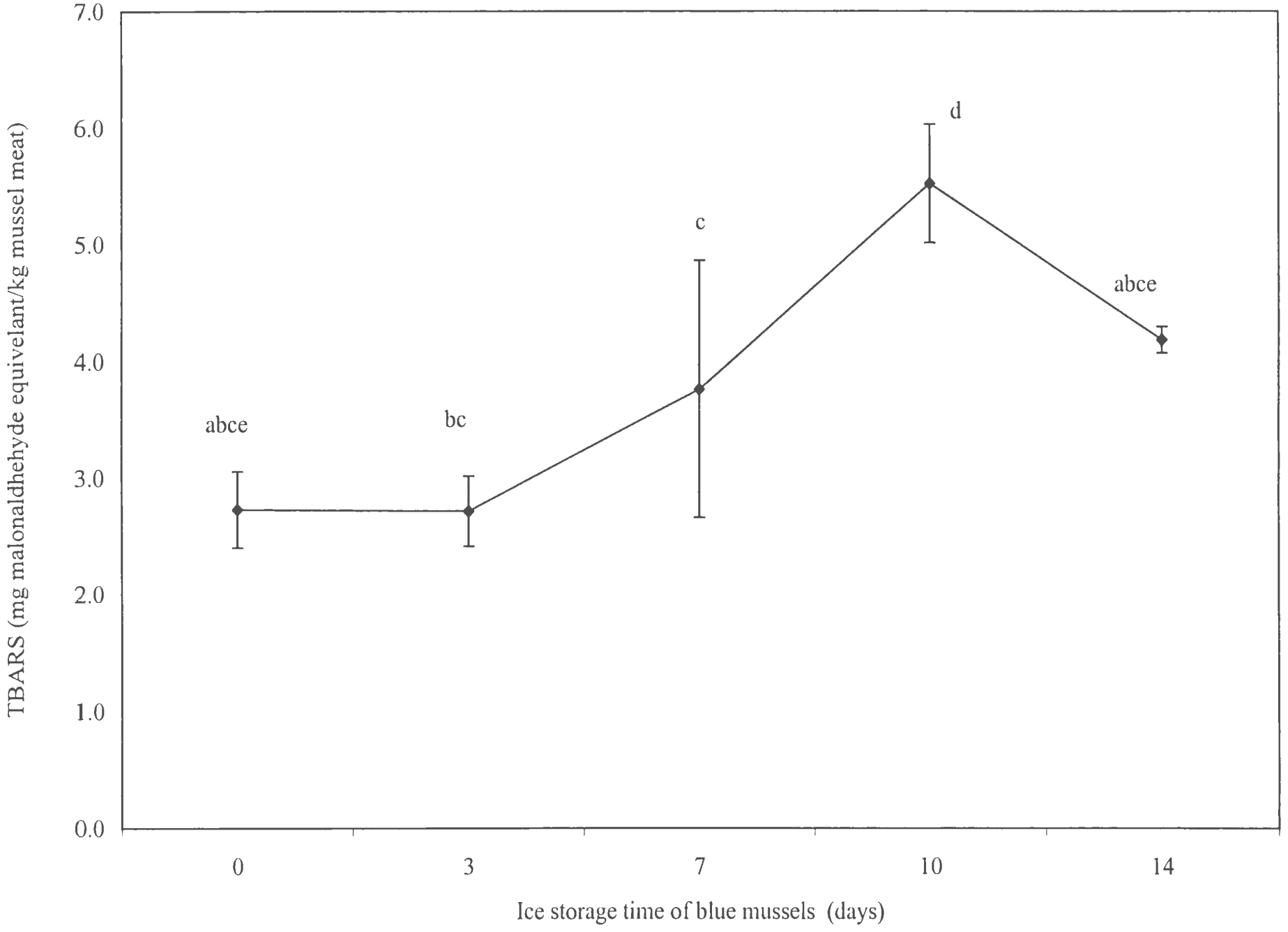

7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
TBARS (mg malonaldhehyde equivelant/kg mussel meat)
abce
bc
c
d
abce
0
3
7
10
14
Ice storage time of blue mussels (days)

Table 5.1 Proximate composition (moisture, protein, lipid, carbohydrate and ash content, g / 100 g meat) of mussels stored on ice for 14 days.

Storage days	Moisture	Protein	Lipid	Carbohydrate	Ash
0	79.86 ± 0.62 [a]	12.64 ± 0.38 [a]	2.55 ± 0.04 [a]	3.55 ± 0.59 [abcd]	1.41 ± 0.34 [abcde]
3	78.98 ± 0.80 [a]	12.67 ± 1.22 [a]	2.44 ± 0.14 [a]	4.42 ± 1.04 [bcd]	1.48 ± 0.33 [bcd]
7	80.95 ± 0.63 [a]	12.02 ± 0.74 [a]	2.49 ± 0.25 [a]	2.68 ± 0.70 [cd]	1.86 ± 0.40 [cde]
10	80.38 ± 0.95 [a]	12.27 ± 0.14 [a]	2.48 ± 0.07 [a]	2.71 ± 0.90 [d]	2.16 ± 0.09 [de]
14	80.14 ± 0.52 [a]	12.88 ± 0.80 [a]	2.44 ± 0.05 [a]	2.01 ± 0.70 [bcde]	2.53 ± 0.42 [e]

(Mean ± S.D., n=3). Means with different letters within each column are significantly ($p < 0.05$) different from each other.

There was no significant ($p > 0.05$) difference in the content of 16:0 fatty acid among mussels stored on ice for 0, 7 and 14 days, but the value was significantly ($p < 0.05$) greater at 3 and 10 days. Meanwhile, total SFA were significantly ($p < 0.05$) higher after 10 days of storage compared to other storage days. Monounsaturated fatty acid (16:1n-7) was significantly ($p < 0.05$) lower after 7 days of storage. Bacterial fatty acid (*ai*15:0) content of ice stored mussels was significantly ($p > 0.05$) higher at 0 and after 14 days of storage compared with other days. No significant ($p > 0.05$) differences were observed in docosahexaenoic acid (DHA), total MUFA, PUFA or BFA throughout the storage period.

The saturated fatty acid (16:0) of ice stored blue mussels was initially (day 0) low at 14.43 %, then increased to an 16 % and remained at this level throughout the storage period. A similar trend was observed for total SFA. Monounsaturated fatty acid 16:1n-7 and total MUFA were significantly ($p < 0.05$) higher after 14 days of storage compared to mussels stored for 0, 3 and 10 days. DHA content of ice stored mussels was significantly ($p < 0.05$) lower than mussels stored for 0 and 3 days. Meanwhile, there was no significant ($p > 0.05$) difference in total PUFA throughout the storage period. Bacterial fatty acid (*ai*15:0) content of mussels increased gradually to its highest levels after 3 days, but thereafter there was no significant ($p > 0.05$) difference in the content of this fatty acid among the stored mussels. Moreover, there was no significant ($p > 0.05$) difference in the total BFA of the stored mussels throughout the storage period.

In general the H_2SO_4 - MeOH technique yielded significantly ($p < 0.05$) higher values for 16:0 (16%), 16:1 n-7 (13%), total SFA (23%), MUFA (23 %) and total BFA (2.5%) than did the BF_3 – MeOH reagent, and the BF_3 – MeOH reagent gave significantly

($p < 0.05$) higher yields for DHA (13%), total PUFA (53%) and the bacterial fatty acids *ai*15:0 (0.2 %) than did the H_2SO_4 – MeOH reagent (Figures 5.4, 5.5, 5.6 and 5.7).

5.3.4 Relationship among storage time on ice, bacterial counts on marine agar and bacterial fatty acids determined by two different methods

Table 5.2 represents the relationship among storage period of mussels (days), total bacterial counts (CFU/g) of mussels stored on ice as determined by conventional agar method, individual (*i*15:0, *ai*15:0, *i*16:0, *ai*16:0, *i*17:0, *ai*17:0 and 17:0 as % of total fatty acids) and total bacterial fatty acids as determined by two transmethylating (BF_3 - MeOH and H_2SO_4 - MeOH) reagents.

The correlation coefficient (r) for CFU/g vs storage days was 0.973 ($p > 0.001$). The highest correlation coefficient for storage period vs. bacteria fatty acids, as determined by the BF_3 – MeOH method was for 16:0 *ai* ($r = 0.646$, $p = 0.017$), whereas for the H_2SO_4 – MeOH, reagent the highest correlation coefficient was for 15: 0 *ai* ($r = 0.830$, $p > 0.001$), followed by *i*17:0 ($r = 0.665$, $p = 0.007$) and total bacterial fatty acids ($r = 0.572$, $p = 0.026$).

The strongest correlation between bacterial fatty acids determined by BF_3 – MeOH and the bacterial counts on conventional agar for stored mussels (CFU/g) was observed with the fatty acid 16:0*ai* ($r = 0.593$, $p = 0.033$). The strongest correlation between bacterial counts on conventional agar method for stored mussels (CFU/g) and bacterial fatty acids, determined by the H_2SO_4 – MeOH reagent, was observed with the fatty acid *ai*15:0 ($r = 0.882$, $p < 0.001$) followed by *i*17:0 ($r = 0.726$, $p = 0.002$) and total bacterial fatty acids ($r = 0.598$, $p = 0.026$). All other variables were either poorly or negatively correlated.

Figure 5.4 Hexadecanoic acid 16:0 and total saturated fatty acids in mussels stored on ice (mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

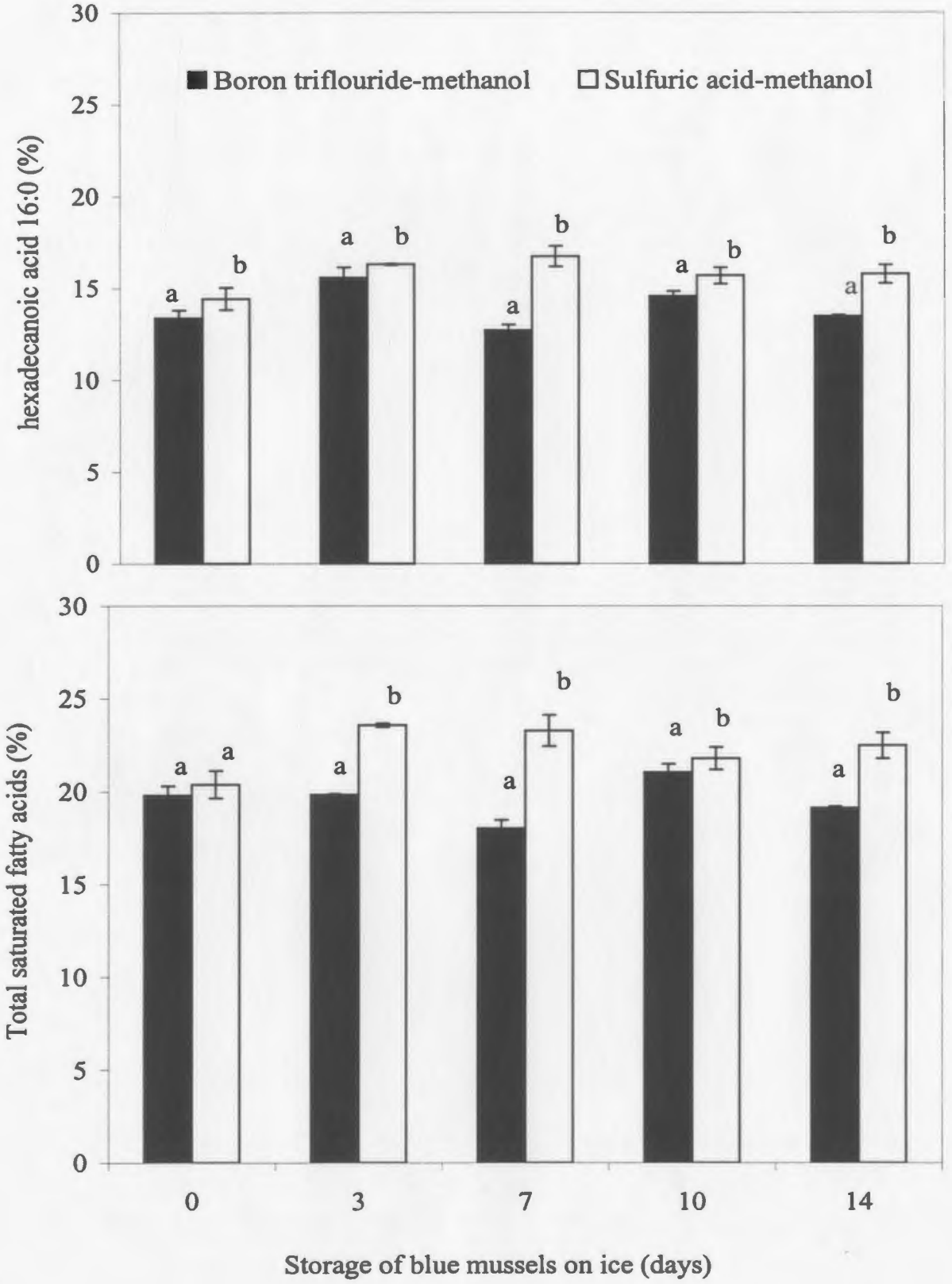

Boron triflouride-methanol
Sulfuric acid-methanol
hexadecanoic acid 16:0 (%)
Total saturated fatty acids (%)
0
5
10
15
20
25
30
a
b
0
3
7
10
14
Storage of blue mussels on ice (days)

Figure 5.5 Hexadecaenoic acid 16:1n-7 and total monounsaturated fatty acids in mussels stored ice. (mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

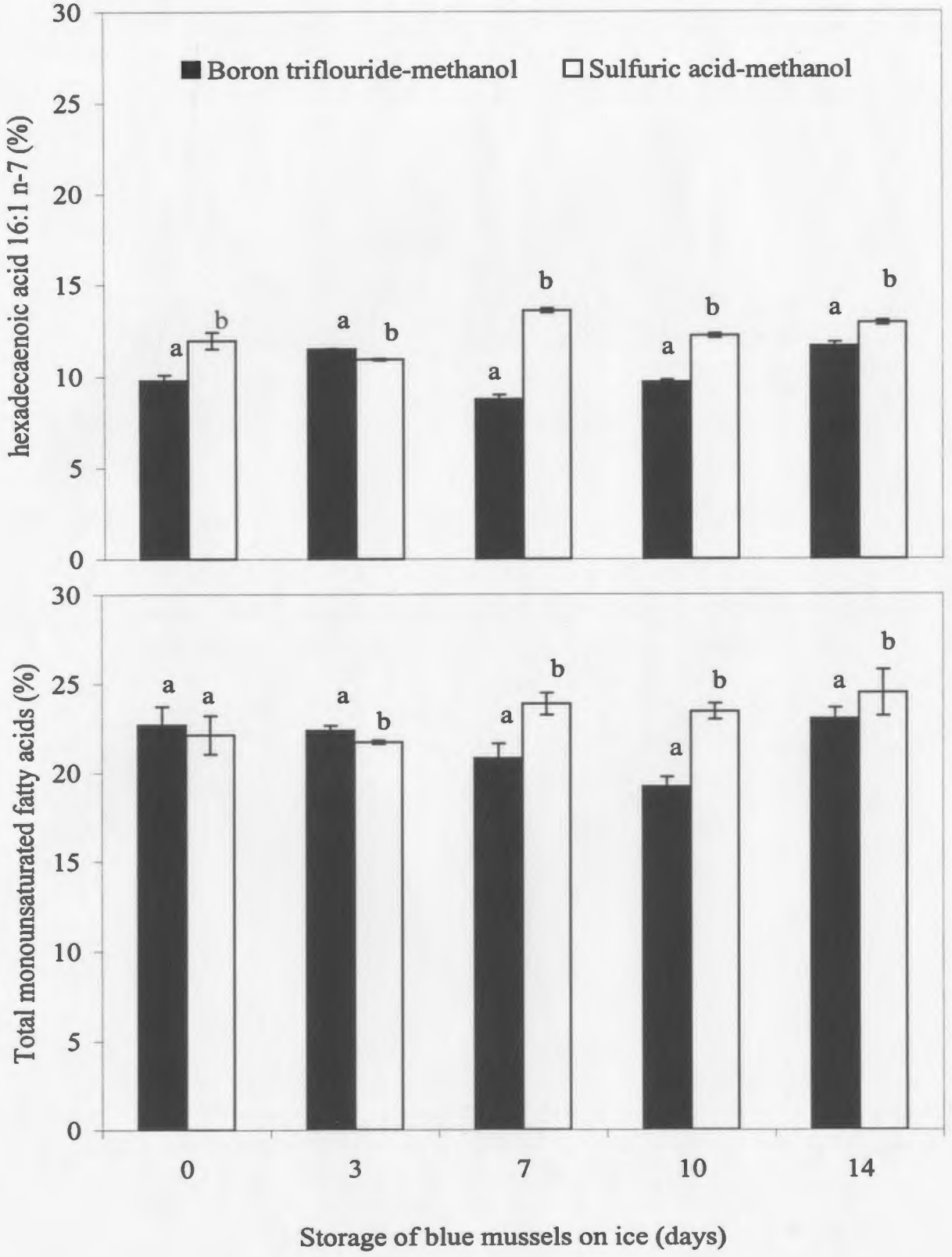

Boron triflouride-methanol
Sulfuric acid-methanol
hexadecaenoic acid 16:1 n-7 (%)
Total monounsaturated fatty acids (%)
0
5
10
15
20
25
30
a
b
0
3
7
10
14
Storage of blue mussels on ice (days)

Figure 5.6 Docosahexaenoic acid 22:6n-3 and total polyunsaturated fatty acids in mussels stored on ice. (mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

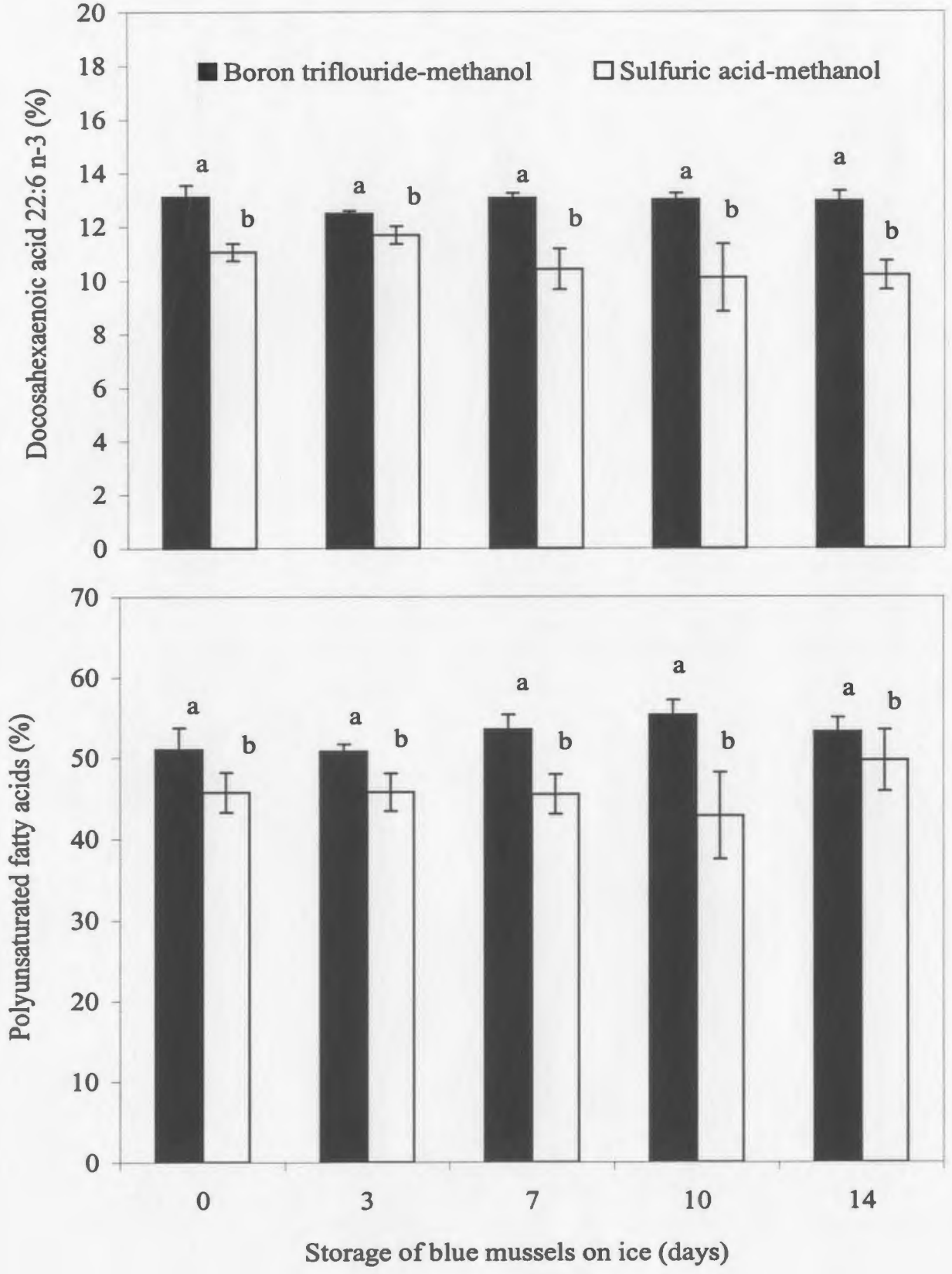

Boron triflouride-methanol
Sulfuric acid-methanol
Docosahexaenoic acid 22:6 n-3 (%)
Polyunsaturated fatty acids (%)
Storage of blue mussels on ice (days)
0
3
7
10
14
a
b

Figure 5.7 Anteiso-pentadecanoic acid *ai*15:0 and total bacterial fatty acids mussels stored on ice. (mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

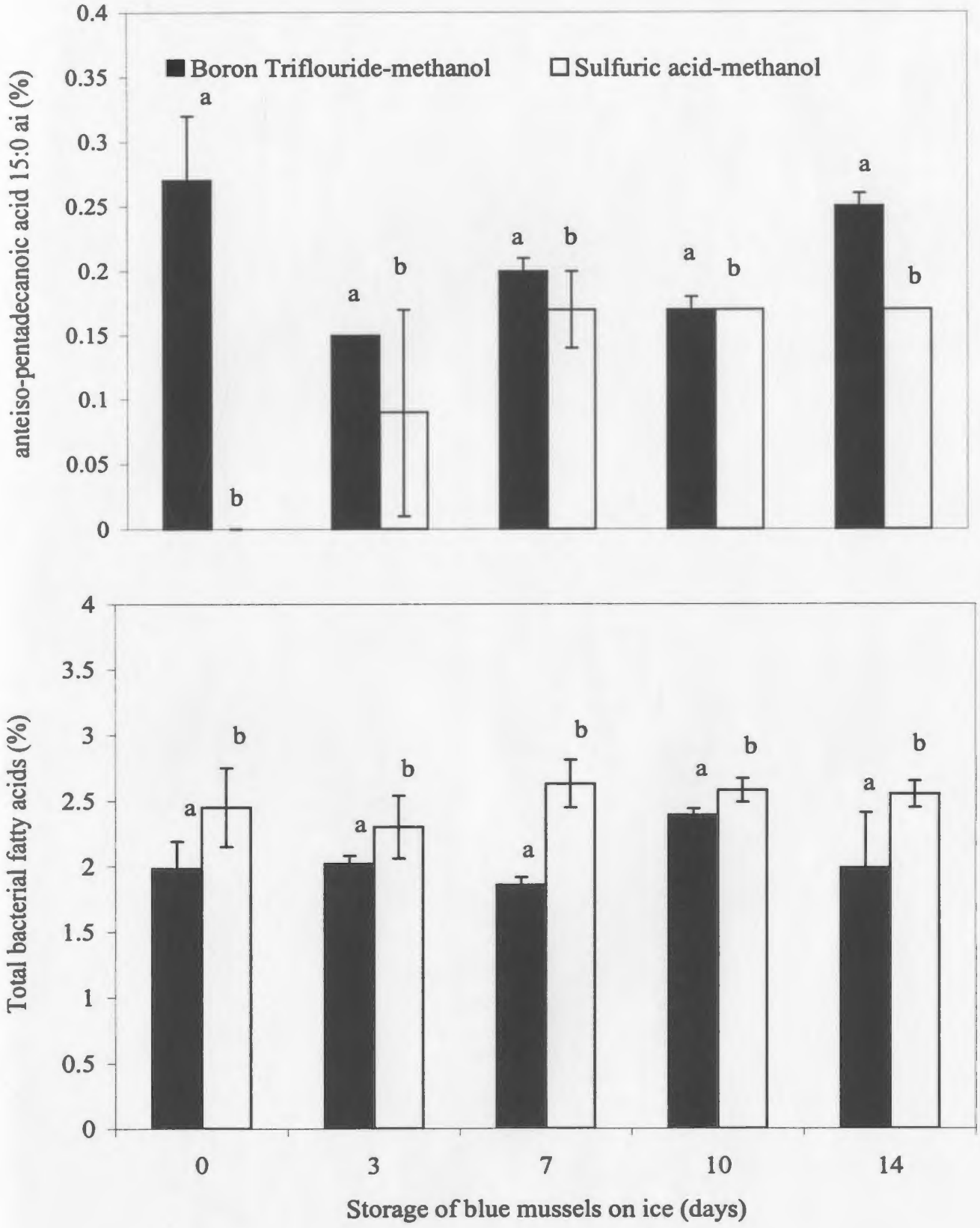

Boron Triflouride-methanol
Sulfuric acid-methanol
anteiso-pentadecanoic acid 15:0 ai (%)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b
a
b
a
b
a
b
a
b
Total bacterial fatty acids (%)
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
a
b
a
b
a
b
a
b
0
3
7
10
14
Storage of blue mussels on ice (days)

Table 5.2 The relationship among ice storage of Newfoundland cultured blue mussels (days), bacterial counts (Log CFU/g) on marine agar and bacterial fatty acids (%). Correlation coefficient r values and level of significance (p)

	BF_3		H_2SO_4	
	Days	Log CFU/g	Days	Log CFU/g
Log CFU/g	**0.973** (<0.001)	-	**0.973** (<0.001)	-
% Total bacterial fatty acids	0.222 (0.426)	0.220 (0.430)	0.572 (0.026)	**0.598** (0.019)
% *i*15:0	-0.292 (0.333)	-0.362 (0.224)	-0.330 (0.229)	-0.307 (0.266)
% *ai*15:0	0.062 (0.841)	-0.099 (0.747)	**0.830** (<0.001)	**0.882** (<0.001)
% *i*16:0	-0.118 (0.701)	-0.177 (0.563)	-0.207 (459)	-0.161 (0.566)
% *ai*16:0	0.646 (0.017)	**0.593** (0.033)	0.102 (0.719)	0.047 (0.868)
% *i*17:0	-0.463 (0.111)	-0.438 (0.135)	**0.665** (0.007)	**0.726** (0.002)
% *ai*17:0	0.250 (0.409)	0.291 (0.335)	0.248 (0.372)	0.304 (0.270)
% 17:0	0.043 (0.888)	0.190 (0.535)	-0.124 (0.686)	**0.563** (0.029)

5.4 Discussion

During the storage of seafoods on ice, various chemical, microbiological and sensory changes lead to reduction in quality and consumer rejection of the product (Grigorakis *et al.*, 2003; Taliadourou *et al.*, 2003). Numerous efforts have been made to develop predictive mathematical models to quantify and predict bacterial growth in seafoods based on various quality changes (Wijtzes *et al.*, 1998; Koustsoumanis and Nychas, 2000). An understanding of these changes may lead to the development of new procedures for maintaining the premium quality of the seafood and the selection of the most appropriate indices to monitor quality changes during storage (Kyrana *et al.*, 1997; Chang *et al.*, 1998; Du *et al.*, 2001; Karungi *et al.*, 2004).

Bacterial counts (CFU/g) in mussels reflect the general microbial content of the seafood and surrounding water as well as hygiene during washing and processing (Hunt *et al* ., 1988). High APC counts (>10^5 (or $\log_{10}$ 5) CFU/g) may suggest to a potential health hazard for consumers (Colby *et al.*, 1995; Anonymous, 1992; Jay, 1996). Various maximum acceptable levels of APC (10^5 to 10^7) in fish and shellfish have been set in different countries (Busta, 1984; Hunt *et al.*, 1984; Colby *et al.*, 1995; Valle *et al.*, 1998). However, it is generally recommended that APC on PCA supplemented with 1% NaCl should not exceed 10^5 or log 5 CFU/g (Anonymous, 1992). Because there is no information on the maximum acceptable levels of microbial counts on MA, log 5 counts were also considered in this study to be the maximum acceptable level. Based on this criterion, the shelf life will only be about 3 days on ice. However, the shelf life should be correlated with sensory analysis in future studies to obtain more accurate estimate of shelf life. Bacteria observed on the marine agar in this study were not identified to genus or

species level. However, the major bacterial genera reported in the literature in mussels and other bivalve include *Pseudomonas*, *Vibrio*, *Moraxella*, *Staphylococcus*, *Listeria* and *Clostridium* (Harihharan *et al.*, 1995; Riquelme *et al.*, 1995). Microbial activity may produce off-odours because of production of undesirable primary metabolites such as trimethylamine from trimethylamine oxide, other amines and ammonia, hydrogen sulphide, carbonyl compounds and organic acids from degradation of lipids and therefore considered to be major quality indicators for refrigerated seafoods (Colby *et al.*, 1995; Jay, 1996; Suvanich and Marshall, 1998; Koutsoumans and Nychas, 1999; Antoine *et al.*, 2002).

pH has been used as an indicator of freshness of seafood, including molluscs. Fresh and good quality bivalves exhibit pH values from 6 to 7 (Jay, 1996). According to this criterion, mussels at day 0, were considered to be fresh seafood products. Bacterial fermentation of carbohydrates in seafoods may lead to the production of organic acids, resulting in a reduction in pH and thus the quality (Colby, 1993; Koutsoumans and Nychas, 1999). However, the observed increase in the pH of ice stored mussels in this study may be explained, at least partially, by the production of alkaline compounds due to proteolysis activity or bacterial alkaline metabolites resulting from the growth of psychrotrophic bacteria at ice storage temperatures (Kyrana *et al.*, 1997; Suvanich and Marshall, 1998).

The thiobarbituric acid reactive substance (TBARS) test is an acceptable indicator of lipid oxidative status in seafoods and has therefore been used frequently (Dulvik *et al.*, 1998; Tang *et al.*, 2001; Saeed and Howell, 2002). However, a decline in TBARS values after 10 days of ice storage of blue mussels should be questioned when this index is used to

monitor lipid oxidation beyond 10 days as it may lead to false low values (Shahidi and Pegg, 1993; Shahidi and Spurvey, 1995). No sensory analyses were performed to determine if oxidative rancidity was developed. However, it appears that lipid were not oxidized during the first 7 days of storage on ice. The large standard deviation for TBARS values of mussels stored in styrofoam containers at day 7 may indicate that some mussels may be very weak or dead due to a high bacterial load, which may have led to further lipid oxidation.

While some studies have examined changes in proximate composition in blue mussels due to processing or seasonal changes (Slabyj, 1977; Slabyj *et al.*, 1978; Orban *et al.*, 2001) it appears that no previous study reported changes in proximate composition of cultured Newfoundland blue mussels during storage on ice. The proximate composition of mussels stored on ice on day 0 (1.4% ash, 79.86% moisture, 2.55% lipids, 12.64% protein and 3.55 carbohydrates) is similar to that reported in the literature for blue mussels cultured in other countries, with some exceptions due to seasonal and reproductive cycle effects (Slabyj, 1977; Krzynowek and Wiggin, 1979; King *et al.*, 1990). The use of whole mussel body, rather than specific parts, for analysis may have hindered detecting changes in protein and carbohydrates contents (Jeong *et al.*, 1999; Hardy *et al.*, 2000). Furthermore, it is possible that the short period of storage on ice (14 days only) was not sufficient for detecting any possible changes (Bayne and Thompson, 1970). Some amino acids may have decreased while other nitrogenous compounds increased. Change in amino acid composition may not have been detected by the Kjeldahl method, which measures only total nitrogenous compounds (Slabyj, 1977). There was a marked increase in the ash content after 14 days of storage corresponding to a relatively low carbohydrate content.

The increase in ash content may not have been a true increase but rather a reduction in carbohydrate content. Nonetheless, increases and decreases of ash content in blue mussels have been observed during various seasons (Slabyj, 1978). Determination of mineral composition may lead to a better understanding of changes in ash content in blue mussels during storage on ice.

Fatty acid data during storage of seafoods at refrigeration and freezing temperatures is limited in the literature, and virtually no information is available for live blue mussels stored on ice. One reason is that other indices are used to follow quality changes during low temperature storage of seafoods (St. Angelo and Spainer,1993; Chang, *et al.*, 1998; Shahidi, 1998; Taliadourou *et al.*, 2004). Another possibility is that 14 days of storage is not sufficient to observe any change. Freites *et al.*, (2002) observed changes in fatty acid composition of *Mytilus galloprovincialis* (Lmk) only after 22 days in the new habitat. Other studies have shown that there are few or no differences in fatty acid composition not only during storage on ice, but also during storage in freezers. For example, Xing *et al.* (1993) reported no changes in fatty acids profiles of fatty fish (Atlantic mackerel) and lean fish (Atlantic cod) during storage in ice and freezers. However, Xing *et al.* (1993) reported that changes in fish species, storage temperature and antioxidants levels might cause oxidation of their constituents PUFA. When Indian mackerel was irradiated and stored at 0-2°C for 14 days, only slight differences in fatty acid composition were reported (Lakshman and Bongirwar, 1999). Cho *et al.* (1999) found that dried mussels and clams stored at 4 and 25°C for 120 days had slight increase in SFA and decrease in MUFA with no major affects on consumers acceptance or rejection of the dried products.The general trend for the fatty acid profile of mussels stored on ice showed no major difference

throughout the storage period, with some exceptions. Even though some differences existed, these would not affect the consumer's perception and therefore market value of the cultivated mussels.

The method and conditions used to measure fatty acids may affect the results. The aim of transmethylation of fatty acids in the extracted lipids of biological origin is to make them more volatile in order to facilitate their determination by gas chromatography. This is usually achieved by reacting the lipid extracts with a mixture of methanol and hydrochloric acid, boron trifluoride, boron trichloride or sulfuric acid with or without an organic solvent, then incubation at various time/temperature combinations. The resulting fatty acid methyl esters are extracted by organic solvents (usually hexane) and used for gas chromatography determination (Parrish, 1988; Blau and Darbre, 1997). The H_2SO_4 - MeOH transmethylation reagent gave lower yields of DHA and total PUFA compared to 14% BF_3 - MeOH for stored mussels. This may be explained, at least partially, by the long incubation time (17 hr at 60°C) which may have caused partial oxidation PUFA in stored mussels. Meanwhile, BF_3 - MeOH transmethylation method gave higher yields of DHA and total PUFA. A similar observation was reported by Budge and Parrish (2003), in which the highest recovery of PUFA methyl esters of blue mussels was obtained with BF_3 - MeOH technique. Meanwhile, the short period of incubation (1.5 hr at 85°C) of BF_3 - MeOH reagent may not have been sufficient for transmethylation of all SFA, MUFA, and bacterial (BFA) which, in part, may explain the low yield of these fatty acids compared with those from the H_2SO_4 - MeOH technique (Mastronicolis *et al.*, 1996; Annous *et al.*, 1997). Base hydrolysis using NaOH in methanol of lipid extract prior to the use of BF_3 - MeOH reagent may improve the yield of BFA methyl esters (Budge and Parrish, 2003).

The high proportion of the bacterial fatty acid *ai*15:0 following transmethylation with BF_3 - MeOH reagent cannot be adequately explained and is probably an anomaly.

One interesting area that has attracted limited attention is the use of bacterial fatty acids to examine the microbial content of refrigerated seafoods, despite the fact that these fatty acids have been used in clinical and environmental studies (Osipov and Turova, 1997; Nichols *et al.*, 2000; Rutters *et al.*, 2002). In this study, an attempt was made to correlate mussels storage time on ice (days), bacterial counts on conventional agar (CFU/g) and their bacterial fatty acids determined by two transmethylation reagents. The aim was to find the best indicator to monitor the microbial changes of mussels during storage on ice. In this study, the bacterial count obtained by the conventional agar method was the best indicator of microbial changes during storage of mussels on ice because there was strong correlation between bacterial counts (CFU/g) and length of the storage period (days). Other potential indicators are the bacterial fatty acid *ai*15:0, *i*17 and total bacterial fatty acids as determined using H_2SO_4 - MeOH transmethylation reagent, due to their moderate to high (0.6 to 0.7) r values and low (< 0.05) p values. The strong correlation between *ai*15:0 and *i*17:0 (%) as determined by the H_2SO_4-MeOH transmethylation reagent, and bacterial counts on marine agar (CFU/g) suggests that most of the bacteria grown on the agar may belong to a bacterial species that has *ai*15:0 and *i*17:0 as its main component. The bacterial fatty acid *ai*15:0 is known to be the major fatty acid in *Listeria monocytogens*, *Staphylococcus*, *Bacillus* and *Corinebacteria*, while *i*17:0 has been observed in *Bacillus*, *Prevotella*, and *Propionibacterium* (Annous *et al.*, 1997; Osipov and Turova, 1997). *Vibrio*, *Listeria*, *Staphylococcus* and *Pseudomonas* bacterial species have been observed in bivalves, including mussels (Hariharan *et al* ., 1995; Jay; 1996, Ray, 1996; Hoi, *et al.*,

1998). However, it is difficult to reach firm conclusions regarding the bacteria that contributed to the spoilage of blue mussels used in this study without further identification. Bacterial fatty acids should be used with caution as indicators for monitoring microbial content of seafoods until further studies are conducted using various seafood samples for different time periods, pure bacterial cultures as references, more rapid lipid extraction and transmethylation methods and more appropriate columns and time-temperature gradients in gas chromatography or gas chromatography - mass spectrometry. The aim of these studies should be to improve the correlation between various variables and reveal more unknown bacterial fatty acids that can be used as indicators of microbial content of seafoods, including cultured blue mussels.

Chapter VI

Oxidative status of Newfoundland blue mussel (*Mytilus edulis*) lipids in relation to mechanical handling, storage on ice and ascorbic acid treatment

6.1 Introduction

The n-3 polyunsaturated fatty acids (PUFA) are critical components of cell membranes, and are therefore important in the structure, dynamics and control of membrane-associated biochemical functions (Uauy *et al.*, 2000). Seafoods are a major source of PUFA for human consumption (Shahidi, 1998; Anonymous, 2002b). The main PUFA present in seafoods, including blue mussels, are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Newton, 2000; Budge and Parrish, 2003). Epidemiological, clinical and nutritional studies have demonstrated that consumption of seafoods has beneficial effects in the alleviation of various clinical disorders, including cardiovascular disease, diabetes, obesity, arthritis, asthma, depression, hyperactivity and some types of cancer (Gill and Valivety, 1997; Shahidi, 1998; Anonymous, 2002c).

Lipid oxidation in seafoods contributes to the loss of freshness and n-3 PUFA, depending on the storage duration and temperature, natural content of antioxidants, type of chemical or physical treatment and packaging (Bell *et al.*, 2000; Gieseg *et al.*, 2000; Hamre and Sandnes, 2003b; Bragadottir *et al.*, 2004). Therefore, every effort is made to control lipid oxidation in seafoods from harvesting until they reach the consumer. Proper application of synthetic antioxidants, natural antioxidants and/or use of inert gas or vacuum packaging and storage at low temperatures can control the oxidation of seafood lipids (Fair, 1995; Khalil and Mansour, 1997; Li *et al.*, 1998; Young *et al.*, 2000; Abdel- Aal, 2001;

Harpez *et al.*, 2003). Ideal synthetic and natural antioxidants for food applications should meet certain criteria such as safety, ease of incorporation, effectiveness at low concentration, absence of undesirable odor, flavor and color (Colby *et al.*, 1995).

The application of synthetic and natural antioxidants to control lipid oxidation in seafoods is well established (Kelleher *et al.*, 1992; Rammannathan and Das, 1992b; Jensen *et al.*, 1998). Frigg *et al.* (1990) studied the effects of a tocopherol dietary supplement on oxidative stability of trout fillets and found that supplementation effectively controlled lipid oxidation. Similarly, Akhtar *et al.* (1998) were able to control lipid oxidation in rainbow trout during refrigeration and freezing using surface application of tocopherol and oleoresin rosemary. Gaitlin III *et al.* (1993) incorporated natural (α-tocopheryl acetate) and synthetic antioxidants (ethoxyquin and butylated hydroxytoluene) in the feed to control lipid oxidation in channel fish. In the later study, only the natural antioxidants provided additional protection against lipid oxidation. Tang *et al.* (2001) examined the antioxidant activity of tea catechins and α-tocopherol on lipids of fresh minced red meat, fish and poultry stored for 10 days at 4°C and found that antioxidant treatment controlled lipid oxidation in the stored meats. More recently, Undeland *et al.* (2003) used aqueous extracts from some seafood muscles, which contained antioxidants, to inhibit lipid oxidation in cod muscle membranes.

L-Asc (Asc) is an effective antioxidant that can help to maintain iron- and copper-containing enzymes in their required reduced form (Halliwell and Whiteman, 1997). It can scavenge free radicals in biological systems, including lipids, thus prevent their oxidative damage. It acts synergistically with tocopherols to reduce lipid peroxy radicals and regenerate tocopherols for further antioxidant reactions (Halliwell *et al.*, 1995).

Meanwhile, AsA can also exhibit pro-oxidant properties depending on concentrations and the presence of metal ions in the system (Halliwell and Whiteman, 1997; Taso, 1997; Yen *et al.*, 2002). In 1993, a new technique was developed by Thed *et al.* (1993) to introduce Asc to aquaculture fish species. In this technique, fish were kept in a temperature-controlled water tank and exposed to various concentrations of Asc for a specific time, then harvested and frozen to evaluate the effectiveness of Asc in controlling lipid oxidation during storage. This technique has been used to control lipid oxidation in Channel catfish (Thed and Erickson, 1994) and Norwegian herring fillets (Harmre *et al.*, 2003a).

Polyunsaturated fatty acids constitute about 50% of the total lipids in Newfoundland cultured blue mussels (*Mytilus edulis*) (Budge and Parrish, 2003). Despite this high content of PUFA in mussels, very little is known about lipid oxidation during mechanical handling or storage on ice and almost no information is available about the effects of Asc treatment on lipid oxidation of blue mussels. Therefore, the objectives of this study were to examine the effects of storage on ice, mechanical handling and Asc treatment on lipid oxidation of blue mussels. Other variables that can be affected by Asc treatment such as pH, microbial growth, antioxidant content and fatty acid composition were also measured.

6.2 Materials and Methods

The experimental set up is described in section 2.13.4. Microbial analyses were carried out as described in section 2.4 and 2.5, pH measurement as described in section 2.3, TBARS as given in section 2.6, lipid extraction as described in subsection 2.10.2, fatty

composition as described in subsections 2.8.2 and 2.9.1, antioxidants capacity as described in section 2.12 and statistical analyses as given in section 2.14.

6.3 Results

6.3.1 Effect of mechanical handling on lipid oxidation in blue mussels

Mussels were obtained from two Newfoundland packaging plants in Charles Arm (CA) and Fortune Harbor (FH). Three different batches of mussels were collected from each plant during mechanical handling (separation, sorting, washing and packaging), (one at the beginning (0 h), two at the middle (after 30 min) and three at the end (after 1 h). There were no significant ($p > 0.05$) differences in TBARS values among the three batches collected from each processing plant. However, mussels obtained from FH had average TBARS values of 2.18 ± 0.03 mg malonaldehyde equivalent / kg meat, while mussels from CA had an average value of 1.3 ± 0.01 mg malonaldehyde equivalents / kg meat (Figure 6.1).

6.3.2 Effect of storage on ice on lipid oxidation in blue mussels

There was a gradual increase in TBARS values of stored mussels from CA and FH up to day 7, after which TBARS values started to decline (Figure 6.2). TBARS values of FH mussels were significantly ($p > 0.05$) higher than those of CA mussels throughout the storage period.

Figure 6.1 Thiobarbituric acid reactive substances (TBARS) values (mg malonaldehyde equivalents / kg meat) of three batches of mechanically handled blue mussels; one at the beginning of handling (0 h), two at the middle (after 30 min.) and three at the end (after 1 h). obtained from two aquaculture sites Charle Arm (CA) and Fortune Harbor (FH) (mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

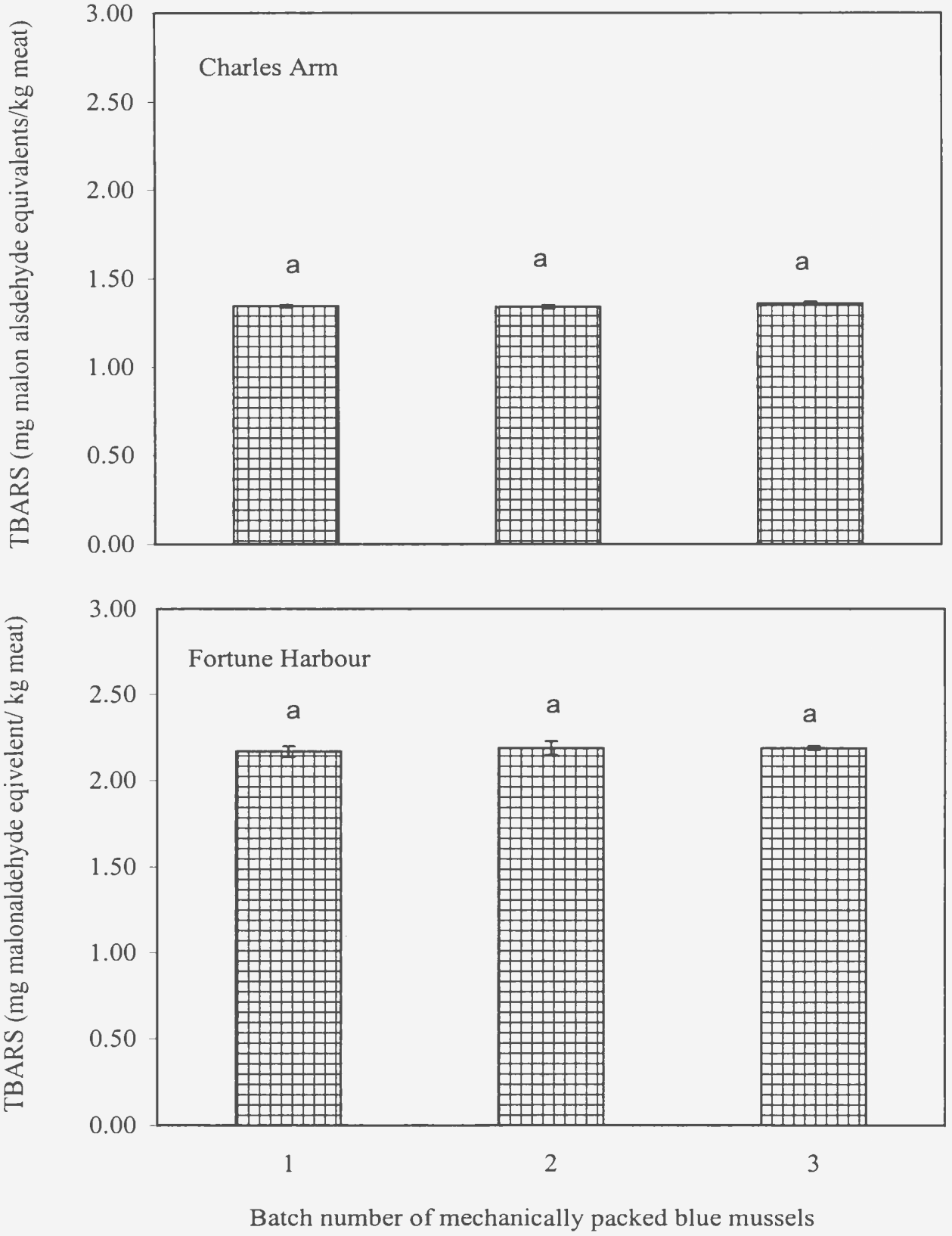
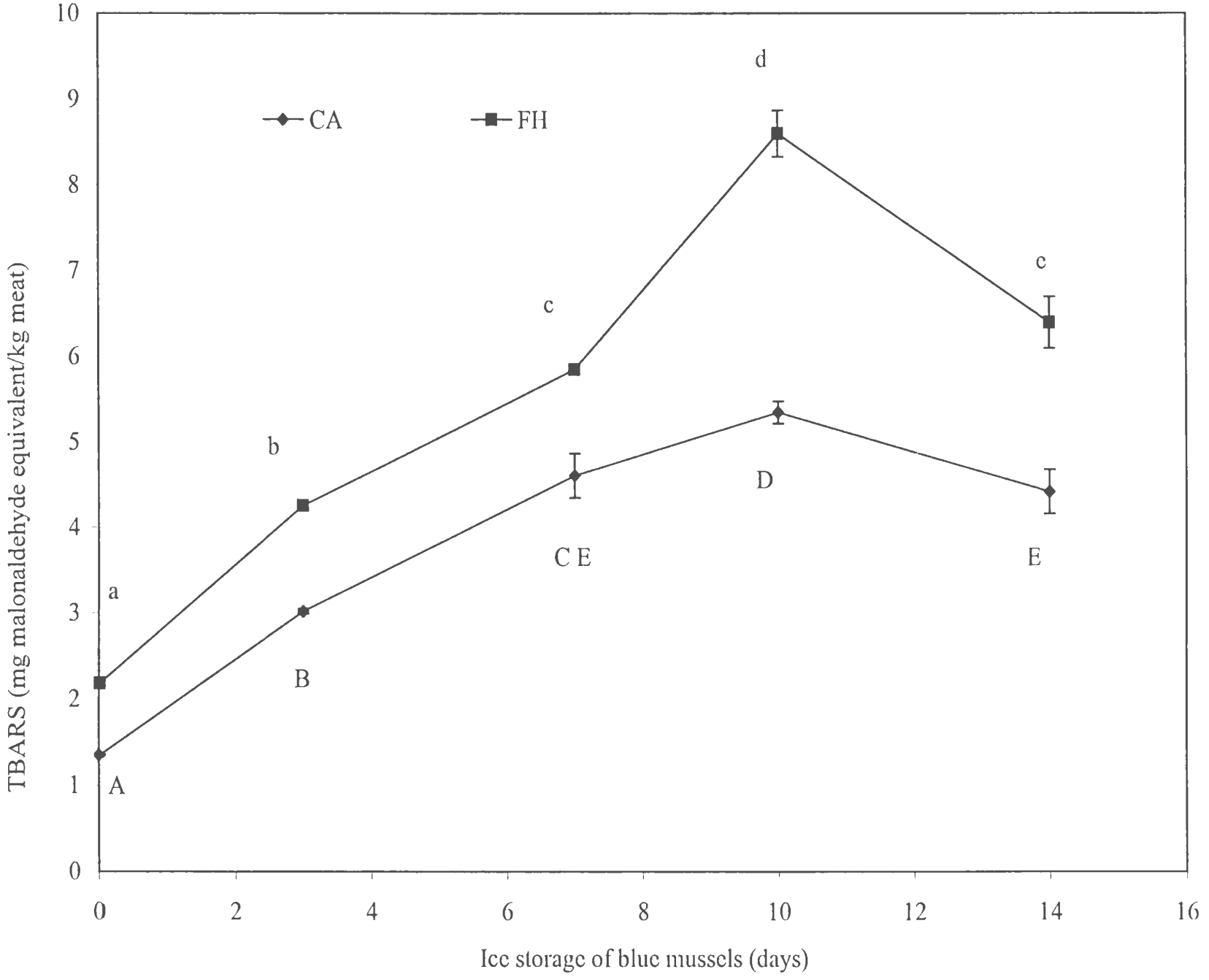

Charles Arm
TBARS (mg malon alsdehyde equivalents/kg meat)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
a
a
Fortune Harbour
TBARS (mg malonaldehyde eqivelent/ kg meat)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
a
a
1
2
3
Batch number of mechanically packed blue mussels

Figure 6.2 Thiobarbituric acid reactive substances (TBARS) values (mg malonaldehyde equivalents / kg meat) of blue mussels obtained from two aquaculture sites Charles Arm (CA) and Fortune Harbor (FH), and stored on ice for 14 days (mean ± S.D., n=3). Means with different letters (a, b, c or A, B, C) are significantly ($p < 0.05$) different from each other.


TBARS (mg malonaldehyde equivalent/kg meat)
Ice storage of blue mussels (days)
CA
FH
a
b
c
d
c
A
B
C E
D
E
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
16

6.3.3 Effect of Asc treatment on pH values of seawater and blue mussels

The pH of filtered seawater without blue mussels was near neutrality at 0 time (7.34 ± 0.02) and after 24 h (7.37 ± 0.02) (Figure 6.3), whereas the pH of seawater with blue mussels decreased from 7.35 ± 0.02 at 0 h to 7.16 ± 0.02 after 24 h. After exposure of mussels for 24 h to NaAs at cold room temperature, pH increased from 6.77 ± 0.02 at 0 h to 7.14 ± 0.01 after 24 h. The pH of filtered sea water decreased considerably after the addition of ACS grade Asc to 4.06 ± 0.01 but increased to 4.25 ± 0.01 after 24 h. Similarly, the addition of ultra-pure grade Asc reduced the pH of filtered seawater containing mussels to 3.94 ± 0.04, but pH increased to 4.19 ± 0.01 after 24 h. Changes in pH values of mussels exposed to various grades of Asc and stored on ice for 10 days are presented in Figure 6.4. Blue mussels exposed to ultra-pure Asc had significantly ($p < 0.05$) lower values of pH at day 0. Thereafter, no significant ($p > 0.05$) differences were observed among the pH values of all groups after 5 and 10 days of storage.

6.3.4 Effect of Asc treatment on microbial content of mussels stored on ice

There were no significant ($p > 0.05$) differences in total bacterial counts in mussels among the Asc treatments at 0 day and after 5 and 10 days of storage. Bacterial counts in mussels with or without Asc treatment increased throughout the storage period indicating that Asc treatment does not prolong shelf life in stored mussels. The shelf life (days on ice to reach log 5 CFU/g) for the stored mussels for all groups was about 5 days.

Figure 6.3 pH of filtered seawater after adding 10,000 μmol / L of various forms of Asc at 0 h and after 24 hours. (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other. FSW: filtered seawater; FSW + mussels: mussels in FSW; NaAs: sodium ascorbate; Asc (ACS): ACS grade ascorbic acid and Asc (Ultra): ultra-pure grade ascorbic acid

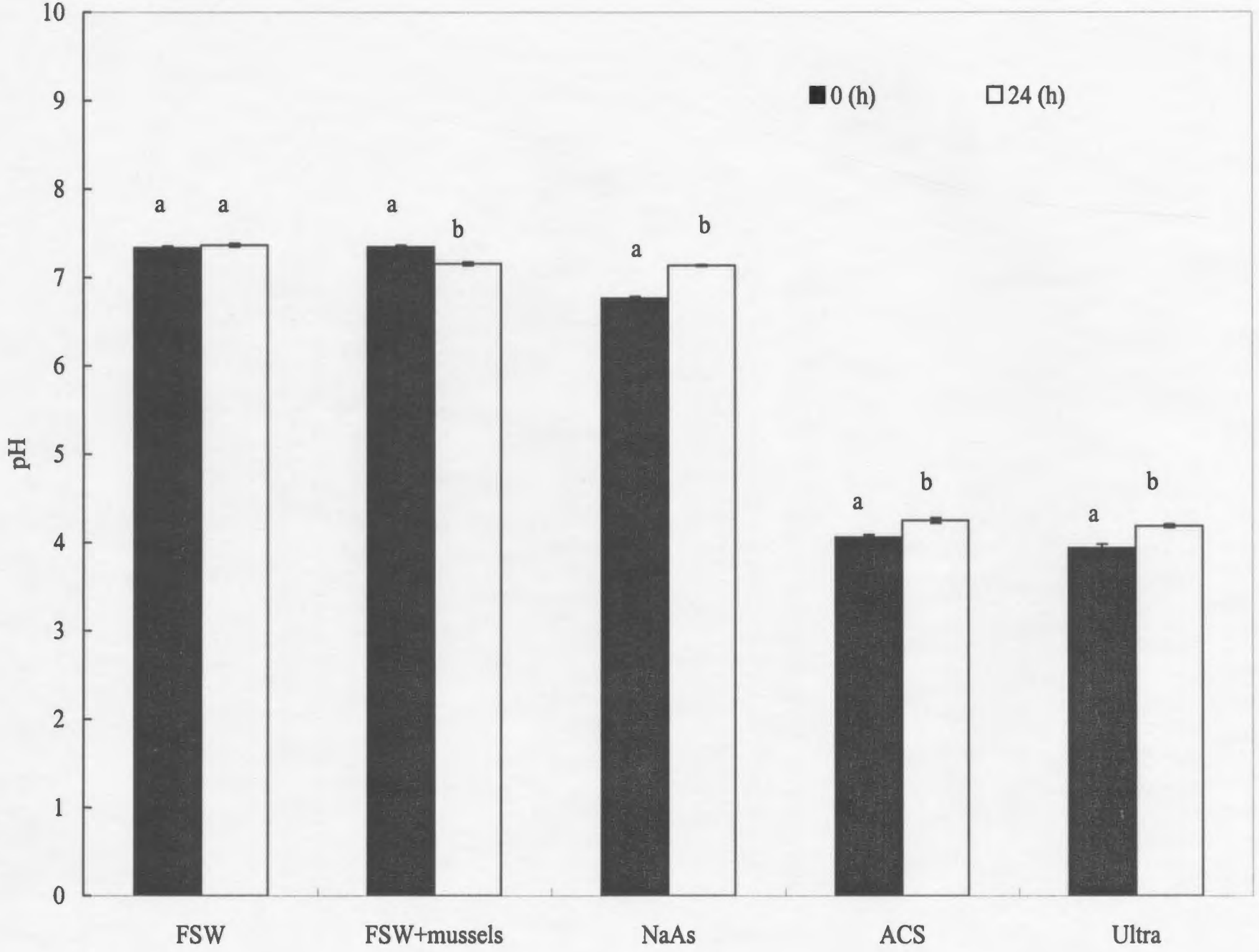

10
9
8
7
6
5
4
3
2
1
0
pH
0 (h)
24 (h)
a
a
a
b
a
b
a
b
a
b
FSW
FSW+mussels
NaAs
ACS
Ultra

Figure 6.4 pH of mussels after exposure to 10,000 μmol / L of various grades and forms of Asc and subsequent storage on ice. (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

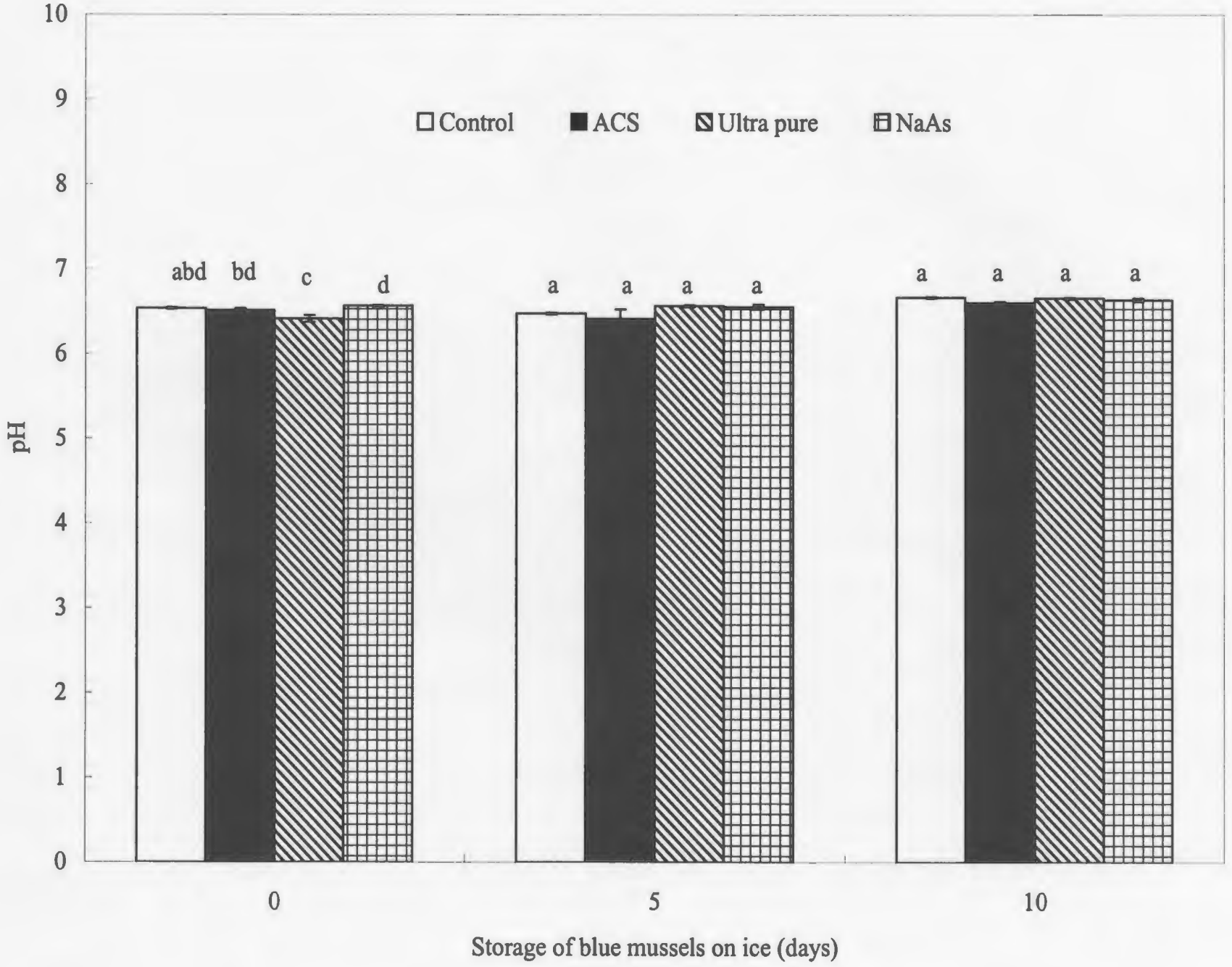
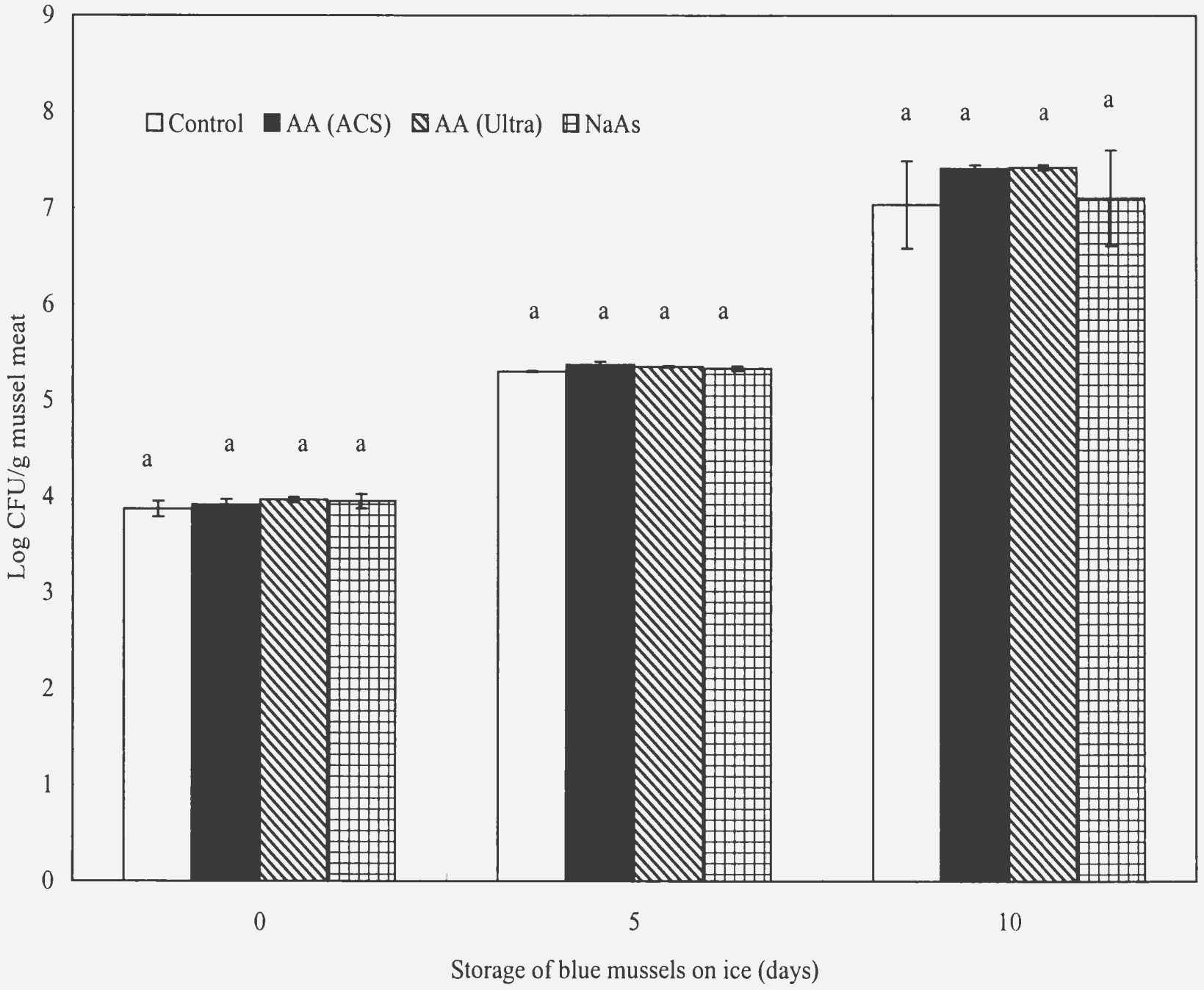

10
9
8
7
6
5
4
3
2
1
0
pH
Control
ACS
Ultra pure
NaAs
abd
bd
c
d
a
a
a
a
a
a
a
a
0
5
10
Storage of blue mussels on ice (days)

Figure 6.5 Aerobic plate count (Log CFU / g) in mussels exposed to 10,000 μmol / L of various forms and grades of Asc and stored on ice for 10 days. (mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.


Control
AA (ACS)
AA (Ultra)
NaAs
Log CFU/g mussel meat
0
1
2
3
4
5
6
7
8
9
a
a
a
a
a
a
a
a
a
a
a
a
0
5
10
Storage of blue mussels on ice (days)

6.3.5 Effect of Asc treatment on lipid oxidation in mussels stored on ice

TBARS values for mussels with or without Asc treatment were not significantly ($p > 0.05$) different from one another at 0 day (Figure 6.6). However, after 5 days of ice storage, all blue mussels exposed to Asc had significantly ($p < 0.05$) lower TBARS values compared to the control. The lowest TBARS values were for mussels exposed to NaAs followed by mussels exposed to ACS grade ascorbic and finally mussels exposed to ultra-pure grade Asc. This indicated that NaAs initially was more effective than other treatments in controlling lipid oxidation in ice stored mussels. However, a reverse trend was observed after 10 days of storage, when TBARS values for all treatments were significantly ($p < 0.05$) higher than in the control. This indicated that all Asc forms eventually had a pro-oxidant activity on the lipids of stored mussels. NaAs followed by ACS grade Asc then ultra-pure Asc exposures exhibited the highest pro-oxidant activity.

6.3.6 Asc antioxidant capacity in seawater and blue mussels after their treatment with various forms and grades of AsA

Asc antioxidant capacity, expressed as μmol Asc equivalent / L of seawater or g meat, (AscAC) in filtered seawater was initially 10,000 μmol (Asc equivalent / L of seawater) for all treatments, but decreased considerably after 24 h to 1192 ± 54.44 μmol / L for NaAs, 906 ± 95.25 μmol / L for ACS grade and 658 ± 89.06 μmol / L for ultra-pure grade. There were no significant ($p > 0.05$) differences in AscAC in stored mussels among the various treatments at 0 day and after 5 and 10 days (Figure 6.8).

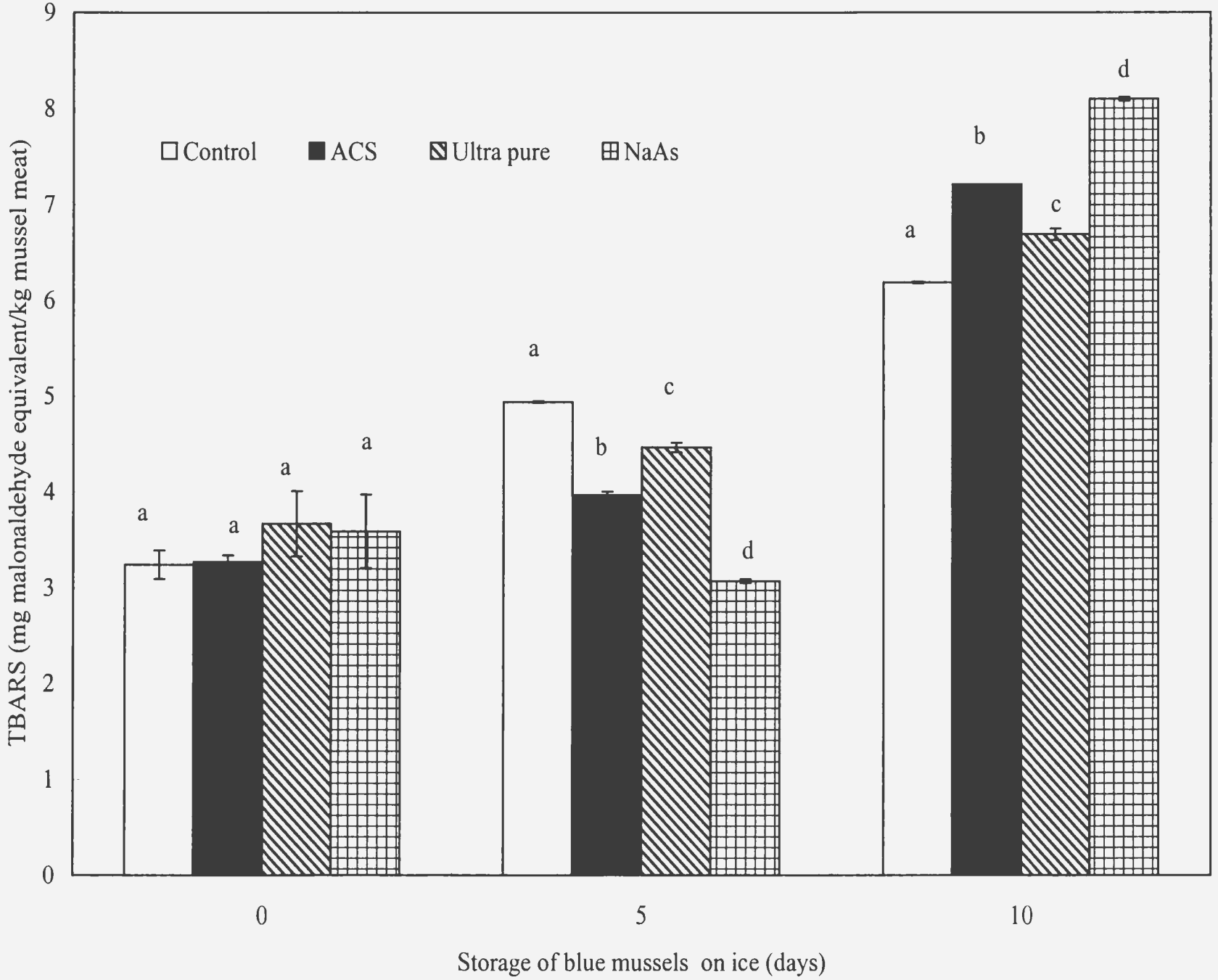

TBARS (mg malonaldehyde equivalent/kg mussel meat)
0
1
2
3
4
5
6
7
8
9
Control
ACS
Ultra pure
NaAs
a
a
a
a
a
b
c
d
a
b
c
d
0
5
10
Storage of blue mussels on ice (days)

Figure 6.6 Thiobarbituric acid reactive substances (TBARS) values (mg malonaldehyde equivalents / kg meat) of mussels treated with 10,000 μmol / L of various grades and forms of Asc and stored on ice for 10 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

However, AscAC in NaAs treated mussels decreased from initial concentration of 35.19 ± 8.27 to 13.19 ± 3.9 μmol / g meat after 10 days of storage, in ACS grade Asc treatment decreased from 33.13 ± 9.21 μmol to 16.06 ± 0.89 μmol / g meat and in ultra-pure grade Asc treatment decreased from 27.38 ± 4.9 μmol / g meat. These results indicate that Asc loss in mussels treated with various forms and grades of Asc and stored on ice ranged from 40 to more than 60 %.

6.3.7 Tocopherol antioxidant capacity in blue mussels after their treatment with various forms and grades of Asc

There were no significant ($p > 0.05$) differences in tocopherol antioxidant capacity (TAC), expressed as μ mol Trolox equivalent / g meat, among mussels treated with various forms and at each sampling day throughout the storage period (Figure 6.8). However, Asc treated samples showed significant ($p < 0.05$) reduction in their TAC after 5 and 10 days of storage.

TCA in control samples decreased from 0.35 ± 0.04 μ mol Trolox equivalent / g meat at day 0 to 0.34 ± 0.05 μ mol Trolox equivalent / g meat after 10 days of storage. In NaAs exposed mussels from 0.27 ± 0.04 μ mol Trolox equivalent / g meat to 0.20 ± 0.03 μ mol equivalent / g meat, ACS grade 0.30 ± 0.03 μ mol Trolox equivalent / g meat to 0.23 ± 0.03 μ mol Trolox equivalent / g meat and ultra-pure Asc treated mussels TAC decreased from 0.32 ± 0.04 μ mol Trolox equivalent / g meat to 0.26 ± 0.06 μ mol Trolox equivalent / g meat.

Figure 6.7 Asc antioxidant capacity (Asc equivalent/ g meat) in mussels treated with 10,000 μmol / L of various grades and forms of Asc and stored on ice for 10 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

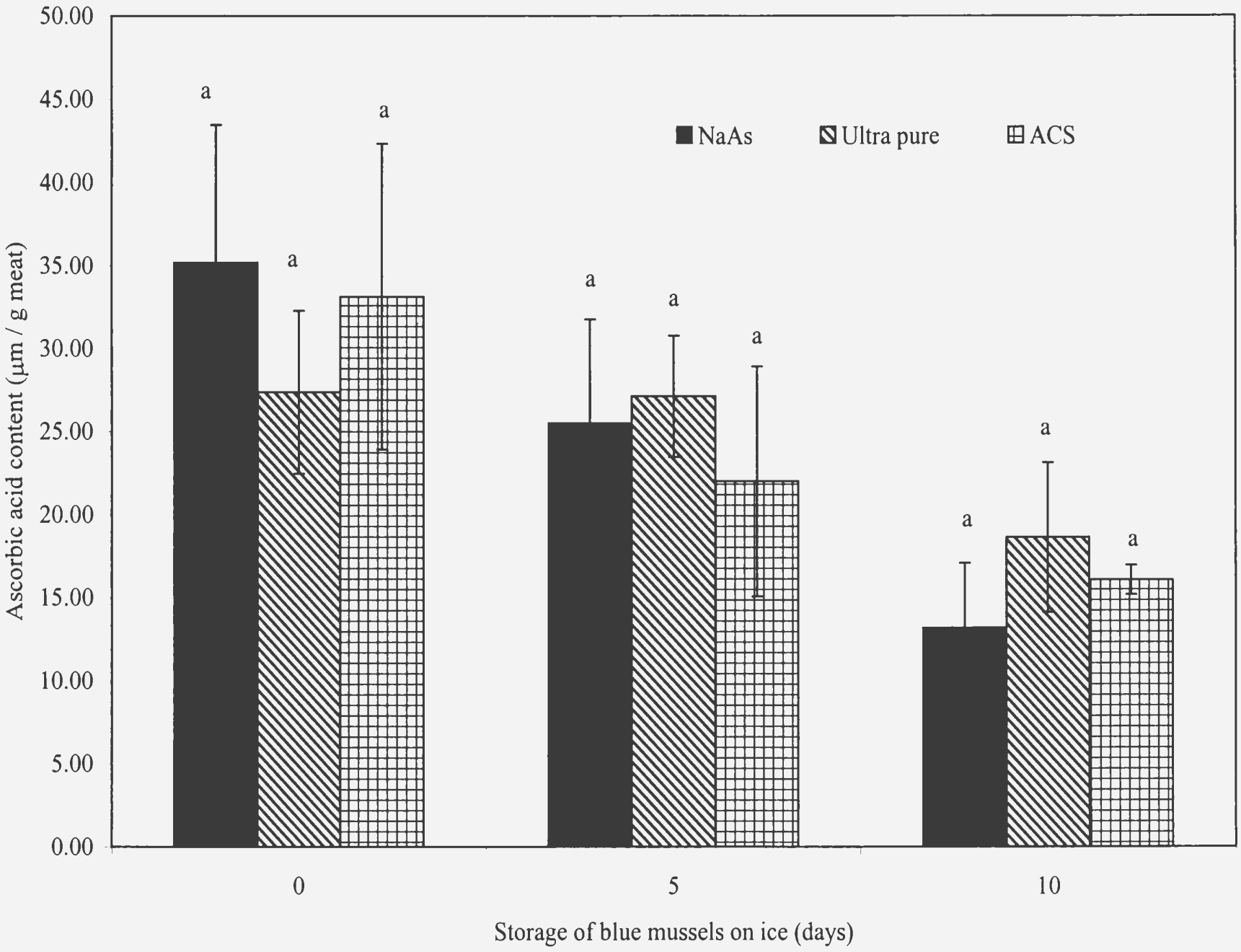
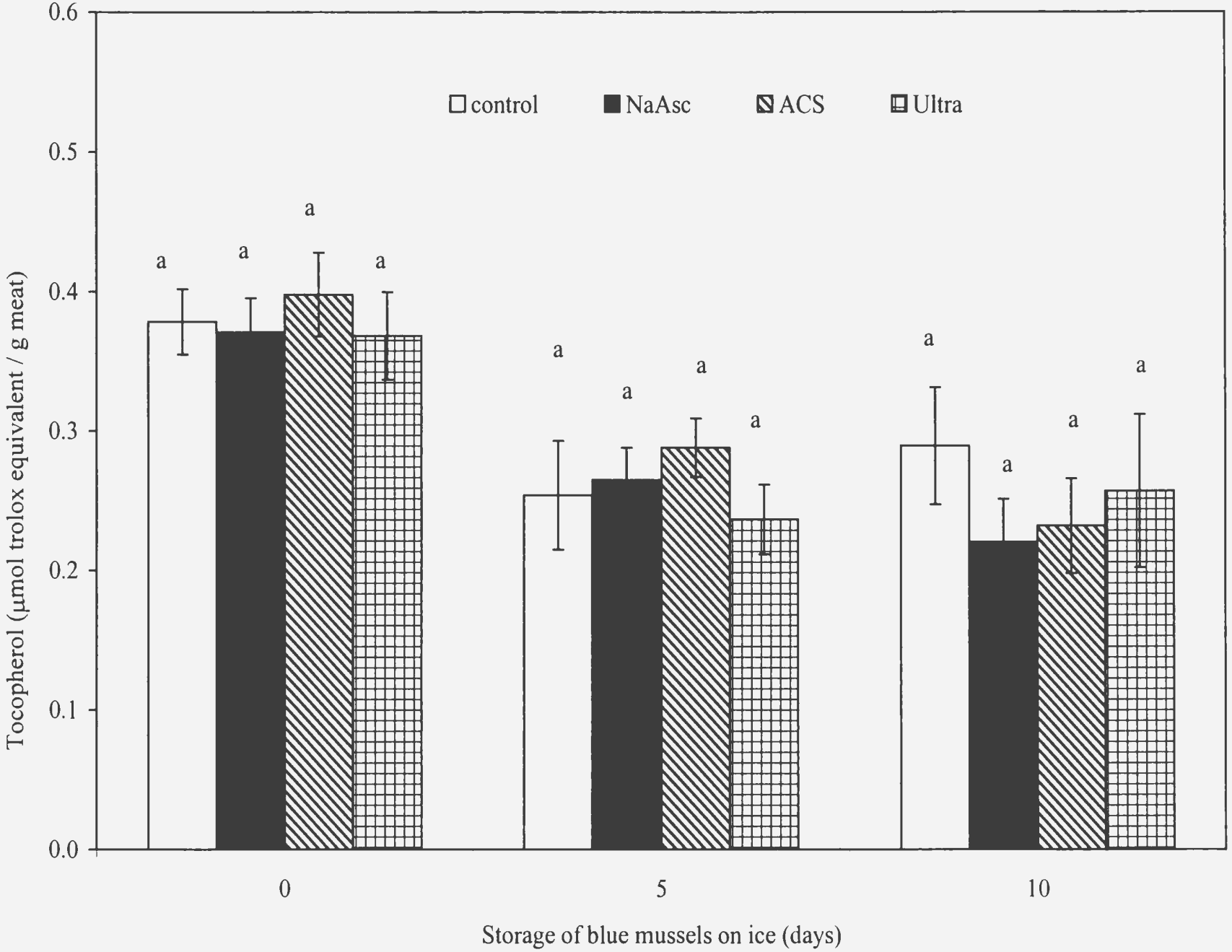

50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Ascorbic acid content (µm / g meat)
NaAs
Ultra pure
ACS
a
a
a
a
a
a
a
a
a
0
5
10
Storage of blue mussels on ice (days)

Figure 6.8 Tocopherol antioxidant capacity (μm Trolox equivalent / g meat) in mussels treated with 10,000 μmol / L f various forms and grades of Asc and stored on ice for 10 days. (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.


0.6
0.5
0.4
0.3
0.2
0.1
0.0
control
NaAsc
ACS
Ultra
a
Tocopherol (µmol trolox equivalent / g meat)
0
5
10
Storage of blue mussels on ice (days)

6.3.8 Fatty acids profiles of blue mussels after the treatment with various grades and forms of Asc

The content of 16:0 fatty acid the in the control was significantly ($p < 0.05$) higher than the treated mussels at each sampling day throughout the storage period (Figures 6.9, 6.10 and 6.11). Similarly, control and ACS grade Asc treated mussels contained higher ($p < 0.05$) amounts of the monounsaturated fatty acid 16:1n-7 throughout the storage period than did other treated mussels. Eicosapentaenoic acid (EPA; 20:5n-3) was the major polyunsaturated fatty acid detected in control and Asc treated mussels. The initial amount of EPA in the Asc treated mussels was significantly ($p < 0.05$) higher than in the control at day 0, after which there were no significant ($p > 0.05$) differences among all treated mussels with one exception. The EPA content in ACS grade Asc treated mussels was higher ($p < 0.05$) than in other treatments after 5 days of storage. No significant ($p > 0.05$) differences were observed in major saturated, monounsaturated, and polyunsaturated fatty acids and the total SFA, MUFA and PUFA in mussels treated with various Asc forms and grades during their storage on ice for 10 days (Appendices 6.1, 6.2 and 6.3), with few exceptions.

6.4 Discussion

Mussels removed from the marine habitat are under constant stress due to inadequate change in their natural environmental temperature and anaerobiosis. Various methods and techniques are used to measure stress in live blue mussels (Bayne and Thompson, 1970; Harding *et al.*, 2002).

Figure 6.9 Major saturated fatty acid, hexadecanoic acid (16:0) (% of total fatty acids) in mussels exposed to 10,000 μmol / L solution of various grades and forms of Asc and stored on ice for 10 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

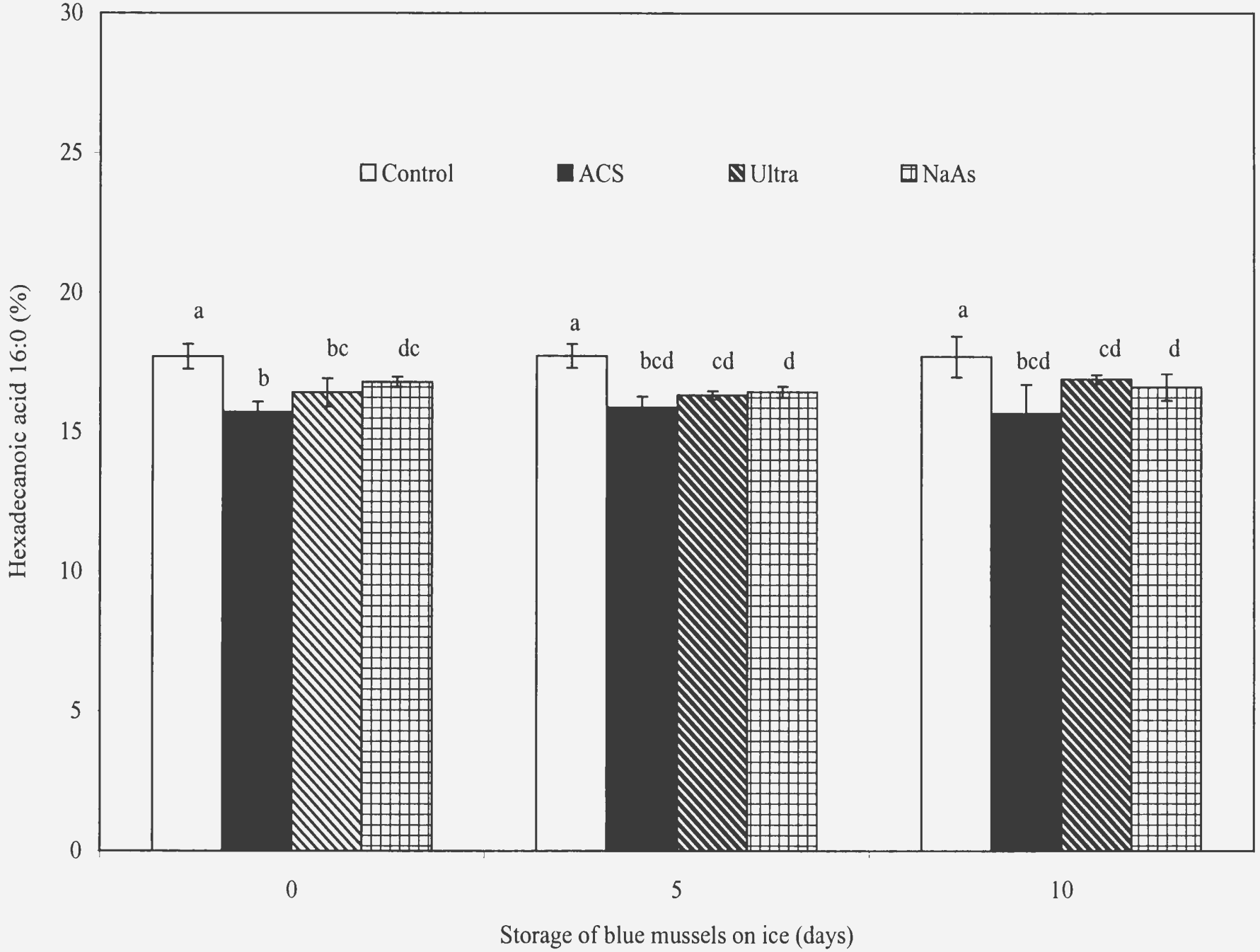

30
25
20
15
10
5
0
Hexadecanoic acid 16:0 (%)
Control
ACS
Ultra
NaAs
a
b
bc
dc
a
bcd
cd
d
a
bcd
cd
d
0
5
10
Storage of blue mussels on ice (days)

Figure 6.10 Major monounsaturated fatty acid, hexadecaenoic acid (16:1n-7) (% of total fatty acids) of mussels treated with 10,000 μmol / L of various forms and grades of Asc and stored on ice for 10 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.

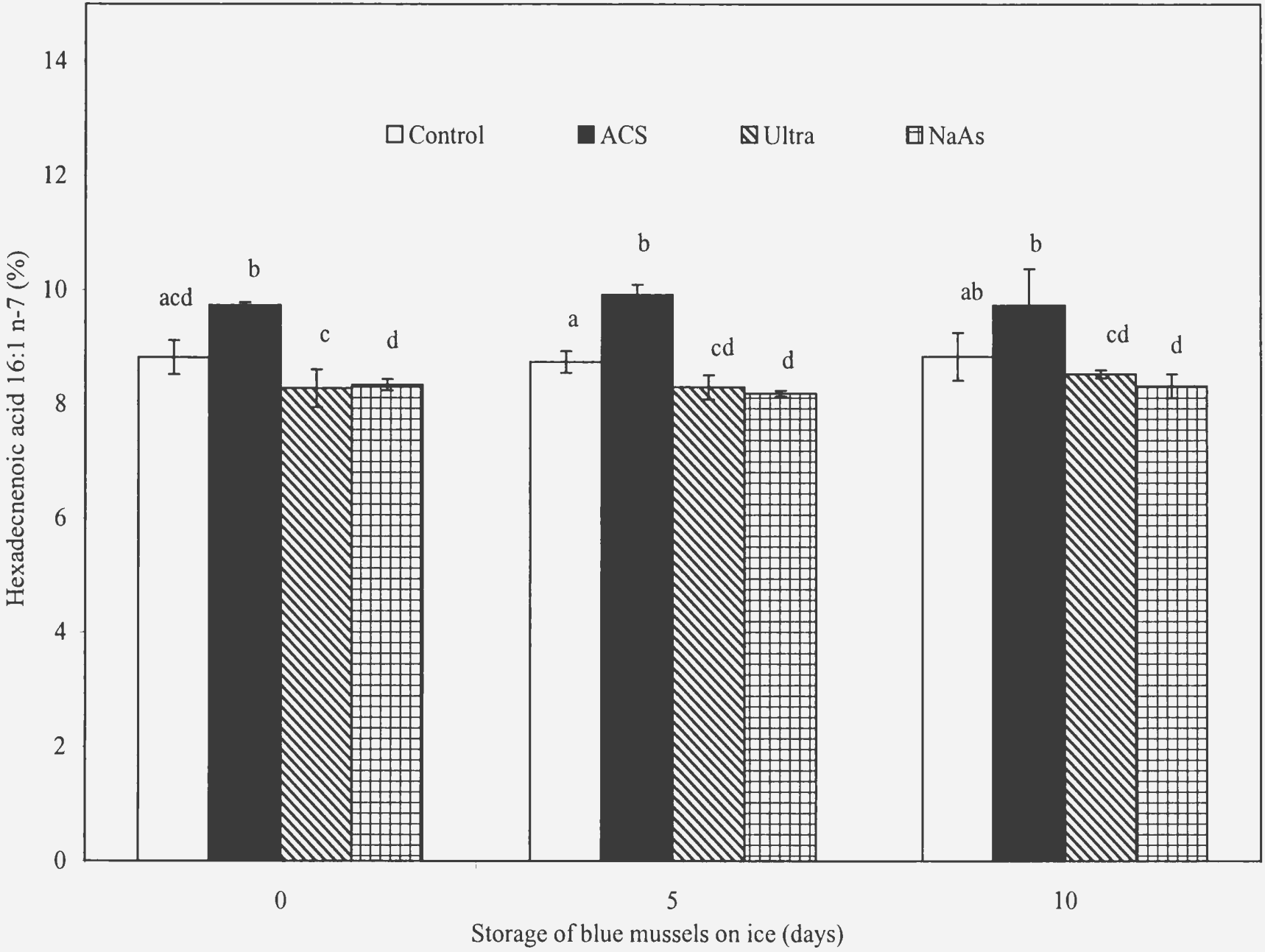
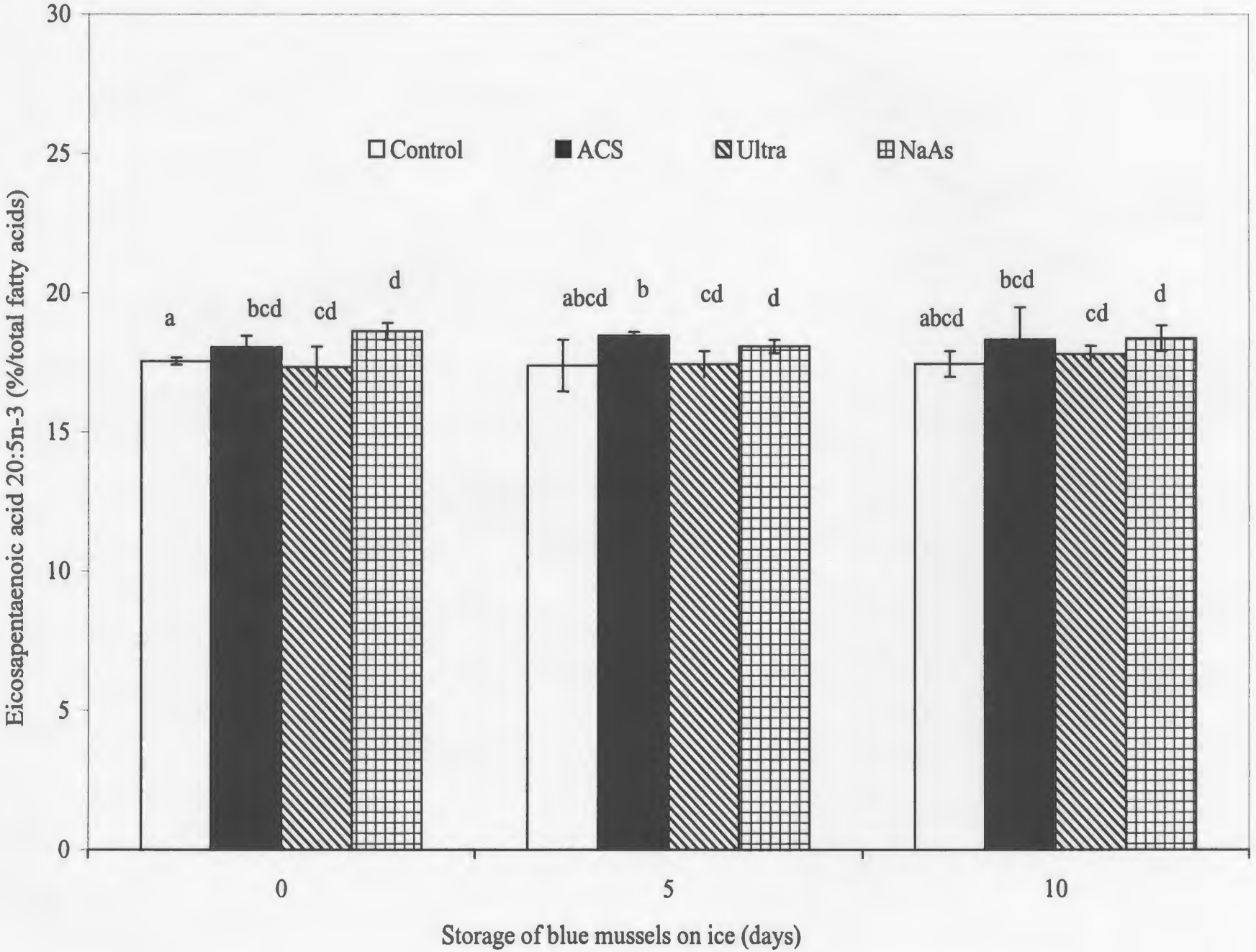

Control
ACS
Ultra
NaAs
Hexadecnenoic acid 16:1 n-7 (%)
0
2
4
6
8
10
12
14
acd
b
c
d
a
b
cd
d
ab
b
cd
d
0
5
10
Storage of blue mussels on ice (days)

Figure 6.11 Major polyunsaturated fatty acid, Eicosapentenoic acid (20:5 n-3) (% of total fatty acids) of mussels treated with 10,000 μmol / L of various forms and grades of Asc and stored on ice for 10 days (Mean ± S.D., n=3). Means with different letters are significantly ($p < 0.05$) different from each other.


30
25
20
15
10
5
0
Control
ACS
Ultra
NaAs
a
bcd
cd
d
abcd
b
cd
d
abcd
bcd
cd
d
0
5
10
Eicosapentaenoic acid 20:5n-3 (%/total fatty acids)
Storage of blue mussels on ice (days)

Measurement of thiobarbituric acid reactive substance (TBARS) is commonly used to evaluate oxidative stress in seafoods (Tang *et al.*, 2001; Saeed and Howell, 2002). Antioxidants also affect the oxidative status of seafoods and their measurement was therefore included in this study (Undeland *et al.*, 1999; Bell *et al.*, 2000: Gieseg *et al.*, 2000). Tocopherol activity in lipid extracts have been measured with spectrophotometric, chemiluminescence and HPLC methods while Asc activity has been determined by spectrophometric, flow injection, capillary electrophoresis, chemiluminescence or HPLC procedures (Lewin and Popov, 1994; Popov and Lewin, 1996; Eitenmiller and Landen, 1999 a and b; Levine, 2000). The luminol-enhanced chemiluminescence method used in this study to examine AscAC and TAC in stored mussels is based on the oxidation of luminol (3-aminophthahydrazide) using a peroxy radical in the presence of water or alcohol (Wheatley, 2000). This reaction yields a luminol radical or luminol dianion that emits light, which is then detected by a luminometer. Antioxidants capacity in mussel TCA and lipid extracts was related quantitatively with standard Asc or Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid) as decribed by Lewin and Popov (1996), Amarowicz *et al.* (1999) and Amarowicz *et al.* (2002).

Examination of TBARS values from both processing plants suggests that their mechanical handling does not adversely affect the oxidative status of the mussels. This can be explained, at least partially, by the short period of time used in processing and immediate packing on ice. If larger batches of mussels are to be handled, requiring several hours of mechanical handling, further oxidative stress related studies should be conducted. TBARS values of mussels stored on ice from both plants for 14 days increased gradually up to 10 days of storage, and thereafter declined. This decline in TBARS values after 10

days of storage should be considered when this index is to be used to monitor lipid oxidation beyond 10 days of storage as it may lead to false low values (Shahidi and Pegg, 1993; Shahidi and Spurvey, 1995). No sensory analyses were performed to determine if oxidative rancidity was developed. However, that lipids were not oxidized for the first 7 days of storage. The relatively large standard deviation among TBARS values of mussels during the later period of storage may indicate that some mussels were weak or dead due to high bacterial load, while others were resistant.

The pH of filtered seawater used in this study was close to neutrality at 0 h and after 24 hr of mussels treated with various forms and grades of Asc. The decrease in pH values after the addition of NaAs, ACS and ultra-pure Asc can be explained by dissociation of acid salts and the acid in aqueous medium (Anonymous, 1983a; Anonymous, 1983b). Addition of 5 g of Asc to 1 L of water (pH = 7) can reduce the pH to 3, while addition of 50 g to 1 L of water will decrease the pH to 2 (Anonymous, 1983a). In the current study, 2 g / L was used to obtain 0.01 M of NaAs and 1.76 g per litter was used to obtain 0.01 M of ACS and ultra-pure grades Asc solutions. Buffering capacity of flittered seawater may not be effective in resisting the change in pH due to Asc addition. Acetic acids was used to control the pH of seawater during the addition of sodium ascorbate (Thed *et al.*, 1993). In this study, pH values increased for filtered seawater containing mussels after 24 h, perhaps due to the excretion of basic compounds such as ammonia (Jay, 1996; Kyrana, *et al.*, 1997). The pH of all treated and control mussels was about 6.5, except for ultra-pure grade Asc treated mussels, which was around 6.4 and is an anomaly. After 10 days of storage, the pH increase in all mussel samples can be explained, at least partially, by the production of

alkaline compounds due to proteolysis or psychrotrophic bacterial growth (Kyrana *et al.*, 1997; Suvanich and Marshall, 1998).

None of the Asc treatments rendered any anti-bacterial activity. Asc, at specific concentrations and upon prolonged feeding periods boosts the immune system of fish, and therefore decreases their susceptibility to clinical disorders, bacterial attack and diseases (Erdal *et al.*, 1991; Gabaudan and Verlhac, 2001). Furthermore, Asc is also known for its antimicrobial activity in foods (Anonymous, 1983a). Nonetheless, when ground chevon, obtained from goats, was blended with various concentrations of Asc and stored at 4°C for 9 days, samples treated with 600 ppm Asc had lower lipid oxidation but had no influence on bacterial growth (Verma and Sahoo, 2000).

Oxygen is required for metabolic activities in all living cells of aerobic organisms. Reactive oxygen species (ROS), however, may be generated in the presence of various initiators such as metal ions, heat, visible light and UV (radiation) light. The reaction of ROS with various cell components will degrade or inactivate certain essential molecules (Halliwell *et al.*, 1995). Under normal conditions, living cells control the adverse effects of free radicals using antioxidant systems, such as antioxidant enzymes (superoxide dismutase), α-tocopherol and phenolics (Arouma, 1998). Lipid oxidation in the human body caused by free radical reactions may damage tissues and cells, leading to various diseases and clinical disorders. In fact, almost any disease is likely to be accompanied by increased formation of ROS (Haliwell *et al.*, 1995).

The susceptibility of seafoods to oxidative damage is determined by the balance between endogenous pro- and antioxidants as well as exposure to physical, chemical and environmental variables (Sole *et al.*, 1994; Undeland *et al.*, 1999; Bell *et al.*, 2000; Gieseg

et al., 2000). In this study, an attempt has been made to increase the balance of water soluble antioxidants through physical treatment by various forms and grades of Asc. This technique has been successfully used to control lipid oxidation during freezing of channel catfish for up to six months (Thed and Erickson, 1994; Erickson, 1997). In a recent study, however, exposing herring filets to 0.2 g / L Asc in tanks or the fishing vessel was ineffective in controlling lipid oxidation throughout 14 weeks of storage in freezers (Harmre *et al.*, 2003). Spraying the same fish with 20 g / L Asc after harvest and filleting did control lipid oxidation for the first 9 weeks of storage, after which the fillets were unaffected by the treatment. This indicates that many variables, including concentration and application technique, play an important role in the effectiveness of Asc as an antioxidant.

The current study, to the best of my knowledge, is the first to report the use of water-soluble natural antioxidants to control lipid oxidation in live bivalves stored on ice. Asc and its salts are highly susceptible to oxidation in aqueous solution, especially at high pH (Anonymous, 1983a; Anonymous, 1983b). This may explain, in part, considerable decrease in the antioxidant capacity of NaAs, ACS and ultra-pure grades Asc in filtered seawater after 24 h of treatment of mussels. Furthermore, this reduction may also be partially attributable to the particle absorption of Asc by approximately 25 mussels in each container.

Asc absorbed by blue mussels exhibited antioxidant activity in controlling lipid oxidation during the first 5 days of storage. Thereafter, they become pro-oxidant. The strongest ($p < 0.05$) antioxidant activity at day 5 was observed for NaAs followed by ACS grade Asc and finally ultra-pure grade Asc. This can be explained by the fact that Asc and

tocopherol antioxidant capacities were not significantly ($p > 0.05$) lower at day 5 of storage of control and treated mussels. After 10 days of storage, the reverse trend was observed and NaAs was more ($p < 0.05$) pro-oxidant followed by ACS grade Asc and finally ultra-pure grade Asc. This trend of pro-oxidant activity corresponded with the reduced levels of antioxidant capacities of Asc and tocopherol in stored mussels, an observation that has been also reported in other studies (Brannan and Erickson, 1996; Bragadottir *et al.*, 2004). The loss of Asc is probably greater at refrigeration or ice temperatures. Furthermore, Asc can act synergistically with tocopherols to protect against lipid oxidation up to certain limits and at specific Asc : tocopherol ratios and concentrations. Therefore, changes in these conditions may actually accelerate oxidation rather than inhibiting it (Undeland *et al.*, 1999; Bell *et al.*, 2000; Gieseg *et al.*, 2000). Another explanation for pro-oxidant activity of Asc absorbed by mussels is their relatively high iron content (Anonymous, 2002a; Anonymous 2002c). Asc at certain concentrations and in the presence of other water soluble antioxidants such as glutathione peroxidase can keep the iron in the hemoglobin and myoglobin (both considered as potential pro-oxidants in seafoods) in the reduced form (Halliwell and Whiteman 1997; Richards *et al.*, 1998; Undelland *et al.*, 2003). However, a reduction in Asc, glutathione and other endogenous antioxidants, as well as the presence of free iron or other minerals, due to prolonged storage and cells death may increase lipid oxidation (Ramannathan, 1992 a; Romeo, 1997; Taso, 1997; Undeland *et al.*, 2000; Yen *et al.*, 2000). In fact, the iron – ascorbate system is used in biological systems to initiate lipid oxidation *in vitro* (Halliwell and Whiteman 1997; Sista *et al.*, 2000).

Exposing mussels to various grades and forms of Asc did not change, to any significant ($p > 0.05$) level the major saturated, monounsaturated and polyunsaturated fatty

acids as well as total SAF, MUFA and PUFA in stored mussels. Although some significant ($p < 0.05$) differences were observed in the 16:0 and a 16:1n-7 fatty acids, they are not sufficient to affect the consumer or industry acceptance or rejection of the treated mussels.

Chapter VII

Summary and Recommendations

7.1 Summary

This research demonstrated that aerobic and psychrotrophic plate count in mussels stored at low temperatures or on ice on marine agar were 1-3 log CFU/g higher than counts on plate count agar or plate count agar supplemented with 1 % NaCl. Furthermore, linear models were established to predict bacterial counts of stored mussels on marine agar using bacterial counts obtained from plate count agar. Storage temperatures and the agar type used for the microbial counts can affect shelf life estimation.

The general trend for all variables (chlorophyll, temperature, salinity, microbial content of aquaculture sites and mussels, fatty acid composition of mussels and plankton samples and lipid composition of mussels) examined at four stations during 2000 and 2001 was that there were no major differences among the four stations throughout the sampling period, with a few exceptions. Even when some differences were observed among the four stations, they could not be directly related to the biochemical composition of the cultivated mussels, which is determined after several weeks of exposure to various environmental variables prior to sampling. Furthermore, the observed differences in the biochemical and microbial variables of cultured mussels among the four stations should not affect either marketing or consumer perception of the product. Therefore, the values of all variables measured at the aquaculture sites and of biochemical and microbial variables of cultured mussels were averaged and plotted against sampling dates to obtain the seasonal and annual variations in these variables and in the quality of the harvested mussels. One of the most important factors that may affect site selection is the seasonal cycle in the

temperature of seawater at the proposed site. This cycle may affect chlorophyll *a* content or the algal species present and their fatty acid compositions and microbial characteristics of water, which in turn can affect microbial content, lipid and fatty acid composition, meat content and shell size of cultivated mussels. The occurrence of high levels of n-3 PUFA and phytosterols in Newfoundland cultured mussels may add to their market value. The optimum growth period in terms of meat content and shell size may be achieved within one year of cultivation. The optimum harvest time based on nutritional value and shelf life may be May to June (prior to spawning) or September to October (if the mussels have started to recover from spawning).

Storage of cultured mussels on ice affected their microbial composition and lipid oxidation considerably, but had less effect on pH, proximate and fatty acid compositions. This indicates that microbial growth and lipid oxidation can be used as indicators to monitor the quality of mussels during storage on ice. In fact, measurement of microbial growth by conventional agar method was the best technique to monitor product deterioration during storage. Other potential indicators are the bacterial the fatty acids *ai*15:0 *i*17:0 and total bacterial fatty acids.

Mussels stored on ice were more susceptible to lipid oxidation than fresh mussels as reflected by their higher TBARS values. The current short mechanical handling practice in the mussel industry, which includes washing, sorting, washing again and packaging, does not affect their oxidative status. Furthermore, exposing live mussels to specific concentrations of Asc may control lipid oxidation during their storage on ice for only 5 days, after which the Asc become a pro-oxidant. Finally, exposure to Asc did not prevent microbial growth during the storage on ice.

7.2 Recommendations

1) The use of marine agar instead of plate count agar to enumerate aerobic and psychrotrophic bacteria in Newfoundland cultured blue mussels to evaluate the microbial content and estimate shelf life is recommended.
2) Further identification of bacterial species in cultured blue mussels is needed.
3) Temperature of cultured mussels should be carefully monitored during storage because abuse temperatures can considerably reduce shelf life.
4) In order to select future mussel aquaculture sites to produce high quality seafood product, the temperature, chlorophyll *a* and flushing rate should be monitored frequently during all seasons and several years.
5) The use of short chain 2- and 3-hydroxy bacterial fatty acids to monitor microbial status of cultivated mussels should be evaluated using various samples at different storage periods, pure bacterial cultures as references, more rapid lipid extraction and transmethylation methods, more appropriate columns and time temperature gradients in gas chromatography or gas chromatography - mass spectrometry and sensory analysis in order to develop rapid techniques.
6) If larger batches of mussels are to be handled mechanically for several hours, then further oxidative stress related studies should be conducted using different evaluation methods.
7) The use of ascorbic acid and other natural antioxidants such as rosemary extracts to control lipid oxidation in cultured mussels stored on ice should be evaluated using different concentrations and at various pH's and temperatures.

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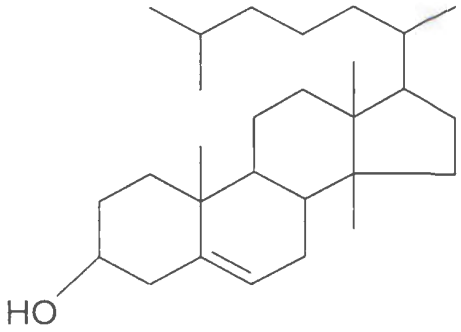
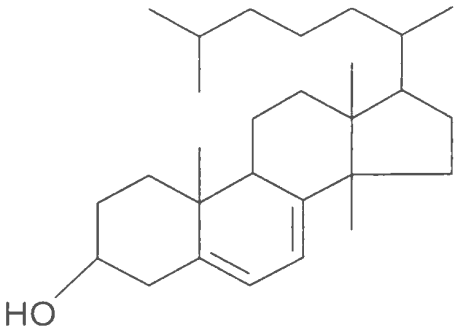
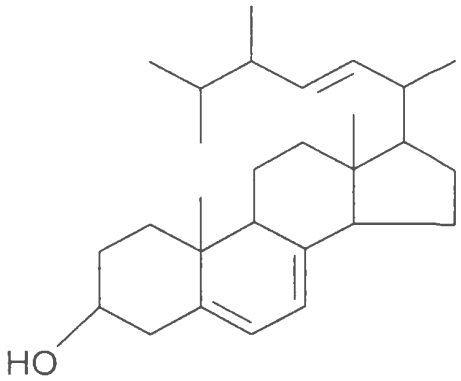
Appendix 1.1 Chemical structures of sterols



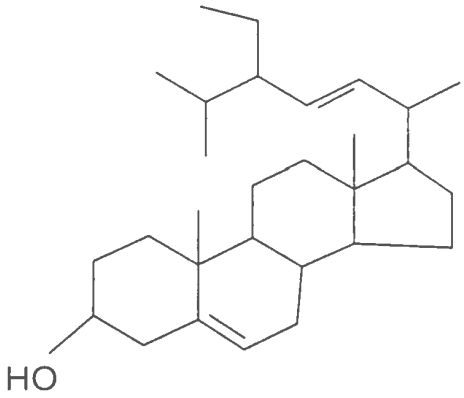
Cholesterol



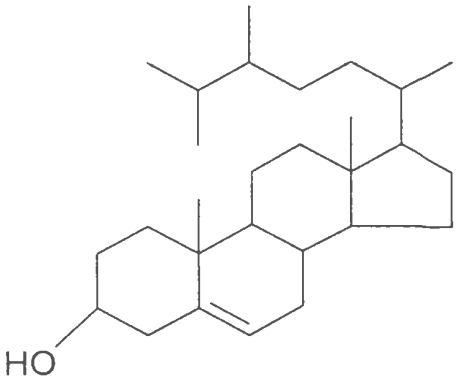
7-dehydrocholesterol



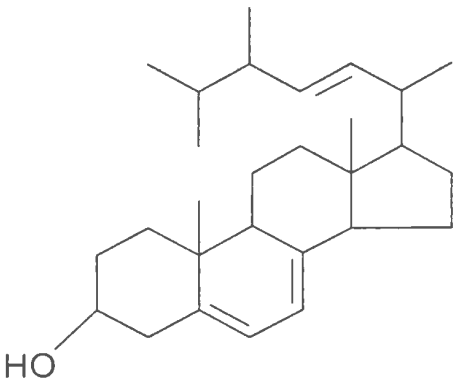
Ergosterol



Stigmasterol

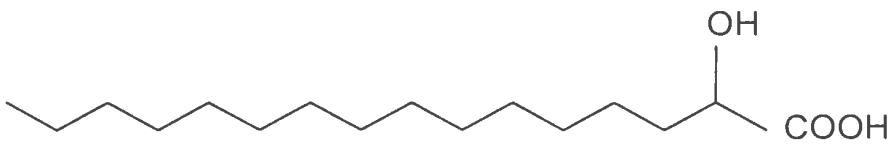


Campesterol

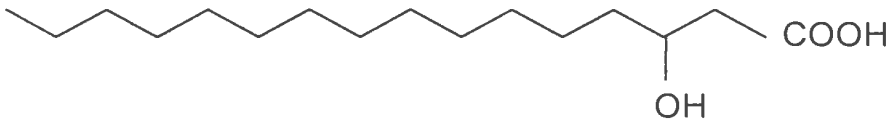


Brassicasterol

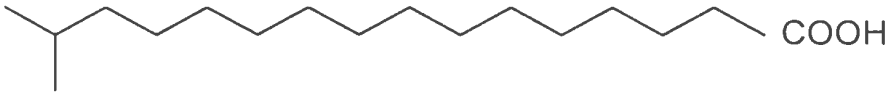
Appendix 1.2 Chemical structures of some bacterial fatty acids



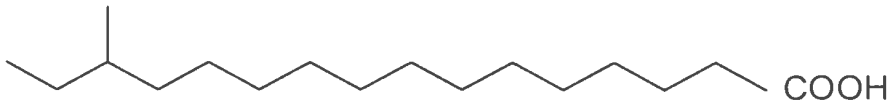
2-OH 16:0



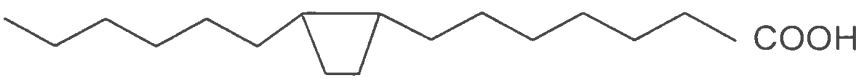
3-OH 16:0



Iso 16:0

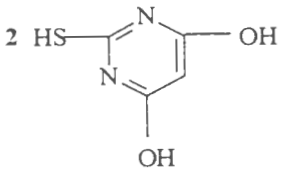


Anteiso 16:0

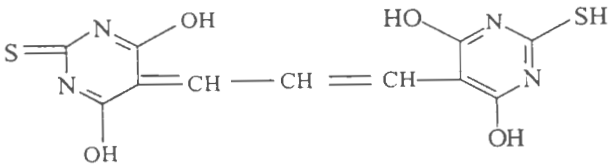


Cyclopropane 15:0

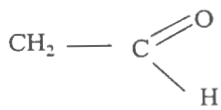
Appendix 1.3 Chemical reaction of 2-thiobarbituric acid with malonaldehyde



Thiobarbituric acid (TBA)

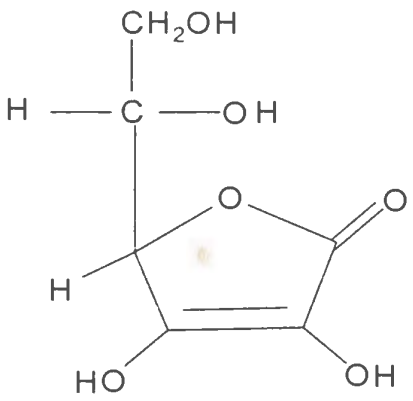


TBA-MA complex

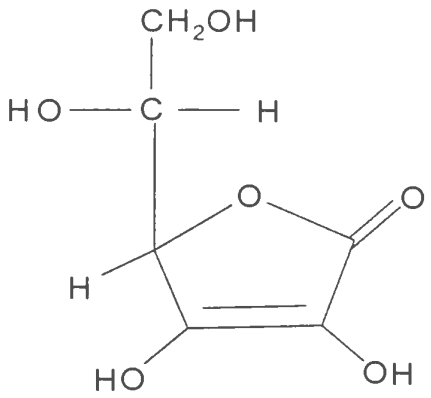


Malonaldehyde(MA)

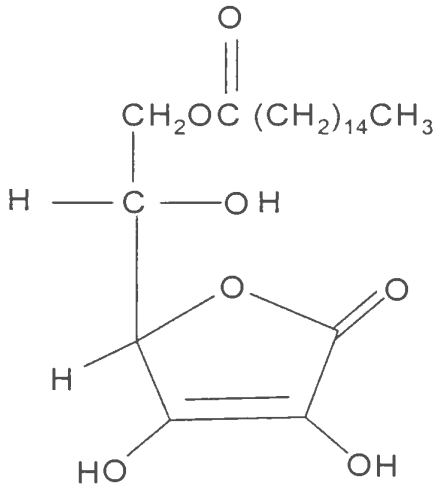
Appendix 1.4 Chemical structure of ascorbic acid and its derivatives



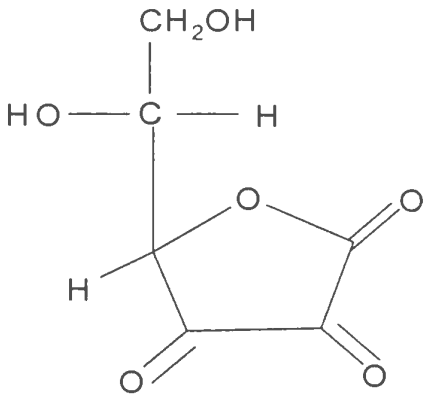
L-Ascorbic acid



Isoascorbic acid

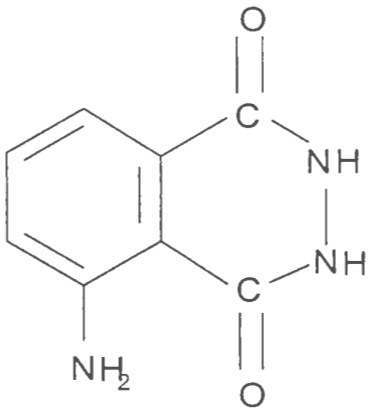


Ascorbyl palmitate

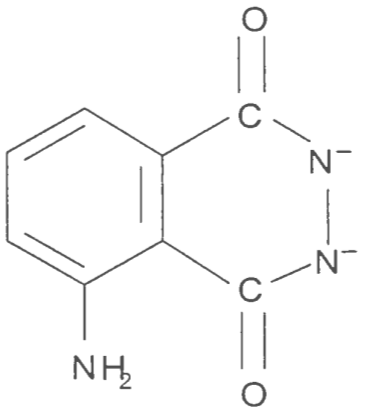


Dehydroascorbic acid

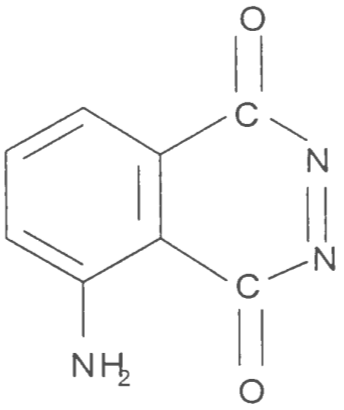
Appendix 1.5 Chemical structure of luminol, luminol dianion and Trolox



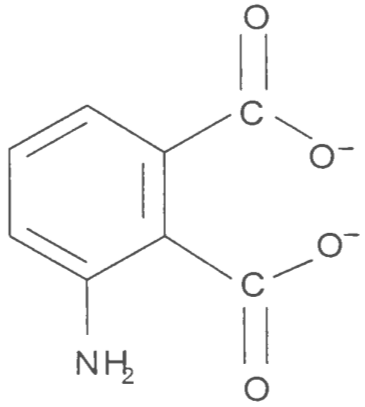
Luminol



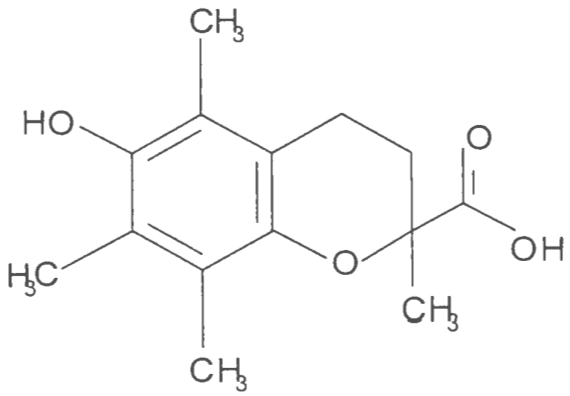
Luminol dianion



Diazaquinone



3-Aminophthalate



Trolox

Appendix 4.1 Typical fatty acid composition (%) of plankton samples obtained from two stations in Charles Arm blue mussel (*Mytilus edulis*) aquaculture site in October 2000 (mean and S.D., n=3).

	I		II	
Fatty Acid	Mean	S.D.	Mean	S.D.
14:0	6.29	0.42	5.53	1.14
*i*15:0	0.72	0.12	0.85	0.11
*ai*15:0	0.04	0.07	0.36	0.13
15:0	0.44	0.04	0.88	0.17
*i*16:0	0.30	0.05	0.40	0.24
*ai*16:0	0.21	0.09	0.33	0.15
16:0	28.64	1.27	24.92	4.26
16:1 n7	2.48	0.46	4.59	0.36
16:1 n5	0.98	0.06	0.56	0.05
*i*17:0	0.36	0.01	0.63	0.11
*ai*17:0	0.00	0.00	0.12	0.12
16:2 n4	0.13	0.04	0.41	0.07
17:0	0.31	0.26	0.72	0.13
16:3 n4	0.09	0.08	0.40	0.35
16:4 n3	3.43	0.41	0.00	0.00
16:4 n1	0.03	0.05	0.37	0.04
18:0	1.20	0.04	2.35	0.45
18:1 n9	0.00	0.00	4.07	0.59
18:1 n7	3.01	0.50	3.12	0.36
18:1 n6	0.00	0.00	0.00	0.00
18:1 n5	0.33	0.11	0.27	0.23
18:2 n6	1.82	0.35	1.58	0.11
18:2 n4	0.00	0.00	0.27	0.11
18:3 n6	0.00	0.00	0.05	0.08
18:3 n4	0.00	0.00	0.00	0.00
18:3 n3	0.78	0.11	1.35	0.02
18:4 n3	1.67	0.27	2.72	0.12
18:4 n1	0.00	0.00	0.00	0.00
20:0	0.12	0.11	0.04	0.07
18:5 n3	0.00	0.00	0.00	0.00
20:1 n9	4.31	3.59	3.02	0.19
20:1 n7	1.90	3.11	0.38	0.08
20:2 n6	0.30	0.05	0.55	0.12
20:3 n6	0.06	0.06	0.00	0.00
20:4 n6	0.25	0.04	0.63	0.11
20:3 n3	0.06	0.05	0.00	0.00
20:4 n3	0.40	0.09	0.73	0.02
20:5 n3	11.04	0.98	12.79	1.97
22:0	0.05	0.05	0.00	0.00
22:1 n11	0.00	0.00	0.00	0.00
22:1 n9	0.00	0.00	0.00	0.00
22:1 n7	0.00	0.00	0.00	0.00
21:5 n3	0.14	0.02	0.31	0.39
23:0	0.14	0.11	0.28	0.09
22:4 n6	0.12	0.01	0.07	0.12
22:4 n3	0.00	0.00	0.00	0.00
22:5 n6	0.00	0.00	0.00	0.00
22:5 n3	0.38	0.02	0.59	0.20
24:0	0.00	0.00	0.00	0.00
22:6 n3	27.06	1.08	23.11	4.87
24:1	0.41	0.08	0.65	0.15

Appendix 4.2 Typical fatty acid composition (%) of plankton samples obtained from two stations in Fortune Harbour blue mussel (*Mytilus edulis*) aquaculture site in October 2000 (mean and S.D., n=3).

	I		II	
Fatty Acid	Mean	S.D.	Mean	S.D.
14:0	6.75	0.81	6.03	1.37
*i*15:0	1.41	0.29	1.02	0.40
*ai*15:0	0.40	0.36	0.57	0.11
15:0	1.21	0.15	1.36	0.06
*i*16:0	0.24	0.20	0.32	0.02
*ai*16:0	0.72	0.44	0.24	0.21
16:0	17.01	1.47	17.30	2.85
16:1 n7	8.10	0.65	5.59	0.42
16:1 n5	0.23	0.20	0.13	0.22
*i*17:0	0.68	0.08	0.73	0.16
*ai*17:0	0.22	0.20	0.17	0.16
16:2 n4	0.62	0.57	0.52	0.20
17:0	0.84	0.26	0.98	0.23
16:3 n4	1.67	1.06	0.96	0.52
16:4 n3	0.91	0.90	0.18	0.32
16:4 n1	0.61	0.54	0.17	0.30
18:0	2.75	0.11	2.54	0.43
18:1 n9	4.90	4.25	3.87	3.35
18:1 n7	4.89	3.09	4.09	1.82
18:1 n6	0.00	0.00	0.00	0.00
18:1 n5	0.58	0.58	0.48	0.07
18:2 n6	2.02	0.19	1.81	0.06
18:2 n4	0.17	0.15	0.29	0.02
18:3 n6	0.00	0.00	0.00	0.00
18:3 n4	0.00	0.00	0.00	0.00
18:3 n3	1.18	0.10	1.32	0.11
18:4 n3	3.94	0.61	4.58	1.25
18:4 n1	0.00	0.00	0.00	0.00
20:0	0.18	0.17	0.07	0.12
18:5 n3	0.00	0.00	0.00	0.00
20:1 n9	0.71	0.47	0.88	0.31
20:1 n7	0.36	0.13	0.17	0.18
20:2 n6	0.67	0.51	0.78	0.13
20:3 n6	0.00	0.00	0.18	0.17
20:4 n6	0.80	0.03	0.69	0.07
20:3 n3	0.20	0.21	0.16	0.15
20:4 n3	1.42	0.09	1.52	0.09
20:5 n3	14.16	0.17	15.66	1.23
22:0	0.23	0.22	0.07	0.12
22:1 n11	0.00	0.00	0.00	0.00
22:1 n9	0.00	0.00	0.08	0.13
22:1 n7	0.00	0.00	0.00	0.00
21:5 n3	0.28	0.25	0.57	0.14
23:0	1.05	0.41	0.46	0.49
22:4 n6	0.22	0.23	0.00	0.00
22:4 n3	0.00	0.00	0.00	0.00
22:5 n6	0.04	0.07	0.09	0.16
22:5 n3	0.64	0.56	0.74	0.64
24:0	0.00	0.00	0.00	0.00
22:6 n3	16.97	2.28	22.23	1.53
24:1	0.00	0.00	0.39	0.12

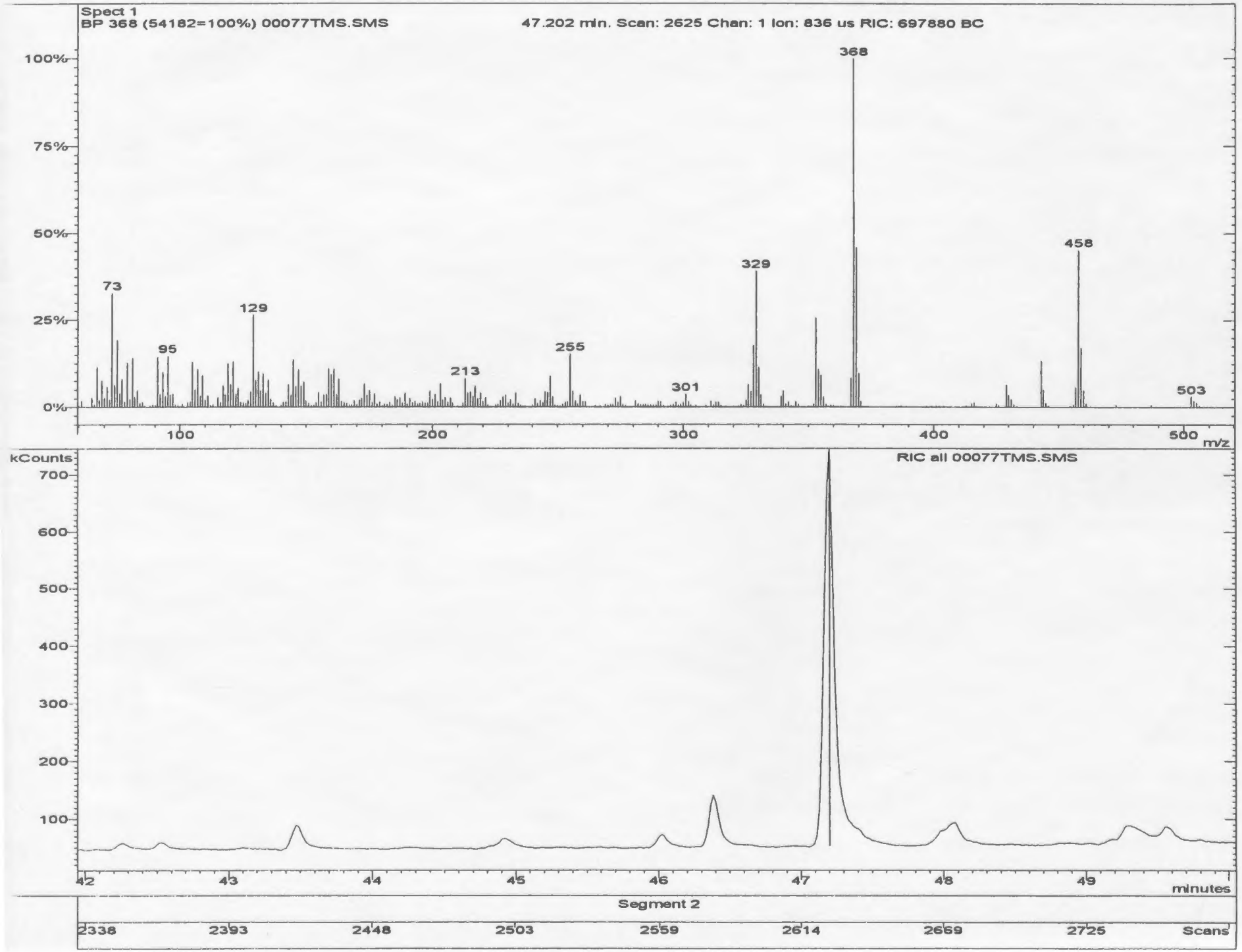
Appendix 4.3 Typical fatty acid composition (%) of cultured Newfoundland blue mussels (*Mytilus edulis*) obtained from two stations in Charles Arm aquaculture site in October 2000 (mean and S.D., n=3).

	I		II	
Fatty Acid	Mean	S.D.	Mean	S.D.
14:0	5.01	0.24	3.90	0.29
*i*15:0	0.12	0.14	0.14	0.11
*ai*15:0	0.00	0.00	0.00	0.00
15:0	0.61	0.02	0.74	0.06
*i*16:0	0.26	0.02	0.29	0.03
*ai*16:0	0.83	0.13	1.16	0.08
16:0	17.10	0.77	14.01	0.49
16:1 n7	2.57	0.22	3.37	0.58
16:1 n5	0.49	0.02	0.25	0.29
*i*17:0	0.98	0.09	1.39	0.11
*ai*17:0	1.22	0.11	1.27	0.85
16:2 n4	0.11	0.13	0.61	0.88
17:0	0.74	0.02	1.06	0.07
16:3 n4	0.84	0.39	0.92	0.45
16:4 n3	8.11	0.69	10.12	1.43
16:4 n1	0.00	0.00	0.00	0.00
18:0	2.79	0.17	3.26	0.37
18:1 n9	0.98	1.03	0.64	0.74
18:1 n7	1.75	0.10	1.61	0.69
18:1 n6	0.00	0.00	0.00	0.00
18:1 n5	0.24	0.07	0.17	0.11
18:2 n6	1.39	0.10	1.22	0.21
18:2 n4	0.04	0.08	0.18	0.12
18:3 n6	0.00	0.00	0.03	0.06
18:3 n4	0.05	0.10	0.08	0.09
18:3 n3	1.84	0.24	1.94	0.35
18:4 n3	3.68	0.75	2.53	0.68
18:4 n1	0.00	0.00	0.00	0.00
20:0	0.00	0.00	0.00	0.00
18:5 n3	0.00	0.00	0.00	0.00
20:1 n9	0.40	0.33	0.00	0.00
20:1 n7	2.29	1.44	3.08	1.09
20:2 n6	0.82	0.10	0.78	0.10
20:3 n6	0.00	0.00	0.00	0.00
20:4 n6	2.72	0.19	4.06	0.40
20:3 n3	0.00	0.00	0.03	0.05
20:4 n3	0.27	0.02	0.24	0.08
20:5 n3	11.28	0.33	13.51	0.61
22:0	0.00	0.00	0.00	0.00
22:1 n11	0.00	0.00	0.00	0.00
22:1 n9	0.00	0.00	0.12	0.25
22:1 n7	0.00	0.00	0.00	0.00
21:5 n3	1.43	0.11	1.55	0.20
23:0	0.07	0.14	0.00	0.00
22:4 n6	0.53	0.05	0.81	0.05
22:4 n3	0.00	0.00	0.00	0.00
22:5 n6	0.00	0.00	0.00	0.00
22:5 n3	0.85	0.07	1.12	0.07
24:0	0.00	0.00	0.00	0.00
22:6 n3	27.57	1.13	23.81	1.28
24:1	0.00	0.00	0.00	0.00

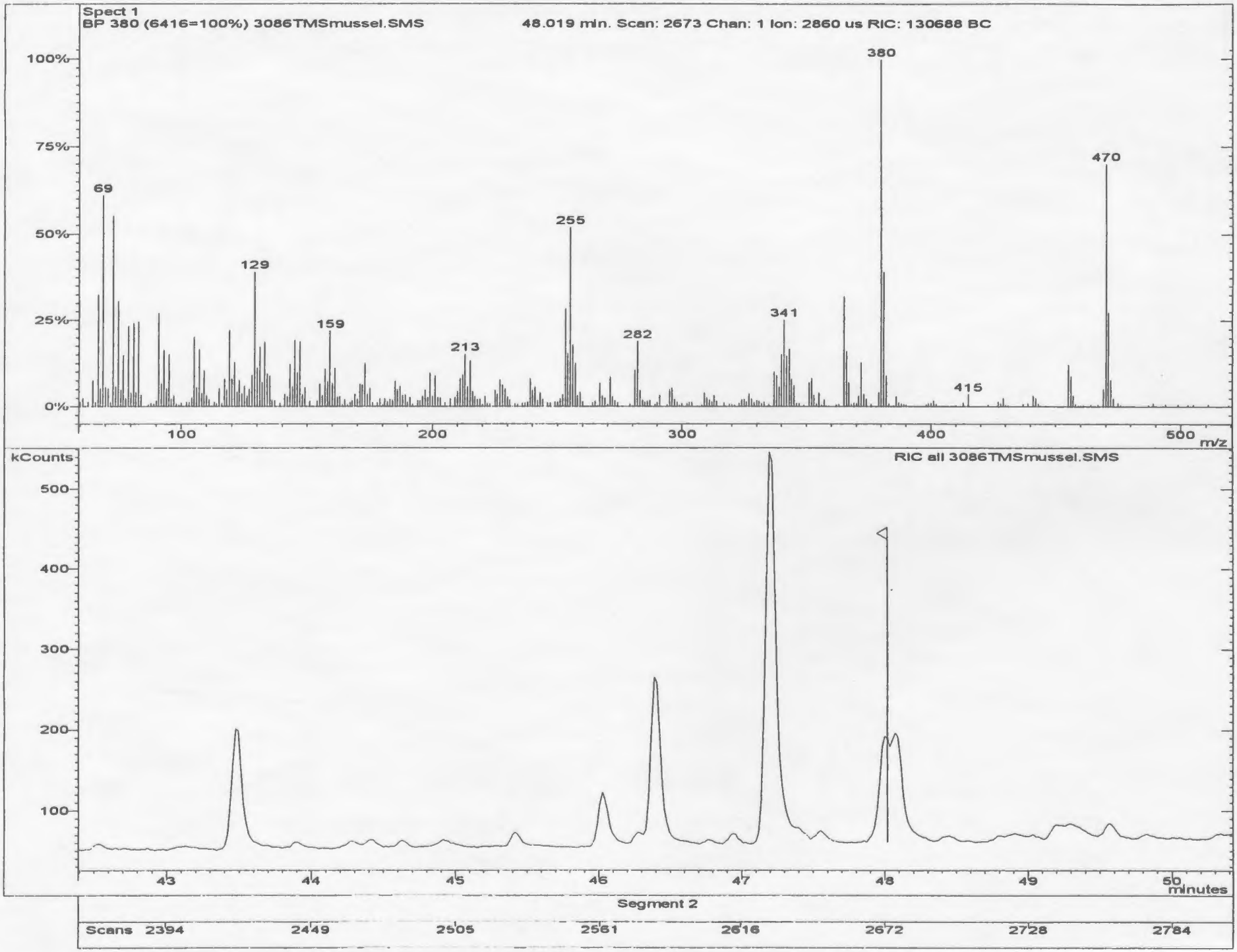
Appendix 4.4 Typical fatty acid composition (%) of cultured Newfoundland blue mussels (*Mytilus edulis*) obtained from two stations in Fortune Harbour aquaculture site in October 2000 (mean and S.D., n=3).

	I		II	
Fatty Acid	Mean	S.D.	Mean	S.D.
14:0	5.09	0.61	5.17	0.82
*i*15:0	0.19	0.13	0.21	0.17
*ai*15:0	0.03	0.07	0.04	0.07
15:0	0.78	0.05	0.85	0.04
*i*16:0	0.25	0.02	0.29	0.01
*ai*16:0	1.01	0.11	1.00	0.11
16:0	14.21	0.45	14.00	0.99
16:1 n7	6.07	1.35	6.07	1.76
16:1 n5	0.26	0.19	0.13	0.26
*i*17:0	1.08	0.11	1.20	0.08
*ai*17:0	0.98	0.66	1.65	0.14
16:2 n4	0.76	0.63	0.47	0.09
17:0	0.85	0.11	0.97	0.06
16:3 n4	0.54	0.45	0.28	0.13
16:4 n3	9.00	1.47	8.96	1.47
16:4 n1	0.00	0.00	0.00	0.00
18:0	2.84	0.29	2.86	0.26
18:1 n9	0.84	0.97	0.59	0.54
18:1 n7	1.95	0.45	1.81	0.54
18:1 n6	0.00	0.00	0.00	0.00
18:1 n5	0.18	0.13	0.24	0.10
18:2 n6	1.33	0.10	1.39	0.17
18:2 n4	0.16	0.11	0.17	0.12
18:3 n6	0.04	0.08	0.14	0.10
18:3 n4	0.17	0.25	0.47	0.68
18:3 n3	1.06	0.06	1.66	0.49
18:4 n3	2.02	0.50	1.69	1.16
18:4 n1	0.00	0.00	0.00	0.00
20:0	0.00	0.00	0.00	0.00
18:5 n3	0.00	0.00	0.00	0.00
20:1 n9	0.53	0.64	0.15	0.29
20:1 n7	2.33	1.25	2.20	0.76
20:2 n6	0.56	0.07	0.64	0.11
20:3 n6	0.00	0.00	0.00	0.00
20:4 n6	3.03	0.70	3.29	0.37
20:3 n3	0.00	0.00	0.00	0.00
20:4 n3	0.13	0.09	0.10	0.11
20:5 n3	14.30	0.73	14.10	0.47
22:0	0.00	0.00	0.00	0.00
22:1 n11	0.00	0.00	0.00	0.00
22:1 n9	0.00	0.00	0.00	0.00
22:1 n7	0.00	0.00	0.00	0.00
21:5 n3	1.09	0.75	1.60	0.04
23:0	0.00	0.00	0.11	0.22
22:4 n6	0.47	0.13	0.66	0.06
22:4 n3	0.00	0.00	0.00	0.00
22:5 n6	0.00	0.00	0.00	0.00
22:5 n3	0.78	0.53	1.04	0.08
24:0	0.00	0.00	0.00	0.00
22:6 n3	25.08	0.69	23.77	0.22
24:1	0.00	0.00	0.04	0.08

Appendix 4.5 Mass spectrum of cholesta-5-en-3β-ol (cholesterol)


Spect 1
BP 368 (54182=100%) 00077TMS.SMS
47.202 min. Scan: 2625 Chan: 1 Ion: 836 us RIC: 697880 BC
100%
75%
50%
25%
0%
368
458
329
73
129
255
95
213
301
503
100
200
300
400
500
m/z
kCounts
700
600
500
400
300
200
100
RIC all 00077TMS.SMS
42
43
44
45
46
47
48
49
minutes
Segment 2
2338
2393
2448
2503
2559
2614
2669
2725
Scans

Appendix 4.6 Mass spectrum of 24-methylcholesta-5, 22E-dien-3β-ol (brassicasterol / crinosterol).


Spect 1
BP 380 (6416=100%) 3086TMSmussel.SMS
48.019 min. Scan: 2673 Chan: 1 Ion: 2860 us RIC: 130688 BC
100%
75%
50%
25%
0%
69
129
159
213
255
282
341
380
415
470
100
200
300
400
500
m/z
kCounts
500
400
300
200
100
RIC all 3086TMSmussel.SMS
43
44
45
46
47
48
49
50
minutes
Segment 2
Scans 2394
2449
2505
2561
2616
2672
2728
2784

Appendix 5.1 Fatty acid composition (%) of blue mussels stored on ice for 14 days determined by 14% BF_3-MeOH transmethylation reagents (mean and S.D., n=3).

	Storage on ice (days)									
fatty acids	0	S.D.	3	S.D.	7	S.D.	10	S.D.	14	s.d
14:0	3.82	0.00	2.75	0.01	2.95	0.08	3.38	0.14	3.56	0.01
*i*15:0	0.42	0.04	0.54	0.02	0.32	0.01	0.43	0.01	0.40	0.02
*ai*15:0	0.27^{ae}	0.05	0.15^{bd}	0.00	0.20^{c}	0.01	0.17^{d}	0.01	0.25^{e}	0.01
15:0	0	0	0	0	0	0	0	0	0	0
*i*16:0	0.07	0.10	0.13	0.00	0.12	0.00	0.12	0.00	0.06	0.10
*ai*16:0	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.01	0.25	0.29
16:0	13.39^{ace}	0.42	15.56^{b}	0.03	12.72^{c}	0.30	14.58^{d}	0.25	13.47^{e}	0.06
16:1 n-7	9.79^{ad}	0.30	11.48^{be}	0.03	8.74^{c}	0.24	9.67^{d}	0.12	11.62^{e}	0.21
16:1 n5	0.31	0.00	0.46	0.01	0.29	0.01	0.00	0.00	0.12	0.21
*i*17:0	0.58	0.03	0.56	0.02	0.53	0.02	0.58	0.01	0.45	0.00
*ai*17:0	0.45	0.03	0.41	0.01	0.47	0.02	0.48	0.01	0.44	0.00
16:2 n-4	0.96	0.02	0.98	0.02	0.72	0.02	0.76	0.05	0.89	0.03
17:0	0.46	0.01	0.37	0.01	0.43	0.01	0.54	0.01	0.39	0.01
16:3 n-4	0.37	0.05	0.58	0.01	0.71	0.07	0.56	0.06	0.57	0.13
16:4 n-3	0.37	0.02	0.10	0.15	0.00	0.00	2.43	0.08	1.50	0.15
16:4 n-1	1.58	0.02	0.97	0.01	1.17	0.11	0.85	0.01	1.23	0.01
18:0	2.59	0.09	1.54	0.00	2.38	0.06	3.08	0.07	2.10	0.02
18:1 n-9	1.55	0.02	1.52	0.03	1.51	0.01	1.52	0.03	1.52	0.03
18:1 n-7	1.14	0.05	1.14	0.00	1.13	0.02	1.17	0.02	1.30	0.01
18:1 n-5	0.16	0.23	0.36	0.00	0.23	0.20	0.32	0.28	0.45	0.02
18:1 n-6	4.31	0.20	2.03	1.89	4.01	0.11	2.70	0.03	2.77	0.01
18:2 n-4	0.16	0.23	0.36	0.00	0.23	0.20	0.23	0.20	0.32	0.00
18:3 n-3	1.17	0.09	1.31	0.00	1.12	0.03	0.99	0.55	1.51	0.02
18:4 n-3	4.41	0.13	4.15	0.23	4.32	0.24	4.49	0.06	4.42	0.12
20:1 n-9	0.72	0.03	0.85	0.00	0.63	0.01	0.65	0.00	0.78	0.01
20:1 n-7	4.41	0.16	4.37	0.93	4.06	0.10	2.99	0.03	4.18	0.11
20:2 delta 5,11	2.97	0.39	2.45	0.02	3.64	0.10	3.70	0.04	2.72	0.03
20:2 delta 5,13	1.31	0.18	0.89	0.02	1.29	0.04	1.20	0.02	0.87	0.01
20:2 n-6	0.94	0.24	0.40	0.00	1.26	0.04	1.70	0.05	1.00	0.19
20:4 n-6	0.48	0.01	0.90	0.00	0.49	0.01	0.63	0.01	0.78	0.02
20:4 n-3	0.96	0.02	1.11	0.01	1.13	0.03	1.23	0.01	1.12	0.02
20:5 n-3	20.41	0.76	22.69	0.12	21.89	0.72	21.14	0.30	21.38	0.54
22:2 delta 7,13	1.58	0.16	0.52	0.05	1.51	0.05	1.87	0.00	1.18	0.08
22:2 delta 7,15	1.07	0.00	1.07	0.04	1.12	0.02	0.59	0.14	1.19	0.02
22:5 n-3	0.71	0.04	0.80	0.14	1.01	0.11	0.82	0.01	0.82	0.03
22:6 n-3	13.11	0.43	12.49	0.10	13.07	0.18	13.00	0.24	12.94	0.38
Σ										
Saturated	19.80^{ae}	0.51	19.85^{ab}	0.04	18.05^{c}	0.44	21.05^{d}	0.45^{a}	19.13^{e}	0.09
Monounsaturated	22.67^{a}	1.05	22.36^{a}	2.90	20.80^{a}	0.82	19.20^{a}	0.54	23.00^{a}	0.62
Polyunsaturated	51.00^{a}	2.75	50.79^{a}	0.90	53.51^{a}	1.87	55.33^{a}	1.82	53.22^{a}	1.77
Bacterial	1.98^{a}	0.21	2.02^{a}	0.06	1.86^{a}	0.06	2.39^{a}	0.05	1.99^{a}	0.42

Means with different superscripts are significantly different ($p< 0.05$) from each other.

Appendix 5.2 Fatty acid composition (%) of blue mussels stored on ice for 14 days determined by 14% H_2SO_4 - MeOH transmethylation reagents (mean and S.D., n=3).

	Storage on ice (days)									
Fatty acids	0	S.D.	3	S.D.	7	S.D.	10	S.D.	14	S.D.
14:0	4.00	0.02	5.61	0.04	4.54	0.20	3.90	0.11	4.36	0.13
*i*15:0	0.45	0.08	0.56	0.01	0.48	0.05	0.44	0.01	0.45	0.01
*ai*15:0	0.00 [a]	0.00	0.09 [b]	0.08	0.17 [cde]	0.03	0.17 [d]	0.00	0.18 [e]	0.00
15:0	0	0	0	0	0	0	0	0	0	0
*i*16:0	0.17	0.06	0.18	0.00	0.18	0.02	0.17	0.01	0.16	0.01
*ai*16:0	0.16	0.03	0.12	0.01	0.16	0.02	0.16	0.01	0.17	0.01
16:0	14.43 [a]	0.60	16.29 [bde]	0.04	16.73 [cde]	0.56	15.66 [de]	0.43	15.75 [e]	0.49
16:1 n-7	11.95 [abd]	0.45	10.91 [b]	0.05	13.57 [c]	0.13	12.18 [d]	0.12	12.88 [d]	0.14
16:1 n5	0.38	0.03	0.44	0.00	0.42	0.01	0.36	0.01	0.39	0.01
*i*17:0	0.67	0.04	0.63	0.00	0.75	0.04	0.75	0.03	0.73	0.03
*ai*17:0	0.60	0.05	0.56	0.01	0.64	0.03	0.62	0.02	0.61	0.03
16:2 n-4	1.02	0.06	1.06	0.21	1.05	0.01	0.99	0.00	1.01	0.00
17:0	0.39	0.03	0.25	0.22	0.41	0.02	0.44	0.02	0.43	0.02
16:3 n-4	0.67	0.07	0.50	0.00	0.64	0.01	0.04	0.07	0.20	0.35
16:4 n-3	0.59	0.05	0.63	0.01	0.73	0.04	1.69	0.03	1.41	0.34
16:4 n-1	1.74	0.18	0.78	0.06	1.52	0.11	1.82	0.08	1.74	0.11
18:0	1.95	0.12	1.67	0.02	2.02	0.08	2.23	0.06	2.39	0.08
18:1 n-9	1.33	0.06	1.10	0.01	1.26	0.21	1.51	0.02	1.59	0.02
18:1 n-7	2.31	0.02	2.29	0.03	2.36	0.06	2.34	0.06	2.33	0.05
18:1 n-5	0.32	0.03	0.32	0.01	0.33	0.01	0.35	0.03	0.36	0.02
18:1 n-6	2.48	0.11	3.20	0.02	2.65	0.03	2.65	0.03	3.15	0.66
18:2 n-4	1.37	0.01	1.28	0.05	1.30	0.02	1.32	0.02	1.39	0.06
18:3 n-3	0.39	0.06	0.36	0.01	0.35	0.01	0.42	0.01	0.39	0.01
18:4 n-3	4.06	0.14	3.54	0.04	3.76	0.14	3.75	0.09	3.88	0.10
20:1 n-9	2.42	0.12	2.51	0.01	2.30	0.02	2.88	0.03	2.49	0.26
20:1 n-7	0.92	0.08	0.84	0.00	0.79	0.01	0.98	0.07	1.09	0.04
20:2 delta 5,11	1.08	0.12	0.45	0.08	1.00	0.02	1.35	0.11	1.25	0.05
20:2 delta 5,13	0.53	0.26	0.00	0.00	0.23	0.01	0.39	0.03	0.36	0.06
20:2 n-6	0.51	0.15	0.55	0.01	0.39	0.01	0.33	0.29	0.40	0.16
20:4 n-6	0.82	0.03	0.87	0.37	0.91	0.04	0.83	0.08	0.80	0.02
20:4 n-3	0.94	0.02	1.12	0.03	0.98	0.04	0.99	0.05	1.12	0.09
20:5 n-3	20.51	0.64	22.20	0.62	21.56	1.24	18.26	0.11	18.71	0.82
22:2 delta 7,13	1.02	0.03	0.52	0.01	0.91	0.02	1.20	0.00	0.00	0.00
22:2 delta 7,15	0.42	0.03	0.32	0.27	0.50	0.04	0.51	0.03	0.54	0.04
22:5 n-3	0.75	0.03	0.71	0.08	0.78	0.03	0.73	1.26	10.17	0.54
22:6 n-3	11.05 [abcd]	0.32	11.69 [b]	0.33	10.42 [c]	0.76	10.08 [d]	3.13	8.06 [e]	1.16
Σ total saturated	20.38 [a]	0.74	23.58 [bcde]	0.10	23.29 [cde]	0.84	21.79 [de]	0.60	22.49 [e]	0.70
total monounsaturated	22.11 [abcd]	1.08	21.70 [bcd]	0.27	23.85 [cd]	0.62	23.42 [de]	0.44	24.47 [e]	1.31
Total polyunsaturated	45.74 [a]	2.01	45.79 [a]	2.13	45.51 [a]	2.43	42.88 [a]	5.31	49.69 [a]	3.80
Bacterial	2.45 [a]	0.30	2.30 [a]	0.24	2.63 [a]	0.18	2.58 [a]	0.09	2.55 [a]	0.10

Means with different superscripts is significantly different ($p < 0.05$) from each other.

Appendix 6.1 Fatty acid composition (%) of blue mussels treated with various types of ascorbic acid and stored on ice (0 day control). (mean and S.D., n=3).

	Control		ACS		Ultra		NaAs	
Fatty acid	%	SD	%	SD	%	SD	%	SD
14:0	2.92	0.10	2.75	0.02	2.67	0.09	2.61	0.03
*i*15:0	0.52	0.02	0.48	0.01	0.24	0.22	0.46	0.00
*ai*15:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15:0	0.25	0.05	0.26	0.01	0.21	0.08	0.15	0.13
*i*16:0	0.11	0.10	0.16	0.01	0.30	0.19	0.18	0.04
*ai*16:0	0.05	0.09	0.11	0.03	0.04	0.06	0.05	0.09
16:0	17.70[acd]	0.43	15.71[b]	0.06	16.41[cd]	0.85	16.79[d]	0.18
16:1 n-7	8.82[acd]	0.30	9.74[b]	0.05	8.28[c]	0.33	8.34[d]	0.10
16:1 n5	0.32	0.01	0.32	0.05	0.30	0.03	0.32	0.05
*i*17:0	0.80	0.03	0.60	0.10	0.78	0.01	0.75	0.01
*ai*17:0	0.34	0.17	0.18	0.10	0.36	0.20	0.37	0..20
16:2 n-4	0.52	0.07	0.63	0.08	0.58	0.08	0.50	0.10
17:0	0.74	0.02	0.55	0.20	0.71	0.02	0.70	0.01
16:3 n-4	2.78	0.58	2.57	0.02	2.62	0.08	3.22	0.11
16:4 n-3	0.33	0.01	0.24	0.01	0.35	0.02	0.31	0.01
16:4 n-1	0.08	0.14	0.20	0.00	0.19	0.17	0.17	0.15
18:0	3.70	0.26	2.42	0.01	3.60	0.14	3.59	0.04
18:1 n-9	0.35	0.04	0.38	0.02	0.35	0.01	0.34	0.04
18:1 n-7	1.76	0.06	1.90	0.01	1.84	0.06	1.81	0.04
18:1 n-6	3.12	0.15	2.85	0.02	2.38	0.72	2.95	0.01
18:1 n-5	0.40	0.06	0.42	0.04	0.44	0.01	0.40	0.04
18:2 n-6	1.54	0.07	1.66	0.01	1.53	0.03	1.57	0.04
18:2 n-4	0.29	0.03	0.42	0.01	0.36	0.03	0.34	0.01
18:3 n-6	0.36	0.07	0.29	0.24	0.44	0.01	0.45	0.05
18:3 n-3	1.06	0.01	1.13	0.01	1.06	0.01	1.10	0.03
18:4 n-3	3.23	0.19	3.84	0.06	3.06	0.10	3.54	0.05
18:4 n-1	0.37	0.01	0.59	0.01	0.31	0.05	0.38	0.05
20:0	1.00	0.10	1.09	0.02	1.01	0.03	1.00	0.01
20:1 n-9	3.21	0.14	2.82	0.05	3.17	0.11	3.11	0.10
20:1 n-7	1.32	0.08	1.32	0.07	1.34	0.01	1.27	0.01
20:2 delta 5,11	1.90	0.12	2.00	0.10	2.01	0.01	1.93	0.02
20:2 delta 5,13	0.47	0.03	0.65	0.09	0.57	0.07	0.50	0.05
20:2 n-6	0.58	0.05	0.89	0.06	0.67	0.03	0.60	0.02
20:3 n-3	0.40	0.04	0.44	0.05	0.41	0.02	0.41	0.06
20:4 n-6	1.31	0.04	1.24	0.01	1.33	0.05	1.35	0.03
20:4 n-3	0.12	0.20	0.42	0.20	0.22	0.19	0.23	0.20
20:5 n-3	17.55[a]	0.13	18.06[a]	0.41	17.34[a]	0.74	18.63[a]	0.30
22:2 delta 7,13	1.95	0.13	2.09	0.01	1.97	0.07	1.97	0.01
22:2 delta 7,15	0.14	0.24	0.46	0.01	0.34	0.29	0.44	0.03
21:5 n-3	0.23	0.40	0.66	0.11	0.52	0.51	0.19	0.33
22:5 n-6	0.23	0.10	0.19	0.11	0.22	0.19	0.35	0.16
22:5 n-3	1.10	0.10	1.09	0.10	1.07	0.04	0.99	0.12
22:6 n-3	13.34	0.36	12.84	0.68	13.50	0.63	14.33	0.32
Σ								
Saturated	24.57[a]	0.84	22.23[a]	0.12	23.9[a]	1.19	24.14[a]	0.39
Monounsaturated	19.3[a]	0.98	19.75[a]	0.31	18.1[a]	1.42	18.54[a]	0.41
Polyunsaturated	49.65[a]	2.87	52.97[a]	1.95	50.67[a]	3.2	53.5[a]	1.81

Values are mean of three determinations with the standard deviations (SD). ACS and Ultra; ACS grade and Ultra pure ascorbic acid; NaAs, sodium ascorbate.

Appendix 6.2 Fatty acid composition (%) of blue mussels treated with various types of ascorbic acid and stored on ice for 5 days (mean and S.D., n=3).

	Control		ACS		Ultra		NaAs	
Fatty acid	%	SD	%	SD	%	SD	%	SD
14:0	2.92	0.08	2.79	0.05	2.67	0.06	2.73	0.03
*i*15:0	0.52	0.01	0.49	0.01	0.50	0.02	0.48	0.03
*ai*15:0	0.08	0.001	0.09	0.01	0.06	0.02	0.08	0.14
15:0	0.25	0.05	0.26	0.01	0.19	0.02	0.29	0.16
*i*16:0	0.12	0.10	0.15	0.02	0.17	0.02	0.14	0.01
*ai*16:0	0.05	0.09	0.00	0.00	0.03	0.06	0.14	0.01
16:0	17.73 [acd]	0.45	15.88 [b]	0.37	16.32 [a]	0.51	16.43 [a]	0.20
16:1 n-7	8.74 [acd]	0.19	9.92 [b]	0.18	8.30 [c]	0.21	8.19 [d]	0.05
16:1 n5	0.32	0.03	0.36	0.01	0.30	0.01	0.31	0.00
*i*17:0	0.80	0.03	0.61	0.02	0.76	0.02	0.72	0.01
*ai*17:0	0.34	0.16	0.25	0.11	0.39	0.01	0.30	0.13
16:2 n-4	0.52	0.08	0.64	0.08	0.56	0.07	0.49	0.03
17:0	0.74	0.04	0.42	0.25	0.71	0.02	0.68	0.01
16:3 n-4	2.99	0.79	2.57	0.03	2.32	0.05	3.04	0.06
16:4 n-3	0.33	0.03	0.25	0.00	0.34	0.02	0.32	0.00
16:4 n-1	0.09	0.15	0.14	0.13	0.25	0.04	0.23	0.00
18:0	3.71	0.38	2.46	0.06	3.69	0.09	3.48	0.04
18:1 n-9	0.35	0.06	0.37	0.01	0.34	0.02	0.36	0.01
18:1 n-7	1.76	0.13	1.92	0.03	1.70	0.04	1.67	0.02
18:1 n-6	3.13	0.12	2.88	0.05	2.83	0.06	2.95	0.02
18:1 n-5	0.41	0.08	0.40	0.00	0.43	0.01	0.41	0.02
18:2 n-6	1.55	0.16	1.68	0.02	1.51	0.02	1.56	0.03
18:2 n-4	0.29	0.05	0.42	0.06	0.33	0.05	0.32	0.04
18:3 n-6	0.37	0.08	0.03	0.07	0.38	0.05	0.41	0.05
18:3 n-4	0.09	0.06	0.05	0.08	0.09	0.01	0.06	0.06
18:4 n-3	3.24	0.27	3.93	0.05	2.99	0.05	3.41	0.04
18:4 n-1	0.37	0.03	0.60	0.01	0.34	0.01	0.38	0.01
20:0	0.00	0.00	1.09	0.03	0.99	0.03	0.91	0.01
20:1 n-9	3.22	0.27	2.79	0.06	3.15	0.07	3.04	0.03
20:1 n-7	1.32	0.08	1.27	0.03	1.34	0.04	1.21	0.01
20:2 delta 5,11	1.90	0.17	1.92	0.03	1.95	0.06	1.81	0.02
20:2 delta 5,13	0.47	0.05	0.54	0.01	0.52	0.08	0.46	0.02
20:2 n-6	0.58	0.07	0.84	0.01	0.61	0.05	0.56	0.02
20:3 n-3	0.41	0.05	0.45	0.02	0.42	0.01	0.39	0.00
20:4 n-6	1.32	0.07	1.26	0.03	1.34	0.03	1.31	0.03
20:4 n-3	0.12	0.21	0.43	0.01	0.34	0.01	0.35	0.00
20:5 n-3	17.40 [a]	0.93	18.48 [a]	0.13	17.45 [a]	0.47	18.09 [a]	0.24
22:2 delta 7,13	1.96	0.18	1.76	0.57	1.98	0.04	1.85	0.02
22:2 delta 7,15	0.15	0.25	0.11	0.19	0.45	0.01	0.44	0.03
21:5 n-3	0.24	0.42	1.32	0.69	0.00	0.00	0.50	0.08
22:5 n-6	0.30	0.09	0.35	0.08	0.29	0.06	0.22	0.13
22:5 n-3	1.10	0.04	1.06	0.01	1.17	0.10	1.13	0.06
22:6 n-3	13.73	0.19	13.15	0.16	13.60	0.42	13.61	0.27
Σ								
saturated	24.61 [a]	0.96	22.48 [a]	0.52	23.86 [a]	0.69	23.84 [a]	0.44
monounsaturated	19.25 [a]	1.11	19.91 [a]	0.5	18.39 [a]	0.5	18.14 [a]	0.14
polyunsaturated	50.19 [a]	4.16	52.69 [a]	2.05	50.18 [a]	1.67	51.95 [a]	0.95

Values are mean of three determinations with the standard deviations (SD). ACS and Ultra; ACS grade and Ultra pure ascorbic acid; NaAs, sodium ascorbate.

Appendix 6.3 Fatty acid composition (%) of blue mussels treated with various types of ascorbic acid and stored on ice for 10 days (mean and S.D., n=3).

	Control		ACS		Ultra		NaAs	
Fatty acid	%	SD	%	SD	%	SD	%	SD
14:0	2.91	0.29	2.59	0.16	2.86	0.02	2.49	0.07
*i*15:0	0.54	0.02	0.46	0.03	0.51	0.00	0.31	0.27
*ai*15:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15:0	0.26	0.06	0.18	0.01	0.28	0.00	0.29	0.13
*i*16:0	0.12	0.10	0.00	0.00	0.17	0.00	0.06	0.10
*ai*16:0	0.05	0.09	0.00	0.00	0.07	0.06	0.03	0.05
16:0	17.70[a]	0.74	15.66[a]	1.04	16.89[a]	0.16	16.60[a]	0.48
16:1 n-7	8.84[a]	0.42	9.74[a]	0.64	8.53[a]	0.07	8.32[a]	0.21
16:1 n5	0.33	0.02	0.34	0.02	0.29	0.03	0.22	0.19
*i*17:0	0.82	0.00	0.60	0.05	0.75	0.01	0.76	0.01
*ai*17:0	0.35	0.16	0.53	0.22	0.33	0.13	0.42	0.07
16:2 n-4	0.53	0.06	0.56	0.05	0.44	0.01	0.56	0.08
17:0	0.76	0.01	0.20	0.34	0.68	0.01	0.70	0.01
16:3 n-4	2.75	0.60	2.52	0.13	3.29	0.06	2.80	0.06
16:4 n-3	0.34	0.02	0.08	0.14	0.31	0.04	0.31	0.01
16:4 n-1	0.09	0.15	0.06	0.10	0.24	0.05	0.08	0.14
18:0	3.81	0.24	2.47	0.16	3.27	0.04	3.72	0.10
18:1 n-9	0.36	0.05	0.36	0.02	0.32	0.01	0.23	0.20
18:1 n-7	1.81	0.06	1.92	0.12	1.65	0.02	2.36	0.05
18:1 n-6	3.22	0.17	2.87	0.18	3.19	0.03	3.04	0.06
18:1 n-5	0.41	0.07	0.41	0.03	0.40	0.01	0.39	0.04
18:2 n-6	1.59	0.11	1.69	0.13	1.48	0.01	1.45	0.01
18:2 n-4	0.30	0.04	0.38	0.01	0.32	0.06	0.34	0.02
18:3 n-6	0.38	0.08	0.55	0.03	0.36	0.05	0.45	0.04
18:3 n-3	1.09	0.05	1.14	0.07	1.04	0.01	1.10	0.03
18:4 n-3	3.33	0.29	3.86	0.22	3.33	0.04	3.43	0.10
18:4 n-1	0.38	0.03	0.58	0.03	0.35	0.01	0.37	0.02
20:0	0.00	0.00	1.10	0.07	0.93	0.03	1.00	0.05
20:1 n-9	3.31	0.15	2.82	0.19	2.91	0.02	3.14	0.09
20:1 n-7	1.36	0.04	1.29	0.09	1.31	0.09	1.29	0.03
20:2 delta 5,11	1.95	0.10	1.44	0.76	1.77	0.09	1.89	0.05
20:2 delta 5,13	0.48	0.03	0.65	0.20	0.47	0.10	0.50	0.02
20:2 n-6	0.60	0.05	0.97	0.28	0.55	0.05	0.62	0.02
20:3 n-3	0.42	0.04	0.45	0.03	0.35	0.00	0.27	0.24
20:4 n-6	1.35	0.02	1.24	0.07	1.36	0.05	1.31	0.03
20:4 n-3	0.12	0.22	0.13	0.23	0.32	0.00	0.12	0.20
20:5 n-3	17.46[a]	0.46	18.34[a]	1.16	17.81[a]	0.30	18.38[a]	0.46
22:2 delta 7,13	2.01	0.11	2.12	0.12	1.76	0.01	1.93	0.02
22:2 delta 7,15	0.15	0.26	0.70	0.38	0.61	0.37	0.90	0.55
21:5 n-3	0.25	0.43	0.21	0.36	0.40	0.35	0.18	0.32
22:5 n-6	0.00	0.00	0.00	0.00	0.27	0.00	0.08	0.14
22:5 n-3	1.13	0.09	0.62	0.54	1.03	0.02	1.22	0.46
22:6 n-3	13.55	0.24	13.08	0.86	13.42	0.32	14.48	0.92
Σ								
saturated	24.68[a]	1.33	22.00[a]	1.44	24.23[a]	0.25	24.1[a]	0.83
monounsaturated	19.64[a]	1.13	19.75[a]	1.39	18.6[a]	0.3	18.99[a]	1.01
polyunsaturated	50.25[a]	3.3	51.37[a]	5.77	51.28[a]	1.9	52.77[a]	3.78

Values are mean of three determinations with the standard deviations (SD). ACS and Ultra; ACS grade and Ultra pure ascorbic acid; NaAs, sodium ascorbate.