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Stability of Shrimp Processing Waters during Refrigerated Storage as a Basis for the Isolation of High Value Compounds

Master's Thesis in the Master Degree Programme, Biotechnology

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THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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Front picture present the two shrimp process waters examined during this project. Main water in the upper jar and steam water in the lower jar.

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Abstract

Huge amounts of water, containing high concentrations of high value compounds today leave the food chain during industrial processing. To stop this waste of high value compounds the water should be cleaned and the molecules recycled back to the food chain. To be able to succeed with this, it is of high importance to know; which high value compounds the waters contain, how to separate the compounds out from the waters and how long these waters can be stored before biochemical changes occurs.

In this project, the storage stability of water from a boiling and peeling process of shrimps was investigated. Two types of shrimp process water, from the local company Räkor & Laxgrossisten AB, were stored in a cold room, for 19 days, at a temperature of 4°C. The two process waters examined were steam water taken from the shrimp boiling machine, and so called “main water”, which is a mixture of steam water and peeling water taken at the end of the process line. Two separate storage trials were carried out of each process waters. In order to monitor the quality and stability of the waters during cold storage (4°C) several parameters were examined in the waters; pH, total protein content, ionic strength and color, but also break down components from e.g. lipids and proteins, like primary and secondary products formed during lipid oxidation as well as total volatile nitrogen and odor.

Large differences were found between the steam water and the main water in terms of protein concentration and stability over time. The average initial protein concentration for the steam water was found to be 15mg/ml. Its pH was stable around 9 with a small decrease during the last days of storage. The peroxide value (PV) increased from day 0-8 and then decreased from day 9-18. The malondialdehyde (MDA) concentration was stable during the whole storage period; in average 0.7µmol/L. For the olfactory sensory evaluation no significant change could be found during the first 10 day of storage, which was the same for the TVB-N test, but for the two last taken samples (at 13 and 18 days) clear changes in smell and TVB-N value were found. Color and ionic strength did not change significantly over time. The initial pH of the main water was in average 8.58, but at day 18 the pH decreased to 7.20. The protein concentration for the main water was stable for the first 10 days, with an average protein concentration around 1.5mg/ml. The PV increased from day 0-4 and then decreased from day 4-18. The MDA concentrations increased between 4 and 13 days of storage, but were still low even after 18 days of storage, around 2.5µmol/L. Between day 5 to 18 the TVB-N value increased almost linearly and strong odor changes took place between day 4-8, according to the sensory test. Color did not change significantly, while ionic strength, like TVB-N, changed almost linearly from day 4-18.

Overall, the steam water was remarkably stable for all parameters tested during the first 10 days of cold storage, and some parameters like MDA and total protein content, were even stable beyond 18 days of cold storage. The main water was less stable with respect to all parameters tested, probably due to higher microbial growth and less astaxanthin.

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1 Introduction

The introduction will start with a background, to why this project is important and how it can change the marine industry compared to how it looks today. Further down in the introduction an explanation of the NoVAqua project, which this project is a part of, will be given, followed by the aim and the questions that were to be answered during the project. At the end of the introduction a short session about restrictions and limitations of the investigation will be mentioned.

1.1 Background

Access to food is today a big problem in many places around the world, which give rise to both nutritional problems and starvation, causing death of millions of children and adults every year (Wfp.org, 2015). At the same time, people in the industrial part of the world, produce large quantities' of food, because of high consumption and high technology equipment. One example of an over-used food source is fish and certain crustaceans. Overfishing, i.e. that more fish is caught than reproduced, can lead to devastating consequences for the fish fauna and also to people on the cost lines, where fish is a main source of nourishment and economical income, if this overfishing problem does not start to reduce (Owen, 2005; Nationalencyklopedin, 2016f).

To solve this overfishing problem, fish farming methods were developed to help the oceans to regenerate the fish population and to withstand increased consumption of fish because of increased human population on earth (Lucas, 2015). Fish farming has increased a lot the last decades, and Norway together with China are two examples, with their large fish farms (Brown, 1983; Lucas, 2015). During the last decade scientists has risen the question that fish farming may not be as environmentally friendly as people in general think, but it is more preferable than to stop eating fish or loose several fishing spices in a near future (Stauth, 1998; Khater et al, 2014). Additionally, it provides a more sustainable economy for some countries and can increase the nutritional intake for the population in developing countries near the coastline (Khater et al, 2014).To be able to feed farmed fish, pelleted formulated feed has been developed. These feed are often exported over large distances and are not friendly for the water surrounding the fish farm, since high nitrogen amount can be formed from uneaten feed (Piedecausa, 2010). The feed pellets can contain e.g. fish meal and fish oil from small pelagic fish species as well as soybean and yellow corn, but to reduce the environmental impact, more research must be done within the fish farming area, and particularly on the feed used (Stauth, 1998; Pickova et al, 2007; Khater et al, 2014). Today scientists and industry really want to find a way to add value to the highly nutritious side flows emerging from the seafood industry, which may lead to more nutritious and more sustainable feed sources in future fish farming (Osman et al, 2015).

Herring process water could be one interesting area for the fish feed industry, since it contains large amounts of high value compounds, as proteins, fatty acids and antioxidants, but nowadays it either goes directly out with the drain or with help from chemical flocculants becomes sludge for biogas production (Gringer et al, 2015). During the last years some articles have been published dealing with different methods on how to isolate high value compounds from herring process water in an effective way (Gringer et al, 2015; Osman et al, 2015). This could lead to (Fjerbaek Sotoft et al, 2015):

- Better utilization of high value compound from the marine area
- A reduction of the environmental impact from fish processing, since less nutrients are discarded
- More environmental friendly and natural feed to the fish farmers

The research, previously conducted, to take care of as much compounds as possible in herring process water, can also be applied to the shrimp process water. In shrimp industries, where shrimps are boiled and peeled, large amounts of high value compounds leave the shrimps and follow the water out in the drain (Pérez-Santín et al, 2013). These compounds, e.g. proteins, lipids, small peptides and antioxidants, are valuable both for the company that can earn money on it and from an environmental perspective, since the nutrients can be used in different areas, like aquaculture. Various compounds may also in a longer perspective be isolated and used as ingredients in food for human consumption (Pérez-Santín et al, 2013).

1.1.1 The NoVAqua Project

NoVAqua (Extracting Novel Values from Aqueous Seafood Side Steams) is a project between the companies Räkor & Laxgrossisten AB, Fisk Idag AB, Bio-Aqua AB and Skretting as well as the academic institutions Chalmers University of Technology and DTU (Danmarks Tekniske Universitet), where Chalmers coordinates the project and perform large parts of the research. Räkor & Laxgrossisten AB stands for shrimp waters and hosts pilot scale trials during which the flotation technique of Bio-Aqua is tested. DTU in Denmark, perform e.g. volatile and amino acid analyses, and Fisk Idag are involved in the evaluation of process waters as stabilizers for frozen food.

Räkor & Laxgrossisten AB (Sannäs) is a company located at Hisingen in Gothenburg, Sweden. It is the only factory in Sweden, that peels and boils shrimps and the company has a large variety of products out on the market. Some of the products produced are brined shrimps, “Räkröra” and “Skagenröra”. Other non-shrimp based products are also produced like mayonnaise, potato salad and beetroot salad.

Fisk Idag AB is a company located with their office in the harbour of Gothenburg, Sweden, and their production on Öckerö, which is an island in the archipelago of Gothenburg. Fisk Idag is a supplier of fish and seafood, which has been stablished since 1992. Majority of the products are produced in the factory on Öckerö. Here are fish boiled, fried, steamed and frozen after wishes from customers. Also different kinds of seafood salads, sauces and stocks are produced by the factory of Fisk Idag.

The main task of the NoVAqua project is to evaluate the composition of two types of waters from the shrimp peeling process, and try to find a method with great isolation capacity for high value compounds. The waters to examine are steam and main process water. The steam water is the water from the shrimp boiling machine. This water has a strong pink/orange color and a strong aroma/smell. The main water is a mixture of both steaming and peeling waters collected at the end of the process line, but without the peel, which is filtered away by a 0.1mm filter. This water is light pink and has a lighter aroma/smell.

The results from the NoVAqua project may, for Räkor & Laxgrossisten AB, lead to advantages from both an environmental and economical point of view, since the shrimp process water can be clean before going out with the drain. High value compounds can also be isolated from the process water and sold to companies within different industrial areas, like the aquafeed sector. Furthermore, the shrimp process water itself can be reused e.g. to stabilize frozen food.

The project described in this report is a subproject to NoVAqua, and it involves stability testing of both “main water” and “steam water” from shrimp processing, with focus on break down compounds. This is particularly important to evaluate if end-uses are within the food industry. During this research several methods will be used to look at lipid oxidation, total volatile basic nitrogen (TVB-N), total protein content, pH, ionic strength and color change over time.

1.2 Aim

Since the shrimp process waters contain a number of different compounds that can have impact on the storage stability, it is of high interest to measure biochemical changes taking place over time prior to further separation of compounds like proteins, lipids and antioxidants. The aim of this project was therefore to look at the storage stability of shrimp process waters, during up to 18 days, at refrigerated temperature.

In this stability monitoring of main and steam shrimp process waters, the more specific questions to be answered were: how protein concentration, lipid oxidation, TVB-N, pH, ionic strength, odor and color, change over time at 4°C.

1.3 Restrictions and Limitations

Numerous different compounds have impact on the stability of the shrimp process waters and due to this several methods and experiments should be performed to evaluate as many compounds as possible. But since this research project has a time limit, only the stability of some compounds will be tested (lipids and proteins) with the development of lipid oxidation and TVB-N as main quality indexes, which can be seen as a limitation of this project.

Another parameter that would have been interesting to examine is the ratio between diameter and height of the container used for storage of the shrimp water, since different ratios may have different impact of some break down compounds, like lipid oxidation. If the ratio (diameter/height) is high, oxygen may have a more operative role in the creation of break down compounds compared with a small ratio. In this study the container size is not included as a variable in the research; however storage of the different shrimp water batches were done in identically shaped containers.

To be able to get an overall picture of the stability of main and steam shrimp process waters, tests would be done on the waters every month for a year, but for this project only two batches of each waters could fit into the time limitations given.

Samples taken over time from the containers were subsequently stored in a -80 freezer, to allow them to be analyzed at the same time. This may be a limitation if some of the compounds change at -

80°C compared to direct analysis. However, most biochemical reactions are known to be very slow or absent at this temperature.

2 Theory

The theory is divided into five sections. In the first section an overall description of shrimps and more detailed information about the shrimp *Pandalus borealis,* can be found. A description of the boiling and peeling process of shrimps at Räkor & Laxgrossisten AB are available in the second section. In the third part of the theory, background information about two of the most important macromolecules (lipids and proteins) is given, together with some fact about lipid oxidation and protein degradation. In the last two parts of the theory, information about all equipment and methods used in this project can be found.

2.1 Shrimp

Shrimps belong to the group crustacean, and can be found in various sizes and appearances. The length can be from a few millimeters up to 20cm, but the average length is around 8cm. The shrimp is a close relative to e.g. crabs and lobsters, and are today present in waters all around the world. It can be found in all oceans, but also in fresh water lakes and streams. In Scandinavia about 45 different spices can be found, one lives in fresh water and the rest in marine environment. (Nationalencyklopedin, 2016a; Jacobsson et al, 2010)

This crustacean is a special kind of hermaphrodite, since it lives as a male the first two years and is then transformed to be a female for the rest of its life (Jacobsson et al, 2010). The female shrimp produce 1500-14000 eggs (Jacobsson et al, 2010; Britannica Academic, 2016). Shrimp mostly consume smaller animals and plants, and several shrimp species are important food sources for human consumption in the world. Some common shrimp species are *Crangon vulgaris* (Sand shrimp)*, Crangon vulgari, Peneus setiferus* and *Pandalus borealis* (Pink shrimp) (Britannica Academic, 2016; Jacobsson et al, 2010; FAO, 2016).

2.1.1 *Pandalus borealis*

Pandalus borealis (Pink shrimp) is one of the most important shrimps in the North Atlantic, and in Europe it can be found outside countries like Sweden, Norway, the Nederlands, Germany, Great Britain and Greenland (FAO, 2016). *Pandalus borealis* can also be found and caught on both the east and west coast of North America (FAO, 2016). It is fished at a deep from 20m down to 1330m and it prefers a 3% salinity of the water and temperature between 1-8°C (FAO, 2016; Jacobsson et al, 2010). In raw state *Pandalus borealis* is transparent with a light pink color, but when boiled it appears as pink. The pink color of *Pandalus borealis* in boiled state is stronger the deeper it is caught (Jacobsson et al, 2010).

Pandalus borealis is the species people in Sweden and other countries in Scandinavia talks about when use the word shrimp. In Swedish it is named “Nordhavsräka” and it can be captured at 50-500m deep in both Kattegat and Skagerrak at the Swedish west coast (Jacobsson et al, 2010).

2.1.1.1 Nutritional Content of Pandalus borealis

The nutritional content of *Pandalus borealis* can be seen in Table 1. The shrimp is high in proteins and minerals, and is at the same time low in fat and contains no carbohydrates. The fat is mostly in

either monounsaturated or polyunsaturated form, but saturated form can also be found. The vitamins identified in higher amounts in *Pandalus borealis* are Vitamin E, Vitamin B12 and folate, and minerals like calcium, iodine, magnesium, phosphor, potassium and sodium are just some minerals found in the shrimp meat. (Livsmedelsverket, 2016a)

Table 1. Nutritional content per 100g of *Pandalus borealis* in raw state (Livsmedelsverket, 2016a).

Nutrient (unit)	Per 100g of Raw Shrimps
Energy (kcal)	77.3
Energy (kJ)	323.4
Carbohydrates (g)	0
Fat (g)	0.64
Protein (g)	17.63
Fiber (g)	0
Water (g)	78.7
Ash (g)	2.2
Saturated fatty acids (g)	0.09
Fatty acid 14:0 (g)	0.01
Fatty acid 16:0 (g)	0.07
Fatty acid 18:0 (g)	0.01
Monounsaturated Fatty acid (g)	0.16
Fatty acid 16:1 (g)	0.04
Fatty acid 18:1 (g)	0.09
Polyunsaturated Fatty acid (g)	0.18
Fatty acid 18:2 (g)	0.01
Fatty acid 20:4 (g)	0.01
EPA (Fatty acid 20:5) (g)	0.09
DHA (Fatty acid 22:6) (g)	0.06
Cholesterol (mg)	147
Vitamin E (mg)	3.93
Thiamine (mg)	0.04
Niacin (mg)	0.99
Niacin equivalents (mg)	4.2
Vitamin B6 (mg)	0.04
Vitamin B12 (µg)	3.52
Folate (µg)	14
Phosphorus (mg)	147
Iodine (µg)	20
Iron (mg)	0.22
Calcium (mg)	21
Potassium (mg)	88
Magnesium (mg)	37
Sodium (mg)	630
Salt (g)	1.58

Selenium (µg)	23
Zink (mg)	1.12
Waste (Peelings etc.) (%)	62

2.2 Boiling and Peeling Process of Shrimps at Räkor & Laxgrossisten AB

Räkor & Laxgrossisten AB is the only factory in Sweden that both boils and peels shrimps. This boiling and peeling process of shrimps is normally performed every Thursday and Friday, and the amount processed depends on the ordering for the week. In average around 12 tons of shrimps are boiled and peeled every processing day. For this process a large amount of water is needed.

The process, which can be seen in Figure 1, starts with fresh shrimps on ice coming to the factory. The shrimps (*Pandalus borealis*) are then placed on a band that first goes to the boiler, for boiling in water. Later on the shrimps leave the boiler at a temperature around 90°C and go for peeling. On the way to the peeling area the shrimps are also cooled down by water. High temperature boiling water, called steam water, also leaves the boiler. Every hour around $1m^3$ of steam water leaves the boiling tank.

In the peeling area, the shrimps are placed and shacked between rubber rollers and exposed for large amounts of water. The peels detach and leave the shrimp through holes in the rubber roller plate. The peeled shrimp then continue on the band, where fans and color laser reject peels and recognize shrimps with aberrant color. At the end of the band, workers go through the shrimps a last time, before continuing to the food product production. For the peeling process around $30m^3/h$ of water is used. After use, the main process water is mixed with the steam water, from the boiling tank, and filtered through a 0.1mm filter to be separated from the peels, and then it's passed out with the drain after been treated and cleaned at the factory. The peels are gathered and transported away for biogas production.

The steam and main process water can be seen on the front page, were the steam water has a strong orange color that sometime can go to a red or even purple color tone, and it has a strong aroma/smell of boiled shellfish. The main water color is more light pink, but can also, depending on season, have a more yellow or brownish color tone, and it has a much lighter aroma/smell, compared to the steam water, of seafood in fresh state.

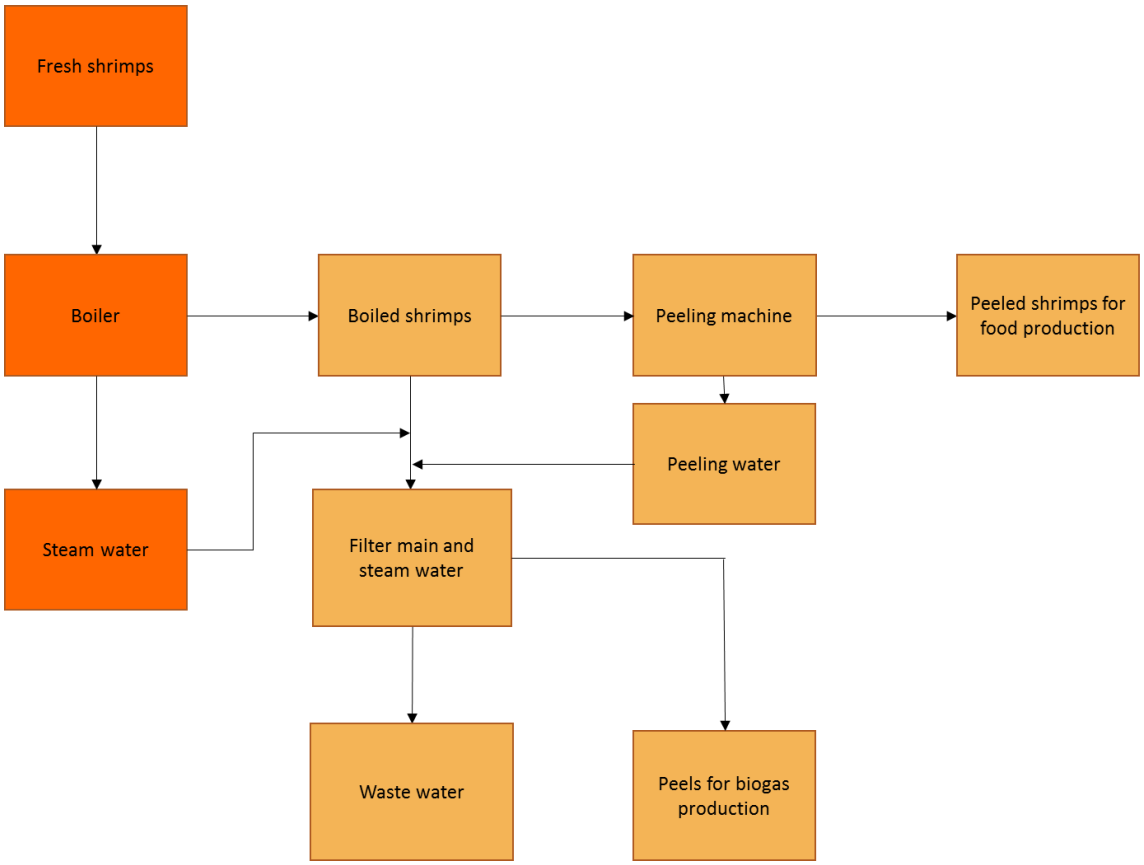

Figure 1. Schematic drawing of the boiling and peeling process of shrimps at Räkor & Laxgrossisten AB.

2.3 Proteins, Lipids and their Degradation

According to livsmedelsverket, shrimps contain around 17.5% proteins, and also some fat (around 8.5%). These macromolecules can over time be changed and be involved in formation of break down compounds, which can lead to sensory spoilage of food, but also to decreased nutritional value and even toxicity (Etienne, 2005; Fernández et al, 1997).

2.3.1 Lipids

Lipids are a wide group of molecules, insoluble in water but soluble in non-polar solvents like chloroform, diethyl ether and hexane (Coultate, 2009). Substances included in the lipid group are e.g. free fatty acids, triglycerides, steroids, glycolipids, esters, phospholipids and carotenoids (Alberts et al, 2008).

2.3.1.1 Lipid Composition and Structure

The basic building block of many lipids is the fatty acid, which structure can be seen in Figure 2. It has a carboxyl head and a carbon tail where the length and amount of double bonds can vary a lot (Andersson, 2015; Coultate, 2009). Lipids usually consist of one or more carbon chain tail bound to a head group, but this is not always the case (Alberts et al, 2008). For the steroids, multiple carbon rings forms the structure, like for the cell-membrane-lipid cholesterol or the male steroid hormone testosterone, which both have a four-ring steroid structure (Alberts et al, 2008). It is the carbon chain of the lipid, which makes the substance insoluble in polar solvents, e.g. water (Coultate, 2009).

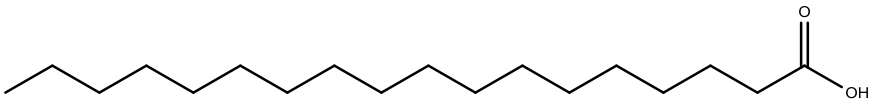

Figure 2. The figure visualizes the basic structure of a fatty acid.

When people out on the street talks about lipids the word fat is normally used. Fat or oil, is another word for triglycerides (Figure 3a), which consists of three fatty acids linked to a glycerol molecule by ester bonds (Alberts et al, 2008; Andersson 2015). The fatty acid, bound to the glycerol, can vary a lot, since hundreds of different fatty acids today exist (Alberts et al, 2008). Some examples of common fatty acids are palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1) (Andersson, 2015; Coultate, 2009). The description (16:0) for palmitic acid defines a fatty acid with 16 carbons and zero double bonds, and the (18:1) fatty acid has 18 carbons and 1 double bond (Coultate, 2009). The position of the double bond is often shown by the number "n", and this number is the sum of the carbons counted from the carboxyl group to the first carbon within the double bond (Andersson, 2015). Fatty acids can be divided in different groups due to the size of the carbon chain or the number of double bonds. If the fatty acid only has single bonds between the carbons in the chain, it is called a saturated fatty acid, Figure 3c. If one or more double bonds instead occur in the carbon chain, the fatty acid belongs to the monounsaturated or the polyunsaturated fatty acid group respectively (Alberts et al, 2008), as can be visualized in Figure 3b. For those lipids having one or more double bonds, the position of the hydrogen surrounding the bond can be described by addition of a cis or a trans in the name (Nationalencyklopedin, 2016b; Coultate, 2009). The lipid is called trans if the hydrogens of the carbons attached to the double bond are located in opposite direction (Coultate, 2009), see lower structure in Figure 3b. The lipid is instead called cis if the hydrogens are located in the same direction from the double bonded carbons (Coultate, 2009), as can be seen in the upper structure of Figure 3b.

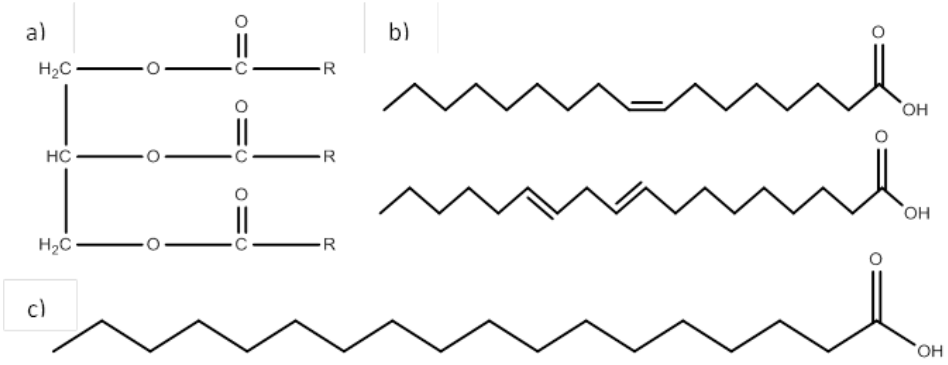

Figure 3. a) define the structure of a triglyceride. R is a fatty acid connected to the glycerol with an ester bond. In b) the structure of a cis unsaturated fatty acid (upper right corner) and a trans polyunsaturated fatty acid (middle structure on the right side) are shown. c) describe the basic structure of a saturated fatty acid.

The most important lipid in the cell is the phospholipid, since it is involved in the construction of the cell membrane. The phospholipid forms a double layer membrane with their hydrophilic parts pointing out and their hydrophobic parts pointing in. The structure of a phospholipid is similar to a triglyceride, but here two non-polar carbon chains are bound to a glycerol, instead of three, and a

head consisting of a phosphate and a polar group is also available. The polar/hydrophilic group can be e.g. an alcohol or choline. (Alberts et al, 2008; Coultate, 2009)

2.3.1.2 Lipids as Essential Components in the Human Diet

In human nutrition, lipids, in terms of triglycerides, are necessary as energy source, but over consumption could lead to obesity, i.e. excess storage as adipose tissue in the body (Coultate, 2009). Lipids are likewise essential as a signaling molecule and for formation of phospholipid-double-layer membrane, separating the inside from the outside of the cell (Alberts et al, 2008). Studies on rats eating a total fat-free feed have also declared a wide range of acute symptoms affecting skin, vascular system and reproductive system (Coultate, 2009).

Humans have the ability to synthesis saturated lipids with up to 18 carbons in the chain length, and unsaturated fatty acids with a double bond between carbon 9 and 10 (Coultate, 2009). Higher or more complex unsaturated fatty acids, like alpha-linoleic acid (ALA) and linolenic acid, must therefore be obtained through the food (Spickett et al, 2015). ALA can to some degree be converted to longer, more unsaturated fatty acids, like EPA and DHA. The conversion is however low. Saturated fat is mostly found in products like bacon, burgers, egg, cheese and other products from the animal kingdom (National Cancer Institute, 2013). To get the two essential polyunsaturated fatty acids it is recommended to eat fish and plant oil, like sunflower oil and corn oil (Livsmedelsverket, 2015). Two especially important unsaturated fatty acids are DHA and EPA, which especially can be found in fish, shellfish and algae. These are so called long chain polyunsaturated fatty acids (LC-PUFA), which are enriched in brain, retina and is important in the signaling process for inflammation, blood clotting and smooth muscle movement (Andersson, 2015).

2.3.1.3 Rancidity – Lipid Oxidation

Rancidity is an indicator of the deterioration of fats and oils, which give rise to an unpleasant smell (Coultate, 2009). This rancidity is divided into two mechanisms, and the first mechanism is lipolytic rancidity, mostly affecting dairy product (Coultate, 2009). Here the microbial flora release lipase, which work as a catalyst and breaks down triglycerides to short-chain fatty acids (Coultate, 2009). Too much free fatty acids can give, for example butter, a smell of over-ripe cheese (Coultate, 2009). The second mechanism, oxidative rancidity or lipid oxidation, is a big problem during storage of various kinds of food and it can also influence the product in many ways (Coultate, 2009; Fernández et al, 1997). It can impact the quality of the food, e.g. odor, color, taste and texture, but it can also impact the nutritional value and generate toxic products (Fernández et al, 1997). To initiate lipid oxidation, oxygen and free radicals (molecules with unpaired electrons) are required but also ions like iron and copper can catalyze the lipid oxidation by speed up the formation of new radicals (Engström et al, 2014; Nationalencyklopedin, 2016c). LC-PUFA, as can be found in e.g. salmon and herring, has a higher risk to be attacked by free radicals because of their multiple double bonds in their fatty acid chains and therefore also easier go through lipid oxidation (Engström et al, 2014; Fernández et al, 1997; Öhrvik et al, 2012). Also shellfish, like shrimps and scallops, are sensitive due to a large part of their fatty acids being polyunsaturated (Öhrvik et al, 2012).

Normally a balance between formation and degradation of radicals is found in live animals, but if an increased formation of radicals occurs, this will have big impact on the animal itself

(Nationalencyklopedin, 2016c). The radicals mostly attack the DNA, proteins and the phospholipids, and change the structure of the nitrogen bases and oxidize the unsaturated fatty acids (Nationalencyklopedin, 2016c; Tullberg et al, 2016). This leads to dysfunctions of the cell (Tullberg et al, 2016). These kinds of reactions can also take place in post mortem tissue.

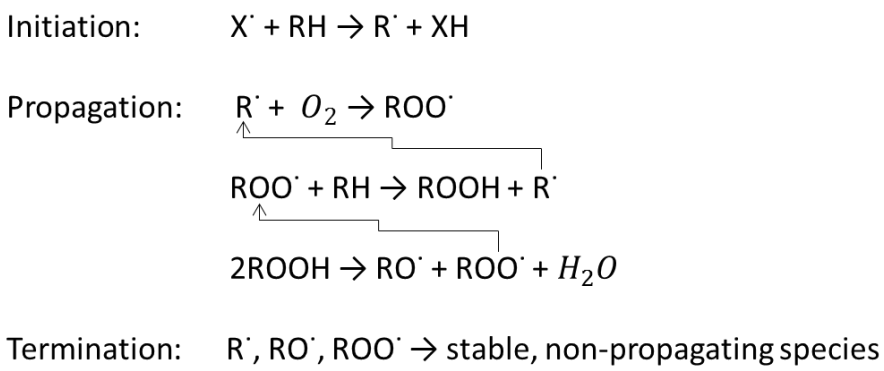

Figure 4. The three phases of lipid oxidation.

The sequence of reactions present during oxidative rancidity are normally divided into three phases, Figure 4, and for each phase different methods can be used to determine the degree of oxidation of the product (Coultate, 2009; Fernández et al, 1997; Engström et al, 2014). In the initiation reaction, free radicals (X·) react with PUFA (RH) to form highly reactive PUFA radicals (R·) (Coultate, 2009). These can further react, in the propagation step, with oxygen and form reactive peroxy radicals (ROO·). Peroxy radicals can continue and react with other unsaturated fatty acids to form hydroperoxides (ROOH), which is the primary product in the lipid oxidation reaction (Coultate, 2009, Fernández et al, 1997). The lipid oxidation can be measured by a peroxide value test where the amount of hydroperoxides in the sample is estimated. Hydroperoxides can later on easily be cleaved by metal ions into secondary compounds like pentanal, hexanal and malondialdehyde (MDA) (Fernández et al, 1997). MDA is a common secondary product from lipid oxidation and can for example be measured in a so called TBA test (Fernández et al, 1997). Other examples of secondary oxidation products are the aldehydes 4-hydroxy-trans-2-hexenal (HHE) and 4-hydroxy-trans-2-nonenal (HNE), Figure 5 (Tullberg et al, 2016). HHE is a derivate from n-3 PUFA and HNE is a derivate from the n-6 PUFA (Tullberg et al, 2016). Both HHE and HNE are hydroxy alkenals and have been shown to be genotoxic and carcinogenic, when found in biological systems (Tullberg et al, 2016). When the amount of free radicals in the fat reaches a specific point, radicals starts to react with each other and form stable end-products. This is known as the termination reaction in the oxidation of unsaturated fatty acids (Coultate, 2009).

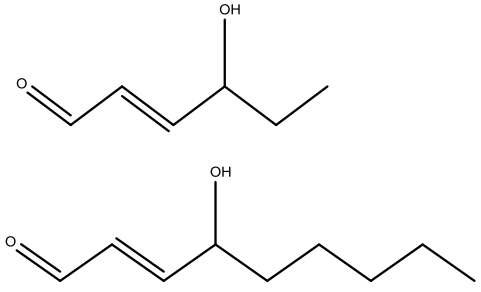

Figure 5. Molecular structure of 4-hydroxy-trans-2-hexenal (HHE), upper structure, and 4-hydroxy-trans-2-nonenal (HNE), lower structure.

2.3.2 Proteins

Proteins are the building block for life and in this section, a background about the basic protein structure, the central dogma of biology, and how many amino acids that are essential in human diet are defined. Some information of protein degradation and marine proteins are also included.

2.3.2.1 Protein Composition and Structure

Proteins are large and complex structures formed by thousands, up to millions of amino acids, bound together by covalent peptide bonds, hydrogen bonds and disulfide bridges. There are basically 20 amino acids combined in various order forming these proteins with unique sequence and highly specific properties. Each type of protein differs in its sequence and number of amino acids. (Alberts et al, 2008)

A protein consists of a polypeptide backbone with attached side-chains, Figure 6. The polypeptide backbone can be found in all amino acids, and the R is the side chain specific for each of the amino acid. The side chain can be positive, negative or uncharged depending on amino acid, and it can also be hydrophilic or hydrophobic. The backbone has an amino terminus (N-terminus) carrying an amino group and a carboxyl terminus (C-terminus) carrying a carboxyl group. A protein is always read from N- to C-terminus and it is also the amino and carboxyl groups that, through a condensation reaction, link two amino acids together by forming a peptide bond. (Alberts et al, 2008)

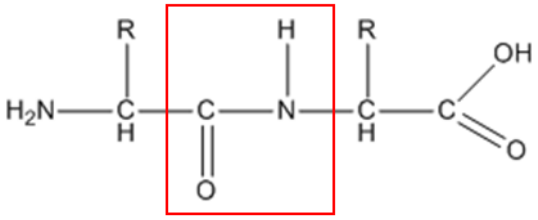

Figure 6. The primary structure of a protein. The red square shows the peptide bond holding together two amino acids in a protein formation.

Proteins are divided in four distinct protein structures (Alberts et al, 2008):

- Primary structure
- Secondary structure
- Tertiary structure
- Quaternary structure

The primary structure of the protein is the linear amino acid sequence, Figure 6, attached together through covalent peptide bonds (Alberts et al, 2008; Coultate, 2009). The secondary protein structure is primary structures bound together with hydrogen bonds to form either alpha helixes or beta sheets (Coultate, 2009). Alpha helix is a spiral conformation of primary structures where each N-H group in the backbone binds, with a hydrogen bond, to the C=O backbone group (Alberts et al, 2008). Beta sheets are parallel or antiparallel flats with primary structures, which are bound together with hydrogen bonds (Alberts et al, 2008; Coultate, 2009). The tertiary protein structure is a three

dimensional organization of a peptide chain including alpha helixes and beta sheets, and the quaternary protein structure describes how different tertiary structures are positioned in relation till each other forming the complete protein structure (Alberts et al, 2008; Coultate, 2009).

2.3.2.2 The Central Dogma of Biology and Marine Proteins

Proteins build up everything and are the basic component for life. This formation of protein by the cell has because of its importance for life been named “The central dogma of biology”. The central dogma of biology refers to the transcription of DNA to RNA, and the translation of RNA to protein, Figure 7. When a cell need a specific protein, DNA coding for the specific protein forms a RNA copy in the nucleus of the cell. The RNA, called messenger RNA (mRNA), goes out from the nucleus and work as a template for direct synthesis of the protein. Ribosome organelles binds to the mRNA template and start to translate the RNA to protein by reading three bases at the time and bind specific amino acids, transported to the ribosome by transfer RNA (tRNA). Each triple base combination codes for a specific amino acid. (Alberts et al, 2008; NCBI, 2007).

Figure 7. Central Dogma of Biology.

The body cannot produce all amino acids, which makes it necessary to get certain essential amino acids through the food. There are 8 amino acids which are regarded essential.

Fish and shellfish contain between 17 to 22% (w/w) and 7-23% (w/w) protein respectively. The three largest protein groups are the myofibrillar, sarcoplasmic and stroma muscle protein (Kim, 2013). Myofibrillar proteins are the biggest protein group in fish and shellfish and mainly consists of myosin, actin and tropomyosin, which are the proteins involved in muscle contraction (Coultate, 2009). Sacroplasmic proteins are mainly myoglobin, hemoglobin and cytochrome proteins (Kim, 2013). The last large protein group, stroma or connective tissue protein, are mainly built of collagen and elastin, which is strong and in the elastin case, also elastic (Coultate, 2009; Kim, 2013).

2.3.2.3 Protein Degradation

Protein degradation is another word for proteolysis, and it is when proteins are decomposed from long polypeptide chains to shorter peptides or completely decomposes to amino acids (Bender, 2014; Nationalencyklopedin, 2016d). This can happen both enzymatically, where the proteins is hydrolyzed by specific enzymes, called proteases, but also due to alkaline and acidic environments (Alberts et al, 2008; Nationalencyklopedin, 2016d; Wolff et al, 1986).

When food is contaminated by bacteria, the protein amount is often negatively affected, since the bacteria can break down proteins with proteases and increase the level of peptides, (Nationalencyklopedin, 2016d). Fish and shellfish contains several different proteases, many of them are acidic; i.e. specifically active at low pH values.

2.4 Experimental Equipments and Techniques

In this section, background information about the different equipment used, are described.

2.4.1 Spectrophotometer

The spectrophotometer was one of the most important laboratory equipment in the 1950s to 1970s at hospital-based clinical laboratories and other research laboratories, and it relies on the old knowledge that gas and liquid in some way will absorb specific wavelengths of light and let some wavelength just pass through. It is still fundamental equipment and several automatic research tools, used today, are based on the same principle. (Dondelinger, 2011)

Figure 8 demonstrates a general description over how a spectrophotometer works. The first component in a spectrophotometer is the light source, which produce a constant light in the correct wavelengths. Depending on the situation different light sources are used, e.g. if ultraviolet light is needed a hydrogen lamp sending out light at wavelength 200-450nm can be used. The light will continue to a filter, also called monochromator, which just let a narrow part of the spectrum to pass. To guarantee the light through the sample a combination of mirrors and lenses are often used. The light passes through the filter and then goes through the cuvettes containing the sample. The cuvettes are rounded or rectangular and can be made of glass, plastic or quartz. The most important thing for the cuvettes is to be completely transparent for the light used in the analysis. When light has passed through the sample, in the cuvette, some light has been absorbed. The light passing can then be detected by a detector giving electronic outputs, which is proportional to the intensity of the light leaving the sample. (Dondelinger, 2011)

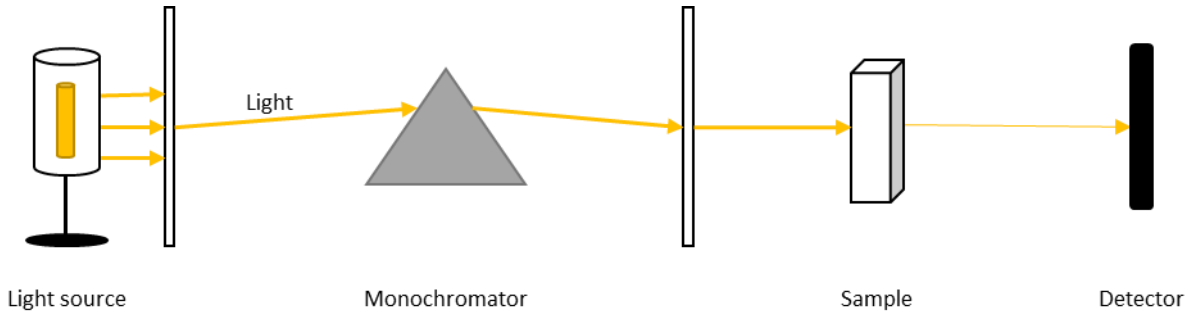

Figure 8. Schematic path of the light through different parts of a spectrophotometer.

When using a spectrophotometer, a blank must first be used to set the machine to zero, which means that everything in the blank is transmitted and then the absorbance of each sample could be measured. Today, by the way, a wide range of spectrophotometers and cuvettes are available at the market, which for example can split the light into a reference sample and the sample itself. Two commonly used cuvettes to this basic spectrophotometer are the flow through cuvette and the continuous cuvette, where the last one measure the absorbance of the sample when continuous flows of sample pass through the cuvette. (Dondelinger, 2011)

A wide range of methods within different areas, today use spectrometry as the last step to verify a color, often from a reagent, of the sample. From this e.g. the total protein amount or the amount of

primary oxidation products, tested by a peroxide value test, can be visualized (Markwell et al, 1978; Schmedes et al, 1989).

2.4.2 Colorimeter

A colorimeter is used to measure intensity of color in a sample and compares it with a blank (Cheesbrough, 2005). It can be used to measure color of food products like tomatoes, salmon, chocolate and bakery products, but also to measure color of plastic and building material, and to determine the concentration of a substance in the body (Konica Minolta; Cheesbrough, 2005). A colorimeter is based on visible light spectrum, with light wavelengths from 400 to 700nm (Cheesbrough, 2005; AATCC, 1997). A light, from a lamp, is sent through the sample, common at an angle of 45°, Figure 9 (AATCC, 1997). The color of the sample is then measured by 3 or 4 filters, measuring red, green, blue and sometimes yellow color, which is compared to a blank (AATCC, 1997; HunterLab, 2008). The blank can be a solution with known concentrations, but it can also be a white plate depending on the examination.

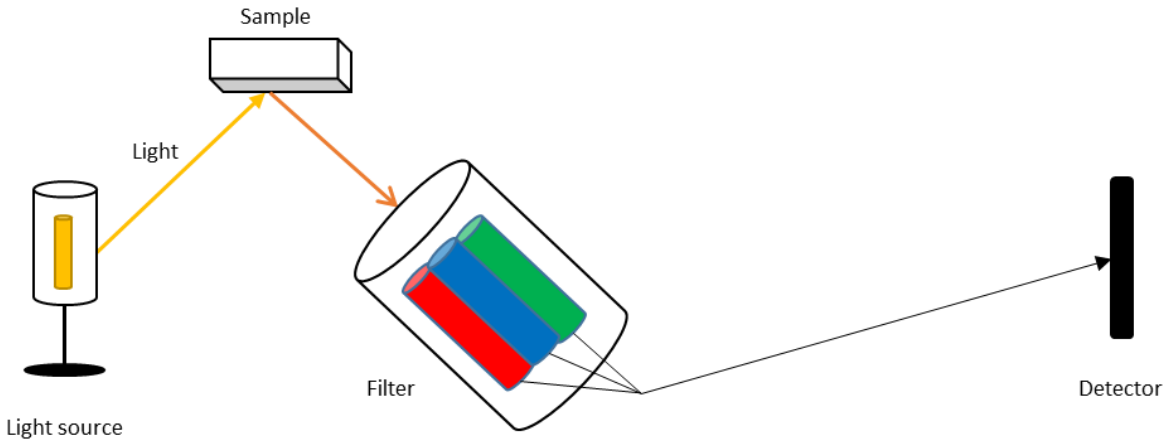

Figure 9. Schematic draw of a colorimeter, with three filters for red, green and blue color.

One of the most common color scales for measurement of color, by a colorimeter, is the so called Hunter **L, a, b** Color Scale, Figure 10. This color scale is organized in a cube formation (Figure 10) with **L, a** and **b** in different perpendicular directions. If **L** is equal to 100 it means that everything is spread and if **L** is equal to 0, the sample is black. Depending on the sign on **a** and **b** the color is different. A positive **a** means red color tone and a negative **a** means green color tone. The same can be seen for **b**, where positive is yellow color tone and negative is blue color tone. (HunterLab, 2008)

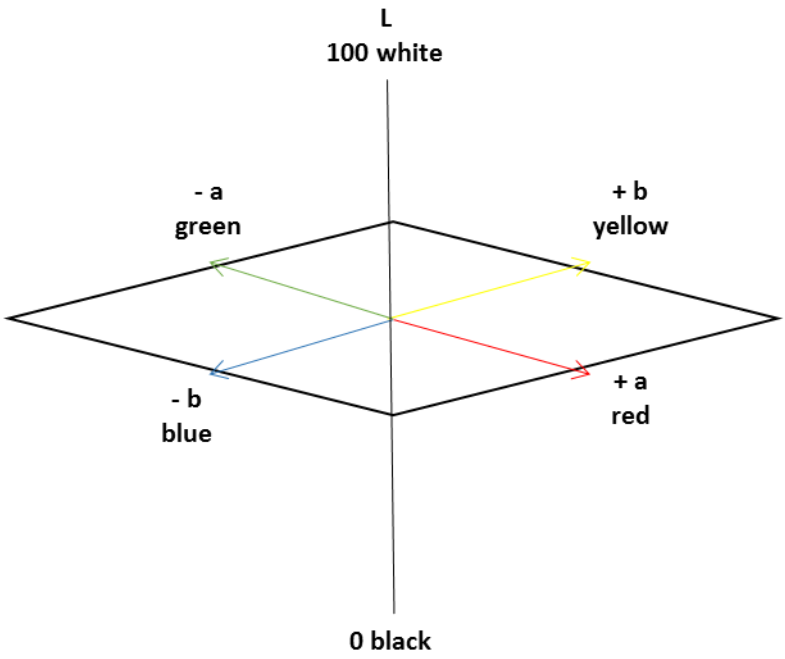

Figure 10. Hunter L, a, b Color Scale.

A modern type of colorimeter is the Chroma Meter CR series, which can be used in color measurements in everything from solids, pastes and granulates, to powders and even liquids. It's a small, portable, reliable and flexible apparatus, which is especially used in food industry to look for color differences. Difference in color can depend on natural variation, but also on low quality and bad processing of a product. The Chroma Meter CR-400 is placed on the object examined. Some photos are taken on the sample and then filtered for colors, which is matched to a blank. (Konica Minolta)

A colorimeter have many similarities to a spectrophotometer, since both measure color change compared to a blank and both can measure color within specific areas of wavelengths. A great difference between a colorimeter and a spectrophotometer is that the colorimeter has filters, which filter out wavelength within a 40nm band. Spectrophotometers are often more specific and can measure color at a specific wavelength. A spectrophotometer also has a prism or other kinds of tools, used to divide the light into continuous spectrum, and it is in general more expensive, less rugged and more technical skills has to be required to be able to use it, compared to a colorimeter. (Cheesbrough, 2005)

2.4.3 pH Meter

The electrode containing pH meter equipment is a simple fast and very used tool to get the pH of any solution. At the tip of the stick an electrode is located, which has the ability to measure the potential of hydrogen in the solution (Omega.com). When the electrode of the pH meter is positioned in the sample, the electrode recognize a potential difference compared to the reference potential that rise in the initial pH meter solution, from what the pH meter is calibrated (Coleparmer.com). This pH change, because of differences in hydrogen ion concentration in the sample, can be calculated and visualized on the pH meter screen (Omega.com). It is important to know that the pH meter equipment is temperature sensitive, and therefore manual temperature measurement equipment or

automatic temperature measurement equipment, built into the pH meter, can be used (Barron et al, 2006; Omega.com). It can therefore have a positive impact on reduction of eventual errors in the measurement, if the temperatures of the calibration buffers are close to the temperature of the samples tested (Omega.com; Barron et al, 2006).

Before start the pH measurement, buffers are used to calibrate the pH meter, to prevent small errors. The pH of the buffers can vary, but often there are three buffers. One is always at pH 7, which is the isopotential point, and the other buffers are often at pH 4 and pH10. When measuring pH it is preferable to have buffers close to the values in the tested samples (Omega.com).

2.4.4 Conductivity Meter

Conductivity comes from the word condu'ctus, which is the Latin word for collect and combine (Nationalencyklopedin, 2016e). Conductivity is the ability for a gas, metal or solution to pass an electric current. In a solution, e.g. sea water, anions and cations carry current, but in solid metals the current is instead carried by electrons. Acids, bases and salts are examples on current carrier ions. Every substance has some kind of conductivity, but it can vary a lot. In aqueous solutions the conductivity can be low, as in pure water, and high as in concentrated chemicals. The conductivity for a solution depends on temperature, concentration and mobility of ions, so these must be kept in mind, when measuring the conductivity. (Radiometer analytical handbook)

A conductivity meter normally contains 2-4 electrodes, and over these electrodes a current will be applied, which give rise to a voltage between the electrodes that can be measured. When the conductivity meter is positioned into the sample, the negative anions in the solution will go to the positive electrode and the positive cations will go to the negative electrode. By knowledge of the voltage, current and the cell constant at the specific temperature, the conductivity can be calculated by hand or by the conductivity meter and shown on the display. The unit for conductivity is S/cm, where S comes from the conductance (1/electric resistance) and cm from the cell constant, which is the ratio between effective area of the electrodes and the distance between the electrodes. The accuracy when measuring conductivity depends on several factors including polarization, contamination, geometry, frequency change and temperature. (Radiometer analytical handbook)

2.4.5 Liquid Chromatography Coupled with Mass Spectrometry (LC-MS)

LC-MS is a widely used method to separate and detect molecules in a sample based on mass to charge ratio (m/z) (Harris, 2010). It can be used in many areas as for example is proteomics and metabolomics research, but also in drug and food control, when measuring the amount of aldehydes formed during lipid oxidation (Tullberg et al, 2016; Harris, 2010; Hoffmann, 2007). Prior to MS the molecules are often separated, to be able to visualize the different compounds later on, in the mass spectrum, except when using a tandem MS where a chromatographic separation is not needed, due to the high accuracy of the method (Niessen, 2006; Harris, 2010; Pitt, 2009). If no separation of the analyzed product is done before MS detection, the probability that some molecules overlap increase, which can give untrustworthy results (Harris, 2010; Niessen, 2006). Another very important reason to separate compounds before MS examination, is that many of the interesting chemicals etc. has isotopes, and to be able to even separate the isotopes a separation before MS is necessary (Harris, 2010). This first separation can today be done by different kinds of chromatography, like (gas

chromatography (GC), liquid chromatography (LC) or supercritical fluid chromatography (Niessen, 2006; Harris, 2010). The name of each chromatography method indicates what kind of mobile phase is included in the method (Niessen, 2006).

Today several different types of LC-MS equipment are available depending on the budget, the accuracy and what is analyzed. A basic schematic drawing for a LC-MS is visualized in Figure 11. The first part, with the LC column included, of Figure 11, belongs to the LC, and the second part belongs to the MS of the method.

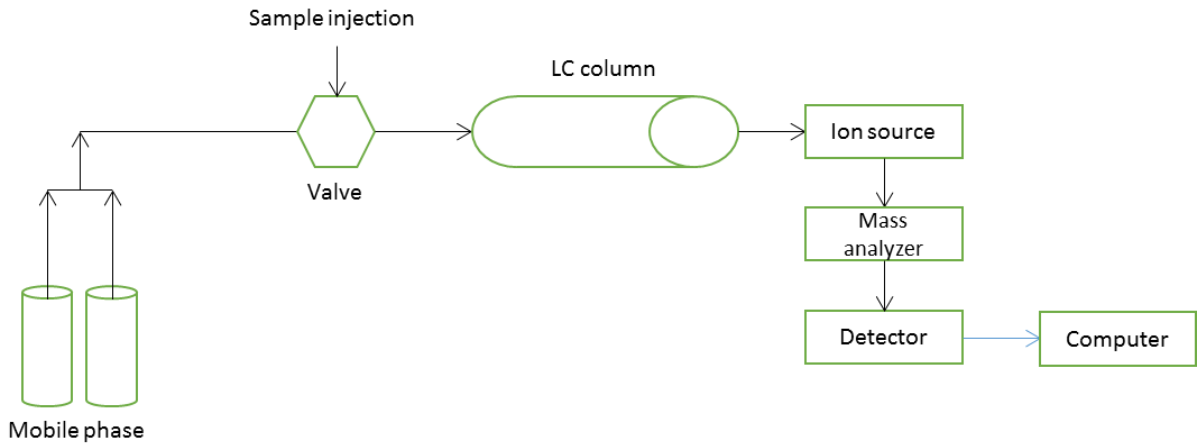

Figure 11. Basic schematic draw of LC-MS process.

2.4.5.1 Liquid Chromatography (LC)

In LC the components in the sample are separated, by pumping a solvent/mobile phase, containing the sample, through a column (LC column). In the column, the analytes from the sample, will be separated based on the absorption to the stationary phase. Different interactions give different retention times through the column, which can be seen later in the detection step. (Harris, 2010)

Depending on the problem to be solved the mobile phase setup can differ. The simplest way is to use an isocratic elution, where a single solution or the same amount and concentration of a solvent mixture, is pumped in to the column during the whole process (Harris, 2010). If a complex sample is analyzed, this can give rise to problems, since the peaks of the molecules with higher retention time gets wider (Harris, 2010). An improvement is to have a stepwise elution, where the mobile phase content is the same but the solvent concentration increase in steps and holds constant in between (Harris, 2010). Stepwise elution is an improvement but differences in peak wide can still occur between analytes with high and low retention time (Harris, 2010). For a complex solution, the best option is to choose a gradient elution (Harris, 2010). Gradient elution is when the mobile phase continuously changes during the separation. It is fast and gives great peaks with similar width, but some disadvantages with the gradient elution are that more complex equipment is necessary and it must be able to pump at least 2 solvent. A gradient elution experiment can also be difficult to redo on other equipment setups (Pitt, 2009; Harris, 2010).

In the LC setup the mobile phase is mixed with the sample in the so called valve and injected into the column. The function of the valve is to mix the sample with a mobile phase solvent under high

pressure, so the sample can enter the column in a homogeneous shape and in a small volume (Harris, 2010).

When passing the valve the sample enters the column, called the heart of the LC system (Niessen, 2006). The size can vary depending on the amount of samples to be injected thus analytical, semi preparative and preparative grade columns are used. For an analytical column the length is between 100-300mm and the diameter is 3-4.6mm (Niessen, 2006). The inner wall of the column is where the stationary phase can be found. The stationary phase most commonly consists of spherical and microporous particles of silica, which is permeable to solvent and has a large surface area of several 100 meter per gram (Harris, 2010). It is normally sensitive to pH above 8, but by adding ethylene bridges to the silica complex, analytes in solutions up to pH 12 can be separated (Harris, 2010). When entering the column, analytes bind with different forces to the column depending on their affinity to the coated particles in the column wall (Chemir, 2016). This difference between analytes in a sample, gives rise to variation in speed through the column, which lead to a separation detectable by a detector (Harris, 2010).

When performing a LC sepeartion, either reversed or normal phase chromatography is chosen, before start the LC examination of a sample. Normal phase chromatography is when the stationary phase is polar, for example silica particles, and the solvent non-polar or less polar (Harris, 2010). Reverse phase, the most used phase, is when the stationary phase is non-polar or weakly polar and the solvent is more polar (Harris, 2010; Niessen, 2006). Typical solvents used in normal and reverse phase LC are water, methanol, ethanol and chloroform etc. (Pitt, 2009).

2.4.5.2 Mass Spectrometry (MS)

LC is often connected with some kind of MS, to improve the probability to identify analytes, depending on the mass (m) to charge (z) ratio (Niessen, 2006).

The mass spectrometry is divided in three distinct parts, according to Figure 11: (Hoffmann, 2007)

- Ion source
- Mass analyzer
- Detector

2.4.5.2.1 Ion Source

To analyze the sample by mass spectrometry, the content first must be ionized, where mainly natural molecules are ionized in gas phase (Hoffmann, 2007). Today a large variety of ionization methods are available, for gas, liquid and solid samples (Hoffmann, 2007). Some of these ionization methods are:

- **Atmospheric pressure chemical ionization (APCI)**
- Electrospray ionization (ESI)
- Electron ionization (EI)
- Matrix-assisted laser desorption ionization (MALDI)
- Chemical ionization (CI)
- Secondary ion mass spectrometry

Atmospheric pressure chemical ionization (APCI) is a method where the liquid solution from e.g. a LC setup enters a nebulizer, where a nitrogen beam converts the solution into a thin fog. Droplets from this fog are then transferred through a desolvation/vaporization chamber. The droplets from the mobile phase start to evaporate, due to passing through this well-controlled heater at atmospheric pressure. After desolvation, the sample left from the mobile phase is carried through a corona discharge electrode were the molecules are ionized. These ions can then be accelerated to the mass analyzing part of the mass spectrometry, for separation and detection. APCI is equivalent to, the so often used, chemical ionization method (CI). The difference between the APCI processes and CI is that the APCI process is under atmospheric pressure, which not a standard chemical ionization is (Hoffmann, 2007).

2.4.5.2.2 Mass Analyser

When the molecules have been ionized into gas phase, the mass analyzer continues the process and separates the ions after the mass to charge ratio (m/z) (Hoffmann, 2007). To do this separation a large variety of methods can be used, but all mass analyzing methods are based on static or dynamic electric or magnetic fields, which can be used either alone or combined, for optimal separation (Hoffmann, 2007). The main principle with the mass analyzing part of the MS is the movement of ions traveling through a magnetic or electric field, which can be affected depending on the mass to charge ratio (Ho et al, 2003). Some examples of often used mass analysers are:

- **Quadrupole**
- Time-of-Flight
- Ion Trap
- Orbitrap
- Fourier transformer (FT) ion cyclotron resonance spectrometers.

Quadrupole setup is one of the most used mass analyzing processes, and it can be found in both simple single quadrupole and more advanced triple quadrupoles (Harris, 2010). This single quadrupole system composed of four parallel metal rods forming the edges on a block structure (Ho et al, 2003) Each rod has an equal distance to each other, and over the four metal rods a voltage is located (Ho et al, 2003). Before the ions entering the quadrupole, the ions are accelerated by usage of a high speed turbomolecular pump or an oil diffusion pump (Harris, 2010). The accelerated ions will pass into the metal rod block, where each pair of rods is connected electrically, and an opposite DC voltage with radio frequency (RF) are also applied together with an AC voltage, which are placed diagonally over the rods of the quadrupole (Ho et al, 2003). When ions pass through the rods the voltage and RF can be controlled, so just ions with a specific mass/charge ratio can pass and be detected by a detector (Ho et al, 2003). The other m/z ions are sorted out on the way, through the long sides of the quadrupole (Ho et al, 2003). In tandem quadrupole system, also called the triple quadrupole, three quadrupoles in a row are used (Ho et al, 2003). In the first quadrupole a separation is done as in the single quadrupole, described above (Ho et al, 2003). In the second quadrupole, which is called collision cell, the ions are collided with gas molecules and fragmented into smaller pieces, which are separated in the third quadrupole. Same as in quadrupole number one, and then detected by a detector (Ho et al, 2003).

2.4.5.2.3 Detector

When ions passed through the mass analyzing step, a detector recognizes them and transforms it into usable signals. Several types of detectors are today available, and which to choose depends on the instrumental design and what is examined. Ion detectors are normally divided into two classes, where the first class counts ions of a single mass at the time. The second class, which photographic plate belongs to, can instead count multiple masses and detect all ions simultaneously along a whole plane. Some examples of ion detectors are: (Hoffmann, 2007)

- **Electron multiplier**
- Electro-optical ion detector
- Photographic plate

Electron multiplier is the most used detector in mass spectrometry (Hoffmann, 2007). The task for the electron multiplier detector is to detect every ion passed through the mass analysing filter (Gates, 2014). Two types of electron multipliers are today used in mass spectrometry (Hoffmann, 2007). The first group is called discrete-dynode electron multiplier and this instrument normally contains 12-20 so called dynodes, and the second electron multiplier group is called continuous-dynode electron multiplier (CDEM) (Hoffmann, 2007). The general function for the electron multiplier detector is to detect the ion from the mass analyzer, by multiplication of the electron signals, which is later detected by an output electrode and interpreted by a computer (Hoffmann, 2007; Gates, 2014). In general the ion from the mass analyzer starts to go to the first dynode, where it smashes into the wall and cause secondary electrodes to be emitted from the surface (Hoffmann, 2007; (Gates, 2014). The angle from the first dynode then focuses the electrodes to the second dynode where more electrodes are emitted, and then pass further to the third dynode and so on (Gates, 2014). Since the potential over each dynode increase for every dynode a series of amplifications will occur, and this continues until the output electrode recognize the highly increased multiple electrons, formed by the dynodes, and a computer starts to interpret the data (Gates, 2014). Normally if one ion goes into the electron multiplier a signal around 10^6 electrons goes out from the detector for interpretation (Gates, 2014).

2.5 Analytical methods

In this last section of the theory, background information of the performed methods can be found.

2.5.1 Sensory Analysis

Sensory analysis is an important and widely used evaluation method for product evaluation in food industries (Lawless & Heymann, 2010). It is done by evaluating how the products are perceived by the human senses; sight, smell, sound, taste and touch (Lawless & Heymann, 2010). This evaluation technology is important when developing new products and is highly essential for the quality control and shelf life evaluation, since if a product shall be passed out on the market it must give the right signals to customers during sensory testing (Edelstein, 2013; Lawless & Heymann, 2010). Example of a field were sensory analysis can be highly important is in the healthy food branch, where nutritionists and dietarians develop health food recipes (Edelstein, 2013). The senses work as a guard for the body, and if the senses do not accept the food it will be difficult for the body to utilize the benefits in the healthy food (Edelstein, 2013).

A sensory analysis can focus on appearance, odor and texture; both in a quantitative and qualitative manner. It is a strong tool to use when following up changes in product overtime (Edelstein, 2013).

2.5.2 Protein Analysis Using the Lowry Method

The Lowry method is a well-known colorimetric assay to estimate total proteins in e.g. fish samples (Cammack et al, 2006), and it is named after Oliver Howe Lowry, a pharmacologist and biochemist during the 20th century (Cammack et al, 2006). Briefly the mechanism is when blue color occurs after the reaction between peptide bonds with Cu^{2+} in an alkaline copper sulfate solution, and when Folin-Ciocalteau reagent reduced by Cu^{+}, make aromatic amino acids, such as tryptophan and tyrosine residuals in the protein, to oxidize as well (Cammack et al, 2006; Lowry et al, 1951). When using the Folin-Ciocalteau reagent, it is highly important to avoid strong light since this reagent is light sensitive (Agbor et al, 2014).

Several modifications of the Lowry method today exist for determination of total protein in a sample, but in general three reagents are prepared; reagent A, reagent B and phenol reagent (Harrington, 1990). Reagent A is an alkali solution that is mixed with reagent B including copper sulfate, to form reagent C (Cammack et al, 2006; Harrington, 1990). Reagent C is later mixed with the homogenized sample to start the reaction between Cu^{2+} and the peptide bonds of the sample proteins (Cammack et al, 2006; Harrington, 1990). After the incubation, Folin-Ciocalteu phenol reagent is added, which during a second incubation gives blue color when reacting with the ring structures of the aromatic amino acids, together with the reaction between peptide bonds and Cu^{2+} described above. This blue color can be measured by a spectrophotometer at 660nm (Cammack et al, 2006; Harrington, 1990). A darker blue color can be caused by high amounts of tryptophan and tyrosine, which can cause to high values when evaluate the protein concentrations with a standard curve built with serum bovine albumin (Cammack et al, 2006; Harrington, 1990).

2.5.3 Peroxide Value Test

To analyze lipid hydroperoxides, the lipids must first be extracted from the sample, which is commonly done by chloroform:methanol; such as in the method of Bligh and Dyer. This method was published 1959 by the Canadian journal of biochemistry and physiology. It is a well-known and used method to separate lipids, from non-lipids, in for example fish (Bligh & Dyer, 1959). This method has, since it was first published, been modified several times depending on aim of the specific research (Schmedes, 1989; Cavonius, 2014; Lee, 1996). The lipid extraction method from Bligh and Dyer were originally used for low lipid containing samples, but later on, Lee et al (1996) found a better extraction of high lipid samples by increasing the chloroform ratio in the chloroform:methanol solution (Cavonius, 2014; Lee, 1997). In the Bligh and Dyer extraction method, the sample is, in general, first homogenized, if necessary, and mixed with a chloroform:methanol solution, which has shown a good ability to penetrate cells and separate the lipids from the rest (Cavonius, 2014; Bligh and Dyer, 1959). After addition of salt water, the solution is separated into a two-layer-phase, where the non-polar compounds (e.g. hydroperoxides) are extracted in to the lower chloroform phase, and the polar compounds exists in the upper methanol:water phase (Bligh and Dyer, 1959; Schmedes, 1989; Cavonius, 2014).

As been described under rancidity, hydroperoxides (ROOH) are the biggest and most important primary reaction product obtained during lipid oxidation, since it can be decomposed to both volatile and non-volatile products and it can also react further to other products during the oxidation. (Fernández et al, 1997; Coultate, 2009; Steele, 2004). Hydroperoxide is an organic compound, where one of the hydrogen carbons has been replaced by a –O–OH group, visualized in Figure 12 (Cammack et al, 2008). Hydroperoxides are today a primary product of lipid oxidation, but also a part of a wide range of chemicals; like tert-butyl hydroperoxide, ethyl hydroperoxide and cumene hydroperoxide (NCBI, 2016a; NCBI 2016b). Since a large variation of potential decomposed products can be formed from hydroperoxide, this compound is considered as a freshness indicator as well as an index for lipid oxidation, which concentration can be measured by a peroxide value (PV) test (Schmedes et al, 1989; Steele, 2004; Lee et al, 1996).

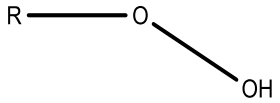

Figure 12. Structure of hydroperoxide.

The traditional method for determination of the peroxide value is by a titration of oil that contains potassium iodide in a chloroform:acetic acid solution. The hydroperoxide in the solution will oxidize the iodide to iodine, which can be determined by titration with sodium thiosulfate. To avoid chloroform extraction, methods to extract the peroxide value without use of chloroform are also available today. (Steele, 2004)

Another method, described by Schmedes et al (1989), to determine the peroxide value, is to add a Fe (II) chloride solution and let the peroxides in the chloroform phase, from the sample, oxidize Fe (II) to Fe (III). Fe (III) will then form a violet complex when mixed with ammonium thiocyanate solution, which absorbance is read in a spectrophotometer at 500nm. By insertion of the absorbance into a standard curve, made from cumene hydroperoxide (CPO), and some calculations, the peroxide value in µmol peroxide per liter sample can be found (Schmedes et al, 1989; Undeland et. al, 2002).

2.5.4 Total Volatile Basic Nitrogen (TVB-N) Test

In fish, shellfish, meat and other muscle products, spoilage is often measured as total volatile basic nitrogen compounds (TVB-N) comprising e.g. trimethylamine-nitrogen (TMA-N) and ammonia. TMA-N is volatile amines that are responsible for the fishy odor in fish and shellfish. TMA-N is produced from trimethylamine oxide (TMAO) by a small number of specific spoilage bacteria (Etienne, 2005). Ammonia can result as a byproduct during protein breakdown (Eghtesad et al, 2013). Apart from TMA-N and ammonia, TVB-N also measures methylamine and dimethylamine (DMA). Methylamine can be result of microbiological activity when storing the fish at too high temperature or due to thermal breakdown of endogenous compounds during processing of the product/fish (Rawdkuen et al, 2010). DMA can be formed from TMAO during frozen storage via enzymes. The total amount of these four compounds in the fish gives rise to TVB-N (fao.org, 2001; Ali et al, 2010). TVB-N is the most widely used parameter to evaluate the quality of fish and shellfish. The amount of TVB-N normally increases during the first week of storage, but some studies have shown a leakage of volatile amines, mainly ammonia, during fish storage on ice (Etienne, 2005). TVB-N can be measured in a large range of different ways and depending on method and equipment used, the TVB-N value

for the same sample can differ with a factor 2 (fao.org, 2001). TVB-N is not a particularly good index of bacterial spoilage, but it is together with TMA-N the best known chemical indicator that is easy to perform (Etienne, 2005; fao.org, 2001).

One way to measure TVB-N is by use of the Conway unit method described by Rawdkuen et al (2010). First, sample is mixed with TCA, to precipitate macromolecules, and then the sample is added to the outer ring of a Conway unit. To the inner ring the gas-absorbing reagent, boric acid, is then added, and to start the reaction a highly alkaline and saturated potassium carbonate solution is placed into the outer ring followed by closure of the lid and incubation for 1h at 37°C (Ali et al, 2010; Rawdkuen et al, 2010; Cammack et al, 2006). During incubation the volatile nitrogen compounds leaves the outer ring solution, due to reaction with the saturated potassium carbonate (Rawdkuen et al, 2010). The volatile nitrogen will then be absorbed by the gas-absorbing boric acid in the inner ring (Chakravarti et al, 2005). The coloured boric acid is from the beginning pink, due to the methyl red compound in the boric acid solution, but during incubation it will turn to green depending on the amount of absorbed nitrogen. After incubation, droplets of 0.02M hydrochloric acid (HCl) are added to the inner ring to slowly increase the pH until the green color goes back to pink (Rawdkuen et al, 2010; Cammack et al, 2006). From the amount of HCl added, the TVB-N value can be calculated (Rawdkuen et al, 2010; Cammack et al, 2006).

3 Materials and Methods

In this section the most important reagents and equipments used in the project are mentioned, followed by a method part, were the execution of each method is described in detail.

3.1 Materials

Shrimp process water (*Pandalus boreals)* was obtained from the local company Räkor & Laxgrossisten AB. Two types of water were given, steam water from the boiler and main water, which is a mix of steam and shrimp peeling water.

2.6-di-tert-butyl-4-methylphenol (BHT) and 2-4-dinitrophenylhydrazine (DNPH) were from Sigma-Aldrich. Potassium sodium tartrate tetrahydrate and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich. Folin-Ciocalteu phenol reagent was also purchased from Sigma-Aldrich. Ammonium thiocyanate, barium chloride dehydrate, cumene hydroperoxide (CPO) and iron (II) sulfate heptahydrate were bought from Sigma-Aldrich. 2-Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) reagent were bought from Sigma-Aldrich and Fisher Scientific respectively. Potassium carbonate reagent was from Fisher Scientific, methyl red and bromocresol green reagents were from Sigma-Aldrich. For more information about all chemicals used in this report, please see Appendix for each specific method (Appendix C – Appendix H).

3.2 Methods

3.2.1 Storage Trial Setup

Five liter of each shrimp process waters was taken from Räkor & Laxgrossisten AB and stored in a cold room at 4°C, in 70% ethanol washed 15L containers. Samples were taken every day for the first 10 days, at day 13 and at day 18 to get extreme values for the waters. The samples taken were placed at -80°C, until examination with different methods. Thawing was done under running cold tap water. Results between different days were then compared to see if any biochemical changes occur during the storage at 4°C. The investigation was made in two replicates, with three weeks between, to see if any composition and stability changes of the shrimp waters between different batches could be found.

The first process water (sampling 1) was retrieved 18th of February, 2016, and the sampling period was performed between 18th of February and 7th of March. The second sampling period was performed 3 weeks later between 10th of March and 28th of March. The methods used to evaluate biochemical changes over time were total protein with the Lowry method, peroxide value test (PV test), MDA evaluation with LC-MS, total volatile basic nitrogen (TVB-N) by the Conway method, sensory analysis of odor, color testing, pH and ionic strength measurements.

3.2.2 pH Measurement

To examine the pH change over time a pH measurement was performed with a calibrated PHM210 Standard pH meter (Radiometer analytical, Villeurbanne Cedex, France). The pH was measured in duplicates and samples held at a temperature of 4°C.

The thawed samples and the calibration buffer for the pH meter were placed on ice. When a temperature close to 4°C occurred, the pH meter was first calibrated with buffers at pH4 and pH7, and then the pH of each sample was measured.

3.2.3 Ionic Strength Measurement

To examine the ion strength change over time a conductivity measurement was performed with a CDM210 four electrode conductivity meter (Radiometer analytical, Villeurbanne Cedex, France). The thawed samples were held at room temperature during the conductivity test.

Different concentrations of sodium chloride (NaCl) were mixed for the standard curve construction, as can be seen in Appendix C. The conductivity was then measured and plotted on the y-axis against the known salt concentration on the x-axis. Data were expressed as NaCl equivalents. It was ensured that the samples covered all four electrodes, to get a reliable result.

3.2.4 Olfactory Sensory Analysis

To evaluate differences in odor between different days of sampling, sensory testing of odor was performed, with five participants from the department as panelists. A training session proceeded the actual analysis, during which the panel agreed on the descriptors to use. A sensory evaluation list for the sensory panelist was also prepared before test day, where odor intensity of the process waters were graded on a line to evaluate how strong the specific odors/descriptors were. A comment line for other descriptions of the odor was also available. Sensory evaluation list, for steam and main water, can be found in Appendix A.

The tests for the main and steam shrimp process waters were divided in two sessions, one process water type the first day and another the second day. For each water type, both replicates/samplings were investigated at the same time. Regarding the main water, samples from day 10, 13 and 18 were excluded before the test start, since they already were spoiled and had a strong microbial odor. From the remaining samples day 1 and day 3 were also excluded to limit the size of the test set. For steam water 7 dates from each sampling was chosen; day 0, 4, 6, 8, 10, 13 and 18.

At the test day, 80mL of each sample were placed into cleaned and similar 250ml E-flasks with screw caps and a code, unknown for the test panelist. The samples were then placed on a tray with a thin layer of ice at the bottom, and in a room with neutral odor. The flasks were placed in a random order, on a line, and allowed to stand for 30min, before start of the analysis. One exception from the random order was that the oldest samples were placed on the second half of the line, to avoid getting the oldest first, which could saturate the nose.

During examination one panelist at the time evaluate the samples by first swirling it a little before opening the cap and puting the opening right under the nose. The panelist should then individually write a cross on the line (1-100), to grade how strong a specific smell was, according to evaluation list in Appendix A. If other odors were recognized it could be written on the comment line. The panelist had to wait a few minutes before examined the odor of the next sample, to give the nose a chance to recover. When the first panelist was finished, the samples had to stay for 30min, with caps on, to allow for a new equilibrium between sample and head space before the next panelist.

3.2.5 Colorimetric Test

To look for color changes over time in main and steam shrimp waters, a measurement with a Chroma Meter CR-400 (Konica Minolta) colorimeter was performed. The color was measured according to the Hunter color measurement system, see colorimeter in theory for more information.

For the color measurement performance, samples of each date were placed in transparent glass jars, with a white lid on top. The Minolta colorimeter was started, calibrated with a white plate and placed under the glass jar containing the sample. The sample was shaken before taking five measures of each sample with the Minolta colorimeter. The given numbers of a*, b* and L* were recorded.

3.2.6 Primary Lipid Oxidation Products by the Peroxide Value (PV) Test

In the peroxide value (PV) test, described by Undeland et al (2002), the amount of primary products formed during lipid oxidation is measured. Prior to PV-analysis, lipids were extracted by the method of Lee et al (1996), modified by Undeland et al (2002). Lipid hydroperoxides hereby end up in the chloroform phase. The samples were extracted, in duplicates and a blank extraction of pure water was also carried out to be included in the PV analysis.

One mL of each sample was mixed with 10mL ice cold chloroform-methanol solution (1:2) and vortexed for 10s. 3.08mL ice cold 0.5% sodium chloride solution was then added to the tubes and vortexed for 30s, followed by centrifugation in a table top centrifuge for 6min at 2000 x g and 4°C. The chloroform phase of each sample was then separated, by use of a Pasteur pipette, and transferred into new glass tubes. The tubes were stored in the -80°C freezer until evaluation by peroxide value test. During the whole extraction process the samples were kept cold to avoid further oxidation.

Chemicals used, to be able to accomplish the Bligh and Dyer extraction from Lee et al (1996), for the process water of shrimp, can be found in Appendix F.

For the PV test a stable ammonium thiocyanate solution was first made by dissolving 7.5g ammonium thiocyanate in distillate water up to 25mL. “Solution 1” was prepared by dissolving 0.2g barium chloride dehydrate, in 25mL 0.4M hydrochloric acid. Solution 1 is only stable in cold storage. A chloroform-methanol solution at ratio 1:1 was also prepared to later be used in both PV test and standard curve formation. Solution 2, which was made fresh every time, was prepared by dissolving 0.1g iron (II) sulfate heptahydrate in 10mL distillate water.

For the standard curve, 0.3805g 80% cumene hydroperoxide (CPO) was dissolved in 100mL chloroform-methanol solution (1:1) to form a 20mM CPO solution (A). The CPO solution (A) was then diluted 1000 times in chloroform-methanol (1:1) to a 20µM CPO solution (B). Solution (B) was used as a stock solution, to be diluted into known concentrations of CPO for the standard curve formation, according to Appendix G. 2mL of each CPO concentration was transferred into glass tubes, and then 33.5µL ammonium thiocyanate solution was added and vortexed for 4s. 3mL of solution 1 was mixed with 3mL of solution 2 and vortexed for 1min followed by centrifugation at 3250 rpm for 3min in room temperature, to get the iron (II) chloride solution. 33.5µL of fresh made iron (II) chloride

solution was added to each CPO sample and vortexed for 4s. The tubes were incubated for 20min in room temperature and then the absorbance was measured by a Cary 60 UV-Vis spectrophotometer (Agilent Technologies) at the wavelength of 500nm. From the CPO stock solution absorbance and the known concentration, a standard curve could be designed.

For analysis of the samples, the chloroform phases were taken from the -80°C freezer and held under running cold water for 1min. 2mL were then transferred to new tubes and held on ice. To the tubes 1.33mL ice-cold chloroform-methanol solution (ratio 1:1) and 33.5µL ammonium thiocyanate solution were added and vortexed 4s. The analysis then proceeded as described above. From the absorbance given, the CPO standard curve and the equation below, the peroxide value, in µmol per liter of sample, could be calculated.

$$\mu mol\ peroxide/L\ sample = \frac{\mu mol\ peroxide\ /L\ chloroform * B * C * tentative\ extra\ dilution * 1000}{L\ shrimp\ water}$$

- B is the amount chloroform used per 1mL sample in the first extraction
- C is the dilution of the chloroform phase during the peroxide value test
- Tentative extra dilution is an extra dilution, if you expect high peroxide values in your sample
- L shrimp water is the amount sample used in the extraction, before the PV test is performed

In Appendix G all chemicals needed for the peroxide value test of main and steam water can be visualized, and in Appendix I an example calculation of the peroxide value is performed.

3.2.7 Secondary Lipid Oxidation Product (MDA) Measurement by LC-MS

In order to analyze the secondary lipid oxidation product MDA, it was extracted, followed by a measurement by a LC-MS method reported by Tullberg et al (2016).

First 3 stable solutions were prepared:

- 2.6-di-tert-butyl-4-methylphenol (BHT) in methanol (0.1g/mL)
- 0.02M ethylenediaminetetraacetic acid (EDTA)
- 0.25M hydrochloric acid (HCl)

A 2mg/mL saturated solution of 2-4-dinitrophenylhydrazine (DNPH) in methanol was also prepared; it was vortexed 1min during preparation to dissolve as much DNPH as possible.

In the experimental performance, 500µL of each sample was placed in a separate tube. To each tube 20µL BHT (0.1g/mL) and 40µL EDTA (0.02M) were added, followed by an acidification with 500µL hydrochloric acid (0.25M). The samples were then vortexed for 10s, left for 5min to precipitate and centrifuged at 16000 x g for 2min. After centrifugation the supernatants were transferred to new tubes, and mixed with 25µL DNPH before vortexing and then incubated for 1h at room temperature. When incubation ended, two extractions with dichloromethane were done. In each extraction 0.5mL dichloromethane was added to the incubated samples, followed by vortexing 10s and centrifugation at 16000 x g for 2min. The lower part was transferred to a new tube and put under nitrogen gas to evaporate the liquid. The dried sample, after evaporation, was diluted in 250µL methanol, vortexed and incubated for 5min before centrifugation 16000 x g for 2min. 200µL from the supernatant of the centrifuged tubes were transferred to vials for LC-MS examination.

For MDA standards, a stock of 1.1.3.3-tetraethoxypropane (TEP) (1mM in 1% sulfuric acid) was used. The concentration of MDA in each of the five standards used can be found in Appendix D. To confirm that DNPH itself did not interfere with these metabolites in the LC-MS, a DNPH blank was also included.

The LC-MS analysis was done by using an Agilent 1260 HPLC system, containing a binary pump, an auto sampler, a column oven, and an UV-detector, which was attached to an Agilent 6120 quadrupole (Agilent Technologies, Waldbron, Germany). The column used for separation was a Phenomenex Luna (4.6mm in diameter × 250mm length, 3µm thickness) (Phenomenex, Macclesfield, UK), and the flow rate of the mobile phase through the system was 0.7mL/min. Nitrogen gas was used for the nebulization and for drying and vaporization at temperatures of 350°C and 450°C. The remaining settings and conditions were set according to Grosjean et al (2000). Before start of the LC/APCI-MS analysis, two mobile phase solutions A and B were prepared. Mobile phase A was methanol with 20mM acetic acid and B was water with 20mM acetic acid. Each sampling took 40min and a gradient program for the mobile phase was fallowed, according to the article by Tullberg et al (2016).

The ions from the analysis were collected at the mass/charge ratio peak 234.0 for MDA, and detected by a UV detector at 264 nm. The area of the three metabolites could then be examined, and by using the standard curve made from the standards, with known concentrations, the concentration of MDA in each sample was defined.

In Appendix D the standard curve and all chemicals used for extraction and determination of the metabolite (MDA) formed during lipid oxidation, can be found.

3.2.8 Protein Determination

For the shrimp water experiment the method by Lowry (1951), modified according to Markwell et al (1978), was used to determine the total protein content in both the steam and main water, which makes it possible to evaluate the change of the total protein content over time and to compare different batches of the shrimp process waters. It is important to do this test in at least duplicates and also to have a blank with just water, following the samples through the method.

Before starting the analysis, a 1M and a 0.1M sodium hydroxide (NaOH) solution were mixed for the dilution step and for the standard curve formation. A reagent A and a reagent B solution were also mixed before start the Lowry, according to Appendix E. A 2mg/mL stock solution was then prepared for the standard curve, by dissolve 0.01g bovine serum albumin (BSA) in distillate water, up to a volume of 5mL. The BSA stock solution was then mixed, to a wide range of concentrations, with 0.1M NaOH, according to Appendix E.

For the shrimp process water examination, samples from each date was taken from the -80°C freezer and placed under running tap water to thaw. 0.9mL sample was transferred to separated glass tubes and mixed with 0.1mL (1M) NaOH solution. The tubes were vortexed and incubated for 15min,

followed by a 1:10 dilution with 0.1M NaOH. The steam process water was further diluted by mixed 0.5mL of the 1:10 diluted steam process water with 4.5mL 0.1M NaOH, which gave a 1:100 dilution.

1mL of each 1:10 diluted main and 1mL of each 1:100 diluted steam process waters were separately mixed with 3mL new made reagent C (1 part Reagent B into 100 parts Reagent A) and incubated in room temperature for 30min. The same procedure was done for the BSA standard curve concentrations. During incubation a phenol reagent was prepared by mix 1 part Folin-Ciocalteu phenol with 1 part distillated water. Important to turn the lights off during this step since Folin-Ciocalteu phenol reagent can be light sensitive (Agbor et al, 2014). After 30min incubation, 0.3mL phenol reagent was added to each tube followed by 45min incubation at room temperature. The absorbance was then measured at the wavelength of 660nm, by a Cary 60 UV-Vis spectrophotometer. By insert the absorbance from each shrimp process water sample into the standard curve and by taken the dilution steps into account, the total protein concentration of each sample could be determined.

All chemicals used to perform and measure the total protein concentrations in the steam and main process water, by Lowry method, are found in Appendix E.

3.2.9 TVB-N test

For the TVB-N analysis, described by Rawdkuen et al (2010), homemade Conway cells were used. For the Conway cell, a smaller jar, 30mm in diameter, was glued at the bottom of a larger jar, 70mm in diameter. It is important to make sure the large jar has a tight lid (Figure 13). The test was made in duplicates and a blank with distillate water was also included.

Figure 13. Pictures of homemade Conway cells used for the TVB-N experiment. Smaller jars are glued into larger jars. The larger jars are screwed on top of each other forming tight lids.

A 4% TCA solution was prepared, and 4mL of each sample were then mixed with 6mL TCA (4%) solution in glass tubes. The tubes were vortexed for 1min, and centrifuged in a table top centrifuge at 3000 x g for 15min at room temperature. A 1% boric acid solution containing 0.165% methyl red and 0.0825% bromocresol green, and a saturated potassium carbonate solution were also made for the test.

2mL supernatant from the centrifuged sample was added to the outer ring of the homemade Conway cells and 2mL boric acid (1%) solution were added to the inner ring. 2mL saturated potassium carbonate was transferred to the supernatant in the outer ring and the lid was then directly closed and incubated for 60min in 37°C. During incubation, a 0.002M hydrochloric acid (HCl) solution was prepared.

After incubation 200µL of 0.002M HCl, at the time, was transferred to the inner ring of the incubated homemade Conway cells. HCl was added to the inner ring until a color change from green to pink appeared, and from this added volume, the TVB-N value in mg/100mL process water was calculated, according to the formula below. It is important to not add too small amounts of HCl at the time, since a distinct color change then could be harder to recognize.

$$TVB\text{-}N\ (mgN/100g)\ = \frac{((Vs - Vg) * (NHCl * AN) * Ve * 100)}{Ws}$$

- Vs is the titration volume of 0.002M HCl (mL) added to the sample
- Vg is the titration volume of 0.002M HCl (mL) added to the blank.
- NHCL is the molality of HCl used (0.002M)
- AN is the atomic weight of nitrogen (14)
- Ws is the weight of the sample (g)
- Ve is the volume used of 4% TCA solution, for the extraction

Chemicals needed to measure the amount of total volatile basic nitrogen, for steam and main process water, can be found in Appendix H.

3.2.10 Replication and Statistical Analysis

Each analysis was performed in duplicate on each sample type. To evaluate the significance between the data from each date and within each sampling, for all parameters of main and steam process water, the Minitab 17 program was used. In Minitab 17 a two-side ANOVA combined with a Tukey test including a confidence level at 0.95 was performed and visualized by raised letters above each standard deviation in Appendix B. The Tukey test was mainly used to investigate when a significant difference between the sample and the control (i.e. the fresh non-stored sample), of each parameter and sampling, occurred.

4 Results and Discussion

In section 4, the result are described and visualized in graphs under each subtitle. A discussion is also included within each result, to evaluate and draw conclusions from the data found.

4.1 pH

When storing shrimp process waters in a 4°C refrigerator, a change of the pH may occur, which could be an indicator of that something has changed the freshness of the water. Figure 14 presents the pH recorded during 18 days storage, at 4°C, for both main and steam process water. The pH for both waters decreased during 18 days of storage. However the relative change in pH for the steam water (from 9.0 to 8.4) was less compared to the main water (from 8.6 to 7.2), which could be due to the higher amount of protein in the steam water, providing buffering capacity.

The pH of steam water, Figure 14A, from sampling 2 was higher than from sampling 1 and during the first 10 days it was quite stable around pH 9. However, after 18 days of storage it had reached 8.73. The pH for sampling 1 of steam water, was also stable for the first 10 days, around 8.9, followed by a decrease during the last two measurements, were the last measurement (day 18) gave a pH of 8.44. No significant differences ($p<0.05$) in pH were found, within each sampling, during the first 10 days, neither for the first nor the second steam water sampling. However, day 13 and 18 were significantly different ($p<0.05$), both compared to each other and to the first 10 days (Appendix B).

In graph 14B, the same measurement was performed for the main water. Sampling 2 was slightly higher at start (pH 8.63) compared to sampling 1 (pH 8.53), same as in the steam water case, but for both samplings of main water, the pH decreased much faster. Already the third, respectively fourth day of sampling, a significant difference ($p<0.05$) from the 0 day (control) sample had occurred in the main water (Appendix B). After 18 days, the pH for sampling 1 and 2 had decreased to around 7.20.

The pH of the shrimp process waters were high, compared to the pH of shrimp muscle (*Pandalus borealis*). Both waters started off at a pH around 9, while pH 7.7 was recorded in chilled, boiled and peeled shrimps *Pandalus borealis,* packed in modified atmosphere (Mejlholm et al, 2005). In the article by Zeng et al (2005), the pH of cooled shrimps was 7.4, which further conclude that the pH of the shrimp muscle itself is lower than the process waters used in this project. This difference is not unexpected due to the fact that the pH of tap water used for processing of shrimps was around 8.5. Also calcium carbonate of the shells may be released elevating the pH.

During cold storage of shrimps an increase in pH has been shown in the article by Zeng et al (2005), which correlated with an increase in microbial growth and TVB-N value (Zeng et al, 2005). However, the results of this master degree project showed that the pH decreased during storage. The decrease in pH of the process waters could be due to the microbial growth, seen in section 4.8. In microbial tests made in parallel to this MSc project, a much higher microbial growth in the main water was shown, compared to the steam water, and this may be correlated to the larger decrease in pH for the main water compared to the steam water.

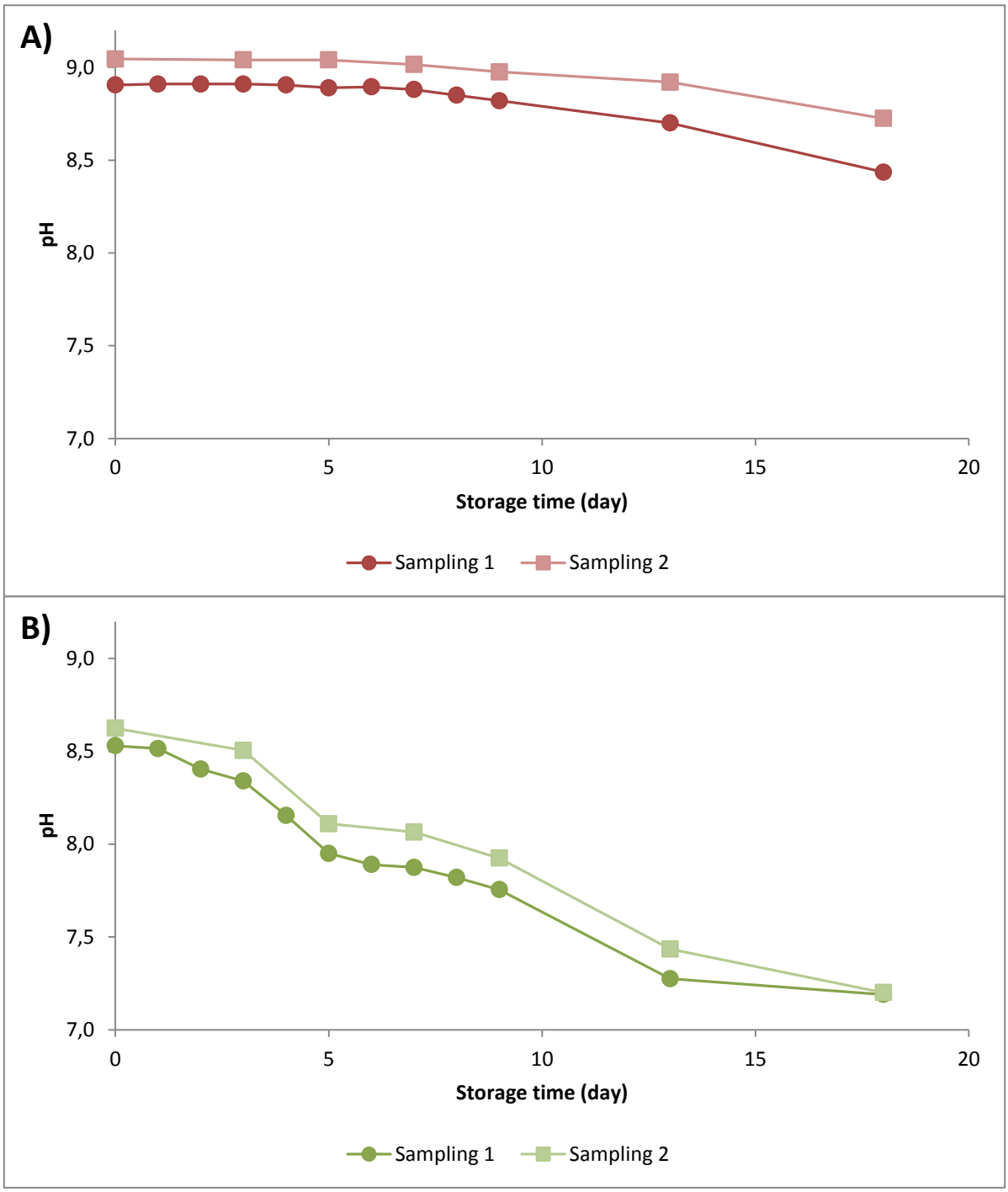

Figure 14. A) The pH change over time in steam water, B) pH change over time in main water. ● describes the result from the first sampling and ■ describes the result from the second sampling. The standard deviation (n=2) for each sampling points included as a bar.

4.2 Ionic Strength

The ionic strength (%) was examined to evaluate if any changes during refrigerated storage, of the shrimp process water, occurs. In Figure 15, the ionic strength in main (graph B) and steam (graph A) process water over time are visualized. As can be seen in graph A, the ionic strength values recorded for the steam water were higher in sampling 2 compared to sampling 1. Sampling 1 started off at 0.73% and sampling 2 at 0.92% NaCl equivalents. Over time the ionic strength for steam water was relatively stable for both samplings, and significant ($p<0.05$) changes, from the 0 day, took place at day 8, for sampling 1, and at day 18, for sampling 2 (Appendix B).

In the main water, as in the steam water, the ionic strength was higher in sampling 2, which is not surprising due to the fact that the main water also includes the steam water, and that the main and steam water within each sampling came from the same batch of shrimps. The ionic strength was however, lower in main water. In sampling 1, the ionic strength for the 0 day was 0.02% compared to 0.04% in sampling 2. The ionic strength values for main water exhibited higher changes (from 0.02 to 0.08% and from 0.04 to 0.12%) compared to the steam water (from 0.72 to 0.80% and from 0.92 to 0.95%), with a significant difference after 4 and 5 days of storage for samplings 1 and sampling 2, respectively.

When comparing the graphs for pH and ionic strength, an inverse correlations appears, were the ionic strength increased over time and the pH, instead, decreased. Even more interesting is the fact that a more sharp increase in ionic strength, as can be seen in the main water, correlated with a more sharp decrease in pH, for the same water. This can be compared to the steam water were both the ionic strength and the pH changes were smaller.

The most reasonable explanation to the increased ion strength, during refrigerator storage, is the bacteria available in the shrimp process waters, seen in section 4.8. The bacteria concentration increased during storage, more in main than steam process water, as can be seen in Figure 23 and 24. This may give an increased change in ion concentration, due to release of ions during protein degradation. Also enzymatic proteolysis, caused by proteases, could have influenced the ionic strength.

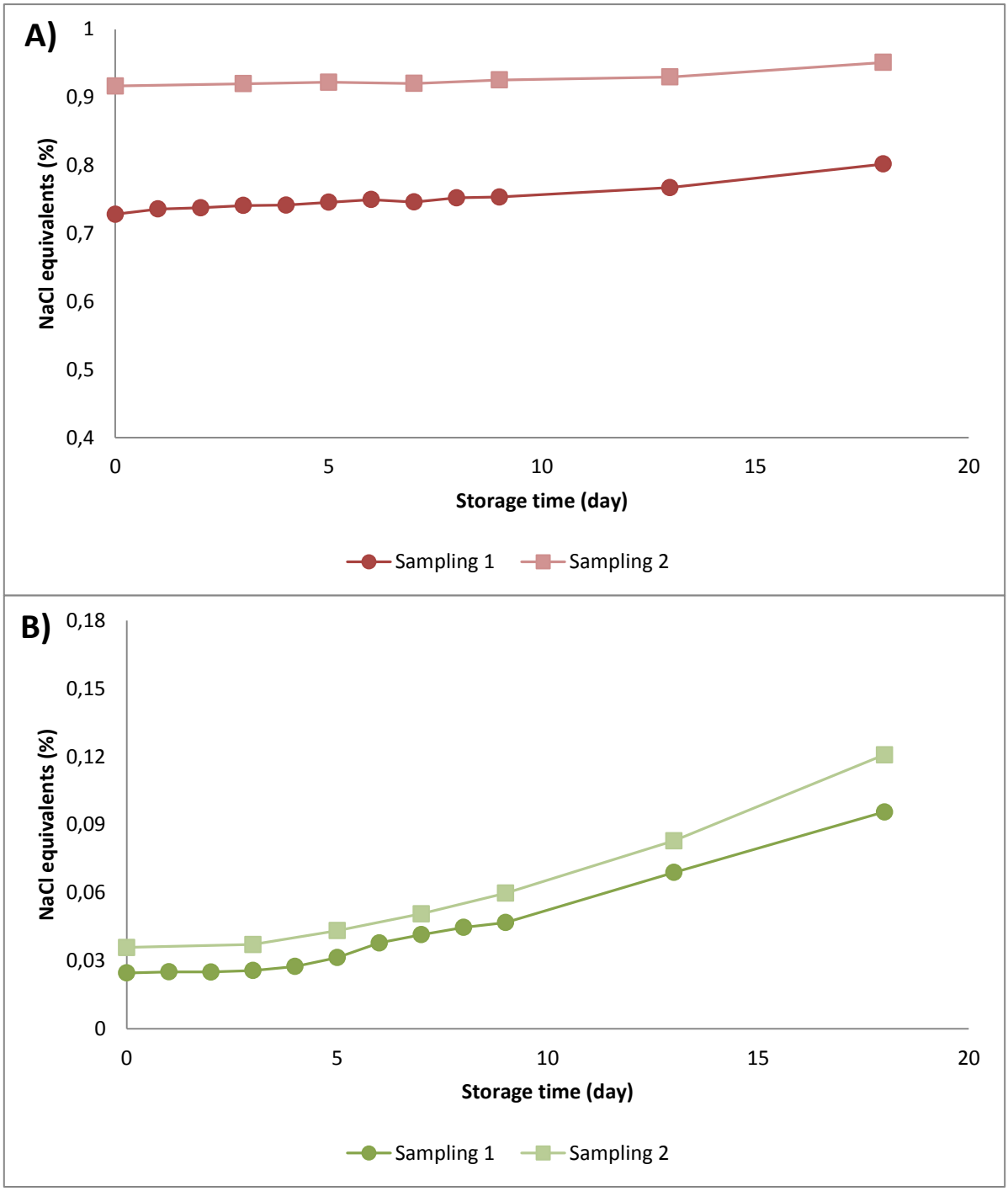

Figure 15. A) The ionic strength (%) over time in steam water, B) ion strength over time in main water. ● describes the result from the first sampling and ■ describes the result from the second sampling. The standard deviation (n=2) for each sampling point included as a bar.

4.3 Sensory Evaluation of Odor Changes

In the sensory test, both samplings of main and steam water were evaluated to determine if any differences between each sampling could be found, and to determine when the human nose perceive changed intensity of certain odor characteristics; “shellfish”, “fishy”, and “boiled shrimp”, agreed upon in a training session, during storage.

From the results a big variation in odor of shellfish, fishy and boiled shrimp were perceived among the panelists, which gave a result with large variation. This makes it hard to draw any detailed conclusions on how the shrimp process waters changed in odor from one date to another. However some conclusions could be drawn.

In Figure 16A, the difference in fishy and shellfish odor is shown over time in main water for both sampling 1 and sampling 2. A clear difference between both samplings could be recognized. Day 0 and day 2 of sampling 1 had strong smell of shellfish with no or small elements of fishiness. The same dates for sampling 2 indicates less shellfish odor and instead an increased smell of fishiness. Small changes within each sampling occurred until day 6, when some panelist started to comment that the water did not smell so good, and that something had happened with the water. At day 6 the fishy smell also started to increase and the shellfish went in the opposite direction. When evaluating the sample of day 7, all participants sensed a dramatic increase in the strength of fishy aroma in both samplings, compared to earlier dates, and words like “old”, “spoiled” and “bad smell”, showed up in the comment area. In the two last days (day 8 and day 9) the smell of shellfish was low and the fishiness was high for all participants. The whole panel now commented that the main process water was “spoiled”, “microbial” and “extremely bad”. Significant difference over time was only observed in sampling 1 for fishy odor (Appendix B). The other storage points could not be separated from each other because of the large standard deviation.

In graph 16B the difference in shellfish and boiled shrimp odor between each day for steam water is shown for both sampling 1 and sampling 2. No clear difference between the two samplings could be found, and also not regarding the comments in the evaluation list. There were also no significant ($p<0.05$) changes seen during the first 10 days. However the two extreme storage points (day 13 and day 18) decreased significantly ($p<0.05$), from the 0 day, and comments like “sour”, “little microbial” and “changed smell” were mentioned from all panelists, compared to the words “good”, “fresh”, “not bad” and “sea” that were given in the first 10 days of storage.

The change in odor of the waters could be correlated to both the changes in TVB-N and in lipid oxidation, which is further discussed under section 4.5 and 4.7.

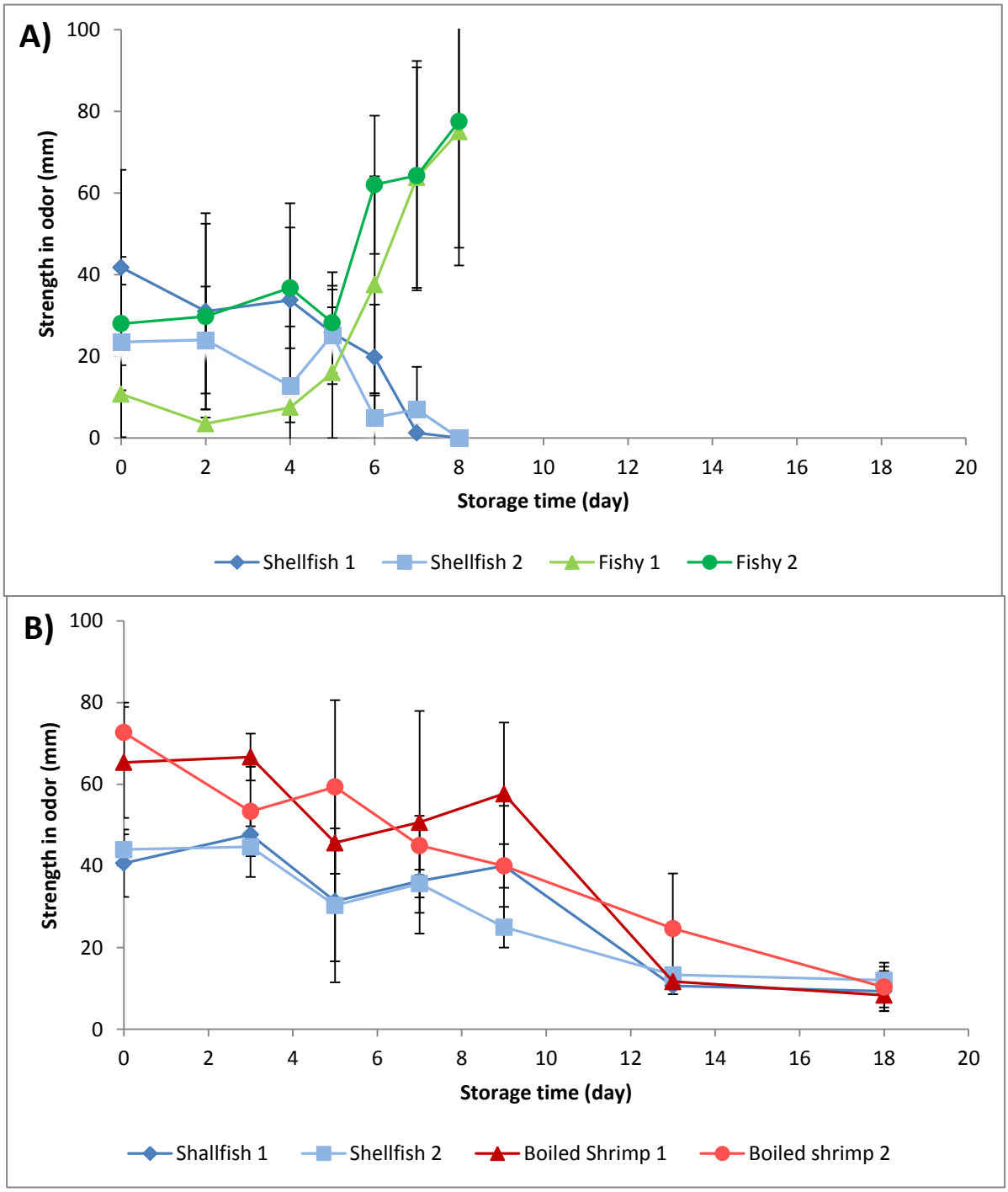

Figure 16. A) The change in odor for main water, and B) steam water. ● describes the smell of boiled shrimps in steam water of sampling 2. ▲ describes the smell of boiled shrimps in steam water of sampling 1, ■ describes the smell of shellfish in sampling 2 and ◆ describes the smell of shellfish in sampling 1. ● describes the result of fishy odor, in sampling 2 and ▲ describes the smell of fishy odor, in sampling 1. The standard deviation for each sampling points included as an error bar. n=3 for steam water and n=4 for main water.

4.4 Color Measurement

To investigate if the color was affected during storage of main and steam shrimp process water, a small color test were done for sampling 1, with L*, a* and b*-values being visualized in Figure 17. In the result for steam process water, Figure 17A, all values were stable over the whole storage period with value around 45, 3 and 6, respectively.

In graph 17B, the color change of main water is shown. The result was similar as for the steam water, but the L-value was lower, around 37. The a-values were around 7 and the b-values were found in the interval 3-6. No significant changes ($p<0.05$) were seen overtime. That the main water just had slightly lower L*-values, but visually was much lighter than the steam water, could be due to the fast particle sedimentation in main water, impacting the result by giving a stronger measured color to the main water.

To find positive a* and b*-values, were not surprising, due to the orange and red color tone for the main, but especially the steam water. The color comes from the pigment astaxanthin.

Earlier studies have shown color changes in shrimps during different kinds of storages, but from our knowledge no study about color change in shrimp process water has been performed. The color change in shrimps is mainly caused by the instability of the antioxidant astaxanthin (Bak et al, 1999). In the article by Niamnuy et al (2008) color change was investigated in shrimps (*Penaeus indicus*), which were dried and then stored under different oxygen conditions (air or vacuum) and storage temperatures (4, 15 and 25°C). The smallest changes were in the sample stored under vacuum and 4°C. In another article by Armenta & Guerrero-Legarreta (2009) the change in astaxanthin concentration, extracted from fermented shrimps (*Litopenaeus vannamei*), during storage under different strengths of light (dark, reduced light and full light), temperatures (5, 25 and 45°C) and oxygen availabilities (air, reduced oxygen, non-oxygen conditions) were investigated. The conditions were evaluated separately, and the smallest change occurred during dark storage, low temperature and absence of oxygen, where just a very small decrease in astaxanthin concentration was seen after 8 weeks storage. Since the shrimp process water in this project was stored in 4°C, darkness and in presence of air, the change in astaxanthin concentration and color can be supposed to not affect the water so much as if the water had been stored in light and higher temperature in combination with the air (Niamnuy, 2008; Armenta & Guerrero-Legarreta, 2009). The relatively low lipid oxidation during storage, which can be found in section 4.5 could also explain the lack of redness loss in this study. A higher lipid oxidation degree had produced more free radicals, which had made more astaxanthin to work as an antioxidant to protect the product against the free radicals. This could lead to a decreased amount of astaxanthin in the product and therefore an increased color change of the product (Bak et al, 1999).

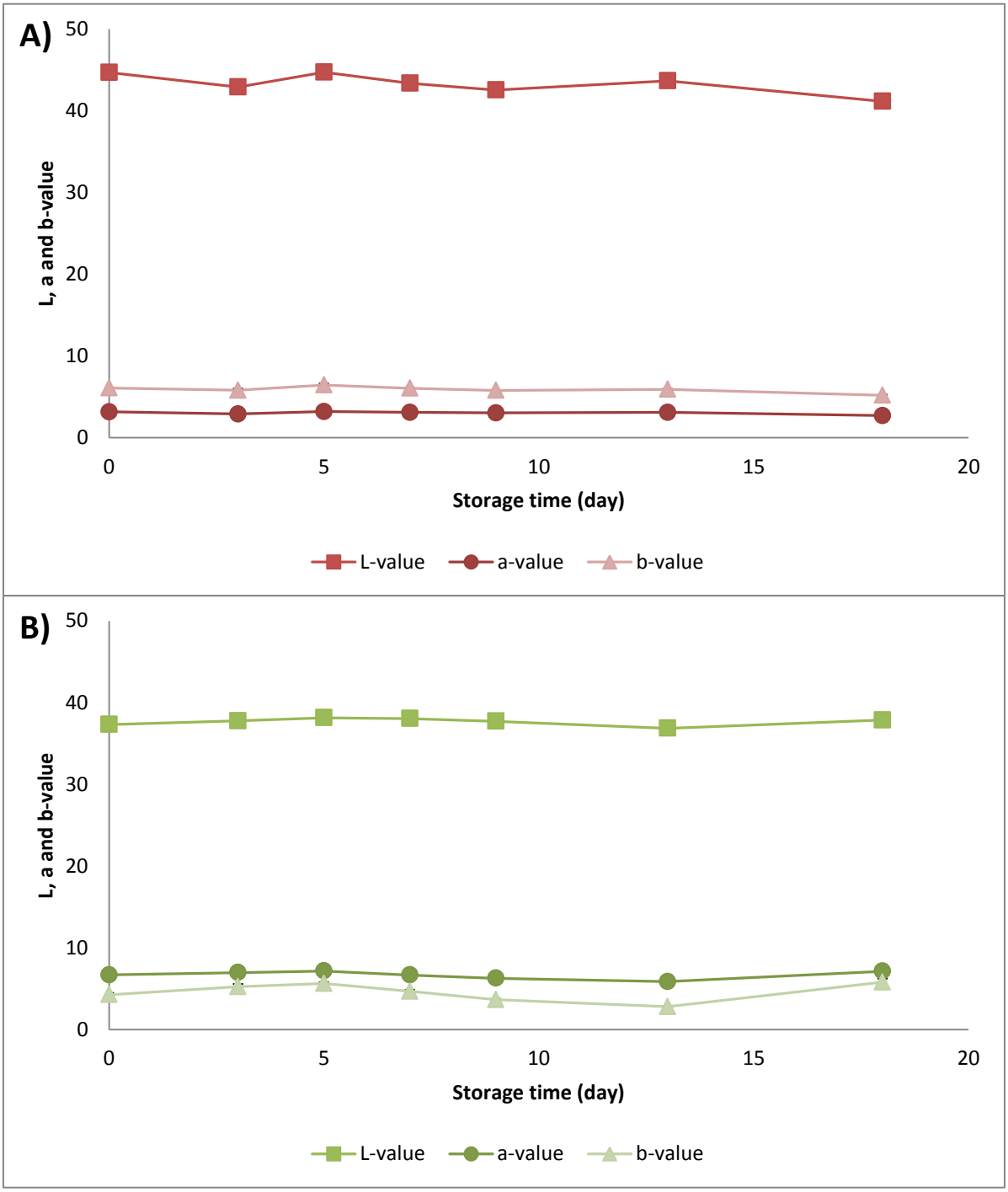

Figure 17. A) The color change over time measured in L, a and b-values for sampling 1 of steam process water, B) The color change over time measured in L, a and b-values for sampling 1 of main process water. ● describes the a-value result, ■ describes the L-value result and ▲ describes the b-value result. Both A) and B) only have results from the first sampling. The standard deviation (n=5) for each sampling point was included as a bar.

4.5 Primary and Secondary Lipid Oxidation Products; PV and MDA

Lipid oxidation, an inevitable phenomenon during food storage, could affect both the flavor as well as the healthiness of the product. It is crucial to analyze lipid oxidation through measurement of both primary and secondary products, since it is a complex reaction. A PV test was performed to investigate the change in lipid hydroperoxide concentration in the process waters, which is a primary product in the lipid oxidation reaction. The results from the PV test were visualized in Figure 19.

For the steam water the PV increased from day 0-8, and then decreased from the day 9-18. For the main water the PV increased from day 0-4 and then decreased from day 4-18. A problem with the PV test, especially for the steam water, was that the color of the steam water highly impacted the results, as can be seen in the lower part of Figure 18, which could make the absolute data untrustworthy. Relative changes can however still be monitored.

Figure 18. Upper jar shows the main shrimp process water and lower jar shows the steam shrimp process water.

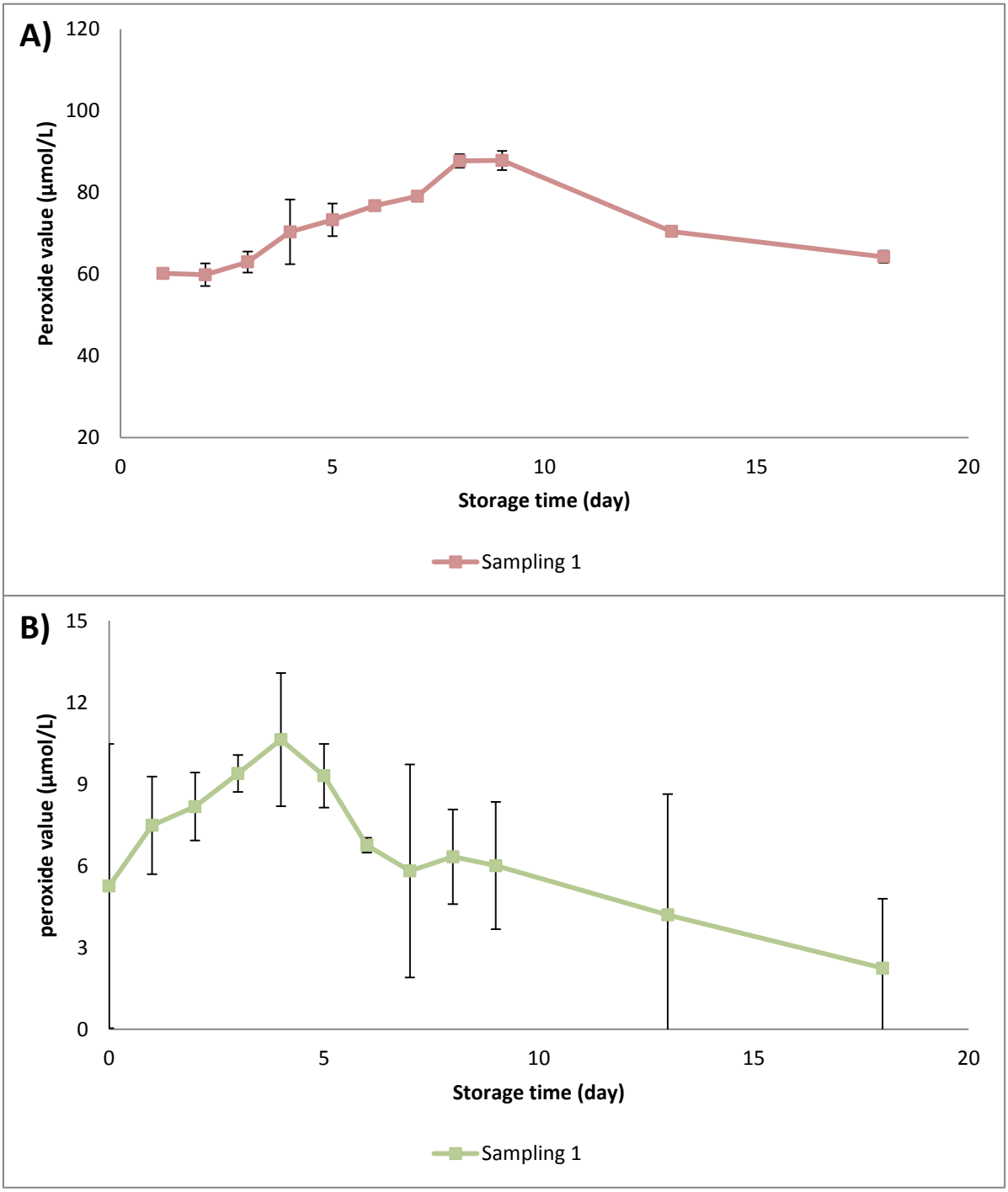

Figure 19. A) The peroxide value change over time measured in steam process water, and B) the peroxide value change over time measured in main process water. ■ describes the first sampling examination. The standard deviation (n=2) for each sampling was included as an error bar.

Figure 20A, presents the change in MDA concentration for sampling 1 and sampling 2 in steam water. An increased MDA value during storage indicates the production of secondary lipid oxidation products. No significant differences ($p<0.05$) within each sampling could be seen over time (Appendix B). The MDA concentration of the first sampling and the second sampling ranged from 0.69 to 0.85µmol/L and from 0.29 to 0.42µmol/L, respectively.

In graph 20B, were the MDA concentration of the main water of sampling 1 and sampling 2 are shown, a clear increase with prolonged storage time could be seen. In the control (day 0) the MDA concentration was low (0.23µmol/L for sampling 1 and 0.60µmol/L for sampling 2), but already after the second test date (day 3) an indication of increased concentration could be visualized. Sampling 1 was here 0.46µmol/L and sampling 2 was 0.80µmol/L. The concentration of MDA continued to increase for both samplings, until around day 13. After this, the MDA concentration started to level out. The MDA concentrations at day 18 for sampling 1 and 2, in main water were 1.82 and 3.19µmol/L, respectively. A significant increase ($p<0.05$) from the control could be seen already day 3 in sampling 1 and from day 7 in sampling 2 (Appendix B). As been described in section 2.3.1.3, primary products transform into secondary products during the lipid oxidation reaction, which means that the peroxide value should increase at the beginning, and when the primary products then starts to decrease, the secondary products e.g. MDA increase instead. This trend was seen in the main water, where the peroxide value (Figure 19B) increased for the first 5 days and then started to decrease. At the same time the MDA concentration (Figure 20B) had a higher increase from day 5, which could be due to the breakdown of the primary products to secondary products. No correlation between the PV and MDA concentration change could be seen for the steam water. Here, the peroxide value increased until day 10 before it decreased. The MDA value was constant during the whole sampling period.

When comparing the PV at day 18 with a similar study on herring process water, from presalting of herring, and a lipid oxidation stability test on catfish sausages during 12 days of refrigerator storage, it seems to be low (Undeland, 2014; Intarasirisawat et al, 2014). Since the main water at day 18 contained high amounts of bacteria (section 4.8) and had a strong bacterial smell, this may indicate that bacteria are a bigger reason to spoilage of the main water compared to lipid oxidation after extended storage. However, when comparing the fishy odor change, Figure 16, with the MDA change of main water, a correlation could be identified, which may signal that the small lipid oxidation increase was more involved in the sensory change of the main process water, early on during the storage.

MDA was recently measured in cod liver oil during gastrointestinal (GI) in vitro digestion with human GI-juices, using the same LC-MS method as was used here (Tullberg et al, 2016). The MDA concentration at start was then around 0.2µmol/L, which is similar to the MDA concentration of the steam and main process water; at start of storage; 0.24-0.85µmol/L. These comparisons conclude the relevance of the start values, since the MDA value should be low, for the control, regardless which product examined.

The shrimp process water field is still an untapped area, and earlier research within this area has mainly been on molecular composition of shrimp process water or to extract the right aroma from the cooking juice (Cros et al, 2006; Vandanjon et al, 2002; Pérez-Santín et al, 2013; Guillou et al, 1995). Still the shrimp itself or the peels are the most research parts, i.e. to extract the natural

astaxanthin from the peels of the shrimp. Former investigations on lipid oxidation in shrimp process water have not been found, but older studies has shown that the cooking juice, which is the same as the steam water, are rich in proteins, minerals and antioxidants, but also contained some lipids, due to the leaching from the shrimps during boiling (Pérez-Santín et al, 2013; Heu et al, 2003). That no significant ($p<0.05$) increase of MDA could be found in steam water, despite lipids in the water, can be due to the high amount of the antioxidant, astaxanthin, which can be found both in the shrimp and also in the steam water (Pérez-Santín et al, 2013). The study from Pu & Sathivel (2010) confirms that astaxanthin impact lipid oxidation, since it significantly decreased the PV when adding extracted astaxanthin from crayfish to flaxseed oil. The addition of astaxanthin in that study also show an effective antioxidant activity when heating the oil from 30 to 60°C, which can be comparable to the boiling process for shrimps (Pu & Sathivel, 2010).

For the main water, where the MDA concentration was similar to that of the steam water control, but then increased more with time than for steam water, Figure 20B, the lower concentration of astaxanthin, indirectly monitored as lower orange color of the main water, can be a reason.

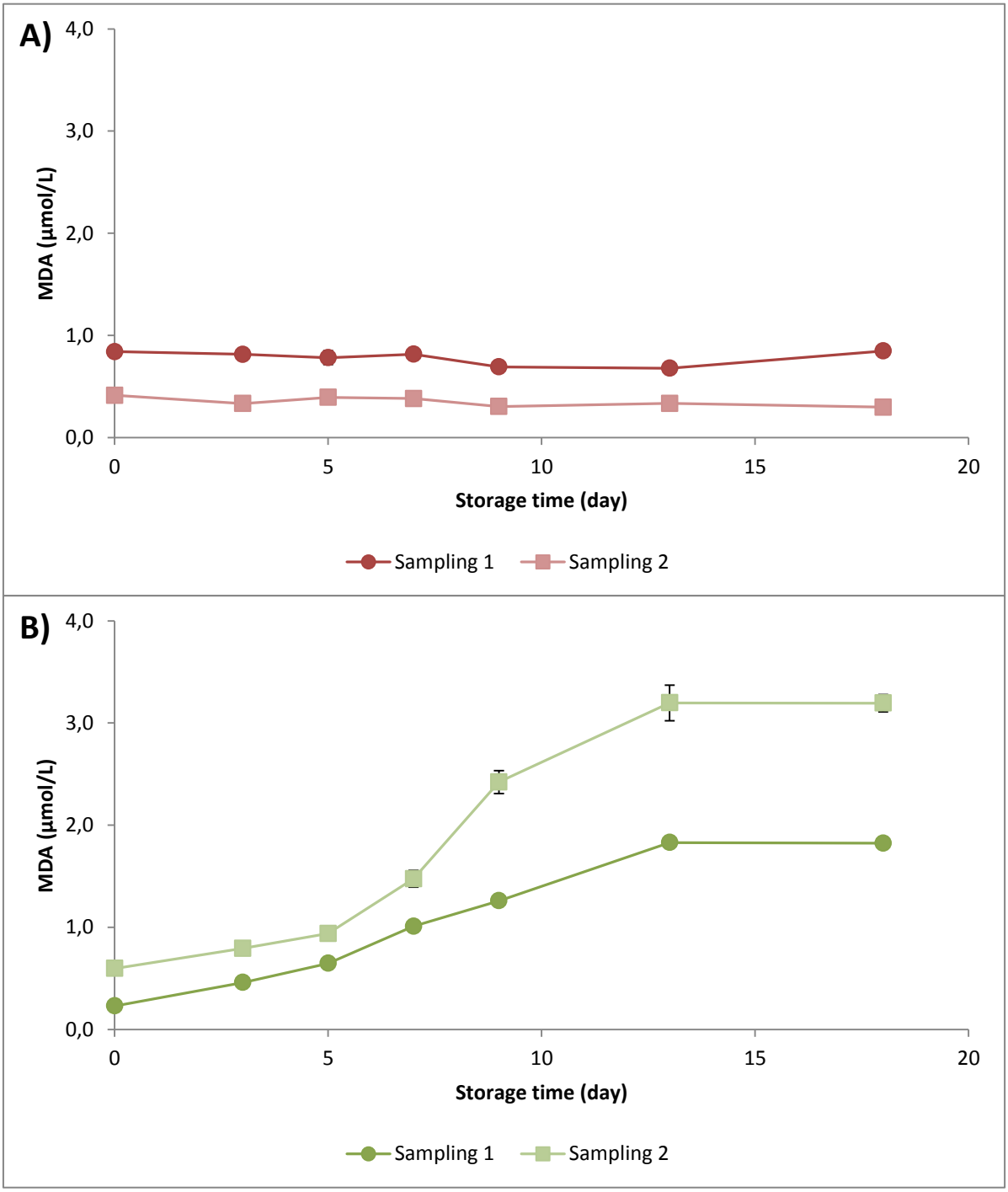

Figure 20. A) The MDA change over time measured in steam process water, and B) is the MDA change over time measured in main process water. ● describes the first sampling and ■ describes the second sampling examination. The standard deviation (n=2) for each sampling was included as an error bar.

4.6 Total Protein

If the numbers of bacteria increase in the stored process waters, the amount of protein can decrease, due to the bacterial breakdown of proteins and usage of protein as a nutrient. Therefore the protein breakdown could indicate spoilage of a product. It could however also indicate enzymatic hydrolysis of the protein. In Figure 21, it could be seen that the amount of proteins was higher in the steam water (graph A) compared to the main water (graph B), which is realistic since main water is much more diluted than the steam water.

Figure 21A shows that the amount of protein measured in the control was 17.3mg/ml for sampling 1 and 13.6mg/ml for sampling 2, respectively. The protein concentration fluctuated during the whole 18 days of storage, but the values did not vary significantly. When comparing the protein concentration of the steam water control with earlier measurements within the department, a 2 fold and even 3 fold differences could be found. This might be due to the seasonal variations of the process waters as well as the performance of the methods used, which could cause an underestimation of the protein concentrations.

The protein concentration of the main water, Figure 21B, followed a different trend from the steam water. The protein concentration of main water at day 0 (control) was 1.7mg/ml for sampling 1 and 1.3mg/ml for sampling 2. The protein concentration values fluctuated the first 10 days of sampling, before it started to decrease during the last two extreme point measurements (day 13 and day 18). At the last test day (day 18) the protein concentration of the main process water had decreased to 1.3mg/ml in sampling 1 and to 1.0mg/ml in sampling 2. Day 13 and day 18 show a significant difference ($p<0.05$) between each other and the control. Main water had both a lower concentration of protein and a higher concentration of bacteria, than steam water, which makes it reasonable to have a faster decrease in protein content compared to the steam water.

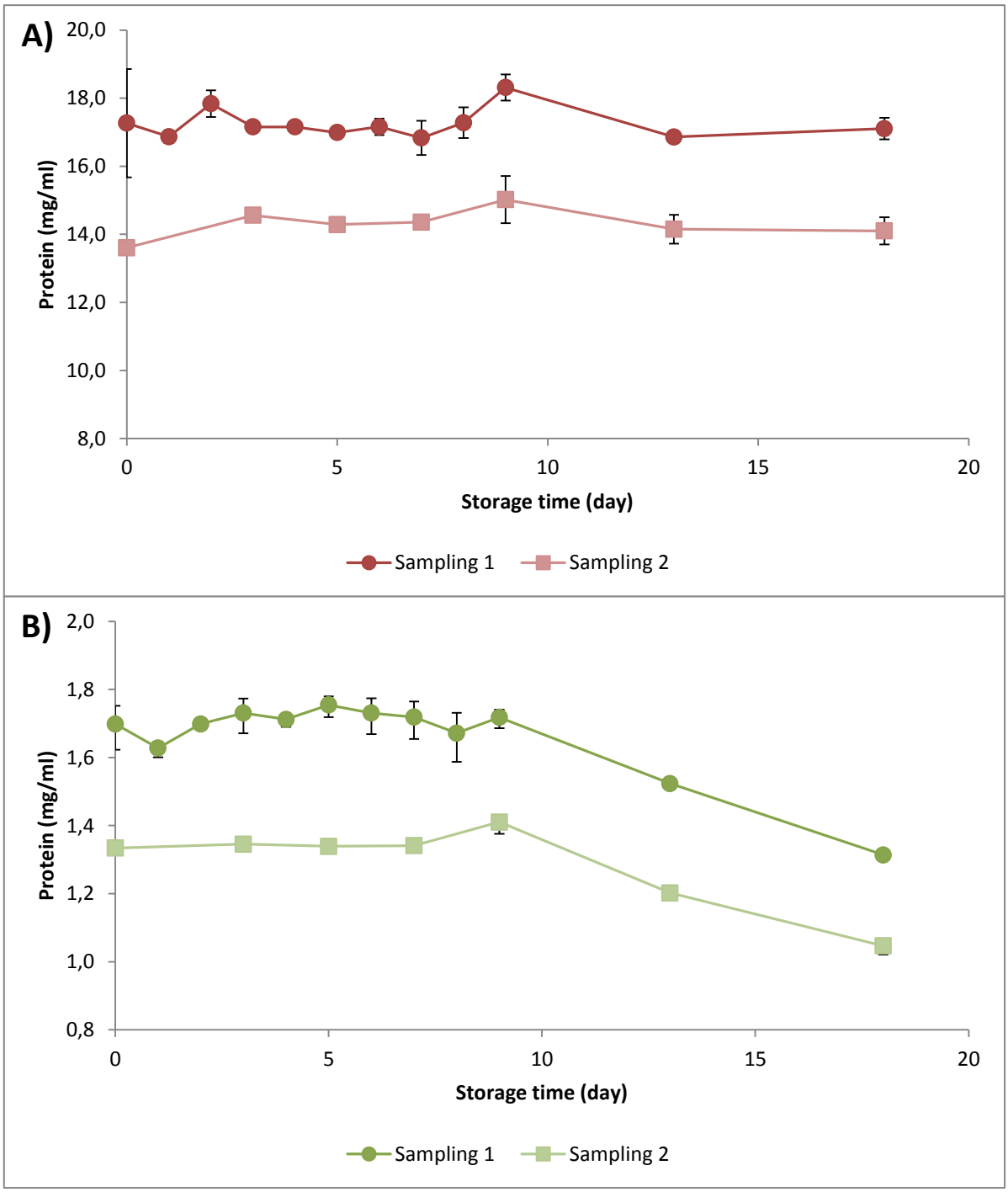

Figure 21. A) The protein change over time measured in steam process water, and B) in main process water. ● describes the first sampling and ■ describes the second sampling examination. The standard deviation (n=2) for each date was included as a bar.

4.7 TVB-N Analysis

One of the most used methods to investigate the degree of freshness of muscle food, is the TVB-N analysis where the total volatile basic nitrogen is examined. Figure 22 presents the TVB-N values for sampling 1 and sampling 2 of steam (graph A) and main (graph B) shrimp process water.

The TVB-N amount at day 0 for both sampling 1 and 2 of steam water was found to be 5.88mg N/100ml. Similar results were given in the article of Pérez-Santín et al (2013), where another kind of test was performed to investigate the TVB-N concentration of steam water from shrimps (*Penaeus spp*). In their article, the concentration TVB-N in fresh steam water from *Penaeus spp* was 10mg N/100ml (Pérez-Santín et al, 2013). As can be seen in Figure 22A the TVB-N values, in steam water, were stable during the first 10 days, and no significant difference could be identified in this time period. When measuring TVB-N day 13 and day 18 a changed concentration started to appear. In sampling 1 a significant change ($p<0.05$) appeared already day 13 with an even more significant change at day 18, compared to sampling 2 were a significant change ($p<0.05$) just appeared at day 18 (Appendix B), compared to the control. The TVB-N value at day 18, for sampling 1, was 16.8mg N/100ml and for sampling 2, 10.5mg N/100ml. However, the TVB-N values, given from the two extreme points, day 13 and day 18, were still below the acceptable limit for TVB-N in shrimps (Zeng et al, 2005). The acceptable value of TVB-N for fish and shrimps are 30mg N/100g, but during the years, investigations have shown TVB-N values over 30mg N/100g for shrimps in the fresh state (Zeng et al, 2005). Different methods also give different result, which must be kept in mind when comparing to other results and to acceptable limits for food.

In Figure 22B the TVB-N concentration in sampling 1 and 2 of main water is plotted. The concentration at day 0 for both samplings was lower than the steam water, 0.84mg N/100ml, which correlates with a less strong smell of the main water. The stronger smell of steam water give rise to more volatile compounds, which may impact the TVB-N result. The TVB-N value was quite stable for the first days, but after 6-7 days significant ($p<0.05$) changes (Appendix B) occurred for both samplings of the main water. This increase of the TVB-N concentration continued for every sample taken and at day 18, the TVB-N value had increased to 10.92mg N/100ml for sampling 1 and 13.44mg N/100ml for sampling 2.

The TVB-N concentration in process water from marine food has also been examined for herring process water in the report by Undeland (2014), by use of the same method as in this report. Here the TVB-N was compared depending on the time the herring had been in contact with the brine, which differ to this project were the TVB-N was measured after storage time without the shrimps present in the water. In a 3% herring salt brine, the TVB-N concentration was found to be 4mg N/100mL for the control (0 day) (Undeland, 2014), which is in the same range as the control for the main and steam shrimp process waters. In the report by Undeland, a sample was also taken after 22h contact with herring fillets, for the 3% herring salt brine, and the TVB-N concentration had then increased to 12.58mg N/100mL. The different numbers between the two studies could depend on the differences in nature of the samples examined.

The change in TVB-N value and the changed odor recognized through a sensory test often goes hand by hand, since more volatile nitrogen in the sample also gives a changed smell of the sample. If comparing the results for the steam water found in the sensory analysis, Figure 16A, with the results in Figure 22A and the significance given from TVB-N test in Appendix B, a remarkable similarity could be seen. In Appendix B, for the TVB-N of steam water, a significant difference can be visualized only for the extreme points (day 13 and day 18), which agree with the sensory test were only a clear change in smell occurs for the last two samples, compared to the 0 day sample. In the main water, a significant increase in TVB-N value occurs around day 6-7, Appendix B. In the sensory test of main water, some panelist started to sense a changed smell at day 6, and at day 7 all panelists commented, on the comment line, that a different/bad smell has been formed.

The main water did not have the same stability as the steam water, but was still under the acceptable limit of TVB-N for shrimps, which can be discussed since the main water during the last days had an extremely bad smell, and still it was not over the limit (Zeng et al, 2005). This may be due to a leakage through the lid during incubation or that volatile basic nitrogen was giving a stronger odor sensation when available in the shrimp process waters, compared to the actual shrimp. The result can also reflect that other compounds than TVB-N were responsible for the strong smell.

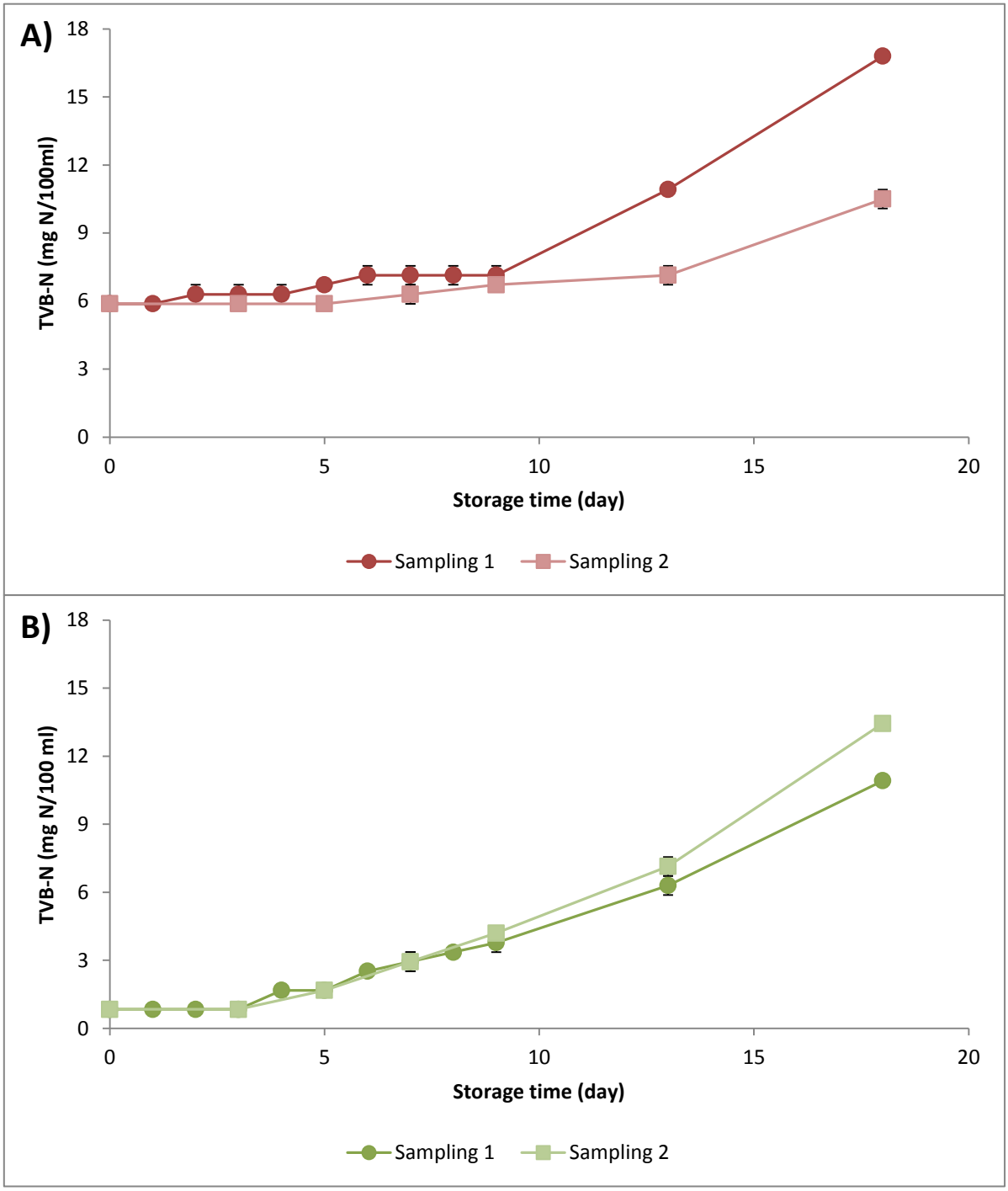

Figure 22. A) The TVB-N change over time measured in steam process water, B) The TVB-N change over time measured in main process water. ● describes the first sampling and ■ describes the second sampling examination. The standard deviation (n=2) for each date was included as a bar.

4.8 Bacterial Growth

To end up this result and discussion session, 2 graphs from the parallel microbial study, performed by Bita Forghani Targhi, will be shown (Figure 23 and Figure 24) and discussed, to compare these outcomes with the chemical and sensory results of the present MSc project.

In Figure 23, two types of aerobic bacteria are shown that were grown on iron agar plates at 20°C. They represent both sulfide producing bacteria and non-sulfide producing bacteria. In the main water both the sulfide and non-sulfide producing bacteria were higher at day 0 compared to the steam water, and the amount then increased fast to a stable bacteria concentration, after 7 days of storage. For the steam water the non-sulfide producing bacteria were low and constant for the first 3 days, but then the amount started to slowly increase until day 18 were the non-sulfide bacteria were as many as in the main water. The sulfide producing bacteria in steam water were stable on a low level during the whole sampling period.

The biggest difference between the main and steam waters in Figure 23 were the sulfide producing bacteria, which is a bacterium that highly impacts the odor and gives it a bad smell. The bad odor appeared around day 5-6, which correlated with the change in sulfide producing bacteria, due to the fast increase up to day 5 and then it was stable for the rest of the sampling period. The sensory evaluation change also correlated with the change of non-sulfide producing bacteria, but since sulfide producing bacteria are known to release an egg like smell, which was in fact picked up in the oldest main water samples, it is more feasible to believe that those bacteria had bigger impact on the odor change.

In Figure 24, psychrotolerant bacteria grown on H&M medium at 15°C are shown, for both main and steam water. The main water had a higher concentration of these bacteria, at day 0, compared to the steam water. The psychrotolerant bacteria concentrations in main water slowly increased during the sampling periods, until day 13, where it levelled out. In the steam water, the concentration increased more rapidly during storage, and at day 18 the concentration of psychrotolerant bacteria in steam and main water were close to the same. The psychotolerant bacteria test show how much marine bacteria that were left and also reflect bacteria from the processing line.

The bacteria concentration was higher in the main water than the steam water, which can have an impact on the odor of the process waters, and the TVB-N values. A large concentration of bacteria could also impact the nutritional value, degrading proteins, and it may also impact the ionic strength and the pH by increasing the ions exposed to the water from i.e. protein degradation, and by producing acid, respectively.

An explanation to the higher concentrations of bacteria in main water, compared to steam water, could be due the larger transport distances through the pipes in the factory, for the main water, which can give more openings for bacterial contamination. Another reason could be that the natural antimicrobial substances had been more diluted in the main water, which increased the possibility for the bacteria to grow.

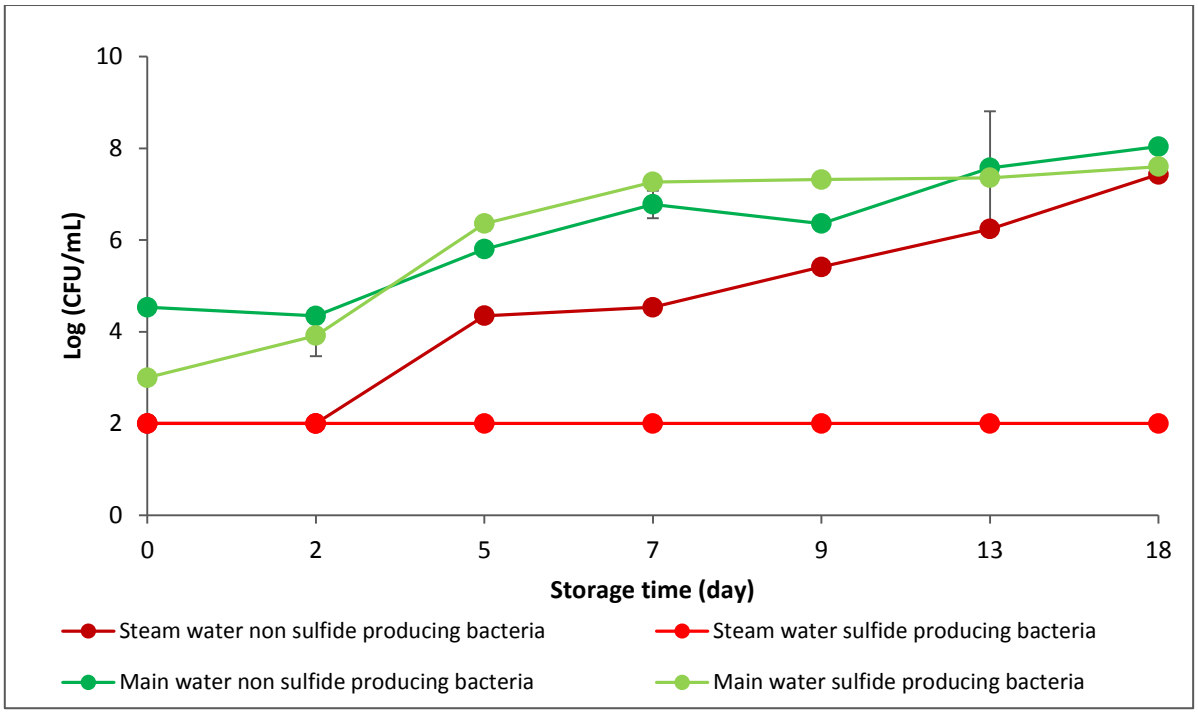

Figure 23. ● describes the change of sulfide producing bacteria in steam process water for sampling 1. ● describes the change of non-sulfide producing bacteria in steam process water for sampling 1, ● describes the change of sulfide producing bacteria in main process water for sampling 1, ● describes the change of sulfide producing bacteria in main process water for sampling 1. These aerobic bacteria were grown on iron agar at 20°C.

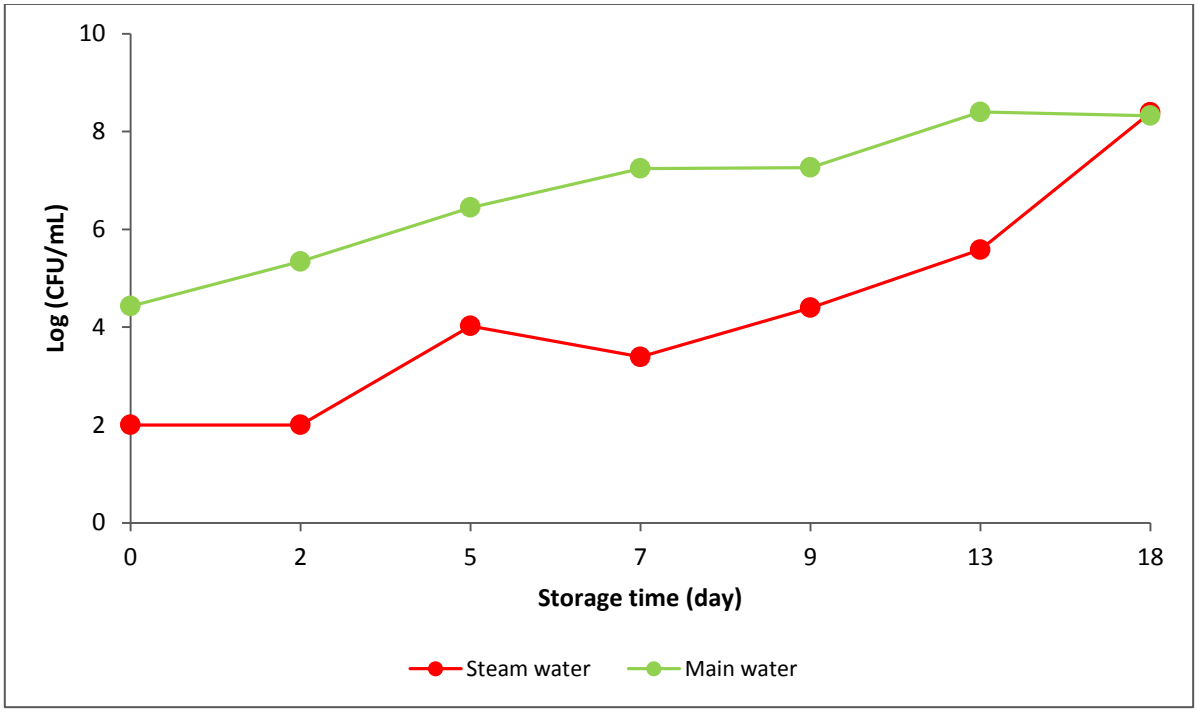

Figure 24. ● describes the change of psychrotolerant bacteria in steam process water for sampling 1, ● describes the change of psychrotolerant bacteria in main process water for sampling 1. These psychrotolerant bacteria were grown on H&M medium at 15°C.

5 Conclusion

Several compounds can have an impact on the stability of the shrimp process waters and therefore, several methods and experiments have been performed to evaluate the stability during the storage trial.

The steam water showed a high stability during this evaluation and in some tests even the longest stored samples (day 13 and day 18) were very similar to the control (0 day). The pH was stable for the first 10 days and started to decrease after this. The ionic strength was also stable the first 10 days, before slightly starting to increase. In the sensory evaluation no big differences could be recognized over the first 10 days, but during the last two days a clear loss of shellfish and boiled shrimp odor was found. Regarding the color, no significant decrease or increase over time was found, which also were the case for lipid oxidation (PV and MDA) and total protein. The TVB-N content was stable during 10 days but it exhibited an increment on the 13^{th} and 18^{th} day of storage. From this investigation it could be concluded that the steam water is a very stable water, since it could be stored for 10 days at 4°C, without any large chemical or physical changes, probably due to the boiling procedure, which kills most of the bacteria, and its high amount of antioxidants.

The main water was stable up to 4 days of storage, before starting to change. The pH was relatively stable the first 4 days, before decreasing dramatically. The same could be seen for the ionic strength, but in the opposite direction. The color did not show any significant changes over time, and the sensory evaluation revealed a changed odor from day 6; fishiness then rapidly increased and shellfish decreased. The PV peaked at day 4 and in the MDA test the concentration increased from day 7. Furthermore, the protein concentration was stable except for the two last storage points (day 13 and 18), which exhibited lower protein concentrations. The TVB-N values significantly increased from day 6 or 7 depending on the sampling, which like the changes in MDA could be correlated to the changes in the sensory test. To conclude, the main water was just stable for a few days under 4°C storage before it started to change both chemically and microbially.

Overall, it can be concluded that steam and main water varied remarkably in terms of stability due to large differences in their composition and in bacteria amounts available. This study gives an important basis for further value adding to the waters, and how fast after generation of the water that such secondary processes must take place.

6 Future Attempts

In this project, several methods have been performed to evaluate chemical changes that can impact the quality of the shrimp process water over time, during cold storage. Several parameters have been evaluated, but since this research had a time limit, the stability of some parameters have not been tested, which can be seen as future attempts.

In the future, it would be very interesting to examine the astaxanthin amount and monitor its changes during storage, due to the fact that astaxanthin is the major antioxidant preventing lipid oxidation. It is also the main colorant in the shrimp process waters; therefore, it would be interesting to look for a correlation between color, oxidation and the change in astaxanthin concentration during cold storage.

Vitamin E can be found in high amounts in *Pandalus borealis,* according to Table 1, and therefore it is of high interest to look at this parameter to further evaluate the oxidative stability of the process waters during storage. Loss of vitamin E is regarded an important and early marker of lipid oxidation.

In this project two samplings of each water (main and steam) were performed, which is too little if trying to draw conclusions on how much the process water can differ between shrimps captured throughout the year. Instead samples should then be taken every month over a year, to investigate the impact of different compounds i.e. the protein content, fatty acid content and composition as well as astaxanthin content on the storage ability of the waters. If biomass isolated from the process water in a near future shall be used in fish feed or as a food ingredient for human use, is it of high relevance to know how the macromolecule content and how the storage capacity change over the year.

In future studies, it would also be interesting to evaluate the dimension of the storage container and how that impacts the stability of the waters, since the amount of soluble oxygen could be affected by the container dimension.

In this project the PV test was suffering from interference by endogenous red color, due to the colorimetric method used. In future tests another method has to be used, since the PV is a great indicator of lipid oxidation, but the test should not measure in the red color range. Also the sensory testing could be repeated to get more reliable results. A larger panel is to be recommend, since more people with the right practice in sensory evaluation will give a more accurate and reliable results.

7 References

AATCC. 1997. Color Technology in the textile industry. Second Edition. Committee RA36 Color Measurement test methods American Association of textile Chemists and Colorists. 1997.

Agbor, G A et al. 2014. Folin-Ciocalteau Reagent for Polyphenolic Assay. Int J Food Sci Nutr Diet. 2014, 3(8), pp 147-152.

Alberts, B et al. 2008. Molecular Biology of THE CELL, Fifth Edition. Garland Science, Taylor & Francis Group, 2008.

Ali, M Y et al. 2010. Post mortem variation in Total Volatile Base Nitrogen and Trimethylamine Nitrogen between Galda (Macrobrachium rosenbergii) and Bagda (Penaeus monodon). BanglaJOL. 2010, 28, pp 7-10.

Andersson, M. Docent. Department of Biology and environmental science. University of Gothenburg. Engineering of plant lipids, lecture, 2 December 2015.

Armenta, R E & Guerrero-Legarreta, I. 2009. Stability Studies on Astaxanthin Extracted from Fermented Shrimp Byproducts. J. Agric. Food Chem. 2009, 57, pp 6095-6100.

Bak, L S. 1999. Effect of modified atmosphere packaging on oxidative changes in frozen stored cold water shrimp (Pandalus borealis). Food Chemistry. 1999, 64(2), pp 169-175.

Barron, J J et al, 2006. The Effects of Temperature on pH Measurement. Technical Services Department, Reagecon Diagnostics Ltd. Retrieved 12 April 2016, from http://reagecon.com/pdf/technicalpapers/Effects_of_Temperature_on_pH_v4-_TSP-01-2.pdf

Bender, D A. 2014. A Dictionary of Food and Nutrition. Fourth edition. Oxford University Press.

Bligh, E G & Dyer, W J. 1959. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Canadian Journal of Biochemistry and Physiology. 1959, 37(8), pp 911-917.

Britannica Academic. 2016. Retrieved 13 February 2016, from http://academic.eb.com.proxy.lib.chalmers.se/EBchecked/topic/542046/shrimp

Brown, E E. 1983 World Fish Farming: Cultivation and Economics. Second Edition. MA: Springer US; 1983, pp 1-3 & 82-91.

Cammack, R et al. online 2006. Oxford Dictionary of Biochemistry and Molecular Biology (2 ed). Oxford University Press. doi 10.1093/acref/9780198529170.001.0001

Cavonius, L R et al. 2014. Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. Anal Bioanal Chem. 2014, 406, pp 7313-7322.

Chakravarti & Bhattacharya. 2005. a handbook of CLINICAL PATHOLOGY. Fifth edition. Academic Publishers.

Cheesbrough, M. 2005. District Laboratory Practice in Tropical Countries. Second edition, Part 1. Tropical Health Technology. Cambridge University Press. 2005, pp 152-153.

CHEMIR. 2016. Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis. CHEMIR Evans Analytical group. Retrieved 25 March 2016, from http://www.chemir.com/liquid-chromatography-mass-spectrometry.htmL

Coleparmer.com. 2016. 430 pH Meter, Instruction Manual. Coleparmer. Retrieved 24 January 2016, from http://www.coleparmer.com/Assets/manual_pdfs/58905-00-05.pdf

Coultate, T P. 2009. FOOD, The Chemistry of its Components. Fifth edition. RSCPublishing.

Cros, S et al. 2006. Technical and economical evaluation of an integrated membrane process capable both to produce an aroma concentrate and to reject clean water from shrimp cooking juices. Journal of food engineering. 2006, 77, pp 697-707.

Dondelinger, R M. 2011. Spectrophotometers. Biomedical Instrumentation & Technology. 2011, 45(2), pp 139-143.

Engström, E. & Tullberg, C. 2014. Lab manual: Lipid oxidation in food. Department of Chemical and Biological Engineering. Chalmers University of Technology.

Edelstein, S. 2013. FOOD SCIENCE, An Ecological Approach, Jones & Bartlett Learning. Second Edition. 2013, pp 84-113.

Eghtesad, S et al. 2013. Malnutrition in Liver Cirrhosis: The Influence of Protein and Sodium. Middle East J Dig Dis. 2013, 5(2), pp 65-75.

Etienne, M. 2005. Volatile amines as criteria for chemical quality assessment. Report. SeaFoodPlus. Retrieved 31 January 2016, from http://archimer.ifremer.fr/doc/2005/rapport-6486.pdf

Ewald, G et al. 1998. Differences between Bligh and Dyer and Soxhlet Extractions of PCBs and Lipids from Fat and Lean Fish Muscle: Implications for Data Evaluation. Marine Pollution Bulletin, 1998, 36(3), pp 222-230.

FAO. 2016. Pandalus borealism. Species Fact Sheets. Food and Agriculture Organization of the United Nations. Retrieved 13 February 2016, from http://www.fao.org/fishery/species/3425/en

fao.org. 2001. Non-Sensory Assessment of Fish Quality. FAO in partnership with Support unit for International Fisheries and Aquatic Research, SIFAR, 2001.

Fernández, J et al. 1997. Thiobarbituric acid test for monitoring lipid oxidation in meat, Food Chemistry. 1997, 59(3), pp. 345-353.

Fjerbaek Sotoft, L et al. 2015. Membrane fractionation of herring marinade for separation and recovery of fats, proteins, amino acids, salt, acetic acid and water. Journal of Food Engineering. 2015, 158, pp 39-47.

Gates, P. 2014. Ion Detectors. University of Bristol, School of Chemistry, Mass spectroscopy Facility. Retrieved 28 March 2016, from http://www.chm.bris.ac.uk/ms/detectors.xhtmL

Giera, M et al. 2012. Recent Advancements in the LC- and GC-Based Analysis of Malondialdehyde (MDA): A Brief Overview. Chromatographia. 2012, 75(9), pp 433-440.

Gringer, N et al. 2015. Recovery of biomolecules from marinated herring (Clupea harengus) brine using ultrafiltration through ceramic membranes. LWT – Food Science and Technology. 2015, 63, pp 423-249.

Grosjean, E et al. 2000. LC/DAD/MS Analysis of Carbonyl (2,4-Dinitrophenyl)hydrazones. Agilent Technologies Innovating the HP Way. 2000.

Guillou, A et al. 1995. Effects of silage preservation on astaxanthin forms and fatty acid profiles of processed shrimp (Pandalus borealis) waste. Aquaculture. 1995, 130, pp 351-360.

Harrington, C R. 1990. Lowry Protein Assay Containing Sodium Dodecyl Sulfate in Microtiter Plates for Protein Determinations on Fractions from Brain Tissue. Academic press Analytical Biochemistry. 1990, 186, pp 285-287.

Harris, D C. 2010. Quantitative Chemical Analysis, Eighth Edition. W. H. Freeman and Company.

Heu, M-S et al. 2003. Components and nutritional quality of shrimp processing by-products. Food Chemsitry. 2003, 82(2), pp 235-242.

Ho, C S et al. 2003. Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. Clin Biochem Rev. 2003, 24(1), pp 3-12.

Hoffmann, E D & Stroobant, V. 2007. Mass Spectrometry Principle and Applications, Third Edition. WILEY.

HunterLab. 2008. Hunter L, a, b Color scale. HunterLab. Retrieved 4 April 2016, from http://www.hunterlab.se/wp-content/uploads/2012/11/Hunter-L-a-b.pdf

Intarasirisawat, R et al. 2014. Effects of skipjack roe protein hydrolysate on properties and oxidative stability of fish emulsion sausage. LWT – Food Science and Technology. 2014, 58(1), pp 280-286.

Jacobsson, A S & Solvika, R. Svenska matfiskars och skaldjurs historia. Författares Bokmaskin Stockholm. 2010, pp 107 & 232-324.

José Navas, M et al. 2013. An Overview of the Kjeldahl Method of Nitrogen Determination. Part II. Sample Preparation, Working Scale, Instrumental Finish, and Quality Control. Taylor and Francis Group, LLC. 2013, 43, pp 224-272.

Khater, E-S G et al. 2014. Physical and Mechanical Properties of Fish Feed Pellets. Food Processing & Technology. 2014, 5(10), pp 1-6.

Kim, S-K. 2013. Marine Proteins and peptides Biological Activities and Applications. First edition. Wiley-Blackwell.

Konica Minolta. Color Measurement Solutions for the Food Industry. Retrieved 4 April 2016, from http://sensing.konicaminolta.us/learning-center/case-studies/KMUS_6June_FoodGuideBrochure.pdf

Larsson K et al. 2012. Oxidation of Cod Liver Oil during Gastrointestinal in Vitro Digestion. Journal of Agriculture and Food Chemistry. 2012, 60, pp 7556-7564.

Lawless, H T & Heymann, H. Sensory Evaluation of Food: Principles and Practices. Springer Science & Business Media. 2010.

Lee, C M. Trevino, B & Chaiyawat, M. 1996. A simple and rapid solvent extraction method for determining total lipids in fish tissue. J. AOAC Intl. 1996, 79(2), pp 487-492.

Livsmedelsverket. 2015. Fleromättat fett, omega-3, omega-6. Retrieved 15 February 2016, from http://www.livsmedelsverket.se/livsmedel-och-innehall/naringsamne/fett/fleromattat-fett-omega-3-och-omega-6/?_t_id=1B2M2Y8AsgTpgAmY7PhCfg%3d%3d&_t_q=fisk+omega-3&_t_tags=language%3asv%2csiteid%3a67f9c486-281d-4765-ba72-ba3914739e3b&_t_ip=129.16.106.139&_t_hit.id=Livs_Common_Model_PageTypes_ArticlePage/_e522077d-e183-4f3a-9fc2-0d6c6c19a772_sv&_t_hit.pos=1

Livsmedelsverket. 2016a. Version 2016-02-17. Räkor. Retrieved 20 February 2016, from http://www7.slv.se/SokNaringsinnehall/Home/FoodDetails/1395#

Livsmedelsverkets livsmedelsdatabas. Version 2016-02-17. Sill. Retrieved 27 April 2016, from http://www7.slv.se/SokNaringsinnehall/Home/FoodDetails/1241

Lowry, O H et al. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951, 193, pp 265-275.

Lucas, J. 2015. Quick Guide Aquaculture. Current Biology. 2015, 25(22), pp R1064-R1065.

Markwell, M A K et al. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Analytical Biochemistry. 1978, 87(1), pp 206-210.

Mejlholm, O et al. 2005. Shelf life and safety aspects of chilled cooked and peeled shrimps (*Pandalus borealis*) in modified atmosphere packaging. Journal of Applied Microbiology. 2005, 99(1), pp 66-76.

Moselhy, H F et al. 2013. A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. J Lipid Res. 2013, 54(3), pp 852-858.

Nationalencyklopedin. 2016a. Räkor. Retrieved 13 February 2016, from http://www.ne.se/uppslagsverk/encyklopedi/lång/räkor

Nationalencyklopedin. 2016b. Transfettsyror. Retrieved 28 March 2016, from http://www.ne.se/uppslagsverk/encyklopedi/lång/transfettsyror

Nationalencyklopedin. 2016c. Radikaler. Retrieved 28 March 2016, from http://www.ne.se/uppslagsverk/encyklopedi/lång/radikaler

Nationalencyklopedin. 2016d. Proteinomsättning. Retrieved 16 March 2016, from http://www.ne.se/uppslagsverk/encyklopedi/lång/proteinomsättning.

Nationalencyklopedin. 2016e. Konduktivitet. Retrieved 30 January 2016, from http://www.ne.se/uppslagsverk/encyklopedi/l%C3%A5ng/konduktivitet

Nationalencyklopedin. 2016f. Överfiskning. Retrieved 20 January 2016, from http://www.ne.se/uppslagsverk/encyklopedi/l%C3%A5ng/%C3%B6verfiskning

National Cancer Institute. 2013. Top Food Sources of Saturated Fata among US Population, 2005–2006 Nhanes. Applied Research Cancer Control and Population Science. Retrieved 15 February 2016, from http://appliedresearch.cancer.gov/diet/foodsources/sat_fat/sf.htmL

NCBI. 2007. Central Dogma of Biology: Classic view. National Center for Biotechnology Information. Retrieved 14 February 2016, from http://www.ncbi.nlm.nih.gov/Class/MLACourse/Modules/MolBioReview/central_dogma.htmL

NCBI. 2016a. Hydroperoxide, PubChem Compound, Search result. Retrieved 15 March 2016, from https://www.ncbi.nlm.nih.gov/pccompound/?term=Hydroperoxide

NCBI. 2016b. PubChem Compound Database; CID = 10964. Retrieved 15 March 2016, from https://pubchem.ncbi.nlm.nih.gov/compound/10964#section=Top

Niamnuy, C et al. 2008. Kinetics of astaxanthin degradation and color changes of dried shrimp during storage. Journal of Food Engineering. 2008, 87, pp 591-600.

Niessen, W M A. 2006. Liquid Chromatography – Mass Spectrometry, Third Edition. Chromatography Science Series, volume 97. Taylor & Francis Group.

Omega.com. pH Meter, Introduction to pH measurments. OMEGA. Retrieved 24 January 2016, from http://www.omega.com/prodinfo/ph-meter.htmL

Osman, A et al. 2015. Quantification of biomolecules in herring (Clupeaharengus) industry processing waters and theirrecovery using electroflocculation andultrafiltration. Food and Bioproduct Processing. 2015, 96, pp 198-210.

Owen, J. 2005. Overfishing is Emptying World's Rivers, Lakes, Experts Warn. National Geographic. Retrieved 20 January 2016, from http://news.nationalgeographic.com/news/2005/12/1201_051201_overfishing.htmL

Pérez-Santín, E et al. 2013. Compositional properties and bioactive potential of waste material from shrimp cooking juice. LWT – Food Science and Technology. 2015, 54, pp 87-94.

Pickova, J & Mørkøre, T. 2007. Alternate oils in fish feeds. European journal of lipid science and technology. 2007, 109, pp 256-263.

Piedecausa, M A. 2010. Total ammonia nitrogen leaching from feed pellets used in salmon aquaculture. Journal of Applied Ichthyology. 2010, 26, pp 16-20.

Pitt, J J. 2009. Principles and Applications of Liquid Chromatography – Mass Spectrometry in Clinical Biochemistry. Clin Biochem Rev. 2009, 30, pp 19-34.

Pradeep, D J & Dave, K. A Novel. A Novel, Inexpensive and Less Hazardous Acid-Base Indicator. Journal of Laboratory Chemical Education. 2013, 1(2), pp 34-38.

Pu, J & Sathivel, S. 2010. Kinetics of Lipid Oxidation and Degradation of Flaxseed Oil Containing Crawfish (Procambarus clarkii) Astaxanthin. Journal of the American Oil Chemists' Society. 2011. 88(5), pp 595-601.

Radiometer analytical handbook. Conductivity Theory and Practice, Conductivity meter handbook. Retrieved 29 January 2016, from http://www.analytical-chemistry.uoc.gr/files/items/6/618/agwgimometria_2.pdf

Rawdkuen, S et al. 2010. Assessment of protein changes in farmed giant catfish (Pangasianodon gigas) muscles during refrigerated storage. International Journal of Food Science & Technology. 2010, 45, pp 985-994.

Schmedes, A & Hølmer, G. 1989. A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. J. Am. Oil Chem. Soc. 1989, 66, pp 813−817.

SCS. Electron Ionization. University of Illinois at Urbana-Champaign, School of Chemical Sciences. Retrieved 28 March 2016, from http://scs.illinois.edu/massSpec/ion/ei.php

Sensorik och arom. SP Sveriges Tekniska Forskninginstitut. Retrieved 28 February 2016, from http://www.sp.se/sv/units/fb/sensory/Sidor/default.aspx

Sundhell, P et al. 2012. Fakta om Fisk & Skaldjur. Svensk FISK. Retrieved 7 May 2016, from http://sjomatsframjandet.se/wp-content/uploads/fakta-om-fisk-och-skaldjur_webb.pdf

Spickett, C M & Forman, H J. 2015. Lipid Oxidation in Health and Disease. CRC Press, Taylor and Francis Group. 2015, pp 45-46.

Stauth, D. 1998. News and Research Communications. Oregon State University. Retrieved 20 January 2016, from http://oregonstate.edu/ua/ncs/archives/1998/oct/aquaculture-not-easy-answer-overfishing

Steele, R. 2004. Understanding and Measuring the Shelf-life of Food. Woodhead Publishing. E-book. pp 135.

Tortora, G J & Derrickson, B. 2013. Essentials of Anatomy and Physiology, International student version, ninth edition. John Wiley & Sons, Inc. 2013, pp 314-320.

Tsaknis, J et al. 1999. Determination of malondialdehyde in traditional fish products by HPLC. The Analyst. 1999, 124, pp 843-845.

Tullberg, C et al. 2016. Formation of reactive aldehydes (MDA, HHE, HNE) during the digestion of cod liver oil: comparison of human and porcine in *vitro* digestion models. Food Funct. 2016, 7, pp 1401-1412.

Undeland, I et al. 2002. Added Triacylglycerols Do Not Hasten Hemoglobin-Mediated Lipid Oxidation in Washed Minced Cod Muscle. J. Agric. Food Chem. 2002, 50, pp 6847-6853.

Undeland, I. 2014. Värdehöjning av processvatten från sillindustrin. Teknisk Rapport Projekt 622-27588-2012/SJV Jnr 2012-5964. 2014.

Vandanjon, L et al. 2002. Recovery by nanofiltration and reverse osmosis of marine flavours from seafood cooking waters. Desalination. 2002, 144, pp 379-385.

Wfp.org. 2015. Hunger Statistics. United Nations World Food Programme. Retrieved 20 January 2016, from https://www.wfp.org/hunger/stats

Wolff, S P. Free radicals, lipids and protein degradation. Trends in Biochemical Sciences. 1986, 11(1), pp 27-31.

Zeng, Q Z et al. 2005. Quality Change of Shrimp (*Pandalus borealis*) Stored under Different Cooling Conditions. Journal of Food Science. 2005, 70, pp s459-s466.

Öhrvik V et al. 2012. Fisk, skaldjur och fiskprodukter – analys av näringsamnen. Livsmedelsverket, Rapport I – 2012. Retrieved 30 January 2016, from http://www.livsmedelsverket.se/globalassets/rapporter/2012/fiskskaldjur-och-fiskprodukter---analys-av-naringsamnen-rapport-1-2012.pdf

8 Appendices

In Appendix A, the sensory panel evaluation forms used during the sensory test for steam and main water are available, and in Appendix B, all values found in the graphs of the results, the standard deviations and significant tests are placed. In Appendix C-H, dilution series, standard curves and chemicals used in each method are located. Appendix I contains two example calculations, one for the peroxide value and one for the total volatile basic nitrogen.

Appendix A – Sensory Evaluation List

Main **Sensory panelist** _____ **Date** _____ **Time** _____

Sample code _____

Shellfish
None — *Strong*

Comments _____

Fishy

Comments _____

Mineral

Comments _____

Extra comments ----------

Sample code _____

Shellfish
None — *Strong*

Comments _____

Fishy

Comments _____

Mineral

Comments _____

Extra comments ----------

Sample code _____

Shellfish
None — *Strong*

Comments _____

Fishy

Comments _____

Mineral

Comments _____

Extra comments ----------

Steam **Sensory panelist** _____ **Date** _____ **Time** _____

Sample code _____

Boiled shrimp
None *Strong*

Comments _____

Shellfish

Comments _____

Mineral

Comments _____

Extra comments ----------

Sample code _____

Boiled shrimp
None *Strong*

Comments _____

Shellfish

Comments _____

Mineral

Comments _____

Extra comments ----------

Sample code _____

Boiled shrimp
None *Strong*

Comments _____

Shellfish

Comments _____

Mineral

Comments _____

Extra comments ----------

Appendix B – Numerical Values, Standard Deviation and Significance Test for each Date and Process Water, Evaluated by Different Methods

By use of ANOVA together with the Tukey method, available at the minitap17 software, the significances of some parameters, between the different storage times of process water, were compared. The significance was only tested for different storage times, within the same sampling and water. The significance letters for sampling 1 and sampling 2 of one type of process water can therefore not be compared to each other. For the test, a two-side confidence interval and at a confidence level at 0.95 was used. Significance was tested only in the first sampling for PV and color test, and for both samplings when evaluated the pH, conductivity, MDA, protein content and total volatile basic nitrogen concentration. In this session also the values and standard deviation are included. Sampling 1 was tested, for significance, every day for the first 10 days except for MDA test where just 5 days during the first 10 day period were examined. In the second sampling, just 7 days were examined (day 0, day 3, day 5, day 7, day 9, day 13 and day 18) for each method.

pH Measurement

Table 2. pH in steam water tested during 18 days of storage and for both samplings.

pH*												
Steam water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	8.91± 0.00^{a}	8.91± 0.00^{a}	8.91± 0.01^{a}	8.91± 0.00^{a}	8.91± 0.01^{a}	8.89± 0.01^{a}	8.90± 0.01^{a}	8.88± 0.02^{a}	8.85± 0.03^{a}	8.82± 0.02^{a}	8.70± 0.03^{b}	8.44± 0.00^{c}
Sampling 2	9.05± 0.01^{a}			9.04± 0.01^{a}		9.04± 0.02^{a}		9.02± 0.01^{a}		8.98± 0.00^{ab}	8.92± 0.01^{b}	8.73± 0.01^{c}

*, The pH is visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 3. pH in main water tested during 18 days of storage and for both samplings.

pH*												
Main water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	8.53± 0.01^{a}	8.52± 0.00^{a}	8.41± 0.00^{b}	8.34± 0.01^{c}	8.16± 0.00^{d}	7.95± 0.01^{e}	7.89± 0.02^{f}	7.88± 0.00^{fg}	7.82± 0.01^{g}	7.76± 0.01^{h}	7.28± 0.01^{i}	7.19± 0.01^{j}
Sampling 2	8.63± 0.01^{a}			8.51± 0.02^{b}		8.11± 0.02^{c}		8.07± 0.03^{c}		7.93± 0.02^{d}	7.44± 0.02^{e}	7.20± 0.01^{f}

*, The pH is visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Ionic Strength Test

Table 4. Ionic strength (NaCl equivalence (%)) in steam water tested during 18 days of storage and for both samplings.

NaCl equivalence (%)*												
Steam water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	0.7282± 0.0004^{d}	0.7359± 0.0047^{cd}	0.7377± 0.0001^{cd}	0.7411± 0.0004^{cd}	0.7415± 0.0003^{cd}	0.7458± 0.0004^{cd}	0.7497± 0.0005^{bc}	0.7462± 0.0001^{cd}	0.7522± 0.0003^{bc}	0.7535± 0.0005^{bc}	0.7673± 0.0006^{b}	0.8020± 0.0003^{a}
Sampling 2	0.9165± 0.0043^{b}			0.9199± 0.0034^{b}		0.9221± 0.0013^{b}		0.9204± 0.0030^{b}		0.9255± 0.0021^{b}	0.9298± 0.0013^{b}	0.9512± 0.0021^{a}

*, The ion strengths are visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 5. Ionic strength (NaCl equivalence (%)) in main water tested during 18 days of storage and for both samplings.

NaCl equivalence (%)*												
Main water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	0.0246± 0.0002^{i}	0.0251± 0.0001^{i}	0.0251± 0.0001^{i}	0.0257± 0.0004^{hi}	0.0275± 0.0003^{h}	0.0314± 0.0004^{g}	0.0379± 0.0005^{f}	0.0415± 0.0001^{e}	0.0447± 0.0003^{d}	0.0468± 0.0005^{c}	0.0700± 0.0006^{b}	0.0802± 0.0003^{a}
Sampling 2	0.0359± 0.0004^{f}			0.0372± 0.0004^{f}		0.0433± 0.0006^{e}		0.0507± 0.0013^{d}		0.0598± 0.0007^{c}	0.0829± 0.0008^{b}	0.1208± 0.0018^{a}

*, The ion strengths are visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Olfactory Sensory Evaluation

Table 6. Change in odor of shellfish in steam water, tested during 18 days of storage and for both samplings.

Odor of Shellfish (mm)*							
Steam water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	40.67± 8.26^{a}	47.67± 2.05^{a}	31.33± 14.70^{abc}	36.33± 7.85^{ab}	40.00± 5.35^{a}	10.67± 2.05^{bc}	9.33± 4.92^{c}
Sampling 2	44.00± 3.74^{a}	44.67± 7.36^{a}	30.33± 18.84^{ab}	35.67± 3.40^{ab}	25.00± 4.97^{ab}	13.33± 1.70^{b}	12.00± 4.32^{b}

*, The change in smell of shellfish, of the steam water, is visualized, together with the standard deviation (n=3) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 7. Change in odor of boiled shrimps in steam water, tested during 18 days of storage and for both samplings.

Odor of Boiled Shrimps (mm)*							
Steam water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	65.33± 13.60^{a}	66.67± 5.73^{a}	45.67± 13.53^{ab}	50.67± 27.25^{ab}	57.67± 17.44^{ab}	11.67± 0.94^{b}	8.33± 3.86^{b}
Sampling 2	72.67± 7.32^{a}	53.33± 10.96^{ab}	59.33± 21.25^{ab}	45.00± 7.25^{abc}	40.00± 14.72^{abc}	24.67± 13.47^{bc}	10.33± 4.99^{c}

*, The strength in odor of boiled shrimps, in steam water, is visualized, together with the standard deviation (n=3) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 8. Change in odor of shellfish in main water, tested during 18 days of storage and for both samplings.

Odor of Shellfish (mm)*							
Main water	**Storage time (day)**						
	0	**2**	**4**	**5**	**6**	**7**	**8**
Sampling 1	41.75± 23.93^{a}	31.00± 24.01^{a}	33.75± 23.73^{a}	25.75± 10.61^{a}	19.75± 12.89^{a}	1.25± 2.17^{a}	0.00± 0.00^{a}
Sampling 2	23.50± 14.04^{a}	24.00± 13.08^{a}	12.75± 14.57^{a}	25.25± 12.05^{a}	5.00± 5.43^{a}	7.00± 10.46^{a}	0.00± 0.00^{a}

*, The strength in odor of shellfish, in main water, is visualized, together with the standard deviation (n=4) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 9. Change in fishy odor for main water, tested during 18 days of storage and for both samplings.

Odor of fish, ”fishy” (mm)*							
Main water	**Storage time (day)**						
	0	**2**	**4**	**5**	**6**	**7**	**8**
Sampling 1	10.75± 10.57^{bc}	3.50± 1.50^{c}	7.50± 3.64^{c}	16.00± 16.03^{bc}	37.50± 26.55^{abc}	63.75± 26.98^{ab}	75.00± 32.79^{a}
Sampling 2	28.00± 16.34^{a}	29.75± 22.73^{a}	36.75± 14.79^{a}	28.25± 12.34^{a}	62.00± 16.91^{a}	64.25± 28.07^{a}	77.50± 30.92^{a}

*, The strength of fishy odor, in main water, is visualized, together with the standard deviation (n=4) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Color Measurement

Table 10. L-value in steam water tested during 18 days of storage and for the first sampling.

L-value*							
Steam water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	44.678± 0.129^{a}	42.894± 0.486^{cd}	44.720± 0.196^{a}	43.366± 0.203^{bc}	42.530± 0.171^{d}	43.662± 0.241^{b}	41.146± 0.484^{e}

*, The L-values are visualized in the table above, together with the standard deviation (n=5) for each sample. Different letters after each data denotes significant differences (p<0.05).

Table 11. L-value in main water tested during 18 days of storage and for the first sampling.

L-value*							
Main water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	37.326± 0.310^{bc}	37.768± 0.223^{ab}	38.156± 0.224^{a}	38.054± 0.381^{a}	37.724± 0.276^{ab}	36.860± 0.198^{c}	37.878± 0.274^{ab}

*, The L-values are visualized in the table above, together with the standard deviation (n=5) for each sample. Different letters after each data denotes significant differences (p<0.05).

Table 12. a-value in steam water tested during 18 days of storage and for the first sampling.

a-value*							
Steam water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	3.168± 0.091^{a}	2.900± 0.153^{b}	3.200± 0.097^{a}	3.110± 0.041^{a}	3.042± 0.039^{ab}	3.102± 0.064^{a}	2.706± 0.039^{c}

*, The a-values are visualized in the table above, together with the standard deviation (n=5) for each sample. Different letters after each data denotes significant differences (p<0.05).

Table 13. a-value in main water tested during 18 days of storage and for the first sampling.

a-value*							
Main water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	6.696± 0.080^{c}	6.972± 0.117^{b}	7.182± 0.046^{a}	6.680± 0.042^{c}	6.294± 0.025^{d}	5.868± 0.047^{e}	7.142± 0.139^{ab}

*, The a-values are visualized in the table above, together with the standard deviation (n=5) for each sample. Different letters after each data denotes significant differences (p<0.05).

Table 14. b-value in steam water tested during 18 days of storage and for the first sampling.

b-value*							
Steam water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	6.094± 0.034^{b}	5.812± 0.208^{cd}	6.448± 0.179^{a}	6.056± 0.043^{bc}	5.798± 0.044^{d}	5.916± 0.053^{bcd}	5.204± 0.052^{e}

*, The b-values are visualized in the table above, together with the standard deviation (n=5) for each sample. Different letters after each data denotes significant differences ($p<0.05$).

Table 15. b-value in main water tested during 18 days of storage and for the first sampling.

b-value*							
Main water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	4.282± 0.201^{c}	5.248± 0.341^{b}	5.642± 0.189^{ab}	4.686± 0.196^{c}	3.656± 0.130^{d}	2.806± 0.109^{e}	5.816± 0.416^{a}

*, The b-values are visualized in the table above, together with the standard deviation (n=5) for each sample. Different letters after each data denotes significant differences ($p<0.05$).

MDA Evaluation

Table 16. MDA content (µmol/mL) in steam water tested during 18 days of storage and for both samplings.

MDA (µmol/L)*							
Steam water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	0.842± 0.039[a]	0.816± 0.028[a]	0.783± 0.068[a]	0.817± 0.025[a]	0.692± 0.040[a]	0.679± 0.027[a]	0.849± 0.055[a]
Sampling 2	0.415± 0.013[a]	0.334± 0.017[a]	0.395± 0.063[a]	0.383± 0.064[a]	0.305± 0.019[a]	0.336± 0.024[a]	0.299± 0.021[a]

*, The MDA concentrations are visualized in the table above, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 17. MDA content (µmol/mL) in main water tested during 18 days of storage and for both samplings.

MDA (µmol/L)*							
Main water	**Storage time (day)**						
	0	**3**	**5**	**7**	**9**	**13**	**18**
Sampling 1	0.231± 0.001[f]	0.461± 0.021[e]	0.647± 0.001[d]	1.011± 0.015[c]	1.260± 0.031[b]	1.830± 0.002[a]	1.824± 0.040[a]
Sampling 2	0.598± 0.012[d]	0.795± 0.005[d]	0.940± 0.010[d]	1.475± 0.082[c]	2.423± 0.112[b]	3.197± 0.174[a]	3.194± 0.0843[a]

*, The MDA concentrations are visualized in the table above, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

PV Test

Table 18. Peroxide value (µmol/L) in steam water tested during 18 days of storage and for the first sampling.

Peroxide value (µmol/L)*												
Steam water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	101.50± 0.08^{a}	60.17± 2.74^{d}	59.87± 2.55^{d}	62.97± 7.93^{cd}	70.35± 3.97^{cd}	73.28± 1.04^{bcd}	76.76± 1.25^{bcd}	79.04± 1.65^{bc}	87.68± 2.34^{ab}	87.83± 1.15^{ab}	70.45± 1.47^{cd}	64.24± 1.19^{cd}

*, The peroxide values are visualized, together with the standard deviation (n=2) for each sample. Different letters after each data denotes significant differences ($p<0.05$).

Table 19. Peroxide value (µmol/L) in main water tested during 18 days of storage and for the first sampling.

Peroxide value (µmol/L)*												
Main water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	5.26± 3.69^{a}	7.49± 1.27^{a}	8.18± 0.88^{a}	9.39± 0.48^{a}	10.63± 1.73^{a}	9.31± 0.83^{a}	6.76± 0.19^{a}	5.82± 2.76^{a}	6.33± 1.23^{a}	6.01± 1.65^{a}	4.20± 3.13^{a}	2.25± 1.80^{a}

*, The peroxide values are visualized, together with the standard deviation (n=2) for each sample. Different letters after each data denotes significant differences ($p<0.05$).

Protein Content Test

Table 20. Protein content (μg/mL) in steam water tested during 18 days of storage and for both samplings.

Protein (μg/mL)*												
Steam water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	17266± 1594[a]	16867± 186[a]	17838± 389[a]	17155± 82[a]	17155± 14[a]	16990± 169[a]	17159± 242[a]	16831± 502[a]	17280± 449[a]	18312± 384[a]	16857± 109[a]	17106± 319[a]
Sampling 2	13606± 80[a]			14558± 41[a]		14287± 2[a]		14355± 181[a]		15019± 696[a]	14150± 420[a]	14101± 396[a]

*, The protein content is visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 21. Protein content (μg/mL) in main water tested during 18 days of storage and for both samplings.

Protein (μg/mL)*												
Main water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	1699± 54[ab]	1628± 19[ab]	1699± 1[ab]	1731± 42[a]	1712± 15[ab]	1754± 25[a]	1730± 43[a]	1719± 46[a]	1671± 60[ab]	1718± 23[a]	1524± 7[b]	1314± 12[c]
Sampling 2	1334± 6[a]			1345± 17[a]		1339± 1[a]		1341± 9[a]		1410± 35[a]	1202± 11[b]	1046± 26[c]

*, The protein content is visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

TVB-N Analysis

Table 22. TVB-N (mg N/100mL) in steam water tested during 18 days of storage and for both samplings.

TVB-N (mg N/100mL)*												
Steam water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	5.88± 0.00^{c}	5.88± 0.00^{c}	6.30± 0.42^{c}	6.30± 0.42^{c}	6.30± 0.42^{c}	6.72± 0.00^{c}	7.14± 0.42^{c}	7.14± 0.42^{c}	7.14± 0.42^{c}	7.14± 0.42^{c}	10.92± 0.00^{b}	16.80± 0.00^{a}
Sampling 2	5.88± 0.00^{b}			5.88± 0.00^{b}		5.88± 0.00^{b}		6.30± 0.42^{b}		6.72± 0.00^{b}	7.14± 0.42^{b}	10.50± 0.42^{a}

*, The total volatile nitrogen is visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Table 23. TVB-N (mg N/100mL) in main water tested during 18 days of storage and for both samplings.

TVB-N (mg N/100mL)*												
Main water	**Storage time (day)**											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**13**	**18**
Sampling 1	0.84± 0.00^{f}	0.84± 0.00^{f}	0.84± 0.00^{f}	0.84± 0.00^{f}	1.68± 0.00^{ef}	1.68± 0.00^{ef}	2.52± 0.00^{de}	2.94± 0.42^{cd}	3.36± 0.00^{cd}	3.78± 0.42^{c}	6.30± 0.42^{b}	10.92± 0.00^{a}
Sampling 2	0.84± 0.00^{e}			0.84± 0.00^{e}		1.68± 0.00^{e}		2.94± 0.42^{d}		4.20± 0.00^{c}	7.14± 0.42^{b}	13.44± 0.00^{a}

*, The total volatile nitrogen is visualized, together with the standard deviation (n=2) for each sample. Different letters after each data, on the same row, denotes significant differences ($p<0.05$).

Appendix C – Standard Curve, Dilution Series and Chemicals Used for Ionic Strength Evaluation

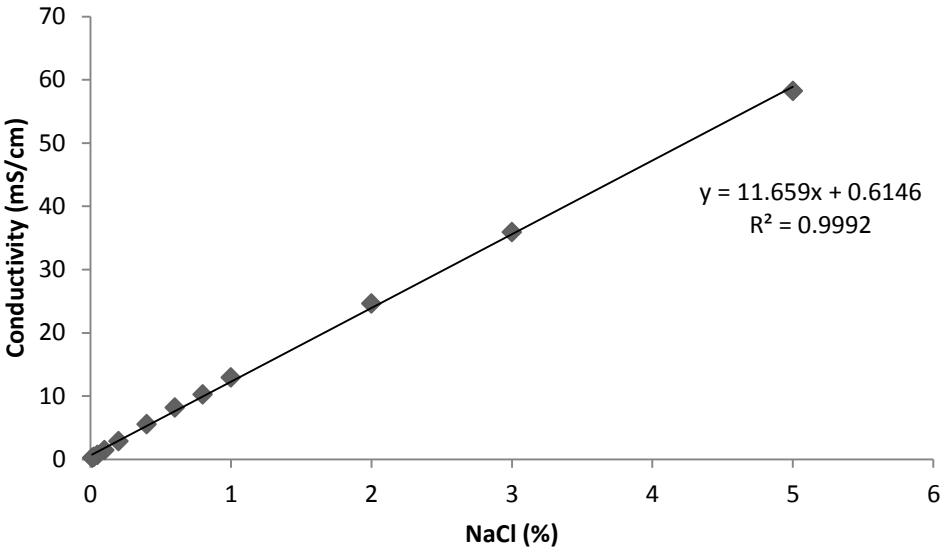

Figure 25. Standard curve formation to examine ionic strengths of main and steam process water. NaCl was used for the standard curve.

Table 24. Dilution series with sodium chloride for ionic strength standard curve formation.

NaCl solution (%)	Mixtures
5	2.5 g NaCl + 47.5mL (distilled H2O)
3	30mL (5% Nal) + 20mL (distilled H2O)
2	33.33mL (3% NaCl) + 16.67mL (distilled H2O)
1	25mL (2% NaCl) + 25mL (distilled water)
0.8	40mL (1% NaCl) + 10mL (distilled water)
0.6	37.5mL (0.8% NaCl) + 12.5mL (distilled water)
0.4	33.33mL (0.6% NaCl) + 16.67mL (distilled water)
0.2	25mL (0.4% NaCl) + 25mL (distilled water)
0.1	25mL (0.2% NaCl) + 25mL (distilled water)
0.05	25mL (0.1% NaCl) + 25mL (distilled water)
0.025	25mL (0.05% NaCl) + 25mL (distilled water)
0.0125	25mL (0.025% NaCl) + 25mL (distilled water)

Table 25. Chemicals used for ionic strength measurement with conductivity meter.

Product name, storage form (size of the storage box and distribution company)	Product number
Sodium Chloide, powder (1kg Sigma-Aldrich)	S9625

Appendix D – Standard Concentrations, Standard Curve and Chemicals Used for MDA Test, Measured by LC-MS

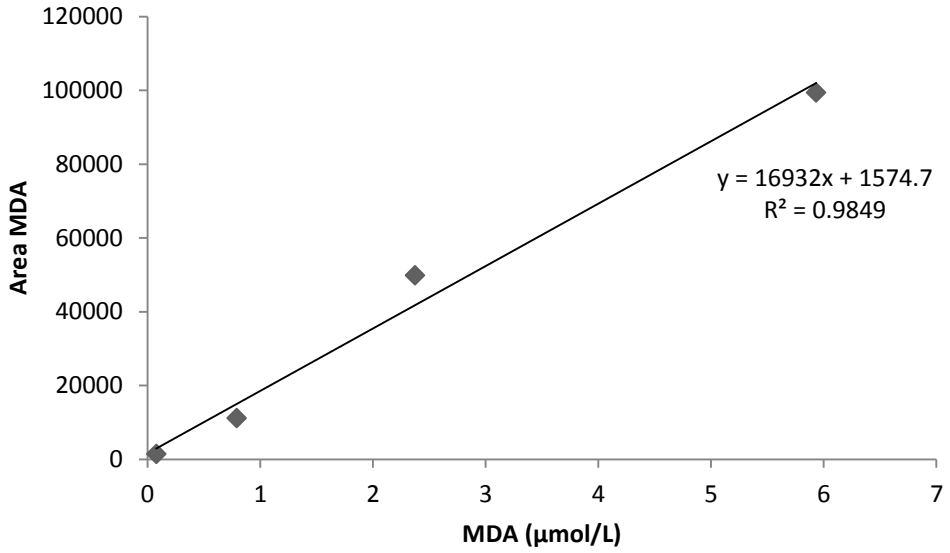

Figure 26. Standard curve formation to examine MDA concentration of main and steam process water, by use of LC-MS. 1.1.3.3-tetraethoxypropane converted to MDA was used for the standard curve.

Table 26. The concentration of MDA/TEP in each standard passed from the extraction and LC-MS analysis.

	MDA concentration [M] (TEP)
Standard 1	0.079
Standard 2	0.791
Standard 3	2.373
Standard 4	5.9325
Standard 5	23.73

Table 27. Chemicals used for extraction and determination of MDA with LC-MS.

Product name, storage form (size of the storage box and distribution company)	Product number
1.1.3.3-tetraethoxypropane (TEP), liquid (0.1L, Sigma-Aldrich)	86570
2.6-di-tert-butyl-4-methylphenol, powder (BHT, 0.1kg, Sigma-Aldrich)	B1378
2-4-Dinitrophenylhydrazine (DNPH) reagent, powder (25g, Sigma-Aldrich)	42210
Acetic acid glacial, liquid (1L, Fisher Scientific)	10171460
Dichloromethane for HPLC, liquid (1L, Sigma-Aldrich)	650463
Ethylenediaminetetraacetic acid (EDTA) synthesis grade, powder (0.5kg, Fisher Scientific)	11394094
Methanol LC-MS, liquid (2.5L, Sigma-Aldrich)	34966
Hydrochloric acid, 37%, liquid (1L, Fisher Scientific)	11362465

Appendix E – Standard Curve, Dilution Series, Reagent Mixture Schedule and Chemicals Used for Protein Content Evaluation

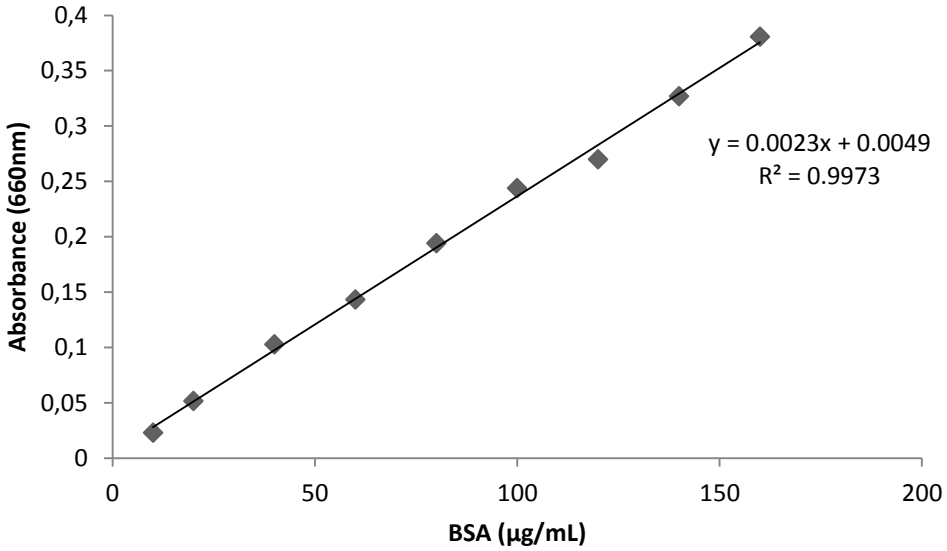

Figure 27. Standard curve formation to examine protein content in main and steam process water. Bovine serum albumin was used for the standard curve.

Table 28. BSA concentration for standard curve during total protein examination by Lowry method.

Bovine serum albumin concentration (mg/mL)	Mixtures
0.160	0.4mL BSA stock solution + 4.6mL (0.1M NaOH)
0.140	0.35mL BSA stock solution + 4.65mL (0.1M NaOH)
0.120	0.3mL stock solution + 4.7mL (0.1M NaOH)
0.100	0.25mL BSA stock solution + 4.75mL (0.1M NaOH)
0.080	0.2mL BSA stock solution + 4.8mL (0.1M NaOH)
0.060	0.15mL BSA stock solution + 4.85mL (0.1M NaOH)
0.040	0.1mL BSA stock solution + 4.90mL (0.1M NaOH)
0.020	0.05mL BSA stock solution + 4.95mL (0.1M NaOH)
0.010	0.025mL BSA stock solution + 4.975mL (0.1M NaOH)

Table 29. The amount of each chemical used, for the reagent preparation step, in the Lowry method.

Reagents	**Mixtures**
Reagent A	2.0% Na_2CO_3
	0.40 % NaOH
	0.16% P- Na-tatarate
	1% SDS
Reagent B	4% $CuSO_4x5H_2O$
Reagent C (FRESH)	1 part reagent B into 100 parts reagent A
Phenol reagent (FRESH)	1 part Folin – Ciocalteu phenol reagent into 1 part distilled water

Table 30. Chemicals used for Lowry method.

Product name, storage form (size of the storage box and distribution company)	**Product number**
Albumin, from bovine serum (BSA), powder (10g, Sigma-Aldrich)	A3059
Cupric sulfate pentahydrate, powder (250g, Sigma Aldrich)	61240
Folin & Ciocalteu's phenol reagent, liquid (0.5L, Sigma-Aldrich)	F9252
Potassium sodium tartrate tetrahydrate reagent, powder (0.5kg, Sigma-Aldrich).	217255
Sodium carbonate anhydrous reagent, powder (1kg, Fisher Scientific)	11388534
Sodium dodecyl sulfate (SDS) reagent, powder (0.5kg, Sigma-Aldrich)	L4509
Sodium hydroxide reagent, pellets (1kg, Fisher Scientific)	11378504

Appendix F – Chemicals Used during Bligh and Dyer Extraction

Table 31. Chemicals used for the Bligh and Dyer extraction.

Product name, storage form (size of the storage box and distribution company)	Product number
Chloroform for HPLC, liquid (2.5L, Sigma-Aldrich)	34854
Methanol LC-MS, liquid (2.5L, Fluka)	34966
Sodium chloride reagent, powder, (1kg, Sigma-Aldrich)	S9625

Appendix G – Standard Curve, Dilution Series and Chemical Used for Peroxide Value Test

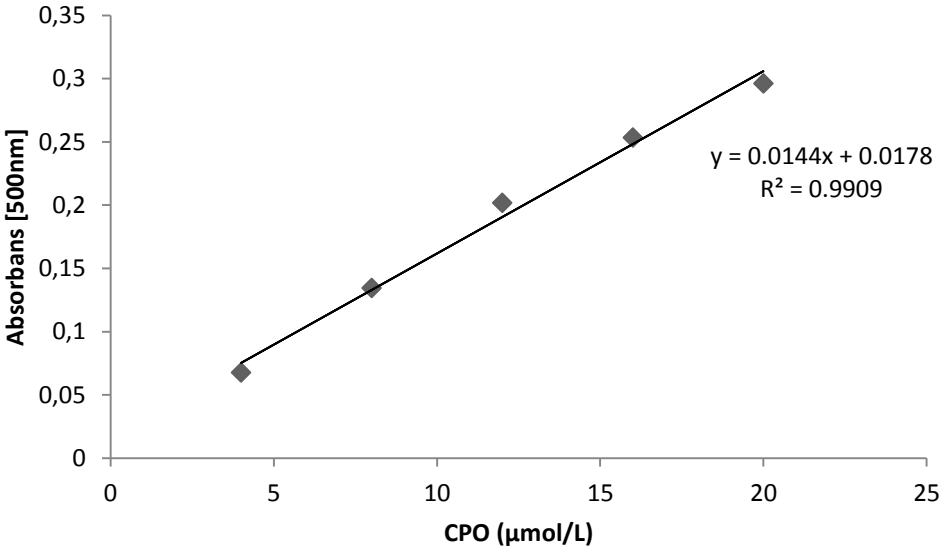

Figure 28. Standard curve formation to examine peroxide value of main and steam process water. Cumene hydroperoxide (CPO) was used for the standard curve.

Table 32. Mix schedule for standard curve construction in peroxide value test.

CPO [µmol/L]	Mixtures
0	5mL Chloroform-methanol (1:1)
4	1mL B + 4mL chloroform-methanol (1:1)
8	2mL B + 3mL chloroform-methanol (1:1)
12	3mL B +2mL chloroform-methanol (1:1)
16	4mL B +1mL chloroform-methanol (1:1)
20	5mL B

Table 33. Chemicals used for peroxide value test.

Product name, storage form (size of the storage box and distribution company)	Product number
Ammonium thiocyanate, powder (0.1kg, Sigma-Aldrich)	A7149
Barium chloride dihydrate, powder (0.1kg, Sigma-Aldrich)	B0750
Chloroform for HPLC, liquid (2.5L, Sigma-Aldrich)	34854
Cumene hydroperoxide (CPO), 80%, liquid (0.1L, Sigma-Aldrich)	C0524
Hydrochloric acid, 37%, liquid (1L, Fisher Scientific)	11362465
Iron (II) sulfate heptahydrate, powder (50g, Sigma-Aldrich)	44970
Methanol LC-MS, liquid (2.5L, Sigma-Aldrich)	34966

Appendix H – Chemicals Used during TVB-N Evaluation

Table 34. Chemicals used to measure total volatile basic nitrogen (TVB-N).

Product name, storage form (size of the storage box and distribution company)	Product number
Boric acid, reagent, powder (0.5kg, Fisher Scientific)	11399873
Bromocresol green solution, liquid (0.5L, Sigma-Aldrich)	02559
Methyl red solution, liquid (0.1L, Sigma-Aldrich)	32941
Potassium carbonate reagent, powder (0.5kg, Fisher Scientific)	11364254
Trichloroacetic acid (TCA) reagent, powder (1kg, Fisher Scientific)	11332605
Hydrochloric acid 37%, liquid (1L, Fisher Scientific)	11362465

Appendix I – Example Calculations

In this session example calculations for PV and TVB-N are visualized. The numbers placed in the formula are constant according to this experimental set up.

Example Calculation for Peroxide Value

$$\frac{B * C * D * tentative\ extra\ dilution * 1000}{L\ shrimp\ water} = \mu mol\ peroxide/L\ sample$$

$$\frac{B * 0.00333 * 1.66 * 1000}{L\ shrimp\ water} = \mu mol\ peroxide/L\ sample$$

- B is µmol peroxide/ L chloroform, and this is the concentration given if the blank absorbance is subtracted from the sample absorbance, and placed into the standard curve.
- C is the amount chloroform used per 1mL sample in the first extraction.
- D is the dilution of the chloroform phase during the peroxide value test.
- Tentative extra dilution is an extra dilution done, if a high peroxide value in the sample is expected.
- L shrimp water is the amount sample used in the extraction, before the PV test is performed.

Example Calculation for TVB-N

$$\frac{((Vs - Vg) * (NHCl * AN) * Ve * 100)}{Ws} = TVB\text{-}N\ (mgN/100g)$$

$$\frac{((Vs - 0) * (0.002 * 14) * 6 * 100)}{4} = TVB\text{-}N\ (mgN/100g)$$

- Vs is the titration volume of 0.002M HCl (mL) into the sample.
- Vg is the titration volume of 0.002M HCl (mL) into the blank.
- NHCL is the normality of HCl used (0.002M).
- AN is the atomic weight of nitrogen (14).
- Ws is the weight of the sample (g).
- Ve is the volume used of 4% TCA solution, for the extraction.