Addis Ababa University

School of Graduate Studies

Microbial, Cellular and Molecular Biology Program Unit

The Effect of Storage Temperature and Time on Bacteriological Load and Physicochemical Quality of Nile Tilapia (*Oreochromis niloticus*) Fillet from Lake Tana, Ethiopia

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Microbiology

By

Tizazu Yitayew

February, 2012

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Dr. Fassil Assefa

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Approved by the Examining Board:

_____ _____

Chairman, Department's Graduate Committee

_____ _____

Advisor

_____ _____

Examiner

_____ _____

Examiner

Declaration

I, the undersigned, declare that this is my original work, has not been presented for a degree in any University, and that all sources of materials used for the thesis have been duly acknowledged.

Name: **Tizazu Yitayew Mengie**

Signature: _____

Place: Addis Ababa

Date of submission: _____

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Table of Contents

List of tables

List of Acronyms and abbreviations

ACC	Aerobic Colony Count
AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of Variance
ATP	Adenosine-triphosphate
CFU	Colony forming unit
DMA	Di-Methyl Amine
FAO	Food and Agricultural Organization
FPME	Fish Production and Marketing Enterprise
ICMSF	International Commission on Microbiological Specifications for Food
LFDP	Lake Fisheries Development Program
SD	Standard Deviation
TMA	Tri-Methyl Amine
TMAO	Tri-Methyl Amine Oxide
TPC	Total Plate count
TVB-N	Total Volatile Basic Nitrogen
TVN	Total Volatile Nitrogen

Abstract

Fish and fish products are one of the food items that are of excellent nutritional source but it is highly perishable unless well preserved. Oreochromis niloticus *(Nile Tilapia) is the most important fish species of Lake Tana fishery. Bacteriological load and physicochemical changes were evaluated during the storage of Nile Tilapia fillets at -18ºC and 5ºC for 30 days. The fish samples were collected from Lake Tana. Enumeration of Aerobic Colony Count, Enterobacteriaceae count and Psychrotrophic count; determination of protein, ash, and moisture contents, pH and TVB-N changes were undertaken. The analyses of all the parameters were carried out at regular 5 days interval on the fish fillets stored for up to 30 days. Data analysis were performed using SPSS version16 and Microsoft excel 2010. There was significant increase in Aerobic Colony Count, Enterobacteriaceae count and Psychrotrophic count on the fillets stored at 5ºC. The frozen condition showed significant effect on inhibiting all of the three groups of microbial counts. The moisture, protein, fat, and ash contents of fresh Nile tilapia fish fillets were 77.98%, 20.26%, 0.523% and 1.322%, respectively. The fillets stored at 5ºC showed significant decreases in protein and ash contents during 30 days of storage. On the other hand, there were no significant changes in moisture and ash contents of fillets kept under frozen condition for about 30 days. However, there was a significant decrease in crude protein content on the later days of storage under frozen condition. Moreover, TVB-N content of the fish fillets increased from 3.92 to 45.04 mgN/ 100g and 5.37 mgN/100g on samples kept at 5ºC and -18ºC, respectively. pH were also increased from 6.23 to 7.60 and 6.81 on the samples stored at 5ºC and -18ºC, respectively. The result shows that Nile Tilapia from Lake Tana had high protein content, low fat content, and high microbial load on fresh fillets, and freezing can keep the qualities of the fish relatively for longer period within safe levels and refrigeration could be able to keep the qualities briefly.*

Key words: *Bacteriological load, Fresh,* Oreochromis niloticus*, Refrigeration, and TVB-N.*

1. Introduction

Fish and fish products are of excellent nutritional source that provide high quality protein, a wide variety of vitamins and minerals. The protein is easily digestible and favorably complements diets provided by cereals and legumes that are typically consumed in many developing countries (FAO, 2010a).

Apart from that, fish foods contain high contents of Omega-3 fatty acids, polyunsaturated fatty acids (PUFAs), that make them relatively healthy to eat and acceptable by many people in the developed world (WHO, 2003). These fatty acids are considered essential for human health as they cannot be synthesized by the body. They play a crucial role in brain memory and behavioral function, and normal growth and development. They have also become popular because they reduce the risk of heart disease, inflammation, cancer, and arthritis (Simopoulos, 1999; 2002).

Ethiopia depends on its inland water bodies for the supply of fish food for its population. In 2001, fish production in the country was estimated as 15,389 tons, which is about 30 percent of the estimated annual total exploitable fish potential of the country. The fish is utilized in fresh, chilled, frozen, cured and canned forms. However, much of the landed fish is prepared into gutted and filleted forms at landing sites. It is estimated that 73% of the total fish landed in Ethiopia is marketed fresh in nearby markets where as the rest reaches distant consumers either chilled or frozen (26%), or dried, smoked and canned (1%) forms. Currently, canning has ceased due to poor product quality and low demand (FAO, 2011a).

Lake Tana is the largest lake in the country covering an area of 3,200 km^2. The lake is oligo-mesotrophic with chlorophyll *a*, average 6.4 μgL^{-1} (Eshete Dejen *et al*., 2004). The major fish groups found in the lake are the cyprinids, with some larger Barbus species, the small pelagic Barbus *trisplopieura, vericorhinus benzo, Garra quadrimaculata* and *G. dembiensis*, one species of cichlid, Nile Tilapia, *Oreochromis niloticus*, and one silurid, African catfish, *Clarias gariepinus* (Boulenger, 1911; Nagelkerke and Sibbing, 2000; de Graaf *et al.*, 2005; Stiassny and Abebe Getahun, 2007). Although the lake is inhabited by these groups, the production is mainly focused on *Clarias gariepnus*, O. *niloticus*, and some Barbus species (LFDP, 1997).

The Lake fish production was estimated to be 1200 ton for the year 1995/1996, with an estimated maximum production of 50 - 70 kg/ha/yr (Mebrat, 1993). However, most of the catch is contributed by the species *Oreochromis niloticus* (Nile Tilapia) which is found in nearly all freshwaters of Ethiopia and accounts for most of the commercial fishery catch in the country (Reyntjens *et al.*, 1998). A recent global level study by Jesupeit (2010) showed a booming of World tilapia production during the last decade, with output doubling from 830000 tonnes in 1990 to 1.6 million tonnes in 1999 and to 3.5 million tonnes in 2008.

Although fish is a very balanced food source, it is highly perishable especially in hot climates and tropical areas. The problem is aggravated where the cold preservation facilities are neither available nor affordable. Consequently, there is a rapid loss in quantity and quality of fish and fish products due to internal chemical and enzymatic reactions, and external microbial activities (Baird-Parker, 2000).

Fish spoilage is very rapid after it is caught (Rigor mortis) and the spoilage process starts within 12 hrs of catching in the tropics (Berkel *et al.,* 2004). It is characterized by a breakdown into different components and the formation of new compounds that change the odour, flavor, texture and general appearance of the fish. The deterioration is believed to be caused mainly by the bacterial activity (Gram and Huss, 1996; Haard, 2002).

It is estimated that post-harvest losses of the annual fishing catch of the world is very high. According to the Food and Agriculture Organization of the United Nations (FAO), post-harvest losses of fish in some developing countries often surpass 50% of the catch and exceed the loss of any other food commodity (FAO, 1986). In Africa, some estimates put post-harvest losses at 20 to 25 percent, and sometimes as much as 50 percent (FAO, 2010b). Reducing these losses could increase protein availability, improve nutritional status; ensure food security and economic gain.

Apart from that, food safety is important because fish are not only prone to rapid spoilage but also to pathogenic infection. The main safety concerns are unhygienic handling during and after fish harvest. In order to alleviate these problems low temperature preservation methods are employed. However, there are some microorganisms that can survive and grow under low

temperature preservation conditions that compromise the nutritional value of fish and fish products (Huss, 1994).

In Ethiopia, a scanty information on postharvest preservation, nutritional quality and microbiological load of fish are available (Shemelis Admassu and Mekonnen Melaku, 2010; Alemu Lema, 2011). These studies focused on the influence of frozen period on the proximate composition and microbiological quality, and the age and sex related variation in nutritive composition and fatty acid analysis profile of Nile tilapia fillets of Lake Zeway. Both the studies focused on total plate count, total and fecal coliforms rather than Enterobacteriaceae which are considered as better indicators of general hygiene status of food products and Psychrotrophs that are able to grow and cause spoilage of fish under cold and frozen conditions (Edwards and Ewing, 1972; Connell, 1990; Dalgaard, 1993; Gram and Huss, 1996).

Although there are a lot of studies on fish biology and diversity, fishery production, management of fish stocks, phytoplankton and zooplankton diversity and production, water chemistry, etc. of Lake Tana (Seyoum Seifu and Kornfield, 1992; Nagelkerke, 1997; De Graaf and Eshete Dejen, 2000; etc.), there is no sufficient work on post-harvest status of the fish in related to the microbiological and physicochemical quality and safety of post-harvest fishing of Lake Tana.

This study was, therefore, designed to evaluate the effect of preservation on microbiological quality and physicochemical quality of postharvest products of *Oreochromis niloticus* (Nile Tilapia) in relation to storage time and temperature.

2 Objectives

2.1 General objective

The study was undertaken to determine the microbiological load and physicochemical quality of Nile Tilapia (*Oreochromis niloticus*) fillet and to evaluate the effect of refrigeration and freezing temperatures on microbial load and physicochemical quality with regard to storage time of fillets.

2.2 Specific objectives

- To determine the effect of temperature on physicochemical quality of fresh Nile Tilapia fillets in relation to storage time.
- To evaluate the effect of low temperature and time on the microbial load of fish fillet.

3. Literature review

3.1 Nile Tilapia (*Oreochromis niloticus*)

The Nile tilapia, *Oreochromis niloticus* Linnaeus (Pisces: Cichlidae) is one of the most important fish species in the inland fisheries of tropical Africa, particularly in the Great East African rift valley lakes (Fryer & Iles, 1972). Besides to its captured fisheries importance, Tilapia is one of the most important species for 21st century aquaculture and is produced in more than 100 countries (Fitzsimmons, 2000). Tilapia culture has expanded worldwide at an average annual growth rate of 14% since 1984, while the overall growth rate of aquaculture was 11%. Tilapia production surged around the world during the 1990s and early 2000s. Tilapias are now the second most popular farmed fishes after carps. The global production of farmed tilapia exceeded 2,002,087 metric tons (Fitzsimmons, 2006).

The species is the most important in the commercial fisheries of Ethiopia and found in almost all Lake of Ethiopia (LFDP, 1998). Nile tilapia prefers to live in shallow water. The lower and upper lethal temperatures for Nile tilapia are 11-12 °C and 42 °C, respectively, while the preferred temperature ranges from 31 to 36 °C. It is an omnivorous grazer that feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. Nile tilapia can filter feed by entrapping suspended particles, including phytoplankton and bacteria, on mucous in the buccal cavity, although its main source of nutrition is obtained by surface grazing on periphyton mats (FAO, 2011b). This fish (O. *niloticus*) found in Lake Tana and it is predominantly herbivore, feeding on macrophytes, algae and detritus (Getachew and Fernando, 1989; Piet *et al.,* 1999).

3.2 Fish spoilage

Spoilage of food products can be due to chemical, enzymatic or microbial activities. Chemical deterioration and microbial spoilage are responsible for loss of 25% of gross primary agricultural and fishery products every year (Baird-Parker, 2000). One-fourth of the world's food supply (Huis in't Veld, 1996) and 30% of landed fish (Amos, 2007) are lost through microbial activity alone.

Fish is a good source of protein and minerals such as calcium, phosphorous and iron, trace elements like iodine (in marine fishes), as well as vitamins A and D (Banks, 1980). The high content of polyunsaturated fatty acids requirement probably helps lower cholesterol levels. Thus, from nutritional point of view, fish is important in the diet of the developing world (James, 1986). Though fish is highly nutritious and tasty, it is very perishable and cannot be kept for long times for consumption.

Maintaining good quality of fish raw material for processing is very important. Therefore, the reasons for quality deterioration leading to spoilage need to be determined carefully. Just after death, fish can be soft for a few hours but then it becomes stiff. This phenomenon is called "rigor mortis". The fish stays in the "rigor mortis" condition for a while, but then its flesh muscles become relaxed again. At that time the fish quality starts to decrease. The quality changes can easily be noticed and consist of changes in color, odour or smell, taste, appearance and texture and are therefore called sensory changes. One of the differences between fish appearance before and after rigor mortis is that the fish muscle is more elastic before rigor mortis. The time of pre-rigor mortis and rigor mortis varies according to species. It also depends on many things like temperature, handling, size and physical condition of the fish. Generally, it is preferred to extend the time before and during rigor mortis. There are some reasons for deterioration of quality and spoilage; they are autolysis, bacteria spoilage, rancidity and mechanical damage (Huss, 1994). Lowering the temperature by icing not only slows down the rigor mortis process, but also reduces the spoilage rate. Therefore maintaining low temperature during the handling and preservation process is very important.

3.2.1 Reasons for spoilage of fish

Fish "spoilage", is a condition, not clearly defined, however, it is referred to as product /fish quality loss, indicated by the deterioration of fish characteristics which include, the colour, flavour, odour or texture; resulting from different spoilage processes that occur during storage (Huss, 1988; 1994; 1995). Essentially, fish spoilage changes, occur as soon as fish dies, and they take place in a sequential process. These processes have been categorized into: autolytic, microbiological and chemical changes.

According to Huss (1995) spoilage of iced cod has been stratified into different phases; to indicate the deterioration of the eating quality of fish during storage in which autolytic spoilage changes predominate in the initial storage period and is succeeded by changes due to bacterial activity, thus, apart from spoilage changes due to chemical reactions of the fish lipids, autolytic and bacterial activity constitute to the main spoilage changes in fish during storage

Autolysis process

The autolysis process relates to enzyme activities in fish (autolysis means self-digestion). Commonly the spoilage due to autolysis occurs first and is followed by spoilage due to bacteria and rancidity but sometimes they overlap (Gram and Huss, 1996). Unlike most fish, autolysis occurs very quickly in some shellfish like lobster and shrimp (Hobbs, 1982).

When the fish dies adenosine-triphosphate (ATP), which is the energy-rich organic compound in its muscle, will mostly be synthesised from glycogen, but also from creatine-phosphate (for finfish) and from arginine-phosphate (for cephalopods) under anaerobic conditions. The glycolysis (glycogen reduction process) still occurs continuously to create the end product of lactic acid. Because the end product of this process is lactic acid, the pH of the muscle will decrease. The ATP concentration gradually decreases and when it goes below 1 μmol/g in the muscle tissue the enzyme ATP-ase is activated. This leads to the stiffing of the muscle which will be constant (rigor mortis). The ATP is gradually degraded during time to some degraded products e.g. adenosine diphosphate, adenosine monophosphate, inosin monophosphate, inosin and hypoxanthin. Hypoxanthin is considered to cause the off-flavour in spoiled fish. When the fish raw material is handled carelessly cells may be broken, which leads to the release of autolytic enzymes and this leads to the production of some spoilage substances. These substances create a very good environment for micro-organisms. Cathepsin, chymotrypsin, trypsin, calpain, cacboxypeptidase, collagenase and TMAO- demethylase are all autolytic enzymes. Therefore, in order to maintain fish quality, enzyme activities should be prevented. Using low temperature is the most frequently used measure to limit enzyme activities (Huss, 1994).

Microbiological

Bacteria are capable of causing spoilage because of two important characteristics. First there are psychotropic bacteria and thus multiply at refrigeration (low) temperatures. Secondly they attack various substances in the fish tissue to produce compounds associated with off- flavours and off-odours. When the fish is alive the bacteria are found on the gill and skin and in the intestines but cannot attack the fish muscle. But when the fish dies the bacteria can penetrate into the flesh muscle of the fish. When fish is preserved by icing the rate of bacterial penetration into the flesh muscle is much slower. Fish spoilage occurs when the enzyme of bacteria diffuses into the flesh muscle and the nutrition substances from the flesh muscle diffuse to the outside. Spoilage will happen more rapidly for fish species with a thin skin layer. The number of bacteria in fish caught in temperate waters can develop even when in ice but the bacteria caught in tropical water grow slowly for one or two weeks in icing preservation (Gram and Huss, 1996).

Not all the growing bacteria are involved in the spoilage process. There are just a few bacteria species that become predominant and are mainly responsible for spoilage. For example in gutted cod, chilled by ice the specific spoilage organism (SSO) is *Shewanella putrifaciens* and in packaged cod fillet it is *Photobacterium phosphoreum* (Connell, 1995). If the fish is preserved by icing or in lack of air the amount of *Pseudomonas* and *Shewanella putrifaciens* bacteria is not very high but *Photobacterium phosphoreum* bacteria becomes quite high. After a certain time in ice in aerobic conditions the *Pseudomonas* and *Shewanella putrifaciens* bacteria will become the predominant bacteria. In general in low temperature (0-5°C), *Shewanella putrefaciens*, *Photobacterium phosphoreum, Aeromonas* spp., and *Pseudomonas* spp. cause spoilage but in higher temperature (15-30°C) other species like *Vibrionaceae, Enterobacteriaceae* and the Gram positive bacteria cause spoilage (Gram and Huss, 1996).

The bacteria produce a high amount of volatile compounds. These are trimethylamine, volatile sulfur compounds, aldehydes, ketones, esters, hypoxanthine as well as other low molecular weight compounds. The bacteria *S. putrefaciens* and some *Vibrionaceae* produce H_2S but *Pseudomonas* and *Photobacterium phosphoreum* do not produce significant amounts of H_2S. The volatile sulphur compounds have a very bad odour so even minimal quantities are considered to affect quality. The low temperature is very important in preservation of raw material. Especially

in the range of 0-25°C the temperature strongly affects the bacteria activity. At 0°C the bacteria grow very slowly. The typical spoilage bacteria like *Shewanella putrefaciens* develop 10 times less in comparison with growing at the optimal temperature. Raising the keeping temperature thus increases the spoilage rate rapidly. Therefore it is important to decrease the temperature to 0°C as soon as possible after catching. For fish in the tropical water area where the ambient temperature is around 25 – 30°C the rate of spoilage can be 25 times higher than when kept at 0°C (Huss, 1994).

Rancidity

Fat oxidation usually occurs after autolysis and bacterial spoilage. The lipid concentration in fish can contribute to the spoilage process in fish. The fats in fish are mainly unsaturated fatty acids that are easily oxidised by oxygen from the atmosphere. High temperature or exposure to light can increase the oxidation rate. For fatty fish preserved in ice, spoilage due to rancidity is mainly caused by oxidation. This produces a bad and unpleasant odour as well as a rancid taste (Hobbs, 1982). Fat fish species like herring, mackerel, and salmon are mostly affected by rancidity. The lean fish fat content is mostly about 0.1-0.9% and the fat fish fat content is higher than 5% (Love, 1982)

Mechanical damage

If the fish is broken by harsh handling, it will be subject to mechanical or physical damage and become bruised and defected in outside appearance. But it is more important that some small cells will break leaving the enzymes free to react with other substances. Mechanical damage gives good conditions for some enzymatic activities. Fish kept in thick layers in a box with ice can cause high pressure between the ice and fish causing cells to break. All careless handling of fish raw material can result in bruised fish. This also opens channels for the micro-organisms to enter the fish flesh and enables quicker spoilage of the fish (Huss, 1994). In general, in order to maintain the fish raw material quality after catching, some measures for handling and preservation are needed to prevent all the quality change processes mentioned above.

3.2.2 Factors that influence the rate of fish spoilage

Effects of time/temperature conditions on microbial growth

The most crucial factors determining the quality of fishery products are time and temperature tolerance. Proliferation of microorganisms requires appropriate high temperatures; while at lower temperatures close to 0ºC their activity is reduced, thereby extending the shelf life of fish products. Temperature is the single most important factor affecting post-harvest quality of the products. It is often critical to reach the desired short-term storage temperature rapidly to maintain the highest visual quality, flavour, texture, and nutritional content of fresh fish. The rate of spoilage is dependent upon the holding temperature and is greatly accelerated at higher temperatures, due to increased autolytic and bacterial action (FAO, 2011c).

Effects of hygiene on fish quality during handling

Apart from the microorganisms that fishes have at the time of capture, more is added via unhygienic practices and contaminated equipment such as storage facilities. This was demonstrated by studies that compared the quality and storage life of completely aseptically treated fish (aseptic handling), washed fish, iced in clean plastic boxes, with clean ice (clean handling) and with un-washed fish, iced in old, dirty wooden boxes (normal handling). A considerable difference was found in the bacterial contamination of the three batches, the latter heavily contaminated with a reduction in storage life compared with the other samples (Huss *et al.,* 1974).

Rough handling

Rough handling will result in a faster spoilage rate. This is due to the physical damage to the fish, resulting in easy access for enzymes and spoilage bacteria. Physical mishandling in the net, such as very large catches, fishermen stepping on fish or throwing boxes, containers and other items on top of the fish, may cause bruises and rupture of blood vessels. When fish is in rigor mortis (a complicated series of chemical changes that result in stiffening of the fish's muscle shortly after death) rough handling can cause gaping (Huss, 1995).

Initial bacterial load

The microflora on tropical fish often carries a slightly higher load of Gram-positives and enteric bacteria but otherwise is similar to the flora on temperate-water fish (Liston, 1980). Basically, bacteria populations on temperate fish are predominantly psychrotrophic reflecting water temperatures of about 10^{o}C while fish from the tropics have largely mesophilic bacteria (Gram and Huss, 1996).

Methods of capture and storage

The fishing gear and method employed determines the time taken between capture and death. Fish caught in gillnets struggle much to escape, and in so doing, they are bruised by the net which increases exposure to microbial entry and subsequent deterioration. Fish caught by hook and line methods, on the other hand, die relatively quickly and therefore bruises and stresses are likely to be minimal. Physical mishandling in the net due to long trawling nets and very large catches accelerates spoilage. The large catches in the net are compacted against each other resulting in the fish getting bruised and crushed (especially small sized fish) by the heavy trawl net. In bulk-storage, the weight of the pile may crush the fish at the bottom, leading to a loss of weight (yield) as well as other physical damage. It has been reported that when haddock is kept in a short, deep pile of about 3 ft, the bottom fish lose 15% of their weight compared to a normal weight loss of 3-8%, which is entirely due to biochemical changes that cause a loss of water holding capacity leading to drip (Regenstein and Regenstein, 1991). Crushing of the fish by ice or other fish can seriously affect the quality of fish by releasing enzymes from the gut into the fish muscle thereby accelerating autolytic processes.

3.2.3 Methods for evaluation of fish spoilage

The spoilage is rather a complex process of physical and chemical changes in the food itself. The contemporary consumer demand for fresh food products and the strict food safety regulations in general, requires appropriate assessment and management of safety and quality of food products, by using methods that can quantitatively relate microbial growth to the characteristics of the products during storage, to avoid unnecessary economic losses (Connell, 2001).

The various methods commonly used for assessment of fish spoilage have been classified into two categories: sensory methods and instrumental (microbiological, biochemical, and physical) methods (Huss, 1995).

Sensory evaluation

Sensory evaluation of food, according to Huss, (1995); Meilgaard *et al.* (1991) is defined as the scientific means of quantifying and interpreting the variations in food characteristics (odour, taste, tactile, appearance) by using human senses of sight, smell, taste, touch and hearing. Trained and experienced taste panel is essential to obtain accurate and reproducible result

Microbiological methods

The major changes in fish freshness for instance unattractive change in food characteristics such as, flavours and odours and colour are largely due to bacterial growth and activity (Connell, 1990; Huss, 1995). Microbiological methods are used to estimate bacterial numbers, in order to determine fish freshness, hygiene and/or evaluate the possible presence of bacteria or organisms of public health importance (Huss, 1994; 1995). Microbiological prediction/estimation of bacterial numbers therefore, in order to serve the purpose of food safety and shelf life determination, is expected to relate quantitatively to the characteristics of the food during storage (Dalgaard, 2002).

There are a lot of microbiological methods to determine fish bacteria like plate count, direct microscopic count, ATP measuring, but the plate count is a traditional and common method with some different media like plate count agar or iron agar (Gram, 1992).

Microbiological quality evaluation of fish aims to quantify the hygienic quality of fish, including temperature abuse and the possible presence of pathogenic microorganisms in the fish. Total aerobic bacteria, also called total plate count (TPC); specific spoilage organisms (SSO) and various pathogenic bacteria are examined using appropriate agar media. Quality levels are based on the plate counts for acceptance or rejection of fishery products for human consumption (ICMSF, 1986).

Chemical methods

The evaluations of food using chemical methods are considered to be more objective than sensory methods especially when it is done accurately using appropriate method (Huss, 1995).

These methods involve determination of the concentration of a specific chemical(s) in the food under study. Chemical methods of food evaluation are normally used to indirectly predict the level of a sensory attribute, which allows for immediate determination of freshness. To use chemical methods to serve this purpose, well set, quantified and standardised tolerance levels of chemical spoilage indicators need to be established (Huss, 1995).

With regard to evaluation of fish quality using chemical methods, the total volatile basic amines (TVB) constitute to the commonly measured chemical indicators. TVB is a general phrase used to include volatile amines such as, trimethylamine (TMA), ammonia (NH) produced by spoilage bacteria; dimethylamine (DMA) and produced by autolytic enzymes during frozen fish storage (Huss, 1988). The concentration of these chemicals in fish tissues can be determined by steam - distillation method (Malle, 1989).

The measurement of the amount of hypoxanthine (Hx) in fish is one of the chemical methods of determining fish freshness. Hx is one of the products of nucleotides degradation mediated by bacterial activity (*Proteus bacterium*) is known to be responsible for bitter, off-flavours of spoilt fish (Huss, 1995). Studies have shown that the degradation of nucleotides progresses vary greatly from one fish to the other but often coincidently progresses with the preserved level of spoilage as may be determined by trained analysts thus the development of the formula for fish freshness based on these autolytic changes that entailed.

K-value, (freshness) can be determined by calculating the ratio of inosine and hypoxanthine to the sum of ATP and all the other products of ATP degradation multiplied by 100 (Haard, 2002; Huss, 1995; Connell, 2001). The interpretation is that the smaller the K-value the more fresh the product is and high Kvalue indicate unacceptable fish product. K-value of 20% has been suggested as a freshness limit and 60% as the rejection point. Nevertheless; its application has not been popular in fish industry due to loss of recognition from the EU system (Huss, 1995).

Other methods such as Peroxide Value (Pv), Thiobarbituric Acid (TBA), Iodine value (Iv) and also constitute to the chemical methods that are used to measure rancidity in fish and fish products but they will not be included herein this study. However, generally, there are a number of limitations associated with traditional methods of food/fish evaluation.

Physical methods

Electrical Properties: It has long been known that the electrical properties of skin and tissue change after death, and this has been expected to provide a means of measuring post mortem changes or degree of spoilage. However, many difficulties have been encountered in developing an instrument: for example, species variation; variation within a batch of fish; different instrument readings when fish are damaged, frozen, filleted, bled or not bled; and a poor correlation between instrument reading and sensory analysis. Most of these problems, it is claimed, are overcome by the GR Torrymeter (Jason and Richards, 1975).

pH and Eh: Knowledge about the pH of fish meat may give valuable information about its condition. Measurements are carried out with a pH-meter by placing the electrodes (glass-calomel) either directly into the flesh or into a suspension of fish flesh in distilled water (Huss, 1995). Measurements of Eh are not carried out routinely, but it is likely that a freshness test can be based on this principle

Measuring Texture: Texture is an extremely important property of fish muscle, whether raw or cooked. Fish muscle may become tough as a result of frozen storage or soft and mushy as a result of autolytic degradation. Texture may be monitored organoleptically but there has for many years been a need for the development of a reliable objective rheological test which would accurately reflect the subjective evaluation of a well-trained panel of judges. Gill *et al.* (1979) developed a method for evaluating the formaldehyde-induced toughening of frozen fish muscle

Alternative "Rapid" methods for food assessment

According to Bourgeois *et al.* (1995); Fung (1995); Mossel *et al.* (1994) the definition of "rapid" methods refers to improved, alternative methods for food evaluation that can enable early, accurate and faster detection, isolation, characterization and or enumerate microorganisms and their products related to food spoilage.

In accordance with Fung (2002) the application of "Rapid" methods for food quality and safety evaluation are a recent development in the general field of applied microbiology. The main characteristic of the various techniques considered as rapid means for food quality assessment is the measurement of the changes in the food characteristics, associated with spoilage due microbial activity such as; the concentration of microbial ATP, Redox potential, odour/flavour, dielectric properties, pH, texture etc. Various studies have shown that there wide range of gadgets, instrumentation and diagnostic kits that can be used to serve this purpose.

3.3 Typical microbiota of fish

The muscle of living fish is sterile. However, the skin, mucus, gills and gut contain significant bacteria, whose composition and quantity vary according to the fish species, temperature and salinity of the water, level of dissolved oxygen, degree of pollution, feed, stress, etc.

The microbiota of marine fish from temperate waters is usually composed of Gramnegative psychrotrophic bacteria, whose growth is possible at 0°C and optimal at around 25°C. The majority belongs to the class γ of proteobacteria: *Pseudomonas*, *Shewanella*, *Acinetobacter*, *Aeromonas*, *Vibrio*, *Moraxella*, *Psychrobacter*, *Photobacterium*, etc., and to a lesser extent to the CFB group *(Cytophaga- Flavobacter-Bacteroides*). Nevertheless, Gram-positive bacteria, such as *Micrococcus, Corynebacterium*, *Bacillus, Lactobacillus* and *Clostridium* may also be present in variable proportions (Gram and Huss, 1996; Wilson *et al*., 2008). The same bacterial genus can be found in tropical marine fish but Gram-positive bacteria, Enterobacteriaceae and Vibrionacea are often dominant (Liston, 1980). Although this environment is partially anaerobic, most researchers have observed a predominance of aerobic bacteria, also present in the surrounding water. This could be due to the collecting techniques, which are not always suitable for strict anaerobes (Burr *et al.,* 2005).

In general, Gram-negative bacteria (*Vibrio*, *Acinetobacter* and *Enterobacteriaceae*) are dominating the microbiota (Ringo and Birkbeck, 1999). These are fermentative bacteria that develop rapidly in the gastro-intestinal tract due to the low pH, the lack of oxygen and the abundance of nutrients. Sometimes, staphylococci have also been found to be the dominant microbiota in the fish intestine. However, both the number and diversity of the microbiota are

probably widely underestimated due to the classical microbiological methods involving growth on agar media that so far have been used.

Enterobacteriaceae

The Enterobacteriaceae family is a group of bacteria that is used to assess the general hygiene status of a food product. This group includes species that originate from the intestinal tract of animals and humans, as well as plants and the environment. High levels of these bacteria are expected in some food commodities such as salad vegetables. The use of sanitizing rinses may reduce but not entirely remove these organisms. Some Enterobacteriaceae can contribute to the formation of histamine (scombrotoxin) in foods such as scombroid fish (mackerel and tuna) and occasionally some cheeses if these are not processed properly and/or stored at an adequate refrigeration temperature. Ingestion of fish with high histamine levels is toxic, and maximum permissible levels of <200 or <400 mg/kg of histamine (depending on the type of product) are set by Regulation (EC) No. 2073/2005 (as amended) (European Commission, 2000).

The test for Enterobacteriaceae has replaced the tests for coliforms that traditionally have been used as indicators of hygiene and contamination after processing. The major problems with the coliform tests are the variability in definition of the term coliforms (they are defined usually by the method used for their detection) and the fact that only lactose fermenting organisms are detected (Edwards and Ewing, 1972). In comparison the family Enterobacteriaceae is well defined taxonomically and methods for their enumeration are based on common properties (British Standards Institution, 1991; 1993). Furthermore, the methods also detect important non-lactose fermenting organisms such as salmonellas.

3.4 Quality Changes during Frozen Storage

The freezing of fish is an effective way of long term preservation and it has been shown that fish stored for up to three months under ideal conditions cannot be distinguished from fresh fish regarding colour, taste and texture (Nielsen and Jessen, 2007). Freezing and frozen storage of fish muscle may, however, lead to denaturation and aggregation of especially myofibrillar proteins. These changes result in altered functional properties, changed textural attributes and reduced water holding capacity and juiciness. The result is a hard, dry and fibrous fish product

with a reduced eating quality. Moreover, liquid losses may also directly result in economic losses (Barroso *et al.*, 1998).

Water is the main constituent of most foods. It accounts for 65-80 % of fish muscle and is solvent for different salts, enzymes and other non-fat food solids. It also functions as a plasticizer of non-crystalline food components (Slade and Levine, 1995). The amount of water and its physical state in foods are important properties, influencing quality, shelf life and processing.

Tropical fish apparently have much longer shelf lives in ice than those from temperate or cold water (Gram *et al.,* 1990). This is because the microorganisms found in fish from cold water can easily adapt the low temperature of ice. Most researches on the spoilage of fish in ice have been carried out on marine species. Less is published on spoilage characteristics of fresh water fish. However, these studies do indicate that, although spoilage patterns of freshwater and marine fish are similar, the storage life of freshwater fish tends to be longer (Shewan, 1977).

4. Materials and Methods

4.1 Description of the Study Area

Lake Tana is located in the Northern highlands of Ethiopia (1800m.a.s.l) and it is considered as the source of the Blue Nile. The Lake was formed by a volcanic blockage that reversed the previously North-flowing river system (Beadle, 1981). The total area of the Lake Tana basin is 16,500 km^2 and the lake itself covers about 3,150 km^2. Numerous seasonal streams and four perennial rivers feed the lake, while only one, the Blue Nile, leaves it. Air temperatures range widely, between 7^oC to 31^oC, whereas water temperatures stay relatively mild, normally between 18^oC and 26^oC (Nagelkerke, 1997).

Lake Tana hosts the only extended Cyprinid species flock in Africa. Fifteen species of large barbs have been described from Lake Tana (Nagelkerke, 1997). The lake also inhabits *Clarias gariepinus* and *Garra* populations. There is one Cichlid: *Oreochromis niloticus.* It is the most wide spread Tilapia species in Africa, and this species contributes much to the fishery of Lake Tana.

4.2 Collection and Preparation of Samples

Nile tilapia samples (average weight of 230gm) caught by gillnets were purchased from the fish marketing association, Lake Tana on July 14, 2011. The fish samples were identified, measured and processed following the methods used by the association (personal communication). The fillets were packed in sterile polythene bags and kept in icebox. The samples were immediately transported to the Biotechnology and Microbiology Laboratory (Bahir Dar University). The samples were stored at 5ºC and -18ºC temperatures within 30 minutes. Part of the fresh Samples was processed for bacteriological and physicochemical analyses within 30 minutes. At each sample analysis during 30 days of storage, the contents of one package from each of the storage conditions were examined bacteriologically and chemically every 5 days.

4.3 Sample Processing and Analyses

Ten gram of each sample was taken in a sterile stomacher bag and poured in 90 ml of sterile 0.1% peptone water and blended for 2 minutes. This homogenate sample was further diluted to

10^{-2} using screw cap vials containing 9 ml of sterile 0.1% peptone water. This was repeated till finding the needed dilutions.

Tilapia fish product samples (fresh and preserved) were analyzed for Aerobic Colony Count, Psychrophilic bacteria count, Enterobacteriacceae, pH, moisture content, ash content, fat content, protein content, and total volatile bases nitrogen (TVB-N) at Biotechnology and Microbiology laboratory, and Food Chemistry Laboratory; Bahir Dar University. These analyses were undertaken every 5 days throughout the preservation period starting from the fresh sample.

4.4 Microbiological Analysis

Microbiological analysis was performed according to the standard procedure for the enumeration of respective group of microorganisms. All equipment and chemicals were sterilized at 121ºC (15lb pressure) for 15minutes.

Aerobic Colony Count (ACC): 100 µL of the diluent of the homogenate sample were spread-plated on plate count agar (oxoid) in duplicate and incubated at 35ºc for 48hrs and counted using colony counter. Plates between 30 and 300 were taken and recorded.

Count for Enterobacteriaceae: 100 µL of the diluent of the homogenate sample were spread-plated on violet red bile glucose agar (VRBG-agar) in duplicate and incubated anaerobically at 30^0C for 2 days.

Psychrotrophic bacteria count: 100 µL of the diluent of the homogenate sample were spread-plated on plate count agar (oxoid) in duplicate and incubated at 10ºC for 5 days

4.5 Physicochemical Analysis

Total Volatile Basic Nitrogen (TVB-N)

Total volatile basic nitrogen (TVB-N) was estimated by steam distillation (Malle and Tao, 1987). Ten gram of each fish fillet sample was added to 90 ml of 7.5% (w/v) aqueous trichloroacetic acid (TCA) and homogenized on stomacher for 2 minutes. Then the homogenate was filtered using whatman filter paper No. 1. Twenty five milliliters of the extract was transferred to Kjeldahl-type distillation tube, and 5mL of 10% NaOH was added to it. Steam distillation was performed using a vertical steam distillation unit, and the distillate was collected into a beaker

containing 15 mL of 4% aqueous boric acid (w/v) and 0.04ml of methyl red and bromocresol green indicator solution up to a final volume of 50ml. Finally, the distillate was titrated with 0.1 N H_2SO_4. The amount of TVB-N was calculated by using the equation:

$$\text{TVB in mg-N/100g} = \frac{a \times b \times 14\ \text{g/mol} \times 300}{25\text{ml}}$$

Where: a = Milliliters of sulfuric acid.

b = normality of sulfuric acid

Measurement of pH Value

The pH values was recorded by using a Schott model pH meter after homogenization of each 10g fish muscle sample in 100ml distilled water by stomacher. The pH meter was calibrated using pH 4 and pH 7.

Determination of Moisture Content (AOAC 925.04, 1998)

To determine moisture content, oven drying method was used. Empty dishes were dried using air drying oven for 1 hour at 105^0C, transferred to the desiccators (with granular silica gel), cooled for 30 minutes, and were weighed. The prepared samples were mixed thoroughly and 5.0g of composite fresh fillet were transferred to the dried and weighed dishes. The dishes and their contents were placed in the drying oven and dried at 105^0C for 3hrs in an oven until constant weights were obtained, and then the dishes and their contents were cooled in desiccators to room temperature and reweighed. The moisture content was determined by measuring the weight of a sample before and after the water was removed by evaporation:

$$\%\ \text{Moisture content} = \frac{\text{Weight of wet sample} - \text{Weight of dried sample}}{\text{Weight of wet sample}} \times 100$$

Measurement of Ash Content (AOAC, 2000)

A dry, tarred porcelain dish containing 2 g wet sample was placed in an open oven in order to carbonize the sample. The carbonized sample was placed in a muffle furnace set at 550ºC for 1 hour and allowed to cool in a desiccator.

Finally the product weighed by an analytical balance, the ash content was determined using a formula:

$$\% \text{ Ash} = \frac{W_2 - W_1}{W_3} \times 100\%$$

Where: W1 = mass of empty porcelain dish

W2 = mass of dish with ash

W3 = mass of original product sample

Measurement of Protein Content (AOAC 955.04, 1998)

Crude protein in fish fillet was quantified by Kjeldahl methods. A digestion flask containing about 5 g sample, to which 10ml of concentrated Sulphuric acid and catalyst mixture Copper sulfate ($CuSO_4$) and potassium sulfate (K_2SO_4) were added. The mixture was exposed to about 370ºC for 3hrs in order to allow digestion. After digestion was completed, formed clear solution was cooled for 30 minutes and diluted with 25ml distilled water. Then it was distilled with Kjeldahl apparatus (Kjeltech system, Hoganas, Sweden) by adding 25ml of 40% NaOH (to nuetrlize) and the distillate was collected in receiving flask with 25ml of boric acid, and bromocresol green and methyl red as an indicator. The distillation was terminated when the volume of receiving flask reached between 200 to 250ml. Finally, the distillate was titrated with 0.1N HCl (Egan *et.al*., 1981). The amount of Nitrogen was calculated using the formula;

$$\text{Nitrogen content (\%)} = \frac{\text{ml of acid added} \times \text{Normality of acid} \times 0.014 \times 100}{\text{Weight of wet product sample (g)}}$$

Crude protein = 6.25 × %N

Determination of Fat Content (AOAC, 2000)

Undertaken only for fresh sample. A clean and dried thimble containing about 5 g of sample which was covered with fat free cotton at the bottom and top was placed in the extraction chamber. Then extraction has been performed for about 6 hours by Soxhlet apparatus. Finally, the fat content was estimated by using the formula:

$$\text{Fat content (\%)} = \frac{\text{Weight of fat} \times 100}{\text{Weight of sample}}$$

5. Statistical Analyses

Statistical analyses were performed using SPSS/16 software for Windows (Dyno Tech Company, Westland, MI) and Microsoft Excel 2010. Numbers of colonies were transformed into $\log_{10}$CFU/g. Data were analyzed using analysis of variance followed by Duncan's multiple range test for multiple comparisons among treatment means at a 5% level of significance for microbiological, TVB-N and pH data. Results were presented as means $\pm$ standard deviation.

6. Results and Discussion

6.1 Microbiological Analysis

In this study, the microbial load of fresh, refrigerated and frozen Nile tilapia fish fillet were evaluated based on Aerobic Colony Count (ACC), Enterobacteriaceae count, and Psychrotrophic counts in relation to different storage time (Table 1).

Table 1. Changes in microbial load (Log mean count, CFU/g) on Nile Tilapia (*Oreochromis niloticus*) fish fillets during refrigeration at 5ºC and frozen condition at -18ºC for 30 days.

Storage time (Days)	Aerobic colony count (CFU/g)		Psychrotrophs (CFU/g)		Enterobacteriaceae (CFU/g)	
	Ref	Deep	Ref	Deep	Ref	Deep
0(fresh)	6.79^{a}	6.79^{a}	6.40^{a}	6.40^{a}	5.74^{a}	5.74^{a}
5	7.30^{b}	5.84^{b}	7.02^{b}	5.63^{b}	7.03^{b}	3.73^{b}
10	8.48^{c}	5.59^{c}	8.60^{c}	5.18^{c}	8.51^{c}	3.69^{b}
15	9.18^{d}	5.34^{d}	9.77^{d}	5.10^{d}	10.36^{d}	3.54^{cd}
20	10.52^{e}	5.34^{d}	10.57^{e}	5.10^{d}	10.07^{e}	3.57^{c}
25	9.65^{f}	5.32^{d}	10.20^{f}	5.07^{de}	9.83^{f}	3.48^{de}
30	8.60^{g}	5.31^{d}	8.79^{g}	5.04^{e}	8.56^{c}	3.47^{e}

$^{a-g}$ Means with the same superscript letters within a column are not significantly different at $P < 0.05$.
Ref = refrigeration temperature at 5ºC; Deep = deep freezing at -18ºC.

Aerobic Colony Count (ACC)

Table 1 show that the fresh fish fillet had initial load of 6.79 log CFU/g aerobic bacteria at harvesting time. This is comparative, slightly higher, to the report of Alemu Lema (2011) that showed the incidence of an initial load 6.3 log CFU/g on male Nile tilapia fish fillets collected from Lake Zeway. In this finding, the recorded value is higher than the range of an initial count 2-6 log CFU/g reported by Chytiri *et al.,* (2004) on fresh water fish. Generally, the initial microbial load of different freshwater fish species varies owing to differences in handling, water quality (pollution level) and temperature. This may be looked with improper filleting and handling of fish that leads to heavy bacterial contamination and this contributes in the reduction of shelf life of the fish fillets.

The data shows that the ACC increased steadily as a function of time from 7.30 log CFU/g within 5 days to the highest count of 10.52 log CFU/g after 20 days of incubation (storage) at refrigeration temperature of 5ºC indicating significant increase by 4 log units (Table 1).

The ACC slightly increased from 6.79 log CFU/g at 0 time to 7.30 log CFU/g after 5 days of incubation (Table 1). This is comparatively slow increase (only 7.5%) to the study of Massa *et al.* (2005) that reported the increase of total aerobic bacteria from about 5 log CFU/g to 7 log CFU/g (40% increase) on flounder stored in ice on day 6. This may be due to the high initial load of bacteria which leads to higher competition for available resources, and reduces the growth rate.

The ACC count steadily increased with time rapidly from 8.48 log CFU/g on day 10 to the highest count of 10.52 log CFU/g at storage time of 20 days. Studies on different fish species reported similar increase in total aerobic bacteria count during storage, but most of the increasing rates were relatively higher than the present study. Alemu Lema (2011) reported the increase of aerobic bacteria from an initial load 6.33 log CFU/g to 7.94 log CFU/g on day 10 and then to 11.33 log CFU/g after 26 days of storage of male Nile tilapia fish fillets kept at refrigeration temperature. Sallam (2007) also reported the increase of total aerobic bacteria from about 3.85 log CFU/g to 9.41 log CFU/g on salmon stored at 0ºC for 15 days. Likewise, Taliadourou *et al.* (2003) reported the increase of total viable count from initial load of 4.9 log CFU/g to 7.5 log CFU/g and 9 log CFU/g after 9 and 16 days, respectively, of sea bass fillets stored in ice. Another study by Jeyasekaran *et al.* (2005) showed a 3 log units increase of total bacterial load from 5 log CFU/g to 8 log CFU/g on Reef Cod (*Epinephelus merra*) stored for 17 days in ice. In general, all these studies showed the steady increase of bacterial loads as a function of time indicating refrigeration temperature (5ºC) may retard but it does not stop the growth of microorganisms on fish fillet as a function of time.

The International Commission on Microbiological Specifications for Food recommends that during fish storage at lower temperature, the total plate count should never exceed log mean 7 log CFU/g of wet weight (ICMSF, 1978). According to the result, Tilapia fish fillet stored at 5^{o}C loses its microbiological quality acceptance level within 5 days of time (microbiological based shelf-life). The initial high load (6.79 log CFU/g) may have contributed to the higher number

(rejection level of ICMSF) of total bacterial count in short storage time. Huss *et al*. (1974) stated that the initial quality of the raw fish, in terms of their freshness, microbiological load and physical damage, is an important factor that influences the quality of the end product.

On the contrary, the aerobic colony count of Nile Tilapia fish fillet stored under frozen condition showed a significant decrease through the time of preservation (Table 1). The bacterial load showed decreases from 6.79 log CFU/g to 5.34 log CFU/g on day 15 and then it showed a relatively constant population on the following days. Similarly, Shimelis Admassu and Mekonnen Melaku (2010) reported a decrease of total bacteria load from 6.4 log CFU/g to 4.3 log CFU/g on Nile tilapia fish fillet samples stored at -18ºC for 30 days.

This shows that the frozen condition has successfully kept the total bacterial count under the recommended level throughout the storage days till day 30. It confirms the inhibitory effect of frozen condition on the growth of microorganisms on fish fillet, but it does not eliminate the microbial population during 30 days of storage.

Enterbacteriaceae

With regard to the count of Enterobacteriaceae from fresh Nile Tilapia fish fillet, it was found to be 5.74 log CFU/g. The initial load of Enterobcteriaceae count was slightly higher compared to the range from 2 log CFU/g to 5.54 log CFU/g which is reported by Elmossalami *et al.,* 1997; Taliadourou *et al.*, 2003 and Popovic *et al.* 2010 on different fish species. This indicates the presence of temperature, handling and processing abuse of fish, and it may indicate the pollution level of the Lake water which is used as washing of fish during processing (Fujioka *et al.,* 1988 and ICMSF, 1998).

The data (Table 1) shows that the Enterobcteriaceae count increased steadily as a function of time upon storage of fish fillets under refrigeration condition. The increase was found to be rapid within 15 days reaching 10.36 log CFU/g showing significant increase which was more than 4 log units, and stabilized afterwards with a slight decrease. Similarly, Sallam (2007) reported the increase of Enterobacteriaceae count from about 2 log CFU/g to 4 log CFU/g (100% increase) on salmon stored at 0ºC for 15 days. Likewise, Taliadourou *et al.* (2003) reported the increase of Enterobacteriaceae count from an initial load of *ca* 2 log CFU/g to *ca* 3.8 log CFU/g (about 90%

increase) after 16 days of sea bass fillets storage in ice. In this study, there was about 80.5% increase during 15 days of storage which was relatively lower than those reports, considering the storage condition.

Depending on the data, it can be suggested that Enterobacteriaceae contributes to the spoilage of Nile Tilapia fish fillets stored under refrigeration temperature. Similarly, Enterobacteriaceae have been reported to be part of the spoilage microflora of Mediterranean fish (Drosinos and Nychas, 1996).

The deep freeze storage condition showed a significant effect on Enterobacteriaceae count. Table 1 shows a 2 log units decrease (from 5.74 log CFU/g to 3.47 log CFU/g) of Enterobacteriaceae count within 5 days storage of fillet under deep freeze (-18ºC). The count reached 3.47 log CFU/g on day 30. Popovic *et al.* (2010) reported that out of 15 fresh fish samples 9 samples with <2 log CFU/g, 4 samples with 2-3 log CFU/g, and the other 2 samples found to have >3 log CFU/g, and all 15 frozen fish samples found to have <2 log CFU/g in the summer time.

The Enterobacteriaceae count is considered as an index of fish quality because it is related to storage in ice, washing and evisceration (Zambuchini *et al*., 2008). Monitoring of these microorganisms has been suggested as a measure of fish safety and quality. Even this study shows a 5-fold increase in Enterobactericeae upon refrigeration temperature compared to the Psychrotrophs and Aerobic Count Count indicating that they are rapidly proliferating under refrigeration.

Although Ethiopia is yet to set the permission level, the Enterobacteriaceae counts are relatively higher on fresh, refrigerated and frozen fish sample compared to levels permitted by Croatian microbiological standards for food and food products < 2 log CFU/g for fresh and frozen fish (Croatian Chamber of Economy, 2009).

Psychrotrophic Count

The initial load of Psychrotrophic bacteria count was found to be 6.40 log CFU/g. It was higher compared to reports by Jeyasekaran *et al.* (2005), Sallam (2007), Cakli *et al.* (2008), and Liu *et al.* (2010).

Psychrotrophic count on the fish muscle kept under refrigeration temperature showed a 4 log units increase reaching to 10.57 log CFU/g upon storage for 20 days, and later showed slight decrease reaching to 8.79 log CFU/g of fish muscle on day 30. Liu *et al.* (2010) reported an exponential increase of psychrophilic bacteria count from initial load of about 3 log CFU/g to about 6 log CFU/g on day 9 and 7.4 log CFU/g (146% increase) on day 13 on tray-packed tilapia fillet samples stored at 0ºC. Sallam (2007) also reported the increase of psychrophilic bacteria from about 3.59 log CFU/g to 10.38 log CFU/g (189% increase) on salmon stored at 0ºC for 15 days. Similarly, Jeyasekaran *et al.* (2005) reported an increase of total Psychrotrophiles from 3 to 6 log CFU/g on Reef Cod (*Epinephelus merra*) stored for 17 days in ice. According to Cakli *et al.* (2008) the psychrophilic bacteria count on sharp snout sea bream stored under refrigeration temperature (4.05ºC) increased from 3.34 log CFU/g and exceeded 6.0 log CFU/g (80% increase) after 8 days of storage. In the present study, the increase was only about 52.6% during 15 days of storage which was lower than those reports. This may be due to the high initial load of bacteria which leads to higher competition with low temperature. However, this shows psychrophilic bacteria are notorious to grow, and cause spoilage and threats to health.

For fresh and refrigerated freshwater and marine fish, the microbial limit of acceptability was established at 6.0 log CFU/g for psychrophilic bacteria (Mol *et al.,* 2007). In the present study, the initial psychrophilic count was slightly higher than this limit. This high initial load made the shelf life of the fish fillet shorter, because these bacteria grow very quickly and accelerate the spoilage of fish under low temperature condition.

On the other hand, Psychrotrophic count of the fish muscle stored at -18ºC showed a significant decrease during the storage for 30 days. Although Psychrotrophic bacteria count showed a significant decrease, it was less shocked compared to the other group of bacteria. Psychrotrophic

count decreased about 1.36 log units (21.3% decrease) while ACC and Enterobacteriaceae count decreased about 1.48 log (21.8% decrease) and 2.27 log units (39.5%), respectively, after 30 days of storage. It significantly decreased from 6.40 log CFU/g to 5.18 log CFU/g of fish fillet on day 10 and it showed relatively constant population of Psychrotrophic bacteria on the later days of storage. The minimum count 5.04 log CFU/g was recorded on the last day of storage. However, the initial load was high relative to the limit. Popovic *et al.* (2010) reported that out of 15 fresh fish samples 1 sample with <2 log CFU/g, 8 samples with 2-3 CFU/g, and the other 6 samples found to have >3 log CFU/g, and from 15 frozen fish, 15 fish found to have 2-3 log CFU/g during summer time. The Psychrotrophic bacteria are the major group of microorganisms responsible for spoilage of aerobically stored fresh fish at lower temperatures (Gram *et al*., 1987; Gram & Huss, 1996).

Table 2. Changes in moisture, protein and ash contents of refrigerated (5ºC) and frozen (-18ºC) Nile Tilapia fish fillets during 30 days of storage.

Storage days	Moisture content (%)		Protein content (%)		Ash content (%)	
	Ref	Deep F.	Ref.	Deep F.	Ref.	Deep F.
0(fresh)	77.98 ± .086[a]	77.98 ± .086[a]	20.26 ± .148[a]	20.26± .148[a]	1.322 ± .012[a]	1.322 ± .012[a]
5	77.96 ± .119[a]	77.97 ± .190[a]	20.15 ±.080[a]	20.28±.029[a]	1.302 ± .004[ab]	1.316 ± .091[a]
10	77.88 ± .012[a]	77.81 ± .088[a]	19.93 ±.033[ab]	20.14±.221[a]	1.305 ± .001[ab]	1.318 ± .000[a]
15	77.59 ± .349[a]	77.78 ± .044[a]	19.71 ±.149[b]	20.10±.170[a]	1.293 ± .009[ab]	1.308 ± .002[a]
20	77.57 ± .458[a]	77.80 ± .294[a]	19.59 ±.254[bc]	19.74±.154[b]	1.282 ± .002[bc]	1.287 ± .005[a]
25	77.44 ± .547[a]	77.76 ± .145[a]	19.71 ±.027[b]	19.74±.195[b]	1.264 ± .007[c]	1.282 ± .001[a]
30	77.30 ± .001[a]	77.74 ± .033[a]	19.36 ±.051[c]	19.71±.129[b]	1.229 ± .006[d]	1.276 ± .013[a]

[a-d] Means with the same superscript letters within a column are not significantly different at $P < 0.01$.

Ref. – refrigeration temperature (5ºC)

Deep F. –deep freezing temperature (-18ºC)

6.2 Physicochemical Analyses

The chemical composition of the fresh fish fillets of Nile Tilapia (*Oreochromis niloticus*) were 77.98% moisture, 20.26% crude proteins, 0.523% crude fat, and 1.322% crude ash. The changes in the chemical composition of the fish fillets stored at 5ºC and -18ºC for 30 days are presented in Table 2.

Moisture Contents

The major component of the fish fillet was moisture (77.98%) and fresh fish fillet moisture content was within the range as previously reported by Love (1970). Shimelis Admassu and Mekonnen Melaku (2010) reported a slightly higher moisture content which was about 79.87% on Nile Tilapia fish from Lake Zeway. Similarly, Murthy *et al.*, (2011) reported a moisture content of 81.34% on Tilapia (*Oreochromis mossambicus*). On the other hand, Castrillon *et al.,*

(1997) reported 60.68% moisture content on a fatty (15.44% fat) fish Sardine (*Clupea pilchardus*) in Spain. According to FAO (1999) moisture and lipid contents in fish fillets are inversely related and their sum is approximately 80% with other components accounting for the remaining 20%.

Although there was a decrease in moisture content with a function of time under refrigeration temperature (5ºC), it was not significant decrease. It only decreased by 0.9% from the initial moisture content of the fish fillet. Zeng *et al.* (2005) reported the decrease of water holding capacity of fish muscle from about 93% to about 87% on shrimp stored in flake ice after 6 days of storage.

Similarly, the moisture contents of fillets stored in deep freeze temperature were relatively constant and not significantly affected by the frozen condition storage for 30 days. The moisture content data shows only about 0.3% decrease which is slightly lower than the loss recorded on samples kept in refrigeration condition which was about 0.9% decrease during 30 days of storage. Similarly, Shimelis Admassu and Mekonnen Melaku (2010) found no significant decrease of moisture content on Nile Tilapia fillets kept under frozen condition for 30days. Mishra and Dora (2010) also found no systematic variation in the moisture content of the flesh with the duration of frozen (-20ºC) storage of Ribbon fish for more than 30 days. This suggests that lowering the storage temperature can lower the moisture content loss. Drip loss decreases with decrease in storage temperature Oyelese (2007).

These little losses of moisture content of fish might be due to loss of water holding capacity of the fish muscle, drip loss, and desiccation as a result of temperature fluctuation during storage. It has been clearly stated that fluctuating cold store temperature (temperature abuse) is a major cause of dehydration (FAO, 1994).

Crude Protein Content

The protein content of the fresh fish fillets were about 20.26%. It can be classified as high protein (15-20%) fish category according the classification given by Stansby (1962). This protein content was relatively higher than the protein content recorded (18.52%) on the same fish species from Lake Zeway by Shimelis Admassu and Mekonnen Melaku (2010). It was also higher than

the protein content (16.72%) reported by Murthy *et al.,* (2011) on Tilapia (*Oreochromis mossambicus*) from natural freshwater body near Mysore, South India.

As indicated in Table 2, the crude protein content of fresh sample showed a significant decrease showing about 2.7% loss after 15 days of storage under refrigeration temperature, and the least crude protein content 19.36% was recorded on the last day of storage. The total crude protein content loss of fillets stored at refrigeration temperature was about 4% during 30 days of storage. Similarly, Reza *et al.* (2009) reported a significant decrease of protein contents of Silver jewfish from 16.30% to 12.10%, Bombay duck from 8.96% to 7.12% , and Ribbon fish from 20.12% to 11.70% after 13 days of storage in ice.

The crude protein content of fish fillets kept under frozen condition showed a significant decrease indicating about 2.56% loss after 20 days of storage. The protein content difference recorded only after 5 days later than that of the difference recorded on samples kept at refrigeration temperature, but the protein content was relatively constant on the later days of storage under frozen condition. Shimelis Admassu and Mekonnen Melaku (2010) reported the decrease of crude protein content from 18.52% to 17.63% which was about 4.7% loss (higher than the present study) on frozen Nile Tilapia fish species sample from Lake Zeway through 30 days of storage.

Different causes could contribute to the decrease of crude protein content on the fish sample stored under refrigeration temperature and frozen condition. This might be due to microbiological and/or enzymatic denaturation. The data shows, the loss of protein content coincides with the high microbial count in the case of refrigeration temperature (Table 1). Drip loss of water soluble protein might be the other reason. Denaturation of protein involves the destruction of its secondary, tertiary and quaternary structures, reducing the protein to a simple polypeptide chain (Careche and Li-Chan, 1997). Proteins can be degraded by Psychrotrophic bacteria on the fish muscle even at low temperatures (Venugopal, 1990; Shewan, 1961; Liston, 1980).

Ash Contents

The ash content of fresh fish sample was 1.322%. There was a significant decrease of ash contents on the fish samples after 20 days of storage at refrigeration temperature. From the initial content, a total of 7% ash content loss was recorded on samples stored at refrigeration temperature for 30 days. Reza *et al.* (2009) reported the decrease of ash content from 2.88% to 2.67% on Ribbon fish stored in ice for 7 days.

About 3.4% loss of ash content was recorded on samples stored under frozen condition through the duration of storage for 30 days. Shimelis Admassu and Mekonnen Melaku (2010) reported loss of ash content from 0.98% to 0.94% on Nile Tilapia fillets stored at -18ºC for 30 days, it was about 4% loss. Beklevic *et al.,* (2005) showed a significant decrease of crude ash content of sea bass from 1.17% to 1.06% stored at -18ºC after 9 months. Drip loss might be the reason for this ash content loss on the fish fillets during storage. There is no tangible finding that shows how the ash content of fish fillet decreases through time when kept under low temperature.

Crude Fat Content

The fat content of the fish was found to be about 0.523%.This low crude fat content of the fish is compensated by its high moisture content *ca* 78%. The results obtained by Zmijewski *et al.* (2006) found a reverse correlation between the fat and moisture contents, which is common for many fish species. The fat content is slightly higher than the fat content of Nile Tilapia from Lake Zeway (0.37%) reported by Shimelis Admassu and Mekonnen Melaku (2010). The fat content is found to be influenced by season and geographical location, with lower lipid content in fish from tropical waters (Argen *et al*., 1991). Fish with fat content below 5% are considered as lean (Stanby, 1982), hence, this fish is considered as a lean fish.

Changes of TVB-N

The results of TVB-N contents of tilapia fish fillet that was stored at 5ºC and -18ºC up to 30 days for the current study are presented in Table 3. TVB-N is a measure of the total amount of a variety of nitrogen containing substances which are produced during storage. The TVB-N content of fresh tilapia fish fillet recorded 3.92 mgN/100g was lower than the typical concentration range of TVB-N in freshly caught fish which is between 5 and 20 mg N per 100 g muscle (Huss, 1988). This may be due to the sample was analyzed quickly in less than 30 minutes, and special character of the fish. The initial TVB-N content was very low compared to the report by Shimelis Admassu and Mekonnen Melaku (2010) which was about 12.04 mg N/100gm on the same species from Lake Zeway since they needed to transport the samples for analysis from Zeway to Addis Ababa which is a distance of about 156 km.

On both storage conditions, the total volatile basic nitrogen (TVB-N) values increased although the increases were with different rate. TVB-N values of samples stored at 5ºC showed a rapid increase almost doubling every 5 days until day 15 reaching 23.80mg N/100g of sample and then it showed a constant TVB-N content for 10 days. However, the TVB-N value showed a jump from 22.96 (25^{th} day) to 45.04mg N/100g on 30^{th} day of storage. This corresponds with the conclusion of Oehlenschlager (1992) TVB-N remains constant for the first days of storage or increases slowly but rises fast later in the spoilage process. This increase in TVB-N might be due to high number of microorganisms (responsible for its development) grown on the fish fillet stored under refrigeration temperature as presented in Table 1 and it also corresponds with decrease in protein.

Similar to the present study, the increase in TVB-N content were reported by different authors. Taliadourou *et al.* (2003) showed the increase of TVB-N content of sea bass fillets stored in ice from 27.18 (on day 1) to 23.69, 26.88 and 34.78mg N/100g after 3,9 and 16 days of storage, respectively. Similarly, Jeyasekaran *et al.* (2005) reported the TVB-N content of fresh Reef Cod fish about 4.29 mg and after 6hrs delay in icing increased to 6.40 mg. The same study showed TVB content progressively increased during storage in ice and attained the acceptable limit of 30–35 mg N/100g set by Connell (1995) on the 19^{th} day of storage in ice. Similarly, Erkan and

Ozden (2008) reported the increase of TVB-N content on gutted sardine (*Sardina pilchardus*) stored in ice from 10.18 (day 1) to 18.05 mg N/100g after 5 days.

Table 3. The changes of pH values and TVB-N contents of Nile Tilapia (*Oreochromis niloticus*) fish fillets stored at -18ºC and 5ºC for 30 days.

Storage days	TVB-N(mg N/100g)		pH value	
	Refrigeration	Deep freeze	Refrigeration	Deep freeze
0	$3.92 \pm .96^{a}$	$3.92 \pm .96^{a}$	$6.23 \pm .028^{g}$	$6.23 \pm .028^{f}$
5	$7.00 \pm .48^{b}$	$4.76 \pm .48^{ab}$	$6.33 \pm .007^{f}$	$6.28 \pm .000^{e}$
10	$11.48 \pm .48^{c}$	$5.48 \pm .42^{b}$	$6.52 \pm .014^{e}$	$6.35 \pm .007^{d}$
15	$23.80 \pm .48^{d}$	$6.44 \pm .48^{c}$	$6.67 \pm .014^{d}$	$6.52 \pm .007^{c}$
20	23.80 ± 1.28^{d}	$8.12 \pm .48^{d}$	$6.89 \pm .007^{c}$	$6.67 \pm .014^{b}$
25	22.96 ± 2.56^{d}	$5.32 \pm .48^{b}$	$7.11 \pm .010^{b}$	$6.69 \pm .007^{b}$
30	45.04 ± 1.92^{e}	$5.37 \pm .16^{b}$	$7.60 \pm .007^{a}$	$6.81 \pm .007^{a}$

[a-g] Means with the same superscript letters within a column are not significantly different at $P < 0.05$. The values are means of triplicate determination ± SD.

Cao *et al.* (2008) reported the increase of TVB-N on oyster stored at 5ºC from 4.25 to 53.33 mg TVB-N/100 on day 18. Liu, *et al.* (2010) reported the increase of TVB-N from initial 6.5 to 61.3 mg TVB-N/100g on day 29 from tray-packed tilapia fillet samples stored at 0ºC. These all reports indicate the variations in initial contents and increasing trends of TVB-N values.

In the present study, the Nile Tilapia fish fillet stored at 5ºC attained TVB-N acceptance level (30-35mg N/100g) between 25th and 30th day of storage. But it does not mean the fish is acceptable until day 25 since development and accumulation TVB-N varies from species to species. Limits of acceptability for total volatile base nitrogen (TVBN) in some fish recommended were within the range 30 to 35 mg /100g (Huss, 1988); however, Ababouch *et al*. (1996) found that 25-35mg TVB-N/100g was the limit of acceptability in sardine, while El Marrakchi *et al*. (1990) found the limit of acceptability in another fish sample to be 25-30mg

TVB-N/100g. These findings suggest that the development of TVB-N in fish may vary widely and is dependent on fish species, the storage condition and a number of intrinsic and extrinsic factors. This suggests the importance to establish TVB-N rejection level for Nile Tilapia considering the possible factors.

Regards to the samples stored under frozen condition, TVB-N showed <1mg N/100gm increase every 5 days until the 15th day of storage and reached its maximum value on day 20 attaining 8.12 mg N/100gm (by 107%). Later on, the TVB-N of Nile Tilapia fish fillet decreased and showed constant values. Shimelis Admassu and Mekonnen Melaku (2010) showed the increase of TVB-N content from 12.04 mg N/100gm to 14.58 mg N/100gm (by 21%) on samples stored at -18ºC for 30 days. This indicates the effect of frozen condition to keep TVB-N content at lower level in Nile Tilapia fish fillet for longer period and it corresponds with the lower number of microorganism found on the fillets.

Changes in Muscle pH

The pH value of fresh Nile Tilapia fillets recorded was 6.23. The pH of live fish muscle tissue is close to neutrality (Huss, 1995). Pedrosa-Menabrito and Regenstein (1988) stated that pH depends on fish species and is usually between 6.2 and 6.5 immediately after *rigor mortis*.

The changes in pH value of Nile Tilapia fillet over the period of storage under refrigeration temperature and frozen condition are presented in Table 3. The pH value significantly increased with time of storage from 6.23 to 7.60 at 5ºC and from 6.23 to 6.81 at -18ºC after 30 days of storage. Ababouch *et al.* (1996) reported the increase of sardine muscle pH from 6.15 to 6.85 which at ambient temperature within 23hrs and from 6.24 to 6.55 the muscle stored in ice for 9.5 days. Erkan & Ozden (2008) reported the increase of pH on gutted sardine (*Sardina pilchardus*) stored in ice from 6.02 (day 1) to 6.20 after 9 days. Likewise, El Marrakchi *et al*. (1990) stated that the pH value of sardine (*Sardina pilchardus*) stored in ice was 5.8 on day 0 and 6.36 on day 9. Liu *et al.* (2010) reported significant increase of pH on tray-packed tilapia fillets stored at 0ºC from about 6.5 to about 7.0 on day 21. Jeyasekaran *et al.* (2005) reported the initial pH of 6.36 on the Reef Cod (*E. merra*) fish began to decrease on the third day of storage in ice, but increased from the seventh day onwards, and attained a pH of 7.47 on the 19th day of storage

resulting from the production of volatile bases by bacteria. Shimelis Admassu and Mekonnen Melaku (2010) also reported the increase of pH from 6.43 to 6.49 on Nile Tilapia samples stored under frozen condition.

Increases in pH indicate the accumulation of alkaline compounds, such as ammonia (member of TVB-N compounds) mainly derived from microbial action. After glycolysis, autolytic changes such as denaturation or breakdown of proteins provide an optimum condition for growth and reproduction of spoilage microflora, which can produce amines raising the product pH (Pedrosa-Menabrito and Regenstein, 1988). The increase of pH values might be attributed to the formation of basic decomposition products, such as ammonia and trimethylamine, which are members of TVB-N. The result shows increase in pH value during storage was associated with the increase in total volatile basic nitrogen (TVB-N) in the fish muscle which in turn correlates with the increase of microbial deterioration of the fish muscle. However, pH alone cannot indicate spoilage; it has to be supported by other microbiological, chemical and sensory analyses (Scott *et al*., 1992).

7. Conclusions and Recommendations

The results show that Nile Tilapia contains high protein and low fat which is an important attribute in commercial food processing. The fish caching and processing technique has not been done under good sanitary conditions since the fresh fillets held high bacteria load. Thus, consumption of raw fish should be avoided. This high bacteria load also had impact on the shelf life of the fillet during storage. During storage of fish fillet at refrigeration temperature, there were significant increases in microbiological load and loss in physicochemical quality. The fillet loses its microbiological acceptability level very shortly. Hence, refrigeration temperature could not be reliable storage condition for longer time unless it is intended for temporary storage. Although there were changes in nutritional and physicochemical quality, frozen condition storage could maintain the fish fillet qualities for longer time. In general, the results obtained showed that storing at frozen condition can significantly improve the shelf life of fish quality compared to refrigeration temperature storage. Besides this, fish catching, handling and processing need to be evaluated and improved to assure quality and safety, and to minimize loss.

Further study is required to address important questions surrounding fish and fish products of Lake Tana. The studies need to include; identification and characterization of specific spoilage organisms, potential pathogenic microorganisms and parasites, and further studies to establish fish quality acceptance levels, detailed quality analysis and addressing the other species.

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