The United Nations University
FISHERIES TRAINING PROGRAMME

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121 REYKJAVIK, ICELAND

FINAL PROJECTS
1999

A COMPARATIVE STUDY OF STORAGE TIME OF WARM AND COLD WATER FISH IN VIEW OF THE CURRENT MARKET DEMANDS

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ABSTRACT

Fillets from warm and cold water fish were purchased from respective processing plants and stored at 0°C and 5°C in commercial styrofoam boxes for as long as acceptable. During storage, sensory, microbiological and chemical tests were carried out. The warm water Nile perch Lates niloticus and the cold water Ocean perch Sebastes marinus stored at 0°C remained acceptable for 2-3 weeks and 1 week respectively. Fillets stored at 5°C reached the limit of acceptability within a week of the trials. The TPC at limit of acceptability was 10^6-10^7/g in plates incubated at 22°C and 10^4 in plates incubated at 37°C for both fish stored at both temperatures. The H2S- forming bacteria, were slightly more in fillets stored at 5°C than fillets stored at 0°C. Plates incubated at 37°C had fewer H2S- forming bacteria than plates incubated at 22°C. Correspondingly, the TVB-N was ≥ 20mg/100g and ≥ 25mg/100g for Nile perch and Ocean perch respectively. The TMA was five times more in Ocean perch (≥50mg/100g) than Nile perch (<10mg/100g) in both storage temperatures. The major bacterial spoilers in Nile perch were identified as belonging to *Pseudomonas* group 1 and 2.

Key words: Nile perch, Ocean perch, bacteria and storage time.

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1 INTRODUCTION

For the last fifteen years, there has been an increase in international fish trade from less developed countries (LDCs) to the more industrialised countries. This trend has led in part, to the evolution of safety and quality regulations, and their imposition on every fish consignment imported. Examples of the regulations include the European Union (EU) Council Directive 91/ 493/ EEC and the United States of America's Food and Drug Administration (FDA): 21CFR. Often, fish consignments from LDCs have been found to have unacceptable levels of microbiological contamination (EU 1997, ´98 &´99). The microbiological contamination of concern has been high loads of unspecified spoilage bacteria and pathogenic bacteria; namely Salmonella spp and Escherichia coli. In the majority of cases, this has led to rejection or incineration of whole consignments of fish exported. More often than not, the exporter from the LDC incurs financial loss and in addition pays for incineration of the consignment.

Unacceptable levels of microbiological contamination have been attributed to the less developed quality management systems in most LDCs, which do not ensure compliance to the safety and quality regulations. This assertion however, may not be entirely true because the standard limits set in the regulations have usually been based on studies carried out on temperate and marine water fish, which may not necessarily apply to tropical freshwater fish. In addition, the methodologies used in establishing the quality limits in importing countries may not be analogous with those used in exporting countries. Admittedly, although most LDCs have inadequately equipped laboratories and may not have enough well qualified and efficient personnel to handle large numbers of samples at any given time, these shortcomings should not be sufficient reasons to impose unnecessary restrictions on LDCs exports. A mechanism should be put in place to critically examine non-compliant consignments for the underlying cause(s).

There has been little research conducted on spoilage trends and microbiological standard limits in tropical fish. Consequently, there is limited information on most fish species in Ugandan lakes, with research only having been carried out on Lake Victoria Nile perch bacteriology and spoilage patterns of Nile perch kept at 0^{o}C and $\leq 20^{o}$C (Gram et al. 1988, Gram 1989, 1990). However, the Food Science and Technology Research Institute (FOSRI) in Uganda has been mandated by the 1992 Act of Parliament to carry out demand driven research work in fish and other related food products. Presently, some preliminary studies on shelf life of Nile perch at different storage temperatures has now been done (FOSRI 1997) but there are still information gaps which need to be filled.

However, there has been considerable research carried out on temperate water fish and spoilage patterns of fish have been well-documented (Shewan 1961, Banwart 1981, Hobbs 1982, Howgate 1982, Connell 1990, Huss 1992, 1994, 1996). Generally, spoilage tends to vary according to species, feeding habits, seasonality, gender, age and possibly geographical location which is partly a basis of this study. Spoilage patterns differ between species of fish; for example round fish deteriorate much faster than flat fish, such as cod and plaice respectively (Howgate 1982). The bacteria that have been implicated in the majority of microbial spoilage cases have the ability to proliferate in sub-environments provided by the skin/shell surfaces, gills and the alimentary canal.

Microbial levels vary depending on water conditions and temperature (Sumner and Magno 1985). Fish from colder waters or temperate with temperatures <10°C, generally yield counts of 10^2-10^4 CFU/cm^2 of skin and gill surface, while fish from the tropics have levels of 10^3-10^6 CFU/cm^2 when incubation temperatures are 35-37°C (Shewan and Murray 1979, Liston 1980, ICMSF 1998). This suggests that bacteria on fish from warm waters are more mesophilic than bacteria on temperate water fish. In addition, it also implies that fish from the tropics spoils much faster than fish from temperate waters by virtue of its high microbial load, if all other parameters are kept constant. When fish is held on ice however, the fish from warm waters keeps longer than fish from cold water. This widely held view is apparently in relation to the relative proportions of psychrotrophic bacteria on fish in waters of different temperatures. Cold waters tend to favour proliferation of high numbers of psychrotrophs on fish, which in turn enhances spoilage and ultimately shortens the shelf life of a fish. Gram et al. (1990) however, disagrees with this hypothesis with regard to Nile perch where psychrotrophs accounted for 10% of the total number of bacteria. In the present study, Nile perch caught in the waters of Lake Victoria is acclimatised to an average temperature 26°C all year around. On the other hand, the Ocean perch probably caught in the west of Reykjanes within the Icelandic EEZ is adapted to an average temperature of 8°C. By implication, the psychrotrophic microflora on Ocean perch would be present in higher numbers than on Nile perch and therefore the former would be expected to spoil faster than the latter.
Essentially, fish spoilage can be attributed to three main causative factors namely; microbial, enzymatic and oxidative rancidity. The most prevalent form in fresh fish, is microbial spoilage (Bligh 1980).

For a decade Uganda has been exporting chilled Nile perch fillets to several countries including those in the EU, which accounts for 80% of total fish exports. In 1997, fish exports contributed over US$ 100 million to the Ugandan government and it was second to coffee among the traditional export commodities (MTI 1997). Chilled fillets for this lucrative market are processed and air freighted the same day, thereby ensuring quality and a safe product. In principle, the fillets should remain in acceptable condition for 12 days, but according to the market vendors in Europe, Nile perch fillets have a shelf life of 5-7 days. Consequently, either the market vendors are inaccurate in their supposition of shelf life or the Ugandan processors are not processing freshly caught fish. Presently, EU has imposed a ban on fish exports from Uganda purportedly for non-compliance on several regulatory demands (1997&1998). According to the Ugandan State Minister for Agriculture in charge of Fisheries, the ban is costing the country US$ 800,000 per week in lost revenue, (Kukunda 1999). The regulatory demands from the EU ranged from procedural laboratory sample traceability to unacceptable levels of chemical and microbiological contamination in the exported fish consignments.

For the purpose of this study, Nile perch (Lates niloticus) a predacious tropical fresh water fish, predominant in Lake Victoria and Ocean perch (Sebastes marinus) a temperate marine fish inhabiting the North Atlantic ocean along the European and American coasts, were chosen. Nile perch is capable of growing to a maximum size of 2 m and weighing 180-240 kg. The market size most preferred by consumers weighs 2-3 kg and 70 cm in length with a fat content of 5-6%. In contrast, Ocean perch on the other hand, attains a maximum length of 90 cm (Bykov 1983). The marketable size

landed at Reykjavik harbour is between 37 - 40cm in length, with a corresponding weight of 685- 891 g (Sigurdsson 1999, pers. comm.) and a fat content of 3-5% (Bykov 1983). Thus the main objectives of this study are fourfold.

1.1 Objectives of the study

- To establish the limit of acceptance for chilled Nile perch fillets on the EU market.
- To test the notion that fish from the tropics has a longer storage time at 0°C than fish from temperate waters.
- To gain knowledge on microbiological methodologies.
- To increase microbiological information on tropical fish especially Nile perch.

2 MATERIALS AND METHODS

2.1 Sample collection

Fillets of Nile perch (total 132) were airfreighted to Reykjavik from Greenfields (U) Limited, Entebbe -Uganda. The Nile perch had been caught between the 3rd and the 6th of October 1999 in Lake Victoria and processed on the 7th October. The average temperature of the fish samples on arrival was 0.35°C after a shipment period of 70 hours via Amsterdam and Brussels. Thus, the fillets were 11-9 days old on arrival. The average weight of an individual fillet was 280 g.

Four-day old Ocean perch fillet samples, (total 147), and with an average weight of 123.5 g, were bought locally from Grandi fish processing plant in Reykjavik. The average temperature of the fillets on arrival was 4.7°C but had been around 0°C prior to processing.

It was deemed necessary to establish the initial microbial load on Nile perch, four hours after capture. An area of 15 cm^2 of the skin of whole 3 kg Nile perch was swabbed twice, once on each side. The exercise was repeated for two more perches thus, providing six sample plates. Subsequent sample preparations proceeded according to the Gram et al.1988 method. The plates were packed in a styrofoam box and surrounded with ice packs to provide chilled conditions, then air-freighted to Reykjavik where the plates were incubated at 37°C and 22°C.

2.1.1 Sample treatment

A total of 47 and 42 fillets of Ocean perch (OP) and Nile perch (NP) respectively, were stored in styrofoam boxes at 5°C. The remainder of the fillets for both species were similarly packed and kept at 0°C. It was envisaged that the fish stored at 0°C might still be acceptable for a maximum of 3 weeks while for the fish stored at 5°C may do so for only one week.

Three types of analysis were carried out per treatment per fish species, over the period of the experiment. They included sensory evaluation, microbiological and chemical assessment.

2.1.2 Sampling plan

For each sampling day, two fillets of NP and four fillets of OP per storage temperature, were aseptically and randomly picked from the styrofoam boxes for all three tests. Sensory evaluation required half the number of the fillets while microbiological/chemical analysis shared the remaining half. The analyses were carried out twice a week, according to the availability of the sensory assessors.

Table 1: Summary of type of tests, parameters analysed and methods used.

Type of analysis	Parameters to be analysed	Methodology
Sensory	Appearance, texture, mouth-feel and flavour after cooking.	Trained panellists
Microbiological	Total plate count and counts of H2S-forming bacteria.	Standard methods e.g. Gram et al. 1987
Chemical	TVB and TMA	Steam distillation

2.2 Sensory methods

Fish quality was assessed by Icelandic Fisheries Laboratories (IFL) trained panel consisting of 10-12 persons. Since the samples were fillets as opposed to whole fish, shelf-life determination was based on acceptability of cooked fish. Cooked samples according to Howgate (1982) give a reliable assessment of freshness. A scale from 0-10 was used (Appendix 1&2). Samples retaining odours and flavours typical for the species were given scores above 6. Fish with slightly off-odours and off-flavours were given scores 4-5. Scores below 4 indicated objectionable strong unpleasant off-odours and off-flavours. Shelf life was established by the duration between the time of capture and the time when the cooked quality score dropped below the limit of acceptability. Samples were cut into 20 g pieces, boiled in aluminium boxes at 98°C for 5 minutes and served to each panellist. Samples were served in duplicates.

2.3 Microbiological methods

Iron Agar according to Gram et al. (1988) was used for Total Plate Count (TPC) and counts of H2S-forming bacteria. A primary dilution was prepared by homogenising 25 g of fillet with 225 g of Butterfield's buffer using a Seward stomacher 400 lab. system. Decimal dilutions were prepared in buffer and 1 ml of appropriate dilutions poured on a plate with Iron Agar and an overlay. Duplicate plates from samples kept at 0°C and 5°C were incubated for 72 and 48 hrs at 22°C and 37°C respectively, as a matter of routine, although Gram (1992a) had suggested 35°C for tropical fish, to select mesophilic organisms and 22°C to select pschrotrophic. The number of black colonies i.e. H2S-forming organisms and white colonies were counted thus constituting total plate count or TPC. Identification and confirmatory tests for the microbes present were also carried out. Prior to identification, there was a need to isolate pure strains from TPC plates. To do this, isolates were streaked on Plate Count Agar (PCA) and incubated at appropriate temperatures. In this case, an incubation temperature of 22^{o}C was used. PCA was prepared according to methods described in Appendix 3.

To establish composition of bacterial flora 25 randomly selected colonies were picked off from a pair of IA plates, which had 25-250 colonies, then streaked on PCA plates for further confirmatory tests.

Confirmatory tests included Gram staining on 1 day old culture (Hucker's modification); motility test on fresh cultures in Nutrient broth by the "hanging-drop" method and microscopic identification of shape and other characteristics; catalase (3% H202); production of oxidase (Kovacs 1956); fermentation/oxidation test for glucose metabolism in MOF medium (Leifson 1963 without 0.5% NaCl). The tubes were incubated at 22°C and acid formation recorded after 7 days (Appendix 3 for detailed procedures).

2.4 Chemical methods

2.4.1 TVB-N determination by steam distillation

200 ml of 7.5% aqueous trichloroacetic acid solution was added to 100 g of fish muscle and homogenised in a Waring blender. The mixture was filtered through a Whatman n^{o} 3 paper. 25 ml of filtrate were transferred into a distillation flask and 6 ml of 10% NAOH were added. Steam was produced by using a Kjeldahl-type distiller (Vapodest Gerhardt). A beaker containing 10 ml of 4% boric acid and 0.04 ml of methyl red and bromocresol green indicator was placed under the condenser for the titration of ammonia. Distillation was started and steam distillation continued until a final volume of 50 ml was obtained in the beaker (40 ml distillate). The boric acid solution turned green when alkalinised by the distilled TVB-N, which was titrated with aqueous 0.25N sulphuric acid solution using a 0.01 ml, graduated microburette. Complete neutralisation was obtained when the colour turned pink on the addition of a further drop of sulphuric acid (Malle and Poumeyrol 1998).

Calculation: (n´ml)(14mg/mol)(0.025/L)(300)(100)/25(1000)=(n´ml)(4.2)mgN/100g.

2.4.2 TMA determination by steam distillation

200 ml of 7.5% aqueous trichloroacetic acid solution was added to 100 g of fish muscle and homogenised in a Waring blender. The mixture was filtered through a Whatman n^{o} 3 paper. Formaldehyde (20 ml) was added to the distillation flask to block the primary and secondary amines.

An aliquot of the TCA extract was reacted with 45% KOH, formaldehyde and toluene, thoroughly shaken, and allowed to stand for 10 min, after which the toluene layer was removed. An aliquot of the water-free toluene extract is reacted with a picric acid solution and the resulting colour is quantitatively measured using a spectrophotometer (Malle and Poumeyrol 1998).

TMA-O colorimetric method (Bystedt 1959)

An aliquot of the TCA, 20-40 drops of TiCl3 were placed into a 100 ml volumetric flask and left standing for 1 hr. When about 10 drops of KNO3 were added, the violet colour disappeared and the flask was filled to the mark with water. All TMA-O was

then expected to have been reduced to TMA (=reduced TMAO + TMA before reduction) which is measured according to TMA-N calorimetric method.

TMA-O = total TMA - TMA before reduction.

3 LITERATURE REVIEW

3.1 General interrelationships between fish deterioration and extrinsic variables

The biochemical composition of fish or intrinsic factors and their interrelationships with post-mortem extrinsic factors, contribute substantially to the perishability of fish as a food commodity because they determine the initial contamination (Huss et al. 1997). The physical, chemical and bacteriological characteristics of fish tend to vary with species, feeding habits, seasonality (Smith et al. 1980), spawning cycles, methods of catching, fishing ground (Bramsnaes 1965, Whittle et al. 1980) size, age, (Ssali 1988) environment, initial microbiological load and possibly geographical location (Shewan 1961). However, temperature and rigor mortis appear to be the major underlying factors.

Howgate (1982) observed that round fish e.g. cod deteriorated much faster than flat fish like plaice. Fish show considerable variations in spoilage during their feeding and spawning cycles. Heavily feeding fish tend to be more susceptible to autolytic tissue degradation than petite feeders (Gildberg and Raa 1980). In addition, the type of food on which they feed may have an effect on their post-mortem spoilage (Bramsnaes 1965). The number and variety of microorganisms are determined by the quantity and origin of food consumed by the fish. A non-feeding fish has low levels of bacteria in the intestines as compared to a heavily feeding fish. When spawning fish tend to use most of their glycogen or energy and the effect of the depletion is reflected in their susceptibility to rapid deterioration (Connell 1990).

The lipid and moisture content of fish tend to rise and fall inversely. This phenomenon seems to affect texture, flavour and possibly the rate of microbial spoilage (Huss 1994, ICMSF 1998). The method of capture is known to have profound effect on the keeping quality of fish. Bramsnaes (1965) noted that fish that had been trawled had a shorter shelf life than fish that had been caught by long line. The difference was attributed to the rate of struggling prior to death, which corresponded with the level of glycogen at the time of death, crushing and anoxia. The more exhausted a fish is when it dies, the lower the muscular reserves of glycogen and the smaller the drop in pH and therefore the higher the level of spoilage. Haddock caught by long line and a trawl net had a glycogen level of 0.12 % and 0% respectively. The drop in pH triggers the fast onset and resolution of rigor mortis which, is the prerequisite for autolytic spoilage (Liston 1980, Huss 1992). The change in pH weakens the wall membranes of cellular components including the lysosomes which contain proteolytic enzymes which, when released digest the surrounding tissues.

The geographical location appears to determine the spoilage insofar as temperature, microenvironments and food types are concerned. Sometimes, the environment/

fishing ground in which fish live becomes hazardous to their well being and this becomes apparent in the post-mortem characteristics. Bramsnaes (1965) citing other authors reported an unusual phenomenon in certain fishing grounds in the North Sea in which the fish flesh was completely broken down as if by digestive enzymes. The phenomenon did not coincide with bacterial spoilage. Spores of the parasitic protozoan *Chloromyxum thyrsites* were thought to have been the cause. There is a similar phenomenon in Lake Victoria commonly known as "*Kaliiro*". During certain periods of the year, fishermen report deterioration of their catches on unprecedented scale without the intervention of bacteria or other causative factor. It is important to note that, it also does not coincide with the increase in bacterial numbers as expected during the thermal stratification of the lake, which takes place during the month of July. The water temperature at this time of the year is much lower (20-22°C) than the normal 26-28°C. In addition, the population of microbes associated with live fish reflects microflora of the environment/ fishing ground at the time of capture and this constitutes to initial microbial load. The level of pollution in the environment or fishing ground corresponds with the risk of spoilage. Bacteria establish themselves on the outer and inner surfaces of the live fish (gills, skin, gastro-intestinal tract) and usually gain entry in the inner parts of the fish where they play a leading role in subsequent spoilage.

Essentially, the intrinsic and extrinsic factors are interrelated during post mortem fish spoilage and their effects are only enhanced by inappropriate handling practices and facilities along the handling chain.

3.2 Spoilage

Botta (1995) defines fish spoilage as a change in a fish or fish product that makes it unsafe, less acceptable, or unacceptable to the consumer for its original intended purpose. The rate of spoilage differs among fish species because of different defence mechanisms and physical barriers like mucus in plaice which contains lysozomes to fend off intruding foreign bodies (Shewan and Murray 1979).

Fish are much more perishable than any other high protein muscle foods. The high degree of perishability is primarily due to the large amounts of non-protein nitrogen NPN) e.g. free amino acids, volatile nitrogen bases i.e. ammonia, TMA, creatine, taurine, uric acid, carnosine and histamine (Mayer and Ward 1991). The readily available NPN compounds support the post-mortem bacterial growth (Connell 1990, Huss et al. 1997). Fish flesh provides an excellent substrate for the growth of most heterotrophic bacteria with compositional attributes that affect bacterial growth and the related biochemical activities. Fish also possess a neutral or slightly acid pH and high moisture content, which permit the growth of a wide range of microorganisms (Huis in't Veld 1996). The poikilothermic nature of the fish allows also bacteria with a broad temperature range to grow (Gram and Huss 1996, Huss et al. 1997). Microbiological contamination therefore has been noted as the main cause of fish deterioration, followed by non-microbiological namely: oxidative rancidity and then chemical or enzymatic denaturation of proteins (Bramsnaes 1965, Liston 1980, Huss 1994). The latter may be a result of microbial activity or change in the cellular microcomponents of fish (Connell 1990). The immune system in live fish confines the bacteria on the surface of the skin and/or within the walls of the viscera. Upon death, there is a migration of bacteria into the interior of the flesh where they degrade tissue

components leading to unpleasant odours and flavours associated with spoilage (Connell 1990, Huss 1994). The unpleasant characteristics of spoiling fish are not caused by chemical changes but are a result of bacterial activity.

The non-microbiological forms of spoilage are usually associated with either enzymatic activity and/or chemical reactions with fish components. When a fish is alive, its life processes are maintained by a complicated and interacting system of chemical reactions mediated by a complex organic compounds and enzymes that enable the reactions to proceed smoothly and under control (Howgate 1982, Huss 1994). After fish dies, the enzymes still remain active and chemical reactions continue (Hobbs 1982) but the balance of reactions is modified for example, the onset and resolution of rigor mortis which lowers and raises the pH respectively (Connell 1990). The change in pH affects the enzymatic and other chemical reactions in fish muscle cell, which may result in flavour and odour changes. Enzymatic hydrolysis of lipids in fatty fish results in sweaty and slightly cheesy odours and flavours (Howgate 1985). The post-mortem endogenous biochemical changes in nucleotides and lipids occur after deaths (Pedrosa-Menabrito and Regenstein 1989, Hultin 1992) reduce the flavours and odours associated with freshly caught fish. Odours and flavours follow definite patterns as the fish spoils. Initially, there is a great difference between species but as spoilage continues the flavours and odours become alike (Howgate 1982). Although oxidative rancidity plays a minor part in the deterioration of chilled fish as opposed to microbiological spoilage, inadvertent exposure of fatty fillets to atmospheric oxygen may lead to deteriorating changes characterised by off flavours and odours (Hobbs 1982).

Fish spoilage essentially, can be attributed to three main causative factors namely microbial, enzymatic or autolytic and chemical spoilage (oxidation) which occur in sequence as shown in Figure 1.

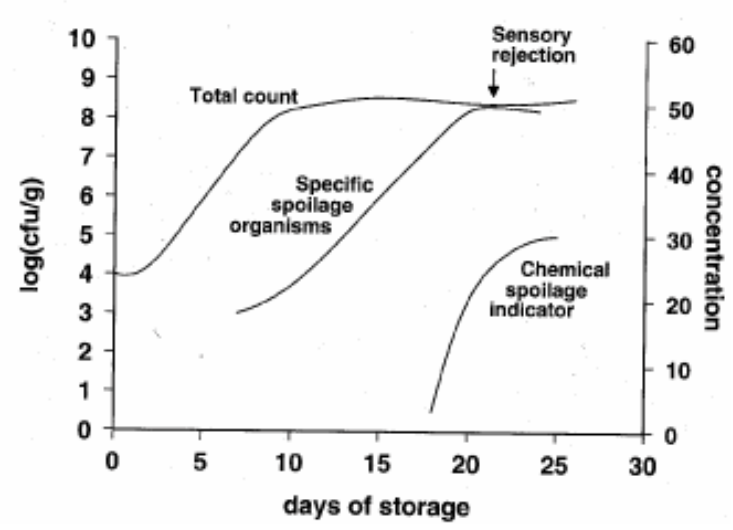

Figure 1: Model of changes in TPC, specific spoilage organisms (SSO) and chemical spoilage indices during chilled storage of a fish product (Modified from Gram et al. 1996).

3.2.1 Microbiological spoilage

The microbiological form of spoilage has been known to be more prevalent than either enzymatic or oxidative rancidity (Liston 1980) accounting for a substantial percentage of spoilage in the fish industry (Shewan 1961). Its prevalence has been attributed to the aforementioned myriad of interactive spoilage intrinsic/extrinsic factors and the wide range of growth tolerance limits of spoilage bacteria (Huss et al. 1997). Bacteria are characterised according to their optimal growth requirements, which include among others water, oxygen, pH, temperature and redox potential (Eh). Their distribution in the environment is therefore dependent on their requirements. Bacteria living on the surface of marine animals are phenotypically capable of utilising amino acids, peptides and other non-carbohydrate sources. Utilisation of these substrates normally leads to the production of slightly alkaline conditions especially in the stored fish products (Liston 1980). Enterobacteriaceae including coliforms are not isolated from fish captured away from the coastline. Its presence on fish implies that the catch was mishandled at the landing beaches (FOSRI 1997). Bacteria from skin and gills are predominantly aerobic although facultative bacteria particularly *Vibro* may occur in high numbers on pelagic fish. Obligatory anaerobic bacteria are common on the surface of fish but occur in significant numbers in the intestines (Bramsnaes 1965). Generally, bacteria on fish from temperate waters are predominantly Gram negative, while those on fish from tropical warm waters are Gram positive (Liston 1980, Huss 1994). According to the ICMSF (1998) review, common bacteria found on living fish in warm waters include *Bacillus, Micrococcus* and *Corynebacterium* which constitute 50-60% of the total microflora. The microflora found on fish from cold waters is slightly different. It is composed of *Psychrobacter (Moraxella) Pseudomonas, Actinobacter, Shewanella, Flavobacterium, Cytophaga* and *Vibro*. The microflora on fresh water fish exhibit a similar pattern of preference except that *Aeromonas* replaces *Vibro*. The pathogenic bacteria namely *Compylobacter jejuni, Yersinia entercolitica, E. coli, Shigella spp and Salmonella spp* have all been isolated from fish taken from waters subjected to human sewage pollution or terrestrial run-off. It is unclear whether the microbes are inherent in the fish or acquired during capture (Shewan 1961, Banwart 1981, Huis in't Veld 1996).

Spoilage bacteria are characterised both by their dominance in the microflora of spoiling fish and their ability to produce spoilage compounds (Gram et al. 1987). The bacteria most commonly identified with spoilage are species of *Shewanella* and *Pseudomonas* (Gram et al. 1992, Huss et al. 1997). The latter has been found dominant in spoiling tropical or fresh water fish (Huss 1994) Accumulated metabolic products of bacteria are the primary causes of the organoleptic spoilage of raw fish. The microbial activity on reduced compounds, produce the characteristic fishy, ammoniac and sulphide odours and change the texture to the slimy and pulpy constitution associated with spoiled fish. For example, when trimethylamine oxide (TMAO) usually present in marine fish but has been also found recently in Nile perch (Gram 1989), is typically reduced to trimethylamine (TMA) by spoilage bacteria, it produces the characteristic "fishy" smell of spoiled fish. Reduction of peptide to ammonia gives the ammoniac and sulphide odours. The release of fatty acids and break down of sulphur-containing amino acids to methyl mercanptan, dimethylsulphide and hydrogen sulphide accords the characteristic smell of spoilt fish (Gram 1992a, Kraft 1992, Gram and Huss 1996). At lower storage temperatures *S.*

putrefaciens dominates and has been consistently isolated from chilled products throughout the world (Gram 1992a, Kraft 1992, Huss 1994).

According to Liston (1980) microflora of different fish species is dominated by Gram negative bacteria, for example, *Pseudomonas, Aeromonas hydrophila*, and *Vibro* bacteria, which dominate in spoiled fish, kept at elevated temperatures of 10-37^{o}C. During storage, specific spoilage organisms (SSO) generally grow faster than other organisms in the natural microflora, produce the metabolites responsible for off-flavours, off-odours or slime (Huss 1994) and finally cause sensory rejection. The sequence of which has been illustrated in Fig. 1.

3.2.2 Autolytic spoilage

The enzymes and other related chemical reactions do not immediately cease their activities in the fish muscle (Howgate 1982). Their continuation initiates other precursor processes like rigor mortis, which is a basis for autolytic spoilage. According to Huss (1994) autolytic changes are responsible for the early quality loss in fresh fish but contribute very little to spoilage of chilled fish and fish products. However, under frozen conditions, autolytic enzymes break down TMAO to dimethylamine (DMA) and formaldehyde (FA). The fish viscera contain proteolytic enzymes responsible for food digestion but when fish die, they attack the organs and the surrounding tissues culminating into a condition known as belly-burst. They are also capable of penetrating the flesh and causing additional damage (Connell 1990). This autolytic tissue degradation tends to be more pronounced in heavily feeding fish than petite feeders (Gildberg and Raa 1980).

3.2.3 Chemical spoilage (oxidation)

Generally, fish have higher degree of unsaturated lipids than most other foods and therefore susceptible to oxidative rancidity (Connell 1990). After death, the lipids in fish are subjected to two major changes, lipolysis and auto-oxidation (Hardy 1980) which constitute important chemical spoilage processes in fish (Huss 1994). The main reactants in these processes involves atmospheric oxygen and fish lipid but the reactions are initiated and accelerated by heat, light (especially UV-light) and several organic and inorganic substances like copper and iron ions. The end products are aldehydes and ketones, which impart the strong rancid flavour normally, associated with spoilt fatty fish (Huss 1994).

3.3 3.3 Factors influencing spoilage

Apart from intrinsic factors such as moisture content, pH, onset and resolution of rigor mortis, chemical composition, redox potential, other factors may influence spoilage in fish, if not eliminated or reduced considerably. These include temperature, fishing method, initial microbial load in association with fishing ground, post-mortem handling procedures and processing facilities. Literally, every stage of handling from harvest to consumption affects spoilage in one way or another.

3.3.1 Temperature

The most important factor influencing the composition of microflora is temperature. After capture and death, fish is normally in chilled brines or ice giving rise to changes in the microflora. Typically, bacterial populations on fish from temperate waters are predominantly psychrotrophic reflecting water temperatures of ≤10^{o}C in the main water mass, while fish from the tropics, especially pelagics have mesophilic bacteria (Gram and Huss 1996). This can lead to rapid spoilage if chilling is delayed. On the other hand, because the natural incidence of psychrotrophic bacteria on tropical fish is low, rapid chilling and sustained low temperature storage can result in long shelf life for tropical species (Gram et al. 1989). The microflora of marine fish are predominantly halotolerant , able to grow over a wide range of salt concentrations but display optimal growth at 2-3% salt concentration. This is enhanced by the common use of ice to chill the fish, thus exposing the bacterial populations to decreasingly saline conditions during storage. This seems to favour the survival and growth of halotolerant species e.g. *Vibro spp*. (ICMSF 1998). Temperature is the primary factor controlling spoilage in fish because it has direct effect on the growth of microorganisms. Connell (1990) reported that increasing the temperature from 0^{o} C to 5^{o}C at least doubled the rate at which cod and similar species spoil (Fig. 2). Mayer and Ward (1991) contend that temperature contributes immensely to the perishability of fish as evidenced by the effective inhibition of bacterial growth on fish harvested from warm waters by refrigeration. Chilling exerts selective pressure on the bacterial populations on the surface of the fish resulting into mesophiles failing to grow and psychrotrophs increasing in abundance. Poulter and Nicolaides (1985) observed that psychrotrophs were able to grow and utilise the non- protein nitrogen (NPN) compounds relatively rapidly at 0^{o}C. They also found that when fish from temperate regions were held at 0^{o}C there was a bacterial lag phase from 1-5 days and with exponential growth from 6-14 days. Sensory evaluation of shelf life showed a rapid deterioration from about 12 days onwards.

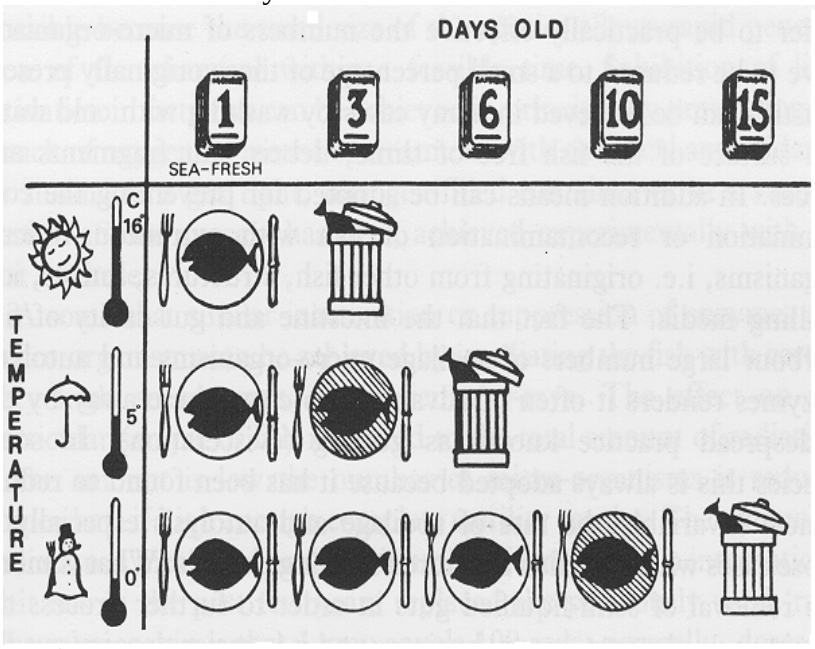

Figure 2: The effect of temperature on storage life of fresh temperate fish. (Connell 1990).

This corresponds to accumulation of bacterial metabolites e.g. hydrogen sulphide. The counts on tropical fish handled properly, chilled rapidly and held at $0^{o}C$ show some extension of the lag phase and marked decrease in growth rate (Mayer and Ward 1991). This results in a shelf life of ≥30 days for a number of species (Poulter and Nicolaides 1985, Gram et al. 1989). The effect appears to be due to both the low concentration of bacteria on tropical fish that are able to grow at low temperatures and an intrinsically lower growth rate for the naturally occurring psychrotrophic species (Deveraju and Setty 1985). Storage at adequately low temperature will not prevent spoilage, but will limit spoilage to psychrotrophic microorganisms. Generally, they are largely comprised of Gram negative rod-shaped non-spore forming bacteria *Pseudomonaceae* and some Gram positive rods lactic acid bacteria notably *Lactobacillus. Pseudomonas* and particularly *Shewanella putrefaciens* are the most common spoilage organism in fish. Gram and Huss (1996) attributed their importance in fish spoilage, to their wide distribution in the environment, their ability to utilise a wide range of materials as substrates for growth and the ability to contaminate a product from many sources.

3.3.2 Rigor mortis

In live fish, there are different enzymes naturally present in fish flesh and engaged in normal processes like tissue building and muscular contraction and relaxation. After fish die, they become involved in predominantly degradating reactions. One of these reactions is the gradual hydrolysis of glycogen to lactic acid, resulting in the fall of pH from the normal neutral to acidic i.e. 7 to 5.8-6.8 depending on the species and condition of the fish. The decline in pH and the imbalance in the biochemical reactions within the fish musculature brought about by the post mortem continuation of enzymatic activity (Hobbs 1982), initiates a phenomenon referred to as rigor mortis. It involves the stiffening of the fish muscles, which subsequently may pose processing problems. It affects the quality of the fish, as the texture of the flesh is rendered firmer because of its tendency to lose moisture. From the microbiological viewpoint, it is an advantage to have a low pH as it retards bacterial activity but in practice, the degradation of NPN compounds raises the pH during the post-rigor considerably. The increase in pH from 6-8.0 in some species provides a favourable environment for bacterial proliferation (Connell 1990). The change in pH affects the enzymatic and other chemical reactions in fish muscle cell, which may result in flavour and odour changes.

3.3.3 Method of capture

Fish caught by hook and line die or are killed relatively rapidly when brought into the air (ICMSF 1998) and the method minimises stressed and its associated deterioration attribute. Fish caught by a seine net, has a better quality index than fish caught in a trawl net, which tends to compact the fish and in so doing presses out guts with their high bacterial contents. The spilled bacteria utilise the available substrate and their metabolic products constitute deterioration. Gillnetting on the other hand entails fish struggling, which in turn quickens the onset of rigor and subsequent deterioration (Mayer and Ward 1991, Sorensen and Mjelde 1992). Bacteria may gain access through puncture wounds and bruises during the death struggle and may multiply rapidly in these localised areas. The degree of struggling before death reduces the levels of glycogen in the fish muscles, which has a negative impact on quality as far

as the texture is concerned. A method that inflicts stress or struggle hastens the onset of spoilage.

3.3.4 Initial microbial load

The initial microflora on the surface of the fish is directly related to the water environment while the flora in the gastro-intestinal tract corresponds to the type of food and the condition of the fish (Liston 1980). According to Huss et al. (1997) intrinsic and extrinsic factors determine the initial bacterial contamination. This is further enhanced by, the poikilothermic nature of the fish, the presence of TMAO which increases the redox potential, the presence of large amounts of NPN and low carbohydrate content which increases the pH in the flesh post-mortem. Bacterial numbers range from 10^2 to 10^6 CFU/cm^2 on the skin, 10^3-10^5/g on the gills and in the intestines from very few in non-feeding fish to 10^7 or greater in feeding fish (Liston 1980, Mayer and Ward 1991). During storage however, the counts on a given morphological entity, may increase by a minimum of one logarithm (Liston 1980). Regardless of the differences in the initial microflora, the spoilage patterns of fish during ice storage are usually quite similar and are caused by *Pseudomonas sp*. and *Shewanella putrefaciens* (Gram and Huss 1996).

3.3.5 Post-mortem handling practices and storage procedures

Thrower (1987) and Ward and Baj (1988) have documented various post-harvest practices and stowage procedures, which enhance microbiological contamination, and subsequently influence spoilage. During capture operations, fish come in contact with nets, ropes, deckboards, human hands and clothing. This contact continues during packing and storing operations below deck. Excessively handled fish may carry significant numbers of Gram positive bacteria, some of which may be spoilage bacteria or pathogenic. Most of these Gram positive bacteria are naturally derived from human, avian, and terrigenous sources.

Gutting of fish is one of the practices, where fish is exposed to agents of spoilage. In some species, gutting is avoided especially on board fishing vessels either because the fish is too difficult e.g. spiny redfish-*Sebastes sp*. or too small and numerous to handle in the time available (Connell 1990). Although Nile perch and other fish species in Uganda are fewer per boat they are not gutted because the fishing vessels are not adequately equipped to handle the gutting and subsequent operations (Masette 1993).

Offloading operations provide opportunities for bacterial contamination via offloading equipment, pumps, conveyors, baskets, and boxes that redistribute surface contamination (Huis in´t Veld 1996). Insufficient cleaning may lead to bacterial build up which in turn will act as a source of subsequent contamination. Public auction markets where fish may be displayed on/in wooden, metal or plastic containers in the open, potential bacteriological dangers abound. For example, exposure of catch to direct sunrays especially in the tropics where ambient temperatures are more than 20^{o}C permits multiplication of spoilage bacteria. Delays in chilling when ambient temperatures are high can significantly shorten shelf life during subsequent storage (Liston 1992).

The type of production material and design of the containers may not allow them to be cleaned and disinfected effectively. The presence of vermin e.g. rodents, birds, domestic animals and people (handlers and auctioneers) are additional sources of contamination. NARO (1997) reported similar sources of contamination around most landing sites in Uganda.

At the fish processing plant, further handling occurs during wet processing operations like sorting, filleting and trimming. These operations transfer Gram positive bacteria usually associated with humans, directly from fish skin and gut to filleted flesh surfaces. It has been estimated that fillets and other products from fresh fish processors usually carry counts of 10^3-10^5/g or more (Liston 1980, Gram et al. 1988). If the product is refrigerated later on, the dominant bacteria on the product after a day or two of storage will be Gram negative because of the inability of Gram positive bacteria to grow competitively under refrigeration (ICMSF 1998). The bacteria may also be transferred from the processing environment namely, contaminated surfaces, knives and machines.

3.4 Quantification of spoilage

With the current market demands, it is imperative to have reliable methods of detecting contamination or remaining shelf life of individual consignments under storage or on display to avoid unnecessary financial losses. Zdzislaw et al. (1990) and Dainty (1996) further recognise the importance of rapid and effective means of identifying the causes of spoilage as a basis for instigating quick remedial action. Loss of freshness followed by spoilage is a complex combination of microbiological, chemical and physical processes influenced by species type and different storage conditions (Howgate 1982). A single spoilage index can not therefore be used as a measure of freshness but rather a combination of selected indices representative of different changes that occur during spoilage. Some particular feature of the spoilage process is measured and used as an index of spoilage. The various methods for assessing spoilage in fish can be placed in four broad categories namely, sensorial, microbiological, chemical and physical. It is only through a co-ordination of instrumental and sensory analysis, that optimal information on a given food can be obtained (Botta 1995). Each of these methods measures different spoiling indicators or freshness in fish and/or fish products. Sensory evaluation gives direct measurements while the other three give indirect measurement, though the measurements are closely related.

3.4.1 Sensory evaluation

Jellinek (1985) defined sensory evaluation of food as the use of senses which he preferred to call biological detectors, to detect variations in odour, taste, tactile, appearance and other characteristics of a given food. The testing methods applied should give reproducible results that can be analysed statistically. According to Howgate (1982), sensory methods are the most satisfactory way of assessing freshness of fish and fish products. This is because they can be applied to all fish species, do not require elaborate laboratory facilities, they are quick non-destructive unless sample is being cooked, and they are closely allied to the criteria the consumer uses in evaluating acceptability. However, the author points out one major disadvantage, i.e. they are difficult to standardise and the results are subject to

personal whims and bias of a taster. Dainty (1996) stated that while the sensory methods were appropriate and cheap, their reliance on trained panels to minimise subjectivity made them costly and unattractive for other routine requirements. In addition, without supplementary studies, sensory does not establish the cause of spoilage. Jellinek (1985) suggested solutions based on rigorous training of tasters and formulation of tests to ensure reproducibility of results, which can be verified statistically. Botta (1995) agreed with Jellinek (1985) on the objectivity of sensory evaluation by affirming the use of technologically sound procedures involving both a structured category scale and a highly trained tasters who have been taught to function as analytical instruments. There are several grading methods used to assess freshness in fish and fish products. They are the EEC system, Torry system and other hedonic scales, which entail scores ranging from 0-10 or 0-5 or the other way round.

3.4.2 Microbiological methods

Bacterial activity is the prime cause of spoilage in fresh fish since they give rise to undesirable flavours and odours (Connell 1990). The measurement of bacterial numbers therefore, should provide a direct index of freshness but unfortunately, there are both practical and theoretical limitations. For example, bacteriological tests generally require 2-3 days or more to complete (Howgate 1982, Connell 1990). Dainty (1996) however, suggested some more rapid alternatives, which included epifluorescent microscopy, flow cytometry and electrical impedance. Another disadvantage of microbial counts is that, not all species of bacteria present on fish cause spoilage (Hobbs 1982). Hence, counting the total number of bacteria present is only an approximate measure of the numbers of relevant organisms and may be misleading as an index of freshness. Huss et al. (1997) contends that unless supported by sound scientific experiments, assessment of spoilage based on bacterial numbers should not be used because off-odours may be due to lipid fraction or enzymatic degradation of protein. Nevertheless, it has been generally agreed that if fish has more than 10^6 bacteria /g of flesh, there is a possibility that spoilage is well advanced and if the count exceeds $10^8/cm^2$ of skin, the fish will be inedible (Howgate 1982). There are three methods of measuring the presence or absence of bacteria in a fixed quantity of food: Total Plate Count (TPC), Most Probable Number (MPN), Instrumentation (e.g. ATP unit, microscopy, turbidomentry and conductance, identification using gene technology) (Einarsson 1999).

3.4.2.1 Total Viable Count / Total Plate Count / Standard Plate Count / Aerobic Plate Count

TVC, TPC, SPC, APC all mean the number of bacteria (cfu/g) in a food product under specified standard and uniform conditions of culturing (Connell, 1990; Huss, 1994). For the present study, TPC will epitomise the rest.

TPC measures the fraction of the microflora able to produce colonies in the medium used under the incubation conditions. Thus, the temperature during incubation of plates has influence on the number of colonies developing in the sample. The TPC method does not differentiate between types of bacteria in spite of the wide variation in their biochemical activities in a sample (Huss 1994). In addition, it is irrelevant as an index of spoilage, in some semi-preserved fish products because of the unusually high numbers of non-spoilage bacteria e.g. lactic acid bacteria or in fresh fillets stored

aerobically. It is also valueless with regard to assessment of the present state of sensory quality unless the methods used are sensitive, selective, accurate and rapid (Zdzislaw et al. 1990, Huss et al. 1997). However, high counts of bacterial are likely to increase the risk of spoilage (Howgate 1982). Zdzislaw et al. (1990) suggested that hydrogen sulphide-forming bacteria or other indicator organisms were better indices of spoilage than TPC.

3.4.2.2 Most Probable Number (MPN) or Multiple-Tube Fermentation Method

It is one of the oldest methods used to estimate the quality of water with regard to the presence of coliform organisms (Klein 1996). Interpretation is based on the production of gas bubbles in the small Durham tubes by the action of the coliform bacteria in fermenting a lactose medium (Mulvany 1969). Coliforms are facultative anaerobes, gram negative, non-sporing, rod shaped bacteria that ferment lactose with gas formation within 48 h at 35°C (Klein 1996). Several tubes are inoculated with sample made into various dilutions. The number of positive and negative tubes is recorded and the number of organisms is estimated consulting the MPN table for the number that satisfies the number of positive tubes. The number obtained is multiplied by the dilution factor to get MPN/g of product. To use MPN method, at least 3 dilutions are required. Ideally the least dilute should be all positive and most dilute all negative. Since it does not always happen, the highest dilution in which all samples are positive and the two succeeding are selected for estimation of MPN (Banwart 1981). Although, it is easier and simpler to do than TPC, it is less precise and it does not detect the presence of bacteria some in samples (Mulvany 1969). Experientially TPC is easier and simpler to do than MPN.

3.4.3 Chemical methods

Chemical methods, considered to be more objective than sensory methods, involve chemically analysing a sample to determine the concentration of a specific chemical(s) within a sample. The concentration is used to indirectly measure or predict the level of a specific sensory attribute, which allows for the immediate determination of freshness (Botta 1995). Several chemical tests indirectly related to bacterial activity have been often employed for assessing freshness or levels of spoilage in fish and other seafood products (Zdzislaw et al. 1990). The proposed tests have been used to establish quantities of different spoilage compounds. According to Howgate (1982) only three have stood the test of time as reliable i.e. determination of trimethylamine (TMA), total volatile bases (TVB) and hypoxanthine (Hx). K-value has also been proposed as an ideal indicator of freshness (Zdzislaw et al., 1990. The first two are related to bacterial activity and the third is the end product of a series of enzymatic reactions in the fish flesh. The relationship between these three frequently used methods is illustrated by Connell (1990) in Figure 3 to depict spoilage trends in cod. There are other methods such as Peroxide Value (Pv), Thiobarbituric Acid (TBA), Iodine value (Iv) and Anisine, which measure rancidity in fish and fish products but they will not be considered in this study.

3.4.3.1 Trimethylamine (TMA)

TMA is a volatile nitrogenous volatile base formed by the reduction of trimethylamine oxide (TMAO) by certain species of aerobic bacteria, which utilise the

oxygen in anaerobic conditions. In other words, TMAO acts as the terminal electron acceptor (Huss et al. 1997). It has also been hypothesized that some intrinsic enzymes may be partly responsible for its production (Connell 1990). TMA has been known to increase with bacterial numbers during ice storage of fish. Over the first few days, there is little change but then the concentration rises at an increasing rate. Because of the delay, TMA is not a suitable method for discriminating between batches of fish that have been less than 6 days in ice or thereabouts. TMA is only produced in fish that have adequate amounts of TMAO (Howgate 1982). Freshwater fish until recently (Gram 1989) were thought not have TMAO. Flatfish, some pelagic fish and shellfish only have low amounts of TMAO, though it may increase during storage. Although TMA production is related to bacterial growth, it is not a direct measure of bacterial spoilage because not all bacteria present on spoiling fish reduce TMAO to TMA. It also varies with individual fish within a species of the same storage time and freshness (Botta 1995).

3.4.3.2 Total Volatile Bases (TVB)

During post-mortem storage of a wide variety of seafood, microbiological spoilage causes the formation of volatile bases principally ammonia, DMA and TMA (Zdzislaw et al. 1990, Botta 1995). These bases can be distilled, collected and made to neutralise acids. The amount of acid used is a measure of the total bases distilled and it corresponds with the level of spoilage or freshness albeit indirectly. (Howgate 1982). There are several procedures used for the determination of TVB content and each procedure gives a different result for the same fish sample. The difference has been attributed to the way bases are released and distilled from the fish (Howgate 1982). There are other shortcomings associated with the use TVB as an index of freshness. It can only be used in those species where TMA is present. It varies with different procedures and between fish in a batch. As shown in Fig. 3, the TVB value is much higher than TMA or Hx for the same storage period and this has been accredited to the presence of ammonia which is present even in freshly caught fish. Although a significant increase in TVB coincides with bacterial spoilage, it is not a sensitive index for fish freshness. However, it may be used for fish near the limit of acceptance, which Zdzislaw et al. (1990) reported as 30 mg/100 g and a slightly lower value of 20 mg/100 g for fatty fish. Virtually all changes in TVB are due to TMA component, which is a major constituent of volatile bases.

3.4.3.3 Hypoxanthine (Hx)

Hypoxanthine is a normal constituent of fish flesh though present in very low concentrations in live fish. It is the end product of a series of enzymatic reactions going on in the fish flesh namely:

ATP → ADP → AMP → IMP → Inosine → HxR → Hx

Unlike TMA and TVB, it increases in most species soon after death and in the early days of storage (Howgate, 1982). It can be used to discriminate between batches of fresh fish. Hx concentration with storage time and it is more variable between species than TMA or TVB. Generally, the measurement of Hx content is a better index of freshness and gives a better indication of spoilage over a wide range of qualities than TMA or TVB. It is applicable to a wider range of species and products in which the

limit of acceptance has been restricted to ≤ 4 μm/g. However, it is not commonly used because most international quality standards prefer the other two tests.

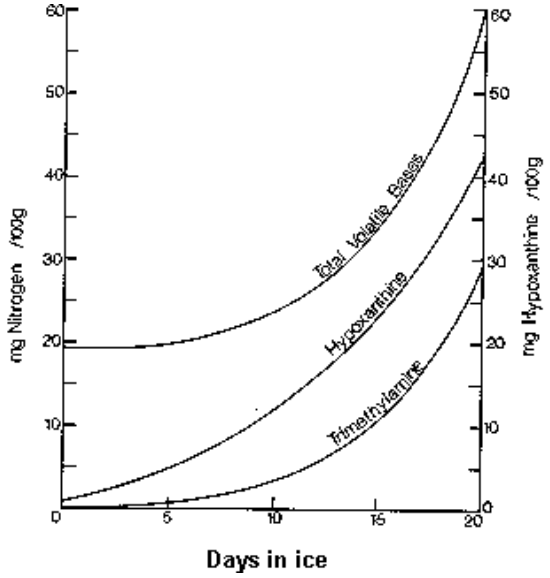

Figure 3: The changes in concentrations of TVB, Hx and TMA with the degree of spoilage of cod. (Connell 1990).

3.4.3.4 K-value

K-value may be defined as the ratio of inosine and hypoxanthine to the sum of ATP and all the other products of ATP degradation times 100 as listed above in section 3.4.3.3.

$$K\% = \frac{Hx + Ino}{ATP + ADP + AMP + IMP + Ino + Hx} * 100$$

According to Zdzislaw et al. (1990) many fish accumulate inosine instead of hypoxanthine. Immediately after capture, the K-value does not exceed 10% but increases gradually due to enzymatic degradation because of autolysis. A rapid increase is observed later, after an inflection point due to bacterial activity. A K-value of 20% has been suggested as a freshness limit and 60% as the rejection point. During ice storage the time when different fish species reach a K-value of 20% may vary by as much as 10 days.

3.4.4 Physical methods

The sensory, chemical and microbiological methods have been found to have operational disadvantages, namely they are destructive, require laboratory facilities and take time to complete. These disadvantages have rendered them unsuitable for use in various places in the fishery industry. Both at the auction markets and during inspection of raw material in factories rapid and reliable measurements are required. Importers or supermarket chains also require documented evidence to attest to the safety and quality of the product (Howgate 1982, Botta 1995). This need for rapid and practical methods to meet consumer demands has led to the development of physical

methods like "electronic noses" (Ólafsdóttir et al. 1997). There are currently several types of gadgets in the fish industry used to measure freshness, e.g. the Torry meter, Intelectron Fishtester and RT-Freshness Grader (Zdzislaw et al.1990, Heia et al. 1997). The principle involved in all these gadgets is the dielectric properties present in skin or muscle, which change in a systematic way after the death of the fish. The change or particular combination in these properties is displayed as a number on a small screen. This reading gives an indirect measure of the freshness of the fish. Because of the variation between fish within a batch, the latest version of the meters store up to 16 measurements before an average value is displayed (Howgate, 1982). There are several advantages of physical methods. They are fast with little or no handling of fish, can be used for all species though more useful for some than others and can measure quality over a wide range of freshness (Botta, 1995, Heia et al. 1997). Certain defects in the fish for example physical damage will give a lower reading. Since these defects are associated with faster spoilage, the resultant reading is an advantage to the quality manager. There are disadvantages associated with these freshness testers. The initial readings differ between species and water content, lipid content, condition factor, state of maturity seem to cause the variation in freshness readings of freshly caught fish (Huss 1992). In addition, Heia et al. (1997) highlight the unreliable results with thawed fish and recommends that these gadgets can only be used with unfrozen samples.

3.5 Comparison of Methods

Sensory, microbiological, chemical and physical methods measure different features of spoilage or freshness. Physical methods and electrical testers in particular, are the only freshness indicators, which have a linear function with storage on ice (Heia et al. 1997). On the other hand, sensory methods are the only methods, which use the same criteria as the ultimate consumers of the product and give direct measurements as opposed to the rest of the methods that give indirect measurements. However, the results from all the methods are closely related and convertible as shown in Table 2.

Table 2: Comparison of methods of assessing quality of boxed iced cod (Howgate 1982).

Time in ice (days)	Sensory score	TMA-N mg /100 g	TVB-N mg/100 g	Hypoxanthine mg/100 g	GR-Torry meter reading
2	9	0.1	19	2.4	14
5	8	0.3	20	5.1	13
8	7	1.9	22	8.8	11
11	6	5.0	27	14.0	10
14	5	11	37	21	8
17	4	24	56	30	6
20	3	45	85	43	4

4 RESULTS AND DISCUSSION

In the present study, all spoilage indicators showed that there was some of form of spoilage in both fish albeit in small quantities at the beginning of the study. The sensorial, chemical and microbiological indices indicated similar values although, the tropical fresh water fish Nile perch (NP) was 9-13 days post-mortem and the temperate fish Ocean perch (OP) was 4 days post-mortem. The values for both species were within the EU acceptable quality and safety limits: score 8-7, TPC $\leq 10^3$, TMA $\leq$ 10-15 and TVB-N of < 35-40 mg/100 g. After 3 days however, there was a dramatic increase in all parameters under investigation except chemical indices and the trend was similar in both species. The increase was further exacerbated by the effect of storage temperature on spoilage rate. Generally, fish stored at 0°C kept longer than fish kept at 5°C irrespective of species although it was not always apparent. This comparison is shown in Table 3.

Table 3: Sensory description of Nile perch and Ocean perch fillets during storage at 0°C and 5°C.

Keeping time (days) &T°C		Sensory description of each fish using Torry scheme	
Day*	Temp.°C	Ocean perch (OP)	Nile perch (NP)
0	0	Lack of characteristic flavours of species.[8]	Muddy, metallic, burnt and bland or no sweet taste or no liver taste. [7&6]
	5	Same as above.[8]	Same as above. [7&6]
3	0	Condensed milk, caramel or toffee-like odours.	Meaty, rancid, sour and TMA. [5&4]
	5	Absolutely no flavours. [6]	Inedible ∴not presented to the panellists. [3,2&1]
6	0	Trace of off-flavours, some sourness but no bitterness. [5]	Inedible ∴not presented to the panellists. [3,2&1]
	5	Some off-flavours and some bitterness. [4]	

*Note that OP and NP were 4 and 9-13 days post-mortem at 0 day of the trials respectively.
[ ] Scores based on Torry scheme (Appendix 1&2).

The NP fillets stored at 0°C were barely edible by the third day of storage, which corresponded with 16-19 days post-mortem, and the OP was on the borderline of acceptability, i.e. 7 days post-mortem. In essence, the warm water fish had kept longer than cold water fish at chilled temperatures. This result is in agreement with previous studies conducted with ice storage (Shewan 1961, Bramsnaes 1965, Shewan and Murray 1979, Liston 1980, Gram, 1992b, ICMSF 1998) although it was in slight disagreement with Shewan and Murray (1979) and Gram et al. (1988) on the actual storage time of Nile perch. The former reported 29 days and the latter 23 days as shown in Table 4 compared to < 19 days for NP in the present study. The differences may be attributed to the sampling procedures. Whereas Gram et al. (1988) washed fish thoroughly with methanol prior to aseptically filleting, the fillets for the present study were purchased from the open market. Actually, the plant mentioned in section

2.1 where the NP fillets were purchased complies with the EU regulation 493/91 that does not allow disinfectants on fishery products. In addition, the aseptically removed fillets were placed in clean plastic bags prior to ice storage, whereas the handling procedures for the present study were simulated commercial practices. However, the average of the authors' sample 2&3 agrees with the present study. Shewan and Murray (1979) do not include the details of their methodology in the report so the differences can not be elucidated.

Table 4: Shelf life and bacterial status studies of Nile perch (*Lates niloticus*) (Gram et al. 1988).

Fish sample	Storage temperature	Initial count TVC	Initial count H2S	Final count TVC	Final count H2S	% H2S	Shelf life
1)Whole fish	20-30°C	$3 \cdot 10^3$	<10	$3 \cdot 10^7$	$6 \cdot 10^6$	20	13 hours
Fillet	20-30°C			$7 \cdot 10^7$	$2 \cdot 10^7$	30	13 hours
2) Whole fish	0°C	10^4	10^2	$4 \cdot 10^8$	$5 \cdot 10^6$	1	27 days
Fillet	0°C	10^2	<10	10^8	$5 \cdot 10^4$	0.01	23 days
3)Whole fish	0°C	10^5	$<10^2$	$2 \cdot 10^8$	$6 \cdot 10^5$	0.1	15 days
Fillet	0°C	$5 \cdot 10^5$	$<10^2$	$2 \cdot 10^8$	$<10^4$	0	14 days

Sensory evaluation, as aforementioned has to be confirmed by chemical and microbiological objective methods of assessing levels of spoilage. Although the present microbiological method (TPC) i.e. direct measurement of bacterial numbers present on fish does not demonstrate its freshness, it gives an indication of the risk of spoilage involved. However, if SSO like H2S-forming bacteria were brought into the picture, then bacterial load would correspond with spoilage.

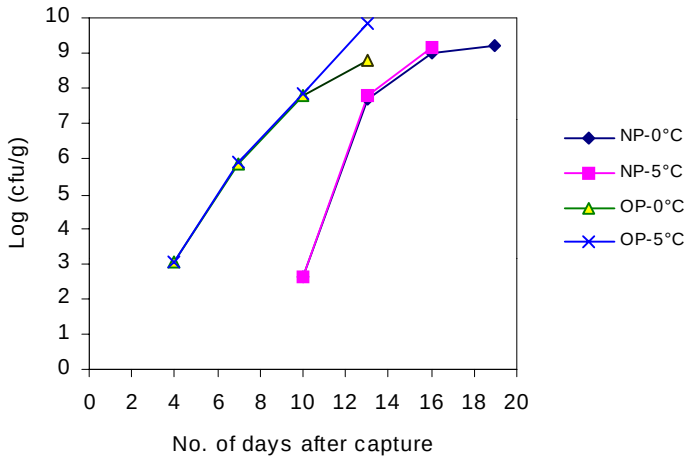

Figure 4: Bacterial growth on plate agar (without NaCl) incubated at 22°C during storage of NP and OP fillets kept at 0°C and 5°C.

The total plate count (TPC) with plates incubated at 22°C did not show clearly the differences between fillets stored at 0°C and 5°C up to the third day of storage (Fig. 4) but the panellists were able to differentiate between them, hence reject them accordingly. This scenario is what makes sensory assessment an indispensable method of quality evaluation as previously noted (Howgate 1982, Botta 1995). The incubation temperature of 22°C selects for psychrotrophic bacteria responsible for fish spoilage at low temperatures. The change of incubation temperature to 37°C, resulted in lower bacterial counts compared to counts at 22°C (Fig. 5).

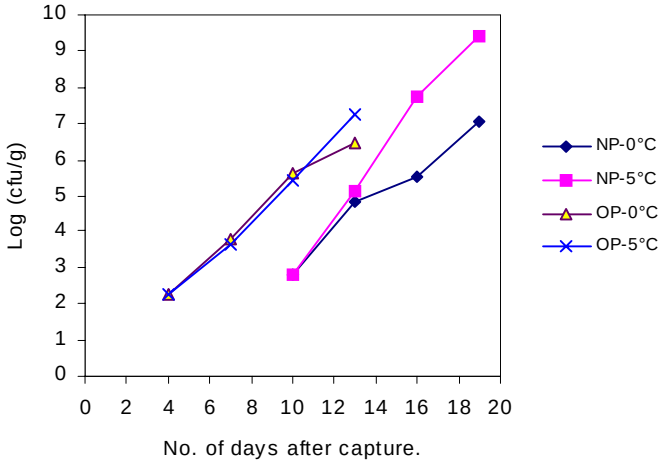

Figure 5: Bacterial growth on plate agar (without NaCl) incubated at 37°C during storage of NP and OP fillets kept at 0°C and 5°C.

It is generally accepted that fish with a microbial load of $>10^6$/g is likely to be in the advanced stages of spoilage or the upper limit of microbiological acceptability (Shewan 1961, Liston 1980, Howgate 1982, Connell 1990) but according to Gram et

al. (1992). The total counts of bacteria on/in fish rarely indicate the sensoric quality of the fish.

Using this criterion, NP and OP samples stored at 0°C and 5°C, reached the upper limit of acceptability on day 7 and 12 after capture in Fig. 4, which almost corresponds with the day of rejection. In Fig. 5 however, the upper limit was reached long after rejection. Fish stored at 5°C ought to have been rejected on day 11 and 14 for OP and NP respectively according to the stipulated limit and similar samples kept at 0°C on day 11 and 17 respectively. There was not a major change in the storage time of OP, whether stored at 0°C or 5°C. There was however an increase of 3 days for NP stored at 0°C as compared to 5°C. The difference in storage time may be attributed to the nature of psychrotrophic spoilage bacteria, which tend to dominate at low incubation temperatures of 22°C and at 37°C for mesophiles. The results of the present study are in agreement with Liston (1980) who noted that the counts, when incubation temperature is ≤ 35-37°C are ten times lower than when it is at 20-25°C. This further emphasises the psychrotrophic nature of the fish microflora as a function of temperature.

As Hobbs (1982) observed not all bacteria present on fish are spoilers but there are certain active spoilers which are the major players in fish spoilage. These bacteria have the ability to reduce trimethylamine oxide (TMAO) to TMA and produce hydrogen sulphide from sodium thiosulphate and/or cysteine incorporated in the growth culture medium. When decomposing the thiosulphate or the amino acid - cysteine, these bacteria form black colonies due to precipitation of FeS. Their log growth rate as a function of storage time has been illustrated in Fig. 6 and Fig. 7. The number of H2S-forming bacteria on plates incubated at 22°C is higher at any storage time than on plates incubated at 37°C probably, because the H2S-forming bacteria are psychrotrophic in nature. Gram et al. (1987) identified the majority of black colonies or H2S-forming bacteria in the study, as *Alteromonas putrefaciens* currently known as *Shewanella putrefaciens* and a few of them as *Vibrionaceae* due their fermentative glucose metabolism. In the same study, the H2S-forming bacteria increased with storage time as shown in Table 4.

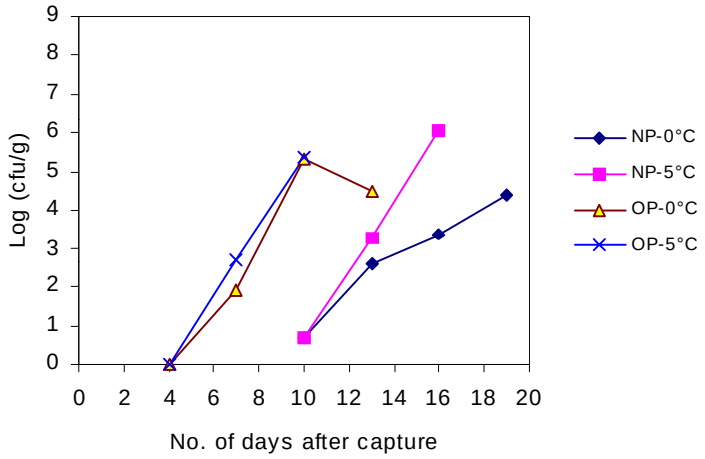

Figure 6: Growth of H2S -forming bacteria on IA agar incubated at 37°C during the storage of NP and OP fillets kept at 0°C and 5°C.

From figure 6, the criterion of 10^6 as the upper limit of product acceptability, does not correlate with results of the sensory evaluation, which further illustrates the dominance of psychrotrophic bacteria during spoilage of chilled fish. Incubation of plates at 37°C is probably not appropriate for assessing the microbial spoilage of chilled fish products. However, Fig. 7 which shows the growth rate of H2S -forming bacteria at 22°C correlates with the sensory results and is therefore an excellent procedure for assessing spoilage of chilled fish.

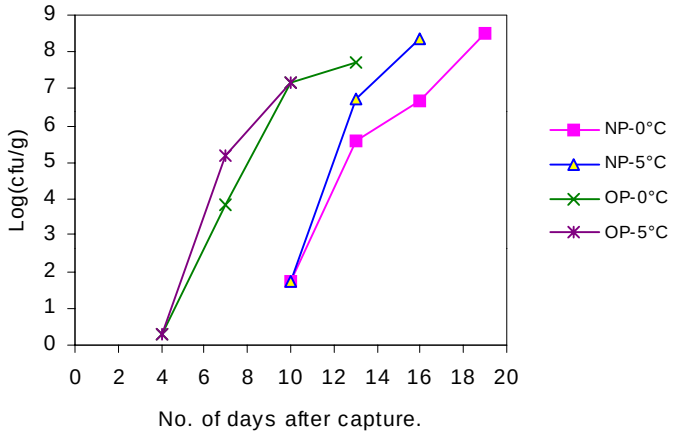

Figure 7: Growth of H2S -forming bacteria on IA agar incubated at 22°C during the storage of NP and OP fillets kept at 0°C and 5°C.

As aforementioned a single spoilage index can not be used as a measure of spoilage but combination of microbiological and sensory selected indices can complement each other to give a comprehensive picture of the different changes that occur during spoilage. In line with the complimentary concept, chemical indices were studied during the storage of OP and NP at 0°C and 5°C.
Figure 8 shows the development of one of the chemical indices used in the present study to compliment sensory evaluation. As Connell (1990) and Howgate (1982) noted, TVB-N is even present in fresh fish because of ammonia, which is one of the major components of nitrogenous volatiles. In the present study the initial TVB-N value was below 20 mg/100 g in both species as shown in the figure below.

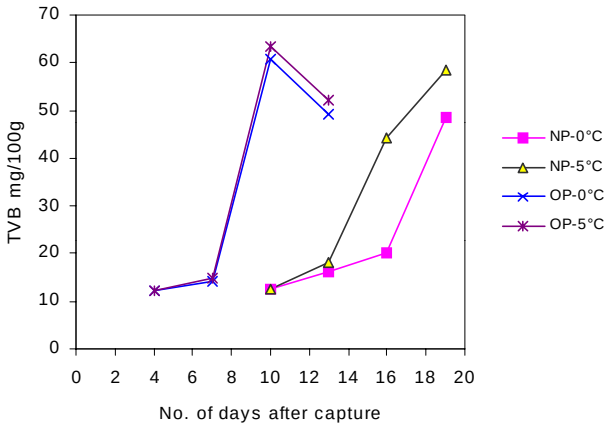

Figure 8: Development of TVB-N during storage of Nile perch and Ocean perch fillets kept at 0°C and 5°C.

According to Icelandic Regulation No. 233, appendix 5, article 2 regarding the handling, processing and distribution of seafood and Ugandan 1998 Quality Assurance Regulations, both species meet the safety and quality standards, which by coincidence require compliance of 25mg/100g. Based on these regulations, which bear the hallmark of the EU Directive 493/91, the storage time for OP fillets kept at both temperatures, was around 8 days compared to 15 and 18 days for NP fillets kept at 5°C and 0°C respectively. These storage times correlate with the results of sensory evaluation albeit marginally.

In both species, there is a varying gradual "lag phase" (3 days in OP at both storage temperature; 3 and 6 days for NP stored at 5°C and 0°C respectively) where TVB-N remains below 20 mg/100 g. Essentially, this period corresponds with the exponential growth period of spoilage bacteria in Fig. 7, which would ideally entail a sudden increase in TVB-N but since the spoilage bacteria were low in number, the corresponding TVB-N value was equally low.

Because TVB-N measures volatile bases in their totality, it does not relate directly to bacterial spoilage. It is from this standpoint that Connell (1990) and Connell and Shewan (1980) suggested the use of TMA as a more specific chemical index of microbiological spoilage. In Fig. 9, the spoilage trend of OP as indicated by the development of TMA is similar to the TVB-N trend in Fig. 8. Thus, substantiating the observation made by Howgate (1982) that most changes in TVB-N are due to the TMA component. In addition, the TMA in OP was noticed in both treatments earlier on during storage albeit in small quantities < 2mg/100g. At the end of its storage time i.e. day 7, there was almost a vertical increase in TMA content which corresponded less accurately with the TPC (Fig.4) but more accurately with H2S-forming bacteria (Fig. 7). The TMA development trend in Nile perch however, was totally different. The presence of TMA became apparent at the end of storage time i.e. on days 16 and 19 at 5°C and 0°C respectively. Even when the samples had been rejected on day 19, the TMA content was still within the acceptable EU limits of 10-15mg/100g (Connell, 1990) though counts of H2S-forming bacteria which normally reduce TMAO to TMA were quite high (Fig.7). The results therefore strongly indicate that there is much less TMAO in NP than in OP (Fig. 9). Gram et al. (1989) however, found >25 mg /100 g from fresh Nile perch. This trend demonstrated that H2S-forming bacteria in NP relied on compounds other than TMAO as an electron acceptor
.
This inconsistency in TMA as a spoilage indicator further demonstrates the need for the use of complementary indices in assessing spoilage in fish and fish products.

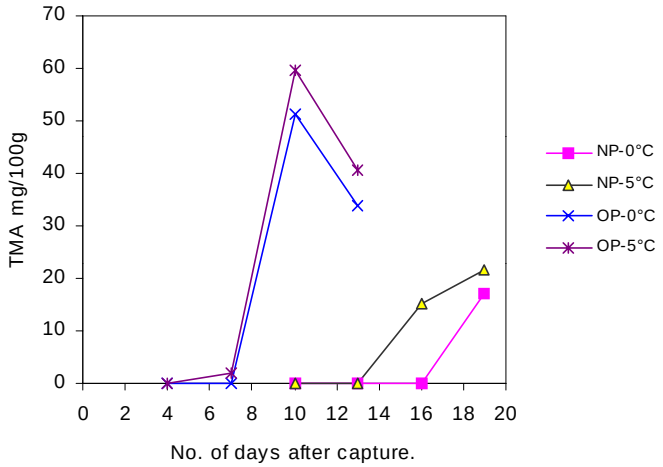

Figure 9: Development of TMA during storage of Nile perch and Ocean perch fillets kept at 0°C and 5°C.

The presence of TMAO in marine fish serves a regulatory function but its presence in warm water fresh fish has not been elucidated. Duly it is reduced to TMA by some spoilage bacteria, which Gram et al. (1987) tentatively identified as *Alteromonas putrefaciens* currently referred to as *Shewanella putrefaciens* among other spoilers.

Table 5: Composition of bacterial flora in Nile perch fillets during storage at 0°C.

	% Composition of psychrotrophic bacterial flora on iron agar				
Strains identified	* Skin swabs on freshly caught NP	Nile perch fillets stored at 0°C			
		10 days	13 days	16 days	19 days
Pseudomonas 1&2	64	92	88	92	84
Pseudomonas 3&4/ Shewanella	0	8	0	8	8
Vibrio/Aeromonas	20	0	0	0	4
Enterobacteriaceae	8	0	0	0	0
Pres. Kurthia spp	8	0	12	0	0
Psychrobacter (Moraxella)	0	0	0	0	4

* Average (%) composition for three fish swabbed on each side (see 2.1).

From Table 5, it is evident that most of the bacterial flora in Nile perch fillets were Gram negative as previous studies had indicated (Huss 1994, Gram et al. 1990, Liston 1980). *Pseudomonas* 1&2 identified by their Gram negative rods, motility, positive oxidase nature and inability to ferment glucose, constituted a major fraction of the bacterial flora in Nile perch fillets during storage at 0°C. This was in total agreement with Gram et al. (1990), Gram and Huss (1996), Gram (1992), Gram et al. (1988, 1989) and Liston (1980). This was probably due its abundance on the raw material where it accounted for 64%. However, Liston (1980) ascribes the dominant position in the microflora during storage, to its rapid growth at low incubation temperatures (0-5°C) with a generation time of 10-30 hrs. as suggested by Shewan and Murray (1979). The author also recognises its ability to utilise a variety of

compounds including NPN in the fish muscle juice quickly and efficiently as one of the characteristics to ensure dominance. Some species of *Pseudomonas* were responsible for the objectionable off-odours detected during the last days of storage. According to Liston (1980), they were due the breakdown of S-containing compounds e.g. cysteine and methionine by *P. fluorescens, P. perolens, P. putida* and *P. putrefaciens*. Admittedly, the swabs ferried from Uganda to Icelandic Fisheries Laboratories for the present study were not quite reliable because in some plates, the medium had spilled over. Nevertheless, some effort was made to pick 25 intact colonies, to establish their identity.

Pseudomonas 3&4/ *Shewanella*, characterised by their inability to ferment glucose and their inert reaction in OF medium, were the second most common though they were originally not found on the fish skin. Their presence in the spoiling fish stored at 0°C agrees with previous studies cited by Gram et al. (1990) and Huss (1994). In this present study, the identification tests were not comprehensive enough to differentiate between *Pseudomonas* 3 and 4 or *Shewanella*. However, since these bacteria were only 8% of the total flora, it is likely that *Shewanella putrefaciens* did not play an important role in the spoilage of NP fillets. This is in agreement with results obtained by Gram et al. (1990).

On the other hand, *Vibrio/Aeromonas* characterised by their Gram negative rods, motility, positive oxidase nature and their fermentative reaction in OF medium, were second in the skin swab samples, though they did not show up again until the last days of storage. According to Gram et al. (1990), *Aeromonas* is only of great consequence in the spoilage of Nile perch kept at ambient temperatures and not in ice. Although, it was not possible to differentiate the genera further in the present study, there was a possibility that it constituted only of *Aeromonas*, because in an elaborate and comprehensive bacteriological study on Nile perch, Gram et al. (1990) did not come across *Vibrio*.

Enterobacteriaceae constituted 8% of the microflora, found on the Nile perch skin. This result is consistent with the NARO (1997) report, which indicated the presence of certain species of *Enterobacteriaceae* like *E. coli* and *Salmonella spp*. at some fish landing sites along the shores of L. Victoria. It was therefore not surprising to find them in the skin swabs of the present study. Huss (1994) reported that these particular bacteria are distributed world-wide and can multiply and survive in the environment for several weeks. However, they are seldom encountered in fresh spoiling marine fish but may be found in fish caught in polluted water (Banwart, 1981).

The presumptive *Kurthia spp.* a Gram positive, motile, oxidase positive and non-fermentative rod was found in the initial samples and on day 13. It is rare in fish and fish products but has been found in stagnant water, fresh and spoiling meat and meat products. *Kurthia zopfii* is not known to cause spoilage but its importance might be as an indicator of mishandling (Banwart, 1981).

Psychrobacter, formerly known as *Moraxella*, Gram negative, aerobic, non-motile and non-fermentative rods were not found in the initial swabs but were detected in the samples that were on the verge of rejection. Gennari et al. (1989) isolated similar strains in fresh and spoiled sardines.

Magnússon and Martinsdóttir (1995) carried out a similar study on iced Ocean perch fillets. The composition of the microflora found is shown in Table 6.

Table 5: Composition of the bacterial flora in thawed Ocean perch fillets kept on ice.

Bacterial strain	1 day in ice	16 days in ice
1. Gram-positive	44	0
Micrococcus / Staphylococcus	4(4)	0
Coryneforms	16	0
Lactobacillus	12	
Pediococcus/Streptococcus/Leuconostoc	12	
2. Gram-negative	52	100
Psychrobacter/ Acinetobacter	16	0
Flavobacterium/ Cytophaga	4	0
Pseudomonas/Shewanella/Alcaligenes	0	100 (8)
Enterobacteriaceae	4	0
Vibrio/ Aeromonas	28(4)	0
3. Unidentified	4	0

Source: Magnússon and Martinsdóttir (1995).

The most predominant group of spoilers was identified as *Pseudomonas/Shewanella/Alcaligenes* but the major spoiler in the group was suspected to be *Shewanella* because 75% of isolated strains on day 16 reduced TMAO to TMA.

5 CONCLUSION

Nile perch (warm-water fish) and Ocean perch (temperate-water fish) fillets stored at 0°C in styrofoam boxes (simulated commercial conditions) kept for 16-19 days depending on the day of capture and 7 days respectively. Thus, tropical fish kept longer than temperate fish under commercial simulated storage temperatures. Storage of fillets at 5°C in display cabinets during marketing lessens the shelf life of chilled fish products. High temperature (5°C) does not favour proliferation of psychrotrophic spoilage bacteria, which probably accounted for the different spoilage rates between fillets stored at 0°C and 5°C. *Pseudomonas* 1 and 2 were the major spoilers in chilled Nile perch fillets during storage. There was less TMAO in NP than in OP by a factor of five, at the end of shelf life. TVB-N was a better indicator of spoilage than TMA. The concept of complementary indices was reaffirmed, as it was not possible to use a single spoilage index as a measure the level of spoilage or freshness in the present study.

6 RECOMMENDATIONS

- Uganda fish processors vying for the lucrative EU market ought to process only freshly caught NP which is probably less than 2 days post mortem. In addition, they should endeavour to export the consignment, on the day of processing.
- Temperature of the display cabinets in the market place (EU) should be maintained at < 5°C to benefit from the consequential extended storage time.

- The EU regulations regarding quality/safety limits should be based on individual species, considering that not many species are involved in the international fish trade or Uganda for that matter.
- When carrying out a storage time study on chilled products, it would be advisable to take samples on a daily basis for meaningful spoilage trends.
- This study should be repeated without the shortcoming of sample procurement experienced in the present study, if far-reaching conclusions are to be made.
- Uganda ought to synchronise their microbiological methods with those recommended in the EU.

ACKNOWLEDGEMENTS

I am greatly indebted to the Director of United Nations University Fisheries Training Programme Tumi Tomasson who made the pursuance of this study possible, my supervisor Hannes Magnusson whose fish microbiological knowledge was an indispensable asset. Thor Asgeirsson's computer acumen is also appreciated. The laboratory personnel of Icelandic Fisheries Laboratories especially Jarmila and librarians are also thanked. Last and not least, thanks to Gavin Begg and all UNU fellows for the input and company respectively.

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APPENDIX 1: QUALITY SCORES FOR COOKED NILE PERCH.

Odour	Flavour	Texture	Colour	Grade	Score
Typical of Nile perch, fresh, broth-like	Typical of Nile perch, cooked	Succulent, elastic	Ivory-like, white	First quality	9
Steamed green vegetables	Cassava or sweet potatoes, weak broth.	Easy to chew & swallow	Clear	First quality	8
Neutral	Neutral, tasteless	Mealy & succulent initially	White	First quality	7 & 6
Weak rotten	Weak rotten	Soft, mealy & dry	Pale white/ greyish	Second quality	5 & 4
Rotten, urea, sour, stinking	Rotten, sour, nauseating	Very soft, like butter, very mealy	Greyish, dark	Rejectable	3, 2, & 1

APPENDIX 2: QUALITY SCORES FOR COOKED TEMPERATE FISH E.G. COD.

Odour	Flavour	Score
Strong seaweedy	Fresh, sweet flavours characteristic of the species	10
Some loss of seaweediness	Some loss of sweetness	9
Lack of odour or neutral odours.	Slight sweetness and loss of flavour characteristic of the species	8
Slight strengthening of the odour but no sour or stale odour; wood shavings, woodsap, vanillin etc.	Neutral flavour, definite loss of flavour but no off flavours	7
Condensed milk, caramel or toffee-like odours.	Absolutely no flavour, as if chewing cotton wool.	6
Milk jug odours, or boiled potato or boiled-clothes-like odours.	Trace of off-flavours, some sourness but no bitterness.	5
Lactic acid and sour milk, or byre-like odours	Some off-flavours and some bitterness	4
Lower fatty acids e.g. acetic, some grassness or soapiness	Strong bitter flavours, rubber-like flavour, slight sulphide-like flavours.	3
Ammoniacal (TMA & lower amines) odours	Distinct sulphide flavours	2
Strong ammoniacal (TMA) and some sulphide odours	Strong bitterness, but not nauseating.	1
Strong ammonia and faecal, indole and putrid odours	Strong off flavours of sulphides, putrid, tasted with difficulty	0

Fresshness score sheet for iced cooked red fish

Score	Odour	Flavour
10	Initially weak odour of boiled cod liver, fresh oil, starchy	Boiled cod liver watery, metallic.
9	Shellfish, seaweed, boiled meat, oil, cod liver	Oily, boiled cod liver sweet, meaty characteristic.
8	Loss of odour, neutral odour	Sweet and characteristic flavours but reduced in intensity.
7	Woodshavings, woodsap, Vanillin	Neutral
6	Condensed milk, boiled potato	Insipid
5	Milk jug odours boiled clothes- like	Slight sourness, trace of off-flavours, rancid
4	Lactic acid, sour milk TMA	Slight bitterness, sour, off-flavours, TMA rancid
3	Lower fatty acids (e.g. acetic or butyric acids) composed grass, soapy, turnipy, tallowy	Strong bitter, rubber, slight sulphide rancid

APPENDIX 3: MICROBIOLOGICAL PROCEDURES AND METHODS.

Preparation of PCA:

Weigh 23.5 g of plate count agar. Add 1 liter of deionized water. Heat while stirring with magnetic stirrer. Bring to boil or until the liquid becomes clear. Decant in dispenser bottles for autoclaving at 121°C for 15 min. Pour 15 ml of agar on plates. Leave at room temperature for two days, then pack in poly-bag for refrigerated storage until required.

1. Gram staining using Hucker method.
Hucker crystal violet
Solution A
Crystal violet (85% dye content) 2.0 g
Ethyl alcohol 95% 20.0 ml

Solution B
Ammonium oxalate monohydrate 0.2 g
Distilled water 20.0 ml

Preparation of necessary four solutions:
a. Ammonium oxalate-crystal violet solution: Mix equal parts of solutions A and B (Sometimes the crystal violet is so concentrated that gram-negative organisms do not properly decolourize. To avoid this difficulty, the crystal violet solution may be diluted as much as tenfold prior to mixing with equal parts of solution B)
b. Lugol's solution, Gram's modification: Dissolve 1.0 g of iodine crystals and 2.0 g potassium iodide in 300.0 ml of distilled water.
c. Counterstain: Dissolve 2.5 g safranin to 100.0 ml 95% ethyl alcohol. Add 10.0 ml alcohol solution of safranin to100.0 ml of distilled water.
d. Ethyl alcohol: 95%.

Staining procedure: Stain the heat-fixed smear for 1 min with the ammonium oxalate-crystal violet solution. Wash the slide in water; immerse in Lugol's solution for 1 min. Wash the stained slide in water; blot dry. Decolourize with ethyl alcohol for 30 sec, using gentle agitation. Wash with water. Blot and cover with counter-stain for 10 sec, then wash, dry, and examine as usual.
Cells that decolourize and accept the safranin stain are gram-negative. Cells that do not decolourize but retain the crystal violet stain are gram- positive.
Preferably stain vigoruosly growing cultures from nutrient or other agar free of added carbohydrates. Use positive and negative culture controls.
Identification of isolates:
25 colonies from the total plate count plates, kept at 0°C and incubated at 22^{o}C and skin-swabbed plates ferried from Uganda, were streaked on Plate Count Agar (PCA). The streaked plates were incubated at 22°C for 24 hours. Colony size, shape and colour were documented before the Gram staining, catalase, oxidase, motility, and oxidation/fermentation tests were carried out.

2. Catalase test

Plates were flooded with 3% hydrogen peroxide solution. Then bubble formation was observed using a hand lens or unaided sight. Colonies exhibiting no evidence of gas formation are catalase negative. The alternative method was to transfer a loopful of colony to a slide, mix with 2.0 to 5.0% hydrogen peroxide and observe as above. The test is a qualitative procedure for determining the presence of catalase enzyme activity in bacteria. The enzyme catalyses hydrogen peroxide to water and oxygen which is then utilized by the bacteria. When the test is positive, it implies that the bacteria in question is oxidative-aerobic and the negative result means anaerobic.

3. Oxidase test

A DrySlide ™ made by DIFCO laboratories Detroit MI 48323-7058 USA, was used to determine the oxidase reaction of bacteria, a helpful tool in the bacterial identification procedure. The slide is impregnated with an aromatic amine compound (N,N,N´,N´- tetramethyl-p-phenylenediamine dihydrochloride) that is dimethylated at its two amino groups. The reagent is colourless in the reduced state and dark purple in the oxidized state.The oxidase test is a qualitative procedure for determining the presence or absence of cytochrome c oxidase activity in bacteria. This activity is dependent on the presence of an intracellular cytochrome oxidase system that catalyses the oxidation of cytochrome c by molecular oxygen. The cytochrome c, then serves as the terminal electron acceptor in the organism's electron transport system (Steel 1961). In other words, the cytochrome oxidase produced by the organism does not directly oxidize the reagent, but rather oxidizes cytochrome c, which in turn oxidizes the reagent to form a purplish-blue coloured compound.

Organisms containing cytochrome c as part of their respiratory chain are oxidase positive and turn the reagent purplish-blue; organisms lacking cytochrome c leave the reagent colourless within the time limits of the test, and are oxidase negative.

Procedure:

A loopful of pure strain from streaked plates was rubbed directly onto a portion area of the slide using a platinum loop (nichrome or any other iron containing loop give false results). The reaction area was examined for development of colour within 20 seconds.

4. Fermentation and oxidation test

Preparation of oxidation/fermentation medium(OF)

Casetone	1.0 g
Yeast extract	0.1 g
Ammonium sulphate (NH4)2 SO4	0.5 g
Tris buffer	0.5 g
Bacto-agar	3.0 g
Phenol red indicator	10.0 ml
Distilled water	950 ml

Procedure:

All the above components were weighed into a 2000 ml flat bottomed flask and heated with a magnetic stirrer until the mixture boiled. The pH was adjusted to 7.8-8.0. The flask was then cooled for 20 min before 190 ml were decanted into each of the 5 dispenser 250 ml bottles for sterilization at 121°C for 15 min. After sterilization, 10 ml of 20% glucose was added to each dispenser bottle before measuring out 5 ml

into previously autoclaved small test tubes. The test tubes were then cooled for 5-10 min in water. For each plate, only one colony (strain) was sampled and duplicate tubes with loosely fitting lids for each strain, were labeled and inoculated accordingly. One of the tubes was covered with 2ml of paraffin to prohibit oxygen entry and two tubes acted as the control. All the tubes were then incubated at 22°C for 7 days. Growth in OF medium was observed after 3 and 7 days

5. Motility test
A loopful of pure strain from streaked plates was inoculated into vials containing 5 ml sterile nutrient agar. The vials were incubated at 22°C for 24 hours. A "Hanging drop" method using a light microscope Leitz Laborlux S made by Leitz Wetzlar Germany 513558, was used to establish the motility of the strain at a magnification of 400. The strain that could move from point A to B was designated as motile or positive and that which was oscillating at the same locality was non-motile or negative.
Preparation of nutrient broth:
Weigh 2.4 g of nutrient broth. Add 300 ml of deionized water. Heat while stirring with magnetic stirrer for 5 min to dissolve all the ingredients. Decant in 8ml vials and autoclave at 121°C for 15 min. Keep closed at room temperature until required.

Determination of TVB using the Flow Injection/ Gas Diffusion (FIGD)

Six standards of ammonia in the range of 0-200 micromoles per liter (approximately equivalent to 0-3 mg TVB per 100g fish flesh) are prepared by taking appropriate dilutions of a 0,5 mM stock solution of ammonium chloride or trimethylamine crystaline hydrochloride in 7,5% trichloroacetic acid (TCA) solution. 100 microliter quantities of these standards (and samples) are injected into the FIGD manifold (a Rheodyne 5020 low pressure injection valve supplied by Anachem, Luton with a 100 microliter sample loop) which is then closed.
Preparation of the sample:
One part of minced fish muscle is blended with two parts 7,5% TCA solution and filtered through Whatman No. 1 paper.

FIGD mechanism of operation.
The flow of 1.0 M NaOH from the peristaltic pump (Ismatec, 4-channel with variable flow rate) carries the injected liquid (standard or sample) through the mixing coil alkalising it and releasing its contained nitrogen in the form of ammonia gas. On flowing through the gas diffusion cell,(a laboratory-built gas diffusion cell with channel dimensions 240 mm x 1,5 mm x 0,2 mm with a microporous, chemically inert and acid resistant PTFE membrane RS No. 8003525), the released ammonia passes through the gas permeable membrane into a 0,3 g/L solution of bromothymol blue (BTB) indicator flowing from the peristaltic pump. The color change caused in the indicator produces a proportionate response from the detector, a photometer incorporating a red light-emitting diode connected to a Perkin-Elmer 56 recorder.

TVB-N is determined as Nitrogen
(fish with a 80% moisture content)

$$\text{mg TVB-N /100 g sample} = \frac{\mu M/l\ (TVB\text{-}N) \times 10^{-3} \times 14\ g/mol \times 100g}{357{,}14}$$

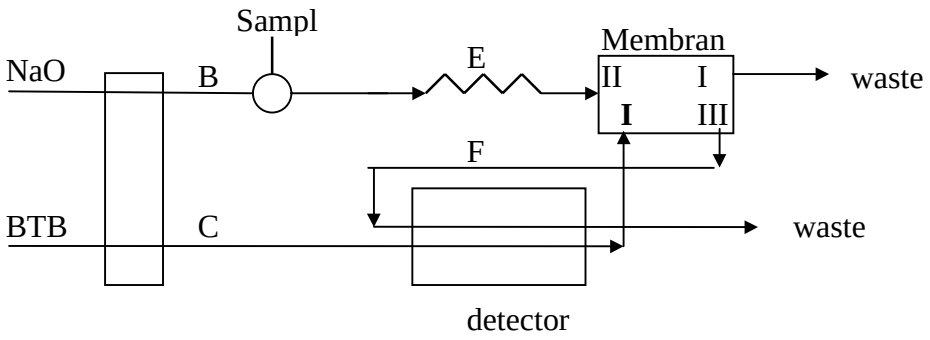

Figure 1: A schematic diagram of the Flow Injection/Gas Diffusion apparatus used for the determination of TVB-N.

APPENDIX 4. BACTERIAL IDENTIFICATION SCHEME FOR GRAM-NEGATIVE BACTERIA.