Masters thesis

“Rapid” (alternative) methods for evaluation of fish freshness and quality

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Faculty of Business and Science
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MAY 2010

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Submitted for partial fulfilment of the requirement of the degree of

Masters of Science in Natural Resource Science - Fisheries Science

Printed copies: 10

Number of pages: 77

Appendices: 7

University of Akureyri

Faculty of Business and Science

Department of Natural Resource Sciences

UNITED NATIONS
UNIVERSITY
Fisheries Training programme

MAY 2010

Declaration

I hereby wish to declare that this work is done by me and was not done and submitted at any place before.

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Lillian Chebet

It is hereby confirmed that this master thesis is satisfactory to M.Sc.-degree from the faculty of Business and Science, department of Natural Resource Science.

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Prof. Hjorleifur Einarsson

Abstract

The quality of fish and fishery products is a major concern in fish industry worldwide. Traditional methods for fish assessment are associated with various limitations characterized by; delays, cost implication less accuracy and for sensory methods, psychological and physiological limitations of analyst's posse challenges to fish industry. The aim of this thesis was to select, test simple, cheap and "rapid" (alternative) methods for evaluation of fish freshness and quality at landing sites. The measurement of Redox potential (E_h); Dye-Reduction Time (DRT), ATP and pH were chosen for this purpose. These methods were assessed using bacterial cultures and cod fillets in a model system and; field trials of method on Lake Victorian Nile perch (*Lates niloticus*) and cod (*Gadus morhua*) were conducted.

The results of the measurement of DRT, ATP and E_h demonstrated a high feasibility of application for assessment of fish quality. Regression analysis of DRT; ATP showed a significant correlation coefficient, r was = 0.8 with the spread plate counts in cod fillets. The measurement of E_h demonstrated real-time result on spoilt fish with E_h 0 mV; with varying initial E_h value of high quality fish (between +100-+300 mV) but does not give an account of the marginal levels of quality deterioration. High bacterial numbers (> 7log CFU/g) are required to cause a significant change in E_h of fish. However, swab method of measuring ATP showed unreliable ATP values; pH as well demonstrated unreliable results.

Keywords: Rapid methods; fish freshness; redox potential dyes and electrodes; resazurin.

Acknowledgement

I appreciatively acknowledge the technical guidance and supervision of Prof. Hjörleifur Einarsson. His tireless and valuable supervision from the beginning to the end of this work has not only registered a success in this work, but has imparted a lot of knowledge in me.

I am at the same time indebted to UNU-FTP; Dr. Tumi Tómasson and Mr. Þór H. Ásgeirsson (Directors) for offering full scholarship, after nurturing my career in the six months fellowship Training programme a year before.

Similarly, I am thankful to Ms. Sigríður Kr. Ingvarsdóttir of UNU-FTP for her good administration.

I extend my appreciation to the Management of Fisheries Training Institute for giving me courage to pursue this programme. Special thanks goes Ms. Gertrude Abalo; the Principal Fisheries Training Institute for accepting my request to attend this programme.

I am also indebted to the Director, Greenfields Uganda Limited., fish establishment; Entebbe –Uganda for accepting my request for fish samples treatment and storage in their establishment. Further I am grateful to the staff members of this establishment for working hand in hand with me until the end of the experimental work.

I extend my appreciation to the Management of Fisheries Department for their kind response to request to carry out the study experiments in their laboratory. And I am particularly indebted to the staff members of Uganda Fish Laboratory (UFL) for their valuable assistance during the experimental work.

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1 Introduction

The quality of fish and fishery products has become a major concern in fish industry all over the world (Huss et al., 2003). Fish, being one of the exceptionally perishable foods and as a result of globalization of food trade fish products tend to be more susceptible to rejection due to poor quality especially if the initial raw materials are of poor quality despite the technological developments in fish production (FAO, 2009; Huss et al., 2004). Furthermore, various outbreaks of food-borne illnesses, among which fish has been implicated as one of the vehicle foods, in various countries in the past years (Huss, 1995) have led to strict food quality/safety rules and regulatory system worldwide. This is clearly reflected in the current EC food regulations where the responsibility of food safety and quality is placed on food business operators and those operating fish businesses must carry out organoleptic examinations to ensure that fishery products comply with food quality and safety criteria (E C regulation No. 178/2002; E C regulation No. 853/2004).

In many occasions fish is sold and priced on freshness criteria for instance; the fresh Nile perch at Lake Victoria in Uganda. The fish is brought on shore at designated landing sites where it is graded by fish inspector in to different price group's based on freshness using sensory analysis. Freshness is one of the most important attributes of fish quality; and at the same time, it is not a single attribute however, it can be measured by different analytical methods. These methods can be divided into two categories: sensory methods (e.g. quality index method) and instrumental methods (e.g. chemical, physical and bacteriological analysis). All these methods are associated with operational limitations.

Sensory analysis is appropriate and indeed indispensable for product development. But, its reliance on highly trained panel to minimise subjectivity makes it costly, requires trained and a larger number and controlled enhanced psychological and physiological status of panellists to obtain meaningful results (Huss, 1995; Olafsdóttir et al., 1987). Therefore sensory analysis is unattractive for routine requirements (Huss, 1995; Dainty, 1996).

The instrumental methods as well require highly trained personnel and they are generally slow, requires at least two days or more to complete and often require expensive equipment and chemicals. Traditional bacterial enumeration methods are not only slow but are also dependent on significant number of colony-forming units (Adams and Moss 2008; McMeekin and Ross, 1996). Chemical methods may not be a useful especially during the early time of fish storage due to the fact that the spoilage metabolites at that time are below detection levels

(Huss, 1995; McMeekin and Ross, 1996). Traditional techniques therefore are increasingly being complemented and or replaced by more rapid alternatives, such as Dye-reduction techniques, using redox dyes such as resazurin, methylene blue and others; electrical methods like the Torry meter and Fischtester VI (Huss, 1995; Huss et al., 1992), ATP determination (Kyriakides and Patel, 1994), immunological methods like enzyme linked immune-sorbent test (Patel, 1994; Fung, 1995; Fung, 2002; Bourgeois and Mafart, 1995; Adams and Moss, 2008) and electronic noses (Olafsdóttir et al., 1997). However, as rapid methods" are increasingly gain momentum in food industry the need for validation against traditional methods e.g. against sensory and microbiological assessment before application for food evaluation is vital.

In previous study (Chebet, 2008) two instrumental methods, ATP and Dye-Reduction Time measurement showed a high potential for "rapid" and cheap evaluation of fish quality. Therefore, this work is a follow up of the previous study and explores other methods that can be used to serve this purpose.

1.1 Aim of the study

The aim of this study was to select and test some simple, cheap and "rapid" methods suitable for freshness evaluation of fish in fish industry and at landing sites. In order to do fulfil the aim the work was divided into four main tasks:

1. Review of the available literature on rapid methods for food freshness evaluation and selection of 2-3 of these methods
2. Evaluation of the methods in model system using bacterial cultures
3. Evaluation of methods using fish fillets
4. Trial studies of the methods in the field on Nile perch and cod.

The choice of these fish species was made basing on its high commercial value in Uganda and Iceland respectively and its availability throughout the year and the possibility of evaluating fish freshness during storage

Specific objectives

1. To identify alternative methods for evaluation of fish in plant and at landing sites
2. To determine their application in fish industry
3. To determine their correlation with the classical (sensory and microbiological) methods of fish freshness evaluation.

2 State of the art

2.1 Fish and factors influencing fish spoilage

The biochemical composition of a food (intrinsic factors) and their interrelationship with extrinsic factors during storage, contribute significantly to their freshness and quality partly for the reason that they determine and enhance the initial microbial growth (Huis in't Veld, 1996). With regard to fish, the inherent characteristic of the presence of non-protein nitrogen components, such as, trimethylamine-oxide (TMAO), creatine, methionine, free amino acids, cystine, histamine, carnosine, volatile nitrogen bases such as urea especially in cartilaginous fishes support microbial growth and the production of their related metabolites; responsible for fish spoilage during storage (Huss, 1988; Huss, 1994).

TMAO is one of the major non-protein nitrogen (NPN) fractions found in almost all marine fish species, but in varying quantities (1 to 5%) of the dry muscle weight. The variation in the amount of TMAO in fish muscle depends on; fish species, the fishing ground and season (Huss 1995). With regard to the amount of TMAO in various fish species, elasmobranchs and squid, are known to contain the highest quantities of TMAO (75-250 mg N/100g), whereas cod has a relatively low amount (60-120 mg N/100g) while flatfish and pelagic fish have the least. However, the concentration has been found to be more in the dark muscles whereas in demersal fish, higher concentrations are in the white muscle (Huss 1995). However, early studies of Gram et al., (1989) have found that Nile perch and tilapia from Lake Victoria comprises of 150-200 mg TMAO/100g of fresh fish.

According to Huss (1988) the source of TMAO in fish is known to be from the biosynthesis of certain species of zooplanktons. These organisms, posses an enzyme, mono-oxygenase, which oxidizes TMA commonly found in marine plants as are many other methylated amines (mono-methylamine and dimethylamine) to TMAO thus, the plankton eating fish obtain their TMAO from these zooplanktons. However, the reduction of TMAO to TMA is not independently the function of TMAO-reducing bacteria, but other systems too have been found to participate in its reduction. Further, the TMAO-reducing system is known to exist in dark muscles of some pelagic fish species and certain fish species are able to convert TMAO to TMA but this is regarded as of minor importance.

With reference to Gram and Huss, (1996) the highly nutritious properties of fish flesh provides an excellent substrate for the growth of most heterotrophic bacteria and the composition affects the bacterial growth and related biochemical activities. Further, the poikilothermic nature and its aquatic surroundings of fish favour the proliferation of a wide range of bacteria on fish. Further, the ultimate pH of after death is normally neutral or slightly acidic pH and therefore favours the growth of a wide range of microorganisms.

Likewise, the presence of highly unsaturated fatty acids in triglycerides (long–chain fatty acids and the phospholipids) in fish in general and especially in the pelagic fish species have important part in the spoilage processes of fish under aerobic storage (Huss, 1994).

The physical, chemical and bacteriological characteristics of fish tend to differ with species, feeding habits, seasonality spawning cycles, methods of catching, fishing grounds, size, age, microbiological load and geographical location (Shewan, 1977; Huss, 1995). Nevertheless, temperature and rigor mortis are the main underlying factors in fish spoilage. Round fish deteriorate faster than flat fish like plaice (Huss, 1995).

According to Huss (1988), storage of fish caught during their heavy feeding and or spawning seasons show wide variations in spoilage rate. Heavily feeding fish tend to be more susceptible to autolytic tissue degradation than the petite feeders. The type of feed /food on which fish is feeding on may similarly have an effect on their spoilage rate during storage. Non-feeding fish have been found to have low levels of bacteria in the intestines as compared to the heavily feeding fish.

Spawning fish tend to use most of their glycogen and the effect of depletion is reflected in their susceptibility to rapid deterioration during storage due to faster onset and resolution of rigor mortis (Connell, 1990; Huss, 1994).

The fishing grounds and geographical locations exclusively tend to determine fish spoilage insofar as temperature, food types, the level of pollution; microenvironments are concerned Shewan (1977). A somewhat similar factor commonly known as "*kaliiro*" has more often than not been experienced in the waters of Lake Victoria. This phenomenon occurs in the month of July, time during which fishermen report high spoilage of catches without any implication of bacterial intervention. The water temperatures during this time of the year are known to be much lower between 19-23^{o}C compared to the normal temperatures, 26-30^{o}C (Personal observation).

Further, bacteria associated with fish vary according to their optimal growth requirements, which among others include; oxygen or redox potential (E_h), pH, temperature and water activity thus, their distribution in the environment therefore, is dependent on their requirements. According to Huss, (1988) bacteria living on the surface of marine fish are phenotypically capable of utilizing amino acids, petites and non-carbohydrate sources for growth. Their activity on these substrates normally leads to the production of slightly alkaline conditions in stored fish products.

Bacteria which colonise fish skin surfaces and gills are predominantly aerobes however, facultative bacteria particularly *Vibrio* may occur in high counts on pelagic fish. According to Huss, (1995), obligate anaerobic bacteria are not common on the surfaces of fish but their occurrence is significant in the intestines.

Additionally, the method of fishing as well has implication on the level of glycogen level at the time of fish death, more exhausted fish, for instance those caught by trawl method exhibit lower glycogen reserves in the muscle cells thus, higher ultimate pH at the resolution of rigor mortis and therefore, more prone to higher rate of spoilage as high pH favours microbial proliferation (Huss, 1995).

Again, the geographical location, and or the type of waters, has a remarkable influence on the type of micro fauna that will grow on the fish. With reference to Mossel, (1977) microorganisms associated with food spoilage are found and can thrive under a wide spectrum of environments.

According to Gram et al. (1989) the micro-fauna found in fish from warm waters (tropical waters) differs from those from cold waters. The predominant bacteria on the surface of fish in warm waters consist of the Gram positive bacteria; *Bacillus, Micrococcus, and Carnobacterium* which constitute to 50-60% of the total microflora. Whereas those found on cold waters comprise of Gram negative bacteria; *Psychrobacter, Moraxella; Pseudomonas ssp; Actinobacter, Shewanella, Flavobacterium, Cytophaga* and *Vibrio.*

According to FOSRI, (1997) *Enterobacteriaceae,* together with coliforms predominate in fish caught along the coast line. However, with reference to (Gram and Huss, 1996; Gram et al., 1990) *Enterobacteriaceae,* prevalently grow and dominate over *Pseudomonas spp.* in products under chill storage (5-10^{o}C). Nevertheless, the common spoilage organisms associated with seafood stored in ice and air, whether of temperate, sub-tropical or tropical origin, are the gram negative Psychrotrophic bacteria such as *Pseudomonas* and *Shewanella*

putrefaciens. Other pertinent spoilage bacteria include *P. phosphoreum* commonly isolated in packed fish and other meats; *Vibrionaceae*, associated with fish at elevated temperatures anaerobic spoilers such as L*actobacillus spp, Leuconostoc spp* (Huss, 1995).

In general, the growth of specific spoilage bacteria and the accumulation of their metabolic - bye products constitute to the major spoilage changes in fish during storage. Essentially, freshly caught fish are usually characterised sensorially by 'fresh fish flavours' (sweet, sea weedy); during storage, a period is reached where the odours and flavours are described as neutral or non-specific, (the first indications of off-odours and flavours) which progressively become more pronounced and ultimately renders the fish unacceptable for consumption (Gram and Huss, 1996).

2.2 Fish spoilage

Fish "spoilage", is a condition, not clearly defined, however, it is referred to as "product /fish quality loss, indicated by the deterioration of fish characteristics shown in Table 1which include, the colour, flavour, odour or texture; resulting from different spoilage processes that occur during storage (Huss, 1995; Huss, 1994; Huss, 1988). Essentially, fish spoilage changes, occur as soon as fish dies, and they take place in a sequential process. These processes have been categorized into: autolytic, microbiological and chemical changes. According to Huss (1995) spoilage of iced cod has been stratified into four phases; to indicate the deterioration of the eating quality of fish during storage in which autolytic spoilage changes predominate in the initial storage period and is succeeded by changes due to bacterial activity, thus, apart from spoilage changes due to chemical reactions of the fish lipids, autolytic and bacterial activity constitute to the main spoilage changes in fish during storage (Figure 1).

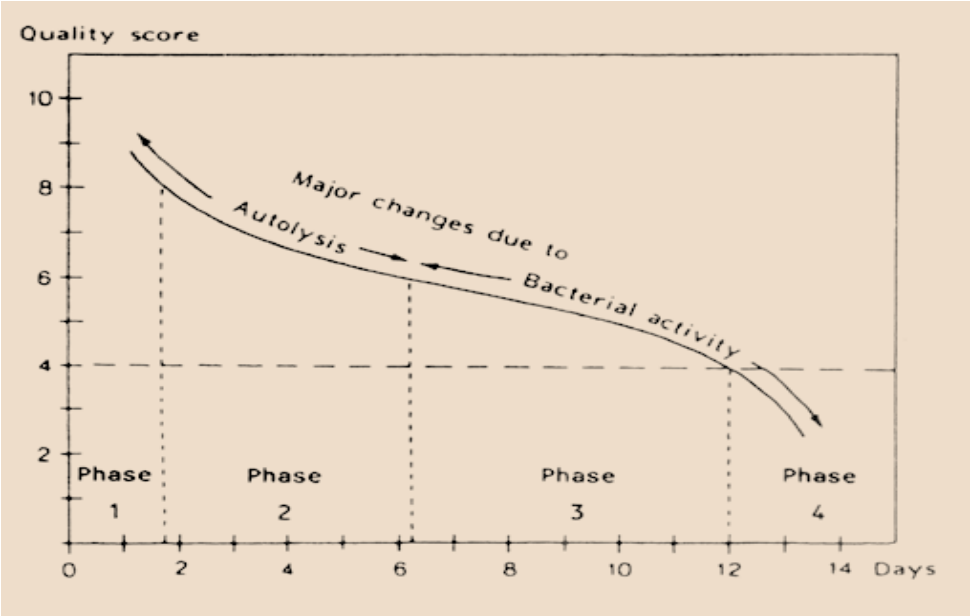

Figure 1: *Spoilage changes and eating quality of iced cod (0 °C) (Huss, 1995)*

Table 1: Spoilage processes, spoilage indicators in fish during storage

Spoilage process	**Spoilage indicators/signs**					**Reference**
	Slime formation	Change in texture	Discoloration	Gas formation	Off-odours/ off-flavours	
Physical changes	-	+	+	-	-	Haard (2002);
Autolytic changes	-	+	+	-	+	Huss (1994);
Chemical (Lipid oxidation)	-	+	+	-	+	
Microbiological growth	+	+	+	+	+	Huss(1995); Huss (1988)

2.2.1 Autolytic spoilage changes

With reference to Gram and Huss (1996) the initial quality loss in fish is basically due to autolytic changes and is not related to microbiological activity. The autolytic spoilage changes

precede the other changes responsible for the loss of fish quality during storage. Autolysis is principally enzymatic reactions, which take place in fish tissues (Table 2). Enzymes and other related chemical reactions do not immediately cease their activities in the fish muscle after fish death (capture). Their continuation initiates other processes like rigor mortis; which is the basis for autolytic spoilage in fish (Huss, 1995).

Most important in the autolytic spoilage changes is the degradation of fish nucleotides (ATP-related compounds) i.e. Adenosine triphosphate (ATP) degrades to adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), Inosine (Ino) and hypoxanthine (Hx), associated with bitter fish flavours (Haard, 2002; Huss, 1995). Generally, the biochemical changes due to enzymatic activity related to freshness deterioration in fish are change in the flavour and colour (Haard, 2002). However, the presence of ATP intermediate nucleotides, monophosphate (IMP) and inosine (Ino) is associated with the desirable sweet flavour in fresh fish (Huss, 1995).

Unlike in the degradation of other nucleotides, which are known to be principally as a result of enzymatic activity; the ultimate degradation of ATP intermediate nucleotides, (IMP and inosine) to hypoxanthine (Hx) is attributed to bacterial activity (Haard, 2002). The presence of Hx is usually characterized by undesirable, bitter flavour, an indication of spoilt fish product under chilled storage (Huss, 1988). However, with the exception of the loss of ATP, associated with rigor mortis, none of the intermediate nucleotides are regarded to be related to the perceived changes in the texture of the fish during autolysis (Huss, 1995).

According to Haard (2002); Huss (1995); Gram and Huss (1996), the degradation of ATP catabolites progress in the same way in most of the fish species although the rate of degradation may vary from fish to fish however, their progression coincide with fish spoilage as perceived by a sensory analyst.

Further, in connection to autolysis, the activity of proteolytic enzymes, which include digestive enzymes such as proteinases in ungutted fish, result into a condition referred to as "belly-burst" more especially in small fishes and heavily feeding fish and crustaceans (Connell, 1990; Haard, 2002). The seepage of proteolytic enzymes, which include digestive enzymes such as proteinases, in the fish viscera in to the organs and the surrounding tissues of the fish lead into quality defect or damage in fish. However, its contribution to the spoilage of chilled fish and fish products is known to be minor (Huss, 1994). Nonetheless, under frozen conditions, autolytic enzymes break down trimethylamine-oxide (TMAO) to dimethylamine

(DMA) and formaldehyde (FA), associated with undesirably, dry and hard fish texture (Huss, 1995).

Table 2: Autolytic and chemical changes in fresh fish during storage

Reaction	**Case in point**	**Consequences/Spoilage**	**Reference**
Physical changes: (rigor mortis); Mechanical handling during slaughter	Gaping and rupture of muscle connective tissues	-Low water holding capacity and softened /sogginess of the flesh texture -Change in dielectric properties	Haard (2002);
Digestive enzymatic changes	Digestive proteinases-midgut gland proximity to the flesh	-Seepage of digestive proteinases-Rapid fish quality loss	Huss (1995);
Fish muscle enzymatic changes	Amylase-anaerobic glycolysis	-Formation of lactic acid -flesh texture change, -Sugar phosphate precipitation -flesh browning; -low pH, -Flesh texture toughening	
	Lipases & Phospholipases glycerol-fatty acids hydrolysis	-Formation of free fatty acids (FFA) -Off-odours, -Destruction of vitamins, amino acids, -Formation of ammonia (NH_3), -Changes in texture and water holding capacity	Huss (1994);
Nucleotides degradation		-Inosine and Inosine monophosphate (IMP) - Ammonia (NH_3)	
Enzymatic and or chemical changes	Lipid oxidation	-Formation of volatile alcohols and aldehydes, ketones, esters,	
	Degradation of hydroperoxides	-Browning /yellow discolouration; -Texture changes; Formation of ions (Cu and Fe) and antioxidants e.g. ascorbic acid, citric acid etc	Huss (1988)

2.2.2. Microbiological spoilage

Microorganisms exist on the skin/slime, in the gills in the gut of live and newly caught fish. According to Huss (1995) the proportion of microorganisms on the surface and gills/ guts of fish is; 10^2-10^7cfu (colony forming units)/cm^2 and between 10^3 and 10^9 cfu/g respectively. But due the strong defensive mechanism of live fish, they are not able to cause spoilage.

According to Gram and Huss (1996) spoilage of fish under aerobic conditions becomes apparent when specific spoilage bacteria (SSO) reaches the values of 10^8-10^9/g flesh or cm^2. Normally, this occurrence is after a lag phase, the time-span of which mainly depends on temperature before bacteria enter into exponential growth particularly in tropical fish species stored under low temperatures (0^oC) (Gram et al., 1990; Huss, 1988).

According to Gram and Huss (1996); Huss (1994); Huss (1995) microbial spoilage of fish may take diverse forms which manifest itself as changes in the sensory characteristic such as; off-odours and flavour, and taste; formation of slime, visible pigmented and non-pigmented colonies, discolouration and gas production; that finally cause sensory rejection. However, most important of these changes is the formation of pungent, volatile odours resulting from the degradation of fish components by microbial action (Table 3).

The degradation of ATP intermediate nucleotides by *Proteus mirabilis, S. putrefaciens, Pseudomonas spp* or Enterobacteriaceae to hypoxanthine (Hx), associated with bitter taste in chilled fish, as mentioned above.

Essentially, the initial bacterial growth on fish under aerobic conditions are primarily aerobic organisms using carbohydrates and lactate as energy source with the ultimate formation of CO_2 and H_2O as end products (Huss, 1988). As result of proliferation of aerobic organisms in fish during fish storage, partial anaerobic microclimates/conditions on the surface of the fish is formed which subsequently favours the growth of facultative anaerobic bacteria and TMAO-reducing bacteria (spoilage bacteria) for instance *Shewanella putrefaciens, Photobacterium phosphoreum* and *Vibrionaceae,* as well as some obligate anaerobes.

According to Huss (1988); Gram et al. (1989/1990); Dalgaard et al. (1993) the utilization of TMAO by these organisms lead to the formation of trimethylamine (TMA), one of the most important compounds responsible for off-odours, "fishy" odours at later stages of spoilage in many marine fish. *Shewanella putrefaciens* is also known as one of the potent spoilage bacterium in addition to *Enterobacteriaceae,* responsible for the breakdown of the sulphur–

containing amino-acids, cystine and methionine that results in the formation of hydrogen sulphide (H_2S) and methyl mercaptane (CH_3SH) and dimethylsulphide $(CH_3)_2S$ respectively. According to Huss (1988); Gram and Huss (1996) these compounds are characterised by volatile foul-smelling, pungent off-odours, that can be detected organoleptically even in very low concentrations. Whereas, *Pseudomonas spp, Enterobacteriaceae* and anaerobic spoilers leading to the production of fruity, sweet-smelling esters, ketones, aldehydes, and putrid sulphur compounds, non-H_2S sulphides (Gram and Huss, 1996; Huss, 1995; Huis In't Veld, 1996).

Furthermore, the formation of ammonia (NH_3) and other lower fatty acids are among the fish spoilage off-odours resulting from bacterial degradation of urea in cartilaginous fishes; amino acids during prolonged anaerobic storage; are collectively indications bacterial spoilage although a small amount of NH_3 is formed during autolysis (Huss, 1988). According to Huss (1988) the main NH_3–producing microorganism is Bacteriodaceae (*Fusobacterium*) however, generally, the production of the various off- odours associated with spoilage are as a result of specific bacterial activity on various fish components (see Table 3).

2.2.3. Chemical spoilage (degradation of fish lipids)

The chemical spoilage associated with fish during storage is mainly due to fish lipid degradation (auto-oxidation). In general, fish have high degree of unsaturated lipids than other food commodities (Huss, 1995). During fish storage, fish lipids are known to susceptible to oxidative rancidity.

According to Huss (1994); Haard (2002) fish lipids are subjected to two main changes, lipolysis and auto-oxidation. The main reactants in these processes involves atmospheric oxygen and fish unsaturated lipids, leading to the formation of hydroperoxides, associated with tasteless, flavour and accompanied by brown yellow discolouration of the fish tissue (Huss, 1994).

Up on further degradation of hydroperoxides is the formation of strong rancid flavours e.g. aldehydes and ketones, usually associated with spoilt fatty fish species (Huss, 1995; Haard, 2002; Ashton et al., 2002). However, these reactions are initiated and accelerated by heat, light, especially the UV-light and several inorganic and organic substances such as copper,

iron ions and several antioxidants with the opposite effect, such as; alpha-tocopherol, ascorbic acid, citric acid, carotenoids (Huss, 1994).

2.3 Spoilage of Nile perch (*Lates niloticus*)

The micro-fauna on newly caught Lake Victorian Nile perch, (*Lates niloticus*) are microorganisms belonging to the genera; *Moraxella, Alcaligens, Acinetobacter, Pseudomonas, Aeromonas, Micrococcus* and other Gram-positive organisms: According to Gram et al. (1990) the proportions of Gram-negative and positive species were 39% and 61% respectively. According to Gram (1989), 40-90% of the bacteria found on Nile perch were able to grow at 7^{o}C. With reference to Gram et al. (1989) the numbers of psychrotrophic bacteria have been found to increase after prolonged storage time of fish on ice in the order of 10^{9}/g flesh, while the fish was still acceptable.

The spoilage of Nile perch at ambient temperature (20-30°C), is characterized by, strong rotten, fishy, hydrogen sulphide off-odours; occurring in 11-17 h. The production of these off-odours is attributed to the activity of *Aeromonas,* spp., known to reduce trimethylamine - oxide (TMAO) to TMA and has also been noted to produce hydrogen sulphide from amino acids (Gram et al., 1989/1990).

In connection to TMAO, early studies of Gram et al. (1989) have found that Nile perch and tilapia from Lake Victoria comprises of 150-200 mg TMAO/100g of fresh fish. In spite of the presence of TMAO, 90% of the predominant spoilage microflora isolated in spoiled iced Nile perch, are known to comprise of *Pseudomonas,* which are non-TMAO–reducers. However, its predominance in spoiled Nile perch stored on ice is known to occur along side with *Shewanella putrefaciens* (Gram and Huss, 1996).

Pseudomonas is characterised by the production of fruity off-odours in spoiled fish and none of these compounds, (trimethylamine or H_2S) are produced *Shewanella putrefaciens* and *Aeromonas* spp., isolated in iced Nile perch. However, the role of *Shewanella putrefaciens* in the iced spoilage of Nile perch, and in tropical water fish in general, is considered insignificant for the reason that, their growth, to detectable levels of spoilage, are known to occur only very late in the storage of fish (Gram et al., 1990).

Table 3: Substrate and typical spoilage products of SSO in fish stored in various conditions.

Storage conditions	**Specific spoilage organism**	**Substrate**	**Typical spoilage compound**	**Reference**
Iced fish Ambient storage temperatures; iced & lightly preserved fish CO_2 packed fish	*Shewanella putrefaciens* *Enterobacteriaceae* *Vibrionaceae* *Photobacterium phosphoreum*	TMAO	TMA	Gram and Huss (1996); Huis in't Veld, (1996); Jay et al. *(*2005);
Iced fish Ambient storage temperatures ; iced & lightly preserved fish	*Shewanella putrefaciens* *Enterobacteriaceae* *Vibrionaceae*	Cystine	H_2S	
Iced fish Ambient storage temperatures ; iced & lightly preserved fish CO_2packed fish	*Shewanella putrefaciens* *Pseudomonas ssp.* *Enterobacteriaceae* *Photobacterium phosphoreum*	Inosine & IMP	Hx	Adams and Moss (2008); Huss (1995);
Iced fish	*Shewanella putrefaciens* *Pseudomonas ssp*	Methionine	CH_3 SH, $(CH_3)_2$ S	Huss (1988); McMeekin and Ross (1996);
Iced fish Ambient storage temperatures ; iced & lightly preserved fish	*Shewanella putrefaciens* *Enterobacteriaceae*	Carbohydrates Lactate	Acid	Huss (1995);
Iced fish Sous-vide (Cooked) & Iced fish Ambient storage temperatures ; iced & lightly preserved fish	*Pseudomonas fragi* Anaerobic rods *Pseudomonas ssp* *Enterobacteriaceae*	Monoaminomono carboxylic acids Amino acids (glycine, serine, Leucine), Amino acids	Fruity ethyl esters, off-odours Ketones, aldehydes, esters Non-H_2S Sulphides	Dalgaard et al., (1993);
Iced fish Ambient storage temperatures ; iced & lightly preserved fish	Anaerobic spoilers *Pseudomonas ssp* *Enterobacteriaceae*	Urea	NH_3	
Iced fish	Anaerobic spoilers	Acetate, Urea	Acetic, butyric and propionic acid	Gram and Huss (1996);

2.4 Shelf life of chilled fish species

Various studies have demonstrated that the shelf life (keeping time) of fish vary from to fish species to another but temperature seems to be one of the most important factors controling the rate of spoilage and therefore the shelf life. According to Huss (1995) the relative rate of spoilage (RRS) for fresh fish stored at similar temperatures is similar in all fish species. In spite of this fact, the general model for the determination of the RRS of fish and shelf life of fish, is estimated that the average relative rate of spoilage (RRS) of tropical fish species, stored at 20-30^{o}C tend to be about 25 time higher than at 0^{o}C. This is more than twice as high as approximated RRS using the the general model and can not account for the change in the spoilage microflora of tropical fish (Huss, 1995). Thus, the relative rate of spoilage for tropical fish (RRS) = $0.12 * t\ ^{o}C$, allows for the determination of the RRS, of fish stored at various temperatures (Figure 1).

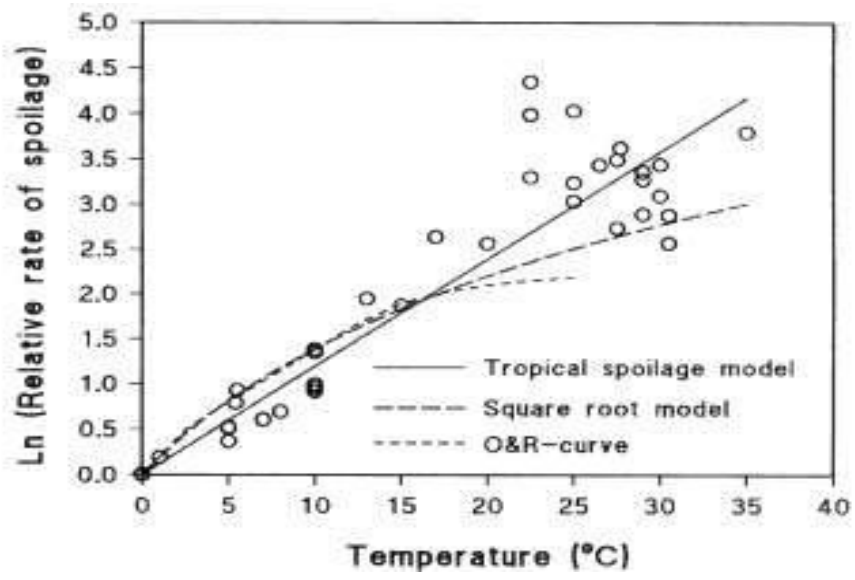

Figure 2: Natural logarithm of the relative rate of spoilage of tropical fish species plotted against storage temperatures from (Dalgaard and Huss, 1994).

However, various authors, using these equations to determine the shelf lifes of various fish species fish have remarked that tropical fish species generally tend to have longer shelf life on ice, than the temperate fish species (Huss, 1995). Tropical water species, have been estimated to have a shelf life stretching from 6-40 days on ice whereas the temperate fresh water species

keep for 2-24 days on ice however, variation exists among the different fish species (Gram; 1989). Lake Victorian Nile perch (*Lates niloticus*) in particular, is reported to keep for 13-32 days on ice, whereas the marine species are known to keep for 8-17 days on ice (Gram et al., 1989).

Although the spoilage and spoilage microflora have been distinctly defined, the reason behind the long keeping time of tropical fish species is still not very clear. The various theories put across to elucidate this phenomena are based on the difference in bacteria flora. Gram et al. (1990) remarked that the keeping time of tropical fish species is difficult to affirm due to the unclear definition of spoilage given on a "tropical" fish species; this is because experiment conducted to assess the shelf life of tropical fish have been carried out using different sensory and bacteriological analyses.

The reviewed literature on spoilage trials of tropical fish spp. have remarked that the overall sensory, chemical and bacteriological changes occurring during spoilage of tropical fish spp stored at low temperatures are similar to those described for temperate spp. According to Gram and Huss (1996) Psychrotrophic bacteria belonging to *Pseudomonas spp.,* along with *Shewanella putrefaciens,* constitute to the main spoilage flora of iced stored fish. *Shewanella putrefaciens* is characterized by the the production of TMA and sulphides (H_2S) whereas spoilage by *Pseudomonas spp.,* is characterized by the absence of these compounds and the occurrence of sweet, rotten sulphydryl odours, not typical of temperate marine fish, widely studied. However, in spite of the presence of TMAO, known to occur in most marine fish, and in some tropical marine fish species e.g. in Nile perch (*Lates niloticus*) (Gram et al,.1989). *Pseudomonas spp*.is known to be incapable of reducing TMAO to TMA (Huss, 1995; Gram and Huss, 1996).

Shewan (1977); Gram et al. (1989) attributed the long keeping time of tropical water fish to the low numbers of psychrotrophic spoilage bacteria due to the long lag phase of 1-2 weeks during storage, contrary to temperate water fishes in which the spoilage microflora grow immediately during storage. Whereas, Huss (1995) remarked that the low bacterial counts claimed by various studies are attributed to either inappropriate media, too high incubation temperatures (30^oC) that can not allow psychrotrophic spoilage bacteria to growth on agar plates. Other authors have attributed the long keeping time to the absence of TMA and TVB, characterized by pungent undesirable off-odours during fish storage. Huss (1995) and Gram and Huss (1996) considered this to be an indication that spoilage is dominated by

Pseudomonas spp. which are associated with non-pungent undesirable off-odours during fish storage.

2.5 Traditional methods for evaluation of fish spoilage

The spoilage is rather a complex process of physical, chemical changes in the food itself and due microbial activity which occur during storage. The contemporary consumer demand for fresh food products and the strict food safety regulations in general, requires appropriate assessment and management of safety and quality of food products, by using methods that can quantitatively relate microbial growth to the characteristics of the products during storage, to avoid unnecessary economic losses (Connell, 2001; McMeekin and Ross, 1996).

The various methods commonly used for assessment of fish spoilage have been classified into two categories: sensory methods and instrumental (microbiological, biochemical, and physical) methods (Huss, 1995). However, the most commonly used of these methods for evaluation of wet fish freshness in fish industry is sensory method. This is because its reliability that has been found to coincide with the need to assess the freshness of wet fish within the short time as possible considering its perishability (Connell, 1990).

2.5.1 Sensory evaluation

Sensory evaluation of food, according to Huss, (1995); Meilgaard et al. (1991) is defined as the scientific means of quantifying and interpreting the variations in food characteristics (odour, taste, tactile, appearance) by using human senses of sight, smell, taste, touch and hearing. Studies have shown that assessment of food freshness/ characteristics using sensory methods are capable of giving objective and / reliable results when assessments are done under controlled conditions. Generally, trained and experienced taste panel is essential to obtain accurate and reproducible result (Connell, 2001; Alejandra et al., 1992). Sensory methods are divided into two groups; discriminative and descriptive tests however, the most commonly used is the descriptive test which measures the difference or absolute value indicating the different quantitative levels (Meilgaard et al. 1991; Huss, 1995). There are

several grading methods used to assess freshness in fish and fish products for instance the EU scheme and the Torry system (Huss, 1994).

Nonetheless, other new sensory schemes exist like the quality index method (QIM), originally developed in Tasmania (Bremner et al,. 1985). QIM is a tool, for estimation of the quality attributes in a more objective way, based on the significant parameters for raw fish (Frederiksen, 2002); with a score system ranging from 0-1; 0-2; 0-3; 0-4 or more, demerit points (Jonsdottir,1992). The main advantages of the QIM method when compared to EU scheme is that QIM is species specific and confusion about attributes is minimised. Each fish species has its own characteristic sensory attributes (flavour, appearance, odour, and texture) which change with time and temperature after harvest (Martinsdóttir, 2002). QIM schemes have been developed for species such as European cuttlefish (S*epia officinalis*) Arctic charr (*Salvelinus alpines*) fresh cod (*Gadus morhua)* fillets (Martinsdóttir, 2002).

However, sensory methods in general are known to be irrationally expensive due to the high training requirement of the panel; cost of running, need for individual scheme for individual fish species given the different spoilage patterns and physiological and psychological limitations of the analyst (Connell, 2001).

2.5.2. Microbiological methods

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

According to Bourgeois and Mafart (1995) the various ways that can be used to determine bacteriological contamination in food/fish include; Total Plate Count (TPC), Most probable number (MPN) and other instrumental methods e.g. (ATP, microscopy, turbidometry, conductance, others). Total Viable Count / Total Plate Count/ Standard Plate Count/ Aerobic

Plate Count SPC, APC; all mean the number of bacteria (colony forming units, cfu/g or ml) in a food product under specified standard and uniform conditions of culturing. In general, these methods rely on the estimation of the fraction of the microflora able to produce colonies in the medium used under specified incubation conditions (Huss et al., 2004). Therefore, the temperature during incubation of the plates has greater influence on the number of colonies developing in the sample thus, in the examination psychrophilic bacteria, pour plating and a 3-4 day incubation period at 25^{o}C is recommended other than at 30 or 37^{o}C (Huss, 1995; Huss, 1994). The enumeration of bacterial counts in food can be done using a variety of medium, thus the classification of these methods for instance; plate count agars (PCA) are commonly used for enumeration of bacteria (Huss, 1995; ICMSF, 1998). However, it is recommended that a more nutrient rich agar, such as (Iron Agar, Lyngby) be used when analysing seafood to obtain reproducible results (Huss et al., 1987).

Most Probable Number (MPN)

The MPN technique, more often referred to as Multiple-Tube Fermentation method is one of the oldest methods used to enumerate low numbers of viable microorganisms Kyriakides and Patel (1994). This method is usually performed by inoculation of replicate tubes of an appropriate liquid medium with three different dilutions/ sizes of the material to be studied and incubation with 48 h at 35^{o}C. The medium used is normally designed to ensure clear identification of whether growth or no growth has occurred and the number of positive at each sample (Adams and Moss, 2005). The interpretation, of coliforms for instance is based on the production of bubbles in the small Durham tubes by the action of bacteria that ferment lactose with gas formation within 48 h at 35^{o}. The number of positive and negative tubes is recorded and the organism is estimated consulting the table for the number that satisfies the positive tubes which is multiplied by the dilution factor to obtain the MPN/g or ml of the product.

2.5.3. Chemical methods

The evaluations of food using chemical methods are considered to be more objective than sensory methods especially when it is done accurately using appropriate method (Huss, 1995). These methods involve determination of the concentration of a specific chemical(s) in the food under study. Chemical methods of food evaluation are normally used to indirectly

predict the level of a sensory attribute, which allows for immediate determination of freshness. To use chemical methods to serve this purpose, well set, quantified and standardised tolerance levels of chemical spoilage indicators need to be established (Huss, 1995).

With regard to evaluation of fish quality using chemical methods, the total volatile basic amines (TVB) constitute to the commonly measured chemical indicators. TVB is a general phrase used to include volatile amines such as, trimethylamine (TMA), ammonia (NH_3) produced by spoilage bacteria; dimethylamine (DMA) and produced by autolytic enzymes during frozen fish storage (Huss, 1988). The concentration of these chemicals in fish tissues can be determined by steam distillation method (Malle & Poumeyrol, 1989).

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

K-value, (freshness) can be determined by calculating the ratio of inosine and hypoxanthine to the sum of ATP and all the other products of ATP degradation multiplied by 100 (Haard, 2002; Huss, 1995; Connell, 2001).

The interpretation is that the smaller the K-value the more fresh the product is and high K-value indicate unacceptable fish product. K-value of 20% has been suggested as a freshness limit and 60% as the rejection point. Nevertheless; its application has not been popular in fish industry due to loss of recognition from the EU system (Huss, 1995).

Other methods such as Peroxide Value (Pv), Thiobarbituric Acid (TBA), Iodine value (Iv) and also constitute to the chemical methods that are used to measure rancidity in fish and fish products but they will not be included herein this study. However, generally, there are a number of limitations associated with traditional methods of food/fish evaluation. Some of these limitations are listed in Table 4.

Table 4: Strength and limitations associated with the traditional methods of fish evaluation.

Methods	Advantages	Disadvantages	References
Sensory methods	Fast, Non destructive Accurate under controlled conditions	Costly, require training, psychological Physiological status of the analyst Difficult to standardise	Huss, (1995); McMeekin and Ross, (1996), Dainty (1996)
Microbiological methods	Can be tuned to enumerate various types of bacteria	Not fast Require highly trained personnel Not all bacteria are spoilers Valueless in fresh products that are merely contaminated Bacterial counts don not correlate well with sensory and chemical methods on odour changes MPN method does not represent other spoilers	Huss (1995); McMeekin and Ross (1996); Huis in't Veld (1996); Gram and Huss (1996); Adams and Moss (2008)
Chemical methods	They are fast Excellent for marginal quality asessment Correlate well with sensory methods	Volatization of stored samples cause analytical errors, TMA shows up at later spoilage stages Not all fish have TMAO/TMA TMAO/TMA quantities vary within and between fish spp Not all bacteria produce TMA at same rates/quantities Can not differentiate the source of NH_3 bacterial/autolytic valueless in fresh fish with high NH_3 e.g. fatty fish	Huss, (1995); Huss, (1988);

2.6 Alternative "Rapid" methods for food assessment

Essentially, the objective of food assessment is to avoid the ingestion of contaminated food; to control the nutritive value of food by detecting the elevated levels of food adulteration and in the end to ensure the safety of the consumer (Mossel et al, 1994; Mossel, 1977). To fulfil this

purpose, the need for objective and reliable methods of evaluation is essential. However, although the traditional methods of evaluation play a considerable role in food industry, they are associated with shortcomings characterized by delays in producing results, accuracy/objectiveness and other related loop holes (Table 3). Because of these shortcomings advances towards the development and application of various techniques, considered to be cheap and "rapid" for the assessment food freshness/quality has been made in food industry. According to Bourgeois et al. (1995); Fung (1995); Mossel et al. (1994) the definition of "rapid" methods refers to improved, alternative methods for food evaluation that can enable early, accurate and faster detection, isolation, characterization and or enumerate microorganisms and their products related to food spoilage.

In accordance with Fung (2002) the application of "Rapid" methods for food quality and safety evaluation are a recent development in the general field of applied microbiology. The main characteristic of the various techniques considered as rapid means for food quality assessment is the measurement of the changes in the food characteristics, associated with spoilage due microbial activity such as; the concentration of microbial ATP, Redox potential, odour/flavour, dielectric properties, pH, texture etc. Various studies have shown that there wide range of gadgets, instrumentation and diagnostic kits that can be used to serve this purpose; a list of some of these techniques is shown in Table 5.

2.6.1 ATP determination technique

The measurement of the concentration of adenosine triphosphate (ATP) in foods is considered as a "rapid method" for the assessment of bacteriological quality of food. The basis of this technique is the inherent existence of ATP molecule in all living organism. With reference to Huss (1995) ATP is a nucleotide, found in all living cells, including bacteria and is the universal agent for the transfer of free energy molecule. In accordance with Kyriakides and Patel (1994); Adams and Moss (2000) a number of organisms including bacteria have evolved a mechanism for producing light by the activity of enzymes (luciferase) on substrates known as luciferin. For this reaction to occur, the presence of ATP and magnesium ions is required to produce one photon of light at the expense of the hydrolysis of one molecule of ATP through a series of intermediates reaction categorized into four (4) phases:

1) Luciferin + Luciferase +ATP = 2) Luciferin - Luciferase -AMP + PP + O_2; 3) =Oxyluciferin* -Luciferase-AMP + HO_2 and 4) = Oxyluciferin + Luciferase +AMP+ light emission.

In accordance to Hartman et al. (1992); Bourgeois et al. (1995) the amount of light emitted is directly proportional to the quantity of ATP and therefore the number of viable cell in the medium. With reference to Adams and Moss (2005) the detection of light emission, as low as 10^2-10^3fg (which correspond to 10^2-10^3 bacterial cells) can be achieved using photomultiplier tubes depending on the sensitivity of the instrument.

According to Kyriakides and Patel (1994) ATP technique of bacteriological assessment of food quality correlate well with bacterial counts in food however, caution must be taken in samples whose somatic cells dominate in the sample to avoid alteration of the measurement.

2.6.2. Redox potential (E_h) technique

The measurement of the variation in Redox potential (E_h) is considered to be one of the "rapid" ways for the estimation of bacteriological quality of food (Huss, 1988; Bourgeois and Mafart, 1995).

Metabolically active micro-organisms; especially aerobic micro-organisms are capable altering the E_h their substrate (i.e. food), leading to lower E_h values (Jay, 1992; Jay et al. 2005). According to Huss (1995) and Huss (1988) the measurement of redox potential can be done by using an appropriate instrument such as redox electrodes and expressed in millivolt. The common electrodes used to serve this purpose are, silver-silver Chloride electrodes and platinum electrodes with calomel reference electrodes (Huss, 1995). Similarly, the Dye-reduction technique constitute to one of the ways of determining the bacteriological quality of food by estimating the detection time indicated by the dye discolouration time in hours. According to Bourgeois and Mafart, 1995); Adams and Moss (2000/2008; Atherton and Newlander (1977) the common redox dyes used to serve this purpose include; Resazurin, methylene blue, 2, 3, 5-triphenyltetrazolium Chloride (TTC); expressing the result as; Dye-Reduction Time in hours (h). According to Atherton and Lawlander (1977); Grimaud et al. (2007) longer Dye-Reduction time is used to indicate lower bacterial numbers and shorter Dye-Reduction time has been found to indicate higher bacterial numbers. However, the principle of operation of redox electrode and dye-reduction techniques in the method of

microbial estimation is based on the variation in the E_h of the food due to microbial activity and their related metabolic by-products, associated with spoilage.

According to Gil et al. (2008) reproducible result using redox potential technique, are obtainable once precautionary procedural measures are taken to avoid the influence of atmospheric oxygen, since oxygen constitute to one of the considerable redox couples that influence the measured redox potential a medium. In the application of electrode technique it is recommendation that, thorough cleaning of the electrode before and between individual measurements as well as calibrating them in specified medium (Thermo Fisher Scientific USA, MA).

2.6.2. Electronic nose techniques

The electronic nose technique is used to detect and quantify the concentration of volatile compounds (bio-amines) in fish, which occur at during spoilage. According to Olafsdóttir (2005) the principle of operation of the Electronic nose involves the transfer of the total headspace of the sample to a sensor array that detects the presence of volatile compounds in the headspace and a pattern of signals is provided that are dependent of the sensors' selectivity and sensitivity and the characteristics of the volatile headspace. The electronic nose technique is considered produce as objective information as can be obtained by sensory panellist regarding the freshness of fish during storage (Olafsdóttir, 2005; Olafsdóttir et al., 1997). The application of electronic nose technique apart from fish is reported to have demonstrated success in other products such as; pharmacy, environment and plastic and packaging for purposes of quality control. However, the use of the electronic nose requires calibration on the quantification of various volatile compounds in different samples in order to build a database of reference that can be produce reproducible information.

2.6.3. Electrical technique

The change in the electrical properties, such as conductance; capacitance or impedance is one of the pertinent indicators of food quality deterioration due to microbial growth. According to Adams and Moss (2000/2008) microbial growth in a substrate is known to lead into change in the chemical composition of the growth medium and may consequently lead to change in the

electrical properties (capacitance, impedance and conductance) of the medium. The measurement of these changes can be done using instruments operating under the same principle and these include; Torrymeter) (UK), RT meter (Iceland) and Fischtester VI (Germany) Huss et al. (1992), Bactometer, Malthus system Fung (1995); BacTrac, and Rapid Automated Bacterial Impedance Technique (RABIT). This method has been applied with success in the analysis of variety of foods such as milk, meat, and fish to test for total counts of aerobic and selected groups of organisms such as coliforms, Salmonella and yeast as well as measurement of niacin (Bourgeois and Mafart, 1995).

2.6.4. Texture measurement

According to Huss, (1995); Bremner et al. (2002) the change in texture, resulting from autolytic, bacterial and chemical changes in fish during storage has a direct relationship to change fish freshness. The measurement of textural changes is considered as one of the ways of determining food quality deterioration. In accordance with Huss (1995) fish texture can be determined by using Texturometer, a hand held device utilizing a cylindrical probe that is exerted on the food product with force, *F* that increases the preset value. The measurement of firmness using this technique has been found to correlate well with the sensory textural attributes like dehydration and firmness. This measurement is considered as a direct extension of the human sensory assessment "figure press test".

2.6.5. Immunological methods

Immunological methods include enzyme-linked rapid methods, referred to as "ready to use kits". With reference to Hartman et al. (1992); Patel and William (2002); Fung (2002) the principle of operation of immunological technique lies on the detection of the presence of gram-negative microorganisms and foodborne pathogens through a chemical reaction between the kit with the substrate (food product) leading to specified indication (colourations). Some of the examples of these kits include; Latex, agglutination kits, QuikAlert kit, SDI RapidChek, Path-CHEK swab, Transia card/plate and Assurance EHEC & Gold EIA (see Table 5).

Table 5: Aternative "rapid" methods for evaluation of food/ fish quality and safety

Fish quality indicator	**Test kit/ Instrument**	**Principle of operation**	**Reference**	**Some of the Examples of company Home page**
Change in electrical properties (conductance, impedance and capacitance)	Torry Freshness ;meter RT-Freshness meter Intellectron FischtesterV1	Measurement of Electrical properties	Huss, (1995); Huss et al. (1992) Petal, (1994) Oehlenschlager (2003	http://www.Distell.com
Muscle texture variation	Texturometer	Probe press test	Huss (1995)	http://www..lloyd-intruments.co.uk
Discoloration	Konica Minolta colorimeter	colour analysis	Huss (1995)	
Dissolved and Volatile organic compounds	Electronic nose and tongue	Detection of volatile compounds	Olafsdóttir, G. J. (2005)	http://www.konicaminolta.com
Microbial growth	ATP-Luminometer	ATP Bioluminescent (Light emission)	Bourgeois et al. (1995); Hartman et al. (1992); Patel and Kyiakides (1994)	
Variation in redox potential	Redox dyes/strips: Resazurin or methylene blue Redox potential electrodes	Redox potential In: Redox dye-reduction time, Millivolt	Atherton and Newlander (1977); Jay (1978); Adams and Moss (2008); Bourgeois and Mafart (1995) ; Huss (1995)	http://www.Alpha-MOS.com http://www.hygiena.net
Growth of foodborne pathogens: *Salmonella*, *Listeria* spp, *Campylobacter*, *Staphylococcus*, *E. coli*, *Vibrio Enterobacteria*, *Clostridium* spp, *Enterococcus* spp, *Bacillus* spp, *Aeromonas* spp	Bactometer, Malthus system, RABIT, Bac Trac Latex agglutination kit Assurance EHEC & Gold EIA, SDI RapidChek, Path-CHEK swab, &Transia plate Quik Alert Micro-PRO Test kit	Impedimetric/ Conductance measurement Antigen /Antibody reaction test Immunoassay reaction Enzyme-linked test Cell flow Cytometry	Bolton and Gibson (1994); Huss et al. (1992, 1995); Hartman et al. (1992) Bourgeois et al. (1995); Hartman et al (1992); Patel & Williams, (2002); Fung (1995/2002) Bourgeois and Mafart (1995)	http://www.thermo.com

2.7 Selection of methods

For the purpose of this work, having reviewed the different alternatives of rapid methods, the following methods were selected:

1. Redox potential measurement techniques:

a) Redox dye-reduction technique, resazurin indicator dye (Waldeck GmbH &Co, KG Chroma, Havixbecker StraBe, Munster, Germany).

b) Electrode technique; Glass Combination Redox /ORP Electrodes, Cat. Number: 9778BNWP and 9778SC or Epoxy Sure-Flow Combination Redox/ORP Electrodes Cat Number: 9678BNWP and 987800 (Thermo Fisher Scientific, MA, USA).

2. The pH measurement technique; using pH electrode (ROSS flat pH electrodes (Thermo Fisher Scientific USA, MA).
3. The adenosine triphosphate (ATP)-bioluminescence technique; Luminometer (SystemSure Plus ATP meter, Hygiena LLC UK)

The criterion of selection of these methods was based on the readily availability of the reagents and or instrument in the laboratory and in the market in addition to reports from recent studies on their application for evaluation of the freshness and quality of various foods in food industry.

3 Materials and Methods

The experimental part of the work was divided into four parts; studies on: a) pure cultures of spoilage bacteria, b) chilled cod (*Gadus morhua*) fillets, c) Nile perch *(Lates niloticus)* and d) iced eviscerated cod (*Gadus morhua*). For the purpose of this work, the following analyses were done: Sensory analysis, estimation of bacterial numbers, determination of Resazurin Dye-Reduction Time (DRT), measurement of redox potential (E_h); Adenosine triphosphate (ATP) and pH.

3.1 Preparation of samples

3.1.1. Bacterial cultures

Black and white colony, originally isolated from spoiled cod fillets (stored for 14 d at 0°C) on Iron Agar (Lyngby) Gram *et al*. (1987) at 25°C, were maintained on Iron Agar plates throughout the trial. Prior to an experiment, a subculture from the colonies were streaked onto new Iron Agar plates, incubated at 25^{o}C for 48 hours and checked for purity. One black and one white colony were transferred on a needle to 250 mL Erlenmeyer flasks containing 50 mL of sterile Tryptic Soy Broth (TSB) (Becton and Dickinson, MD, USA) and then incubated for 48 hours at 25^{o}C. To obtain evenly distributed bacterial solutions a series of dilutions (1/10) TSB broth were inoculated in a set of 76, 250 mL Erlenmeyer flasks containing 50 mL, fresh TSB medium and were incubated for another 48 hours at 25^{o}C prior to analyses. The Iron Agar recipe is shown in Appendix 5.

3.1.2. Fish sample preparation: Cod *(Gadus morhua)* fillets

Cod fillets from whole fish at day 3 of storage obtained from a local fish factory, (Brim hf, Akureyri) were individually wrapped tightly in polyethene bags and stored at 0-1^{o}C. Four fillets were analysed at day 3, 5, 9, 11, 14, 16, 18, and 20 days of storage.

3.1.3. Fresh Nile perch *(Lates niloticus)*

Two batches of fresh, whole Nile perch, from Lake Victoria were purchased at the local landing site and storage was done at Greenfields Uganda limited fish establishment in Entebbe- Uganda. One batch was stored on ice for 25 days in insulated fish tubs; replacement of melted ice was done and analyses were done during 25 days of storage as necessary.
Then, batch two, were filleted, and were air blast chilled at -1^{o}C, packed in Styrofoam boxes and stored for 25 days at 0^{o}C. The data of the temperature of the chill room where the fillets were stored was not recorded however; the temperature of the fillets was measured prior to taking samples for analyses and found to be below 1^{o}C at all sampling occasions.
Three fish (whole Nile perch and Nile perch fillets each) were analysed at each sampling day. E_h, ATP, pH, sensory and microbiological analyses were done.

3.1.4. Eviscerated cod *(Gadus morhua)* on ice

A batch of eviscerated cod, stored on ice, (1, 7 and 14 days from catch), were obtained from a local fish factory (Brim hf, Akureyri). The analysis of fish of the respective storage days was done on the same day.

3.2 Analyses

3.2.1. Sensory analysis

Sensory assessment of cod was carried out in the laboratory by two trained students. The fish were blind coded accordingly prior to assessment in an appropriate environment. Analyses were done using the Quality Index method. Demerit scores were recorded in the quality index scheme for fresh cod shown in appendix 6, Table 12.

For Nile perch, sensory assessment was carried out on whole fish, using a modified organoleptic quality index scheme shown in appendix 4, Table 11. The sensory characteristics of Nile perch were assessed by three (3) trained sensory panellists at Uganda Fish Laboratory. The main quality parameters included; the general appearance of the skin, belly, texture, eyes, slime and gills against characteristic attributes such as colour, firmness, clarity and smell. The

demerit scores, 0-5 against the samples presented on each sampling day were averaged to give the quality index score.

3.2.2. Microbiological analyses

In the case of pure culture, one mL of bacterial cultures from the Erlenmeyer flacks were serially diluted (1/10) in 0.4% sterilised Butterfield Phosphate Buffer solution.
In the case of the fish samples, twenty five grams of flesh were aseptically removed from the fillets and put in 225 mL 0.4% Butterfield Phosphate Buffer in a Stomacher® bag with filter (Seward Laboratory, London, UK) and masticated for 4 minutes in a Stomacher Lab Blender 400 (Seward Laboratory, London, UK). Further, serial dilutions (1/10) of sample (fish homogenate) were done in 0.4% Butterfield Phosphate Buffer.
From appropriate dilution a sample was taken and streaked on Iron Agar plates (Lyngby) Gram et al., (1987). Plating was done in duplicate using Eddy Jet Spiral plating machine (IUL Instrument, Barcelona Spain).
The plates were incubated at 23-25^{o}C for two days. Counting of colonies was performed by using Flash and Go automatic colony counter (IUL instrument). The results were expressed as log CFUg^{-1}. Whereas samples for Nile perch fillets were plated in duplicates using pour plate method and counting was done using the colony counter.

In the analysis of DRT; ATP; pH and E_h of bacterial cultures/ solutions and fish homogenate, (1/1) and (1/10) dilutions respectively were used.
For ATP; pH and E_h, measurement was done by inserting the ultrasnap swab pad; pH electrode or E_h electrode into the cultures and measurements were done as required.

3.2.3. Measurement of redox potential (E_h)

The measurement of E_h was done using either Glass Combination Redox /ORP Electrodes, Cat. Number: 9778BNWP and 9778SC or Epoxy Sure-Flow Combination Redox/ORP Electrodes Cat Number: 9678BNWP and 987800 (Thermo Fisher Scientific, MA, USA).
To carry out E_h assay in the fish flesh, three cuts; 1-2 cm deep and 2 cm apart were made in the fish along the dorsal fin using a sterile surgical blade. Then the ORP electrode was

directly inserted into the fish fresh through each cut accordingly (see Figure 3). The E_h results were expressed in millivolt (mV).

Prior to measurement of redox potential (E_h) the operating performance of the electrode was checked using Oxidation Reduction Potential (ORP) standard solution (No. 900001, Thermo Fisher Scientific) with a known E_h (+420±3 mV).The E_h of the samples was recorded when the meter readings stabilised (usually within 5 minutes) and in between each measurement, the electrode was cleaned accordingly as recommended in the electrode manufacture user guide (Thermo Scientific).

3.2.4. Measurement of pH

The pH value in the fish muscle was measured after the temperature had stabilised at approximately 10-12°C. A sterile knife was used to make a 2-3 cut through the skin of the fish (or the flesh of the fillets) and a pH electrode (ROSS flat pH electrode, Thermo Fisher Scientific) was then inserted approximately 2 cm in to the flesh.

3.2.5. Resazurin dye assay

To perform resazurin dye assay, 9 mL of sample bacterial culture (1/1); fish homogenate (1/10) were mixed accordingly, with 1 mL of 0.01% solution of resazurin indicator (Waldeck GmbH &Co, KG Chroma, Havixbecker StraBe, Munster, Germany) in sterilised tubes in duplicates and were incubated in light free water bath at 35^{o}C. Then the dye-reduction time, (in hours) was recorded accordingly (Atherton and Newlander, 1977).

3.2.6. ATP assay

To measure ATP, a sterilized template (5 cm x 10 cm) was aseptically put on the surface of the sample and swabbed using ultrasnap swab pad (SystemSure Plus, Hygiena LLC UK) Measurement was carried out in the Luminometer (SystemSure Plus ATP meter, Hygiena LLC UK) and the meter readings recorded were expressed in relative light units (RLU/cm^2).

3.3 Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significant variation between the studied indices with the predictor indices with MYSTAT 12.0 (SYSTAT, IL USA). The differences between means of the indices examined were determined at significance level of $P<0.05$. Linear regression was done using EXCEL 2007 (Microsoft, USA). Correlation matrix between the studied indices and between and the predictor indices were also prepared.

4 Result

4.1 Bacterial cultures

Figure 2 shows the correlation between SPC and Redox potential (E_h), ATP, pH and Resazurin Dye-Reduction Time (DRT) in bacterial solutions. The DRT was long in samples having low number of bacteria. The DRT decreased rapidly as the number of bacteria increased as was <1 h in bacterial solutions with SPC ≥8 log CFU/mL In bacterial solutions with SPC of 7 log CFU/mL the DRT was between 5 and 6 h.

The E_h in bacterial cultures with SPC above 7 log CFU/ml were inconsistent, within the range of (- 400 to +250 mV). Generally the E_h in the bacterial solutions with SPC 6 to 8log CFU/mL was above -100 mV.

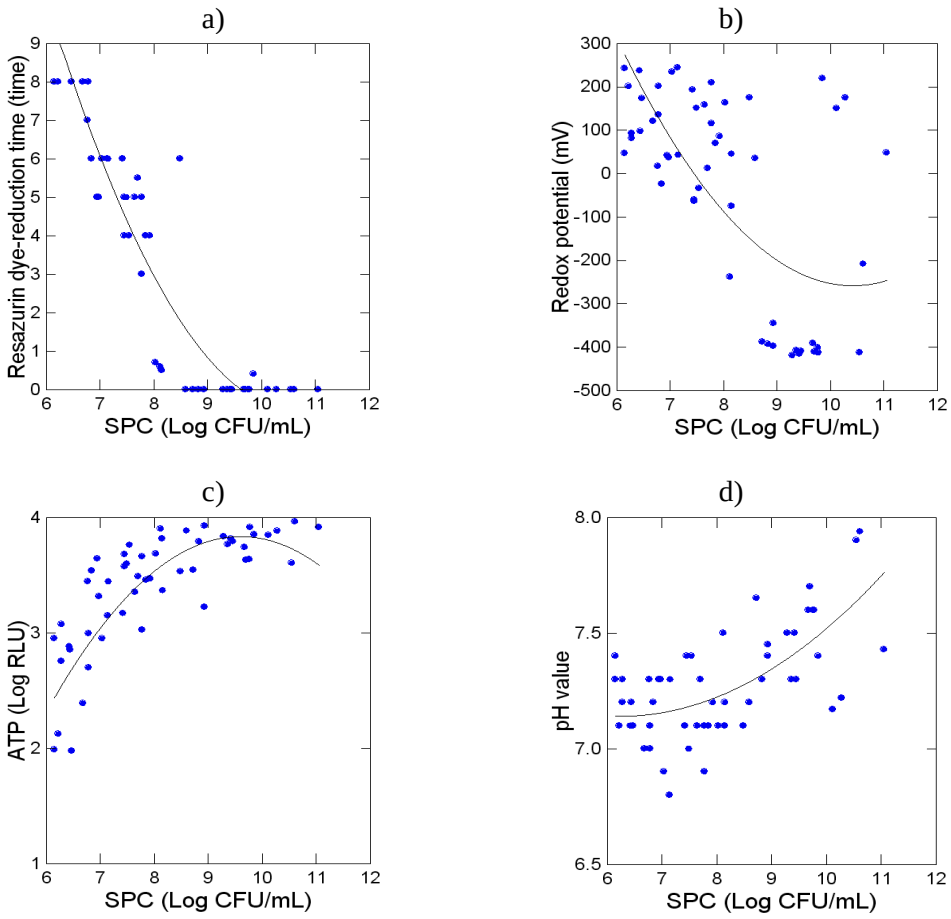

Figure 3*: : Correlation between DRT (a); E_h (b); ATP (RLU) (c); pH (d) and bacterial numbers (SPC) in Tryptic Soy Broth at 25°C.*

The ATP in bacterial solutions with SPC in the range of 6 and 11log CFU/mL was 2 and 4 log RLU respectively. The ATP values in bacterial solutions with SPC ≥8 log CFU/mL were between 3 and 4 log RLU and the RLU values levelled out at high (>8.5 log CFU/mL).
The pH in the bacterial solutions with SPC in the range of 6 and 11 log CFU/ml were between 6.7 and 7.9 however, the values varied greatly.
Linear regression analysis between SPC and DRT; ATP; E_h and pH in bacterial cultures in TSB showed the correlation coefficient of (r) = 0.76; 0.6; < 0.5; and <0.5 respectively (Appendix 7, Table 13).

Table 6 shows the mean values of Resazurin Dye-Reduction Time or detection time (DRT), redox potential (E_h), pH and ATP in bacterial cultures in TSB with different bacterial numbers (SPC). The DRT in bacterial solutions with high SPC (≥ 8log CFU/g) was as short as < 1h whereas the DRT in solutions with SPC 6 log CFU/mL was longer, up to 8 h. However, the DRT was 6 and 5 h in bacterial solutions with SPC of 7log CFU/mL).
On the other hand, the ATP value in bacterial solutions with SPC 6 log CFU/mL was 2log RLU and in bacterial solutions with higher SPC, (≥ 7log CFU/g) the ATP value was between 3 – 4 log RLU.
As shown in Table 6, the E_h values in bacterial solutions with SPC ≥ 8 log CFU/mL was between -400 and + 250 mV. In solutions with SPC 6 log CFU/mL the E_h was between + 50 and +250 mV.
The pH value was higher, (7.2 and 7.5) in bacterial solutions with SPC 6 log CFU/mL than the pH in bacterial solutions with SPC 7 log CFU/mL which was 6.7 and 7.3. The pH in solutions with SPC ≥ 8log CFU/mL was 6.8 and 7.9.

Table 6: Mean values of DRT; E_h; ATP and pH of bacterial cultures inTSB and different bacterial numbers (SPC log CFU/mL).

	SPC (Log CFU/mL)		
	6	7	≥8
DRT (h)	8	5 - 6	< 1
Redox potential(mV)	+50 -+250	-50 - +250	-400 - +250
pH value	7.2-7.5	6.7-7.3	6.8-7.9
ATP (Log RLU)	2	3 - 4	3-4

4.2 Packed cod *(Gadus morhua)* fillets stored at 0^{o}C

Figure 3 shows the changes in SPC, Resazurin Dye-Reduction Time, E_h, ATP and pH of cod fillets during storage at 0°C for 20 days.

The SPC in cod fillets at day 3 of storage was approximately 3log CFU/g; a consistent increase in SPC was observed with increase in storage days and at day 20 of storage SPC was 10log CFU/g.

Cod fillets were judged spoilt at day 14, at that time, the SPC; E_h; ATP and pH were: 8log CFU/g; 4 h; 0 mV; >3log RLU and 7.2 respectively.

The DRT was approximately 8 h in cod fillets at day 3 of storage. A reduction in DRT, to 4 h was observed in cod at day 14 of storage. However, the DRT in cod at day 16-20 of storage was shorter, (between < 1 and 3 h).

The E_h in cod fillets at day 3 of storage was +70 mV and the E_h remained constant at 70 to 80 mV during the first 11 days of storage. At day 14 of storage, the E_h dropped to values close to 0 mV. After day 14 of storage, a further drop in E_h to negative values up to -120 mV was observed.

The ATP value was low during the first week of the storage less than 2log RLU. At day 14 of storage, the RLU value was higher than 3log RLU however; no significant increase in ATP was shown in cod during the later storage time, (16-20) day; the ATP value at that time was between 3 and 4log RLU.

The pH of cod at day 3 of storage was 6.7 and at day 14 of storage, the pH was 7.3 however; the pH of cod showed an increase with increase in storage days.

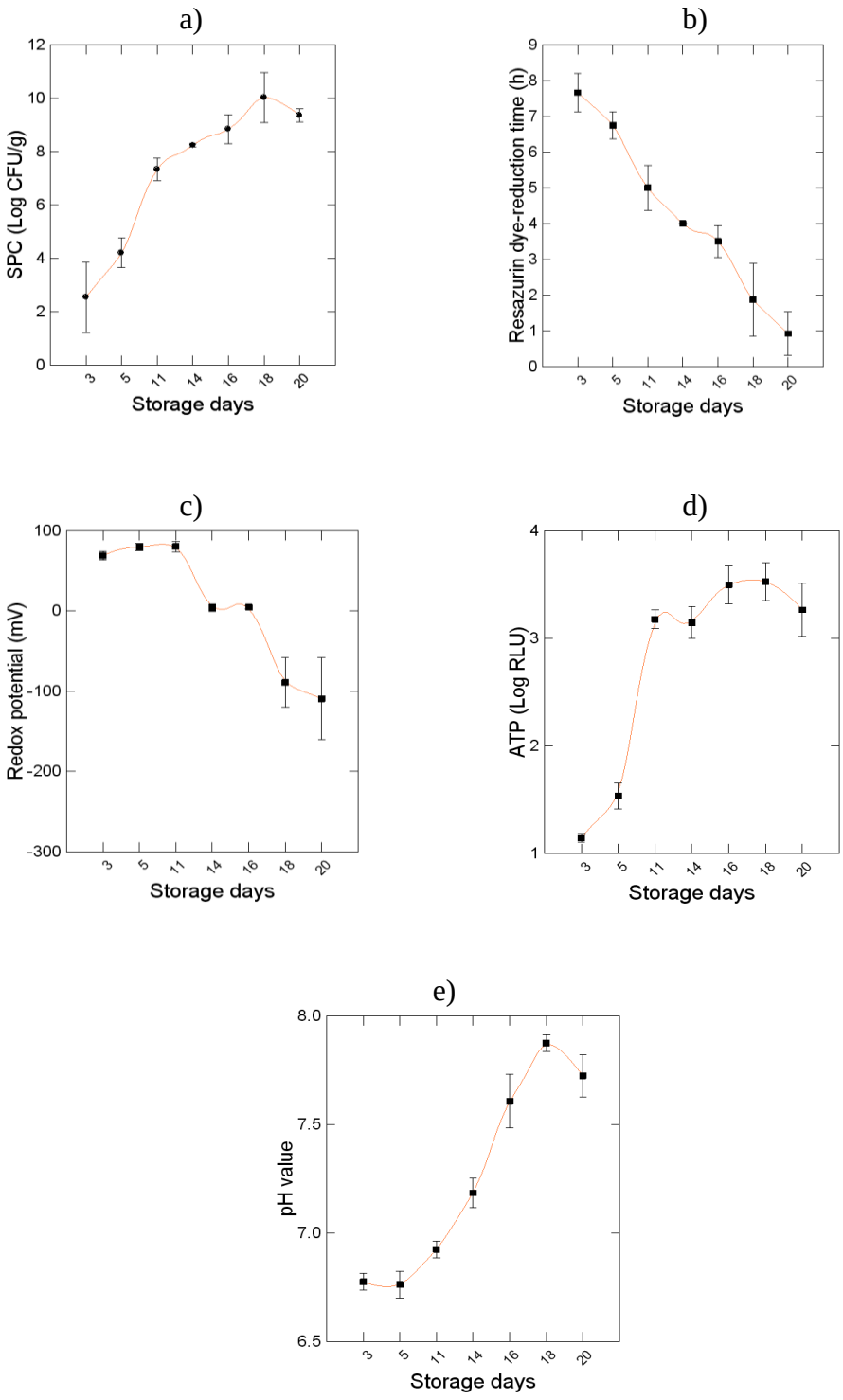

Figure 4: The changes in SPC (a); Resazurin Dye-Reduction Time (b); Redox potential (E_h) (c); ATP (d) and pH (e) of packed cod (Gadus morhua) fillets stored at 0^{o}C for 20 day, (N= 4, bars=s.d).

Figure 4 shows the correlation between SPC and DRT; E_h; ATP and pH in cod fillet stored at 0 ^oC.

Cod fillets were judged spoilt when SPC were 8 log CFU/g and DRT; E_h; ATP and pH at that time were: 4 h; 0 mV; >3log RLU and 7.2 respectively.

The DRT in cod decreased consistently with increase in SPC. In cod with SPC 9 and 10 log CFU/g, the DRT was shorter, (<1 and 3 h) whereas, the DRT in cod with SPC 7 log CFU/g was 5 and 6 h. However, in cod with low SPC, below 4, log CFU/g the DRT was longer, (i. e. 7 and 8 h)

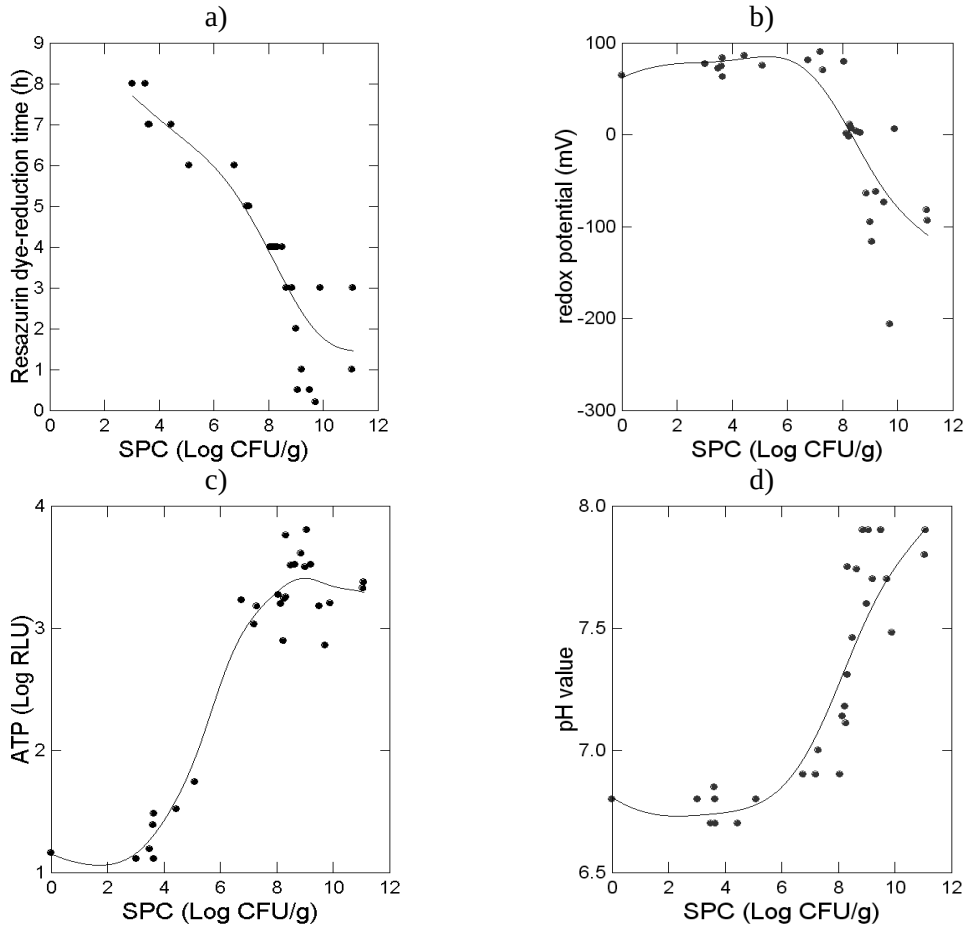

Figure 5: Correlation between spread plate count (SPC) with DRT (a); E_h (b); ATP (c); pH (d) in cod(Gadus morhua) fillets stored at 0^oC for 20 days.

The E_h value was high (70-100mV) in samples with bacterial numbers up to 7 log CFU/g. The E_h showed a sharp fall to 0 mV in cod with SPC 8 log CFU/g and further drop in E_h to negative value, -120 mV was observed in cod with SPC between 9 and 10log CFU/g.

The ATP value was below 2log RLU in cod with SPC below 6log CFU/g; a sharp rise in ATP to 3 and 4log RLU was observed in cod with SPC ≥ 7log CFU/g. The pH showed a consistent increase with increase in SPC; the pH was 6.8 in cod with SPC 4log CFU/g and in cod with SPC 9-10log CFU/g, the pH was 7.9.

Linear regression analysis between SPC and DRT; ATP; pH and E_h in cod stored at 0^oC for 20 days showed a correlation coefficient, r was = 0.8; 0.8; 0.7 and 0.5 respectively (Appendix 7, Table 13).

Table 7 shows the mean values of Resazurin Dye-Reduction Time (DRT), Redox potential (E_h), pH, and ATP in cod and numbers of bacteria (SPC log CFU/g) at different storage times at 0 - 1^oC.
Cod fillets were judged spoilt at day 14 of storage when SPC was 8log CFU/g, at that time, the DRT; E_h; ATP; and pH were: 4 h; 0 mV; >3log RLU and 7.2 respectively.

The DRT in cod at day 3 and 5 of storage was longer; (7 and 8 h) at that time, the SPC was between 3 and 4log CFU/g. At day 16 and 20, when cod was already spoilt, the DRT was as short as less than (<) 1 and 3 h whereas the SPC were 9 and 10 log CFU/g.

The E_h was 70 and 80 mV at day 3 to day 11 of fish storage; at that time, the SPC were below 8log CFU/g. At day 16 and 20, after spoilage, the E_h of cod was 0 and -120 mV respectively, whereas the SPC were > 8log CFU/g.

The ATP value was lower, (< 2log RLU) at day 3 and 5 of storage and at day 16 and 20, when fish was already spoilt, the ATP value was greater than (>) 3log RLU whereas SPC at that time were 9 and 10log CFU/g.

The pH value cod was 6.7 while SPC were 3 and 4log CFU/g at day 3 and 5 of storage. However, at day 16 and 20, when fish was already spoilt, the pH was higher, 7.7 and 7.9; at that time, the SPC were 9 and 10log CFU/g (see Table 7).

Table 7: Mean values of DRT; E_h; pH; and ATP in cod (Gadus morhua) fillets and numbers of bacterial (SPC log CFU/g) at different storage times at 0 ^oC.

	STORAGE DAYS			
INDEX	3 - 5	11	14*	16 - 20
SPC (log CFU/g)	3 - 4	<7	8*	9-10
DRT (h)	7 - 8	5-8	4*	0 - 3
E_h(mV)	70-80	70 - 80	0*	0 to -120
pH value	6.7	6.8	7.2*	7.7-7.9
ATP (Log RLU)	< 2	3	3*	>3

(*) indicate the time (storage days); at which fish was considered spoilt and the corresponding SPC; E_h; pH and ATP values.

4.3 Whole Nile perch *(Lates niloticus)* stored on ice

Figure 5 shows the changes in the changes in Quality index, E_h, ATP and pH of whole Nile perch stored on ice for 25 days.

The Quality Index increased with increase in storage time. The shelf life was found to be 19 days; the quality index at that time was 4 whereas the E_h; ATP and pH values at the time were: 0 mV; >3 log RLU/cm^2 and 6.5 respectively.

The E_h of whole Nile perch at day 1 of storage was approximately +300 mV; which remained rather constant at +300 mV, during the first two weeks (14/15 days) of storage. A sharp drop

to 0 mV was observed at the time fish was judged spoilt at day 19 of storage and further drop in E_h to an average value of -30 mV at day 25 of fish storage was observed.

The ATP value of Nile perch during the first 14 days of storage was below 3log RLU/cm^2. At the time fish was spoilt, (day 19), the ATP value was on average close to 4 log RLU/ cm^2 but with a wide standard deviation between individual fish.

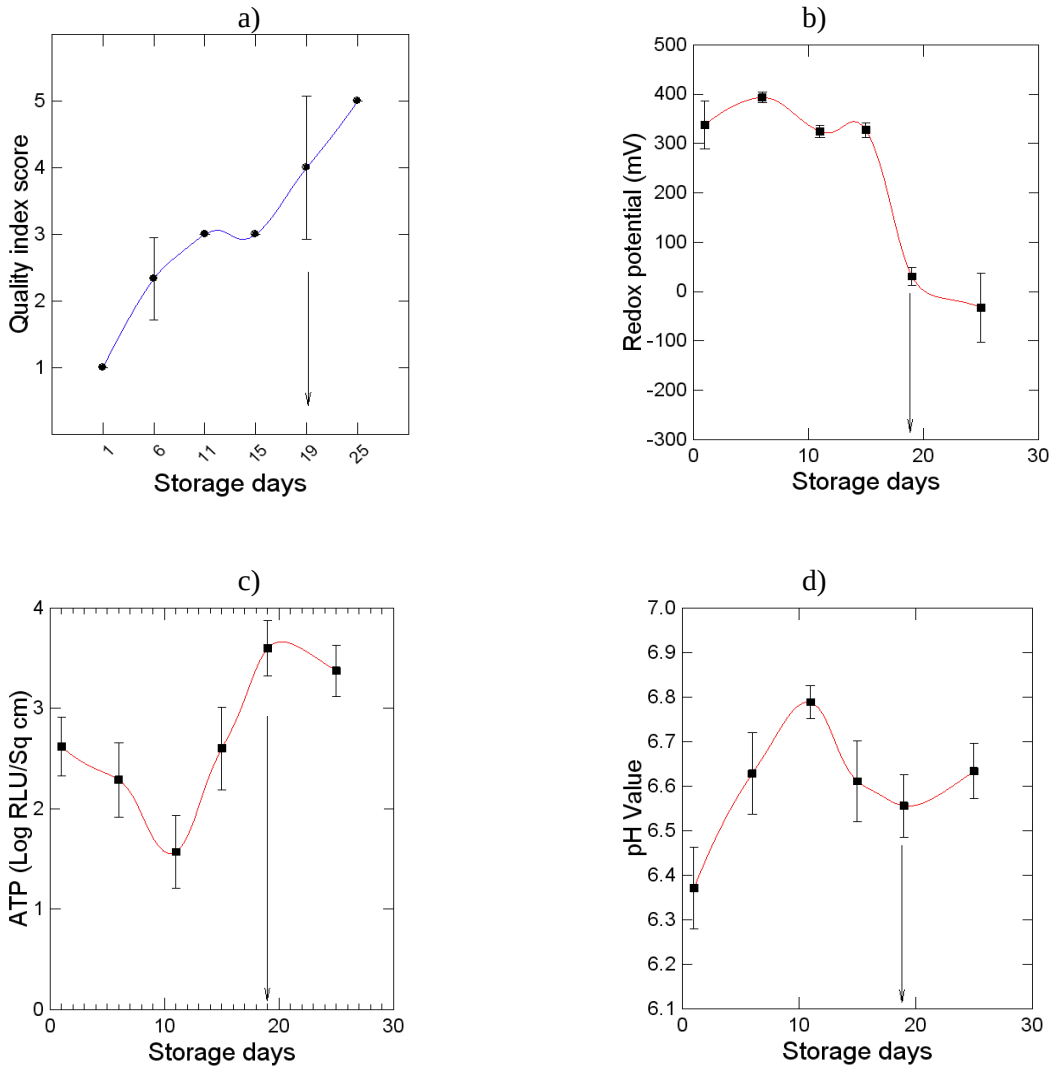

Figure 6: Changes in Quality index (a); Redox potential (E_h) (b); ATP (c); pH(d) of Nile perch (Lates niloticus) on ice during storage for 25 days, (N=3, bars=sd). The arrow indicates the time fish was judged as spoiled.

The initial pH of Nile perch was approximately 6.4. It increased during storage to 6.8 (at day 11) and fell again to 6.6 toward the end of the storage time.

Figure 6 shows the correlation between Quality index and the E_h; ATP and pH of Nile perch during storage on ice for 25 days.

The E_h of Nile perch remained constant at +300 mV; at that time, the Quality index was 1, 2 and 3. However, at Quality index 4, the time when fish was spoilt the E_h of the fish was 0 mV. The E_h of the fish dropped to -30 mV when Quality index was 5, when fish was already spoilt.

The ATP and pH values showed no systematic relationship with Quality index however, at Quality index 3, ATP value was < 3 log RLU/cm^2. The ATP value was > 3 (close to 4log) RLU/cm^2 when the Quality index was 4, at that time, the fish was judged spoilt.

Whereas the pH value was 6.4 when the Quality index was 1 and at Quality index 2, the pH rose to 6.8. At the time Nile perch was judged spoilt, (at Quality index 4), the pH was about 6.5, and there after, at Quality index 5, it rose to 6.7.

Linear regression analysis between Quality index and storage time (25 days) of Nile perch on ice showed a correlation coefficient, r was = 0.9. Whereas the linear regression analysis between storage days; quality index and E_h of Nile perch on ice showed a correlation coefficient, r was = 0.7 and 0.6 respectively.
The linear correlation coefficient, r was = < 0.3 was shown between ATP; pH and Quality index (Appendix 7, Table 13).

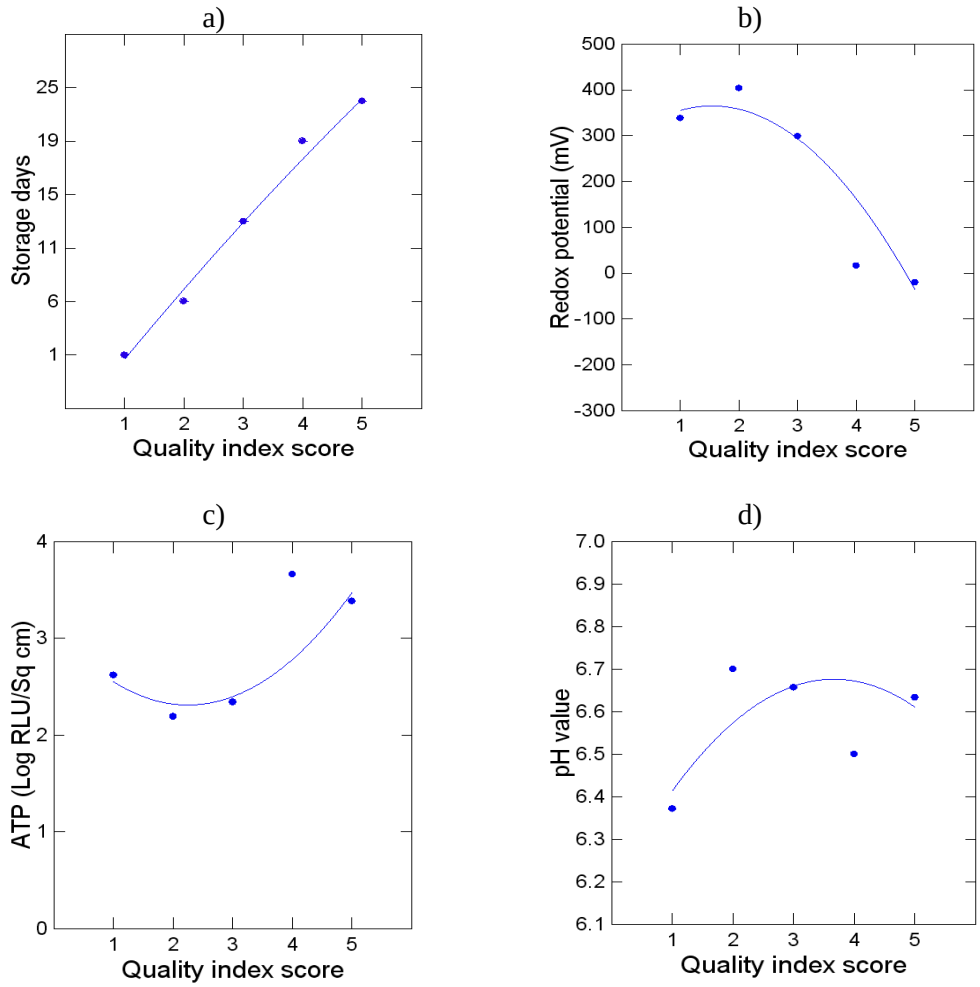

Figure 7: Correlation between Quality index with storage days (a); Redox potential (b); ATP (c) and pH (d) in Nile perch (Lates niloticus) on ice during storage time of 25 days.

Table 8 shows the mean values of E_h; ATP and pH and Quality index in whole Nile perch at different storage times on ice.

The fish was spoilt at day 19 of storage; the Quality index at that time was 4, whereas the E_h; ATP and pH values were: 0 mV; >3 log RLU and 6.5 respectively.

The E_h value was high, (+300 mV) during the first 2 weeks of storage; the Quality indexes at that time were 1; 2 and 3. However, at day 25 of storage, the time when fish was already spoilt, the E_h was approximately -30 mV whereas the Quality index was 5.

The ATP value was below 3log RLU/cm^2 at Quality index 1; 2 and 3 during the first 2 weeks of storage. At day 19 and 25 of storage, when fish was spoilt, the ATP value was higher, >3log RLUcm^2; the Quality index at that, time was 4 and 5.

Whereas the pH of Nile perch at day 1 of storage was 6.4; the Quality index at that time was 1. At Quality index 2, the pH was 6.6, then at day 19 of storage, the time when fish was spoilt, the pH was lower (at 6.5); the Quality index at that time was 4.

Table 8: Mean values of DRT; E_h; pH; and ATP and Qualty index in whole Nile perch (Lates niloticus) at different storage times on ice.

	STORAGE DAYS				
Index	1	10	15	19*	25
Sensory Quality index	1	2	3	4*	5
Redox potential(mV)	+300	+300	+300*	0*	-30
pH value	6.4	6.8	6.6*	6.5*	6.7
ATP (Log RLU/cm^2)	2.7	< 2.2	2.3	>3*	>3

(*) indicate the time (storage days); at which fish was judged spoilt (unacceptable for consumption) and the corresponding E_h, pH and ATP values.

4.3.1 Nile perch *(Lates niloticus)* fillets

Figure 7 shows the changes in SPC; E_h; ATP and pH of Nile perch fillets during storage at 0-1 ^oC for 25 days. The fish was judged spoilt at day 14 of storage; the SPC at that time was 6log CFU/g.

The E_h of Nile perch fillets (at day 0-1) of storage was +100mV and at day 5 the E_h was +300 mV storage and remained constant up to day 14 (time of spoilage); at day 19 of storage, a drop in the E_h value to 0 mV was observed.

The ATP was low (< 3 log RLU/cm^2) and inconsistent throughout the storage period and variation between replicate samples were high (s.d. +/- 2.0 -2.3 log RLU/cm^2).

The pH in fresh fillets was 6.7 which remained constant and at day 25 a rise in the pH to 7.3 was observed.

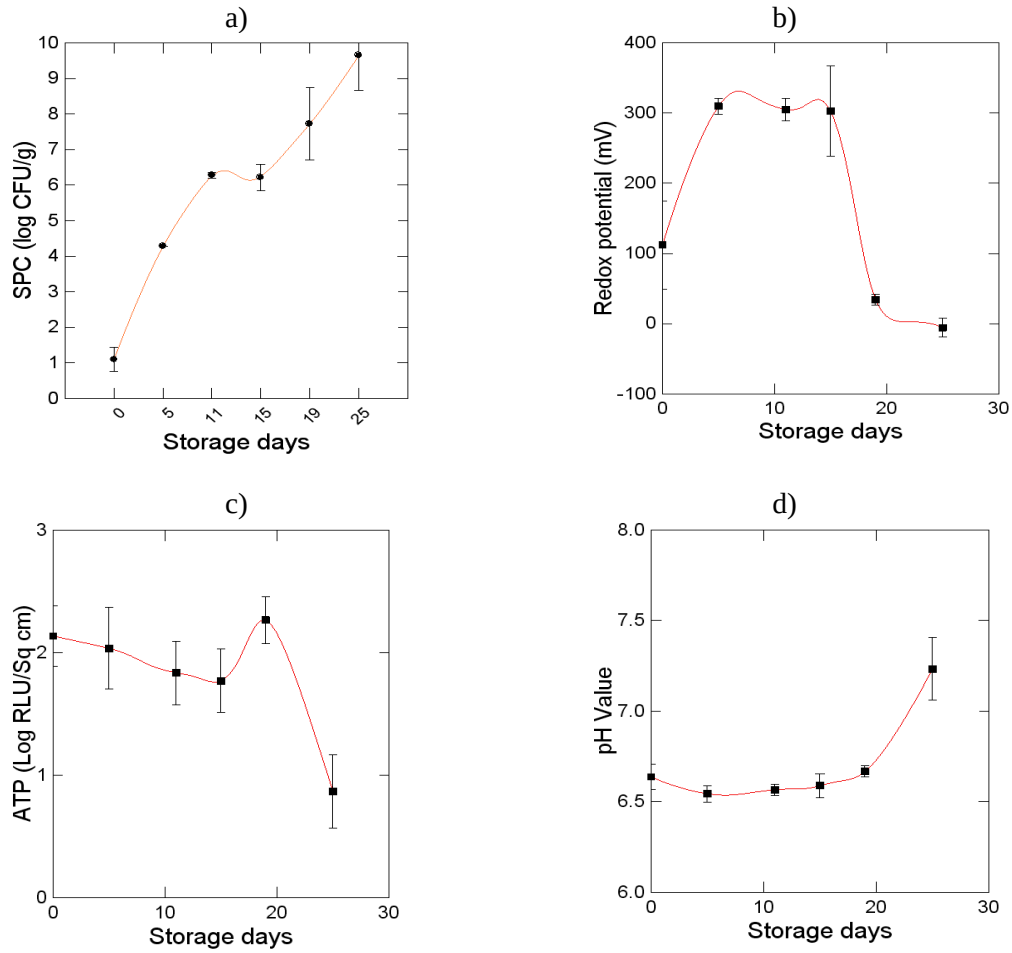

Figure 8: Changes in SPC (a); Redox potential (E_h) (b); ATP (c) and pH (d) of Nile perch (Lates niloticus) fillets stored at 0 -1 ^oC for 25 days, (N=3, bars=sd).

The correlation matrix is shown in Appendix 7, Table 14. Linear regression analysis between E_h; ATP and pH with SPC in Nile perch fillets stored at 0-1 oC for 25 days showed a similar correlation coefficients, r was = < 0.4

Table 9 shows the mean values of SPC; E_h; ATP and pH of Nile perch fillets at different storage times at 0^oC.

The E_h of Nile perch was low (+100 mV) at day 0-1 of storage; the SPC at that time was 1 log CFU/g.

At day 14of storage, when the fish was judged spoilt, the E_h was higher (+300 mV); the SPC at that time was 6 log CFU/g whereas the ATP; pH values were < 2 log RLU/cm^2 and 6.6 respectively.

ATP value was generally low (≤ 2 log RLU/cm^2) at day 1; 5 and 14 of storage; whereas the SPC were low < 7log CFU/g.

The pH was lower, (6.5 and 6.6) at day 5 and at14 of storage, SPC at this time were < 7 log CFU/g. At day 25 of fish storage, the pH was higher, (7.5); the SPC at this time were > 8 log CFU/g, whereas the ATP value was much more lower (< 1 log RLU/cm^2) and the E_h was at -5 mV.

Table 9: Mean values of Redox potential; pH; and ATP and SPC in Nile perch (Lates niloticus) fillets at different storage times at 0 -1^oC for 25 days.

	STORAGE DAYS				
INDEX	0-1	5	14*	19	25
SPC (CFU/g)	1	4	6*	7 - 8	9- 10
Redox potential(mV)	+100	+300	+300*	0	-5
pH value	6.7	6.5	6.6*	6.8	7.5
ATP (Log RLU/cm2)	2	< 2	< 2*	2.3	<1

(*) indicate the time (storage days); at which fish was judged spoilt (unacceptable for consumption) and the corresponding SPC; E_h; pH and ATP values.

4.4 Whole eviscerated cod *(Gadus morhua)* stored on ice

Figure 8 shows the changes in DRT; E_h; ATP and pH, SPC, and Quality index of eviscerated cod stored on ice for 14 days.

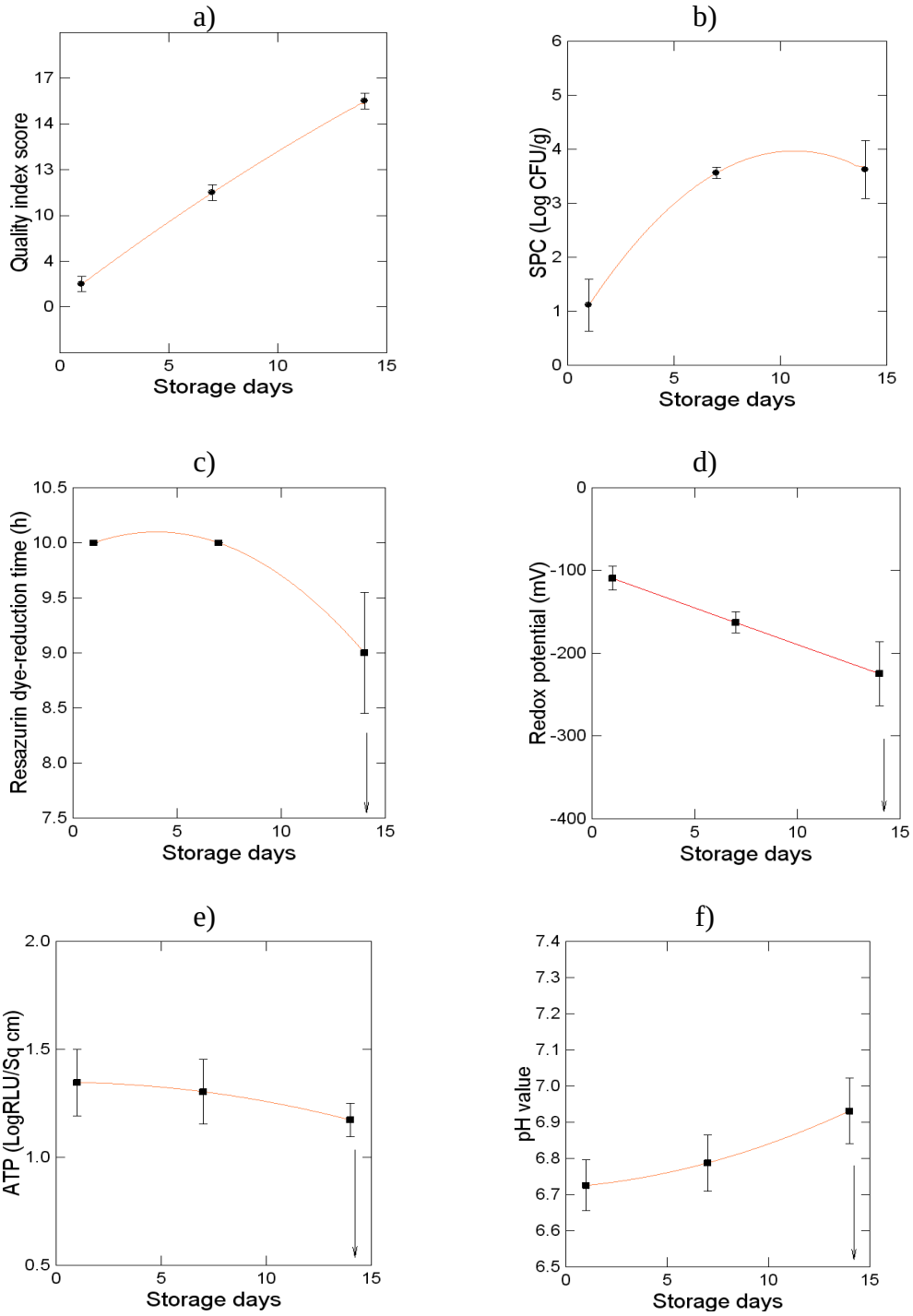

Figure 9: Changes in quality index (a); SPC (b); DRT (c); Redox potential (E_h) (d); ATP (e); pH (f) of eviscerated cod (Gadus morhua) on ice during storage for 14 days, (N=3, bars=s.d). The arrow indicates the time fish was judged spoiled.

The Quality index in cod increased with increase in storage time. At day 1 of storage, the Quality index was below 1 and at day 14, the Quality index was 15. The SPC in cod similarly increased with increase in storage time (i. e. 1 to 4 log CFU/g at day 1 to 14 of cod storage respectively).
Cod was judged spoilt at day 14 of storage at Quality index 15, when DRT, Eh, ATP; SPC and pH were: 8 h; -225 mV; 1.2log RLU; approximately 4log CFU/g and 6.9 respectively.

The DRT in cod (day1 of storage) was 10 h and remained unchanged for the first week. The DRT in cod at day 14 of storage reduced to about 9 h.
The E_h was in the range of (-100 mV and -225 mV) at day 1 and 14 of storage respectively; no positive E_h values were observed. At day 1 of storage, the E_h was -100 mV; the E_h showed a progressive decrease to lower during throughout the storage time.

The pH of cod, at 1 day of storage was approximately 6.7; the pH values showed an increase with storage days; at day 7 and 14 of storage, the pH was 6.8 and 6.9 respectively.
The ATP values of cod at, day 1 and 7 of storage were rather constant at 1.4log RLU /cm^2 of the skin surface. In the later days of storage, (day 14) the ATP reduced to 1.2log RLU/cm^2 of the skin surface.

Figure 9 shows the correlation between Quality indexes (QI) and DRT; E_h; ATP and pH of eviscerated cod stored on ice for 14 days.
The fish was spoilt at Quality index 14; at that time, DRT; Eh; ATP and pH were: 9 h; -225 mV; 1.2log RLU; 6.9 respectively.
The DRT was longer, and remained constant at 10 h at quality index 2 and 7; followed a reduction in DRT to 8 h; the Quality index at that time was 15.
The E_h value reduced with increasing Quality index value and reached E_h value -225 mV at Quality index 15.
The ATP value in whole cod showed inconsistent relationship with Quality index. Whereas the pH showed an increase with increase in Quality index; the pH was approximately 6.7; the Quality index at that time was about 4; at quality index 15, the pH was 6.9.

Linear regression analysis between Quality index and E_h showed a correlation coefficient, r was = 0.5; and similarly lower r was = < 0.5 between Quality index and DRT; ATP and pH respectively (Appendix 7, Table 13).

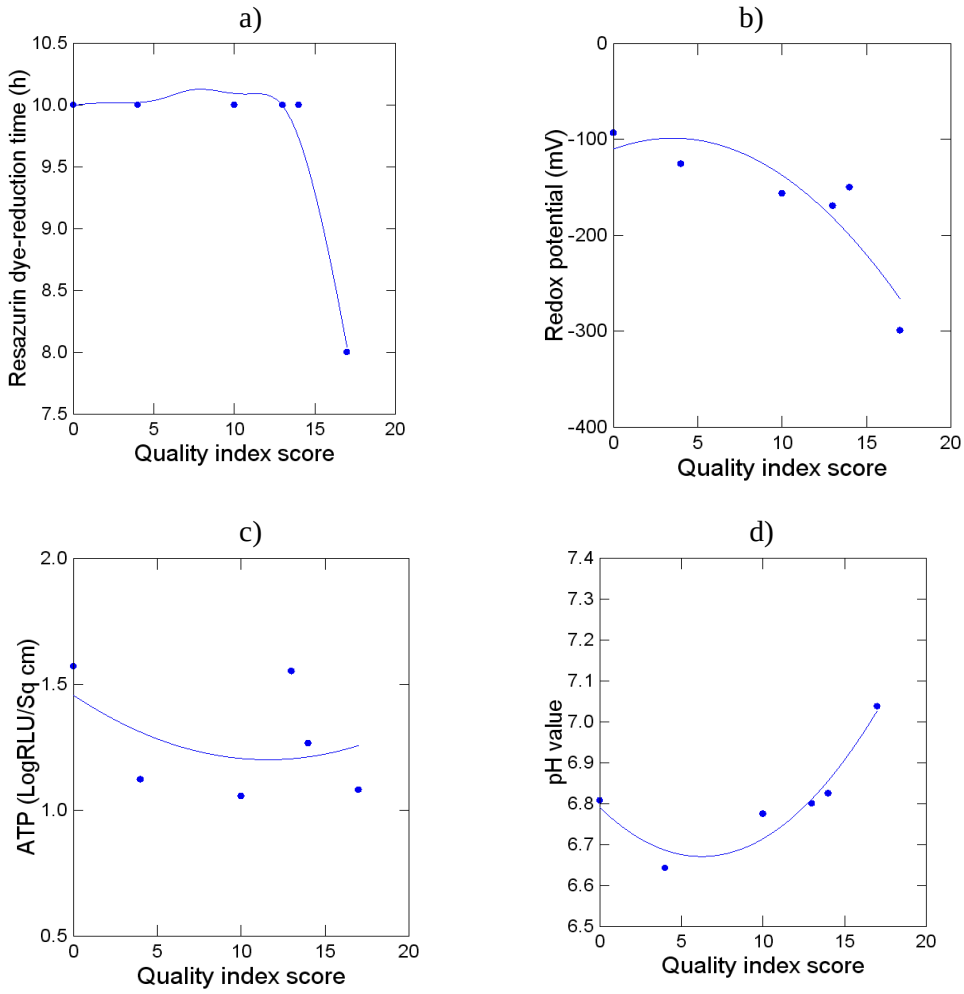

Figure 10: Correlation between quality index with DRT (a); Redox potential (b); ATP (c); pH (d) in cod(Gadus morhua) on ice during storage for 14 days.

Figure 10 shows the correlation between SPC with DRT, E_h; ATP and pH in cod stored on ice for 14 days.

Similarly, the pH and ATP values showed no consistent trend with SPC; where, DRT and E_h showed negative trend with increase with SPC. The DRT was long and constant at 10 h in cod with SPC less than (<) 4log CFU/g. This was followed by a reduction in DRT to 9 h, as the SPC in cod increased to > 4log CFU/g).

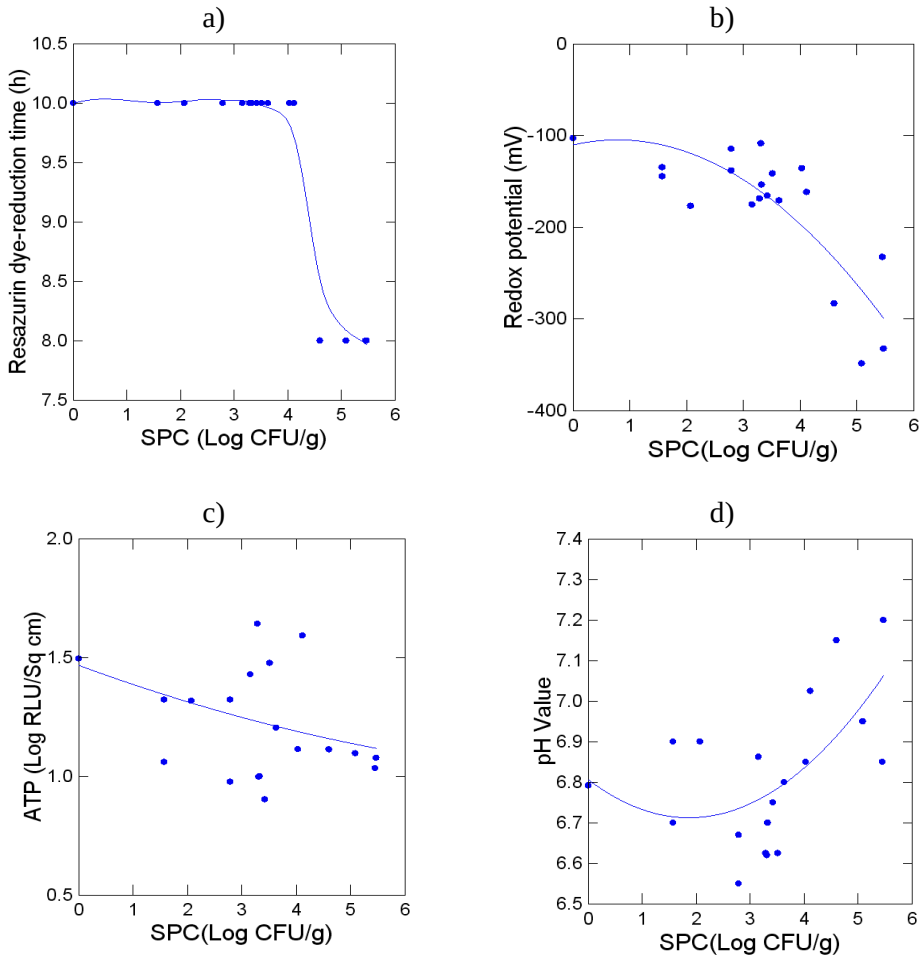

Figure 11: Correlation between SPC with DRT (a); Redox potential (b); ATP (c); pH (d) in cod (Gadus morhua) on ice during 14 days of storage .

Linear regression analysis between SPC and E_h; DRT; ATP and pH cod stored on ice for 14 days showed a correlation coefficient, r was = 0.5; 0.4; 0.2 and 0.1 respectively (Appendix 7, Table 13).

Table 10 shows the mean values of Quality index, SPC, DRT, E_h, ATP, pH in whole gutted cod at different storage times on ice for 14 days.

The Quality index was high (15) at day 14 of fish storage, when the fish was judged spoilt, whereas the SPC was below 4log CFU/g; the DRT at that time was 9 h. The E_h at that time was lower than -200 mV while the pH and ATP values were; 6.9 and 1.2log RLU/cm^2. At day 7 of storage, the E_h was -160 mV and DRT was at 10 h; the quality index at that time was 11,

whereas SPC were < 4 log CFU/g. ATP value during that time was higher, (1.3 log RLU/cm^2) than the ATP value shown at day 14 (1.2 log RLU/cm^2) the time when fish was judged spoilt.

Table 10: Mean values of Quality index, SPC, Resazurin Dye-Reduction Time (DRT), Redox potential(E_h), pH and ATP of eviscerated cod (Gadus morhua) at different storage times on ice.

	STORAGE DAYS		
INDEX	1	7	14*
Sensory Quality index	2	11	15*
SPC(Log CFU/g)	<2	< 4	<4*
DRT (h)	10	10	9*
Redox potential/ E_h(mV)	-100	-160	-224*
pH value	6.7	6.8	6.9*
ATP (Log RLU/cm^2)	1.4	1.3	1.2*

(*) indicate the time (storage days); at which fish was judged spoilt considered spoilt (unacceptable for consumption) corresponding to the SPC; E_h; pH and ATP values

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5 Discussion

The purpose of this study was to select and test rapid methods that could be used outside laboratories e.g. at landing sites in Uganda. The methods chosen were; the measurement of the redox potential (E_h) using two methods: the electrode and resazurin dye-reduction technique; ATP bioluminescence and pH techniques. This was because the various spoilage changes in fish which occur during storage are known to lead to changes in these indices (Huss, 1988; Huss, 1995; Huss, 1994).

The results of Redox potential values of the bacterial cultures /solutions in Tryptic Soy Broth (TSB) were inconsistent. This can be explained by the fact that no attempt was made to hinder the access of oxygen to the samples during measurement of E_h.
The RLU value (ATP) found in bacterial cultures containing approximately 6 log CFU/mL was in the range of 100 to 1000 RLU (= 2-3 log RLU). The levelling out of ATP at high bacterial numbers in bacterial cultures is most likely to be due to saturation as the maximum RLU value of the instrument was set to 4 (log RLU).

The cod fillets showed an increase in bacterial numbers as expected and SPC of approximately 8logCFU/g was reached at day 11. This corresponds well with results of others regarding bacterial proliferation in cod fillets (Gram and Huss, 1996).
The ATP values of cod fillets followed well the bacterial growth. At SPC < 6 log CFU/mL, the ATP value was < 2 log RLU (<100 RLU) and increased to ATP > 3log RLU at SPC > 7log CFU/g of cod fillet, a similar trend and value as was found in the pure culture studies. The consistent increase in the ATP value in cod fillets with increase in bacterial numbers (SPC) suggests a high feasibility of the application of this method for assessment of quality of fish; at that time fish was judged spoilt, the ATP value (in RLU) was above 1000 (or >3logRLU). This result is close to the findings in milk where 7log CFU/mL produced approximately, 2.5 log RLU values (Kyriakides and Patel, 1994).

The results of the resazurin dye-reduction technique showed a significant correlation ($r > -0.8$) with increasing SPC ($P < 0.05$). The found results suggest that SPC close to 7log CFU/g/mL have a DRT of 5-6 hours in fish.

At SPC of 7log CFU/g the DRT was 5-6 h which is very close to the results obtained in the bacterial culture studies. This result shows a significant similarity to the findings on other foods such as meat, where the DRT in samples reduced with increase in bacterial counts (Jay et al., 2005). A well represented profile of the quality deterioration levels in cod fillets indicated by the Dye-Reduction Times (DRT) and as well as a good correlation with storage time of fish provide a good possibility for prediction of the shelf life of fish using resazurin DRT technique. The relatively short detection time (≤4 h) is a good yardstick for discriminating spoilt fish from fresh/acceptable fish.
On Comparison of the results from the bacterial culture studies and the cod fillets studies a remarkable coherence can be seen in both trials i.e. SPC of 7log CFU/g/mL gives DRT of 5-6 hours in both trials.

On the other hand, the pH value of the cod fillets increased as expected (pH approximately 6.8-6.9). The redox potential (E_h) similarly, as expected was high (80 mV) in fish at early storage times and remained high (approximately +70 mV), when the SPC were below 7 log CFU/g. However the redox value fell below 0 mV after storage of more than 14 days.
The storage time for cod fillets stored at 0^oC according to Gram and Huss, 1996 was found to be two weeks.
In this study, cod fillet stored at 0^oC were judged spoilt at day 14 of storage at that time the E_h value was 0 mV. This seems to propose that a fall in the E_h value of fish close to 0 mV the fish product may considered spoilt. This result also shows a close resemblance to the finding of Huss (1988) who found the E_h of cod to be 0 mV at 14/15 days of storage when the fish was judged spoilt

The found results for whole cod stored on ice showed however some unexpected results except for the Quality Index (QI). The QI followed the expected linear increase trend and reached the QI of 16 at day 14 of storage. This corresponds well to results of other, finding the shelf life of cod on ice to be 2-24 days approximately (Gram et al., 1989).
The SPC was at that time found to be approximately 4log CFU/g but that value, compared to the other studies, is low. The DRT was 9 hours and the ATP value was between 10 and 100 RLU. Although lower than expected, compared to the QI, the DRT, ATP and SPC correspond well with the results from the pure cultures and cod fillets.

The result E_h value of cod stored on ice, disagrees with the result on the E_h of Nile perch studies, which showed positive values for the first two weeks; similarly, it also disagrees with finding of Huss (1988) who found that the E_h of cod remained at high positive values for the first two weeks of storage. Perhaps such defiant E_h values might be attributed to other factors such as the difference in the lipid amounts; whose degradation lead into various reducing compound/ anti-oxidant which are potent, influencing factor that lead into lower E_h value in fish (Huss, 1994; Huss, 1988).

The results for the whole Nile perch stored on ice showed a gradual decrease in quality as indicated by increased in quality index score (QI score). The result of sensory assessment in this study showed the shelf life of Nile perch on ice to be 19 days. This finding disagrees with Mhongole (2009); Gram et al., (1989/1990); according to the works of these authors, the shelf life was found to be 4 weeks. Several studies have reported Nile perch shelf life ranging from 13 -33 day (Huss, 1995) however, these authors have not reported shelf life in connection with the E_h changes of fish.
However, in this study, the E_h value at 19 days of fish storage was 0 mV and it remained high, at +300 mV for the first two weeks.

Similarly, the E_h of Nile perch fillets was found to be two week of storage, the E_h value at that time was +300 mV. The bacterial number increased constantly during the storage period and reached 6 log CFU at the time of spoilage (day 14). The redox potential was high, +300mV until day 19, when fish was already spoilt fell to 0 mV.
Whereas the result of the shelf life agrees with the findings of Gram and Huss, (1996) who have found that the shelf life of aerobically stored fish fillets from tropical waters to be two (2) weeks; the result of the E_h value does not agree with the findings of (Huss 1988) who found that E_h 0 mV in cod stored at 0^oC for two weeks.
However, the result for Whole Nile perch agrees with the findings of Huss (1979) who found that the E_h of cod remained constant at positive E_h values for two weeks prior to the fall in E_h to 0 mV. The constant E_h of the fish during the fish two weeks of storage may be attributed to the influence of the poising effect of trimethylamine-oxide (TMAO) and perhaps the predominant spoilage bacteria, *Pseudomonas* spp., which is not a TMAO-reducer as reported by (Gram et al., 1990).

For Nile perch fillets, the prolonged high E_h value perhaps could be attributed to method of fillet treatment (blast-chilled) and the storage temperatures (0^oC) that may have slowed the proliferation of spoilage bacteria (e. g. *Shewanella putrefaciens)* responsible for the production of E_h lowering compounds (H_2S) and reduction of TMAO as mentioned earlier. However, the E_h value at that time was +300 mV thus, showed no significant difference with changes in the quality of the fish ($P>0.05$).

The result of pH value of Nile perch in this study was generally unstable thus suggesting that pH measurement might not be an appropriate technique for fish quality evaluation.

The ATP was high from the start (approximately 2 and 3 log RLU/cm^2). At storage day 15 the ATP value was less than 1000 RLU and the redox potential +300mV and at the time of spoilage (day 19) of storage, it reached approximately 3.6log RLU.

The values for ATP (RLU) were around 2log RLU but fell at the last sampling day. However, although ATP values in Nile perch showed a clear distinction between spoilage and good quality fish, (i.e. low ATP values of < 2log RLU/cm^2 and >3log RLU/cm^2 for good quality and spoilt fish respectively), the ATP values were inconsistent. Perhaps this may take into account of the method used to obtain samples (surface swab method)

Very few studies have been done using direct redox potential measurements to evaluate fish quality. The findings of this study show that this method has great potential for evaluation of fish quality, it is rapid, requires less than 10minutes to perform. Initial cost is the electrode and the meter but the running cost in very low (however it requires one operator).

6 Conclusion and recommendations

The purpose of this study was to select and test rapid methods that could be used outside laboratories for example at landing sites in Uganda. Among the methods chosen and tested in this study were measurements of the redox potential (E_h) using two methods: the electrode and Resazurin Dye-Reduction technique; ATP bioluminescence and pH techniques; conclusively, the findings of this study demonstrates a high feasibility of using these methods as yardsticks for the estimation of bacteriological quality of fish:

- The Dye-Reduction method proved suitably reliable for assessment of fish quality, but not suitable outside the laboratory.
- The ATP technique is equally a suitable and rapid means for fish quality assessment
- The Electrode technique of measuring the Redox potential is equally a rapid means for evaluation of fish freshness, but however, is not a good method for estimating the shelf life of fish because it does not show a clear profile of all the quality deterioration levels of fish.
- The surface swab method pf obtaining samples determine ATP is not recommended due to the irregular results associated with it.

Proposed scale for assessment of fish quality using resazurin Dye-Reduction is as follows:

1. Fresh fish: DRT 7-8 h = Acceptable
2. Fresh fish at marginal level: DRT 5-6 h= Still good and fit for consumption
3. Spoilt fish: DRT 4 h and or less = unacceptable fish product.

Recommendations:

- Identify ways of outside laboratory application of resazurin dye
- Find appropriate application of the electrode technique in fish industry that can exploit the advantage of the “Real-Time test “associated with the electrode method.
- Further studies on the assesment of these methods and perhaps using larger sample size of samples is recommended.
- And above all the sensory methods of fish assessment should be strengthene (adequately training of the panellist) since it is the basic means for evaluation in fish industry

7 References

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8 Appendixes

Appendix 1

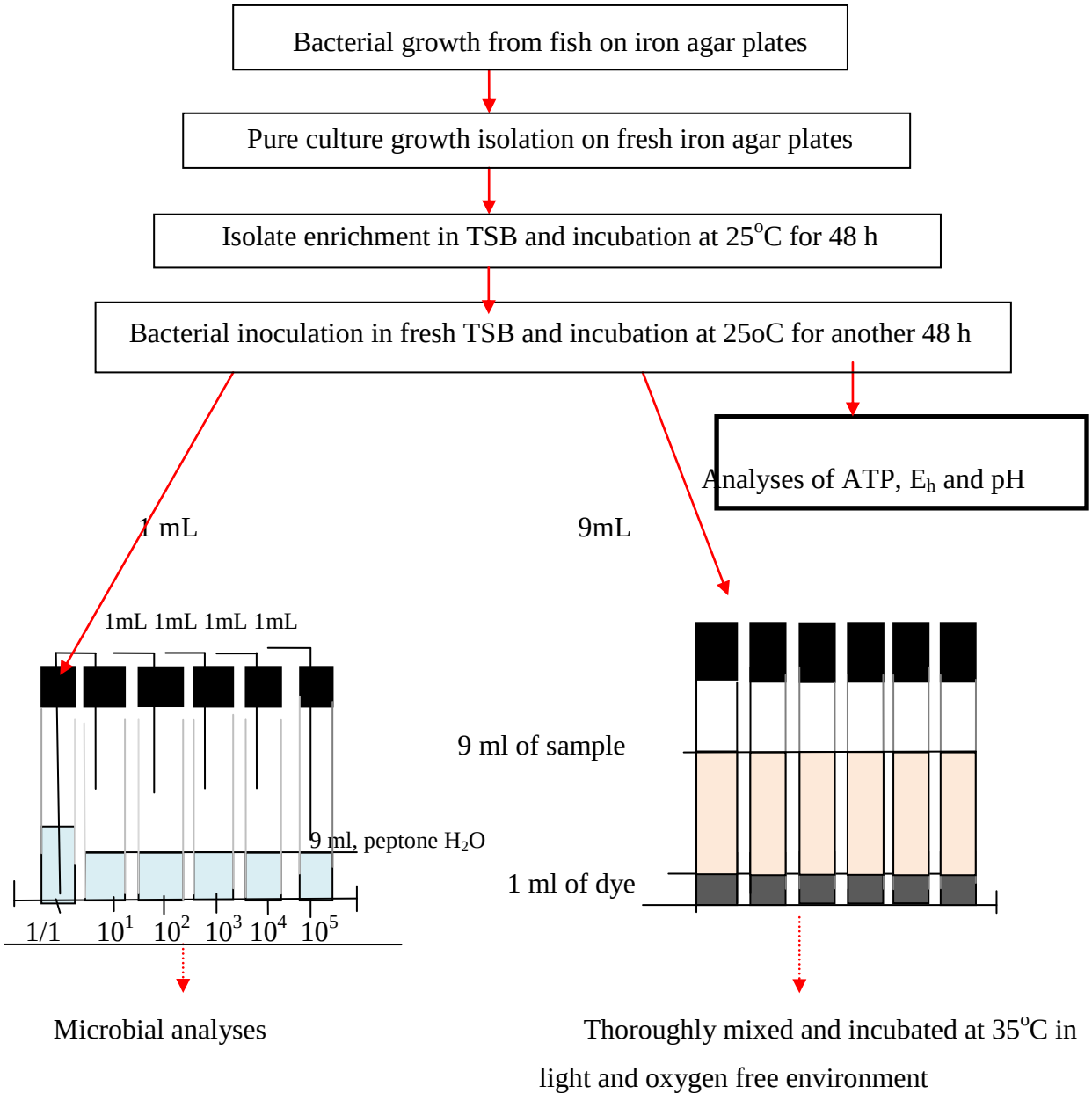

Figure 12: Bacterial culture preparation protocol for calibration of ATP, pH redox potential with microbiological analyses.

Appendix 2

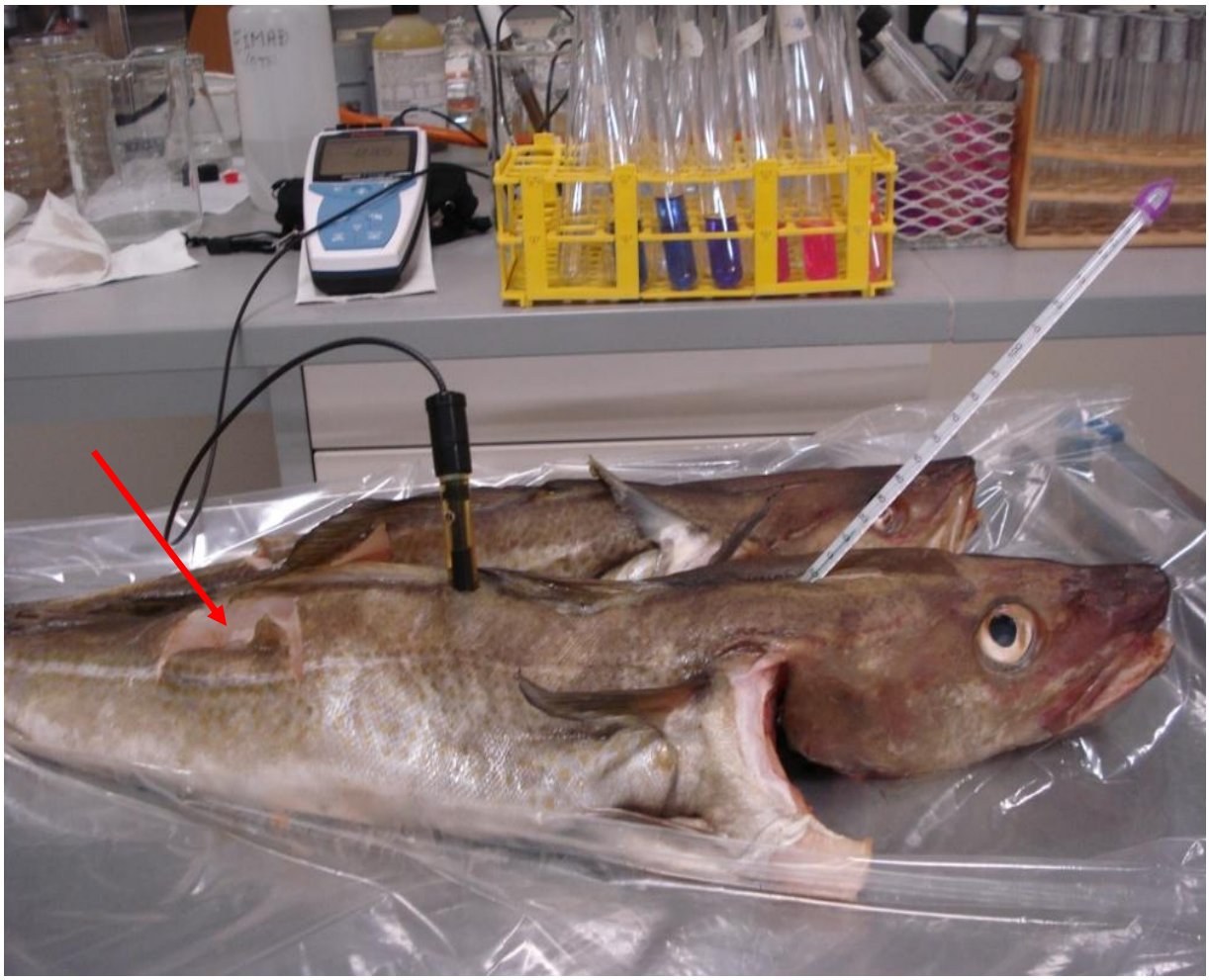

Figure 13: Measurement of the redox potential of cod. The arrow indicate the region from which flesh sample was taken for microbiological analysis

Appendix 3

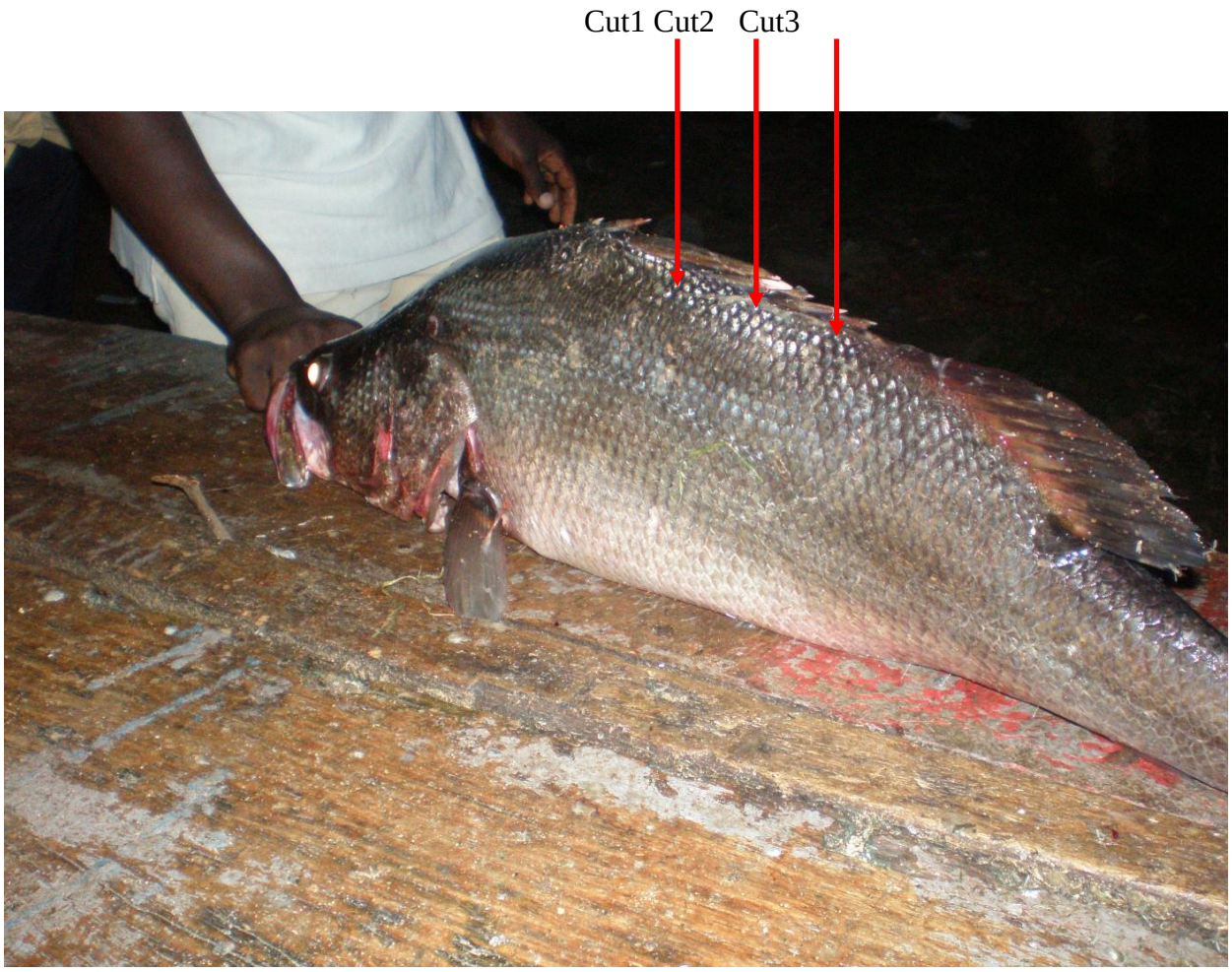

Figure 14: The order of cuts made for the mesurement of redox potential (E_h), ATP and pH of Nile perch

Appendix 4

Table 11: Modified organoleptic assessment scheme for Nile perch stored on ice (Connell 1989)

Quality parameter		**Quality score (QI)**					
General appearance		0	1	2	3	4	5
Skin	Colour	Shinny/silvery	Very bright	Bright	Fairly bright	Pale/dull	Very pale dull
	T exture	In rigormortis (hard and stiff)	Very firm and elastic	Firm and elastic	Less firm and elastic	Soft (post rigormortis)	Very soft and non elastic
Eyes	Shape	Highly convex /protruding	Protruding	Fairly protruding	Fairly Sunken/convex	Concave /sunken	Very concave/sunken
	Colour	Very clear	Clear	Fairly clear	Cloudy and bloody	Very cloudy and bloody	Opaque and or bloody
Gills	Colour	Maroon red	Red	Bleached red	Fairly bleached	Bleached	Greenish
	Smell	Fresh, sea weedy	Neutral	Fairly of odour	Musty/ sour	Stale meat/Rancid	Sulphidy/ rotten egg smell
Slime	Thickness	No slime	Slime present	Minor slime	Minor slime and clear	Less clear and thick	Very thick and Opaque

Appendix 5:

Iron agar (IA) for total viable counts (Psychrotrophic counts) including detection of black colonies (H_2S) Gram et al., (1987)

Recipe/ composition

1. Peptone 20g
2. Lab lemco powder 3g
3. Yeast extracts 3g
4. Ferric citrate 0.3g
5. Sodium thiosulphate 0.3g
6. Sodium chloride 5g
7. Bacto agar 12g
8. Cystine 0.6
9. 990ml distilled water

N.B: Sterilize at 121^{o}Cfor 15 minutes

Appendix 6

Table 12: Modified organoleptic assessment scheme for cod stored on ice (Connell 1989)

Quality parameter	Characteristic	Score
Skin colour	Shinny/silvery with transparent slime	0
	Fairly bright with cloudy slime	1
	Discoloured with cloudy slime	2
	Dull with thick/opaque slime	3
Belly	Firm	0
	Less firm	1
	Soft	2
Texture	In rigor mortis (hard and stiff)	0
	Firm and elastic	1
	Less elastic	2
	Soft (post rigor mortis)	3
Eyes: Shape	Convex	0
	Less convex	1
	Flat	2
	Sunken	3
Eyes: Clarity	Very clear	0
	Clear	1
	Bloody	2
	Cloudy	3
Gills: Colour	Maroon	0
	Red	1
	Bleached	2
	Greenish	3
Gills: Smell	Fresh, sea weedy	0
	Odourless	1
	Off-odour	2
	Stink /rancid	3
Sum of score =		Min. 0 and Max. 20

Appendix 7

Table 13: Correlation matrix and the respective slope and intercepts of DRT, E_h, ATP and pH with SPC, QIS and storage days of fish

		Resazurin dye-reduction time (DRT)			Redox potential (E_h)			ATP			pH		
Sample	Predictor	Corr-Coef	Slope	Intercept	Corr-Coef	Slope	Intercept	Corr-Coef	Slope	Intercept	Corr-Coef	Slope	Intercept
Bacterial cultures	SPC	0.764	-2.29	22.1	0.453	-1.27	984	0.584	0.321	0.771	0.367	0.107	6.41
Cod fillets	SPC	0.816	-0.93	11.3	0.497	20.1	0.49	0.81	0.308	0.535	0.672	0.133	6.30
	Storage days	0.894	0.38	8.91	0.676	10.6	138	0.82	0.141	1.009	0.831	0.067	6.43
Nile perch	QIS	-	-	-	0.604	-104	548	0.2	0.258	1.890	0.093	0.004	6.49
	Storage days	-	-	-	0.656	-17.8	459	0.3	0.051	2.024	0.063	0.005	6.54
N.perch fillets	Storage days	-	-	-	0.199	-7.8	274	0.289	0.04	2.257	0.383	0.021	6.45
	SPC	-	-	-	0.109	-17.9	281	0.249	-0.10	2.412	0.092	-12.3	188
Whole gutted cod	Storage days	0.32	-0.08	10.2	0.419	-8.87	-100.6	0.082	10.01	1.37	0.25	0.02	6.69
	QIS	0.31	-0.07	10.3	0.515	-8.83	-80.2	0.01	-0.01	1.39	0.19	0.01	6.69
	SPC	0.38	-1.43	16.6	0.492	-29.6	-83.8	0.21	-0.07	-1.46	0.13	0.03	6.73