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INFLUENCE OF THAWING AND POST THAWING STORAGE ON MICROBIAL SUCCESSION AND BIOGENIC AMINE DEVELOPMENT ON FROZEN ATLANTIC MACKEREL

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ABSTRACT

At abused temperature handling, scombroid fish including mackerel develops toxic biogenic amines commonly associated with scrombotoxicosis. The present study examined the influence of two thawing methods: fast (FT) and slow (ST) thawing on bacterial diversity and formation of biogenic amines on frozen mackerel stored up to 36 hours and 12 days at ambient temperature (30°C) and refrigerated condition (2-5°C) accordingly. The FT was achieved by immersing frozen mackerel in tap water initially at 18°C for 2 hours, while in ST, polystyrene boxes containing frozen mackerel were kept at 30°C for 12 hours. Total viable counts (TVC), hydrogen sulphide (H_2S) producing bacteria and pseudomonads counts were enumerated on culture method while their identification and diversity was performed by sequencing purified 16S rRNA amplicons obtained from extracted DNA from the samples. Formed biogenic amines were quantified using HPLC. FT mackerel exhibited higher ($p<0.0001$) bacterial growth at 30°C than ST mackerel but the difference was not apparent at 2-5°C. The levels of the biogenic amines were insignificantly influenced by thawing methods though their formation during ambient storage was realized at a lower TVC of log 4 in ST and log 6 for FT after 12 hours. At 2-5°C they were first quantified on the 8th day for ST and delayed to 10th day for FT at a much higher total viable log counts. Moreaxellaceace group of bacteria dominated the bacteria in both treatments with low relative abundance of higher BA producers save for Enterorbactericeae at 30°C than 2-5°C. Because of the low amine levels even when the fish was spoiled, their use as spoilage indicators may not be dependable.

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1. INTRODUCTION

Fish is nutritionally important as a source of first class protein, essential fatty acids and other viable micro nutrients in human nutrition. This can only be realized with proper handling and preservation of fish immediately after harvesting. In the developed nations the fish value chain is characterized by better logistics culminating to a short fish value chain maintained at low temperatures. This is not true for many developing countries including Kenya whose fishing industry is largely controlled by artisanal fishermen (70%) (SDF 2012; Odoli *et al.* 2013). Kenya lands about 10000mt of fish annually from marine waters majorly deep fried and sold along the streets and restaurants (KCDP 2013). As an economic activity, artisanal deep frying (3-15 kg per processor) has been growing rapidly and the artisanal fishing effort no longer meets the raw material demand. This has created a huge supply gap being filled by imports mainly from Asia of frozen mackerel (KCDP 2013). After thawing, fish is mainly handled at ambient conditions (23-35°C) without use of ice for long time.

These conditions may be perfect for the growth of both pathogenic and spoilage bacteria which are mainly controlled by deep frying process but may not destroy heat stable biogenic amines in scombroid fish such as mackerel, if it has been formed during storage. Biogenic amines are generated by the action of decarboxylase enzyme from certain bacteria, breaking free amino acids freely existing or from proteolytic microorganisms (Zarei *et al.* 2011). The amino acids histidine, tyrosine, tryptophane, ornithine and lysine undergo decarboxylation to form toxigenic histamine, tyramine, tryptamine, putrescine, and cadaverine respectively while spermidine and spermine forms from putrescine (Visciano *et al.* 2012). Currently there is no threshold toxic dose for biogenic amines but the US Food and Drug Administration and the European Commission have set levels of histamine at 50 and 100 ppm respectively of fish as a potential health hazard (European Commission 2005, FDA 2011).

1.1 Problem statement and justification

A recent inspection audit in Kenya by European Union food and Veterinary Office (FVO) raised a deficiency on temperature control and lack of clear methodology for monitoring biogenic amines (BA) particularly histamine in the industry in fish and fish products (EC, 2013). The rate of their formation are dependent on a combination of many factors (Ekici & Alisarli 2008) including the diversity of the fish micro flora, temperature and time (EFSA, 2011). As an extrinsic factor, higher temperatures accelerates bacterial growth to reach the exponential phase with increased metabolic activities resulting into faster rate of fish spoilage and biogenic amine development in scombroid fish species (FAO/WHO 2013). Both changes in intrinsic and extrinsic conditions with time largely influences the microbial community and activity (Kim *et al*. 2002; Reynisson *et al*. 2009). If BA has reach critical levels in fish, intakes may cause distinct pharmacological, physiological and toxic effects (Zarei *et al.* 2011).

Conventional thawing (air thawing) is the cheapest method used and sometimes water in uncontrolled conditions. Thawing under controlled conditions of water, air or steam, microwave and radio frequency systems used in the industry has established differing effects of fish sensory, chemical and microbial quality (Archer *et al*. 2008) but how thawing and post thawing conditions influences the microbial diversity and development of biogenic amine is still not known. Only high-performance liquid chromatography (HPLC) is found to be sensitively and simultaneously able to quantify all BA in foods, hence its choice in the study (EFSA 2011). Specific spoilage and pathogenic organisms of food have to a great extent been identified by conventional culturing methods with much success, but this is still limited to the

known methods (Reynisson *et al*. 2008). 16S sequencing techniques are able to unravel the ecology of microorganisms to a higher degree of accuracy. Microorganisms are identified by matching the sequence of 16S rRNA DNA material specific to each microorganism with existing database (Reynisson *et al*. 2009). The throughput of molecular techniques is higher compared to the culture methods (Reynisson *et al*. 2008) hence preffered choice for diversity study. The aim of this study was therefore to establish the influence of slow and fast thawing methods and post thawing storage temperatures 2-5˚C and 30˚C on bacterial load, diversity and their potential to form biogenic amines on frozen Atlantic mackerel (*Scomber scombrus*).

1.2 Goal

To study the influence of thawing methods and post thawing handling storage on development of biogenic amines as influenced by microflora diversity in thawed frozen mackerel.

1.2.1 Objectives

1. To determine the influence of thawing method and post thawing handling on micro-flora and diversity in frozen mackerel
2. To determine the level of formation of biogenic amines in mackerel as influenced by thawing method, post thawing storage and micro flora diversity

1.2.2 Research questions

1. Does microbial diversity and biogenic amine content depend on thawing method?
2. Does post thawing storage conditions influence microflora diversity, rate of formation and amount of biogenic amine on frozen thawed mackerel?
3. Is microflora succession important in biogenic amine production?

2. STATE OF THE ART

2.1 Artisanal Marine fish supply characteristics in Kenya

Kenya has both fresh and marine water sources of fish with fresh water constituting over 90% of annual capture (SDF 2013). Lake Victoria is the largest contributor of gross domestic product (GDP) from the fishery subsector. Limited exploitation of the rich deep marine waters with an estimated potential of 150000mt/y (SDF 2013) is as a result of inadequate investment and low fishing technology. The industry is characterized by artisanal fishery and confined to a small strip of 2.5 to 3.0 nautical miles using craft vessels with an annual catch of 10000mt/y (McClanahan and Mangi 2004, SDF 2012).

Because of the multi species nature and low quantities of the catches, the fish catch rarely reach the industrial processing but are sold at the domestic market. Only crustaceans (crab, lobster and octopus) have been exploited for European export market. On the other hand, the fin fish value chain is long, complex with limited regulations. High levels of postharvest losses from inadequate fish handling, processing and preservation facilities is common. This increases the risks of developing toxins due to spoilage and pathogenic bacterial growth (Odoli *et al.* 2013).

In order to better utilize the catches, deep frying is one of the main methods used in the artisanal fish supply chain. (KCDP 2013). Deep frying's rapid growth has created a supply gap being filled by imports especially of frozen mackerel (Figure 1) from countries like India, Pakistan, Japan, Korea and China (SDF 2013)

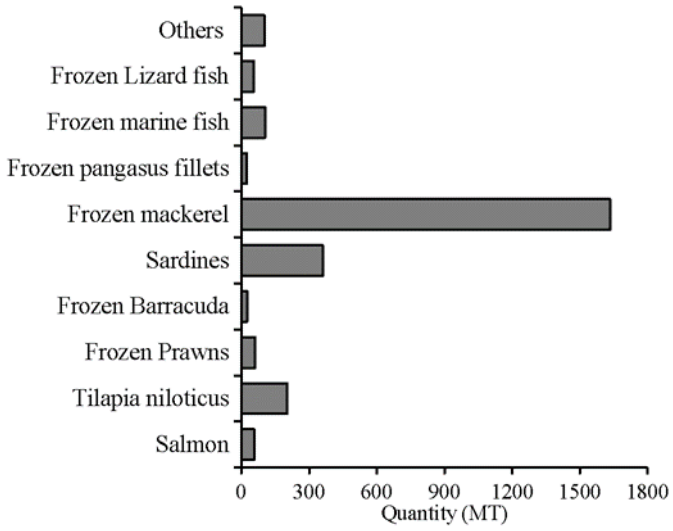

Figure 1. Imports of fish and fishery products in Kenya in 2012 (Source: SDF 2013).

2.2 Thawing and changes during thawing

Freezing of fish and fish products has been used for prolonging shelf life and also controlling foodborne parasites. Other benefits include evening out of fish supply in high and low seasons as well as from high to low producing countries across the globe. Thawing which is the process by which frozen water melts from the frozen fish by absorbing the heat energy is considered important in the value chain in determining the quality and safety of fish products (Dincer *et al*. 2009). The seafood ice completely melts at -1°C in a thawing time influenced by the method, fish size, freezing method (block or individual) among others. Due to low water content, fatty

fish requires less energy of 240kJ while lean fish requires 300kJ per piece (Archer *et al*. 2008). A rapid rise in temperature during thawing has been established to be the result of the melting of ice glaze layer in the fish/food which has higher thermal conductivity than water but after its thawing, the rate slows (Figure 2) (Jason 1982).

Uncontrolled thawing accelerates the rapturing of the cellular structure of the food and leads to higher drip loss. In order to maintain the firm texture, the fish commercially is commonly thawed at just below freezing point in a process known as tempering (Archer *et al*. 2008). There is however no definitive maximum temperature but a few recommendations based on air or water thawing have been advanced as shown in Table 1.

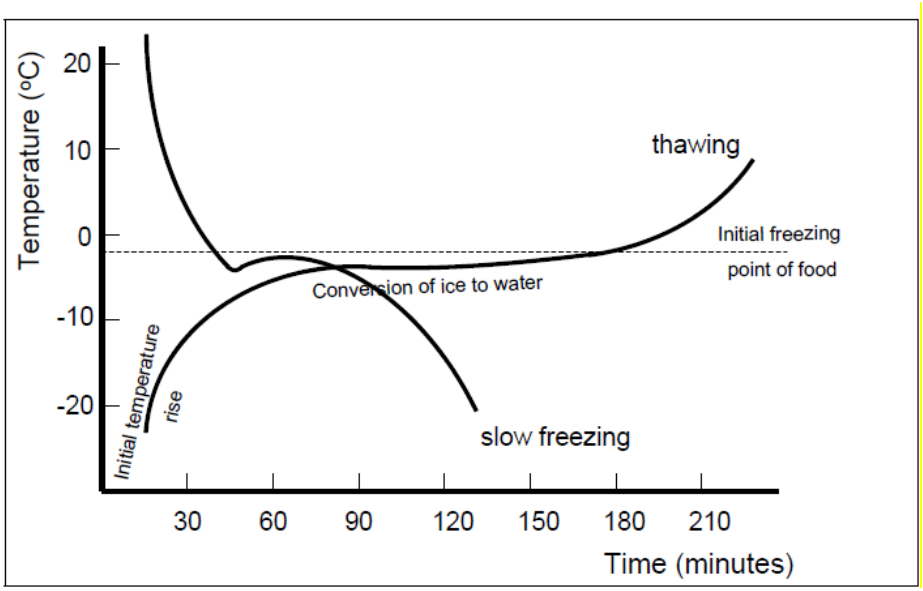

Figure 2. Rise in temperature during thawing in fish muscle (Source: Archer *et al.* 2008).

Table 1. Recommended thawing conditions (Source: Archer *et al*. 2008).

Source	**Maximum Air and Water temperature**	**Maximum product temperature**
CODEX Air thawing of frozen blocks	25ºC	7ºC
CODEX Water thawing of frozen blocks	21±1.5ºC	7ºC
Torry Research Station	20ºC-air blast 15ºC- still air 18ºC- water	2-5ºC
International Institute of Refrigeration (IIR)	20ºC- air 18-20ºC- water	4-5ºC
New Zealand Training manual	16-18ºC- water 12-15ºC- air	4ºC

In spite of air thawing not requiring sophisticated equipment, it is only practicable in small scale due to large space requirement, excessive handling and long thawing time (8-10 hours) depending on temperature (Jason 1982).

During thawing, the temperature of the fish surface gets higher than the interior so subjecting fish to a slower and longer thawing time increases the risks for microbial spoilage as is the case for refrigerated thawing temperatures albeit at slow rate (Dincer *et al*. 2009).

2.3 Microbial diversity and potential to produce biogenic amines in fish

The muscle tissues of fresh fish is considered sterile but the contamination normally occur during evisceration and washing which raptures the cell membranes leading to the transfer of the microorganisms into the flesh (FAO/WHO 2013). Characteristic deterioration follows the path of biochemical changes and microbiological activities culminating to lipid oxidation and growth of bacteria producing undesirable odours and flavours (Raouf *et al*. 2009).

Both Gram-positive and Gram-negative bacteria have been established to produce decarboxylase enzymes though in different amounts and forms (EFSA 2011, Kim *et al*. 2002, Hungerford 2010) and under different study conditions. Rarely have the species been isolated from fresh fish but mainly on spoiled fish stored at above 7-10^{o}C but there is limited formation of BA at below 0°C (Visciano *et al.* 2012).

Those bacterial groups that have been found to poses higher activity includes; *Morganella morganii*, *Morganella psychrotolerans*, *Photobacterium damselae*, *Photobacterium phosphoreum*, *Raoultella planticola* and *Hafnia alvei*; (EFSA 2011, Kim *et al*. 2002) while for the rest of the biogenic amines several families or genera are reported to be involved, such as Enterobacteriaceae, Pseudomonaceae, Lactobacillus, Enterococcus and Staphylococcus (EFSA 2011).

2.4 Biogenic amines: hazard identification and characterization

Biogenic amines (BA) are low molecular weight organic, basic nitrogenous compounds in which one, two or three hydrogen of ammonia are replaced by alkyl or aryl groups and are naturally existing or produced from the decarboxylation of free amino acids in food as shown in Figure 3 (Chong *et al*. 2011). Structurally (Figure 4) they are classified as either aliphatic (putrescine and cadavarine), heterocyclic (histamine and tryptamine) and aromatic (tyramine and phenylethylamine) but are also classified as per the number of amines either as monoamine (tyramine and phenylethylamine) and diamines (histamine, putrescine and cadaverine) (EFSA 2011, Chong *et al*. 2011).

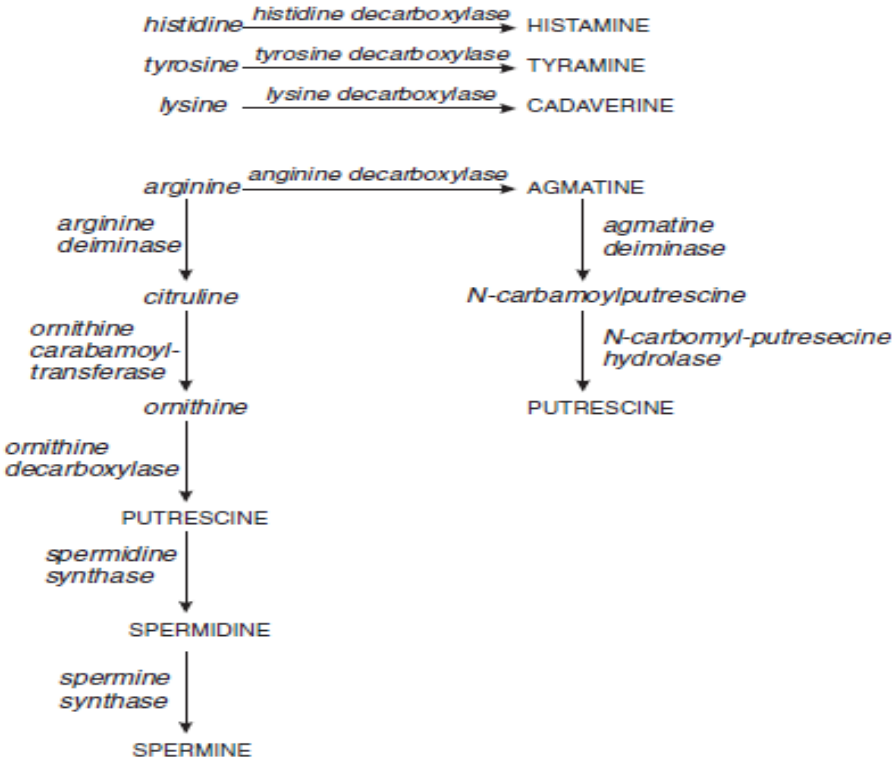

Figure 3. Synthesis of histamine, tyramine, putrescine, spermidine, spermine and cadaverins (Source: Prester 2011).

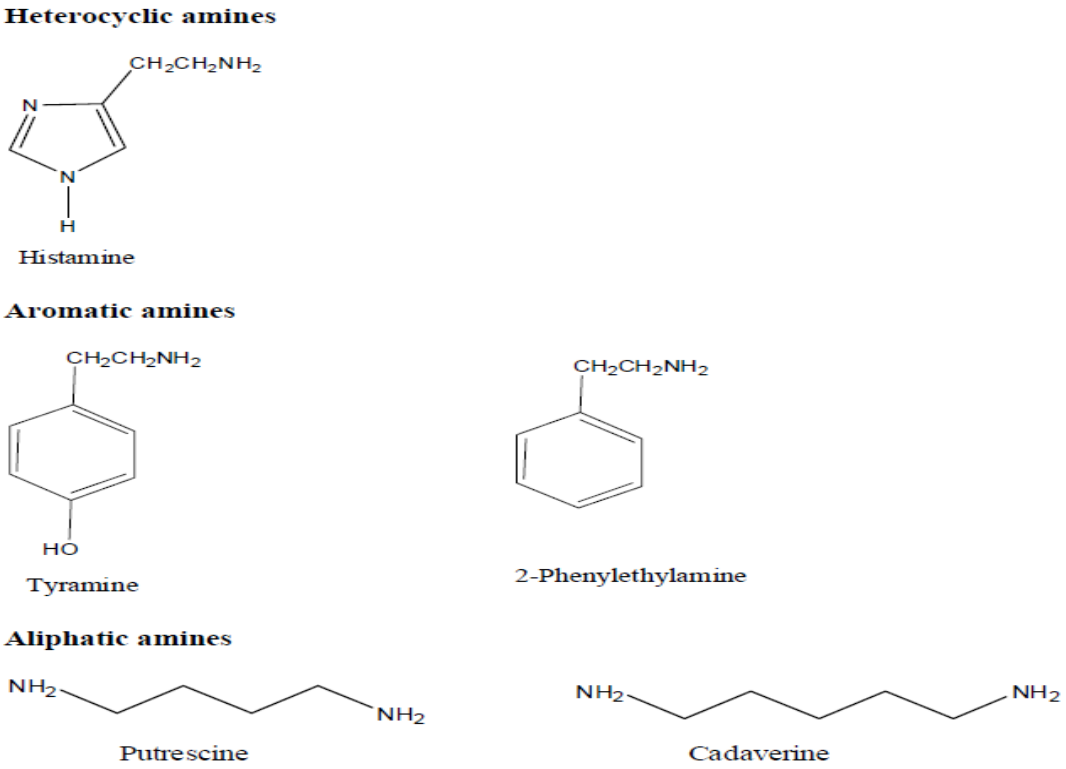

Figure 4. Structure of histamine, tyramine, 2-phenylethylamine, putrescine and cadaverine (Source: EFSA 2011).

Naturally amines exist in the body performing important physiological functions including synaptic transmissions, blood pressure control, allergic response and cellular growth control (EFSA 2011, Ladero *et* al. 2010). However, at high intake, they may result in fish poisoning commonly referred to as scrombotoxicosis. Their outbreaks are believed to be underreported due to misdiagnosis and faster recovery of patients (Lehanea & Olley 2000). Of all the BAs, only histamine and tyramine are regarded to have higher biological activities (Prester 2011). Histamine is derived from the decarboxylation of the free histidine in scombroid family of fish by bacteria with ability to release decarboxylase enzymes during spoilage and fermentation of fish (Chong *et al*. 2011, Hungerford 2010). At elevated concentrations, it elicits intolerances in sensitive individuals or poisoning depending on the exposure (FAO/WHO 2013, EFSA 2011). Tyramine occur naturally as monoamine compound derived from the amino acid tyrosine and can also reach higher levels in spoiled and fermented fish. Cadaverine and putrescine are produced by the decarboxylation of free lysine and ornithine respectively but putrescine can as well be produced by the deamination of the agmatine (EFSA 2011). Other important amines include spermine, spermidine, dopamine and agmatine mainly found in the temperature abused and their presence may act as spoilage indicators (Prester, 2011). Apart from histamine, the rest of the biogenic amines are not well studied in fish and fish products as toxins (Hungerford 2010). Even though they are believed to be histamine potentiators by inhibiting intestinal histamine degrading enzymes (FAO/WHO 2013, Tao *et al*. 2011, Zare *et al*. 2013). When the levels of histamine go beyond the degradation capacity of a healthy person or those under alcohol and certain drugs such as antihypertensive, antidepressants, antihypotonics, antiarrhythmics and other drugs able to inhibit the diamine oxidase medication, then toxicity occur (Chong *et al*. 2011, Ladero *et al*. 2010). Higher concentrations of biogenic amines are considered to be mutagenic precursors in the presence of nitrite forming carcinogenic nitrosamine (Prester 2011, Visciano *et al.* 2012).

2.5 Safety and regulatory limits

Setting safety limits for BA has been hampered by difficulties in performing dose response studies including methodological problems involving translating effect of individual BA's administered intravenously to laboratory animals or volunteers to food intakes. Ingestion limits based on case reports may be impractical since they are obtained from poisoning reports (Ladero *et al*. 2010). Only histamine has been widely investigated and introduced in the regulatory limits. The European Union has set a critical limit based on nine samples in which the average of the histamine is set as 100 ppm and not more than two samples should have levels between 100-200 ppm, however none of the samples should exceed 200 ppm. The limit is set higher for fish that has undergone enzyme maturation in brine but should not exceed histamine level of 400 ppm (European Commission 2005). US Food and Drug Administration (FDA) has a much stricter acceptable limit of 50 ppm for defect action limit and 500 ppm to be toxic levels a limit that is based on data collected from several outbreaks (FDA 2011).

Some studies though have established minimum toxic dose of 75 mg of pure liquid oral histamine able to cause both acute and delayed symptoms in 50% of healthy females. For tyramine it has been suggested that its dietary acceptable levels for adults be 100-800ppm with levels beyond 1000 ppm is considered toxic. For individuals using monoamine oxidase inhibitor (MAOI), even lower levels of dietary tyramine of 60 and 100-250 ppm can potentially cause migraine and hypertensive crisis (Prester 2011; Ekici & Alisarli 2008). Maximum tolerable limits in fish of cadaverine and putrescine has also been advanced to be 510 and 170 ppm respectively (Rauscher-Gabernig *et al*. 2012) but because of the presence of other BAs a maximum total of 750-900 ppm has been proposed (Ladero *et al*. 2010).

2.6 Scrombotoxicosis outbreaks

Yearly scrombotoxicosis is reported in Japan and it is also the country that has reported the highest outbreak with over 4000 cases 1970-1980 involving the consumption of scombroid fish. In the year 1973 the worst incidence of scrombotoxicosis happened in Japan involving over 2000 cases suspected to have eaten dried horse mackerel (Lehanea & Olley 2000). Several authors have indicated high levels in the samples indicating that many are exposed to the toxins without knowing. In an elaborate work that involved investigation of the potential of histamine hazard in fish samples from nine countries Fiji, Japan, China, Netherlands, Germany, Norway, Cambodia and the Philippines, about 9% of the samples were determined to have histamine levels above 50 ppm in which 5 samples out of 159 investigated had above 500 ppm (Tao *et al*. 2011) exceeding the 100 ppm recommended limit in the EU (European Commission 2005).

3. METHODOLOGY

3.1 Experimental design

Thawing and post thawing handling was conducted as illustrated in Figure 5. Two thawing methods were tested, one using water set 18°C as a fast thawing method (FT) and the other using polystyrene box set at 30°C for 12 hours as slow thawing method (ST). Both groups were subsequently stored at refrigeration (2-5°C) for 12 days and 30°C ambient (resembling Kenya's coast ambient temperatures) temperatures for 36 hours. Other details are as described in thawing and storage.

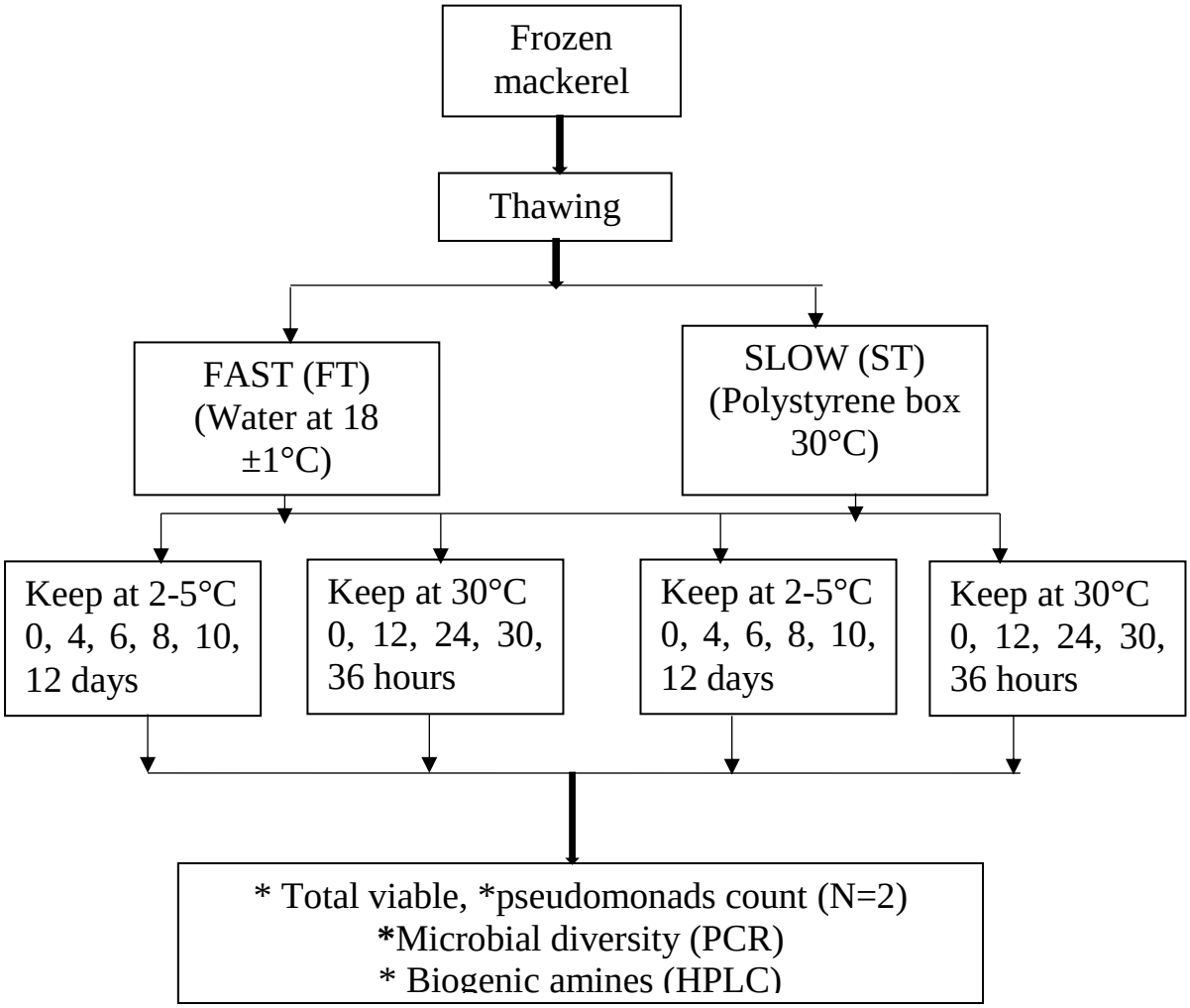

Figure 5. Experimental design.

3.2 Raw Material

Atlantic mackerel (*Scomber scombrus*) of weight 290.30±36g used in this study was harvested in the month of July 2013 in South-East of Iceland using purse Seine. The fish was be-headed and gutted on board and kept below -1°C until landing. It was then frozen and kept at -18°C in 22kg cartons block for 6 months. For the analysis, the blocks were transported in a refrigerated truck (-15 to -18°C) within 7 hours to the laboratory (MATIS, Icelandic Food and Biotech R&D) for subsequent analysis.

3.3 Thawing and Storage condition

Frozen mackerel was thawed using two methods. Slow thawing (ST) was done by keeping the fish in closed polystyrene boxes (Figure 6a) placed in an incubator set at 30°C overnight while fast thawing (FT) was done by immersing frozen fish in fresh tap water (Figure 6b) with initial temperature of 18°C for two and a half hours (time predetermined during thawing trial). After each thawing four pieces of fish were stored in polystyrene box (400x264.5x135mm) and kept in refrigeration (2-5°C). Samples were then collected on 4th, 6th, 8th, 10th and 12th day for analysis. In ambient conditions fish were kept in open polystyrene boxes at 30°C and sampled on the 12th, 24th, 30th and 36th hour. The temperatures were monitored using loggers iButton type DS1922L (Maxim Integrated Products, Sunnyvale, CA, USA) with an accuracy of ±0.5°C, a resolution of 0.0625°C and an operating range of -40–85°C) recording every 10 minutes inside the fish and in both incubator (30°C) and refrigerator (2-5°C) during storage. Analysis were conducted on total viable counts (TVC) and pseudomonads count were in duplicate using two pieces of fillets from pectoro-ventral muscle in each replicate while biogenic amines were determined using HPLC and PCR and 16S RNA sequencing performed on pooled samples of four pieces of mackerel. Other details are as outline below.

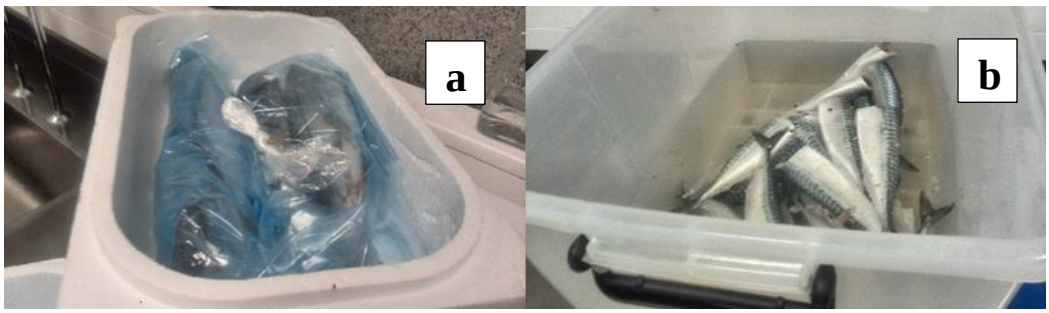

Figure 6. ST in polystyrene box (a) and FT in water at 18°C (b).

3.4 pH

The acid value pH was determined using Radiometer PHM80 Portable pH meter, Denmark by dipping the glass electrode for 1 minute in the homogenate of 5g portion of minced fish sample in 5ml distilled water. The pH meter was calibrated using the buffer solutions (Orion, Thermo Fisher Scientific, Beverly, MA 01915, USA) buffer of pH 7.00 ± 0.01 and 4.01 ± 0.01 (25°C) (Radiometer Analytical A/S, Bagsvaerd, Denmark).

3.5 Cultivation method

Total psychrotrophic viable counts (TVC) (total of white and black colonies) and Hydrogen sulfide producing bacteria (black colonies) were determined on Iron agar (IA) as described by Gram (Gram, *et al*. 1987) with slight modification. 20g of minced sample was aseptically weighed in stomacher bag and mixed with cooled 180 ml of maximum recovery diluent with 1% NaCl instead of 0.5% (MRD, Oxoid, UK). Homogenization was done using Waring laboratory blender (Seward, Stomacher 400, Lab, UK) for 1 minute and serially diluted to appropriate 10 fold dilutions then spread plated on Iron agar (IA) and incubated at 17°C for 5 days. The presumptive pseudomonads were enumerated on modified Cephaloridine Fucidin Cetrimide (mCFC) agar as described by Stanbridge and Board (1994). Pseudomonads agar base (Oxoid, UK) with CFC selective agar supplement (Oxoid, UK) was used and the plates incubated at 22°C for 3 days. The results were expressed as logarithms of the mean numbers of bacteria colony-forming units (log cfu/g of fish).

3.6 Bacterial diversity analysis

3.6.1 DNA extraction

Fractions of tenfold dilutions during the culture method were collected and kept at -20^{o}C until DNA extraction was done as described by Reynisson *et al.* (2009). Template genomic DNA were extracted from one ml pooled from the duplicate of diluted samples. The sample was centrifuged at 11000 x g for 10 minutes to form a pellet and supernatant discarded. The DNA was recovered from pellet using DNA isolation kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions.

3.6.2 PCR amplification

VAMPS primers were used to amplify the v3 to v6 region of the 16S rRNA gene using `5-YCTACGGRNGGCWGCAG-3' forward primer and `5-CGACRRCCATGCANCACCT-3' reverse primer (Sogin *et al*. 2006). Titanium adaptors A and B were attached to the forward and reverse primers respectively along with multiplex identifier (MID) adaptors recommended by Roche to be used in the FLX pyrosequencing. The PCR was performed in a 25 uL reaction volume using FastStart High Fidelity polymerase system (Roche, Madison, WI). The PCR program was as follows: 95°C for 3 min, 35 cycles of 95°C for 40 s, 54°C for 40 s and 72°C for 30 s and a final extension step at 72°C for 7 min. After the recovery of a PCR product from the DNA the rest of the workflow prior to sequencing was done according to manufacturer instructions for FLX amplicon sequencing using the GS Titanium SV emPCR Lib-A kit (Roche, Madison, WI).

3.6.3 16S rRNA sequencing

The 16S rRNA sequences were processed through the Qiime pipeline using the latest Greene gene database (version 12.1). The first steps included quality processing including filtering sequences which were under 200bp and over 1000bp, contained incorrect primer sequences (>1 mismatch). Sequences were assigned to samples through the MID sequences and clustered into Operational Taxonomic Units (OTU) based on 97% similarity in the16S rRNA sequences using Uclust and then assigned phylogenetic taxonomy through RDP classifier. OTU sequences were then aligned with PyNast. Chloroplast sequences were finally filtered from the dataset. Taxa were then summarised through bar plots within the Qiime toolbox. The α-diversity parameters chao1 and Shannon index were calculated within each sample. A β-diversity analysis (diversity between samples) was done by principal coordinate analysis (PCoA) using weighted phylogenetic UnFrac distances between samples and subsampling of 150 sequences from each sample (Lozupone *et al.* 2011). The data was also analysed by SILVAngs pipeline which; an automatic and standardised procedure to analyse bulk 16S rRNA sequence data (Quast *et al.* 2013).

3.7 Determination of biogenic amines

Histamine, cadaverine, putrescine and tyramine were determined from the same samples from microbiological analysis using HPLC as described by (Corbin *et al*. 1989).

3.7.1 Extraction of biogenic amines

Minced fish sample of 25g was weighed into a plastic container and homogenized (Ultra Turrax T25 homogenizer) at 9100 rpm for 60 seconds in 50ml 10% TCA. The extract was then filtered through Whatman 0.45 μm (CAT No.HAWG047S6) filter paper under vacuum into 100ml volumetric flask.

3.7.2 Derivatization procedure

A total of 0.25 ml of filtered sample extract/standard was added to 0.5 ml OPA (o-phthaldialdehyde) reagent in a test tube with a screwed cap then kept in darkness for exactly 3.5 minutes. Two ml ethyl acetate was then added and vortexed (Genie Model K-550, scientific Industries Inc. USA) for 1 minute for complete phase separation. An aliquot from the top phase was pipetted in a vial and in exactly 3.5 min. after addition of ethyl acetate injected for analysis.

3.7.3 Standards & quantification

Exactly 100 mg of each standard was weighed and made to 100 ml with 10% TCA in volumetric flasks. A stock solution was made by mixing the four amines in volumes which are about 10 mg/100 ml in concentration. Dilutions of 1/2-1/4-1/8-1/16-1/32-1/64 were made for a standard curve preparation. Quantification of biogenic amines in the sample was determined by the area measurements obtained from a standard area versus concentration plot and their rates determined using the following formula:

Rate = $(B_c\text{-}A_c)/(B_t\text{-}A_t)$ where B_c is the concentration of the preceding time and A_c is the concentration of the previous time. B_t is the preceding time and A_t is the previous time expressed as ppm/hour or ppm/day.

3.7.4 Instrumentation and chromatographic conditions

HPLC consisted of HP automatic injection system of volume of 5 μl at a flow rate of 1.0 ml/min with a runtime of 40 min. Through a HPLC RP column BDS C18, 5μm (250*4.0mm) with a guard column (Lichrospher RP-18, 5 μm (10*4mm). detector (Varian 9070 fluorescence) was set at excitation and elution 336nm and 440nm wavelengths respectively**.**

3.8 Data analysis

Excel 2013 and XLSTAT 2014 software were used to conduct ANOVA to determine statistical significance of the variation in the quantity of biogenic amines, bacterial load with respect to thawing method, time and temperature treatment. QIIME and SILVAngs pipeline were used to determine diversity of bacteria.

4. RESULTS

In this work, the influence of two thawing methods on bacterial growth and diversity on BA, was examined and summary of all the results is attached in Appendix1.

4.1 Temperature changes during thawing and storage

For the FT group, the fish temperature was 7˚C after 2 hours with thawing water lowering to 12˚C as shown in (Figure 7) while for the ST group the fish attained 2˚C when polystyrene boxes were overnight to attain 30°C (Figure 7).

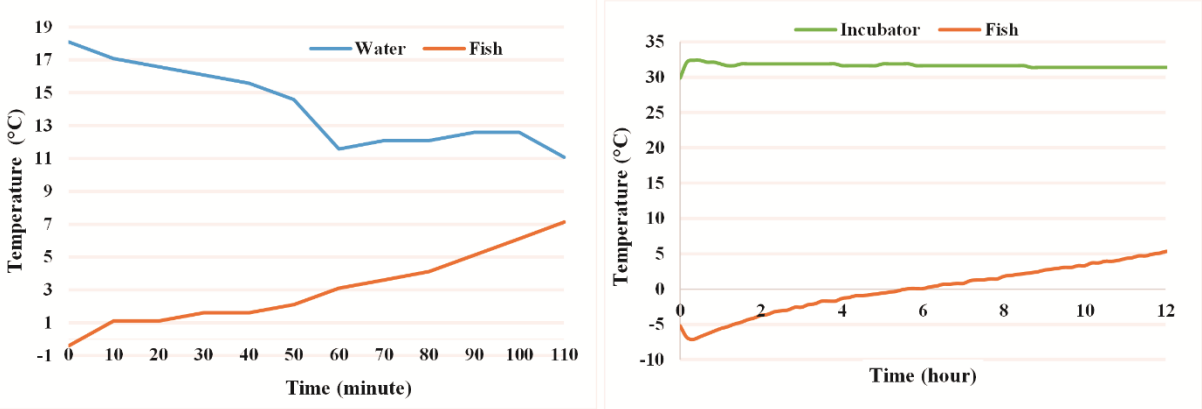

Figure 7. Temperature changes during fast thawing in water at 18ºC (left) and slow thawing in polystyrene boxes kept at 30ºC (right).

4.1.1 Post thawing storage temperature

The changes in temperature during thawing and post thawing storage are shown in Figure 8. The incubator mean temperature during storage for 36 hours was 31.7±0.32˚C and the fish recorded a mean temperature of 27.83±5.5ºC. In refrigeration condition the fish temperature reached a mean for both thawing methods 4.3±5.0˚C while the cooler was consistently low at (4.1±0.74).

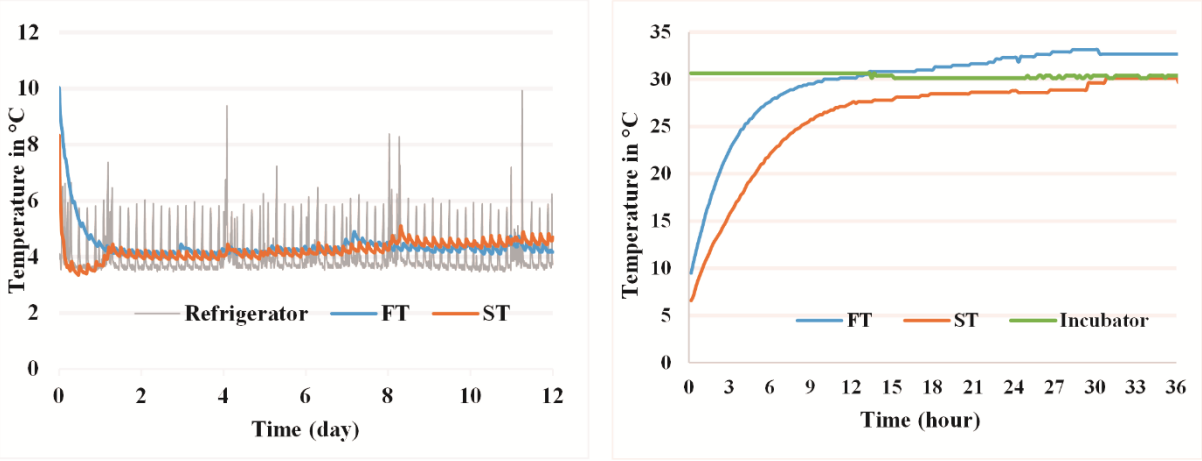

Figure 8. Changes in temperatures in fast and slow thawed mackerel stored at 2-5˚C (left) and 30°C (right).

4.2 pH

At the beginning, the mean difference in the pH was statistically the same, however, it significantly increased ($p<0.001$) in both thawing methods kept at 2-5°C (Figure 9), The pH in the FT group increased from 6.28±0.05 to 6.72±0.08, having a strong correlation with time (adjusted R^2, 0.86) than in ST group whose pH rose from 6.19±0.13 to 6.50±0.04 (adjusted R^2, 0.61). On the other hand, at 30°C the pH changes in the ST group (from 6.19±0.13 to 6.38±0.04) was not significant and produced a non-uniform pattern with time (Figure 9) (adjusted R^2, 0.48) whereas, in the FT group the increase was significant (from 6.28±0.05 to 6.53±0.02) with a stronger correlation (adjusted R^2, 0.89).

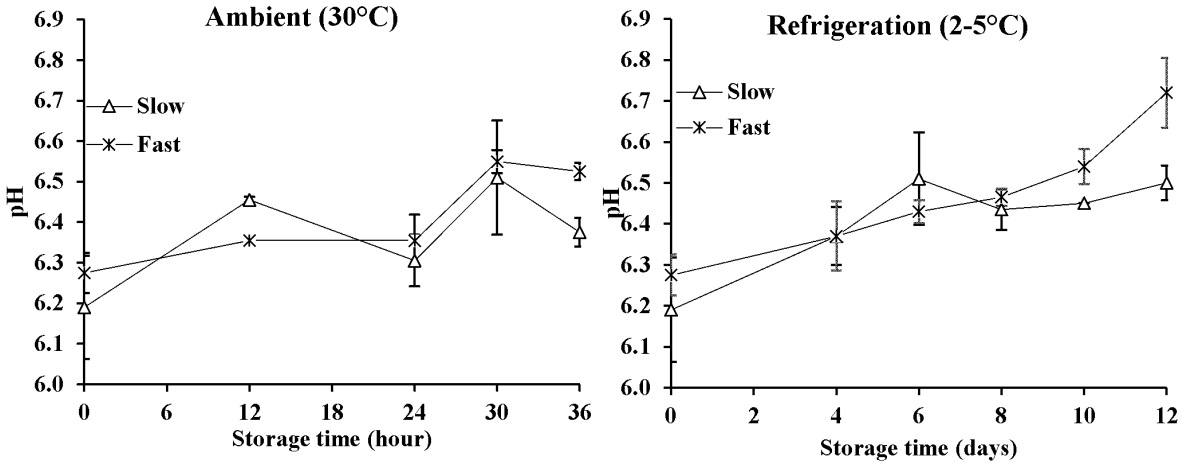

Figure 9. Changes in pH of slow and fast thawed mackerel during storage at 30°C (left) and 2-5°C (right).

4.3 The thawing and microbial count

The microbial load for the FT was significantly higher ($p<0.001$) than ST group kept at 30°C even though both, had low initial counts as shown in Figures 10, 11 and 12 for TVC, H_2S, and Pseudomonads respectively. At 2-5°C only TVC was significantly higher ($p<0.048$) in FT than in ST group.

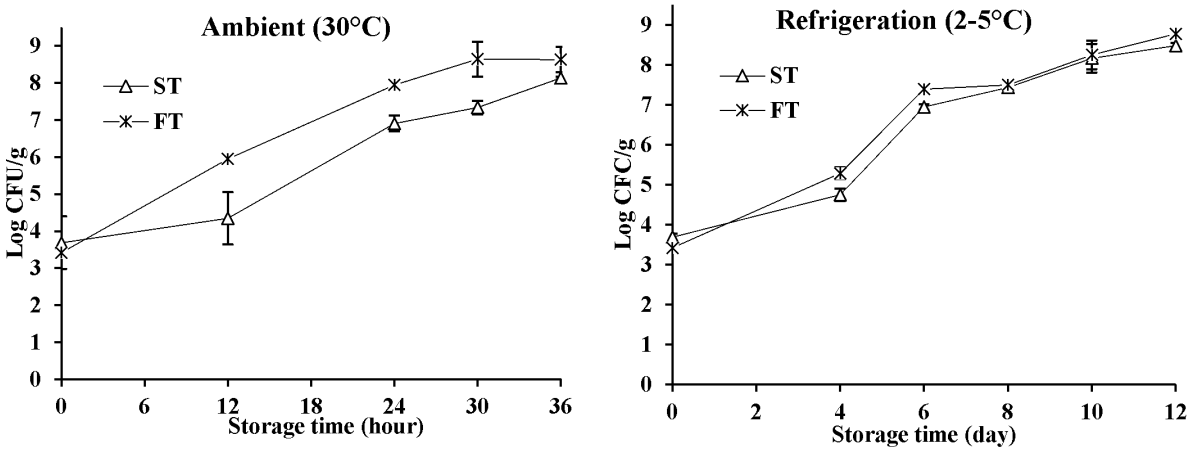

Figure 10. Total viable count of slow and fast thawed mackerel during storage.

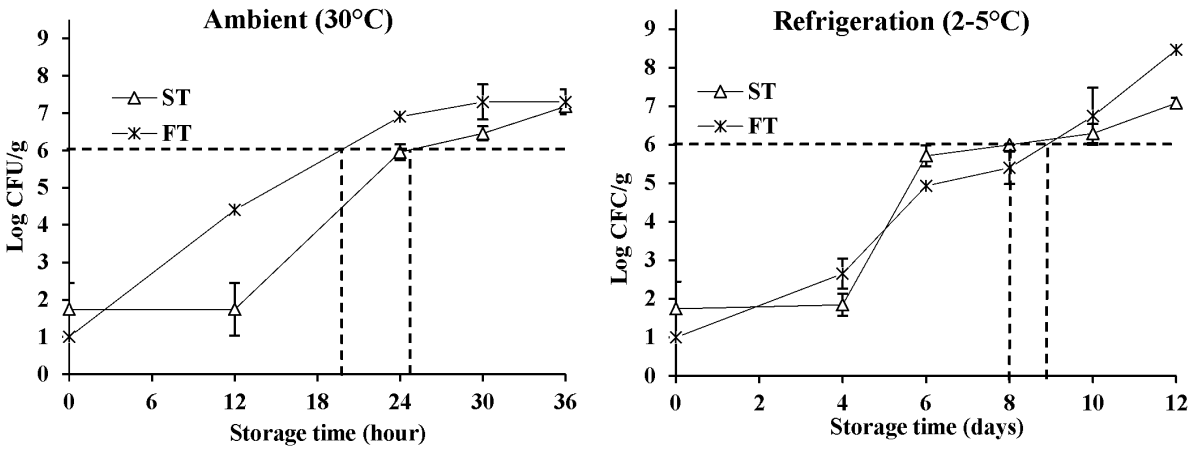

Figure 11. Hydrogen sulphide producing bacteria growth in slow and fast thawed mackerel during storage.

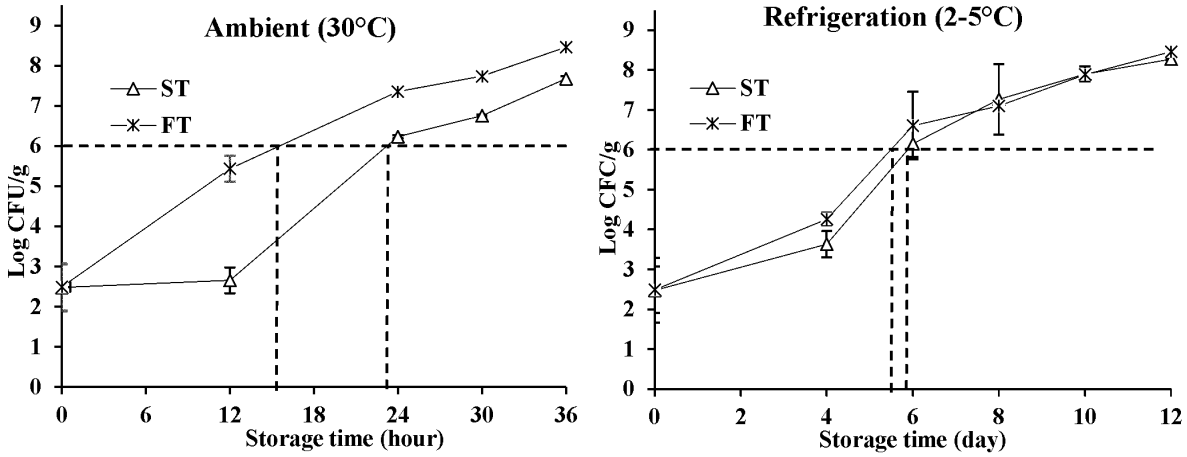

Figure 12. Growth of pseudomonads bacteria of slow and fast thawed mackerel during storage.

These Figures 11 and 12 shows that in this case, Pseudomonas are of greater importance as spoilage bacteria than H_2S producers in this case since they are usually higher.

4.3.1 Proportion of hydrogen sulphide in total viable count

Initially, the proportion of the H_2S-producing bacteria to TVC (Table 2) was 29% and 47% respectively but colonized during storage to constituting above 80%. FT kept at 2-5°C gave the highest proportion of 96%.

Table 2. Percentage proportion of hydrogen sulphide producing bacteria in the total viable count during storage at 2-5ºC and 30ºC.

	2-5°C			30°C	
Day	%ST	%FT	**Hours**	%ST	%FT
0	47	29	**0**	47	29
4	39	50	**12**	40	74
6	82	67	**24**	86	87
8	81	72	**30**	88	84
10	77	82	**36**	88	85
12	83	96			

Percentage proportion from two determination (n=2)

4.4 Influence of thawing and microbial count on Biogenic amines

The estimation of BA was done by extrapolating the standard curve of the amines from several dilutions as shown in Figure 13 for histamine.

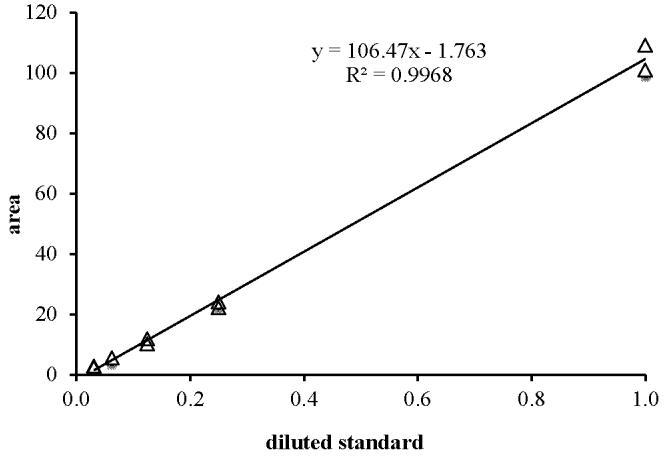

Figure 13. Standard calibration curve for histamine.

The FT and ST did not significantly differ in their levels of histamine, tyramine, putrescine and cadaverine but were dependent on storage time. The formation of the BA during storage time for 36 hours at 30°C and 12 days at 2-5°C are shown in Figures 14 and 15 accordingly.

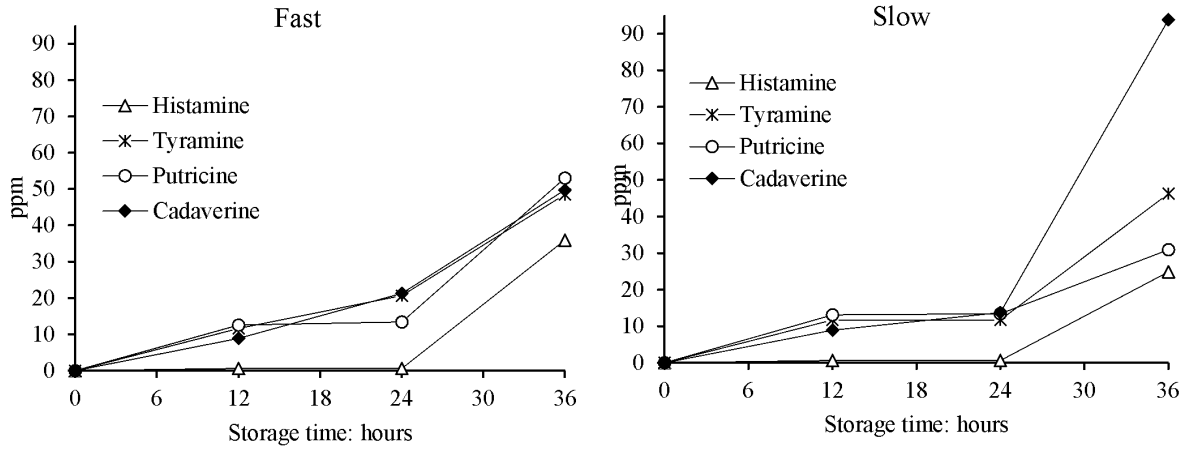

Figure 14. Production of biogenic amine of fast (left) and slow (right) thawed mackerel stored at 30ºC.

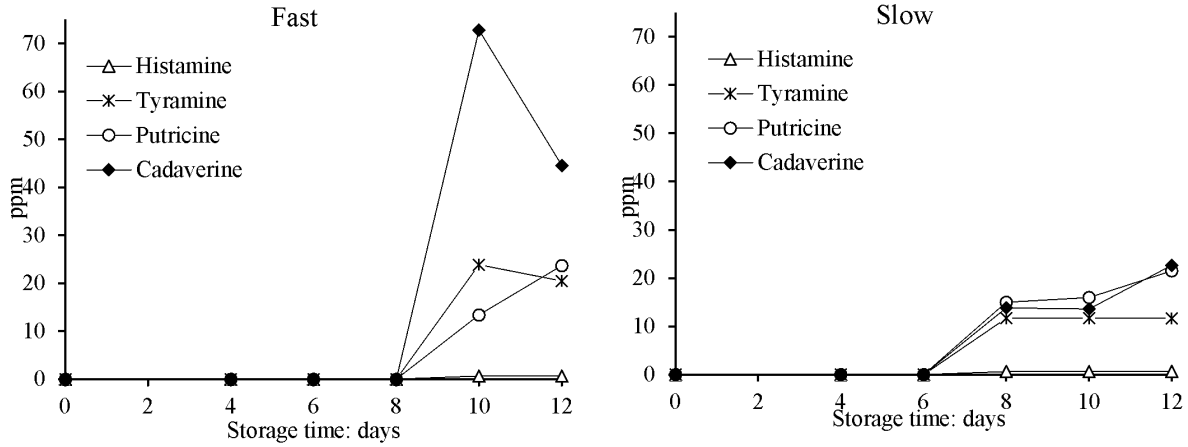

Figure 15. Production of biogenic amine in fast (left) and slow (right) thawed mackerel kept at 2-5ºC.

The influence of high bacterial counts of FT mackerel did not result in increased BA formation. After 12 hours of post thawing storage at 30ºC decarboxylation occurred at relatively low total proportions of H_2S producing bacteria of 40% for ST than 74% for FT. Despite the growth of bacteria at 2-5˚C over time, the production was delayed till the 8th and 10th day when the H_2S producing bacteria was constituting 81% and 82% of the TVC in ST and FT respectively.

The BA values remained low during the entire storage. Only putrescine for FT and cadaverine for both methods kept at 30˚C reached the 50th ppm mark while at 2-5°C, cadaverine went beyond the mark in FT by the 10th day. The rate of BA production as shown in Table 3 indicated that they were not uniform in 2-5˚C in fact with little increase or sometimes reduction. At 30˚C between 24-36th hour the rates were higher.

Table 3. The rate of biogenic amines production in fast and slow thawed mackerel kept at 30ºC and 2-5˚C.

	Hours	Hist F	Hist S	Tyr F	Tyr S	Putr F	Putr S	Cad F	Cad S	Total F	Total S
	0-12	0.05	0.05	0.97	0.97	1.04	1.09	0.74	0.74	2.81	2.85
30°C	12-24	0.00	0.00	0.75	0.00	0.08	0.03	1.03	0.40	1.86	0.43
	24-36	2.95	2.02	2.32	2.89	3.30	1.46	2.38	6.68	10.94	13.05
	Days										
	2-5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2-5°C	4-6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	6-8	0.00	0.30	0.00	5.83	0.00	7.48	0.00	6.92	0.00	20.52
	8-10	0.15	0.00	5.96	0.00	3.35	0.51	18.19	-0.14	27.65	0.37
	10-12	0.00	0.00	-0.85	0.00	2.56	2.76	-7.06	4.54	-5.35	7.30

Hist-histamine, Tyr-Tyramine, Putr- Putrescine, Cad- Cadaverine, F- Fast thawed and S-Slow thawed

All the same the trend for the absolute values of the total BA over the entire storage period at 30°C showed higher values for FT except for cadaverine in ST. No clear pattern was observed at 2-5˚C though (Figure 16).

At the end of the experiment, at 30˚C the total amines were 277ppm for the FT group and 270ppm for the ST group while at 2-5˚C storage a lower total was realized for FT 200ppm and ST 139ppm.

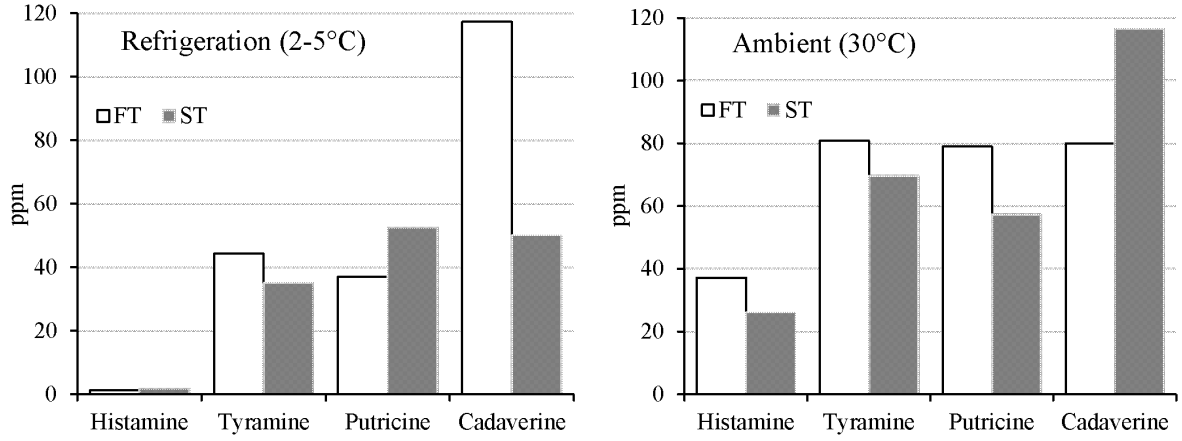

Figure 16. Total accumulated biogenic amines levels in fast and slow thawed mackerel produced during storage at 2-5˚C (left) and 30˚C (right).

4.5 Microbial diversity during thawing and storage

Of the 20 samples in total, 12 samples were successfully analysed by sequencing. Six samples did not yield PCR products mainly from early storage samples while two delivered too few sequences to enable proper analysis. From the 12 samples, a total of 20,271 sequences were analysed by Qiime with an average of 1842 sequences per sample and 586 observed operating taxonomic units (OTU), which were considered as observed species as shown in Figure 17.

To represent the bacterial diversity at beginning of storage, SD1 (ST at 2-5ºC stored for 1 day) as shown in krona chart (Figure 18) with 28 sequences representing 1% of total sequences from this samples was chosen under Qiime pipeline. The *Micrococcaceae* of genus *Rothia* and the genera belonging to typical marine bacteria e.g. SAR group dominated at 17.9% and 14% respectively. Their dominance was, however, run over during storage by rapid growing

spoilage bacteria, as diversity was being reduced when bacteria of *Moraxellaceae* family multiplied from 11% in SD1 to an average abundance of 73% in both thawing and storage conditions.

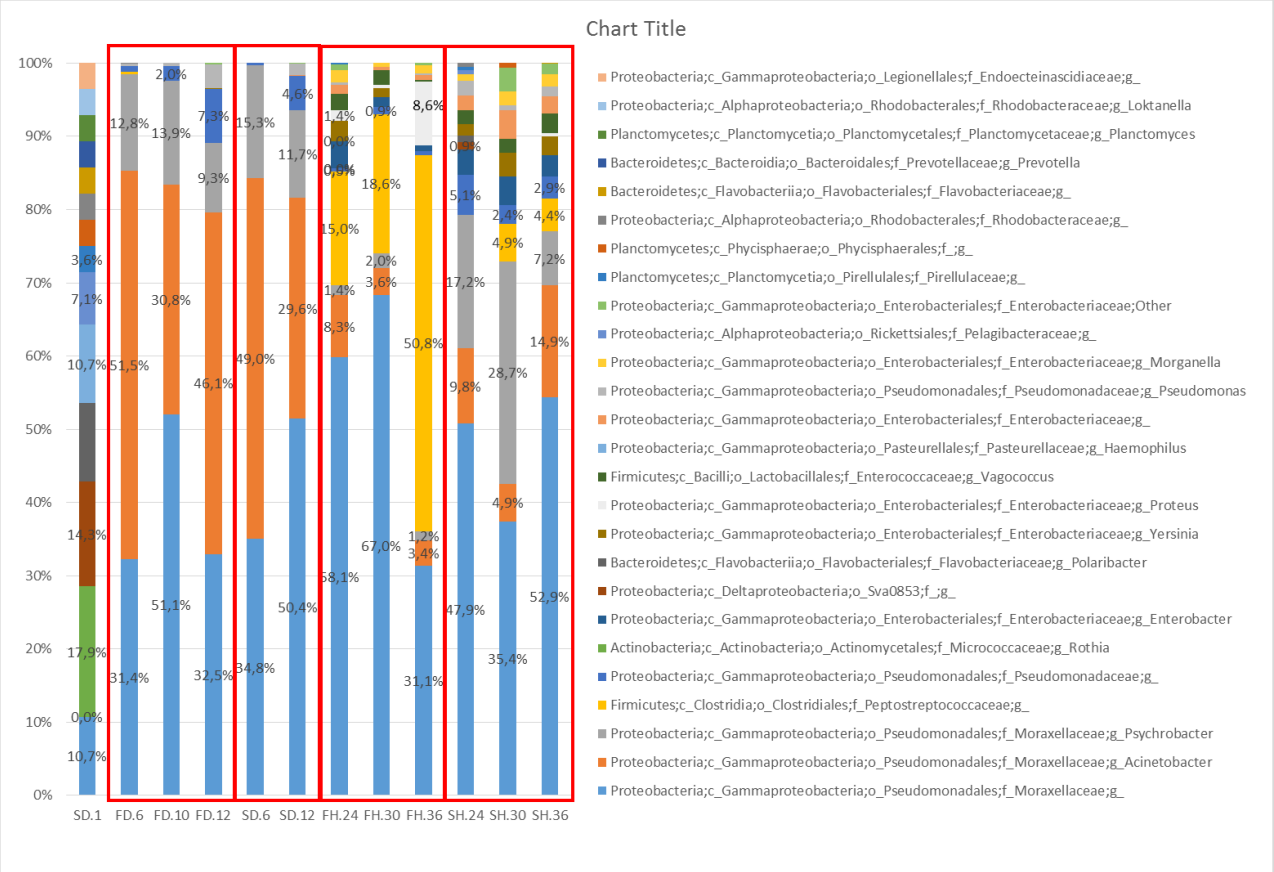

Figure 17. Bar graph presenting 97.7% of all taxa identified in the samples. The remaining 2.7% of the taxa presented contain 72 taxa average 0.28% abundance. c (class), o (order) f (family), g (genus), the labels e.g. FH30 and SD12 corresponds to F fast thawing s stored for H 30 hours at 30°C while SD12; S slow thawing stored for D 12 days at 2-5°C.

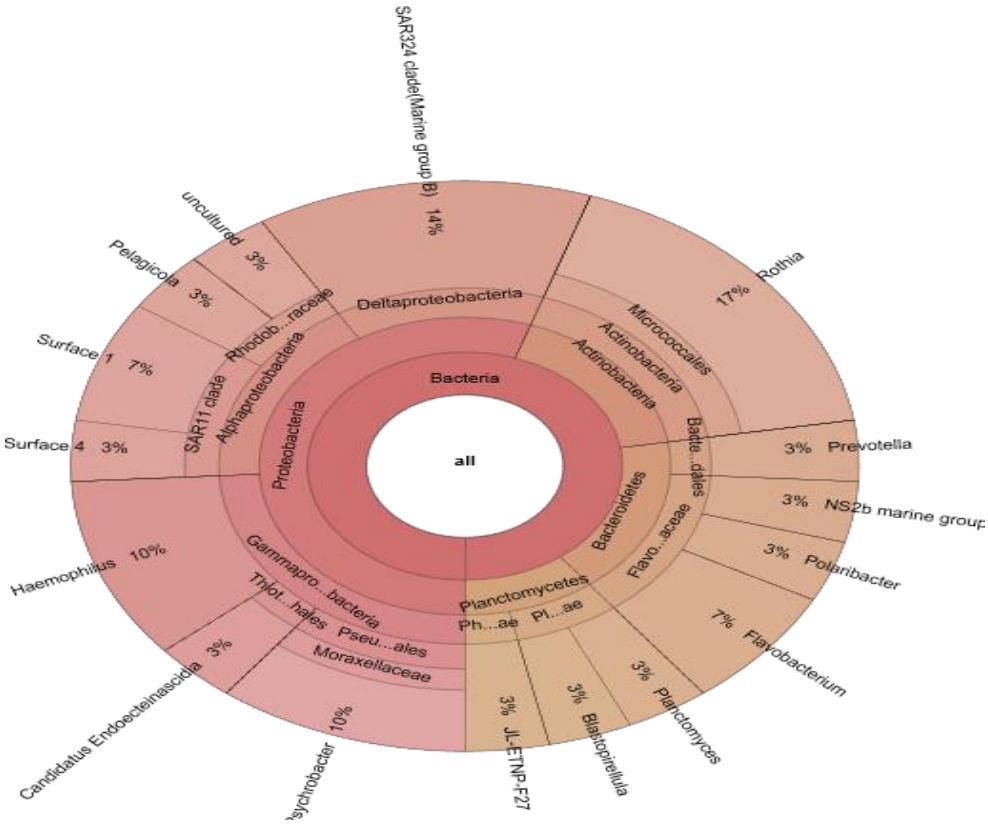

Figure 18. Krona chart showing high diversity of bacteria at the beginning of storage (SD1).

According to Shannon index (Table 4), higher bacterial diversity and richness in the groups SH (ST kept at 30˚C) and FH (FT kept at 30˚C) was observed as compared to SD (ST kept at 2-5°C) and FD (FT kept at 2-5˚C). At the same storage period FD group gave higher diversity than SD group. SH produced, however, a higher and increasing diversity than the FH group which declined with storage time. From principal coordinate analysis (PCoA) (Figure 19) was used to illustrate β-diversity between samples, subsampling 150 sequences from each sample. This analysis shows that FD and SD samples clustered together due to similar bacterial composition as seen by the abundance of Acinetobacter and Psychrobacter genera belonging to Moraxellaceae family based on SILVAngs. Less clustering of samples was observed at ambient temperatures even though SH group clustered relatively well together. FH after 36 hours (FH36) sample was differentiated along the first principal component. This sample showed over 50% abundance of a taxa belonging to the family Peptostreptococcaceae and 8.6% abundance of the genus Proteus belonging to Enterobacteriaceae family.

Table 4 Summary of diversity indices calculated; phylogenetic distance (PD) of OTU within samples, OTU richness (Chao1) and both richness and evenness (Shannon) based on 137 subsampled sequences from each sample. Sample SD1 contained 28 sequences and is not included.

Sample ID	Phylogenetic distance (PD)	Chao1	Observed OTUs	Shannon
FD6	1.414	64.533	33.5	3.409
FD10	1.465	57.245	24.3	2.666
FD12	1.184	54.515	27.2	3.095
SD6	0.768	54.931	20.0	2.478
SD12	1.001	56.843	24.9	2.810
FH24	2.242	106.657	39.8	3.911
FH30	1.849	73.094	30.5	3.076
FH36	1.859	95.733	28.6	2.904
SH24	3.286	150.654	54.0	4.620
SH30	3.165	100.697	49.6	4.631
SH36	2.543	147.158	51.3	4.698

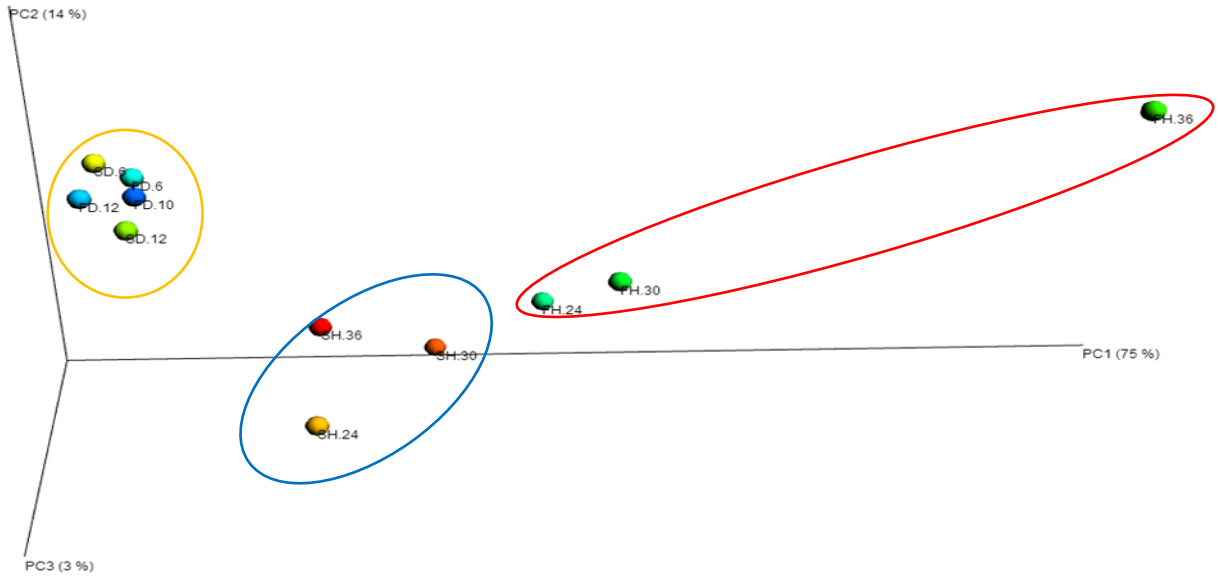

Figure 19. Principal coordinate analysis (PCoA) of weighted unifrac distances between samples derived from bacterial abundances in thawing and post thawing storage analysis. The labels e.g. FH30 and SD12 corresponds to F fast thawing stored for H 30 hours at 30°C while SD12; S slow thawing stored for D 12 days at 2-5˚C Red circle includes FH samples, blue contains SH samples and orange contains FD and SD samples.

5. DISCUSSION

Thawing is an intermediary step in fish processing and therefore determines the quality and safety of fish products (Dincer *et al*. 2009).

5.1 Temperature changes during thawing and storage

The end thawing temperatures of 7°C and 2°C for FT and ST mackerel were in accordance with the general thawing guidelines (Archer *et al*. 2008). It was expected that the initial temperature of fish would be -18°C (the storage temperature prior to experiment) but this was not so partly because the loggers having been placed at the guts' opening, recorded microenvironment temperature created by the contact of fish and the source of thawing energy. The noticeable higher temperature of the fish in the refrigeration condition (2-5°C) was attributed to the polystyrene box insulation property which in turn prevented the fluctuations of the fish temperatures which was apparent in the refrigerator. The higher temperatures of the FT group in ambient 30ºC went beyond the incubator temperature. This could be because of the position of the logger which might have been placed near the heating element.

5.2 pH

During fish spoilage pH increases due to the formation of volatile bases including TMA, DMA, NH_3 and accumulation of peptides and amines (Gram & Huss 1996). The findings in this experiment are consistent with other author's results of increasing pH (Martinsdottir & Magnusson 2001). The pH was also below neutral suggesting that the red muscle of mackerel contained relatively high level of glycogen broken down to lactic acid during storage to maintain the low pH (Oucif *et al.* 2012, Zhang & Deng 2012). Purse seining as a fishing method was used to capture the mackerel used in this study and is generally viewed as a relatively gentle fishing method hence may explain the high post mortem glycogen content (Tzikas *et al*. 2007). It can be concluded from the results that the FT produced a better predictable change in pH than ST mackerel.

5.3 Influence of thawing on microbial count

The low initial counts 3, 1 and 2 log cfu/g of TVC, H_2S and pseudomonads respectively irrespective of the method may be due to the destruction of microorganisms during thawing owing to the growth of ice crystals able to exert pressure on cells. It is also hypothesized that the surrounding of microbial cells becomes diluted during thawing creating osmotic shock hence inhibiting their viability (Archer *et al*. 2008). This may have been more pronounced in the FT than in the ST.

The colonization of the hydrogen producing bacteria in proportion to TVC is believed to be because of their short generation time and hence was above 70%. This is in agreement with findings made by Gram *et al.* (1987).

Based on sensory, high counts of H_2S producing bacteria (6-7 log cfu/g) are required for iced fish to be regarded as spoiled (Chai *et al*.1968, Gram, *et al*. 1987) while at ambient temperatures the rejection may be at lower counts due to accelerated autolytic activities producing off odours (Tzikas *et al.* 2007). Assuming the minimum 6 log cfu/g it is clear that at 2-5°C the ST group gave a shelf life of approximately 8 days (6.00±0.00 log cfu/g) while FT group gave shelf life somewhere between 8 (5.40±0.43 log cfu/g) and10 days (6.74±0.74). A contrary observation

was made at 30°C where ST produced a slightly longer shelf life of about 24 hours when the load was 5.95±0.05 log cfu/g while the FT gave shelf life between 24 hours (4.41±0.13 log cfu/g) and 30 hours (6.90±0.08 log cfu/g). These results are in accordance to the influence of temperature on microbial growth already established by others (Gram *et al*. 1987, Silvia *et al*. 1998), but could also explain the difference between the methods. At the end of thawing (day 0) initial temperatures for FT was 7°C and 2°C for ST group. The low temperature storage (2-5°C) may have had more inhibition of bacterial growth in the FT versus the ST. The elevated temperatures therefore worked for the advantage of the FT for rapid growth than in the ST. This phenomenon has been explained by the fact that the tropical fish (from ambient temperatures) gives longer shelf life than the temperate fish stored at the same condition (FAO 1995).

5.4 Influence of thawing and microbial count on Biogenic amines

As high decarboxylation activities at elevated temperatures at relatively short time was observed and has been reported by many authors (Kim *et al*. 2002, Silvia *et al*. 1998) the delay of formation of BA at 2-5°C may have been attributed to the inhibitory effect of low temperature on decarboxylase enzyme production and activity (Lokuruka & Regenstein 2007).

There seems though to be mixed findings on BA levels from different studies on fish when exposed to both high and low temperatures. Some authors have reported higher values of 200 ppm on fresh mackerel from open markets (Joshi & Bhoir 2011) and some establishing very sharp and high increase in BA at ambient temperatures (Silvia *et al*. 1998, Oucif *et al*. 2012). At 23°C the Indian mackerel was found to have developed over 1690 ppm of histamine after 48 hours (Zare *et al*. 2013).

At low temperatures, early detection of histamine by the 3rd day of storage at 4°C has been reported by some authors on *Chalcalburnus tarichi* developing histamine levels beyond 50ppm (Ekici & Alisarli 2008**).** This was not true in the present study. Low and delayed production patterns agreeing with these results have been reported on mackerel inoculated with *Morgenella morganii* isolates which is a known biogenic amine producer. In that study, the first signs of BA production were at day 6 and reached the final concentration of 45.6 ppm after 14 days at 4°C (Kim *et al*. 2002). Similarly, Indian mackerel stored at 0°C and 3°C resulted in histamine detection on days 9 and 6 and rose slightly to 22.5 ppm and 26ppm after 15 days respectively (Zare *et al*. 2013). The same authors did not however, find the same trend on cadaverine and putrescine which were detected on fresh sample and after 3-day storage, respectively.

Low levels of BA production were observed in gutted Atlantic mackerel kept on ice. It developed histamine of 1.4 ppm, cadaverine of 61 ppm and putrescine of 32 ppm by the 12th day. Those values resonated well with the ones obtained in the present study. However, the same study showed that ungutted samples showed higher values (Lokuruka & Regenstein 2007). The longer storage at lower temperatures do not fully prevent the production of the BA but seems to inhibit production and activity of decarboxylating enzymes (Mendes 2009).

The increase all in all had a positive correlation with time and bacterial growth, a general agreement with these authors. Gutting and de–heading removes gills and guts harbouring high bacterial load (Lokuruka & Regenstein 2007, FAO/WHO 2013, Visciano *et al.* 2012). Further actions of immediate chilling and freezing which has been reported to inactivate Gram negative

bacteria commonly associated with BA production in fish muscle (Takahashi *et al.* 2003) could explain the low content and rate of production of the BA.

Toxicologically, it is clear that the levels of histamine in both the thawing and temperature treatments did not exceed the defect action level limits set by EU of 100 ppm and FDA of 50 ppm (European Commission 2005, FDA 2011) respectively as well as their total being way below 750-900 ppm the suggested limit for the total BA for fish (Ladero *et* al. 2010). Regardless of low BA levels, the fish developed off odours at the end of the storage and would most likely be rejected in a sensory test, as has been established in fish sensory studies (Oucif *et al*. 2012, Raouf *et al*. 2009, Martinsdottir *et al*. 2008).

5.5 Microbial diversity during thawing and storage

Both Gram-positive and Gram-negative bacteria have been established to produce decarboxylase enzymes though in different amounts and forms (EFSA 2011, Kim *et al*. 2002, Hungerford 2010) and under different study conditions. Rarely have the species been isolated from fresh fish but mainly on spoiled fish stored at above 7-10˚C but there is limited formation of BA at below 0˚C (Visciano *et al.* 2012). This explains why there was low and delayed production of BA at 2-5˚C.

Gram negative bacteria from the enteric and marine environment are the main bacteria associated with production of BA in fish (Bjonsdottir-Butler *et al*. 2011). As the production of BA is complementary, it is very difficult to identify which bacteria have the ability to produce multiple decarboxylase enzymes. The rest of the BA may therefore be produced by the histamine producers and so is histamine produced by other bacteria depending on prevailing growth conditions (EFSA 2011).

The bacterial groups that have been found to poses higher histamine (BA) formation of over 1000 ppm in culture broths includes; *Morganella morganii*, *Enterobacter aeroegenes, Raoultella planticola, Photobacterium damselae* (Kim *et al*. 2002, Bjonsdottir-Butler*et al*. 2011, Takahashi *et al.* 2003) while *Proteus vulgaris*, *Erwinia sp., Citrobacter freundii, Citrobacter brakii* and *Hafnia alvei* are classified as low histamine producers of levels between 10-500 ppm (Bjornsdottir-Butler *et al*. 2011, Takahashi *et al*. 2003). It is evident that their abundance was relatively low in the treatments, however, at 30˚C Enterobacteriacea family commonly associated with production of BA at elevated temperatures (Takahashi *et* al. 2003) was higher than at 2-5˚C hence may have been resposible for the BA production.

It may be worth noting that the fish matrix has been cited to inhibit microbial DNA amplification (Takahashi *et* al. 2003) hence the low PCR products.

6. CONCLUSION

The employed thawing treatments in this study did not significantly differ in their effects on biogenic amine production during storage at ambient and refrigeration conditions. However, fast thawing method rather significantly influenced the microbial growth when kept at ambient temperatures (30°C). The rate of formation of the biogenic amines was low as well as their final concentration which were below safe limits even though their increase were highly time dependent. A much higher bacterial load and more time was required at 2-5°C than at 30°C for the decarboxylation activities irrespective of the thawing method emphasising the importance of proper handling both at sea and post-harvest practices in preventing the formation of the BA. In both treatments, the moraxellaceae group of bacteria dominated the fish during storage with low relative abundance of potential BA producers save for slightly higher abundance of Enterobacteriacea at 30°C storage than in 2-5°C.

Because of the low biogenic levels even when the mackerel was obviously spoiled, the use of BA to determine spoilage is not promising and therefore sensory and microbial analysis are still the best indicators of fish spoilage and quality.

7. RECOMMENDATIONS

The aim of the study was to, in proxy, establish the formation of biogenic amines on frozen mackerel being imported in to the Kenyan market. Not the same species was used in the study hence a similar but comprehensive study should be undertaken to evaluate the whole mackerel value chain. This will be able to identify the tropical bacteria responsible for the generation and the levels of the amines in a real chain with different quality of starting material when artisanal handling conditions are incorporated.

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APPENDIX I

Summary of results of microbial counts, biogenic amines of ST and FT thawed mackerel stored at 2-5^{o}C for 12 days and 30^{o}C for 36 hours

		pH S	pH F	TVC S (cfu/g)	TVC F (cfu/g)	H_2S S	H_2S F	Psed (cfu/g) S	Psed (cfu/g) F	Hist S	Hist F	Tyr S	Tyr F	Put S	Put F	Cad S	Cad F
30^{o}C	0	6.19±0.13	6.28±0.05	3.69±0.14	3.42±0.09	1.74±0.71	1.00±0.00	2.48±0.81	2.49±0.59	ND	ND	ND	ND	ND	ND	ND	ND
	12	6.46±0.01	6.36±0.01	4.35±0.25	5.95±0.05	1.74±0.71	4.41±0.13	2.65±0.38	5.43±0.32	0.60	0.60	11.65	11.65	13.06	12.54	8.89	8.89
	24	6.31±0.06	6.36±0.06	6.91±0.10	7.95±0.13	5.95±0.21	6.90±0.08	6.23±0.00	7.35±0.04	0.60	0.60	11.65	20.70	13.42	13.45	13.71	21.27
	30	6.51±0.14	6.55±0.03	7.34±0.04	8.65±0.60	6.45±0.19	7.30±0.47	6.75±0.16	7.74±0.02	_	0.60	_	30.78	_	21.16	_	44.70
	36	6.38±0.04	6.53±0.02	8.14±0.21	8.63±0.01	7.18±0.04	7.30±0.34	7.68±0.12	8.46±0.07	24.85	36.00	46.29	48.51	30.93	53.03	93.91	49.78
2-5^{o}C	0	6.19±0.13	6.28±0.05	3.69±0.14	3.42±0.09	1.74±0.71	1.00±0.00	2.48±0.81	2.49±0.59	ND	ND	ND	ND	ND	ND	ND	ND
	4	6.37±0.07	6.37±0.08	4.75±0.42	5.29±0.15	1.85±0.28	2.65±0.38	3.63±0.33	4.26±0.16	ND	ND	ND	ND	ND	ND	ND	ND
	6	6.51±0.11	6.43±0.03	6.95±0.46	7.39±0.07	5.71±0.27	4.93±0.11	6.15±0.33	6.61±0.85	ND	ND	ND	ND	ND	ND	ND	ND
	8	6.44±0.05	6.47±0.02	7.44±0.41	7.50±0.01	6.00±0.00	5.40±0.43	7.27±0.89	7.10±0.05	0.60	ND	11.65	ND	14.95	ND	13.84	ND
	10	6.45±0.00	6.54±0.04	8.17±0.26	8.25±0.36	6.29±0.25	6.74±0.74	7.90±0.19	7.88±0.08	0.60	0.60	11.65	23.82	15.97	13.39	13.55	72.78
	12	6.50±0.04	6.72±0.08	8.48±0.08	8.78±0.07	7.08±0.13	8.46±0.13	8.27±0.02	8.46±0.13	0.60	0.60	11.65	20.43	21.49	23.65	22.64	44.52

Values of microbial counts and pH are means±sd (n=2) while biogenic amines are from single determination of pooled sample of four (4) pieces mackerel. F- Fast thawed and S-Slow thawed, TVC-total viable count, psed- pseudomonads, Hist-histamine, Tyr-Tyramine, Putr- Putricine and Cad- Cadaverine