

Introduction to Freezing

Chapter 1: Introduction and historical developments in low temperature preservation of fish

1.1.1. Introduction

Several food preservation techniques were evolved in order to enable man to use food produced at various places from age old times. Such methods of preservation of food are sun drying, salting, keeping food in ice, smoking etc.

Fish is a highly perishable commodity as it contains good amount of nutrients and hence there is a greater need to preserve it. Without preservation it is impossible to transport and distribute fish to interior places in good condition.

1.1.1.1. Historical developments in low temperature preservation of foods

In olden days, in places which were covered with snow or ice, the flesh of hunted animals and fish were covered with ice thereby fish was frozen and quality is preserved. However, freezing of fish/ foods by artificial methods is a recent development.

Towards the end of 19th century compressors were developed and because of this machine, novel methods of fish freezing were developed. First patent on freezing of fish using cold air was given to V. Benzamine of England in 1842. In the year 1861, similar patent was awarded to E. Piper of U.S.A. Who used salt ice mixture to freeze food. Russians were pioneers in freezing fish by using machines. They used compressors on board the fishing vessel to freeze fish and also built a freezing plant (factory) in a place called Astrakhan. In 1898 immersion freezing system was developed for freezing fish. Nihoidahl, a scientist from Norway developed spray freezing method. In 1925, a technician named Cook developed contact plate freezer which was further improved by Karensbirdseye in 1929. Similarly methods of freezing and packing it in consumer packs were developed subsequently.

1.1.1.2. Demand for low temperature preserved fish

In most of the processing methods such as salting, drying, canning etc., changes in the sensory characteristics take place. Most people like fish with characteristics as existing in freshly caught fish. Therefore, the principal problem encountered by fishing industry is to preserve fish in such a way that it does not lose its original sensory characteristics. Even for processing by other methods, initial preservation of fish is done by low temperature preservation technique such as icing. Hence **low temperature preservation of fish has become increasingly popular in recent years.**

□ Chapter 2: Structure and function of fish muscle

1.2.1. Introduction

Most of the fish muscle tissue is white but, depending on the species, many fish will have a certain amount of dark tissue of a brown or reddish colour located just under the skin along the side of the body. In pelagic fish, i.e., species such as herring and mackerel which swim more or less continuously, up to 48% of the body weight may consist of dark muscle (Love, 1970). In demersal fish, i.e., species which feed on the bottom and only move periodically, the amount of dark muscle is very small. From technology point of view, the high lipid content of dark muscle is important because of problems with rancidity.

The energy source for ATP generation in the light muscle is glycogen, whereas the dark muscle may use lipids. A major difference is, that the dark muscle contains much more mitochondria than light muscle, thus enabling the dark muscle to operate an extensive aerobic energy metabolism resulting in CO₂ and H₂O as the end products. The light muscle, mostly generating energy by the anaerobic metabolism, accumulates as lactic acid which has to be transported to the liver for further metabolism. In addition, the dark muscle is reported to possess functions similar to those are found in the liver.

1.2.1.1. Lipids

The **lipids present in teleost fish species can be divided into two major groups:** the phospholipids and the triglycerides. The phospholipids make up the integral structure of the unit membranes in the cells; thus, they are often called structural lipids. The triglycerides are lipids used for storage of energy in fat depots, usually within special fat cells surrounded by a phospholipid membrane and a weak collagen network. The triglycerides are often termed depot fat. A few fish have wax esters as part of their depot fats.

The mobilization of energy is much faster in light muscle than in dark muscle, but the formation of lactic acid creates fatigue, leaving the muscle unable to work for long periods at maximum speed. Thus, the dark muscle is used for continuous swimming activities and the light muscle for quick bursts, such as when the fish is about to catch a prey or to escape a predator.

In **elasmobranchs**, such as sharks, a significant quantity of the lipid is stored in the **liver and may consist**

of fats like diacyl-alkyl-glyceryl esters or squalene. Some sharks may have liver oils with a minimum of 80% of the lipid as unsaponifiable substance, mostly in the form of squalene.

Fish lipids differ from mammalian lipids. The main difference is that fish lipids include up to 40% of long-chain fatty acids (14-22 carbon atoms) which are highly unsaturated. Mammalian fat will rarely contain more than two double bonds per fatty acid molecule while the depot fats of fish contain several fatty acids with five or six double bonds.

2.1.2. Proteins

The **proteins in fish muscle tissue can be divided** into the following three groups:

1. **Structural proteins** (actin, myosin, tropomyosin and actomyosin), which constitute 70-80% of the total protein content (compared with 40% in mammals). These proteins are soluble in neutral salt solutions of fairly high ionic strength ($\geq 0.5M$).
2. **Sarcoplasmic proteins** (myoalbumin, globulin and enzymes) which are soluble in neutral salt solutions of low ionic strength ($<0.15M$). This fraction constitutes 25-30% of the protein.
3. **Connective tissue proteins** (collagen), which constitute approximately 3% of the protein in teleost and about 10% in elasmobranch (compared with 7% in mammals).

The majority of the sarcoplasmic proteins are enzymes participate in the cell metabolism, such as the anaerobic energy conversion from glycogen to ATP. If the organelles with in the muscle cells are broken, this protein fraction may also contain the metabolic enzymes localized inside the endoplasmic reticulum, mitochondria and lysosomes. **The fact that the composition of the sarcoplasmic protein fraction changes when the organelles are broken was suggested as a method for differentiating fresh from frozen fish, under the assumption that the organelles were intact until freezing.** The proteins in the sarcoplasmic fraction are excellently suited to distinguish between different fish species, as all the different species have their characteristic band pattern, when separated by the isoelectric focusing method. Fish protein is an excellent source of all essential amino-acids. In diets based mainly on cereals, a supplement of fish can raise the biological value significantly.

1.2.1.3. N – containing extractives

The N-containing extractives can be defined as the water-soluble, low molecular weight, nitrogen-containing compounds of non-protein nature. **This NPN-fraction (non-protein nitrogen) constitutes from 9 to 18% of the total nitrogen in teleosts.** The major components in this fraction are: volatile bases such as ammonia and trimethylamine oxide (TMAO), creatine, free amino-acids, nucleotides and purine bases, and urea in the case of cartilaginous fishes.

in mg/100g weight	Fish			Crustaceans	Poultry	M
	Cod	Herring	Shark species	Lobster	Leg muscle	
amines	1200	1200	3000	5500	1200	
amino – acids	75	300	100	3000	440	
	<10	<10	<10	750	<20	
	20	20	20	100-1000	<20	
	<10	<10	<10	270	55	
	<1.0	86	<1.0	--	<10	
	<1.0	<1.0	<1.0	750	<10	
	400	400	300	0	--	
	0	0	150	100	--	
amine oxide	350	250	500-1000	100	0	

The amount of TMAO in the muscle tissue depends on the species, season, fishing ground, etc. In general, the highest amount is found in elasmobranchs and squid (75-250 mg N/100g) followed by cod (60-120mg N/100g), while flatfish and pelagic fish have the least. Pelagic fish (sardines, tuna, and mackerel) have their highest concentration of TMAO in the dark muscle while demersal, white fleshed fish have a much higher content in the white muscle. Quantitatively, the main component of the NPN-fraction is creatine. In resting fish, most of the creatine is phosphorylated and supplies energy for muscular contraction.

1.2.1.4. Vitamins and Minerals

In general, **fish meat is a good source of vitamin B** and in the case of fatty species **A and D vitamins**. Some freshwater species such as carp have high thiaminase activity so the thiamine content in these species is usually

low. Fish meat is regarded as a valuable source of calcium and phosphorus in particular iron, copper and selenium in general. **Saltwater fish have a high content of iodine.**

Some Mineral constituents of fish muscle

Elements	Content (mg/100 g)
Sodium	30-134
Potassium	19-502
Calcium	19-881
Magnesium	4.5-452
Phosphorus	68-550
Iron	1-5.6
Chlorine	3-761
Iodine	0-2.73

Chapter 3: Postmortem changes in fish

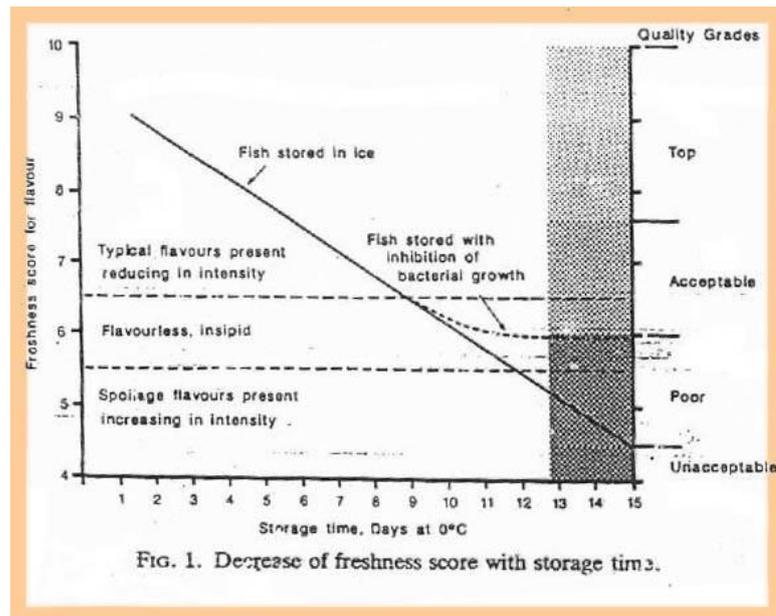
1.3.1 Changes in raw fresh fish

Immediately after death the muscle is totally relaxed and the elastic texture usually persists for some hours, thereafter the muscle will contract. When it becomes hard and stiff the whole body becomes inflexible and the fish is in *rigor mortis*. The rate in onset and resolution of *rigor* varies from species to species and is affected by temperature, handling, size and physical condition of the fish. *Rigor mortis* starts immediately or shortly after death, if the fish is starved and the glycogen reserves are depleted, or if the fish is stressed during fishing.

In *rigor* the fish body will be completely stiff; the filleting yield will be very poor, and rough handling can cause gaping. If the fillets are made from *pre-rigor* muscle, it contract freely and the fillets will shorten following the onset of *rigor*. Dark muscle may shrink up to 52% and white muscle up to 15% of the original length (Buttkus, 1963). If the *pre-rigor* fish is cooked the texture will be very soft and pasty. In contrast, the texture is tough but not dry when the fish is cooked in *rigor*. *Post-rigor* the flesh will become firm, succulent, elastic and best suited for processing.

1.3.2 Changes in eating quality

Changes in the eating quality of chilled fish during storage can be assessed by daily organoleptic examination of the cooked flesh.



Phase 1: The fish is **very fresh and has a sweet, sea weedy and delicate taste**. The taste can be very slightly metallic. In cod, haddock, whiting and flounder, the sweet taste is maximized 2-3 days after catching.

Phase 2: There is a loss of the characteristic odour and taste. The flesh becomes neutral but has no off-flavours. The texture is still pleasant.

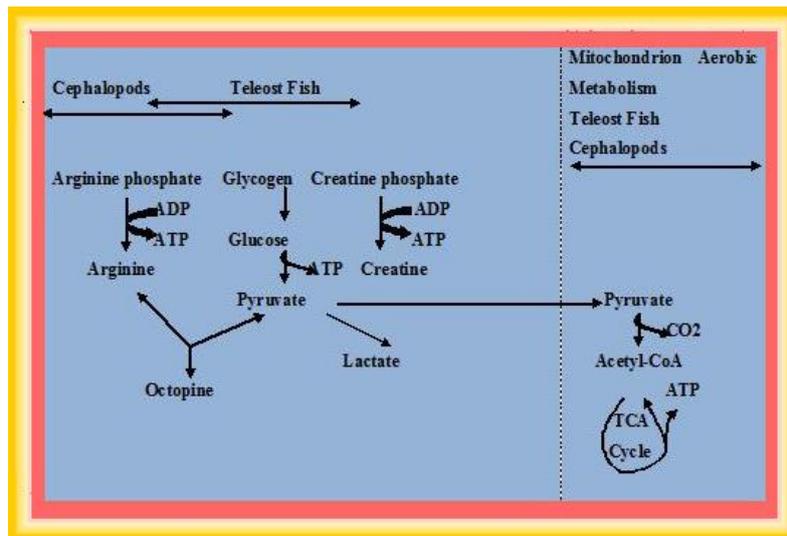
Phase 3: There is sign of spoilage and a range of volatile, unpleasant-smelling substances are produced depending on the fish species and type of spoilage (aerobic, anaerobic). One of the volatile compounds may be trimethylamine (TMA) derived from the bacterial reduction of trimethylaminoxide (TMAO). TMA has a very characteristic “fishy” smell. At the beginning of the phase the off-flavour may be slightly sour, fruity and slightly bitter, especially in fatty fish. During the latter stages sickly sweet, cabbage-like, ammoniacal, sulphurous and rancid smells develop. The texture becomes either soft or watery of tough and dry.

Phase 4: The fish can be characterized as spoiled and putrid.

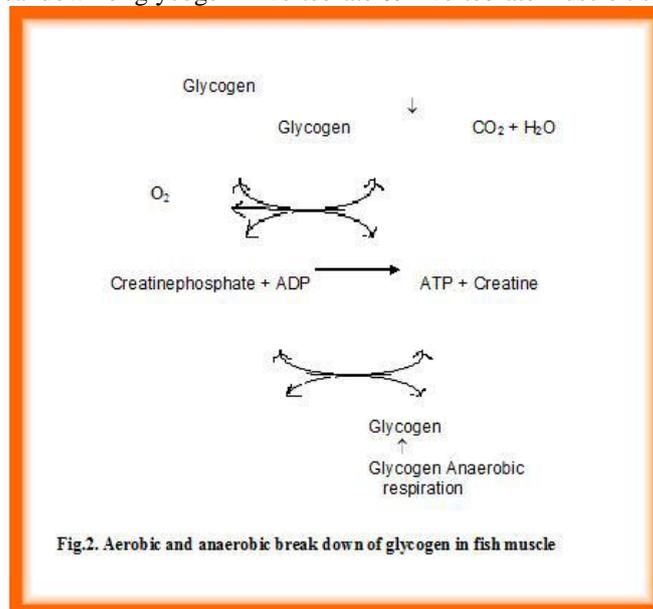
1.3.3 Autolytic changes

Autolysis means “self-digestion”. It has been known for many years that there are at least **two types of fish spoilage: enzymatic and bacterial**.

Muscle enzymes and their activity: At the point of death, the supply of oxygen to the muscle tissue is interrupted because the blood is no longer pumped by the heart and is not circulated through the gills. Since no oxygen is available for normal respiration, the production of energy from ingested nutrients is greatly restricted. Figure illustrates the normal pathway for the production of muscle energy in most living teleost fish (bony finfish). Glycogen (stored carbohydrate) or fat is oxidized or “burned” by the tissue enzymes in a series of reactions which ultimately produce carbon dioxide (CO₂), water and the energy-rich organic compound adenosine triphosphate (ATP). This type of **respiration takes place in two stages: an anaerobic and an aerobic stage**. The latter depends on the continued presence of oxygen (O₂) which is only available from the circulatory system. Most crustaceans are capable of respiring outside the aquatic environment by absorption of atmospheric oxygen for limited period.



Aerobic and anaerobic breakdown of glycogen in vertebrate & invertebrate muscle tissue.



Under anaerobic conditions, ATP may be synthesized by two other important pathways from creatine phosphate or from arginine phosphate. The former source of energy is restricted to vertebrate muscle (teleost fish) while the latter is characteristic of some invertebrates such as the cephalopods (squid and octopus). In either case, ATP production ceases when the creatine or arginine phosphates are depleted. It is interesting to note that octopine is the end-product from the anaerobic metabolism of cephalopods and is not acidic (unlike lactate), thus any changes in *post mortem* pH in such animals are not related to the production of lactic acid from glycogen. For most teleost fish, glycolysis is the only possible pathway for the production of energy once the heart stops beating.

After death, when the regeneration ceases, the ATP is rapidly degraded i.e. after death, the anaerobic muscle cannot maintain its normal level of ATP, and when the intracellular ATP level declines from 7 - 10 $\mu\text{moles/g}$ to $\leq 1.0 \mu\text{moles/g}$ tissue, the muscle enters *rigor mortis*. *Post mortem* glycolysis results in the accumulation of lactic acid which in turn lowers the pH of the muscle. In cod, the pH drops from 6.8 to an ultimate pH of 6.1- 6.5. In some species of fish, the final pH may be lower: in large mackerel, the ultimate *rigor* pH may be as low as 5.8 - 6.0 and as low as 5.4 - 5.6 in tuna and halibut, however such low pH levels are unusual in marine teleosts. In general, fish muscle contains a relatively low level of glycogen compared to mammals, thus far less lactic acid is generated after death. Also, the nutritional status of the fish and the amount of stress and exercise encountered before death will have a significant effect on the levels of stored glycogen and consequently on the ultimate *post mortem* pH. As a rule, well-rested, well-fed fish contain more glycogen than exhausted fish. It is show that bleeding of fish significantly reduced the *post mortem* production of lactic acid.

The *post mortem* reduction in pH of fish muscle has an effect on the physical properties of the muscle. As the pH drops, the net surface charge on the muscle proteins is reduced, causing them to

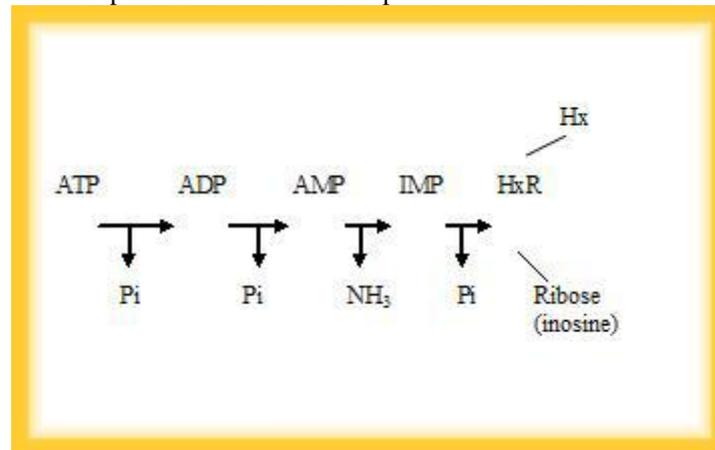
partially denature and lose some of their water-holding capacity. Muscle tissue in the state of *rigor mortis* loses its moisture when cooked and is particularly unsuitable for further processing which involves heating, since heat denaturation enhances the water loss. Loss of water has a detrimental effect on the texture of fish muscle and it has been shown by Love (1975) that there is an inverse relationship between muscle toughness and pH, unacceptable levels of toughness (and water-loss on cooking) occurs at lower pH levels.

1.3.3.2 Autolysis and nucleotide catabolism

Autolysis and nucleotide catabolism

According to the present understanding, rigor mortis is caused by bonding of the myosin heads, extending radially from the thick microfibrils, to the active centers in actin units of the thin filaments. This leads to the formation of a rigid structure of interconnected myofilaments. This reaction between these major muscle proteins is made possible by changes in the regulatory proteins. These changes are in turn induced by an increase in the concentration of Ca^{2+} in the sarcoplasm. In a resting muscle, the concentration of free Ca^{2+} in the sarcoplasm is lower than $10^{-7} M$. This level is maintained because of the action of various calcium pumps, located in the cell membrane and in the sarcoplasmic reticulum, which drives the Ca^{2+} out of the sarcoplasm, against the concentration gradient. The calcium pumps work at the expense of energy from ATP hydrolysis. After the death of the animal, a gradual depletion of ATP in the muscles takes place, due to exhaustion of the creatine phosphate and glycogen reserves. This impairs the action of the calcium pumps and increases the concentration of Ca^{2+} in the sarcoplasm. At ATP concentrations below $10^{-4} M$ and that of Ca^{2+} above $10^{-6} M$, rigor mortis sets in. As the individual muscle fibers contain different quantities of ATP, they do not enter rigor at the same time. Thus, stiffness in a muscle sets in gradually.

The **resolution** of *rigor* is a process still not completely understood but always results in the subsequent softening (relaxation) of the muscle tissue and is thought to be related to the activation of one or more of the naturally-occurring muscle enzymes, digesting away certain components of the *rigor mortis* complex. **The softening of the muscle during resolution of *rigor* (and eventually spoilage processes) is coincidental with the autolytic changes.** Among the changes, one of the first to be recognized was the degradation of ATP-related compounds in a more-or-less predictable manner after death.



Degradation of ATP to hypoxanthine (Hx) and ribose

Freshness is expressed by K-value. This K-value expresses the relationship between inosine and hypoxanthine and the total amount of ATP-related compounds:

$$K(\%) = \frac{\text{HxR} + \text{Hx}}{\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx}} \times 100$$

Very fresh fish, have low K-values. K-values increase gradually which is species dependent. Inosine is said to be more or less flavourless, while **hypoxanthine** has been reported to **impart a bitter flavour in spoiling fish** (Spinelli, 1965). Hx is considered to have a direct effect on the perceived bitter off-flavour of spoiled fish (Hughes and Jones, 1966). It is now widely accepted that **IMP is responsible for the desirable fresh fish flavour** which is only present in top quality seafood.

Chapter 4: Bacteriological changes

1.4.1 Native bacterial flora of fishes

The flesh and body fluids of live healthy fish are generally free from bacteria, i.e. sterile. But, even when the fish is alive, they harbour bacteria, mainly on three sites of their body (i.e.) the slime on the skin surface the gill tissue and the intestine. **Bacteria, which are naturally present on fishes, are called the native bacterial flora of fish.** The population and nature of such flora depend on the waters from where the fish were caught, i.e. whether seawater, brackish water or fresh water. Seawater has more dissolved salts, i.e. a higher salinity than both brackish and fresh water.

Salinity range of water bodies

<i>Water</i>	<i>Salinity range</i>
Sea water	30-36 ppt
Brackish water	10-28 ppt
Fresh water	0.2-1.0 ppt

Depending on the salinity and pollution range of waters, the bacterial flora of fishes varies from water to water. Generally the bacterial population of the fishes from tropical water is in the following ranges .

Bacterial population of marine fishes from tropical waters

Sample	Oil sardine (<i>Sardinella longiceps</i>)	Indian mackerel (<i>Rastrelliger kanagartha</i>)
Skin with slime/cm ²	10 ³ -10 ⁷	10 ⁴ -10 ⁶
Gills/g	10 ⁵ -10 ⁸	10 ⁴ -10 ⁹
Intestine with contents/g	10 ⁵ -10 ⁹	10 ⁵ -10 ⁸

Bacterial population of brackish water fishes

Sample	<i>Pearl spot</i> (<i>Etroplus suratensis</i>)	<i>Milkfish</i> (<i>Chanos chanos</i>)
Skin with slime/cm ²	10 ³ -10 ⁴	10 ³ -10 ⁵
Gills/g	10 ⁴ -10 ⁸	10 ⁵ -10 ⁸
Intestine with contents/g	10 ⁵ -10 ⁸	10 ⁶ -10 ⁸

Bacterial population of fresh water fishes

Sample	<i>Rohu (Labeo rohita)</i>	<i>Mrigal (Cirrhinus mrigala)</i>
Skin with slime/cm ²	10 ⁴ -10 ⁵	10 ⁴ -10 ⁵
Gills/g	10 ⁴ -10 ⁶	10 ⁵ -10 ⁷
Intestine with contents/g	10 ⁵ -10 ⁷	10 ⁴ -10 ⁶

Generally, the bacterial populations on the skin surface are the least, while **bacterial counts in the intestine are the highest.** Bacterial counts in the gill tissue are more or less between the bacterial counts of skin surface and

the intestine. **Bacterial populations exhibit seasonal variations.** During warmer months, the counts used to be higher while during colder seasons, the bacterial counts are lower. Qualitatively, the composition of bacterial flora of the fishes from the three waters differ considerably. **Majority of the bacterial flora of marine fishes are gram negative**, non-spore forming (asporogenous) rods or cocci. Bacterial flora of **fresh water fishes is predominantly gram positive** in nature. The microflora of the **brackish water fishes are evenly composed of both gram +ves and gram –ves.**

From these examples, it can be seen that in the case of marine fishes, not only there is a larger proportion of gram-negative bacteria, but also, there is a large number of bacterial types. But for fresh water fishes, the number of bacterial genera is quite limited.

a. Microbial flora on the skin surface of marine fish:

Gram negative: *Vibrio* , *Pseudomonas* , *Moraxella* , *Acinetobacter* , *Flavobacteria* , *Aeromonas* , *Photobacteria*.

Gram positive: *Arthrobacter* , *Micrococcus* , *Bacillus*.

b. Bacterial flora on the skin surface of brackish water fish:

Gram negative: *Pseudomonas* , *Alcaligenes* , *Flavobacter* , *Moraxella* , *Vibrio* , *Acinetobacter* , Coliforms.

Gram positive: *Micrococcus* , *Bacillus* , *Arthrobacter* , *Lactobacillus* , *Streptococci*.

c. Bacterial flora on the skin surface of freshwater fish :

Gram negative: *Acinetobacter* , *Pseudomonas* , Coliforms.

Grampositive: *Micrococcus* , *Bacillus*.

1.4.2 Factors that influence the growth of microorganisms

There are a number of external and internal factors (intrinsic to the *substrate*, that is, the foods in which they grow) that affect the microbial growth.

Of the different external factors, temperature is the most important one.

(1) Thermophiles (high-temperature loving kind): growth optimum above 45 ° C (35 ° to 65 ° C).

(2) Mesophiles (medium- temperature loving kind): growth optimum above 25° to 37 ° C (15 ° to 45 ° C).

3) Psychrotrophs (cold-temperature tolerant kind): growth temperature is between 0 ° - 25°C (optimum 15 ° - 25 °C).

The bacteria on temperate water fish are all classified according to their growth temperature range **as either psychrotrophs or psychrophiles.** Psychrotrophs (cold-tolerant) are bacteria capable of growth at 0°C but with optimum around 25°C. Psychrophiles (cold-loving) are bacteria with maximum growth temperature around 20°C and optimum temperature at 15°C (Morita, 1975). In warmer waters, higher numbers of mesophiles can be isolated. The microflora on temperate water fish is dominated by psychrotrophic gram-negative rodshaped bacteria belonging to the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella* and *Flavobacterium*. Members of the *Vibrionaceae* (*Vibrio* and *Photobacterium*) and the *Aeromonadaceae* (*Aeromonas* spp.) are also common aquatic bacteria and typical of the fish flora. Gram-positive organisms are *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillus* and Coryneforms can also be found in varying proportions, but in general, Gram-negative bacteria dominate the microflora.

Interaction between growth factors

1) Microbial growth is arrested even when one of the growth factor is controlled. For example, bacterial growth slows down under reduced pH or acidic conditions.

(2) If more than one factor becomes limiting, microbial growth is drastically curtailed or even stopped. For example bacterial growth would stop under acidic conditions combined with low temperature and lower water activity (a_w).

Chapter 5: Spoilage of fish

1.5.1 Spoilage of fish

Spoilage of fish, post mortem, is mainly due to (1) oxidation, (2) autolysis and (3) bacteria. The major cause of spoilage of fish is bacteria, particularly in the case of marine fishes.

The flesh and body fluids of newly caught fish are free from bacteria (except when the fish has bacterial disease). The bacteria present on skin, adhering slime gills and intestine are normally saprophytic. Once the fish is dead, these bacteria invade the fish tissue. There are three main routes for this attack.

1. From the gills into the flesh through the vasculatory (circulatory) system.
2. Through the skin by penetration.
3. Through the peritoneal lining (from the intestinal cavity).

Invasion of bacteria through the first and second routes is faster. Entry through the peritoneal lining can take place only after perforation of stomach and intestinal walls, which normally takes longer time.

The fish muscle contains 15 to 18% protein. Bacteria attack the protein and break it down to peptides and amino acids. Initially, bacteria live and multiply in the fish tissue, utilizing the low molecular weight compounds like carbohydrates and amino acids present in small quantities in the muscle. Due to post-mortem enzymatic break down of the macromolecules in the muscle, enough low molecular weight compounds are formed, which serve as the food of bacteria. Subsequently bacteria elaborate proteolytic enzymes, which break down proteins to peptone, polypeptides, lower peptides and finally to amino acids. Bacteria will metabolize amino acids, in different ways leading to the production of odouriferous and foul smelling compounds like ammonia, hydrogen sulphide, mercaptans, indole, amines and organic acids.

When the fish is left in ambient temperature, which is usually 28 ± 4 °C, tropical fishes get spoiled within 6 to 12 hrs. depending on their size. In order to prevent such spoilage, many methods are in practice. Drying, icing, freezing and use of chemicals are some of the usual methods. The basic principle involved in these methods of preservation of fish is to control the activities of the microorganisms.

Typical spoilage compounds during spoilage of fresh fish stored aerobically or packed in ice or at ambient temperature

Specific spoilage organism	Typical spoilage compounds
<i>Shewanella putrefaciens</i>	TMA, H ₂ S, CH ₃ SH, (CH ₃) ₂ S, Hx
<i>Photobacterium phosphoreum</i>	TMA, H ₂ S
<i>Pseudomonas spp.</i>	Ketones, aldehydes, esters, non-H ₂ S Sulphides
<i>Vibrionaceae</i>	TMA, H ₂ S
Anaerobic spoilers	NH ₃ , acetic, butyric and propionic acid

Substrate and off-odour / off-flavour compounds produced by bacteria during spoilage of fish

Substrate	Compounds produced by bacterial action
TMAO	TMA
Cysteine	H ₂ S
Methionine	CH ₃ SH, (CH ₃) ₂ S
Carbohydrates and lactate	Acetate, CO ₂ , H ₂ O
Inosine, IMP	Hypoxanthine
Amino-acids (glycine, serine, leucine)	Esters, ketones, aldehydes
Amino-acids, urea	NH ₃