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## STUDIES FOR PRESERVATION OF SARDINE IN BRINE

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## I. Introduction

### 1. General Background

A substantial part of the arable lands of the world has been brought under cultivation, forests have been leveled, swamps drained and deserts irrigated. Concurrently, the fishing frontiers have been pushed further out to sea from the immediate coastline of all continents. Many species of fish are pursued over thousands of miles of ocean throughout their range of distribution.

In spite of all of these great advances made in the knowledge of food and nutrition and in its application to raise the level of nutrition, it is unfortunate that, a large majority of the human population still lives on a suboptimal nutritional level in light of the dietary standards recommended by appropriate international and national bodies<sup>1,2)</sup>. This is in regard to both qualitative and quantitative deficiencies in the diet of populations over large areas of the world.

Although deficiency diseases like scurvy and rickets are not common today, there is fairly widespread protein malnutrition in many countries. This occurs particularly in children, in the late weaning and post-weaning periods<sup>2)</sup>. It should be pointed out that the damage that may be done to the body in early life by malnutrition can hardly ever be made up for later in life by correct or even abundant feeding. The vulnerable groups of the population (children and expectant and nursing mothers) have therefore to be looked after with particular care given to their food consumption according to optimum nutrition standards.

Malnutrition is to a great extent the result of an inadequate consumption of high quality protein which is needed to complete and to balance the diets of peoples whose food contains preponderantly vegetables and cereals. Sadly enough, young growing children and expectant mothers suffer most from a lack of proteins containing sufficient quantities, and the correct proportions of the required amino acids<sup>3)</sup>. Milk, eggs, meat, and chicken all contain high quality animal protein but are often too costly and are not universally available. The need is great for a satisfactory, inexpensive animal protein of high quality which would be suitable for incorporation into the diets of people suffering from protein malnutrition.

Fish is as good a source of animal protein, vitamins, and inorganic components as are other flesh foods such as meat and poultry. The amino acid balance in the protein is unexcelled by that of any other foods. The content of B vitamins approximates that of meat, and the mineral content is richer in iodine than that of any other foods. Fish is also a fairly good source of calcium and phosphorus, particularly in the case of small fish which are eaten with the bones. It is also a source of iron and of a trace element like copper<sup>2,4)</sup>. In regard to most of these nutrients, fish would appear to be comparable with meat. Difference species and varieties of fish vary in regard to percentages of protein, fat, mineral matter and vitamins. There are varieties of freshwater fish which contain as low as 0.6% fat.

Those species of fish having a low oil and high protein content and which represent a majority of all species, contain lower calorie content per unit of protein than do meats or poultry<sup>2,4</sup>. Thus these lean fish are an ideal source of animal protein for use in reducing diets.

On the other hand, fish having somewhat higher oil content in the range of 5 to 15%, while of medium oil content for fish, corresponds to an average lean meat with respect to fat content. Furthermore, such fish contains up to 1/3 of its oil in a highly polyunsaturated form with four, five, and six double bonds per fatty acid. This type of oil has been shown to be effective in lowering blood cholesterol. Such fish oils are effective even when included in the diet with much larger quantities of saturated fatty acid<sup>4,5</sup>.

Apart from quality, fish constitutes also quantitatively a good source of protein. The edible portions of freshwater and estuarine fishes investigated contain about 14-25% protein. Also, in marine fishes it varies from 9-26%<sup>2</sup>. Thus qualitatively and quantitatively, fish consumption could in significant measure supplement the low-protein, high cereal diet consumed in many countries of the world. Cereal proteins are rather low in lysine and methionine, both of which fish protein is relatively rich in.

Fish is, therefore, a good food, the consumption of which deserves to be encouraged to promote the level of nutrition in the whole world, and particularly in countries having a high rate of malnutrition among their population.

An analysis of food consumption tables shows<sup>6</sup>, however, that except in a very few countries, i.e., in Norway, Chile and Japan, where figures for daily *per capita* consumption are in the order of 20 g, 87 g and 60 g respectively, fish and fish products constitute a relatively minor proportion of the total diet in the world. Even in the aforesaid countries, where *per capita* consumption is the largest, it provides not more than 2-4% of total calories, 10-20% of total protein and 2-15% of the total fat content of the diet. Figures are worst for developing countries where average daily consumption of animal protein ranges from 8-15 g. These statistics illustrate in part how far behind the former countries are in meeting the dietary requirements of their populations<sup>6</sup>. Therefore, consumption of fish in the world should be stepped-up wherever possible, and particularly in countries where a high-cereal, low-protein diet is consumed, because there appears to be a definite supplementary relation of the proteins of fish to those of cereals. They have also been reported to have a definite value in combatting protein malnutrition in early childhood<sup>2</sup>.

Although a staple diet of recognized value, fish is a highly perishable material and it has always been difficult to preserve and distribute. It undergoes autolytic, microbiological and chemical decomposition quite easily. The consumption of fish oils, which had deteriorate because of oxidation, has been reported to produce injurious results on animal<sup>2,5,7</sup>. Since most countries consuming a low-protein diet are locate in tropical and sub-tropical regions of the world, the consumption of fish in these areas can therefore be stepped-up only if the fish is properly preserved. Moreover, in tropical and sub-tropical countries where inexpensive protein is needed most, the means of preservation, transportation and distribution are least available<sup>3</sup>.

A high priority has to be given to the means of increasing the availability of fishery products on the market. For this purpose, roads are required and appropri-

ate means of transport, storage, and distribution have to be acquired or built, and an efficient marketing organization created. In the field of consumer education, the first step is to familiarize potential buyers with fishery products. In the long run, the program must aim toward breaking down pattern which discourage consumption of fish and which may be traceable to national tradition or religious taboos.

From a nutritional stand point, the most tragic fact is that those whose diets are the poorest are often those least able to buy fish and other animal protein products.

Consumption of fish in developing countries, of course, could be expected to expand substantially if people not used to eating fish (because of unfamiliarity with the products, ignorance of their nutritional value, or because consumption is subject to taboo) were to acquire the habit of including fishery products in their diets. Rigid preferences for certain species and certain forms, with discrimination, at the same time, against other species and other forms have had a depressing effect on the levels of demand<sup>3)</sup>.

Other preferences encountered in developing countries relate to size, fat content, texture, and other physical characteristics of the fish. Large fish are generally preferred to small fish, and consequently fetch better prices in the market. In some countries, however, the government is trying to encourage consumption of small fish. The policy, however, is dictated by the nature of the resources exploited, in which small fish predominate.

Herring and herring-like fishes, small pelagic species such as sardines and anchovies, undoubtedly constitute the dominant group of the world fish catch. This is explained by their mass occurrence in all parts of the globe. The total catch of herring, sardines, anchovies, etc., amounts to approximately one-fourth of the total world sea-fish catch<sup>8)</sup>. At present most of this catch is processed into fish meal or not caught because of a lack of utilization prospects<sup>8,9)</sup>.

Actually it is deplorable that fish processed into fish meal, at the present time, is not wholly converted into direct use as human food. This is primarily due to technical reasons. Furthermore, there is heavy competition between the well-fed domestic animals of the world and human beings in these developing areas. Agriculture is given almost entire credit for the flourishing postwar animal production of Europe. However this can not justify an extensive use of fish protein for animal feeding. Few people realize that several major European countries feed more fish protein to their domestic animals than they do to human beings. It is most surprising to find in this same category South Africa, a country where a large protein of the population is seriously short of protein<sup>10)</sup>.

The United States constitutes the major market for world fish meal, surpassed only by Europe when all individual countries there are added together. The United States' purchase of protein could, in fact, satisfactorily fill the protein gap of the highly protein deficient South American continent. A channeling of the fish protein now used as feed for domestic animals into human use could appreciably improve the poor protein standard in many regions and indirectly enhance productivity. Undoubtedly such fish protein would constitute an excellent basis for world-wide relief<sup>6)</sup>.

This protein deficiency is worth observing in discussions concerning economic balance and surplus problems. On the basis of the experience of Japan and most

European countries it is correct to assume that agriculture in Latin America would hardly be able to provide sufficient protein to feed its rapidly growing population, particularly if the nutritional standard is going to be improved in those many countries which now live on a substandard level in this respect. Fish would not only be the cheapest but also the most efficient way of ameliorating these present conditions. This explains the great interest which today is attached to the development of fishery resources in Latin America.

There are strong and conflicting attitudes towards the use of fish protein for domestic animal consumption. Opponents of this use state "we can not justify feeding fish to animals at all"<sup>11</sup>). Others have made the point that the largest producers of fish meal are often protein deficient countries that export their product to industrial countries, thus precluding domestic use of this source of protein<sup>5</sup>). The argument continues with the fact that exported fish meal brings hard currency to producing countries; Peru's exports of meal and oil in 1990, for example, had a value of more than \$450 million<sup>12</sup>). This is an impressive sum, and its potential for meeting human needs must be recognized. It should also be recognized that in the same terms, the 5.4 million tons of fish converted into meal and oil in 1990 would have benefited more people had it been possible to use that fish as human food. And that limitation is the rub. Approximately 90% of the meal produced is from pelagic species (anchoveta, herring, menhaden, anchovy, pilchard, saury, etc)<sup>9,11</sup>). These species are oily, have dark-colored flesh, strong flavors, and become rancid easily. Their dense schooling behavior favors low-cost capture and the economics of meal production. Although many people use small, oily species by drying, salting, smoking and fermenting to make fish sauces, the 29.9 million tons of fish processed into meal in 1988<sup>12</sup>) makes evident the fact that technological improvements in handling and processing into food products that meet wide acceptability could have a great impact. In some parts of the world, consideration is already being given to work towards this end.

The majority of oily species used for meal production could be processed into food to benefit both protein-deprived populations and manufacturers of meal and oil, who could just as well be processing these species into acceptable human food. The difficulty of using small pelagic species starts with the nature of the fisheries for them which are seasonal and characterized by high volumes landed in short periods. These are often 'glut' traditional markets, and are wasted. Ways of increasing the radius of distribution and of storing the catch, as well as extending the fishing season, are therefore required. The quality of the protein of these small fish is just as good as larger ones but it is not surprising that only a few species are used for human consumption<sup>8</sup>). We must consider the following points: small fish cannot be eviscerated economically on a large scale; they tend to spoil rapidly and the flesh tends to be soft; the fish is bony and the skin fragile. These are the problems of the intrinsic raw material quality that the technologists have to tackle. Present indications and experience suggest that there is no difficulty in the actual catching operation but problems start as soon as the fish leave the water. In order to overcome rapid softening they must be chilled, an expensive process when prices paid for the fish are taken into consideration. Chilled sea water systems are either in use or under investigation in many parts of the world. They show considerable

promise in the reduction of belly bursting and induction of rancidity which are the two major indicators of incipient spoilage. However, more study is required so as to ensure minimum quality loss during storage.

Following landing, fresh fish distribution can be extended over a much greater radius if the raw material is in first-class condition. In many developing countries this can be assured if handling improvements, are introduced, as most catches are made in the immediate vicinity of the landing place. Increasing fresh distribution is mainly affected by the educational process and agreeable economic returns.

Assuming that technological advances can result in the landing of raw material of sufficiently good quality for processing, the range of options, or available products, is, as described previously, very large. It is clear, however, that new technology will be required in order to make full use of the raw material because present processes are generally not capable of massive throughput to produce cheap products.

Unless technological methods are employed to make small fish and their products available to the people without deterioration, and without considerable loss of its nutritive value, its consumption cannot be increased to the extent which may be desired<sup>11)</sup>.

Methods for preserving the quality of the fish from the time they are caught until they pass through the distribution network and reach the consumer have been studied by technologists the world over. Apart from the use of ice, which can preserve fish for only very short periods, the methods used in different countries have been to salt the fish, dry it, or prepare fermented products (pastes and sauces) out of it. Even though tremendous advances have been made, quality assurance is still the foremost problem of an industry that deals with a highly perishable commodity. Fish spoil rapidly. In contrast to animal products, bacteria associated with fish can thrive at ice temperatures, the actomyosin system of fish is more sensitive to denaturation, and the highly unsaturated fats are very susceptible to oxidative rancidity<sup>13,14)</sup>. From a technological standpoint, it would seem appropriate under the circumstances to freeze or otherwise process most fish products rather than attempt to market them in fresh conditions. The consumer, however, prefers fresh fish even though it is many instances impractical to supply it. Quality again has played a role in establishing this preference<sup>14)</sup>.

Because there is a high demand for fresh fish far from local fishing ports, the need for long term fresh fish preservation techniques, which begin from the point of harvest, maintains quality and promote sales, must continue to be addressed. The assurance of fresh fish quality encompasses the degree of freshness and any deterioration throughout the period of harvesting, storage, processing, distribution, and sale. Insufficiency of ice for preservation for the distribution of fresh fish in the marketplace is noted and the need for its replacement or supplementation was affirmed<sup>14)</sup>.

The Peruvian fishery is particularly favored by the upwelling of deep water masses, rich in nutritive substances, in the area of the Humboldt current. Fishers such as sardines and anchovies constitute the dominant groups of the Peruvian fish catch. They appear in big shoals, thus making intensive and profitable fishing possible. The annual landing of sardine in Peru is about 6 thousand tones which represents about 95% of the total catch<sup>12)</sup>. Thus, sardine is the most abundant

species in the country. Unfortunately nearly 90% of the Peruvian fishing yields are processed into fish meal for animal food leaving only a small percentage for direct human consumption. The high percentage of the landed sardine being turned into fish meal is mainly due to the following reasons.

- Small size and high volume caught ;
- Lack of freezing and refrigeration facilities on shore ;
- Bad quality of the sardine due to improper handling and transportation on board.

In view of the prevailing shortage in animal protein which a great part of the population is suffering, it would seem essential to see that greater quantities of the catches would be utilized for direct human consumption<sup>11</sup>. The consumption of fish, available in sufficient quantities, can be expected to help considerably in correcting the state of malnutrition so widely prevalent in Peru today. It would be particularly valuable to provide proteins of high quality comparable with those of meat, milk or eggs. This similarity is indicated by biological experiments as well as by direct amino-acid analysis<sup>2,4</sup>.

Chilling or refrigeration systems on board aims at retaining the fresh fish character. Chilling media are wet ice, mixtures of ice and seawater or ice-cold sea water. With this kind of preservation method, one to two weeks could be the normal storage life<sup>7</sup>. Unfortunately only a few of the Peruvian sardine catcher vessels are equipped with some kind of chilling or refrigeration system. Furthermore, the capture is transported in bulk, on deck, inside compartments with removable shelves. The height of fish in these compartments reaches sometimes one meter or more. Thus, the fish is exposed and rapid decomposition occurs.

The exposure of such conditions, however, reveals the world over the need for a new methodology for fish preservation. However, any new methodology would not only have to extend shelf life in a safe manner and result in a quality product, but it must fit easily into existing seafood industrial technology.

## 2. The Aim of the Present Study

Fish is one of the most perishable foods and needs proper care from the time it is caught until it is served or processed. The handling of fresh fish during this interval determines to what extent deterioration takes place from three sources — enzymatic, oxidative, and bacterial. How rapidly each of these progresses during the spoilage of the fish depends upon, first, the application of basic principles of food preservation and, second, the variables of the species and the fishing methods<sup>15</sup>.

Investigations have shown, that fish muscle contain high amounts of free amino acids<sup>4,16</sup>. So invading bacteria find sufficient free amino acids for growth and reproduction. This fact in addition to its low connective tissue content, and high water content, are responsible for the rapid spoilage in fish<sup>16</sup>. After fish death spoilage organisms on the skin, gills and in the intestinal tract multiply rapidly after a lag phase, to reach numbers exceeding one million per gram wet muscle. Subsequently, the bacterial enzymes convert the TMAO into TMA and decompose the amino acids and proteins forming ammonia, hydrogen sulfide and other undesirable compounds characteristic of microbial spoilage<sup>7</sup>.

The principal aim of fish preservation is to delay, reduce or inhibit microbial



spoilage. In the case of fatty fish, the preservation may also aim at reducing or inhibiting oxidation and other undesirable changes in the fish oils, which are highly unsaturated and capable of going rancid at various stages of processing. Factors affecting life and death of microorganisms are temperature, water activity ( $a_w$ ), pH, and oxygen availability<sup>17)</sup>.

The most important means of preservation of fresh fish in tropical and temperate climates is to chill to about 0°C. As the temperature is lowered, microbial growth slows and eventually stops, thus bringing about a preservative effect. The normal storage life of cold water fish chilled immediately *post mortem* is one to two weeks, while fish from warm tropical waters keep somewhat longer<sup>7)</sup>.

Unfortunately in certain societies where fish is caught abundantly, the availability of fishing vessels equipped with some kind of chilling or refrigeration system is insufficient. Thus, fish are liable to suffer rapid decomposition. The solution to this problem is a challenge and the finding of such a solution is precisely the aim of this investigation.

The growth and metabolism of microorganisms demand the presence of water in an available form. The most useful measurement of the availability of water is water activity ( $a_w$ ). The  $a_w$  in a food may be reduced by increasing the concentration of solutes in the aqueous phase of the food, either by removing water or by adding solutes. Some water molecules are oriented about the solute molecules, and others become absorbed onto insoluble food constituents. In both instances the water becomes less available to enter into reactions.

In curing or salting, it is the addition of solute which lowers  $a_w$  and preserves the foods. The principal preservation action of salt is achieved by the lowering of the moisture content and consequently  $a_w$  of the foods or solution in which it is contained. This is enhanced by the fact that at high salt concentrations most bacterial action is halted or at least greatly retarded, although halophilic bacteria are not so affected. Furthermore, in the brine salting process which is used for salt preservation of various oily fish such as herring and salmon, the fish are kept beneath the surface of the brine. This helps to minimize oxidation of the oils in such fish by keeping oxygen away to a large extent<sup>7)</sup>.

For thousands of years, the acidity of food has been increased; either naturally by fermentation or artificially by the addition of a weak acid, to enhance microbiological stability and preserve the foods. A low pH can also assist in the preservation of foods by directly inhibiting microbial growth, thus in effect prolonging the keeping quality of the food or fish to a considerable extent.

Another way of preserving food is through the use of antimicrobial food additives. Intentional food additives include chemicals which are used for nutritional purposes, consumer acceptance, preservation of quality and processing. These chemicals are needed to efficiently produce an abundance of high quality food. The way they preserve food is closely related to changes in pH and  $a_w$ .

Taking into consideration all the above factors, for the realization of this work, it was decided to test the use of brine alone (NaCl solution) at different concentrations, as well as brine and sea water with some preservatives for fish immersed storage.

## II. Brine in Fish Preservation

Spoilage of fish is brought about chiefly by autolysis and bacterial decomposition. In autolysis, chemical and physical changes are brought about by enzymes contained in the cells of the fish after its death. It begins almost immediately after death and proceeds most rapidly at high temperatures. The lower the temperature, the slower the action. The blood, certain tissues, and glands, such as the kidney, contain very active enzymes. The autolytic enzymes are most active under slightly acid conditions; in alkaline media their activity is greatly reduced. They are most active in dilute solution and do not act in the absence of water. Most enzymes are destroyed or rendered inactive by concentrated salt solutions, therefore salting preserves fish from autolytic decomposition<sup>7,18</sup>.

Sodium chloride in concentrated solution possesses antiseptic properties since it extracts water; salt has therefore been used to keep meat and fish since prehistoric times. In salting fish it is generally very important to select methods which facilitate rapid removal of moisture and penetration of salt; this is especially important in hot climates where the onset of spoilage is rapid unless the preservative effects of the salting process are expedited.

Penetration of salt into the fish depends upon the quality of the sodium chloride. The temperature of the fish and brine during salting is another important factor. Raising the temperature accelerates the rate of penetration of salt into the fish. It also, however, increases the rate of bacterial spoilage. The salt concentration of the solution surrounding the fish is, of course, also an important factor with more rapid penetration and removal of moisture taking place at higher salt concentrations<sup>7,18,19</sup>.

### *Chemical and Sensory Changes in Fish*

From the moment fish is taken from the water a series of deteriorative changes start to occur which eventually will render the fish unmarketable. These changes occur as either the result of microbiological action or can be classified as being some form of more or less pure chemical changes. In most cases, deteriorative changes resulting from microbiological action are the most extensive.

Fish proteins undergo various chemical changes. In some special cases, notably in cold storage of frozen fish, largely chemical alteration in the lipids is involved<sup>4,5,7,16</sup>.

Nucleotide degradation in fish muscle starts immediately after death and their degradation products, apart from having implications in the flavor of fish, are also important as indices of freshness and quality. All the methods of freshness assessment based on the measurement of the products bacterial action are only applicable in the stages of spoilage where bacterial numbers begin to rise sharply. Prior to this, chemical tests have to be based predominantly on the products of autolytic enzyme breakdown. The most important and useful of these has been found to be the decomposition products of adenosine triphosphate (ATP). Thus, a measurement of ATP decomposition products, represented in the so called K value, offers a means of estimating freshness<sup>20,21</sup>.

While many chemical and bacterial tests have been proposed for the measure-

ment of the freshness of fish, none of these are universally applicable. Actually, such tests usually are expected to correlate with various organoleptic conditions.

Volatile base nitrogen (VB-N) is considered quite generally applicable. Although many conflicting results, favorable, unfavorable and others expounding about its variability, have been reported for this test, it usually provides reasonably good correlation with organoleptic freshness<sup>7,21,22</sup>).

Ordinarily the most important *post mortem* change in fish is the changing of the muscle metabolism reactions largely to irreversible ones with the resulting accumulation of lactic acid in the tissue and a decline in its pH. The pH of living fish muscle is not far from 7.0; as a result of *post mortem* accumulation of lactic acid, pH values in the range of 5.8 to 6.2 are reached at peak *rigor* development. Once resolution of the *rigor mortis*, enzymatic changes in the protein and other components cause an increase in amino acids and related compounds. These changes rise the pH and are succeeded by a period of gradually accelerating growth associated with organoleptic changes in the fish<sup>21-23</sup>).

The oldest and still most widespread means of evaluating the acceptability and edibility of fish are the senses of smell and sight, supplemented by taste and touch. The reasons for the preferential use of sensory tests are obvious: no special laboratory equipment is needed; the fish can be examined wherever they happen to be; the tests can be carried out quickly; and many samples can be evaluated in a relatively short time.

The odor of fish ordinarily changes from the moment of capture through storage to the point of inedibility following a general pattern. At the first stage, most fish possess an odor and flavor characteristic of the species. Soon after, much if not all of the natural species characteristic odors and flavors disappear. At the next stage, a group of perhaps quite unrelated odors and flavors, which are referred to as "fishy" begin to accumulate. Later these odors often develop to be quite definite. But from this point on, various amine and other odors and flavors give the fish definitely undesirable properties which certainly drastically lower its quality. At the last stage, where sulfide and other obnoxious odors and flavors predominate, the quality of the fish has sunk to such a low level that by most standards it is considered to be quite inedible.

Another type of odor and flavor which results from oxidation and rancidification of the lipids of fish is more important with certain preserved fish<sup>21,23</sup>).

When brine is used, with or without preservatives, in fish immersed storage, changes in all the above indices and in bacterial counts can be expected to be slowed down, thus extending the shelf life of fish.

### 1. Effect of Fish Amounts on Preservation of Sardine Immersed in Brine

Brine capability for fish preservation related to the amounts of fish and brine solution that can lead to an economic and accurate preservation of the fish were important to determine.

## Materials and Methods

### *Raw material*

Freshly caught sardine (*Sardinops melanosticta*), weighing ca. 85 g each, were purchased from a commercial market in Hakodate and brought to the laboratory.

### *Fish storage and sampling*

Two procedures were used for the determination of brine capability for fish preservation.

In the first place the fish were divided into three lots of 1 and 2 kg, each. Each lot was immersed in 2 l of 4, 10, and 15% brine (NaCl solution) and stored at 0°C for 7 days. At predetermined times, two fish were randomly drawn from each brine sample for K value determination.

In the second place 1, 2, and 3 kg of fish were immersed in 2 l of 10% brine and stored at 15°C during 5 days. The VB-N of the samples was evaluated and compared within storage.

### *K value*

The K value defined by Saito *et al*<sup>24)</sup>, was performed by the method described by Kobayashi and Uchiyama<sup>25)</sup>.

**Muscle extract:** One gram of the muscle was homogenized with 2 ml of chilled 10% perchloric acid at ice temperature. The homogenate was centrifuged at 3,000 rpm for 3 min. The residue was washed with 2 ml of chilled 5% perchloric acid, and centrifuged. After repeating this process twice, the supernatants were combined and immediately neutralized at ice temperature with 10N potassium hydroxide to a pH of 6.4 to 6.8. The neutralized extract was centrifuged and the precipitate of potassium perchlorate was washed twice each time using about 2 ml of chilled neutralized perchloric acid solution, with a pH of 6.8. The supernatant and washings were combined, and the total volume was brought to 10 ml with the chilled neutralized perchloric acid solution.

**Ion exchange resin:** Dowex 1×4 (Cl) was washed with acetone followed by 1N sodium hydroxide, deionized water, and 1N hydrochloric acid in this order. After being washed finally with deionized water, it was stored in a refrigerator.

**Column:** A column (1×18 cm) fitted with a glass filter, at its lower end, was used. The lower end of the column was narrowed to minimize dead space, and thereby to give sharp separation<sup>25)</sup>. It was packed in, 6 cm high, with Dowex 1×4 (Cl).

**HxR, Hx and nucleotides elution:** Two ml of the neutralized extract was adjusted to a pH of 9.4 with 0.5M ammonium hydroxide solution (TB pH test paper) and changed to the column. The column was washed with 20 ml of deionized water which was adjusted to a pH of 9.4 with ammonium hydroxide solution. HxR and Hx were eluted with 50 ml of 0.002N HCl and the effluent (elute A) was collected in a 50 ml volumetric flask, while IMP, AMP, ADP and ATP were eluted with the same volume of 0.6N NaCl in 0.01N HCl (elute B). Elute B was also made up at 50 ml. And both elutes were measured for absorbance at 250 nm to calculate the K value by the following formula:

$$K (\%) = \frac{E_{250nm} A}{E_{250nm} A + E_{250nm} B}$$

### VB-N

The extract for VB-N was prepared as follows; Five grams of skinned fillet from the anterior dorsal region of the fish was blended in a mortar with 45 ml of water for 20 minutes. The extract was filtered through a Whatman No. 2 folded filter paper and used for the analysis.

One ml the filtered extract (Conway method)<sup>26)</sup> was placed in the outer ring of a Conway dish and 1 ml of boric acid (buffer) was placed in the central well. Finally 1 ml of 50%  $K_2CO_3$  solution was added to the fish extract and the Conway dish was sealed hermetically as soon as possible to prevent leaks. Samples disposed of in this way were incubated for an hour and a half at 37°C to help in VB-N liberation. The liberated VB-N, trapped in the central well, was titrated with standard acid (0.01N HCl). Results were calculated as mg volatile base nitrogen per 100 g of muscle using the next equation.

$$VB-N (\text{mg}/100 \text{ g}) = T \times 0.14 \times f \times 1,000$$

## Results and Discussion

### K value

ATP degradation begins from the moment of death. And it is stated that at the resolution of the *rigor mortis* ATP is totally degraded<sup>20,21)</sup>.

Since autolysis begins from the moment of death, IMP disappearance and Hx accumulation proceed throughout most, if not all, of the edible storage life. These processes are primarily autolytic, although augmentation of activity by bacterial enzymes may occur at the later stages.

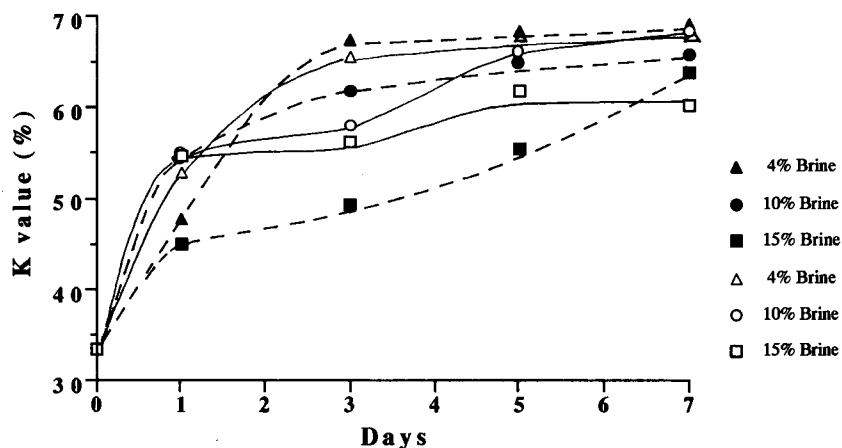


Fig. 1. Effect of the amount of fish immersed in brine on changes in K value of sardine muscle. Lots of 1 kg (open plot) and 2 kg (solid plot) were held at 0°C.

Changes in K value are shown in Fig. 1. The initial K value of the sample was 33%. This value increased during the storage period and on the 7th day differences in K value for the 1 and 2 kg samples immersed in 4% brine were not obvious. Being 65% for the first and 67% for the second. A similar effect was observed for 15% brine immersed samples with a K value of almost 60% on the last day of sampling for both samples. Up to the third day of storage differences in K value were surmised to be due to the differences in the amount of fish immersed in the brine and in the brine salt concentration. The fish amount effect disappeared by the last day of sampling while salt concentration showed its effect until the last day and was surmised to be related to the ionic strength that interfered with enzymatic action<sup>18</sup>).

### VB-N

The VB-N of samples was measured against time. Results are presented in Fig. 2. Clear differences were observed when 1, 2, and 3 kg of fish were stored in 10% brine at 15°C. The VB-N of the samples at the time they arrived at the laboratory was 12.8 mg/100 g. After the first day of storage a slight increase in VB-N was observed in any of the three samples. Later the VB-N for the 3 kg sample increased faster than it did for the 1 and 2 kg samples. Thus on the last day of sampling (5th day), 58.6 mg/100 g of VB-N was recorded for the 3 kg sampling against 43 and 46 mg/100 g for the 1 and 2 kg samples, respectively.

Obviously the most economic alternative is that which offers the highest amount of fish per volume of brine. However, as was mentioned before the VB-N value for the 3 kg sample was considerably higher than those of the 1 and 2 kg samples. In this way 3 kg was discarded as an alternative. Between the other two samples difference was rather small and the 2 kg of fish immersed in 2 l of brine was chosen as the better and most economic alternative to be adopted for the rest of the experiments.

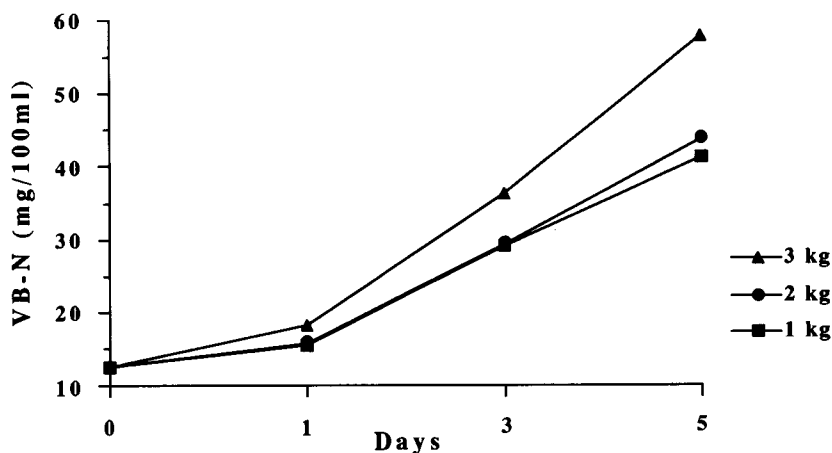


Fig. 2. Effect of the amount of fish immersed in 10% brine on changes in VB-N of sardine muscle. Different amounts of fish were immersed in 2 l of brine held at 15°C.

## 2. Effect of Temperature and Brine Concentration on Immersed Storage of Sardine

The purpose of this study is to find out a new method for fish preservation which could be used instead of chilling or refrigeration based on the fact that there is scarcity of these kinds of systems. As the storage temperature, fifteen degrees was selected in the present study because it represents a moderately cool temperature that could be obtained in the storage compartment of a fishing vessel where refrigeration is used but is inadequate<sup>27)</sup>. This temperature was also selected in that it is consistent with the annual average temperature of towns and the surface of the sea on the Peruvian coast. The effect of this temperature, compared with chilling (0°C), and salt concentration of the brine during sardine immersion was tested in relation to their preserving activity. At the same time permeation of salt into the muscle was observed.

### Materials and Methods

#### *Raw material*

Freshly caught sardine, weighing *ca.* 83 g each, were obtained at Kami-iso in Hakodate Bay, placed immediately into an ice box and delivered to the laboratory. The fish were divided into 6 lots of 2 kg. Two lots each were immersed in 2 l of 4, 10, and 15% brine (NaCl solutions). In this way two sample groups were obtained.

#### *Fish storage and sampling*

One group of the sample was stored at 15°C<sup>27)</sup>. For comparison purposes the other group was stored at 0°C. At predetermined times, two fish were randomly drawn from each lot of the two groups for K value, volatile base nitrogen (VB-N), pH, and muscle salt uptake analyses. Parallel sampling was made on the brine for losses in salt concentration, microbial counts and microbial characterization.

#### *K value and VB-N determinations*

Determinations were performed in the same way as is explained in section II-1.

#### *Salt concentration*

The muscle extract used for NaCl concentration and pH determination was prepared in the same way as that explained for VB-N in section II-1.

The salt content of the fish muscle (S: sample) and the brine was determined by the method of Mohr<sup>28)</sup>. Ten ml of the filtered extract and diluted brine (D: dilution coefficient) were pipetted into 100 ml Erlenmeyer and 1 ml of K<sub>2</sub>CrO<sub>4</sub> (1N) was added. The samples were titrated with 0.1N AgNO<sub>3</sub> standard solution (f: factor) until the first perceptible pale red-brown appeared (T: titration volume). Sodium chloride concentration based on sodium was calculated by the formula below.

$$\text{NaCl}(\%) = 0.00585 \times T \times D \times \frac{100}{S} \times f$$

Table 1. Quality score card for sardine immersed in brine.

Testing subject	Scores of alteration and description of features				
	A Completely fresh, highly acceptable	B Fresh and acceptable	C Fairly acceptable, borderline of acceptability	D Spoiled, unacceptable	E Completely spoiled, totally unacceptable
Overall appearance	Bright, metallic lustre	Bright	Some loss of brightness	Bleaching	Bloom completely gone
Odor	Fresh	Very faint sour	Fishy odor, slightly sour	Strong fishy or sour odor	Putrid
Texture	Firm and elastic	Slightly soft	Some loss of elasticity	Very soft	Totally soft and flabby
Slime	Clear, transparent	Becoming turbid	Opaque and milky	Sticky yellowish	Thick grayish or sticky red

*pH*

pH was measured with a Horiba H7-B pH meter equipped with a glass membrane electrode.

*Sensory evaluation*

The quality of the fish in brine was scored on scales for overall appearance, odor, texture and surface slime. All parameters were scored on a scale of "A" to "E" indicating decreasing quality, using the descriptive score card presented in Table 1. An overall score for quality was based principally on odor. Following assessment on the basis of freshness scores, acceptability was related to the chosen freshness scores as follows.

- A, Completely fresh, highly acceptable ;
- B, Fresh and acceptable ;
- C, Fairly acceptable, borderline of acceptability ;
- D, Spoiled and unacceptable ;
- E, Completely spoiled, totally unacceptable.

**Results and Discussion***K value*

The results are shown in Fig. 3, for 0°C and 15°C, respectively.

The K value for samples immersed at 0°C was nearly 12% once they arrived at the laboratory. After 7 days of storage this value, for all of the three samples, was below 60%, moreover the value for fish immersed in 15% brine was 38%.

The K value represents the percentage of degraded nucleotide in the fish muscle so it offers means of estimating freshness in the early stages as well as in the later stages of storage<sup>21</sup>. On the other hand a K value of 60% is thought to represent the limit for edibility<sup>29</sup>. From this point of view it could be concluded that the



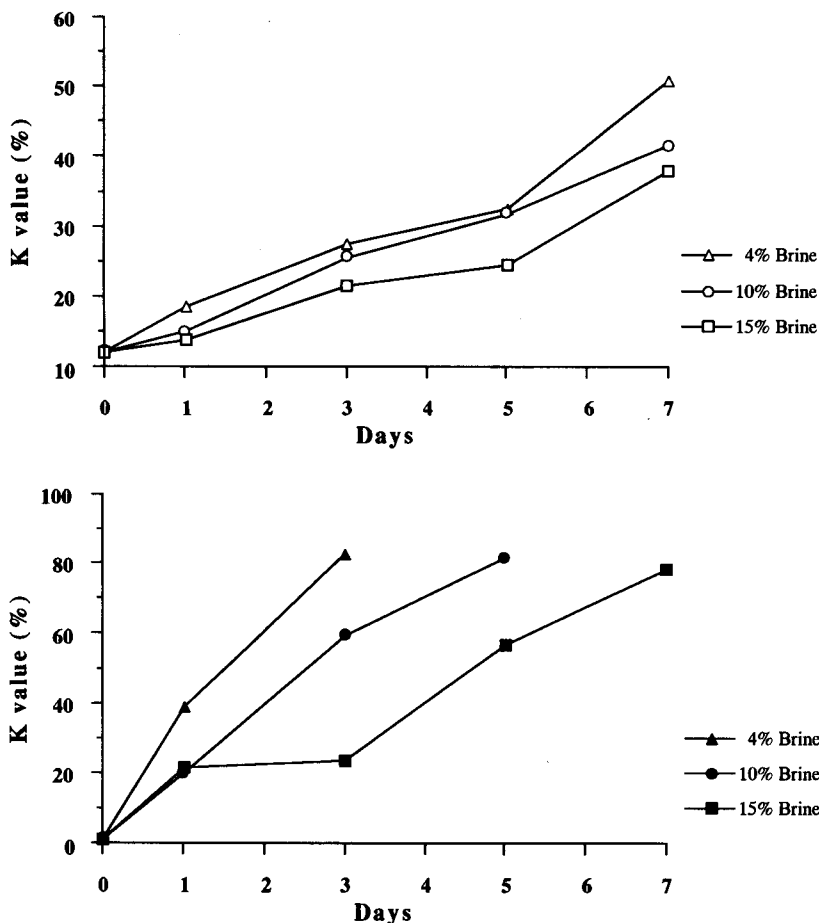


Fig. 3. Changes in K value of sardine immersed in brine. Storage was held at 0°C (top) and at 15°C (bottom).

samples stored at 0°C were acceptable until the last day of sampling, the 7th day. Therefore, at this temperature, the results were in agreement with others published before for many species<sup>30</sup>. Although ATP is degraded and its products accumulate at a steady rate, causing K value increases, during the first five or so days, signs of bacterial decomposition were not observed.

Furthermore, the K value of samples stored at 15°C was about 2% at the time immersion into the brine, but they increased rapidly later on. This behavior was especially observed for samples in 4% and 10% brine which after the 2nd and 3rd day of storage appeared to have values higher than 60%. In addition the 15% brine samples reached 60% after the 5th day of storage, and consequently were considered acceptable up to the 5th day of sampling.

From these results, it is possible to say that K value changes in the samples stored at 0°C were dependant only minimally on salt concentration, thus even

samples immersed in 4% brine appeared to be edible up to the last day of sampling, whereas is samples stored at 15°C, salt had a marked effect in the quality of fish.

### VB-N

It is known that TMA-N (trimethylamine-N) and VB-N values should be used in comparison with sensory evaluations but should not be used as obligatory limits<sup>31</sup>).

Favorable results have been obtained by many authors regarding a correlation between an increase in the total volatile basic nitrogen content and fish spoilage. Tillmans and Otto<sup>23</sup>) found that the total volatile bases increased with the onset of spoilage in such fish as cod, haddock, eel, and sea pike; they suggested an upper limit of 30 mg nitrogen per 100 g for acceptability. Yamamura and Tanikawa<sup>23</sup>) also found the content of VB-N useful as a measure of spoilage, and suggested 30 mg nitrogen per 100 g as the upper limit for acceptability.

As presented in Fig. 4 at 0°C, VB-N increased slowly throughout the storage. The initial value of 12 mg/100 g, resulted in 19, 17 and 15 mg/100 g for samples in 4, 10, and 15% brine, respectively, on the last day of sampling (7th day). As is obvious all the obtained values were lower than the considered sign of initial spoilage (30 mg/100 g)<sup>23,32</sup>), consequently the samples were considered acceptable. These results were in total agreement with results of sensory evaluation.

At 15°C a rapid increment VB-N occurred in the 4 and 10% brine samples after the first day of storage, although higher levels were reached for the 4% samples. The VB-N value of the 15% samples increased gradually and at a lower level than those of 4 and 10%, reaching about 30 mg/100 g on the last day of sampling. Results showed a clear relationship between the outcome of sensory tests for 4 and 10% samples. The fish were sensorily unacceptable when VB-N exceeded the range of 30-40 mg/100 g which is considered to be the limit of acceptability. In the 15%

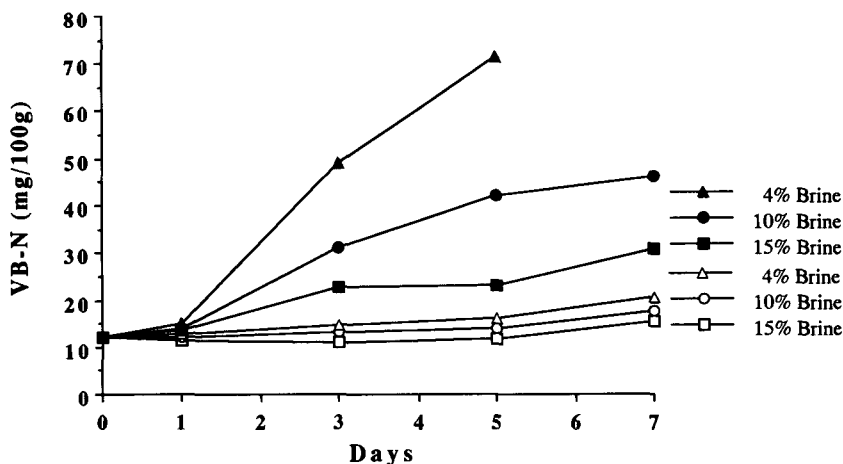


Fig. 4. Changes in VB-N of sardine during immersed storage in brine. Open plot, 0°C; solid plot, 15°C.

samples, on the 7th day of storage, in spite of a VB-N value of about 30 mg/100 g, strong putrid odors were taken to indicate gross spoilage, whereas in the 10% samples, on the 3rd day, a similar VB-N value did not present this characteristic.

Prolongation of sample acceptability was attributed to the salt inhibitory effect on microbial activity because of cell dehydration, decreases of water activity and oxygen solubility in brine and other effects of the same like<sup>18)</sup>.

### *pH*

Changes in VB-N have a close relationship with changes in pH<sup>21,23)</sup> in the fish muscle and therefore, they were measured. Results are shown in Fig. 5. The samples immersed in 4% brine stored at 15°C increased their pH during the first two days of storage, those immersed in 10 and 15% brine also increased, however more slowly and the highest value was reached on the 3rd day. Until this point of storage a good correlation could be seen between these and VB-N values for these samples (Fig. 4). Later, even though the VB-N values continued to increase, the pH did not increase any more. A possible reason for this could be attributed to the salt action which produces equilibrium of the metabolites in fish muscle, such as lactic acid and other related acids.

On the other hand, the pH of the samples stored at 0°C were also observed to raise up to the 2nd and 3rd day of storage. A characteristic of these samples was, that the pH values were comparatively lower than their counterparts of 15°C storage. As is well known, increases in VB-N and consequently in pH are a result of microorganisms growth and activity<sup>21,22)</sup>. Here, this is clearly seen, where the smallest pH changes correspond to samples immersed in the highest brine concentration. So, this salt concentration was surmised to inhibit microbial growth.

Glycogen in the muscle of marine animals is very high. In glycolysis glycogen is decomposed to lactic acid, causing the pH to decrease<sup>21,23)</sup>. During this exper-

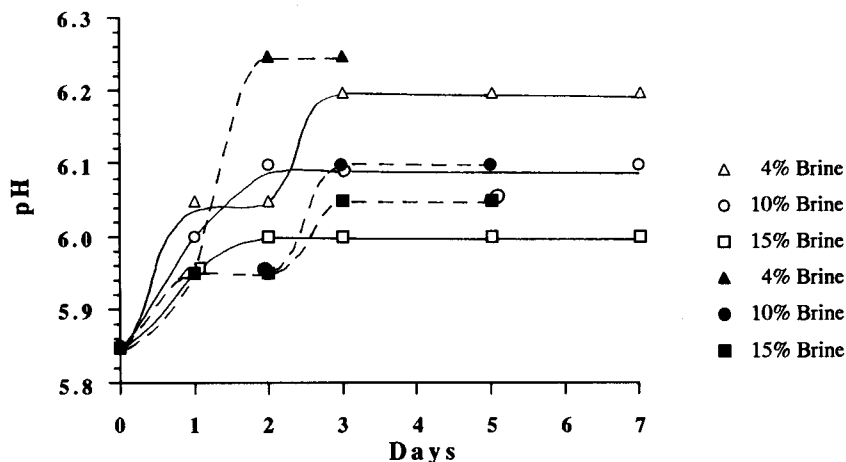


Fig. 5. Changes in pH values of sardine muscle during immersion in brine. Storage was held at 0°C (open plot) and at 15°C (solid plot).

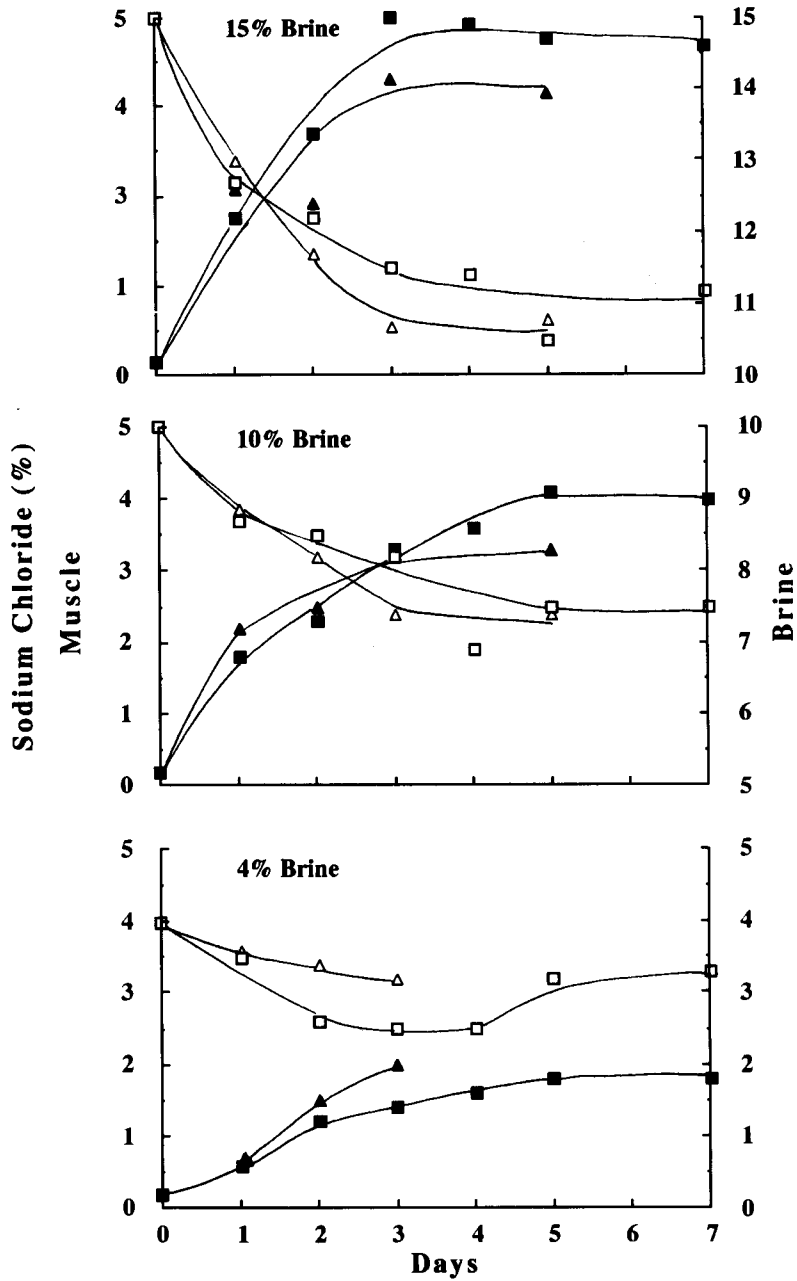


Fig. 6. Changes of NaCl concentration in muscle of sardine and brine during immersed storage held at 15°C,  $\Delta$ : brine,  $\blacktriangle$ : fish muscle; and at 0°C,  $\square$ : brine,  $\blacksquare$ : fish muscle.

iment initial pH of the sample was 5.87. This is a relatively low value and it is suggested to be the effect of the total breakdown of the glycogen, resulting in a high concentration of lactic acid in the muscle.

Differences in muscle between red flesh and white flesh fish are known to exist. Red flesh fish has a higher content of protein and also a higher level of glycogen<sup>21)</sup>. Therefore, after death the lower pH reached for white flesh fish is 6.0-6.4, always higher than that for red flesh fish (5.6-6.0). So, even if pH increases with fish spoilage and with increases in VB-N and related compounds, it has been recognized to be highly dependant on the fish species and to the portion of muscle used for the termination<sup>21)</sup>. In this way pH can be use as an alternative but not as a determinant factor for fish quality determination.

#### *NaCl concentration in muscle and brine*

Salt penetration into sardine muscle is shown in Fig. 6. As aforesaid, the rate of salt permeation into the fish muscle depends upon many factors. The salting method is the most important and then temperature during salting, purity of the salt, and the characteristics of the fish used<sup>18,19)</sup>.

Brine concentration exerted a great influence on salt penetration into the muscle<sup>33)</sup>. During this trial, the amount of salt permeated into the fish muscle at both temperatures was dependent on the brine concentration. Changes in sodium content of fish resulted from varying concentrations of brine. The observed values were higher for samples in the 15, 10, and 4% brine, in a decreasing order.

Even though final salt uptake was almost similar for the samples in the same brine concentration independent of the temperature, results showed storage temperature together with salt concentration to exert an important influence on the rate of permeation. Permeation was faster at 15°C than at 0°C during the first two to three days of storage. Furthermore, at 0°C a clear dependence on the brine concentration with the time in which samples reached maximum concentration was observed, the 3rd day for samples in 15% and the 7th day in 10 and 4% samples, respectively. At 15°C, brine penetration depended on the brine concentration, and reached a maximum on the 3rd day for each sample. Later on values remained constant. A similar result has already been reported for salted fish<sup>34)</sup>.

Samples immersed in 15% brine showed a maximum salt permeation of almost 4% NaCl. Therefore, at 15°C, even if organoleptical assessment showed a sample acceptable up to the 5th day of storage, salt permeation into the muscle could be a limiting factor for consumption.

On the other hand, decreases in salt concentration of the brine corresponded to fish salt uptake. They were larger when salt permeation into the fish was higher.

#### *Sensory evaluation*

At 0°C, none of the characteristics evaluated until the last day of sampling were of grade "D" (Table 2) and consequently all of the samples were considered acceptable until the last day. At 15°C the sensory evaluation indicated differences among the three groups; 15% brine immersed fish remained acceptable for 5 days, whereas 10% samples proved to be from "borderline of acceptability" to "spoiled and unacceptable" between the 3rd and 5th days. Moreover, the 4% sample showed a

Table 2. Sensory quality scores of sardine immersed in brine held at 0°C (top) and at 15°C (bottom).

Testing subject	Before storage	Brine concentration											
		4%				10%				15%			
		Days				Days				Days			
		1	3	5	7	1	3	5	7	1	3	5	7
Overall appearance	A	B	B	C	C	A	B	B	C	A	B	B	C
Odor	A	B	B	C	C	B	B	C	C	B	B	B	B
Texture	A	B	C	C	C	A	B	C	C	A	B	B	C
Slime	A	B	B	C	C	A	B	B	B	A	B	B	B
Acceptability	A	B	B	C	C	A	B	C	C	B	B	B	C

Testing subject	Before storage	Brine concentration											
		4%				10%				15%			
		Days				Days				Days			
		1	3	5	7	1	3	5	7	1	3	5	7
Overall appearance	A	B	D	E	B	B	C	D	A	B	B	C	
Odor	A	C	D	E	B	C	D	D	B	B	C	D	
Texture	A	C	D	E	B	C	C	D	A	B	C	D	
Slime	A	C	D	E	A	C	C	D	A	B	B	C	
Acceptability	A	C	D	E	B	C	D	D	B	B	C	D	

A; Completely fresh, highly acceptable

B; Fresh and acceptable

C; Fairly acceptable, borderline of acceptability

D; Spoiled and unacceptable

E; Completely spoiled, totally unacceptable

much more rapid deterioration, being acceptable only up to the first day and then "spoiled and unacceptable" by the 3rd day of storage.

Based on the sensory scores, we can say that the pattern of spoilage is similar in all of the different salt concentrations, but that spoilage proceeds twice and four times as fast at 4% brine as at 10% or 15%, respectively. The reason is thought to be because of the preservative action of salt.

### 3. Microbial Counts, Isolation and Identification

In general, the bacteria found on living marine fish are predominantly psychrophilic (growing significantly at 0°C), sea water loving (though not necessarily absolutely halophilic), aerobic, and attack proteinaceous materials more actively than carbohydrates.

Genera repeatedly found to be present on marine fish to a greater or lesser extent include *Pseudomonas*, *Achromobacter*, *Vibrio*, *Flavobacterium*, *Corynebacterium*, *Alcaligenes*, *Photobacterium*, *Micrococcus*, *Mycoplana*, *Proteus*, *Bacillus* and (in the intestine) *Clostridium*. Most recent reports, however, indicate an overwhelming preponderance of gram negative rodshaped organisms of the genera *Pseudomonas*, *Achromobacter*, and *Vibrio* in fish and shellfish floras. *Flavobacterium*, Coryneforms, and *Micrococcus* also seem to occur consistently but at much lower levels of incidence. During storage of fish, changes occur in the balance of physiological types among the gram negative organisms. The comparatively slow-growing *Achromobacter* and *Flavobacterium* show a steady decline in relative significance and the rapidly growing *Pseudomonas* types become completely predominant. There is also apparently a shift in balance among *Pseudomonas* types themselves, typified by a reduction in the relative occurrence of typical marine forms and an increase in the proportion of types of *Pseudomonas* (*P. fragi*, *P. putida* and similar species) commonly found on all spoiling "animal protein" foods held under refrigeration, i.e., meat, turkeys, etc<sup>22</sup>).

Movement of bacteria into fish tissues is apparently a slow process and multiplication within tissues is not very rapid. Even when the fish is clearly spoiling, only a small number bacteria can be found in the deep muscle. Consequently, the most widely accepted view is that most of the primary activity of the spoilage bacteria occurs in the surface layers of the fish, and the total effect is largely due to secondary diffusion of bacterial enzymes and products into the deep tissues<sup>22,35</sup>). The tissues and internal organs of healthy fish and shellfish appear to be sterile<sup>35</sup>). Based on this fact, our microbiological assessments were carried out in brine rather in the fish itself.

Penetration of bacteria into cut fish and fillets is certainly more rapid than in the case of whole fish but is still sufficiently slow to justify the application of the above theory to these products also. It is possible that the reason for the primary surface action of the spoilage flora is its obligate aerobic nature.

Microbiological tests during sardine immersed storage were done with the purpose of finding out the kind of microflora present and its relation with each stage of storage under all the studied conditions.

## Materials and Methods

### *Samples*

The same brine used for immersed storage of sardine in section II-2.

### *Microbiological analyses*

Total aerobic counts were determined from 20 ml of brine samples homogenized in 180 ml of sterile 0.85% physiological saline using a standard pour plate technique and duplicate plates. Plates were incubated at 37°C for 24 h. About 30 colonies were randomly picked from each countable nutrient agar plate [beef extract (Difco Laboratories, Detroit, MI, USA) 5 g/l, polypeptone (Nihon Seiyaku, Tokyo, Chiyoda, Japan) 10 g/l, sodium chloride (Kanto Chemical, Tokyo, Nihonbashi, Japan) 5 g/l and agar (Nihon Seiyaku) 15 g/l] and inoculated onto nutrient agar

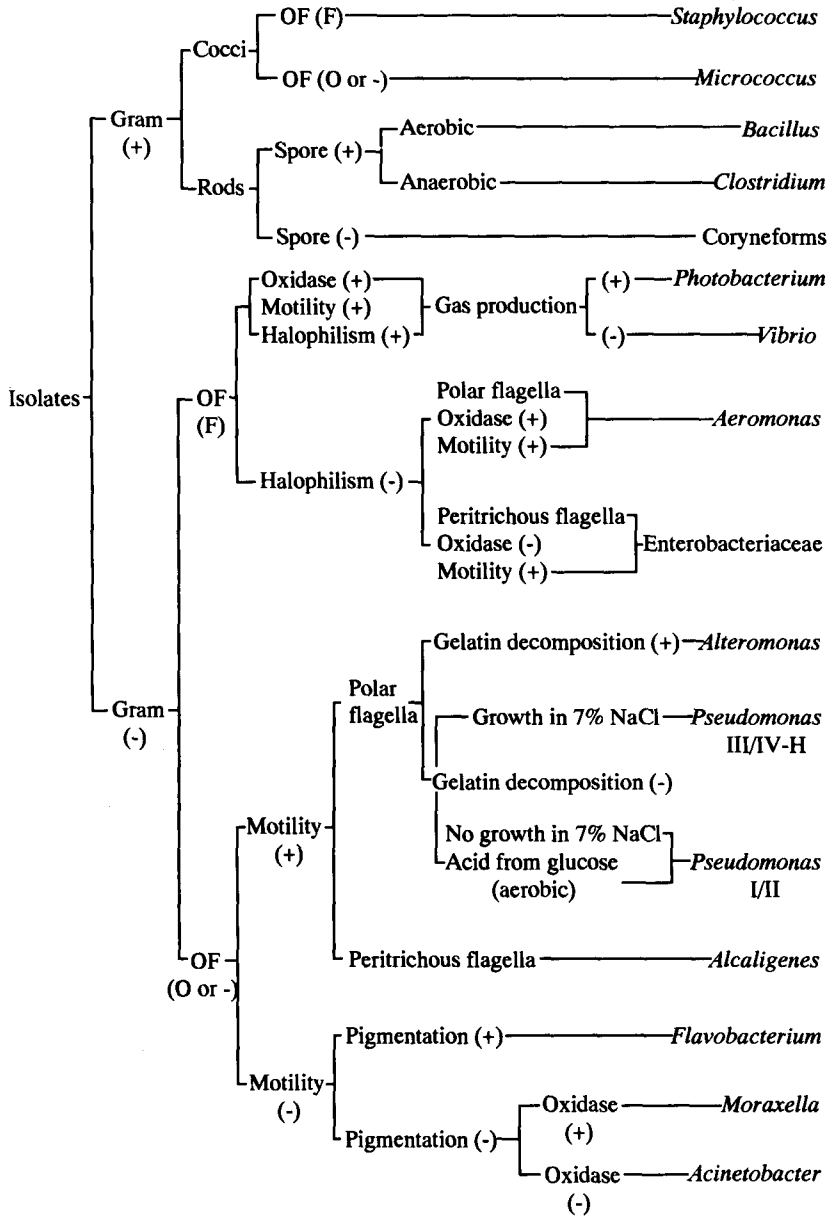


Fig. 7. Identification scheme based on Shimizu, Ezura and Shewan's methods for identification of marine bacteria.



slants. Microorganisms isolated from 15% brine were grown in 10% NaCl nutrient agar, following successively adjustments to lower salt concentrations until they were able to grow in the nutrient agar described above. Isolates, from all the brine samples, obtained in this way were plated on nutrient agar, for three consecutive times to obtain pure cultures. Thus, isolated microorganisms were identified using the scheme shown in Fig. 7.

The tests were conducted as follows: gram test, Hucker method<sup>37)</sup>; shape, phase contrast microscope<sup>38)</sup>; motility, phase contrast microscope and semisolid medium<sup>38,39)</sup>; flagella, Nishizawa and Sugawara method<sup>37)</sup>; spore, Wirtz method<sup>38)</sup>; catalase activity, tested with hydrogen peroxide (3%)<sup>39)</sup>; O/F-test, Hugh and Leifson method<sup>39)</sup>; oxidase activity, Kovac's reagent (N, N, N', N'-Tetramethyl-*p*-phenylenediamine)<sup>39)</sup>; gelatin decomposition, nutrient gelatin stab medium<sup>39)</sup>.

## Results and Discussion

### *Microbial counts*

The number of colony forming units (cfu) in brine during storage of sardine is shown in Fig. 8. The initial bacterial load of brine samples, stored at 0°C, immediately after sardine immersion was *ca.*  $5 \times 10^3$  cfu ml<sup>-1</sup>. In 15% sample, this initial number decreased after the first day and remained very low until the 3rd day. By the 5th day the bacterial numbers increased to 10<sup>2</sup> cfu ml<sup>-1</sup>. Total count on the last day of sampling was almost the same as recorded at the initiation of the storage. In 4 and 10% brine samples the number of cfu gradually increased during the storage and maximum counts were 10<sup>6</sup> cfu ml<sup>-1</sup> and 10<sup>5</sup> cfu ml<sup>-1</sup>, respectively.

At 15°C, also, a marked decrease in microbial count was noticeable during the first 24 hours for 15% brine samples. In this case, also, salt concentration played an important roll in microorganisms growth and the decrease in counts suggested a process of selection for salt tolerant bacteria. Later the counts increased, to reach about 10<sup>6</sup> cfu ml<sup>-1</sup> by the last day of storage. Growth of bacteria occurred more rapidly in the 4 and 10% samples, and by the 3rd and 5th days respectively, and their counts were similar to that of the 15% brine sample on the 7th day.

More or less large populations of bacteria are constantly present on the external surfaces of all marine animals. In the case of free-swimming fish, the numbers of bacteria on the skin range from 10<sup>2</sup> to 10<sup>6</sup> per square centimeter, on the gills from 10<sup>3</sup> to 10<sup>5</sup> per gram, and in the intestine from very few in nonfeeding fish to 10<sup>7</sup> or more per gram in feeding fish<sup>22)</sup>. Thus the initial numbers found during this trial were within the range presented in the literature.

For a short period after death, corresponding rather closely to the onset, duration, and resolution of *rigor mortis*, there is little change in the numbers of bacteria present. This period has been likened to the classical lag phase observed in newly inoculated laboratory cultures of bacteria. It is succeeded by a period of gradually accelerating growth associated with organoleptic changes in the fish, typified by a loss of the characteristic fresh fish flavor. Next the bacterial population enters a phase of more or less exponential growth corresponding to the initial appearance of such well known spoilage indicator substances as trimethylamine and other related bases. This phase is of short duration and is succeeded by a more or

less stationary terminal growth period, during which there is little change in numbers among the surface bacterial populations. Despite the absence of quantitative bacterial change, this is the period of maximum spoilage activity, terminating when the fish is approached putridity. Fish preservation methods aim to lengthen part of the growth curve, usually the lag and accelerating growth phases<sup>22</sup>).

It could be said therefore that the action of salt together with temperature might have delayed resolution of the *rigor mortis* lengthening the lag phase or causing slight growth at 0°C. The same action was observed in 15% samples at

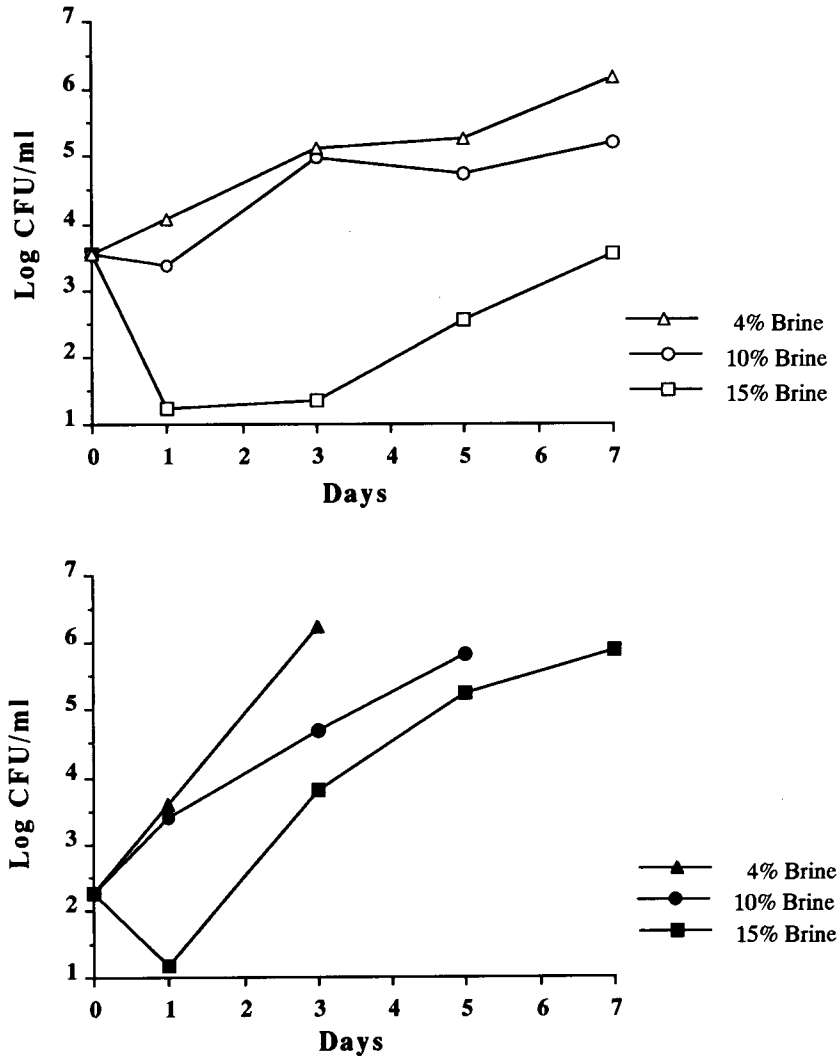


Fig. 8. Changes in viable bacterial numbers of brine during sardine immersion. Storage was held at 0°C (top) and 15°C (bottom).

Table 3. Number of strains isolated from brine during sardine immersion. Storage was held at 0 and 15°C.

Days	Storage temperature							
	0°C				15°C			
	Brine concentration (%)				Brine concentration (%)			
	0	4	10	15	0	4	10	15
Before storage	60	—	—	—	61	—	—	—
1	—	31	30	—	—	32	29	—
3	—	29	27	—	—	31	28	31
5	—	31	30	32	—	—	31	31
7	—	33	31	31	—	—	—	30
Sub total	60	124	118	63	61	63	88	92
Total	365				304			

15°C. Furthermore at this temperature faster increase in microbial numbers correlated closely with faster spoilage of the samples.

#### *Identification of isolated strains*

The number of microorganisms isolated from all of the brines together are presented in Table 3. They were 365 strains from samples stored at 0°C and 304 strains from those at 15°C. All together made a total of 669 strains. Isolated microorganisms were identified to the genus level using the scheme showed in Fig. 7. The results are shown in Tables 4 and 5.

#### *Cocci*

Out of 669 isolates, 156 strains were gram positive cocci. Among them 148 strains were non motile microorganisms shown to be catalase positive and capable of oxidative metabolism of glucose or not able to metabolize it in any way. Therefore they were identified as genus *Micrococcus*. The remaining 8 strains were capable of glucose fermentation and consequently recognized as genus *Staphylococcus*.

#### *Gram positive bacilli*

Ninety nine strains were observed as gram positive bacilli. Twenty six of them able to grow aerobically and which produced spore were characterized as genus *Bacillus*. The other 73 non motile and non spore forming strains were thought to be Coryneforms.

#### *Gram negative bacilli*

a) Glucose fermenters: Twenty three aerobic and oxidase positive strains with polar flagella were recognized as genus *Aeromonas*, and 25 aerobic and oxidase negative strains with peritrichous flagella were classified Enterobacteriaceae.

Table 4. Bacterial genera isolated from brine during sardine immersion. Storage was held at 0°C.

Genera	Before storage	Brine concentration			Total
		4%	10%	15%	
<i>Micrococcus</i>	22	42	33	11	108
<i>Staphylococcus</i>	—	2	2	—	4
<i>Bacillus</i>	4	5	7	3	19
Coryneforms	14	13	18	4	49
<i>Moraxella</i>	2	6	7	11	26
<i>Pseudomonas</i>	10	32	31	23	96
<i>Aeromonas</i>	1	12	4	—	17
<i>Alcaligenes</i>	1	1	—	3	5
Enterobacteriaceae	1	4	7	—	12
<i>Acinetobacter</i>	1	2	4	3	10
<i>Alteromonas</i>	1	1	—	—	2
<i>Flavobacterium</i>	—	1	1	2	4
Unidentified	3	3	4	3	13
Total	60	124	118	63	365

Table 5. Bacterial genera isolated from brine during sardine immersion. Storage was held at 15°C.

Genera	Before storage	Brine concentration			Total
		4%	10%	15%	
<i>Micrococcus</i>	20	7	5	8	40
<i>Staphylococcus</i>	4	—	—	—	4
<i>Bacillus</i>	2	2	1	2	7
Coryneforms	12	7	3	2	24
<i>Moraxella</i>	1	7	33	44	85
<i>Pseudomonas</i>	9	14	29	18	70
<i>Aeromonas</i>	1	4	1	—	6
<i>Alcaligenes</i>	1	3	2	1	8
Enterobacteriaceae	5	7	1	—	13
<i>Acinetobacter</i>	2	4	6	13	25
<i>Alteromonas</i>	1	4	2	1	8
<i>Flavobacterium</i>	2	1	2	—	5
Unidentified	1	3	3	3	10
Total	61	63	88	92	304

b) Glucose non fermenters, motile bacteria : This was the most numerous group with 188 isolated. Strains with polar flagella able to liquefy gelatin were 10, recognized as genus *Alteromonas*, and 166 strains not able to do so were identified as genus *Pseudomonas*. The other 13 strains with peritrichous flagella were genus *Alcaligenes*.

c) Glucose non fermenters, non motile bacteria : Out of a total of 155 isolates, 146 strains did not produce carotenoid pigment. Among them 111 strains were oxidase positive and thought to be genus *Moraxella*. The oxidase negative genus, 35 strains, were *Acinetobacter*. Nine strains having carotenoid pigmentation were

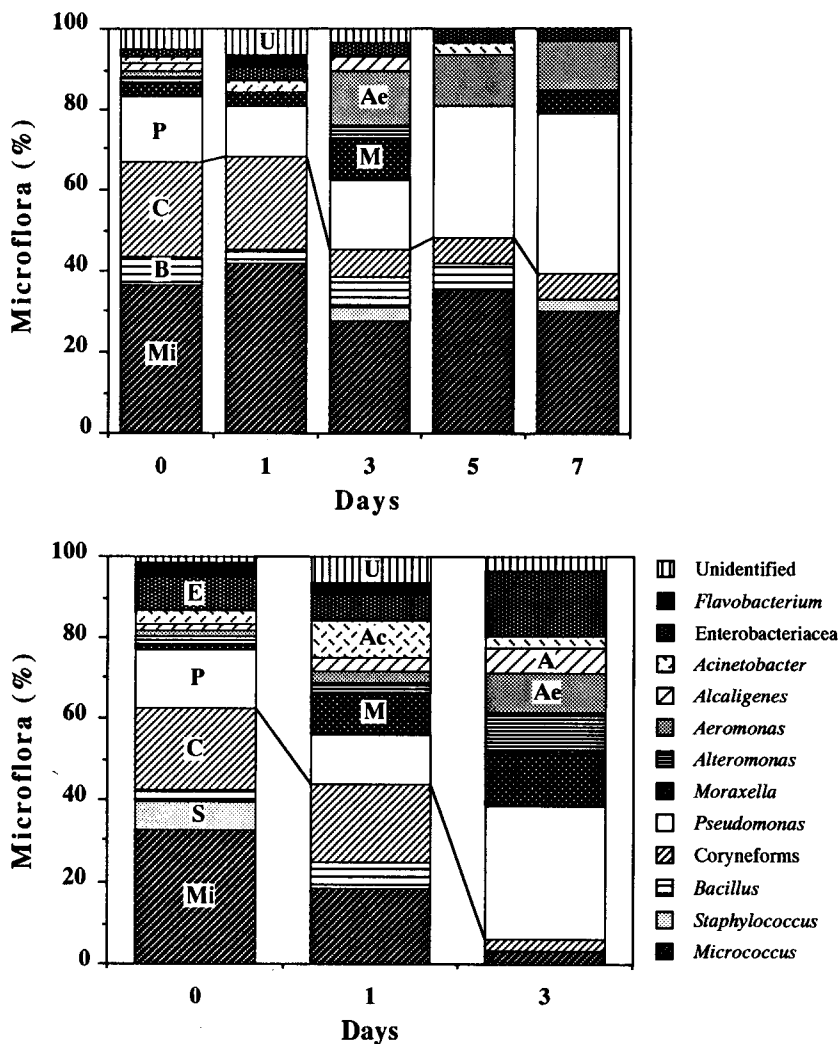


Fig. 9. Changes in microflora of 4% brine during sardine immersion. Storage was held at 0°C (top) and at 15°C (bottom).

classified as genus *Flavobacterium*.

*Changes in bacterial flora*

Microbial flora in the brines of the three different concentrations stored at 0°C and 15°C consisted of thirteen genera (Table 4) and twelve genera (Table 5), respectively. *Pseudomonas* and *Moraxella* species were the most frequently encountered. Other genera identified included *Micrococcus*, *Staphylococcus*, *Bacillus*, Coryneforms, *Aeromonas*, *Alcaligenes*, Enterobacteriaceae, *Acinetobacter*, *Alteromonas* and

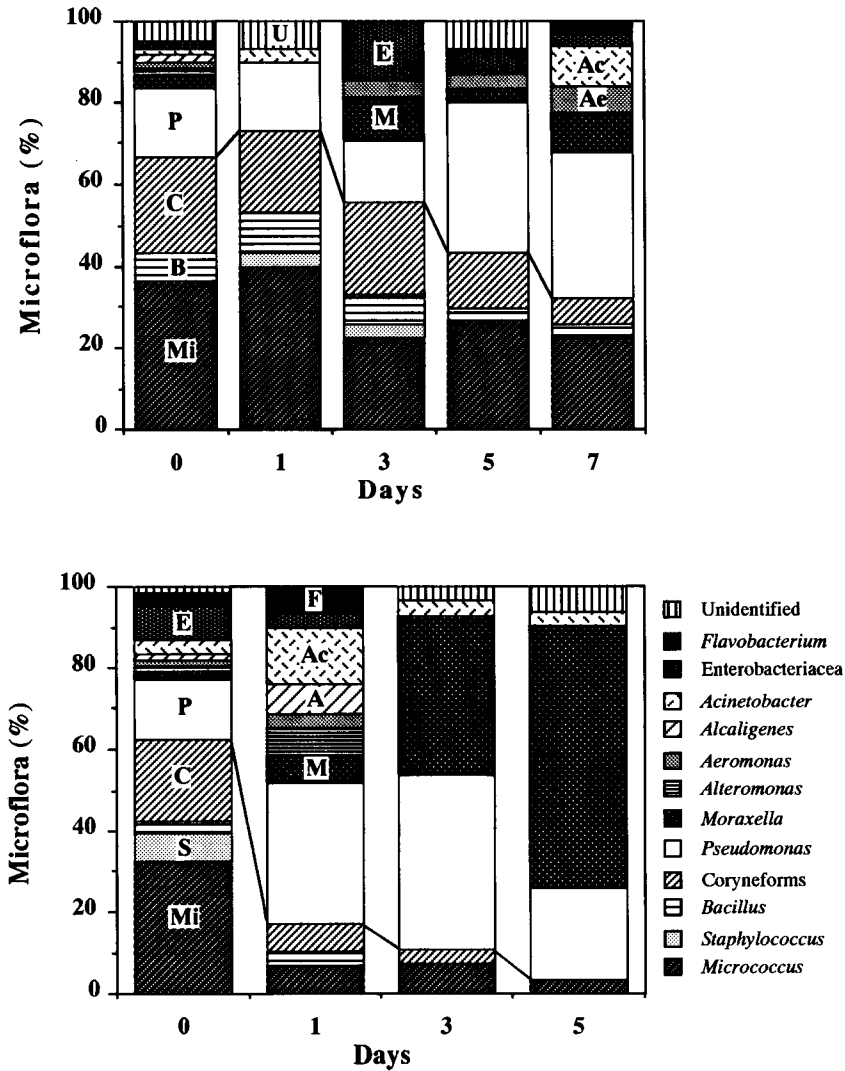


Fig. 10. Changes in microflora of 10% brine during sardine immersion. Storage was held at 0°C (top) and 15°C (bottom).

*Flavobacterium*. These findings coincided, mostly, with those of Liston<sup>22</sup>, Stenstrom and Molin<sup>40</sup>, Gennari and Tomaselli<sup>41</sup>, Gennari *et al.*<sup>42</sup>, and Shewan<sup>43</sup>.

Changes in microflora related to brine concentration and storage time are shown in Figs. 9, 10, and 11. In the case of 4% brine stored at 0°C (Fig. 9), gram positive bacteria belonging to the Coryneforms and to the genus *Bacillus* and *Micrococcus*, made almost 60% of the total microflora on the initial day of storage. By the 3rd and the next days however, gram negative bacteria, such as genus *Pseudomonas*, *Moraxella*, and *Aeromonas* increased and reached almost 65% on the 7th day. Moreover, the percentage of *Pseudomonas* increased continuously from *ca.* 16% to

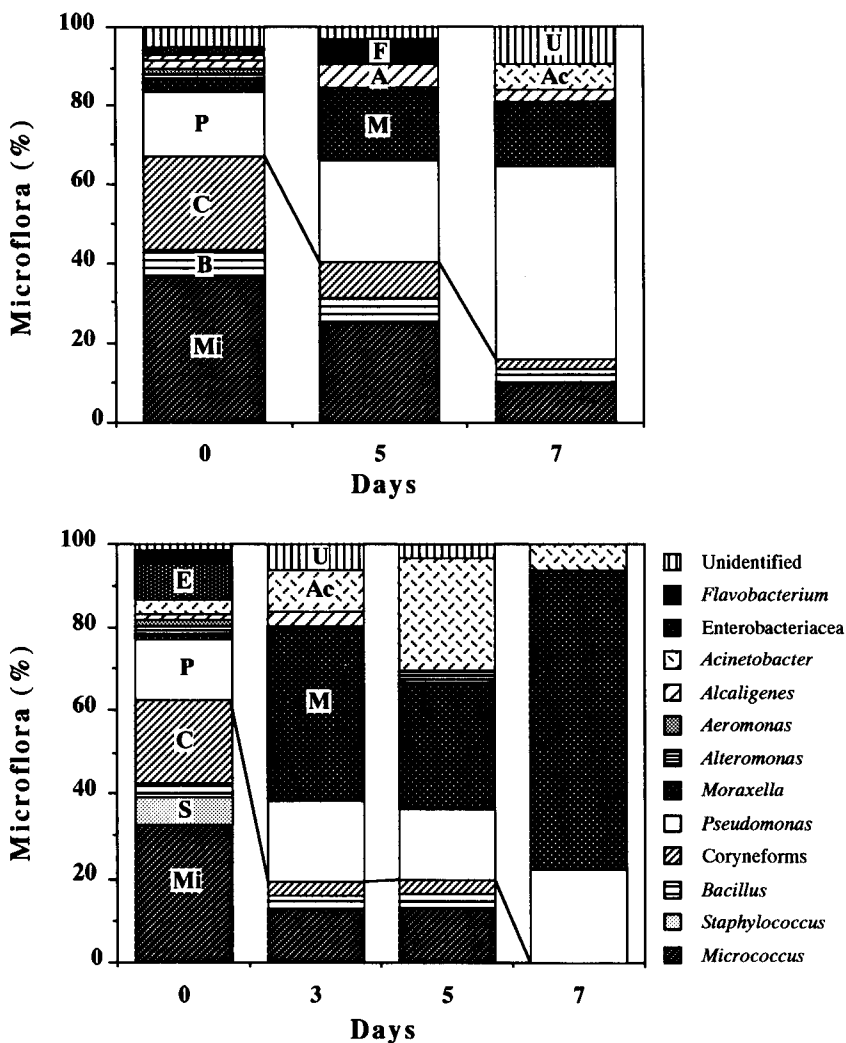


Fig. 11. Changes in microflora of 15% brine during sardine immersion. Storage was held at 0°C (top) and 15°C (bottom).

about 40% and became the predominant flora after the 5th day. Another characteristic of this sample was the constant presence of *Micrococcus* showing always between 25-40% of the total microflora.

In 4% brine stored at 15°C (Fig. 9) the sardine was observed to be completely spoiled on the 3rd day, consequently this was considered the last day of sampling. Changes of microflora in this brine were different to that of samples storage at 0°C. The gram positive bacteria representing ca. 60% of the initial flora decreased to 40% on the first day and was just about 5% on the last day of storage. Simultaneously with these decreases an increase of gram negative bacteria was observed and *Pseudomonas* which reached nearly 35%, became predominant on the 3rd day.

Microflora of 10% brine at 0°C (Fig. 10) showed a similar pattern in changes to that of 4% brine sample stored at the same temperature. *Micrococcus* appeared predominant among the initial flora composed of 60% of gram positive bacteria. The percentage of this bacteria was reduced according to the length of the storage period, and on the 7th day of storage they were only a few percentage whereas gram negative bacteria increased to 70% having *Pseudomonas* as the predominant genus.

In samples of the same brine concentration stored at 15°C (Fig. 10), gram negative bacteria become predominant in a shorter period, the initial 60% of gram positive made only 15% the first day and on the 5th day they represented only a few percentage points. Furthermore, predominance of *Pseudomonas* on the first day was equaled the 3rd day and overwhelmed on the 5th day by *Moraxella* which reached nearly 70%. Some studies in *Pseudomonas* and *Moraxella* isolated from both fresh and spoiled sardine have shown *Moraxella* to exhibit better growth than *Pseudomonas* in 6% NaCl independently of temperature<sup>43</sup>.

Microflora of 15% brine samples stored either at 0 or 15°C (Fig. 11), developed following the same pattern to that of the corresponding 10% brine sample, except that at 15°C it took two days longer to reach almost the same percentages.

Furthermore 15% brine at 15°C showed a continuous increase of the gram negative bacteria, specially, *Moraxella* and *Pseudomonas* between the 5th and the 7th day (Fig. 11). VB-N value for this sample, on the 7th day (Fig. 4), was almost the same as that recorded for 10% brine sample on the 3rd day storage. In spite of similar VB-N values, sensory assessment showed the 10% samples still edible while the 15% sample was considered spoiled. It was supposed, therefore, that differences in sensory assessment between these two sample were due to stronger autolytic activity in 15% samples after a longer period of storage, or to lipolytic processes brought about by *Moraxella* spp<sup>44</sup>, considering their noticeable quantity at this stage of the storage rather than that of another kind of microbial spoilage activity (e.g. protein degradation by *Pseudomonas*).

At the same time, similar VB-N values, although with different microbial counts, were related to the different salt brine concentrations that appeared to inhibit microbial activity, but no longer inhibited autolysis at this stage of the storage.

The predominant gram negative rod floras of living fish are frequently altered by the handling procedures which precede storage so that gram positive organisms such as *Micrococcus* and *Corynebacterium* attain a temporarily important quantitative position in the surface bacterial populations. However during the apparent lag



period and the phase of accelerating growth, qualitative changes in the flora re-establish the predominance of the gram negative rod forms so that by the time logarithmic growth is under way usually over 90% percent of the total bacteria are of this type<sup>22)</sup>. Besides Molin *et al.*<sup>29)</sup> found that in the initial microflora of herring fillets quantitatively the proportion of gram positive organisms (*Coryneforms*, *Micrococcus* spp. and *Staphylococcus* spp.) was higher, they also reported that the spoilage flora of the same fillets stored at 2°C was dominated by *Pseudomonas* spp. and *Moraxella* spp. Thus, the present study agrees with these reports, on the first day of sampling, immediately after immersion of the sardine in the brine (0 day), the aerobic microflora was found to be dominated by almost 55% of gram positive bacteria mostly *Micrococcus* and *Coryneforms*. Thereafter, there was shift in bacterial types, the gram negative bacteria increased sharply and on the last day of sampling almost 100% of the total bacteria were of this type (15°C samples). *Pseudomonas* and *Moraxella* which made up almost 19% of the initial flora, rapidly assumed a dominant position. Other species persisted but at a decreasing level.

From these results it could be concluded that changes in microflora of brine at 0°C showed *Pseudomonas* highly resistant to salt concentration and the same behavior for *Moraxella* although at 15°C. In addition gram negative bacteria displaced the initially predominant gram positive, faster at 15°C than at 0°C. At 15°C the rate of increase of the predominant spoilage microflora<sup>40-43)</sup> bears a close relationship to the brine concentration. By the time the sardine was considered to be spoiled, the microflora of 4% brine was relatively dominated by *Pseudomonas* spp., which reach up to 35% on the third day of storage, and those of the 10 and 15% brine solutions were dominated by *Moraxella* spp., ca. 65 (5th day) and 70% (7th day), respectively. In any case by this time the fish were considered unacceptable (end of shelf life) and the composition of the bacterial flora showed to be composed of nearly 100% gram negative microflora. *Moraxella* is not as frequently involved in spoilage as *Pseudomonas* but their occurrence on fish has been reported<sup>40-46)</sup>. At 0°C increases in the gram negative microflora with respect to salt concentration did not present a special pattern.

The spoilage potential of the microorganisms could be directly linked to the amounts of VB-N compounds produced by them<sup>47)</sup>. Increase in VB-N levels in the brine correlated well with the cell counts of the gram negative flora. When the gram negative spoilage flora reached approximately 100% of total flora, spoilage odors were evident as were increased levels of VB-N.

From these results, it can be concluded that immersed storage in 15% brine at 15°C could give a 5-day shelf life to the sardine. Therefore this preservation system could be used on board when there is no availability of adequate refrigeration systems, as in the case of most Peruvian sardine fishing vessels.

#### 4. Effect of Acetic and Citric Acids on the Growth and VB-N Producing Activity of *Pseudomonas* sp. and *Moraxella* sp.

Since early studies of fish spoilage, it has been known that autolytic activity plays a minor role, i.e. the spoilage is caused by bacteria. *Pseudomonaceae*, *Moraxella*, *Alcaligenes*, *Flavobacterium/Cytophaga*, *Corynebacterium*, *Vibrionaceae*,

*Bacillus* and *Micrococcus* are the microorganisms most frequently isolated<sup>35,40,41</sup>. Fish handling after catch causes contamination with a flora that is difficult to control<sup>48</sup> unless a rapid and adequate storage is done.

Organic acids whether naturally present in foods or intentionally added to them have been utilized for years to control microbial spoilage<sup>49</sup>. The use of an organic acid in raw fish storage after catching may offer interesting prospects for shelf life extension<sup>50,51</sup>. The efficacy of acetic acid as well as of citric acid as antimicrobial agents is well established<sup>49,52,53</sup>. Current data suggest that the mode of action of organic acids is attributed to direct pH reduction of the substrate, depression of the intracellular pH by ionization of the undissociated acid molecule or disruption of substrate transport system by alteration of cell membrane permeability<sup>49</sup>, and therefore depended on pH<sup>49,53,55</sup>.

The effect of treatment with different concentrations of acetic and citric acids was investigated using *Pseudomonas* sp. and *Moraxella* sp. isolated from brine during sardine storage. The objective of this investigation was to determine whether acetic or citric acid better inhibits *Pseudomonas* and *Moraxella* when inoculated in a "nutrient broth" containing 4% sodium chloride; and also sought to determine the minimal concentration of the acids to cause inhibition of bacterial growth and VB-N production.

## Materials and Methods

### *Strains*

*Pseudomonas* sp. P-1 and *Moraxella* sp. M-1 were isolated from 4% brine (section II-3) during storage of sardine at 15°C. They were cultured on nutrient agar that contained beef extract (Difco Laboratories, Detroit, MI, USA) 5 g/l, polypeptone (Nihon Seiyaku, Tokyo, Chiyoda, Japan) 10 g/l, sodium chloride (Kanto Chemical, Tokyo, Nihonbashi, Japan) 5 g/l, and agar (Nihon Seiyaku) 15 g/l (pH 7.2) and stored at 5°C.

### *Effect of the acid on microbial growth*

The effect of different concentrations of acetic and citric acids on the growth and activity of *Pseudomonas* and *Moraxella* was tested by adding 0.02, 0.03, 0.04, and 0.05% of each acid to a nutrient broth containing 4% sodium chloride. Inocula of the strains for the experiments were prepared by growing the microorganisms in nutrient agar slant at 25°C for 24 h. A loopful of these cells was added to a sterile 0.85% physiological saline. The media (100 ml in 200 ml Erlenmeyer flasks) were inoculated with 1 ml of the previously inoculated physiological saline to contain approximately 10<sup>5</sup> cells/ml<sup>56,57</sup> and the cultures were incubated aerobically and statically for 8 days at 25°C. After every two days of the incubation period, bacterial viable count was determined by plating on nutrient agar using the standard pour plate technique and duplicate plates (section II-3).

### *pH and VB-N analyses*

VB-N producing activity of the strains in the media was determined by changes in VB-N using the Conway method (section II-1).

The pH measurements were performed by a Horiba Compact pH-meter C-1 at the time of plating.

### Results and Discussion

This study was initiated to test to what extent acetic or citric acid inhibits the growth of *Pseudomonas* sp. P-1 and *Moraxella* sp. M-1 and the degree of inhibition according to acid concentration.

#### pH

The pH (Fig. 12) of the growth media increased through the incubation period except for that which contained the highest acetic acid concentration (0.05%) and was inoculated with *Pseudomonas* sp. P-1 (Fig. 12A). Besides the pH rate of increase caused by *Pseudomonas* sp. P-1 was faster than that caused by *Moraxella* sp. M-1 at any of the other acid concentrations tested.

When citric acid with larger dissociation constant<sup>58,59</sup>, was contained in the media, the initial value and the rate of increase in pH brought about by any of the tested strains appeared to be larger than those reached when acetic acid was present.

The undissociated molecule of the acid is known to be the active antimicrobial<sup>18,60,61</sup> and also to be responsible for pH values<sup>49,50,55</sup>. Besides it is known that most organic acids are ineffective as microbial inhibitors in the pH range

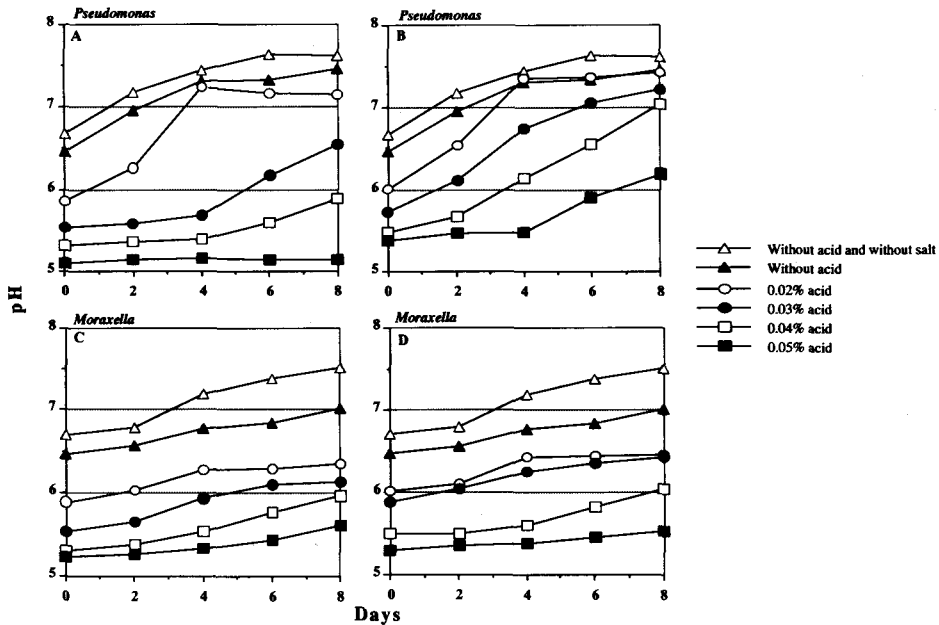


Fig. 12. Changes in pH of nutrient broth inoculated with *Pseudomonas* and *Moraxella*. The broth contains different concentrations of acetic (A and C) and citric (B and D) acids.

5.5 to 6.8 within which all food poisoning bacteria and most spoilage bacteria grow<sup>18</sup>). During our investigation, media containing 0.04% and 0.05% of either acetic or citric acid pH values below 5.5 (5.1 to 5.4) at the initial stage, under this range the action of the acids was evidently stronger than in any other concentration tested. In general the media with acetic acid gave lower pH values than the media with citric acid.

### Bacterial growth

Viable cell counts of *Pseudomonas* sp. P-1 and *Moraxella* sp. M-1 (Fig. 13) in the presence of acetic acid were always lower than their counterparts in the presence of citric acid, as the former has a smaller dissociation constant<sup>58,59</sup>).

A concentration 0.05% of acetic acid in nutrient broth containing 4% NaCl clearly inhibited growth of *Pseudomonas* sp. P-1 (Fig. 13A) and in some degree that of *Moraxella* sp. M-1 (Fig. 13C). The effect of salt was much greater with the presence of acids than when it worked alone. According to Ikawa *et al.*<sup>54</sup>) a similar effect was observed when 5% NaCl working together with 0.04% acetic acid inhibits growth of *Staphylococcus aureus* much more than salt alone. They attributed this effect to the combined action of both, NaCl and acetic acid. Furthermore, it is known that the action of organic acids as antimicrobial agents is generally improved by anions which interfere with the dissociation of the acid molecule; certain specific

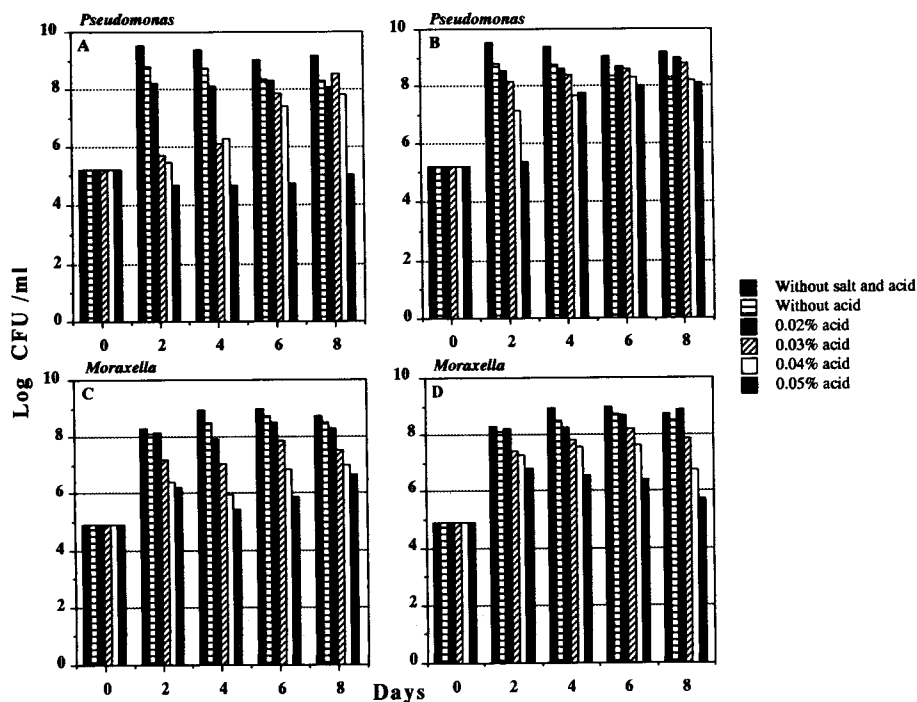


Fig. 13. Viable cell counts of *Pseudomonas* and *Moraxella* in nutrient broth containing different concentrations of acetic (A and C) and citric (B and D) acids.

cations may also significantly increase the effectiveness of organic acids by increasing the solubility of the acid in the microbial cell membrane<sup>62)</sup>.

Many microorganisms use organic acids as a metabolizable carbon source<sup>62)</sup>. From the results in this study, the strains used were supposed to catabolize the acids because they performed better growth in low level, acid concentrations, 0.02-0.03%, than in media without the acids. After 8 days of incubation all the cultures showed growth according to the acid and its concentration. It was observed that the numbers of *Pseudomonas* sp. P-1 were higher than those of *Moraxella* sp. M-1 in all the media tested, except in the medium containing 0.05% acetic acid in which *Pseudomonas* sp. P-1 decreased by the 2nd day and did not show any growth (Fig. 13A).

*VB-N*

VB-N production of both strains inhibited significantly by the addition of acid and salt to the media as compared with production in the media without acid and salt (Fig. 14).

A clear difference in VB-N production by *Moraxella* sp. M-1 and *Pseudomonas* sp. P-1 was observed independent of the tested acid. Values were higher for the latter one, known to have a stronger spoilage activity<sup>40,42,45,48,63)</sup>. VB-N production

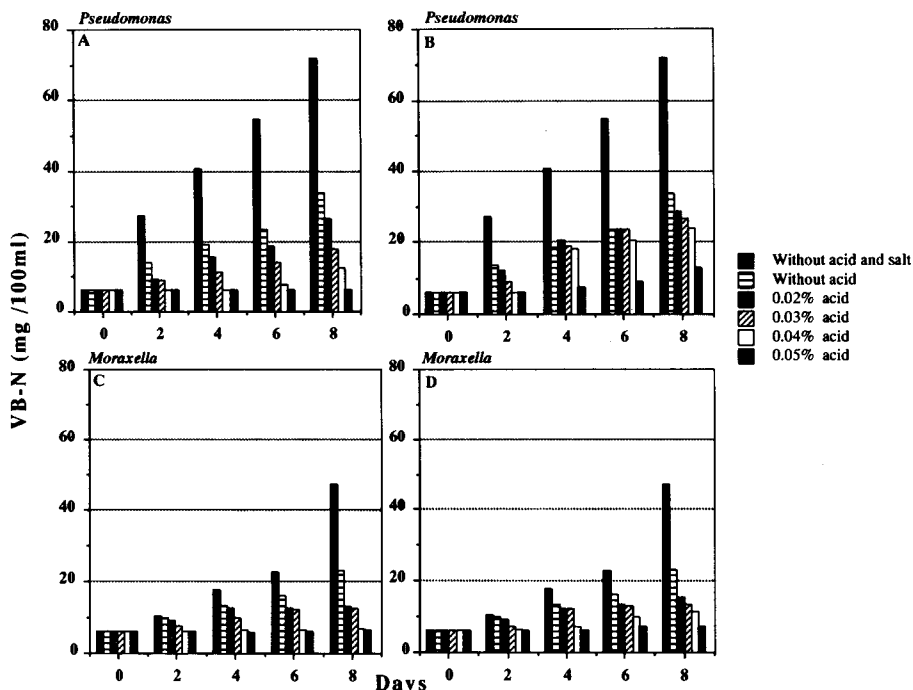


Fig. 14. Changes in VB-N of nutrient broth inoculated with *Pseudomonas* and *Moraxella*. The broth contains different concentrations of acetic (A and C) and citric (B and D) acids.

of the strains was comparatively higher in media containing the citric acid (Fig. 14B and D). Its values increased faster with activity of *Pseudomonas* sp. P-1 than with that of *Moraxella* sp. M-1 (Fig. 14A and C). Besides no significant differences in cell numbers (Fig. 13) between the two strains were observed, and in some cases *Moraxella* sp. M-1 showed larger numbers, *Pseudomonas* sp. P-1 produced higher amounts of VB-N in any of the tested media (Fig. 14).

As mentioned previously, the extent of antibacterial activity of these acids coincided with their degree of undissociation<sup>58,59</sup>. Citric acid with larger dissociation constant was less detrimental to the tested microorganisms than acetic acid. Similar results for *Listeria monocytogenes* were found by Ahamad *et al.*<sup>64</sup> when they were studying inhibition of *Listeria monocytogenes* CA and V7 by acetic, citric, and lactic acids at 7, 13, 21, and 35°C. Moreover, weak lipophilic acids are known to cause leakage of hydrogen ions across the cell membrane, acidifying the cell interior, and inhibiting nutrient transport. Some acids will dissociate to give anions, e.g., lactate, citrate, whose presence does not therefore inhibit energy yielding metabolism. Other acids, e.g., acetic and formic are very effective preservatives since they are not only proton conductors but also may yield inhibitory concentrations of their anions within the cell<sup>18</sup>. This might also explain the stronger activity of acetic acid under our experimental conditions.

From the above, it was decided to test 0.05% acetic acid for fish immersed storage in brine.

## 5. Acetic Acid for Preservation of Sardine Immersed in Brine

In the conversion of live animal to meat for consumption, microbiological contamination occurs as an unavoidable result of manipulation. Although the extent of contamination is highly variable, initial contamination occurs on the surface layers until spoilage is advanced<sup>22,35,65</sup>. Fish is probably the most perishable of all flesh foods and there is no question that spoilage is due primarily to bacterial action<sup>35,50</sup>.

Since early days workers have been experimenting with different chemical compounds to preserve fish<sup>50,51,66</sup>. The use of inorganic and organic acids to control bacterial spoilage of fish or meat has also been suggested<sup>50,52,66</sup>.

Organic acids are known to have good bactericidal activities<sup>49,52,53</sup> and are generally recognized as safe (GRAS) additives (FDA)<sup>53,67</sup>.

The purpose of this present work was to investigate the effect of acetic acid in fish preservation for human consumption when storage was held at 15°C. In this respect organoleptic, microbiological and chemical changes were evaluated.

## Materials and Methods

### *Raw material*

As in the sections before, freshly caught sardine (*ca.* 85 g each) were obtained at Kami-iso in Hakodate Bay. The sardine were placed immediately in an ice box, delivered to the laboratory and immediately divided into six lots of 2 kg.

*Fish storage and sampling*

Two lots were immersed in 2 l of 4, 10, and 15% brine containing acetic acid at a final concentration of 0.05%. For each brine concentration (4, 10, and 15%), one lot was stored at 0°C and other at 15°C.

At regular intervals during storage, two fish were removed for sensory and chemical analyses; pH, K value, and VB-N. At the same time, the brine solution was sampled for microbial analyses.

*K value*

Extract of the nucleotides from the sample was performed as in section II-1. And the analysis was carried out following the method presented in Ando *et al*<sup>(68)</sup>. A 10  $\mu$ l portion of the extracted supernatant was filtered through a Columngard-LCR<sub>4</sub> millipore filter and injected into a high performance liquid chromatographic (HPLC) system constituted by a Jasco BIP-1 HPLC pump equipped with a Jasco UVIDEC-100-V UV monitor and a Hitachi D-2500 Chromato-Integrator. The separation was performed on an Asahipack GS-320 column (7.6 mm i.d.  $\times$  500 mm) packed with a hydrophilic polymer gel and monitored at 260 nm. The mobile phase, 200 mM sodium phosphate buffer at pH 2.9, was used previous filtration through a 0.45  $\mu$ m pore size filter (Nihon Millipore Kogyo K.K.). Its flow rate was maintained at 0.9 ml/min during the separation.

K value was determined by the formula below :

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

*VB-N*

VB-N was performed by the method of Conway (section II-1).

*pH*

pH measurement was done in the same extract used for VB-N (section II-1) by a Horiba Compact pH-meter C-1.

*Sensory evaluation*

Fish were evaluated according to the method presented in section II-2 (Table 1).

**Results and Discussion***K value*

The K value of the different samples are shown in Fig. 15. In samples at 0°C the initial 7% increased gradually during the storage. On the 7th day, samples in 10% and 15% brine had K values of 47.5% and 45.2%, respectively, while samples stored at 15°C had a K value between the range of 70-90%. When compared with samples immersed in brine without acetic acid (section II-2), no differences were observed, and it could therefore be said that the action of the 0.05% acetic acid is void with respect to this parameter.

Since increment in the K value slowed with an increase in salt concentration,

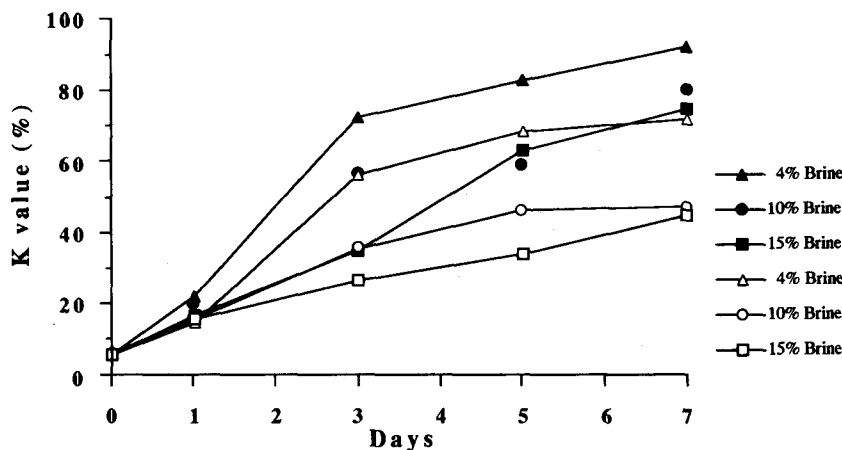


Fig. 15. Changes in K value of fish muscle during immersed storage in brine containing 0.05% acetic acid. Storage was held at 0°C (open plot) and at 15°C (solid plot).

it showed that enzymatic action was depressed by high salt concentration<sup>18)</sup>. The results of the organoleptic test in Table 6 correspond well to the K value pattern.

#### VB-N

Results of changes in VB-N throughout the time of storage are shown in Fig. 16. The VB-N measured immediately after the samples arrived at the laboratory was 10.2 mg/100 g. In 4%, 10%, and 15% brine immersed samples stored at 0°C, this value increased slightly and by the 7th day it was 16.2, 13.8, and 11.4 mg/100 g, respectively. These results could be better attributed to the temperature of storage rather than to the salt or the acid action.

On the other hand, at 15°C, clear increases in VB-N were seen among the samples. Fish immersed in 4% brine had 40 mg/100 g of VB-N on the 7th day, which is considered to represent initial spoilage<sup>23)</sup>. VB-N values for fish immersed in 10 and 15% brine were 26.4 and 20.3 mg/100 g, respectively, and these samples showed to be acceptable until the 7th day of storage. This is in contrast to those at 0°C. The action of salt was apparent and seen to be dependant on the salt concentration. Moreover as compared with the VB-N values recorded in the samples without acid (section II-2) on the last day of sampling, no marked differences were observed at 0°C. On the other hand, at 15°C they could be recognized to a greater extent. Samples stored in 4% and 10% brine without acetic acid were completely spoiled on the 3rd and 5th day, respectively, and those in 15% brine were determined to be at the initial stage of spoilage on the 7th day. Whereas in brine with acetic acid, samples stored in 10 and 15% brine were acceptable until the 7th day and samples in 4% until the 5th day. Thus according to the VB-N values obtained, it could be said that acetic acid extended a shelf life and seemed to be a good alternative for fish immersed storage held at 15°C.



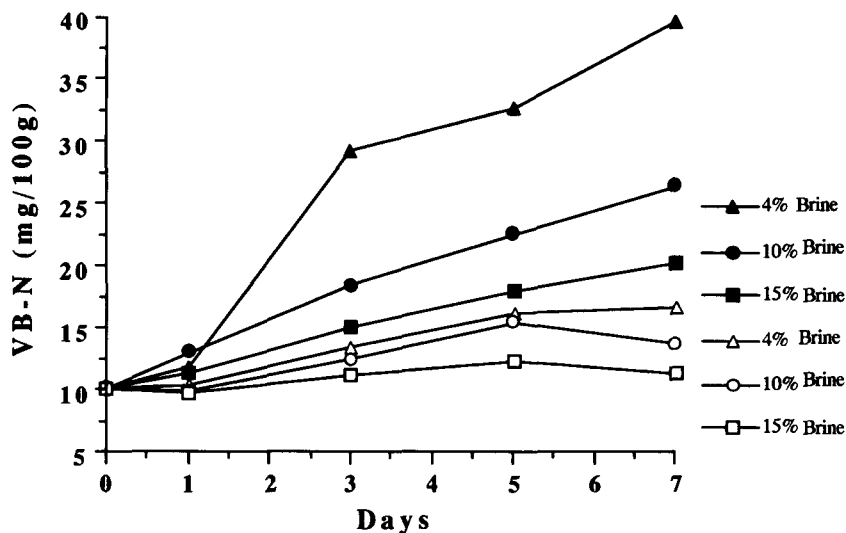


Fig. 16. Changes in VB-N of sardine during immersed storage in brine containing 0.05% acetic acid. Storage was held at 0°C (open plot) and at 15°C (solid plot).

### *pH*

Changes in the pH of sardine during storage are shown in Fig. 17. All samples presented a similar pattern. Their pH values increased steadily from the first day of storage. They reached a peak on the 5th day and then declined by the 7th day. A simple explanation of this change could be assumed on the basis of permeation of acid into the muscle<sup>19)</sup> and changes in muscle structural characteristic. These results seem to be reflected in the sensory evaluation presented in Table 6.

### *Sensory evaluation*

All samples at 0°C, as well as those immersed in 10% and 15% brine stored at 15°C were observed to be acceptable until the 7th day (Table 6). Whereas those in 4% brine stored at 15°C were acceptable until the 5th day. These results showed the effect of acetic acid in extending shelf life of fish of these samples as compared with samples (section II-2) immersed in brine without acetic acid.

## 6. Influence of Acetic Acid on Microbial Counts and Flora of Brine during Sardine Immersion

As it is well known, changes in microflora are related to the storage conditions. Therefore it is important to determine these changes when acetic acid is present in the brine of immersion.

### Materials and Methods

#### Sample

The brine used for sardine immersed storage in the previous section was used for the analyses.

#### Microbiological analyses

These analyses were performed in samples of brine following the procedure presented in section II-3.

Table 6. Overall quality scores and acceptability of sardine immersed in brines containing acetic acid held at 0°C (top) and at 15°C (bottom).

Testing subject	Before storage	Brine concentration											
		4%				10%				15%			
		Days				Days				Days			
		1	3	5	7	1	3	5	7	1	3	5	7
Overall appearance	A	A	A	B	B	A	A	A	A	A	A	A	A
Odor	A	A	A	A	B	A	A	A	B	A	A	A	B
Texture	A	A	A	B	B	A	A	A	B	A	A	A	B
Slime	A	A	A	A	A	A	A	A	A	A	A	A	A
Acceptability	A	A	A	A	B	A	A	A	B	A	A	A	B

Testing subject	Before storage	Brine concentration											
		4%				10%				15%			
		Days				Days				Days			
		1	3	5	7	1	3	5	7	1	3	5	7
Overall appearance	A	B	C	C	D	A	B	B	C	A	A	B	B
Odor	A	B	B	C	D	B	B	B	B	A	A	B	B
Texture	A	B	C	C	D	B	B	B	C	A	A	B	B
Slime	A	B	C	C	C	A	A	A	A	A	A	A	A
Acceptability	A	B	C	C	D	B	B	B	B	A	A	B	B

A; Completely fresh, highly acceptable

B; Fresh and acceptable

C; Fairly acceptable, borderline of acceptability

D; Spoiled and unacceptable

E; Completely spoiled, totally unacceptable

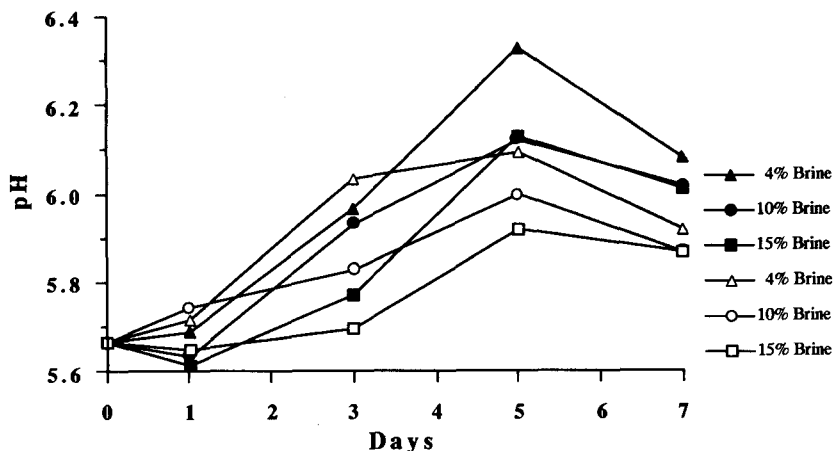


Fig. 17. Changes in pH of sardine during immersed storage in brine containing 0.05% acetic acid. Storage was held at 0°C (open plot) and at 15°C (solid plot).

## Results and Discussion

### *Microbial counts*

Results for microbial counts (Fig. 18) showed quite clearly that the total number of colony forming units (cfu) per ml of brine increased with time in 4 and 10% brines; those at 15°C showing a faster growth than those at 0°C. A major exception to this behavior was 15% brine stored at 0°C in which the action of the acid together with sodium chloride<sup>54)</sup> seemed to cause a strong inhibition of bacterial growth. The same effect was observed on the first day in 15% brine at 15°C. Counts for this sample on the 3rd day coincided with those in 4 and 10% brine stored at 0°C, and on the 5th day with that of the same 10% brine sample at 0°C. Between these two days, counts in 15% brine at 15°C remained at almost  $10^3$  cfu  $ml^{-1}$ . These results are reflected in the sensory evaluation (Table 6).

In comparison with results in previous studies<sup>69,70)</sup> and those in section II-3, an increase in microbial numbers was clearly slower with low counts. Similar effectiveness of acetic acid has been reported in studies of beef<sup>65)</sup> and pork<sup>71)</sup> preservation, and microbial inhibition (section II-4).

### *Identification of isolated strains*

The number of isolates from all of the brines together were 243 strains from samples stored at 0°C and 331 strains from those at 15°C. They made a total of 574 strains (Table 7).

### *Cocci*

Out of 574 isolates, 107 strains were found to be cocci catalase positive bacteria. In addition, since 83 strains of them were glucose non fermenters, they were characterized as *Micrococcus*. The other 24 strains were glucose fermenters and recognized as *Staphylococcus*.

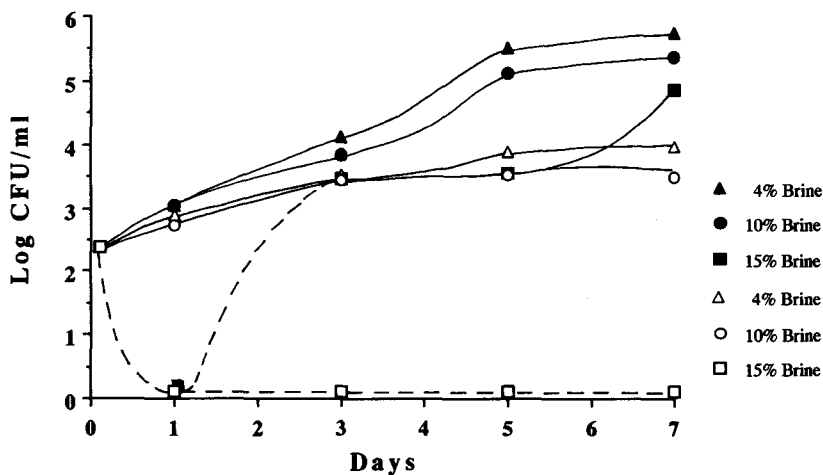


Fig. 18. Viable cell counts in brine containing 0.05% acetic acid during sardine immersion. Storage was held at 0°C (open plot) and at 15°C (solid plot).

#### *Gram positive bacilli*

Twenty eight isolates were gram positive bacilli, 23 of them were catalase positive and spore forming, therefore identified as *Bacillus*. The remaining 5 strains neither motile nor spore forming, according to these characteristics, were identified as Coryneforms.

#### *Gram negative bacilli*

a) Glucose fermenters: This group was constituted for 37 strains and 22 isolates of these showing oxidase positive reaction, motility and polar flagella, were identified as *Aeromonas*. Other 11 isolates were found to be non halophilic, motile by peritrichous flagella and consequently classified as Enterobacteriaceae. The remnant 4 strains were halophilic, motile, fermented glucose without gas and gave oxidase positive reaction, therefore they were recognized as *Vibrio*.

b) Glucose non fermenters, motile bacteria: One hundred forty five strains were grouped here. Seventeen strains having peritrichous flagella were thought to be *Alcaligenes*. Six strains of *Alteromonas* were isolated according to a positive gelatin liquefaction and the presence of polar flagella. Polar flagella were presented by another 122 strains but they were incapable of gelatin liquefaction and therefore recognized as *Pseudomonas*.

c) Glucose non fermenters, non motile bacteria: This was the most numerous group with 244 isolates from among 574 strains. Eighteen strains which colonies were non pigmented and gave oxidase negative reaction, were characterized as *Acinetobacter*. And the oxidase positive, *Moraxella*, were 225 strains. Only one strain of *Flavobacterium* with carotenoid pigment, was found.

These results showed *Moraxella* to be the most abundant group (225 strains) and *Pseudomonas* the second in numbers (122 strains).

Table 7. Number of strains isolated from brine containing acetic acid during sardine immersion. Storage was held at 0 and 15°C.

Days	Storage temperature					
	0°C			15°C		
	Brine concentration (%)			Brine concentration (%)		
	4	10	15	4	10	15
1	30	31	—	30	30	—
3	30	30	—	30	30	30
5	31	30	—	30	31	30
7	31	30	—	30	30	30
Sub total	122	121	—	120	121	90
Total	243			331		

#### Changes in bacterial flora

The flora in the samples stored at 0°C included representatives of 13 genera identified from 243 isolates (Table 8), while the 331 isolates from samples stored at 15°C were classified into 12 genera (Table 9).

Microflora composition in 4% brine (Fig. 19) at 0°C did not present any considerable changes during the storage. *Moraxella* was predominantly constant at 32% until the 5th day, and increased to about 80% on the 7th day. On the contrary, *Pseudomonas* which composed ca. 20% of the microflora until the 5th day decreased to only 3% by the 7th day. It should be pointed out that the 20% of gram positive bacteria on the 1st day of sampling, even showed a decline throughout the remaining the storage period.

In samples of the same brine concentration stored at 15°C (Fig. 19), *Micrococcus* which constituted 20% of the microflora on the 1st day was not isolated on the 5th day and was observed again with 4% of the total microflora on the 7th day. After the 3rd day the gram negative bacteria comprised almost 100% and *Pseudomonas* and *Moraxella* were the predominant flora until the last day of storage.

Ten percent brine samples at both temperatures (Fig. 20) presented exactly the same pattern in microbial evolution, and no severe changes of the flora were observed. Gram negative bacteria were predominant throughout the storage. An almost 70% of the gram negative bacteria was principally composed of *Moraxella* and followed by *Pseudomonas* which remained almost constant independent of storage temperature. *Micrococcus* was a major representative of the gram positive flora and observed to increase, in some degree, from the 3rd to the last day of storage. The changes in flora of these samples showed a striking contrast to the microflora in the samples without acetic acid (section II-3).

Finally, in 15% brine samples stored at 15°C (Fig. 21), gram positive bacteria was 35% of the total isolates on the 3rd day and increased, to such an extent as to reach 75% on the 5th day and 92% on the 7th day. *Micrococcus* became predomi-

Table 8. Bacterial genera isolated from brine containing acetic acid during sardine immersion. Storage was held at 0°C.

Genera	Brine concentration		Total
	4%	10%	
<i>Micrococcus</i>	15	16	31
<i>Staphylococcus</i>	2	3	5
<i>Bacillus</i>	5	1	6
Coryneforms	—	2	2
<i>Moraxella</i>	62	61	123
<i>Pseudomonas</i>	18	21	39
<i>Aeromonas</i>	3	3	6
<i>Alcaligenes</i>	3	3	6
Enterobacteriaceae	3	4	7
<i>Acinetobacter</i>	4	4	8
<i>Flavobacterium</i>	1	—	1
<i>Alteromonas</i>	2	—	2
<i>Vibrio</i>	2	—	2
Unidentified	2	3	5
Total	122	121	243

Table 9. Bacterial genera isolated from brine containing acetic acid during sardine immersion. Storage was held at 15°C.

Genera	Brine concentration			Total
	4%	10%	15%	
<i>Micrococcus</i>	9	14	29	52
<i>Staphylococcus</i>	1	3	15	19
<i>Bacillus</i>	—	3	14	17
Coryneforms	—	—	3	3
<i>Moraxella</i>	42	49	11	102
<i>Pseudomonas</i>	45	27	11	83
<i>Aeromonas</i>	7	9	—	16
<i>Alcaligenes</i>	6	3	2	11
Enterobacteriaceae	3	—	1	4
<i>Acinetobacter</i>	3	6	1	10
<i>Alteromonas</i>	2	1	1	4
<i>Vibrio</i>	—	2	—	2
Unidentified	2	4	2	8
Total	120	121	90	331

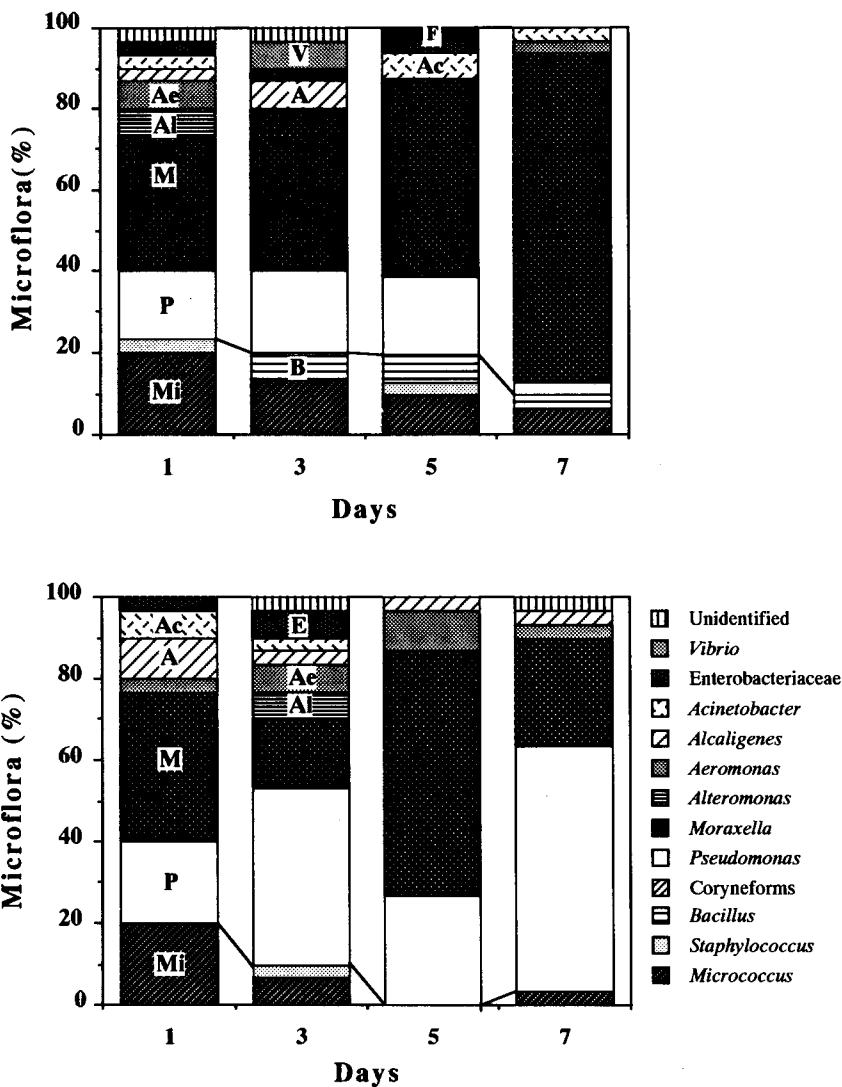


Fig. 19. Changes in microflora of 4% brine containing acetic acid during sardine immersion. Storage was held at 0°C (top) and at 15°C (bottom).

nant with 50% of the isolates on the last day. This data showed an unusual behavior in changes in bacterial flora of fish<sup>41)</sup> or fish related products<sup>63)</sup>. The normal pattern is an increase in gram negative bacteria, specially, *Pseudomonas* and *Moraxella* which are the well known agents of spoilage.

From the findings obtained during this study, it could be said that storage at high temperature (15°C) and immersion in 4% brine containing acetic acid, could give five days of acceptability to the sardine thus stored. Therefore, this may be a

suitable method for preservation of fish destined for human consumption. Although these results show that acetic acid is useful for fish storage at 15°C, further study needs to be carried out on larger scale treatment and the effects on nutritional value.

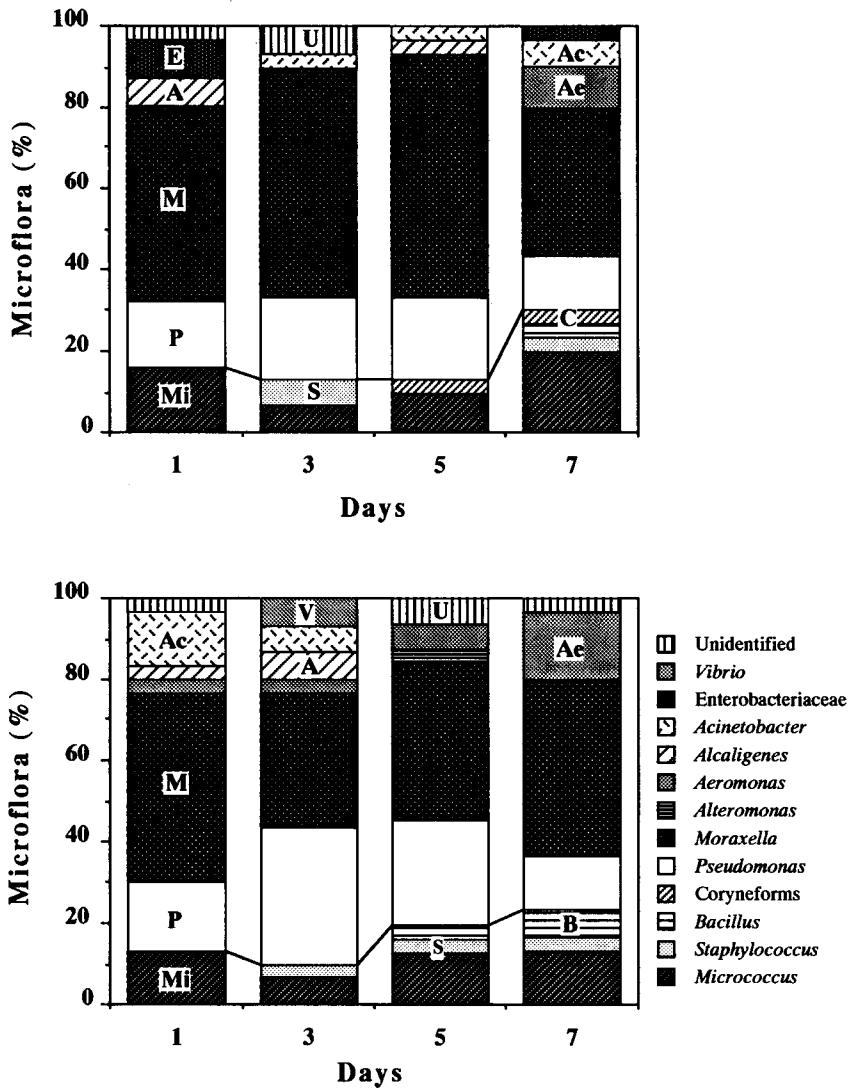


Fig. 20. Changes in microflora of 10% brine containing acetic acid during sardine immersion. Storage was held at 0°C (top) and at 15°C (bottom).



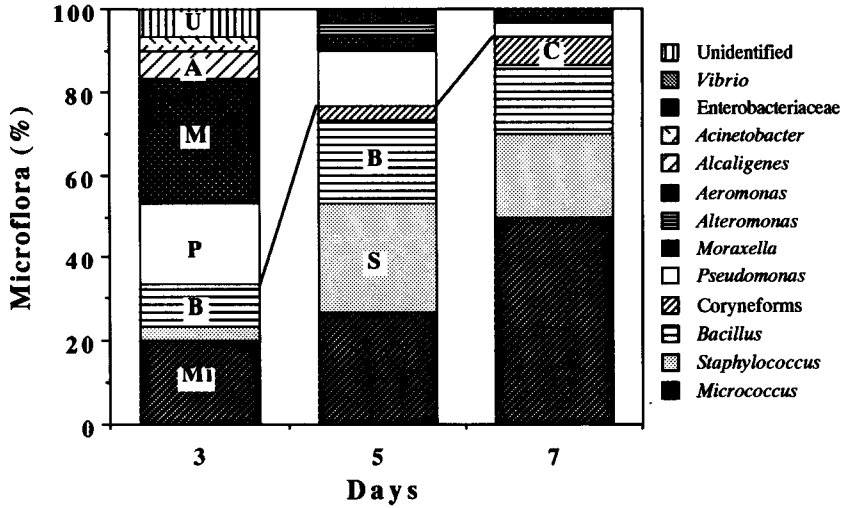


Fig. 21. Changes in microflora of 15% brine containing acetic acid during sardine immersion. Storage was held at 15°C.

### III. A Trial Use of Sea Water for Fish Preservation

Refrigerated sea water has been widely used in some areas in canning plants for storing sardines, herring, pilchards and similar fish prior to processing. It has also been found that herring stored in refrigerated brine showed keeping qualities superior to those of herring held in ice<sup>72)</sup>. Thus the use of sea water for fish storage on board seems to be an ideal, economic, and simple, alternative to be tested as a replacement for brine.

Sea water has a similar salinity to the 4% brine used in the previous chapter, and it could be obtained wherever desired with a low cost. On the other hand, according to the results from sections II-4, 5, and 6 of this study, acetic acid was found to be effective on fish preservation when used together with 4% brine, giving to the fish 5 days of acceptability even if the fish was stored at 15°C. Therefore, it was decided to test the effect of sea water containing acetic acid on sardine preservation under the same temperature.

#### 1. Effect of Acetic Acid on Growth of Bacteria in Peptone Sea Water Media

In sections II-4 and 5 sardine was preserved in brine containing 0.05% acetic acid.

In this section the amount of acetic acid which might be added to the sea water to be used for fish preservation should be again determined. Therefore determination of the optimal concentration was performed testing growth inhibition of *Pseudomonas* sp. growing in a laboratory model.

## Materials and Methods

### *Strain and inocula*

The same strain of *Pseudomonas* sp. P-1 used in section II-4 was used here. Inocula was prepared according to the method described in the mentioned section.

### *Artificial sea water (ASW)*

Artificial sea water was prepared as follows; sodium chloride 30 g/l, potassium chloride 0.7 g/l, magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) 5.3 g/l, magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) 10.8 g/l, and calcium sulfate ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) 1.3 g/l (All the reagents used were from Wako Pure Chemical Industries).

### *Laboratory model*

Previous to the determination of the minimal concentration of acetic acid in a laboratory model, there was the need to determine the optimal concentration of peptone in which *Pseudomonas* sp. P-1, could attain the same level of growth to that of bacteria in 4% brine during immersed storage of sardine (section II-3).

Growth of *Pseudomonas* sp. P-1 was performed in an ASW containing various concentrations of peptone (Polypeptone; Nihon Seiyaku, Tokyo, Chiyoda, Japan); 0.0, 0.001, 0.003, 0.005, 0.01, 0.1, 0.3, 0.4, and 0.5%.

ASW (100 ml) containing the different concentrations of peptone into a 200 ml Erlenmeyer flasks was inoculated with *Pseudomonas* sp. P-1 after sterilization. Cultures were incubated aerobically and statically at 15°C for 3 days. Growth of the microorganism was observed using the standard pour plate technique (section II-3).

### *Determination of the acid concentration*

An appropriate concentration of peptone was used for the determination of acetic acid concentration for the media.

Peptone-ASW medium (100 ml) was prepared in 200 ml Erlenmeyer flasks. Acetic acid was aseptically added to the flasks to make final concentrations of 0, 0.01, 0.02, 0.03, 0.04, and 0.05%. After the acid addition, the media were inoculated with the previously prepared inocula. Growth of the microorganism was observed using the standard pour plate technique (section II-3).

## Results and Discussion

### *Determination of peptone concentration*

Growth of *Pseudomonas* sp. P-1 in various media tested are shown in Fig. 22. In ASW without peptone (control), cell counts decreased throughout the incubation. According to this result it could be undoubtedly concluded that scarcity of nutrients caused the microorganism to die of starvation. On the other hand, it was clear that increases in peptone concentration were responsible for growth acceleration. Major differences in the level of growth were observed in the range between 0.001 to 0.005% (Fig. 22B). This difference became smaller in the range between 0.005 to 0.01% (Fig. 22C), and there was no important difference in the range between 0.1 to 0.5%

(Fig. 22D). At peptone concentration levels from 0.005 to 0.01%, growth of the microorganism approached the microbial growth curve presented in Fig. 22A (the observed growth in 4% brine during immersed storage of sardine) better than it did at other concentrations tested. However, the most similar growth curve to that in Fig. 22A was obtained when the microorganism grew in 0.005% peptone-ASW medium. Therefore, this concentration was used for the determination of the acetic acid concentration.

*Determination of the acid concentration*

All the concentrations of acetic acid tested were seen to inhibit the growth of *Pseudomonas* sp. P-1 (Fig. 23). In the control without acid, growth started from the time of inoculation. On the contrary in samples containing the acid, cell numbers decreased soon after inoculation. Concentration of 0.02 to 0.05% gave a total inhibition of the microorganism within the first 24 hours. Whereas 0.01% acetic acid caused cells to diminish progressively, until they reached *ca.*  $10^2$  cfu  $ml^{-1}$  on the 3rd day. Further tests were not required and 0.01% acetic acid was decided as the lowest concentration to be used in sea water for fish immersed storage.

**2. Sardine Immersed Storage in Sea Water Containing Acetic Acid**

In this section, sea water containing different acetic acid concentrations was

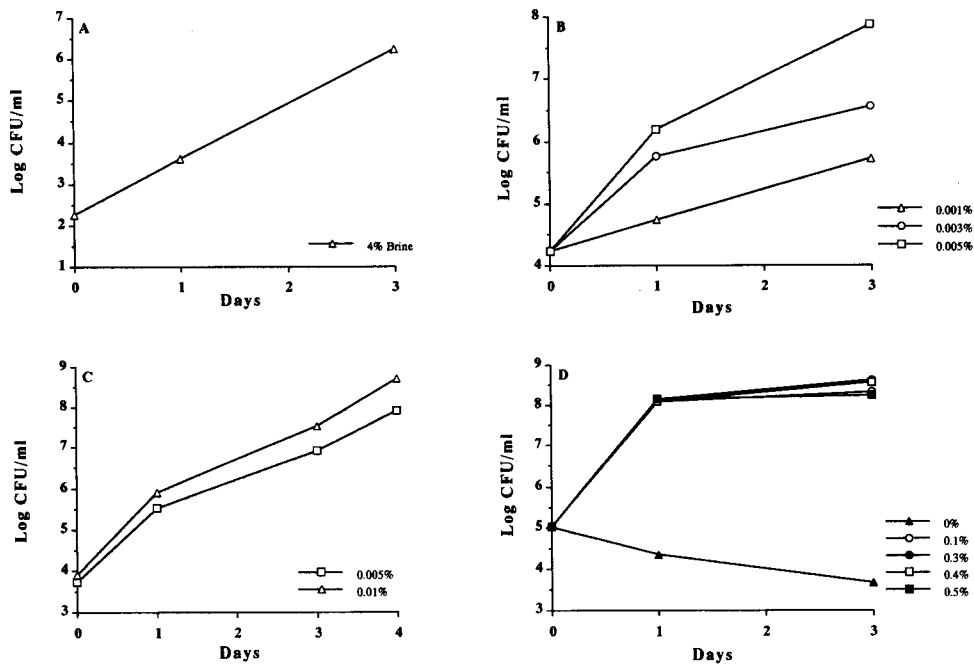


Fig. 22. Growth of *Pseudomonas* in different concentrations of peptone. A, 4% brine without peptone; B-D, peptone-ASW medium.

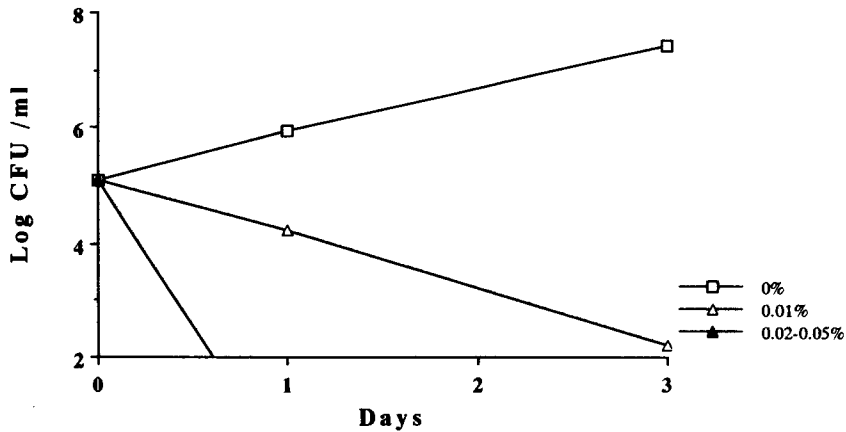


Fig. 23. Effect of different concentrations of acetic acid on *Pseudomonas* sp. growing in artificial sea water containing 0.05% peptone.

tested in fish preservation. The determined acetic acid concentration in the previous section will be the lowest acetic acid concentration to be tested together with sea water.

### Materials and Methods

#### *Raw material*

The sardine, weighing ca. 83 g each, were obtained at Kami-iso in Hakodate Bay and brought to the laboratory (section II-2). The fish were divided into lots of 2 kg and used for the experiments.

#### *Sea water (SW)*

SW for storage of the sardine was obtained at Anama in Hakodate city, filtered through a plankton net (NMG 84) and transported to the laboratory.

#### *Fish storage and sampling*

Each lot of fish was immersed in 2 l of SW in which acetic acid had been previously added to make final concentrations of 0.0, 0.01, 0.03, 0.05, 0.07, 0.15, 0.2, and 0.25%. Storage was held at 15°C.

To make easier presentation of the results, samples were divided into two batches, according to the acid concentration used. Thus, one would be the batch in the low level concentration, 0.01 to 0.07%, and the other the batch in the high level concentration, 0.15 to 0.25%.

Samples were taken to determine microbial counts and VB-N of the sea water in which sardine were immersed and VB-N of the fish. In any case sampling was performed until the VB-N value in the fish muscle reached the designed limit of acceptability (30 mg/100 g).

*Analyses*

VB-N and microbial counts were performed as in sections II-1 and 3.

**Results and Discussion***Microbial counts*

The SW used in this section showed *ca.*  $10^3$  (Fig. 24A) and  $10^4$  (Fig. 24C) cfu  $ml^{-1}$ . Increases in bacteria population (Fig. 24A and C) were seen during the storage in all of the samples. Soon after the samples were immersed, the bacterial counts in the controls, of both batches of samples, were observed to be higher, together with the counts in SW containing 0.01% acetic acid. This difference between the last mentioned samples and the samples containing 0.03% acetic acid concentration or higher, at the initiation of the storage, was related to the inhibiting action of the acid on microbial cells. By the 2nd day no major differences were observed between the control and the samples.

*VB-N*

Values obtained for either SW or fish muscle (Figs. 24B and D, and 25A and B) showed minor increases during the first day. The VB-N values in muscle of all fish samples (Fig. 25A and B) immersed in SW containing acetic acid were acceptable until the second day with the exception of the sample in SW with 0.01% acetic acid. On the other hand, the VB-N of SW with 0.01% acetic acid (Fig. 24B) increased faster than the VB-N of the control SW without acid between the 2nd and 3rd days. This result confirmed the results obtained in section II-4. That is, microbial growth was performed better in nutrient broth containing low acid concentrations than in broth without the acid suggesting that microorganisms use small amounts of the acid for their metabolism.

By the last day the VB-N value surpassed 30 mg/100 ml (or g) in all the SW and fish samples. Therefore, it was concluded that this method would not be efficient in prolonging the 5 days shelf life obtained in the previous chapter and it was decided to proceed in a different manner.

**3. Effect of Pretreatment on Sardine Storage Prior to Immersion into Sea Water**

As the addition of acetic acid to the SW was tested without success for preservation of sardine, it was thought that the pretreatment of the fish should be tested to look for possibility of success. Therefore, such treatment were carried out using high concentrated solutions of acetic acid and NaCl.

When fish is immersed into a concentrated solution, e.g., NaCl solution, it could be logically considered that the high osmotic pressure to which the fish is exposed will be responsible for the movement of the solute into the flesh accompanied by an even more active movement of water from the fish into the surrounding solution. This process will continue, until a kind of barrier is formed in the surface layer of the muscle tissue, limiting further movement from either the outer or the inner layers of the meat, thus bringing about a preservative action.

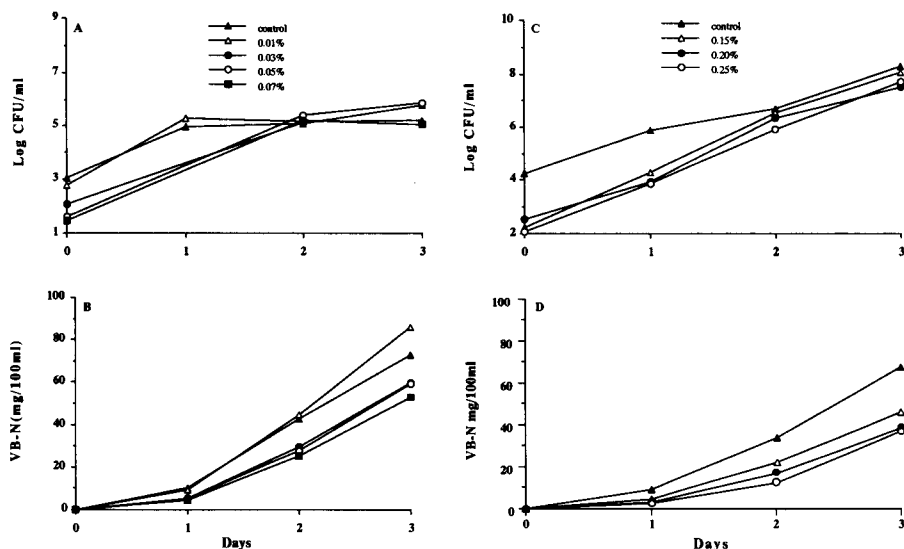


Fig. 24. Microbial counts (A and C) and VB-N (B and D) changes in brine containing different concentrations of acetic acid.

## Materials and Methods

### *Raw material*

Samples were obtained as in the previous chapter (section II-2). Once the fish arrived at the laboratory the sardine were divided into 12 lots of 2 kg.

### *Sea water*

Obtained as in the previous section.

### *Fish storage and sampling*

Samples were grouped into two batches to facilitate presentation of the results.

**Batch 1:** For the first group, two solutions of 3 and 5% acetic acid were prepared using distilled water. Each two lots were immersed in each solution for 1 and 2 minutes, respectively. After being removed, they were finally immersed in 2 l of SW. The last lot without any previous treatment was also immersed in SW as a control.

**Batch 2:** The 2nd group encompasses three different treatments. For the first one a 5% solution of acetic acid (the same as the above) and for the 2nd one a saturated solution of NaCl were used. In the case of the acetic acid and the concentrated sodium chloride solution, two lots were immersed in each solution for 2 min and one of them was rinsed with tap water after that. Consecutively every lot was immersed into 2 l of SW. For the last treatment, the addition of 2% NaCl (40 g/2 l of SW) to the SW to increase its salinity was also used. Fish were immersed after the NaCl had been thoroughly mixed. A control was run as described above (batch 1).

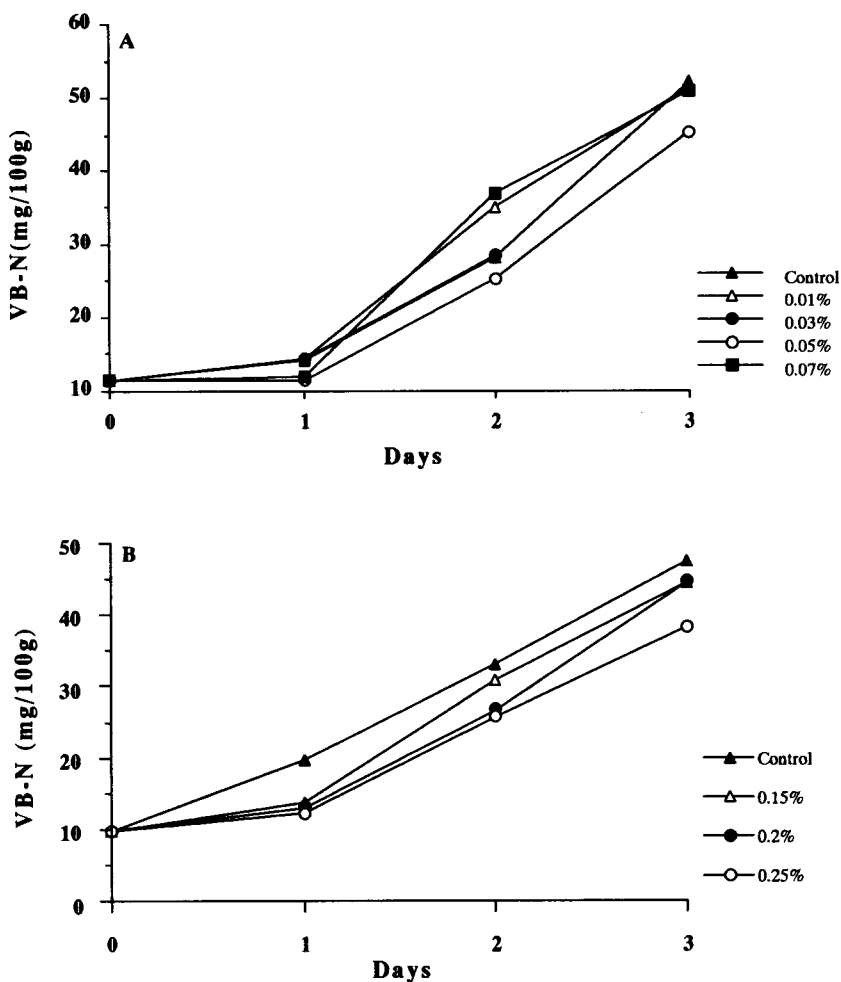


Fig. 25. Changes in VB-N of sardine muscle during immersed storage in sea water containing acetic acid. A, low concentrations ; B, high concentrations.

Storage of all the samples was held at 15°C.

*Analyses*

The methods used were the same as those in the previous section.

**Results and Discussion**

*Batch 1*

*Microbial counts*

In the first group initial microbial count (Fig. 26 A) in the SW used as a control was  $3.6 \times 10^3$  cfu ml<sup>-1</sup>, and that for the treated samples was lower than  $5 \times 10$  cfu

$\text{ml}^{-1}$  in any case. Difference in the initial bacterial load between the control and the samples was attributed to the effectiveness of the pretreatment. On the next day this difference was maintained, although an increase developed at the same rate in all the samples. Furthermore microbial counts of the samples pretreated in acetic acid at all concentrations had almost the same bacterial count by the 2nd day, and on the 3rd day all the samples exceeded microbial counts of the control, in which the bacterial population had already reached the stationary phase.

### *VB-N*

Changes in VB-N of the fish and the SW (in the first group) throughout the storage are shown in Figs. 26B and 27. When the sardine arrived at the laboratory their VB-N was 10 mg/100 g (Fig. 27). Until the 1st day of storage VB-N of the control was somewhat higher than those of the treated samples. However, pretreated samples presented the same rate of increase to that of the control from the first to the 2nd day. In samples pretreated for 2 min in 3 and 5% acid final values were, 40 mg/100 g and 30 mg/100 g on the 4th day of storage, respectively, lower than those of samples pretreated for 1 min for which the values were 50 and 40 mg/100 g, respectively.

Higher VB-N values in the control, although lower microbial counts with respect to the treated samples, forced us to think about differences in microflora.

VB-N of the SW immersion (Fig. 26B), had a very similar pattern to that presented by the fish sample immersed in it. The lowest VB-N was observed for samples pre-immersed in 5 and 3% acid solutions, in this order. Differences, between the control and the treated samples became larger as the storage went on. And the same occurred between the treated samples themselves. Therefore the samples showing lower VB-N were considered to indicate better storage conditions.

### *Batch 2*

#### *Microbial counts*

Results for the 2nd group are shown in Fig. 28A. The initial bacterial load on SW in which samples pretreated in acetic acid had been immersed was nearly ten fold lower than the counts in all the other samples. During the first day parallel increase was observed in all the samples, and on the 2nd day, maximum counts were recorded for most of the samples excepting for those pretreated with the acid solution. In the case of these two samples pretreated with the acid, differences in microbial counts observed between them were attributed to the rinsing process. When fish were rinsed after pretreatment, the bacterial count of the SW was higher than that of the non-rinsed samples. The lowest counts were recorded in non-rinsed samples throughout the storage and a major difference was observed until the 2nd day.

### *VB-N*

After the 4th day of storage VB-N values of about 30 mg/100 g were measured in fish pretreated with 5% acid (Fig. 29), and all the other samples surpassed this value on the 3rd day. These results correlated well with results of microbial counts and VB-N of the SW in which the fish were immersed (Fig. 28A and B). In all of



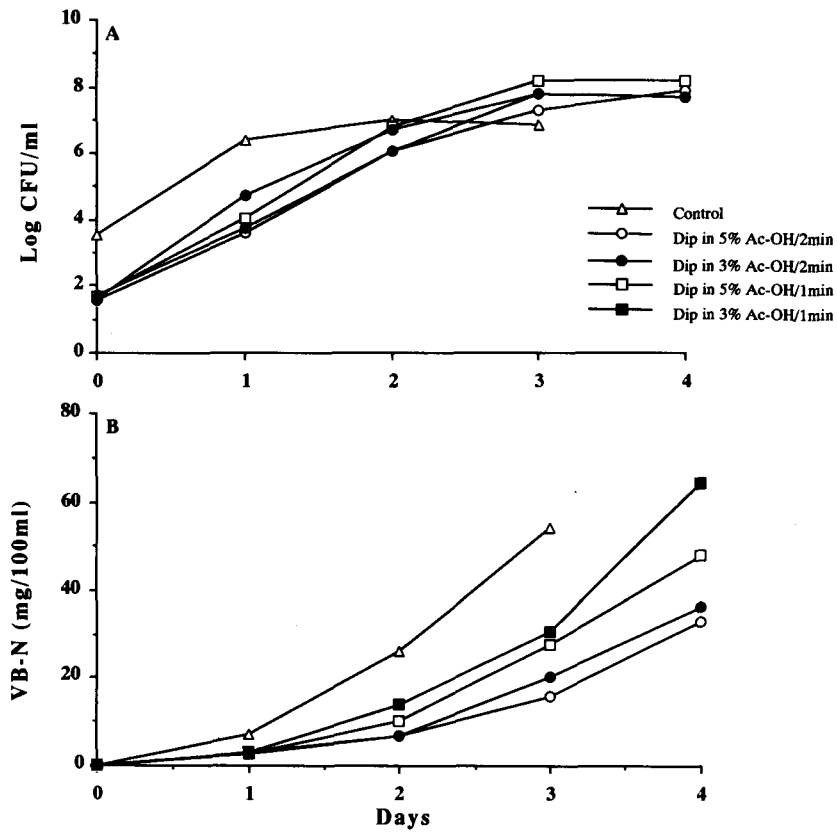


Fig. 26. Changes in microbial counts (A) and VB-N (B) of sea water during fish storage after dipping in acetic acid.

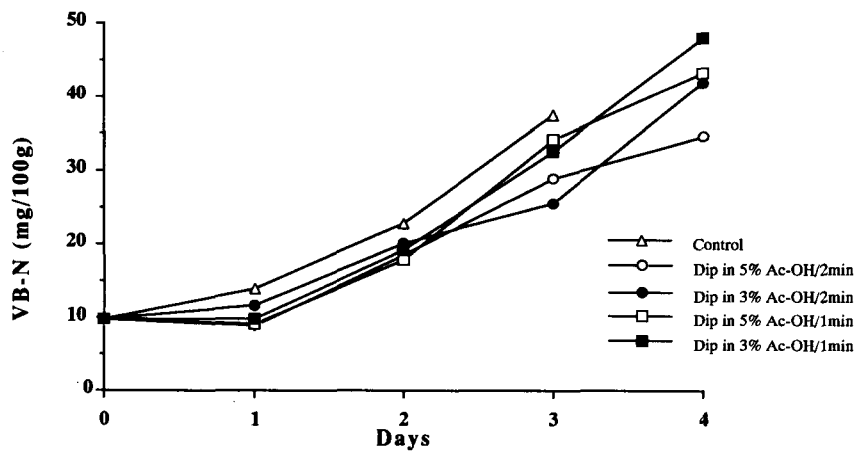


Fig. 27. VB-N of fish immersed in sea water dipping in acetic acid.

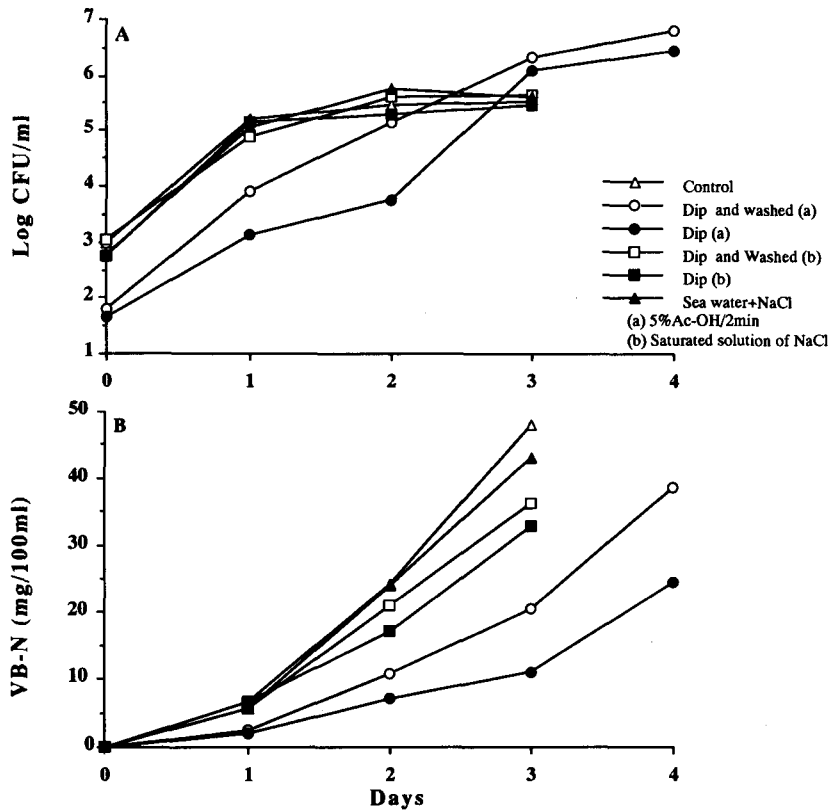


Fig. 28. Changes in microbial counts (A) and VB-N (B) of sea water during fish storage at 15°C, after pretreatment with acetic acid and NaCl.

the samples increases in VB-N were only slightly until the 1st day, thereafter differences in the values became clear, and on the 3rd day the lowest values were recorded in the samples in which fish were pretreated in acetic acid and were immersed in SW. However, even under the best condition only 4th days of acceptability were reached.

Results obtained within these trials showed that none of the treatments tested throughout, was able to prolong the 5 days of acceptability obtained when sardine were immersed in 4% brine containing acetic acid (section II-5). From these results it was supposed that some kind of matter in the SW caused fast spoilage of the fish and the presence of this matter was responsible for the failure of this treatment. Therefore, it was thought that a filtration of the SW could solve this problem. For this purpose it was decided to test filtration systems which were able to remove the suspected matter in the SW.

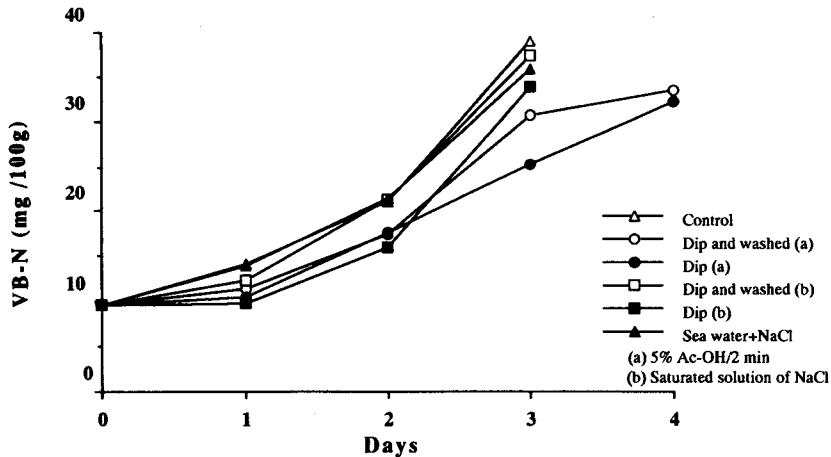


Fig. 29. Changes in VB-N of fish immersed in sea water after pretreatment with acetic acid and NaCl. Storage was held at 15°C.

#### 4. Use of Filtered Sea Water for Sardine Immersed Storage

In line with the conclusion of the previous section, natural sea water was filtered so as to eliminate particles which were supposed to accelerate spoilage of fish. The selected filtration method was the use of activated carbon which is known to be able to bind some kinds of particles within its structure<sup>73)</sup>. Thus, filtered SW was tested in sardine preservation in this section.

#### Materials and Methods

##### *Raw material*

The same sardine being used throughout this investigation (section II-2). Fish were divided into 6 lots of 2 kg.

##### *Sea water and 4% brine*

The same SW used in previous sections was filtered through activated carbon. Brine was prepared as described in section II-1.

##### *Fish storage and sampling*

Two lots of 2 kg were immersed each in 2 l of filtered and non filtered SW. One of the lots was immersed in 3% acetic acid solution for 2 min, prior to immersion into the non filtered SW. And the last lot was immersed in filtered SW containing 0.05% acetic acid as a final concentration.

For comparison purposes 2 more lots were immersed in 2 l of 4% brine. One of the lots was immersed in 3% acetic acid solution for 2 min prior to the final storage.

## Results and Discussion

### Microbial counts

All the samples were judged acceptable only until the 2nd day (Fig. 30A) and their bacterial load should the maximum increase during the 1st day of storage. Later they entered the stationary phase, known to be the period of maximum spoilage activity<sup>22</sup>.

Fish pretreated with acetic acid and stored either in the brine or in the SW were acceptable until the 4th day. As a result of fish pretreatment; immersion into the respective acetic acid solution, brine or SW, lower initial bacterial loads were observed. Between these two samples, counts in brine were lower than in SW. However, the rate at which they increased followed exactly the same pattern until the 3rd day. By the 4th day the numbers recorded were almost the same. Initial differences between these two samples were attributed to higher numbers of bacteria inherent in the SW. However, as it has been reported<sup>74</sup>, initial numbers do not affect the growth rate.

Bacterial growth in the filtered SW containing acetic acid or not, was faster than that in SW containing the fish pretreated with acetic acid. It was clear that filtration of the SW had no effect on the inhibition of bacterial growth.

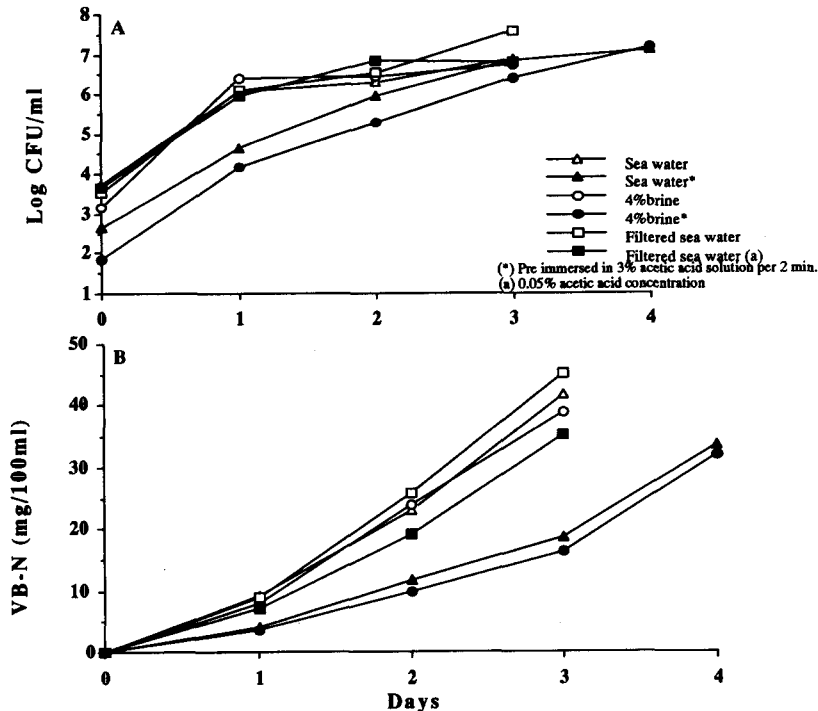


Fig. 30. Changes in microbial counts (A) and VB-N (B) of 4% brine and sea water during sardine immersion.

*VB-N*

The initial VB-N recorded in fish samples (Fig. 31) was around 11 mg/100 g and no major changes were observed until the first day. Thereafter VB-N increased progressively.

The VB-N of the two pretreated samples increased slower and on the 3rd day a marked difference between these and all the other samples was seen to exist. By this time last mentioned samples were determined to be in the borderline of acceptability, whereas the same conclusion was made the next day (4th) for the two former samples.

The VB-N of the immersion solutions (Fig. 30B) was nearly the same as the respective fish sample. The pattern of increase as well as the difference found between VB-N values of the fish samples was reproduced exactly here.

In microbial counts as well, filtration of SW did not slow the rate in the VB-N increase, which is the same as saying that it did not extent the sardine acceptability.

All the described treatments for fish storage, tried in this chapter III, were not able to improve the 5 days of acceptability of fish, obtained in the previous chapter. Therefore, it was concluded that under the proposed conditions SW immersion was not effective in fish storage.

**IV. Use of Food Additives for Sardine Immersed Storage**

Man's first contact with incidental food additives is one of vital concern to us today; the material produced by fire when food is cooked. Spices and salt were known to peoples of all civilizations and probably date into prehistorical times.

Nowadays, the chemicals which are intentionally added to our food constitute a small but important part of the many chemicals which man is adding to his environment. These chemical are needed to efficiently produce an abundance of high quality food<sup>75)</sup>.

Changes in agricultural practices, dietary shifts toward more perishable food,

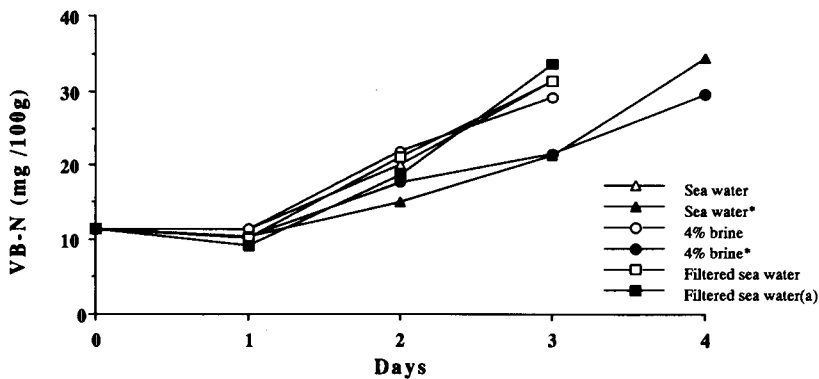


Fig. 31. VB-N of sardine immersed in 4% brine and sea water.

(\*) Pre-immersed in 3% acetic acid solution for 2 min.

(a) 0.05% acetic acid concentration.

increased possibilities of mass contamination through our enlarged distribution systems and the current strong trend toward convenience foods; all are placing greater emphasis upon methods of preservation.

World wide, the growing crisis in the food supply, demands that losses be reduced to a minimum. Hence the use of chemical preservatives, alone or as a supplement to other methods, is essential.

There are preservatives which have general applications, whereas others are more specialized and some of them have dual roles. The benzoates and parabens which have the greatest breadth of activity, encompassing many spoilage bacteria, fungi and yeasts, belong to the first group. While nitrites and nitrates that act not only as preservatives but also improve organoleptic characteristics, belong to the last group<sup>76)</sup>.

Furthermore the benzoates and propionates are relatively inexpensive compounds, whereas sorbates and parabens are relatively more expensive.

### 1. Test of Some Additives in Sardine Immersed Storage

During the present investigation it was seen important to continue looking for a preservation method to improve results obtained in the last section II-6. This time, additives were tested to determine its effect on immersed fish storage. Among the wide variety of preservatives, for the purpose of this study, sodium benzoate, sodium nitrite, and hydroperoxide were selected. This selection was based on their activity and their uses<sup>77-79)</sup>. The selection of sodium benzoate was also made taking into account its low price.

## Materials and Methods

### *Raw material*

The sardine weighing *ca.* 83 g each were obtained as in previous section (section II-2) and divided in 4 lots of 2 kg.

### *Fish storage and sampling*

Solutions containing 0.3% sodium benzoate, 0.1% hydroperoxide, and 0.1% sodium nitrite, were prepared using 2 l of 4% brine as the solvent (All the reagents used were from Wako Pure Chemical Industries, Tokyo, Japan). These preservatives have a better antimicrobial effect at a low pH<sup>76,77,79)</sup>. Therefore, once the desired concentration of the solute was reached, the pH was adjusted to 5 using acetic acid, and fish was immersed into 2 l of the 3 different solutions. Storage was held at 15°C. The concentrations were selected because they are the most common being used at present, or are the limits for uses in foods<sup>77,79)</sup>.

Samples of fish and brine for VB-N and microbial counts were taken at predetermined times.

### *Analyses*

VB-N test and microbial counts were performed as described before (sections II-1 and 3).

## Results and Discussion

### Microbial counts

The initial microbial count was about  $10^4$  cfu  $ml^{-1}$  (Fig. 32A). During the first two days, increase of bacterial counts was faster in the brines containing sodium nitrite and hydroperoxide. These two samples showed maximum numbers on the 4th day. On the contrary, the microbial population of the brine containing sodium benzoate grew slowly up to the 2nd day, later growth accelerated and microbial numbers reached their maximum  $5.5 \times 10^7$  cfu  $ml^{-1}$  on the 4th day. During the 6th and 7th days the bacterial count decreased slightly.

### VB-N

The initial value of VB-N in the fish flesh (Fig. 33) was about 10 mg/100 g. By the 2nd day the values of all the samples stayed below 20 mg/100 g. However, after

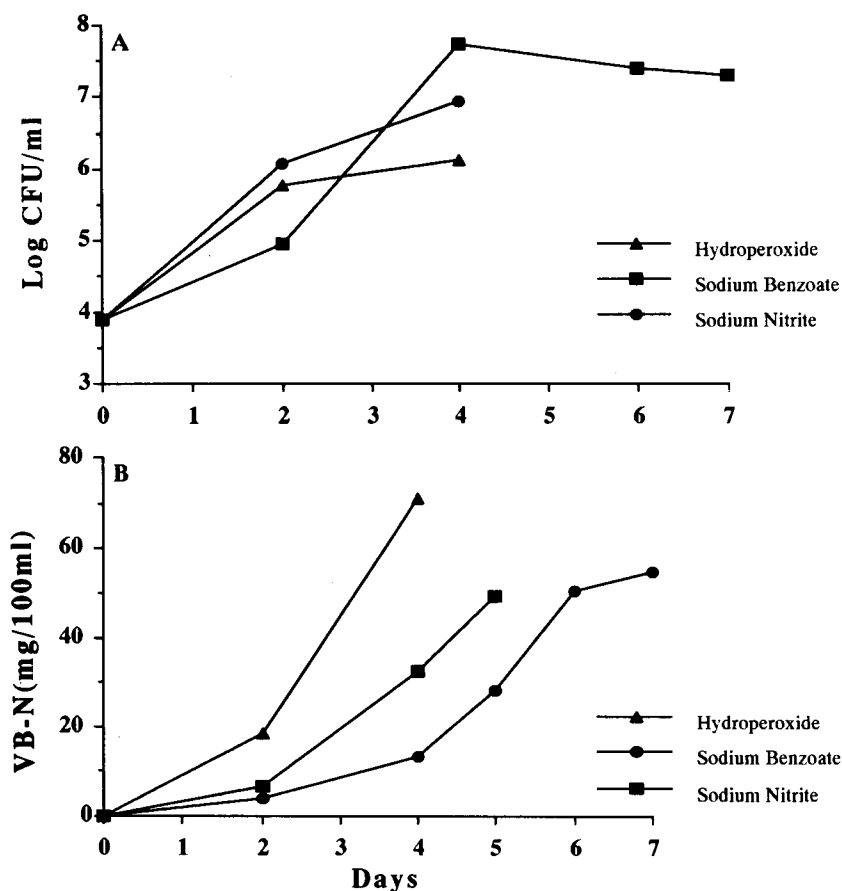


Fig. 32. Microbial counts (A) and changes in VB-N (B) of brine containing preservatives during sardine immersion.

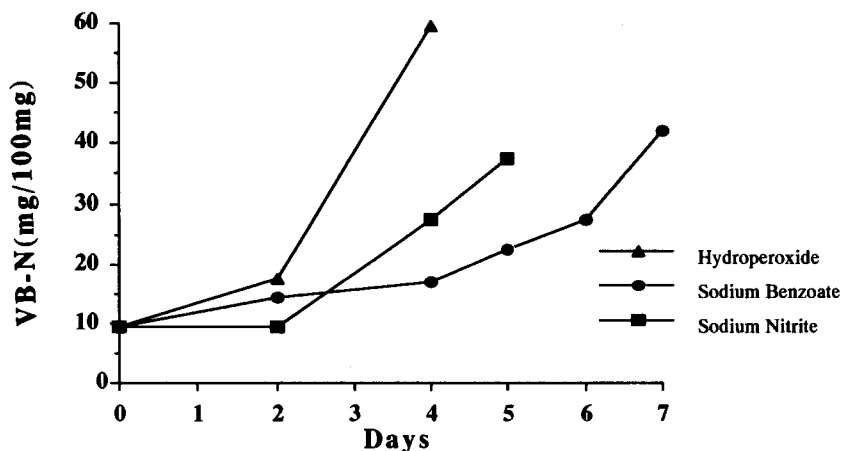


Fig. 33. VB-N of fish immersed in 4% brine containing 0.3% sodium benzoate, 0.1% hydroperoxide, and 0.1% of sodium nitrite. Storage was held at 15°C.

the mentioned day this value increased at a particular rate depending on the kind of preservative added to the brine. It was slowest for the sample in the brine containing sodium benzoate and fastest for the one in brine with hydroperoxide. Thus, on the 4th day the later mentioned sample surpassed by far the considered limit of acceptability, 30 mg/100 g. The sample in sodium nitrite exceeded the same value on the 5th day, whereas fish in sodium benzoate had a VB-N of about 28 mg/100 g on the 6th day. On the 7th day 40 mg/100 g was the signal of fish spoilage.

Changes of VB-N in the immersion solutions containing the preservatives (Fig. 32B) were quite similar to that in the fish. Except that increases in the former were faster than those in the latter.

According to the above results, fish with sodium benzoate was considered acceptable up to the 6th day storage. Therefore, it can be stated that the method described here for preservation of fish at 15°C prolonged by one day the time of storage reached when the fish were immersed in 4% brine containing 0.05% acetic acid (section II-5). Furthermore, it can also be said that the aim and purpose of this investigation was satisfied. As a consequence it was considered desirable to perform a more detailed study in regard to chemical and microbiological changes in the fish and in the solution of immersion

## 2. Concentration of Sodium Benzoate to be Used for Sardine Immersed Storage

Before carrying out any further studies regarding the effects of sodium benzoate on fish storage, it was considered important to determine the best concentration of the preservative to be use in fish preservation. By "best concentration" of sodium benzoate, we mean to find out the lowest possible concentration able to perform the same results obtained in the previous section. This is important from the economi-



cal as well as public health points of view.

For this determination two tests were carried out. One in fish immersed storage and the other one in a laboratory model using some bacterial species.

On the other hand, the majority of previously reported studies about sodium benzoate are concerned more with the mode of action than with the type of microorganism against which it is effective<sup>76)</sup>. Therefore, in this present section the effect of benzoate was tested not only on *Pseudomonas* but also on *Micrococcus* and *Bacillus* strains.

## Materials and Methods

### *Raw material*

Sardine (section II-2) divided into 3 lots of 2 kg were used for the determination.

### *Fish storage and sampling*

Each lot was immersed into solutions of 0.1, 0.2 and 0.3% sodium benzoate. The pH of the solution was adjusted to 5 (section IV-1). Samples were stored at 15°C for 6 days. Sampling was performed on the 0, 4th, and 6th days of storage.

### *Analyses*

VB-N analysis was performed in fish and in the immersion solution where microbial counts were recorded too. The method is described in section II-1.

### *Strains*

*Pseudomonas* sp. P-1, the same strain used in section II-4. *Micrococcus* sp. Mi-1 and *Bacillus* sp. B-1 were obtained from our laboratory's collection.

Inocula of the three strains was prepared as described in section II-4.

### *Microbial counts*

Nutrient broth was prepared in Erlenmeyer flasks to give a final quantity of 100 ml after adding the acid, benzoate and inocula. After sterilizing the nutrient broth and adding the desired concentrations of sodium benzoate, the final pH was adjusted exactly with acetic acid to a pH of 5.0 (0, 0.2, and 0.3% benzoate) and a pH of 6.5 (0.3% benzoate). In the case of *Pseudomonas* sp. P-1 a medium containing 0.15% benzoate was also tested.

After mixing each test culture, a microbial count was performed by using the standard pour plate technique and duplicate plates (section II-4). Test cultures were incubated statically and aerobically at 15°C. Sampling was performed at predetermined times.

## Results and Discussion

### *Effect on fish storage*

#### *Microbial counts*

Immediately after fish immersion into the solutions, the bacterial population

was nearly  $10^3$  cfu  $ml^{-1}$  (Fig. 34A). On the 4th day, the solutions containing 0.1 and 0.2% benzoate presented almost the same bacterial load,  $7 \times 10^7$  cfu  $ml^{-1}$ . By the 6th day the solution containing 0.3% sodium benzoate was observed to have the greatest population, followed by the solution with 0.2 and 0.1% benzoate in decreasing order. At this stage of this study it was still difficult to explain why this occurred. Later investigation helped to answer this question (section IV-4).

#### VB-N

The VB-N of the fish (Fig. 35) increased slower in fish muscle immersed in the brine containing 0.2% sodium benzoate than in the samples in 0.1%. From the 4th to the 6th day increases of this value in the two samples were observed to occur in parallel. Values recorded on the 6th day for these two samples were higher than 30 mg/100 g, whereas fish immersed in the brine containing 0.3% sodium benzoate

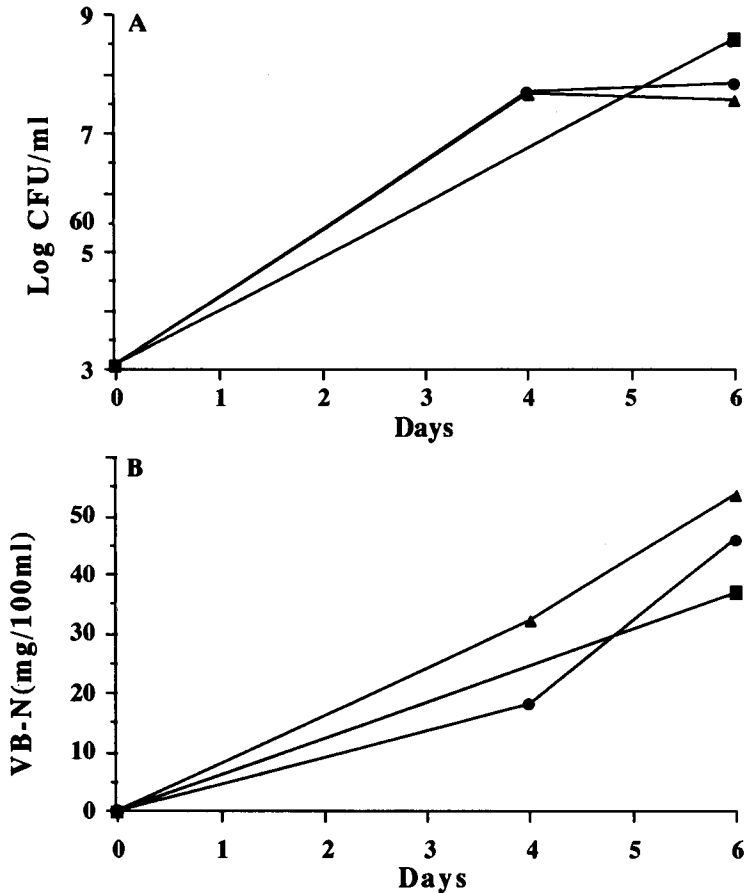


Fig. 34. Changes in microbial counts (A) and VB-N (B) of 4% brine containing sodium benzoate during fish immersion. Symbols are as in Fig. 35.

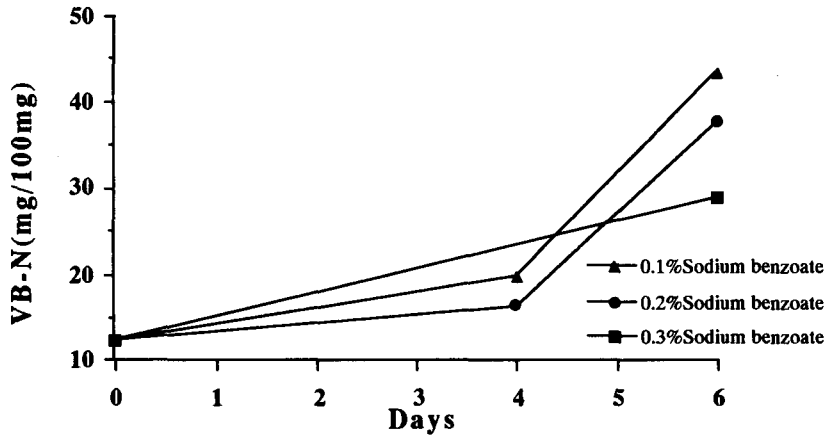


Fig. 35. Changes in VB-N of fish immersed in 4% brine containing sodium benzoate.

showed a value lower than the mentioned samples and below 30 mg/100 g on the 6th day. These results showed that the brine containing 0.3% sodium benzoate is the most adequate to be used for preservation of fish under these experimental conditions.

The VB-N of the immersion solutions (Fig. 34B) showed the same pattern of increase recorded in the respective fish samples. On the last day, the highest value was presented in the 0.1% solution, then the 0.2% and finally, the lowest was again the VB-N of the solution containing 0.3% sodium benzoate.

#### *Microbial counts of Micrococcus sp. Mi-1*

In the medium of pH 6.5 containing 0.3% benzoate (Fig. 36A) some increase in cell numbers occurred during the first two days, but after the 2nd day they stayed almost constant until the last day of sampling.

In the medium of pH 5 without benzoate, cell numbers decreased slightly after the 2nd day. This decrease in cell numbers was also observed in test cultures containing 0.2 and 0.3% benzoate, being somewhat larger in the latter mentioned two samples.

#### *Microbial counts of Bacillus sp. B-1*

Growth was not observed in any of the samples (Fig. 36B). Bacterial numbers remained constant in the medium of pH 6.5 containing 0.3% sodium benzoate. In the medium of pH 5 without benzoate the bacterium decreased gradually during the incubation period. The added acid was responsible for this effect. In addition, inhibitory action of the acid in the medium was improved when sodium benzoate was present at any of the two tested concentrations. In both cases cells of *Bacillus* sp. B-1 diminished abruptly between the first two days. Decrease of about four orders of magnitude was observed.

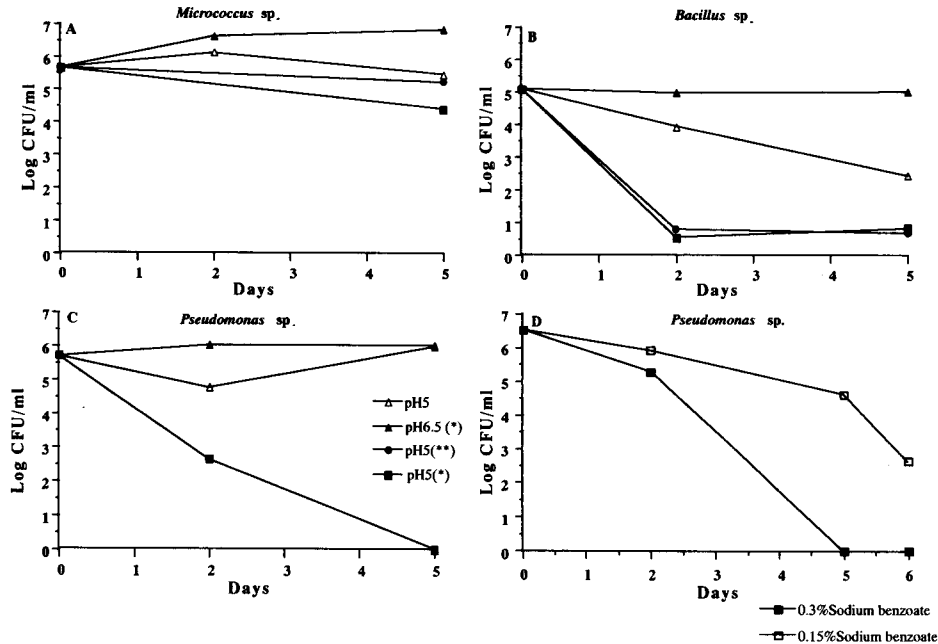


Fig. 36. Behavior of *Micrococcus*, *Pseudomonas* and *Bacillus* in 4% NaCl-nutrient broth containing 0.2% (\*\*) and 0.3% (\*) of sodium benzoate and acidified at pH 5 with acetic acid.

#### Microbial counts of *Pseudomonas sp. P-1*

In the medium of pH 6.5 containing 0.3% benzoate, the same behavior observed for *Bacillus sp. B-1* was observed for *Pseudomonas sp. P-1* (Fig. 36C).

During the first two days of incubation the population of *Pseudomonas sp. P-1* decreased almost one order of magnitude in the medium of pH 5 without benzoate. Thereafter, cells increased to reach the initial number.

Bacterial inhibition attributed to the action of benzoate was clear during the incubation period. Cells of *Pseudomonas sp. P-1* growing in media containing 0.2 and 0.3% sodium benzoate decreased at the same rate and were, finally, completely inhibited by the last day of sampling (5th day).

The effect of the used concentrations of sodium benzoate was clearly observed on microbial growth. In Fig. 36D, the same results as in Fig. 36C were observed when 0.3% sodium benzoate was used. In the presence of 0.15% benzoate, however some decrease in numbers was recorded, it was not of the same degree as that in 0.3% sodium benzoate.

These results confirmed that low pH and 0.3% sodium benzoate in nutrient medium had an inhibitory effect on *Pseudomonas sp.* and *Bacillus sp.*, but has a bacteriostatic effect on *Micrococcus sp.* Furthermore these results and the results of the effect on fish storage showed once again that the best concentration of sodium benzoate to be used for fish preservation is 0.3% in a solution adjusted to a pH of 5.

### 3. Sardine Immersed Storage in Brine Containing Sodium Benzoate at 15°C

Benzoic acid, usually in the form of the sodium salt has long been used as an antimicrobial additive for foods. The sodium salt is preferred because of the low aqueous solubility of the free acid. In use the salt is converted to the acid, the active form<sup>78)</sup>.

Sodium benzoate is generally considered to be most active against yeast and bacteria, and less active against molds<sup>76)</sup>. There are many reports which depict the relationship of pH to inhibitory concentrations of sodium benzoate<sup>76,79)</sup>. It has been also reported that sodium chloride has a considerable synergistic effect with sodium benzoate<sup>76)</sup>. Under regulations of the USA Food and Drug Administration (FDA), sodium benzoate and benzoic acid are generally recognized as safe (GRAS)<sup>76)</sup> for use in foods. In some countries levels up to 0.2 and 0.3%<sup>78,79)</sup> are permitted and are commonly used.

This preservative has especially wide applicability as an antimicrobial agent for foods and it is most suitable for foods and beverages which naturally are in the pH range below 4.0 or 4.5 or can be brought into that range by acid addition<sup>76)</sup>. Mixed in the food in small doses it, is not deleterious to health<sup>78)</sup>.

The inhibitory effect of sodium benzoate on some bacteria that cause foodborne illness or food spoilage has been studied by using different laboratory media as well as food products under various conditions<sup>76,78,79)</sup>. For sodium benzoate to be effective the pH of the substrate must be low since the undissociated acid is believed to be responsible for its antimicrobial action. Currently, virtually no information is available on the effect of sodium benzoate in fish preservation.

Therefore chemical and microbiological changes in 4% brine containing sodium benzoate were evaluated during sardine immersed storage held at 15°C. Chemical, microbiological and organoleptic changes in the sardine were also evaluated.

#### 1) Changes Related to the Quality of Fish and Brine

##### Materials and Methods

###### *Raw material*

The sardine weighing *ca.* 83 g each were obtained at Kami-iso in Hakodate Bay and brought to the laboratory in an ice box (section II-2). Upon arrival they were removed from the ice box and rinsed with tap water. The fish were divided into 6 lots of 2 kg, and used through the experiments. Four lots were immersed in 2 l of 4% brine containing 0.3% sodium benzoate, the other two lots were immersed in 4% brine without sodium benzoate and used as a control. Sodium benzoate to give a final concentration of 0.3% to the brine (4% NaCl) was added and then pH of the solution was adjusted to 5 with acetic acid previous to fish immersion.

###### *Fish storage and sampling*

Samples were stored at 15°C. At predetermined times, two fish were randomly drawn from each lot for K value, VB-N, muscle salt uptake and microbiological

analyses. Parallel sampling was made on brine for changes in salt concentration and microbiological analyses.

#### *Chemical analyses*

Changes of K value in fish muscle were performed as in section II-5. VB-N in fish muscle and in the brine was determined by the method of Conway (section II-1). The salt (NaCl) content of fish muscle and of the brine of immersion was determined by the method of Mohr (section II-2).

#### *Sensory evaluation*

An overall score for quality of fish was determined by the method stated in section II-2, and the quality score card presented in Table 1 was used (section II-2).

### Results and Discussion

#### *K value*

Results are presented in Fig. 37. An initial value of *ca.* 18% was obtained at the time samples arrived to the laboratory. Evaluation during storage showed a steady increase of this value in muscle of the fish used as control. In this case, on the 2nd day the obtained value was about 95%, by far higher than 60%, the considered limit of acceptability. By this time fish was considered to be spoiled according to not only this value, but also by sensory evaluation and VB-N value. On the other hand, fish immersed in the brine containing sodium benzoate had a K value of almost 80% on the 4th day of storage. By the 6th day more than 80% was measured. In spite of this high value, according to other analyses fish were considered still acceptable. Therefore, it was concluded that sodium benzoate causes a rapid ATP degradation to the same degree as acetic acid and in consequence the K value does not constitute a relevant data for this particular kind of storage.

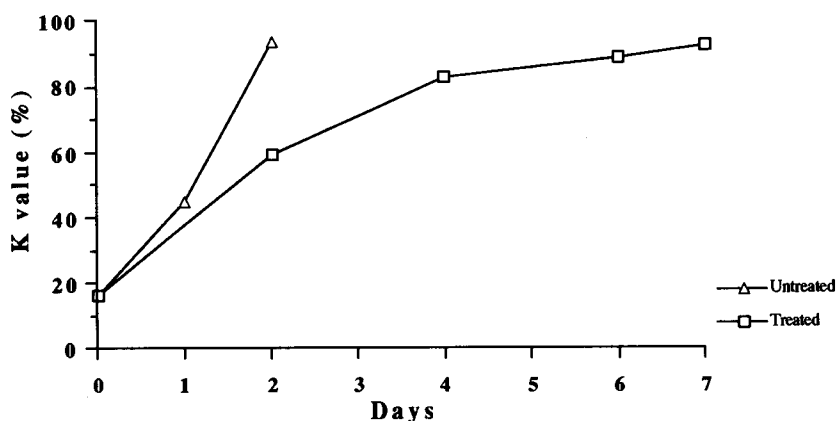


Fig. 37. Changes in K value of sardine muscle, during storage immersion in 4% brine with (treated) and without (untreated) 0.3% sodium benzoate. The pH of the brine was adjusted to 5 with acetic acid.

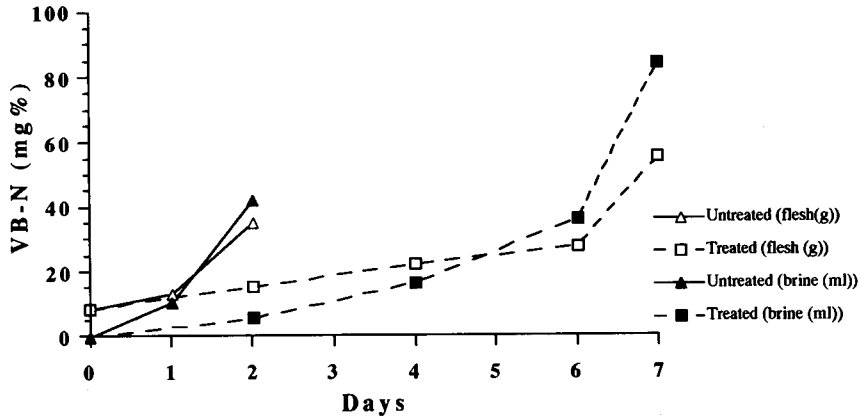


Fig. 38. Changes in VB-N of brine and sardine muscle during immersed storage.

### VB-N

Changes in the total VB-N of the sardine flesh during immersion in brine with or without sodium benzoate, and of the brine themselves are presented in Fig. 38. The VB-N values of fish in the brine containing sodium benzoate increased imperceptibly from an initial 8 to almost 28 mg/100 g on the 6th day of storage. Later on, this tendency changed abruptly and this value suddenly increased to reach nearly 60 mg/100 g. This pattern in VB-N production carried on by bacterial activity<sup>23,35)</sup> was very similar to that observed for microbial growth.

According to this result and to that of sensory evaluation these samples were thought to be acceptable for consumption until the 6th day. However, they were not acceptable on the 7th day when spoilage was evident.

The VB-N of fish used as control stayed almost constant during the 1st day. From the first to the 2nd day it increased very fast so as to reach nearly 40 mg/100 g which has been reported as a sign of spoilage<sup>23)</sup>. A clear coincidence of these results and the outcome of sensory test showed these samples to be spoiled by the 2nd day. These results showed also to be in concordance with results obtained in the two previous chapters.

The VB-N values in the brines were seen to change similarly to that of the corresponding sample of fish. With only small differences showing VB-N of the brine with or without benzoate were observed to be a little higher than that of the fish on the last day of sampling. Here, it could be supposed that bacterial activity was responsible for this difference. It is easier for the bacteria to use nitrogen organic compounds coming out from the fish into the brine than those present in the fish itself. During storage these compounds will increase and larger quantities of them will be available for use by the bacteria. Therefore, it could be expected this difference to be larger as spoilage advances.

### Salt content

A complementary study on salt concentration of fish and brine was done with the purpose of knowing whether or not there is any effect of benzoate on salt

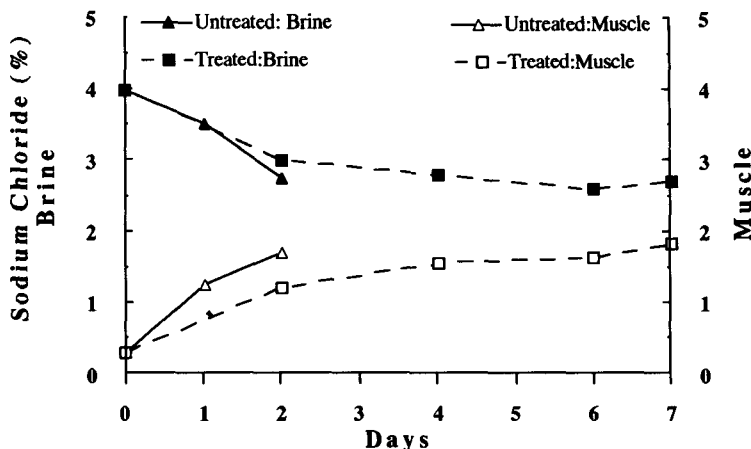


Fig. 39. Changes of NaCl in muscle of sardine and brine during immersed storage. Treated brine contains sodium benzoate.

penetration into fish muscle immersed in the brine. According to the results showed in Fig. 39, rate of increase and final salt concentration were recognized to be the same as those obtained in the previous chapter (III). Therefore it could be said that there is no influence from sodium benzoate on salt penetration.

#### *Sensory evaluation*

Changes in the overall appearance and acceptability scores of both, fish immersed in treated 4% brine containing sodium benzoate and fish immersed in 4% brine (control) are shown in Table 10.

The sensory evaluation showed marked differences between the two samples. Benzoate treated fish remained in good conditions up to the 4th day. Later the evaluated scores declined slightly and it was classified as acceptable or in the borderline of acceptability on the 6th day. This decline continued and the fish was valued "spoiled" on the 7th day of storage.

Samples used as control suffered an extremely fast decomposition. Although they were recognized as acceptable on the first day, they were found to be completely spoiled on the next day. In this case, the rate of change of the different parameters evaluated was very fast. On the first day an average for all of them was "category C". The fish had good appearances although some fishy odor, besides when pressed in the dorsal side the muscle was felt to be slightly soft and the slime still clear in most instances. When evaluated the second day, all the parameters, without exception, had completely decayed making the fish unacceptable.

On the fish immersed in brine containing sodium benzoate, appearance and odor went observed to change progressively while changes in texture and slime went more slowly until the 6th day. Then a sudden decline of all the parameters evaluated indicated that the fish had spoiled on the 7th day. However, it could be say that sodium benzoate extended the shelf life of the treated samples a great deal from 1 day (control) to 6 days (treated sample).



Table 10. Overall quality scores and acceptability of sardine immersed in brine with (treated) and without (control, untreated) sodium benzoate.

Testing subject	Before storage	Control		Treated			
		Days		Days			
		1	2	2	4	6	7
Overall appearance	A	B	D	A	B	C	D
Odor	A	C	D	A	B	C	D
Texture	A	C	D	A	A	B	D
Slime	A	B	D	A	A	C	D
Acceptability	A	C	D	A	B	C	D

A; Completely fresh, highly acceptable

B; Fresh and acceptable

C; Fairly acceptable, borderline of acceptability

D; Spoiled and unacceptable

E; Completely spoiled, totally unacceptable

Although further studies need to be done, an important conclusion from this work is that 4% brine with its pH adjusted to 5 with acetic acid and containing 0.3% sodium benzoate could be a good alternative for sardine preservation even if it is stored at 15°C. Results of this synergistic action<sup>76)</sup> clearly prolonged fish acceptability up to the 6th day of storage.

## 2) Microbial Changes in Fish and in the Brine of Immersion Containing Sodium Benzoate

Sodium benzoate has never been used before in fish preservation. Consequently any aspect involved in the process of preservation must be studied in order to better understand the changes occurring in the fish thus preserved and in the brine of immersion. Certainly the study of the microflora involved and the changes in it during storage became an important point in the study.

### Materials and Methods

#### *Microbiological analyses*

These analyses were performed in samples of fish and brine of the previous section, following the procedure presented in section II-3.

### Results and Discussion

#### *Microbial counts*

The results of colony forming units (cfu) in the sardine and in the brine of immersion are presented in Fig. 40. The initial bacterial load on sardine (surface and flesh) after thoroughly rinsing it with tap water, once it arrived to the labora-

tory, was almost  $10^4$  cfu  $g^{-1}$ . It has been reported that the surface slime of fish is an excellent medium for bacterial growth<sup>32,31)</sup> and that rinsing the fish reduces bacterial counts and improves organoleptic quality.

Any increase in bacterial count of fish immersed in brine containing sodium benzoate was observed until the second day. From the second day, it increased progressively from almost  $10^4$  cfu  $g^{-1}$  (initial load) to nearly  $10^8$  cfu  $g^{-1}$  after 7 days of storage. Between the 6th and 7th day, the rate of increase was observed to be a little faster than in previous days. The lag period observed for treated samples was not observed in the control fish, in which growth of bacteria started to occur immediately after immersion. On the second day, when fish used as control were considered to be spoiled, according to results of VB-N and sensory evaluation, bacterial count was nearly  $10^6$  cfu  $g^{-1}$ .

On the other hand, bacterial population in the brine increased continuously after the sardine was immersed. When benzoate was present, the initial numbers in the brine with sodium benzoate were one tenth of the numbers in the control samples determined at the same time. However the rates of growth were parallel for both samples.

The pattern of microbial growth in the sardine flesh and the respective brine without benzoate was similar during the storage. In the brine with sodium benzoate this similarity was observed from the 2nd day.

Results for fish immersed in the control brine showed no clear relation between the outcome of sensory tests and microbial counts. The fish were sensorily unacceptable before the increase in bacterial count was considered to have a level that shows spoilage<sup>22-35)</sup>. On the other hand, the rate of microbial growth in the brine is similar to that recorded in section II-3 under the same conditions.

It is known sodium benzoate retards trimethylamine formation in muscles of certain fish even though it does not retard bacterial growth<sup>69)</sup>. Therefore, in this study, this could explain the presence of high bacterial number in fish immersed in

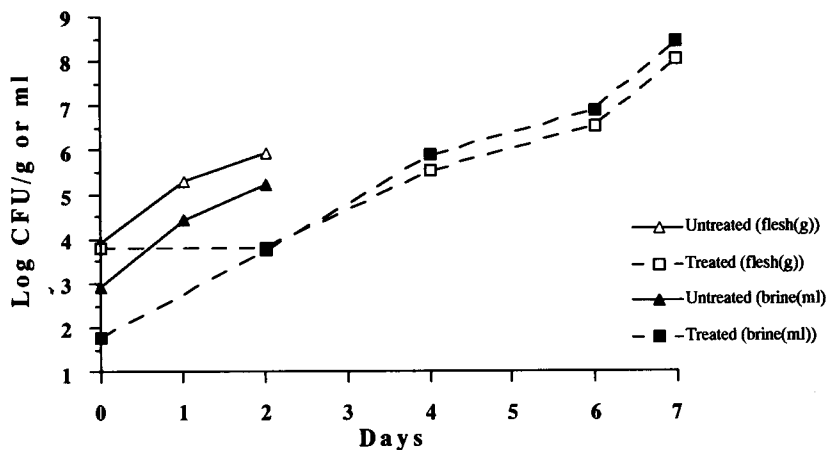


Fig. 40. Changes in microbial counts of brine with (treated) and without (untreated) and fish muscle, immersed storage at 15°C.

Table 11. Number of strains isolated from brine and fish during the immersed storage.  
Storage was held at 15°C.

Days	Brine			Fish		
	Before storage	Control	Treated sample	Before storage	Control	Treated sample
0	116	—	—	117	—	—
1	—	119	120	—	114	—
2	—	115	120	—	112	112
3	—	—	115	—	—	120
4	—	—	117	—	—	122
6	—	—	—	—	—	120
7	—	—	—	—	—	71
Sub total	116	234	472	117	226	433
Total		822			776	

the brine containing sodium benzoate on the 6th day and the lag phase observed between the first two days could be better attributed to the action of the acid (section II-6). Besides, these results did not fit with sensory evaluation, since high bacterial counts were recorded before the appearance of any spoilage characteristic. Differences in results obtained from fish samples in the brines with and without sodium benzoate could only be explained by changes in their microflora.

#### *Identification of microorganisms*

The strains isolated from fish and brine during fish storage made a total of 1598 strains (Table 11). Among them 776 were isolated from the fish and 822 strains from the brine. All of these isolates were identified to the genus level as showed in the section II-3 (Fig. 7).

#### *Cocci*

Cells spherical, gram positive and non motile microorganisms were identified into two genera, *Micrococcus* (261 stains) and *Staphylococcus* (13 strains). Both of them possessed strong catalase activity, but the former was glucose non fermenters and performed better growth in media containing 5% sodium chloride.

#### *Gram positive bacilli*

Among the isolated gram positive rods, two genera were recognized. The spore forming bacteria was classified as *Bacillus* with 15 strains and the remaining, non motile, non spore forming bacteria, 100 strains, Coryneforms.

#### *Gram negative bacilli*

a) Glucose fermenters: Eight strains of oxidase negative, glucose fermenter bacteria, having peritrichous flagella were classified as Enterobacteriaceae. The rod

shaped cells were small and fermentation of glucose occurred with the production of acid and gas. Another 17 strains which fermented glucose with gas production, were classified as *Aeromonas*. These bacteria were oxidase positive and had polar flagella. Nine more strains, also showing, oxidase positive reaction but which fermented glucose without gas production were identified as *Vibrio*.

b) Glucose non fermenters, motile bacteria: The majority of the isolates were in this group. Among 393 strains with polar flagella 18 strains had the ability to liquefy gelatin and therefore considered *Alteromonas*. Out of those strains unable to cause gelatin liquefaction, 368 strains were characterized as *Pseudomonas*. The other 7 strains presenting peritrichous flagella were identified as *Alcaligenes*.

c) Glucose non fermenters, non motile bacteria: Colony pigmentation and oxidase reaction aid in the differentiation of this group. Strains showing production of carotenoid pigment were classified as *Flavobacterium* (41 strains). The remaining strains giving oxidase positive and negative reactions were 408 and 309 respectively. The oxidase positive strains were identified as *Moraxella* and the others as *Acinetobacter*.

#### *Changes in microflora*

Genera identified from fish in brine with sodium benzoate and in the brine corresponded to 14 and 13 genera, respectively, and from fish and brine samples used as control corresponded to 12 and 8 genera, respectively. The numbers of micro-

Table 12. Bacterial genera isolated from brine and fish during the immersed storage.

Genera	Before storage		Control		Treated samples		Total
	Fish	Brine	Fish	Brine	Fish	Brine	
<i>Micrococcus</i>	49	23	24	19	43	103	261
<i>Staphylococcus</i>	—	—	—	—	4	9	13
<i>Bacillus</i>	2	3	6	—	2	2	15
Coryneforms	9	14	12	9	20	36	100
<i>Moraxella</i>	21	28	78	116	95	70	408
<i>Pseudomonas</i>	13	20	31	37	136	131	368
<i>Aeromonas</i>	—	—	1	—	7	9	17
<i>Alcaligenes</i>	—	1	1	1	1	3	7
Enterobacteriaceae	1	—	2	—	3	2	8
<i>Acinetobacter</i>	9	15	58	45	92	90	309
<i>Alteromonas</i>	1	2	1	1	9	4	18
<i>Flavobacterium</i>	11	9	7	2	11	1	41
<i>Vibrio</i>	—	—	1	—	2	6	9
<i>Photobacterium</i>	—	—	—	—	1	—	1
Unidentified	1	1	4	4	7	6	23
Total	117	116	226	234	433	472	1598

organisms belonging to each identified genera are shown in Table 12.

Changes in microflora through the storage period were seen in samples of fish as well as in those of brine. In both, samples with and without benzoate, these changes in either fish and brine corresponded to each other (Figs. 41 and 42).

The initial flora of fish samples showed the gram positive bacteria to be predominant, with almost 52% of the total flora; among them *Micrococcus* was the most numerous bacteria present.

In samples of fish obtained from the control group, increases in microbial counts (Fig. 40) together with changes of microflora (Fig. 41) were observed on the 1st and 2nd days (last of storage). The initially preponderant gram positive cocci showed

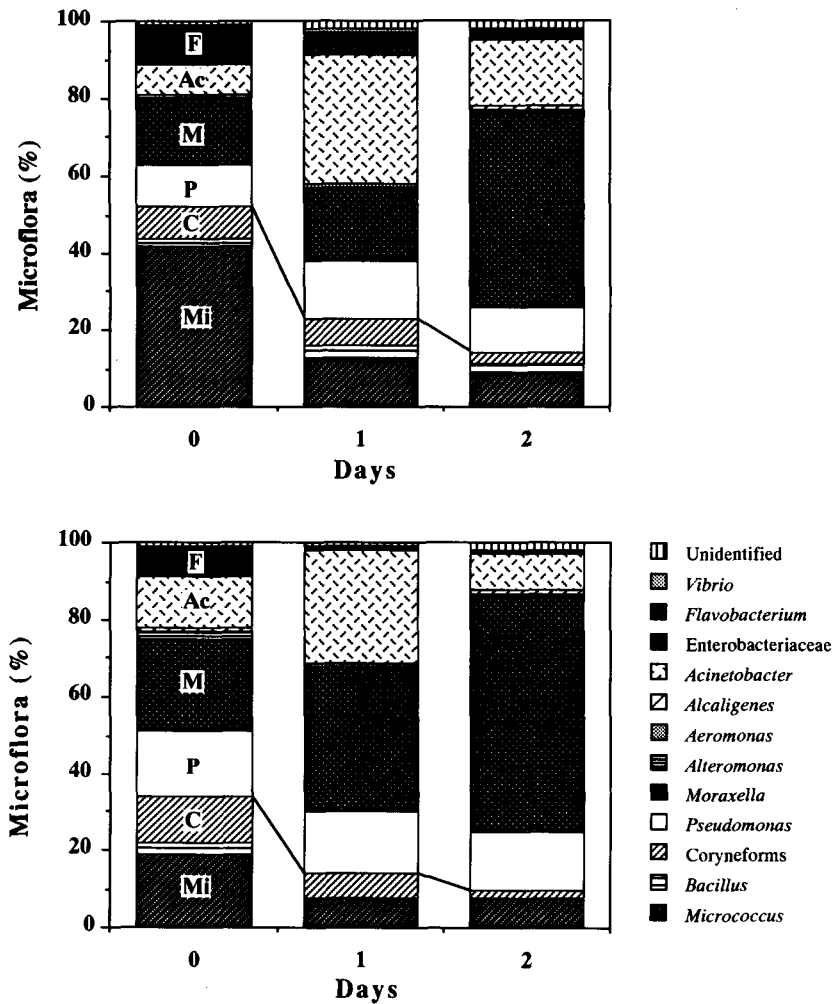


Fig. 41. Changes in microflora of fish (top) and brine (bottom) used as controls during the immersed storage.

a steady decline and were overwhelmed by the gram negative bacteria, thus, the latter group reached about 74% on the first day and nearly 85% on the 2nd day. By this day, *Pseudomonas*, *Acinetobacter* and mainly *Moraxella* represented the most important groups among the gram negative. While these changes in microflora occurred, fish were observed to pass from fresh to unacceptable according to the performed sensory evaluation and VB-N analysis. However, even if as mentioned VB-N signaled the fish at the initial stage of spoilage, *Pseudomonas* recognized as one of the principal agents of spoilage, was present but only constituted about 17% of the microflora. Therefore, a change in the balance of physiological types among the

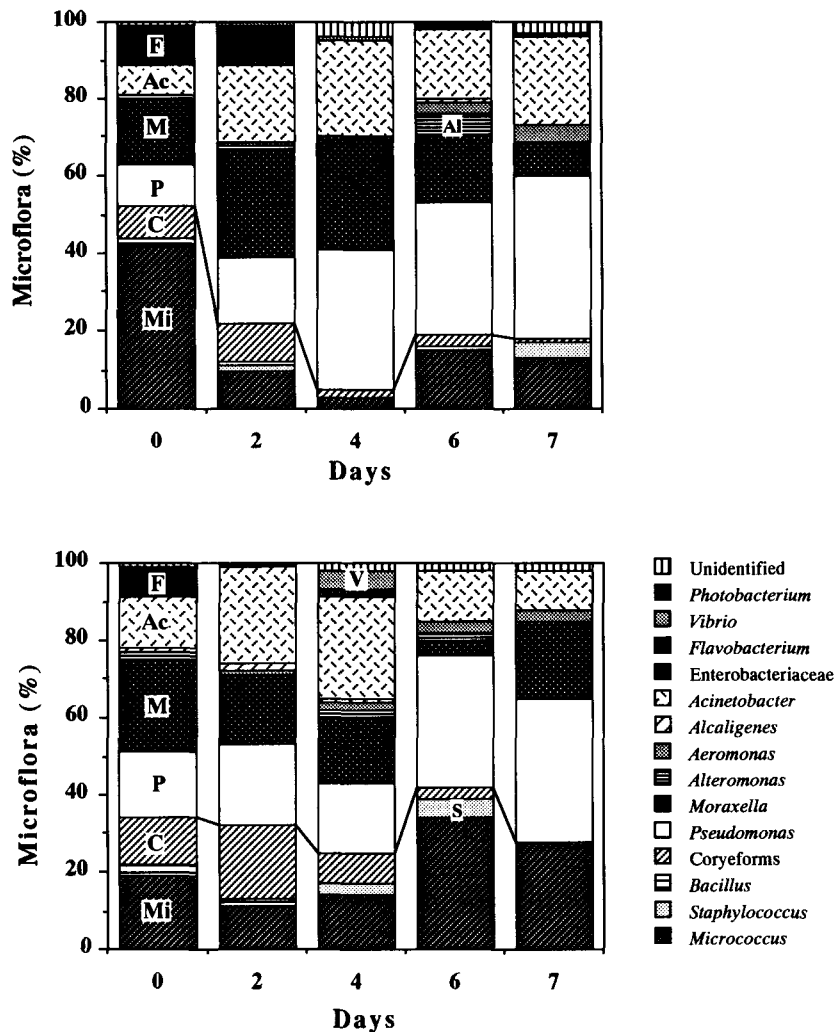


Fig. 42. Changes in microflora of fish (top) and brine (bottom) containing sodium benzoate during immersed storage.

gram negative organisms could still be expected to occur. Thus, the comparatively slow growing *Acinetobacter* and *Moraxella* would be replaced by the rapidly growing *Pseudomonas* and in one or two more days when putrefaction has set in, the latter one would reach higher percentages, approaching results obtained during this study (section II-5) or others published before by different authors<sup>41-43</sup>.

The microflora in the brine of immersion used as a control, presented exactly the same pattern described above with only small differences in percentages. Therefore, the same conclusion could be reached.

On the other hand, microflora of the treated fish (Fig. 42), also showed the initial predominant gram positive bacteria to decrease in numbers and became about 23% by the 2nd day. However, during this period, changes in microbial numbers were not observed, and VB-N values remained below 15 mg/100 g in spite of the gram negative bacteria making up 75% of the total population.

Later this decrease continued, so that gram positive bacteria were only a few percentage points on the 4th day and the gram negative rods constituted the remaining 94%. Although increases in bacterial population and predominance of gram negative bacteria suggested a process of spoilage, increase rate of VB-N was still slow and continued in this manner until the 6th day. By this time the fish was considered still acceptable. From these results, the only possible conclusion to make is related to the effect of sodium benzoate. In other words it could be said that sodium benzoate inhibited bacterial activity, represented in VB-N production of the spoilage microorganisms.

According to the results obtained this effect was observed until the 6th day. By the 7th day a rapid process of spoilage was indicated by a rapid increase in VB-N parallel with increases in bacterial population. Between these two days no major changes in microflora were observed, however, according to VB-N values it was concluded that action of sodium benzoate has been surpassed and the bacteria that had reached the period of maximum spoilage activity, caused fish decomposition.

Microflora of the brine showed a similar pattern of changes and almost the same bacterial flora to that of fish. However, the percentage of gram positive during the storage was somewhat higher, excluding the initial day.

These results showed the efficiency of sodium benzoate on fish immersed storage in brine at 15°C

### 3) Chemical Changes in Brine during Fish Immersion

During the present study, fish immersed storage has been studied. The results obtained suggests, this kind of storage to be a new alternative for sardine preservation on board, therefore as this is not a conventional method, many studies are still needed to learn more about the changes which occur in the brine and in the fish itself. Here an attempt would be made to study the chemical changes taking place in the brine of immersion.

It is known that fish handling and processing procedures occasioned loss of free liquid, thus inducing a loss of organic constituent, e.g. free amino acids; sugars such as glucose; simple peptides, such as anserine, and glutathione; trimethylamine oxide, creatine, as well as fats, lipids, and proteins, all of which the bacteria will

utilize for their growth and metabolism<sup>36,82</sup>).

Action of salt prevents these losses to some degree<sup>82</sup>. However depending on the salt concentration and the temperature of storage this process could be accelerated<sup>19</sup>).

Taking the above factors into account it was decided to evaluate some organic compounds such as lipid, carbohydrate, nitrogen and free amino acids coming out from the fish into the brine and their changes through the storage.

## Materials and Methods

### *Brine*

Samples of brine with and without sodium benzoate in which sardine had been immersed were used for this study (The same samples used in part 1) and 2) of this section).

### *Total lipid*

Lipid from the samples was extracted following the method of Folch<sup>83</sup>. A solution of chloroform-methanol (2 : 1) was added to 10 ml of brine pipetted into a separating funnel, making a proportion of 20 : 1 (v/v). Finally, 20 ml of 0.9% KCl were added to help in the separation and the mixture after being thoroughly agitated was allowed to stand until complete separation. The lower fraction in the funnel was transferred to a previously weighed joint Kjeldahl flask (short neck) and then evaporated in a rotary evaporator until it reached a constant weight.

### *Carbohydrates*

The determination was carried out using 2 ml of sample placed in a test tube, according to the method of Koehler<sup>84</sup>. A solution of 0.2% anthron in 95% H<sub>2</sub>SO<sub>4</sub> was used. Four ml of this reagent were added to the samples contained in the test tubes. Before and during the addition the sample was placed into an ice water bath. Immediately after the addition tubes were transferred to boiling water for 10 min and successively cooled in iced water.

Standard solution of glucose was prepared and treated exactly as described above. Extinction of the standard solution and of the samples at various dilutions was measured in a 1 cm cell against water at a wavelength of 620 nm. Concentration of the samples was estimated using the standard curve built for the standard glucose solution.

### *Total nitrogen*

Kjeldahl method and automatic Kjeldahl analyzer were used for the determination. Sample (2 ml) and subsequently H<sub>2</sub>SO<sub>4</sub> (20 ml) were added together in a digestion flask and this was put into the analyzer for the digestion and distillation process. The distillate was collected for posterior titration with 0.01N NaOH<sup>85</sup>).

### *Amino acids and amino nitrogen analyses*

The brine (5 ml) was mixed with ethanol (20 ml). The mixture was vigorously



shaken and stored at  $-20^{\circ}\text{C}$  until later separation. Once all the samples were collected, they were brought to room temperature and filtered into a separating funnel. The used filters were washed with additional 25ml of 80% ethanol. After that, addition of chloroform and vigorous shaking of the samples was done before letting them stand overnight, to complete separation. The upper fraction was used for free amino acids and amino nitrogen content determination<sup>86)</sup>. The final analysis was performed with an automatic amino acid analyzer (Hitachi L-8500).

## Results and Discussion

### Lipid

Increase in lipid content was observed during the storage in the untreated as well as in the treated brine (Fig. 43). In the former, a sharp increase occurred from the moment of storage until the first day reaching about 0.6 g/100 ml. This could correspond to the time of the highest salt permeation into the muscle, during which major interchanges within the fish and the surrounding brine are supposed to occur. From the 1st to the 2nd day the amount of lipid in the brine stayed constant suggesting that in this respect the system fish-brine has reached the equilibrium. In spite of this apparent stability, the process of spoilage was observed to enter into its developing phase, and fish was no longer considered acceptable. In addition it could be assumed that even though spoilage of fish has set in, the lipid present in the brine was not directly involved in the spoilage process.

Lipid contained in the treated brine increased steadily up to the 2nd day. Between the 2nd and the 6th day the rate of increase, clearly, slowed down, following almost the same tendency of the curve of increases in VB-N values (Fig. 38). This similarity and at the same time the observed period of stability observed, throughout the 2nd to the 6th day, according to the slowly increasing VB-N was interpreted as the preservative effect of sodium benzoate, acting together with the salt present in the brine. From the 6th to the 7th day a fast increase was observed,

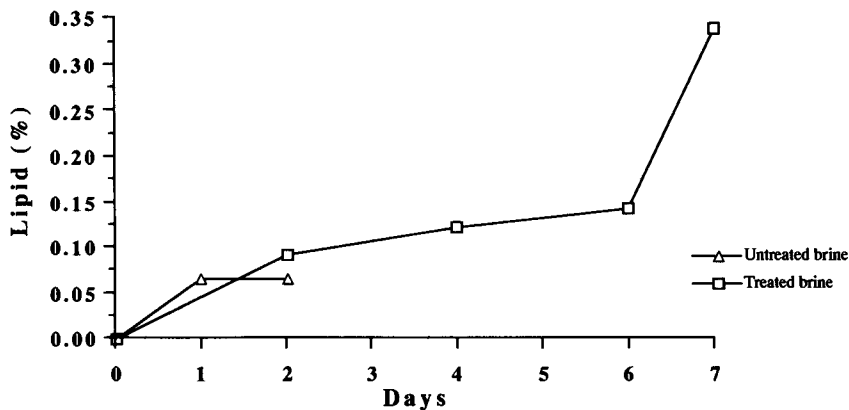


Fig. 43. Changes in lipid content of 4% brine with (treated) and without (untreated) sodium benzoate, during sardine immersion.

with appearance of some spots of lipid floating in the brine surface. During the same period fish entered into a rapid process of spoilage (VB-N, Fig. 38) and softening and disruption of tissues<sup>19)</sup> gave way to losses of lipid in the fish muscle, thus causing increments, into the brine, in a considerable amount. As well as in the untreated sample, spoilage was, again, better attributed to other factors, and the sudden increase of lipid was considered to be a result of the process rather than its cause. Thus the obtained curve of changes in lipid kept closely related to that of changes in VB-N and therefore with changes in the acceptability.

### Carbohydrates

Changes in carbohydrates recorded in brine are shown in Fig. 44. In the untreated brine small increases were observed over the 1st day. By the 2nd day the rate of increase accelerated and about 1 mg/100 ml was observed. The pattern of increase approached that observed for VB-N. However it could not be said for certain if the measured carbohydrates represented the total amount coming out from the fish or if some part was used by the microorganism being in the brine. On the first case they could be considered products of the spoilage of fish, and on the second case they could be assumed to cause brine spoilage and in consequence fish spoilage.

On the other hand, total carbohydrates in the treated brine increased, very fast and constantly, up to the 4th day reaching a maximum value of *ca.* 6 mg/100 ml. From the 4th to the 6th day a sharp decrease occurred, suggesting this compounds were being degraded into compounds of lower molecular weight by either autolytic or microbial action, until almost complete depletion. Thus, the remains recorded on the 6th day were no longer observed by the next day. Making a comparison within these and the results of VB-N obtained for this sample, it could be said that by the time the total carbohydrates reached its lowest level the maximum spoilage activity was observed, with the result that VB-N increased suddenly and sharply (Fig. 38).

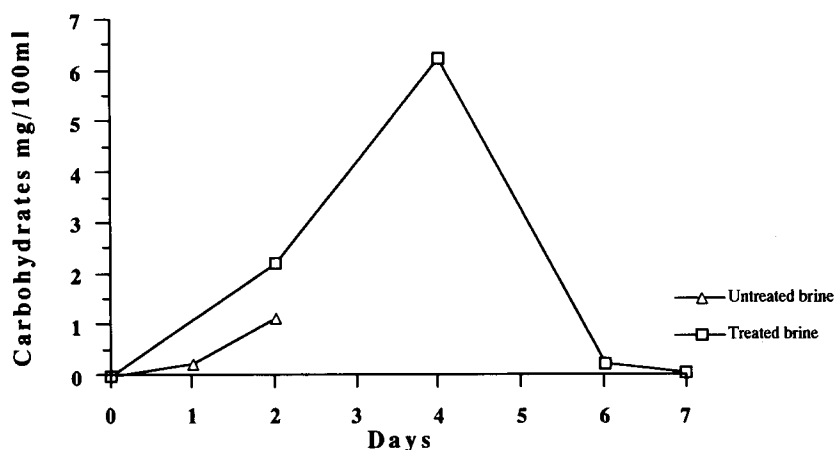


Fig. 44. Changes in carbohydrate content in brine during fish immersion. Storage was held at 15°C.

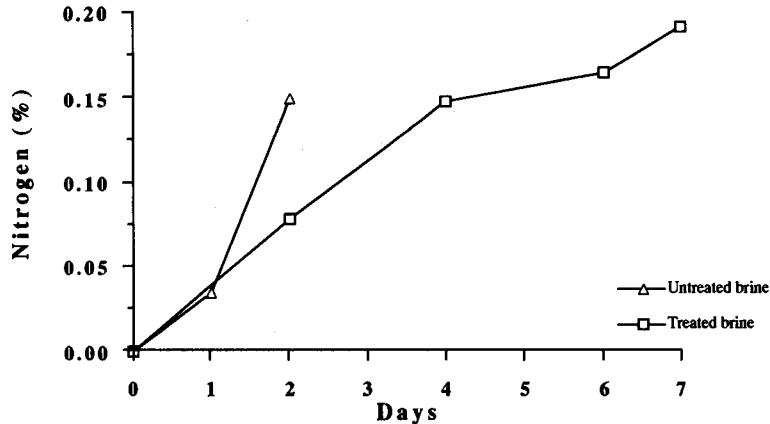


Fig. 45. Nitrogen dissolved in 4% brine during fish immersion. Storage was held at 15°C.

### *Nitrogen*

The determined values of total nitrogen recorded for both samples are presented in Fig. 45. Different from that for changes in lipids and carbohydrates, increases in total nitrogen occurring in the tested samples were observed to be by far larger in the untreated than in the treated brine and undoubtedly thought to be related to the faster spoilage observed in the respectively immersed fish. In this case the nitrogenous compounds excreted into the brine could be used and degraded by the microorganisms present, into simpler ones recognized as signs of spoilage: e.g. VB-N, ammonia and other basic substances. Based on the results of VB-N it was obvious thus this degradation was initiated together with the storage.

In the treated brine as well as in the control one, increase of nitrogen was observed. It increased continuously and on the 4th day the value reached was almost the same as that recorded for the control on the 2nd day. In spite of these similar values, different behavior toward nitrogen bonding compounds utilization by the microorganisms was recognized. That is to say, that marked increases in VB-N were not observed up to the 6th day. This suggests inhibition of microorganisms ability for degradation of this kind of compound, until at least the 6th day, on which fish was still considered acceptable. Later on, parallel with increases in total nitrogen, VB-N increased rapidly, leaving the fish unacceptable.

### *Free amino acids and amino nitrogen*

The amounts of amino nitrogen depend on the amounts of amino acid present. Therefore only results obtained for free amino acid (Table 13, and Figs. 46 and 47) are going to be discussed here and the conclusions to be given would also correspond to the changes in amino nitrogen (Table 14, and Figs. 48 and 49)

Investigation has shown that fish muscle contains high amounts of free amino acid, varying from 1 to 5 g compared to 100 g protein-bound<sup>87)</sup>. In sea food from 7 to 12 g free amino acids were found. So invading bacteria find sufficient free amino acids for growth and reproduction.

Table 13. Changes in the amount of free amino acids in brine.

Amino acid (mg/100 ml)	Untreated brine		Treated brine			
	1st day	2nd day	2nd day	4th day	6th day	7th day
Phosphoserine	0.183	0.550	0.460	1.333	1.450	1.675
Taurine	55.850	95.133	80.620	112.867	127.783	133.400
Aspartic acid	0.283	0.333	4.500	14.217	19.133	16.600
Threonine	1.000	6.933	5.520	18.700	20.383	9.500
Serine	0.250	0.517	5.980	15.000	8.783	0.675
Glutamic acid	2.283	8.317	10.620	31.950	37.167	25.275
Glutamine	0.350	3.133	3.600	10.717	9.567	6.250
Sarcosine	0.133	0.317	0.240	0.533	0.500	0.275
$\alpha$ -Amino adipic acid	0.083	0.183	0.380	1.117	0.917	2.100
Glycine	3.083	5.117	7.900	18.600	40.417	51.500
Alanine	7.067	26.283	16.120	43.517	79.333	98.475
Citrulline	0.150	0.317	0.460	1.117	3.000	2.800
$\alpha$ -Aminobutyric acid	0.100	0.333	0.080	0.800	1.533	2.875
Valine	1.783	13.583	6.800	25.167	52.867	68.400
Cystine/2	0.050	0.217	0.740	2.200	1.417	0.425
Methionine	0.700	4.717	3.500	13.067	25.133	23.650
Cystine	0.133	1.533	0.000	0.000	0.000	0.000
Isoleucine	0.933	8.717	4.700	19.067	37.550	44.900
Leucine	1.900	16.000	8.620	32.783	64.883	77.575
Tyrosine	0.667	1.250	3.960	10.983	12.217	8.875
Phenylalanine	0.783	6.933	3.880	14.933	29.250	35.275
$\beta$ -Alanine	0.100	0.300	0.180	0.483	0.617	0.875
$\beta$ -Amino iso butyric acid	0.150	0.233	0.160	0.317	0.250	0.475
$\gamma$ -Aminobutyric acid	0.283	3.867	0.480	1.350	2.917	4.475
Ornithine	0.467	2.483	1.580	5.650	14.350	12.525
Lysine	1.500	3.000	11.620	26.933	34.283	24.675
Histidine	12.350	4.533	28.120	52.967	63.733	52.500
Arginine	0.333	0.133	4.620	11.267	4.617	0.800
Proline	1.733	4.183	6.560	17.917	29.583	33.800
Carnosine	0.033	0.000	0.000	0.100	0.000	0.000
Tryptophan	0.067	0.583	0.120	1.650	2.483	2.400
1-Methylhistidine	0.017	0.000	0.000	0.067	0.000	0.150
Asparagine	0.000	0.000	2.220	6.117	8.250	4.800
Total	98.517	232.467	228.700	525.250	762.433	789.575

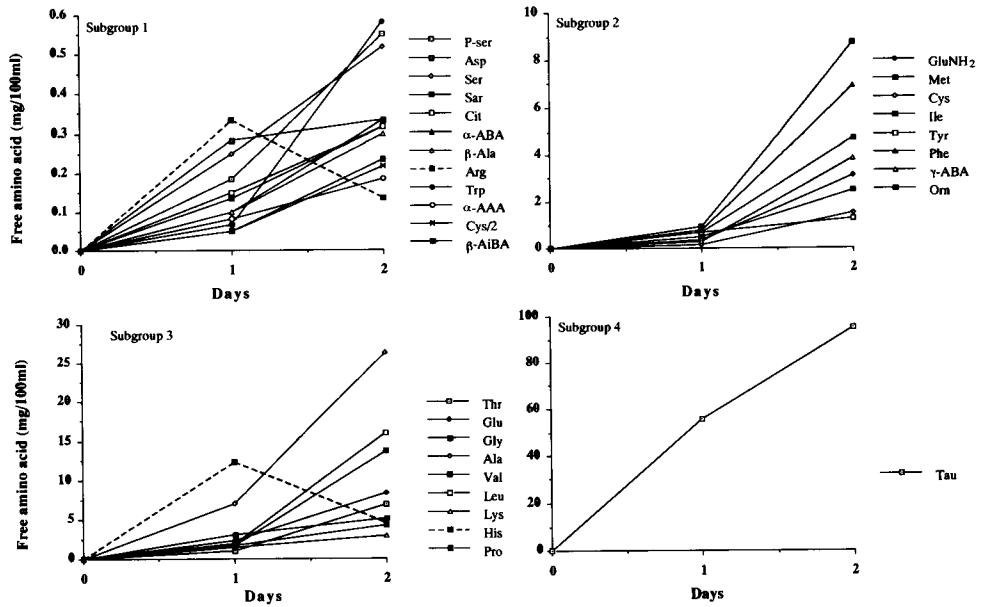


Fig. 46. Changes in the amount of free amino acids in untreated brine (control). Amino acids were divided into four groups according to the recorded concentrations.

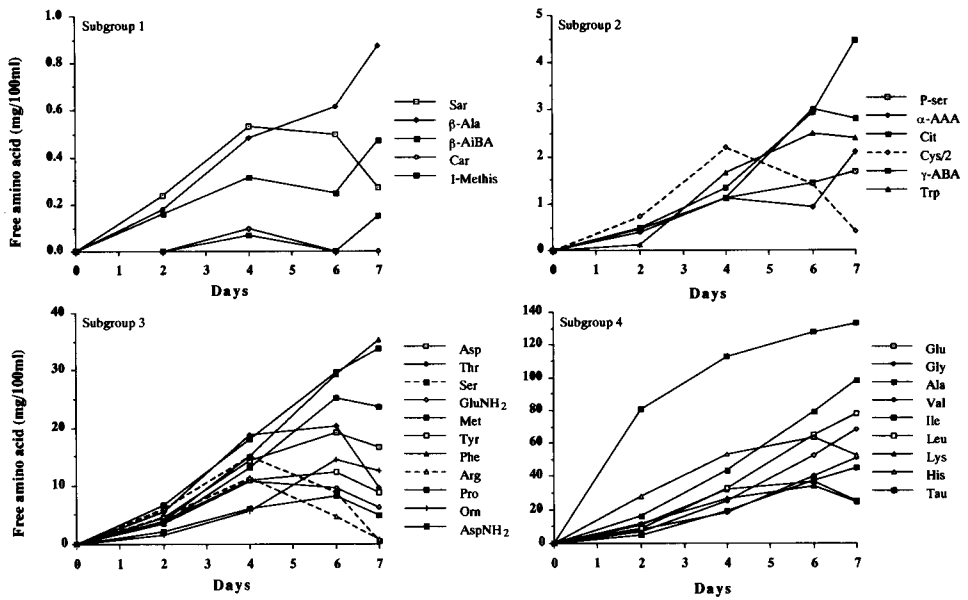


Fig. 47. Changes in the amount of free amino acids in brine containing sodium benzoate during sardine immersion.

Table 14. Changes in the amount of free amino nitrogen in brine.

Amino acid (mg/100 ml)	Untreated brine		Treated brine			
	1st day	2nd day	2nd day	4th day	6th day	7th day
Phosphoserine	0.013	0.040	0.036	0.086	0.110	0.127
Taurine	6.250	10.644	9.020	12.629	14.298	14.924
Aspartic acid	0.028	0.034	0.472	1.495	2.013	1.746
Threonine	0.118	0.815	0.650	2.197	2.398	1.117
Serine	0.033	0.070	0.791	1.990	1.166	0.092
Glutamic acid	0.218	0.791	1.011	3.041	3.540	2.404
Glutamine	0.063	0.600	0.693	2.055	1.831	1.201
Sarcosine	0.018	0.050	0.035	0.083	0.078	0.042
$\alpha$ -Amino adipic acid	0.006	0.017	0.033	0.097	0.080	0.184
Glycine	0.576	0.957	1.474	3.470	7.544	9.607
Alanine	1.108	4.132	2.530	6.837	12.470	15.479
Citrulline	0.033	0.077	0.105	0.264	0.719	0.668
$\alpha$ -Aminobutyric acid	0.009	0.046	0.009	0.106	0.210	0.392
Valine	0.212	1.624	0.813	3.011	6.324	8.180
Cystine/2	0.006	0.023	0.085	0.256	0.165	0.048
Methionine	0.065	0.444	0.328	1.226	2.360	2.222
Cystine	0.012	0.178	0.000	0.000	0.000	0.000
Isoleucine	0.099	0.930	0.501	2.035	4.010	4.794
Leucine	0.201	1.707	0.920	3.503	6.930	8.287
Tyrosine	0.051	0.097	0.305	0.849	0.941	0.686
Phenylalanine	0.066	0.587	0.330	1.265	2.479	2.991
$\beta$ -Alanine	0.013	0.047	0.027	0.078	0.098	0.136
$\beta$ -Amino iso butyric acid	0.007	0.033	0.021	0.044	0.033	0.062
$\gamma$ -Aminobutyric acid	0.038	0.526	0.063	0.181	0.395	0.608
Ornithine	0.100	0.524	0.332	1.196	3.040	2.652
Lysine	0.291	0.572	2.227	5.161	6.569	4.733
Histidine	3.341	1.230	7.613	14.331	17.250	14.207
Arginine	0.103	0.048	1.487	3.621	1.490	0.259
Proline	0.212	0.511	0.797	2.182	3.604	4.114
Carnosine	0.009	0.000	0.000	0.023	0.000	0.000
Tryptophan	0.009	0.079	0.016	0.224	0.342	0.332
1-Methylhistidine	0.003	0.000	0.000	0.019	0.000	0.035
Asparagine	0.002	0.000	0.235	0.649	0.873	0.508
Total	15.849	37.237	36.010	82.424	123.626	133.619

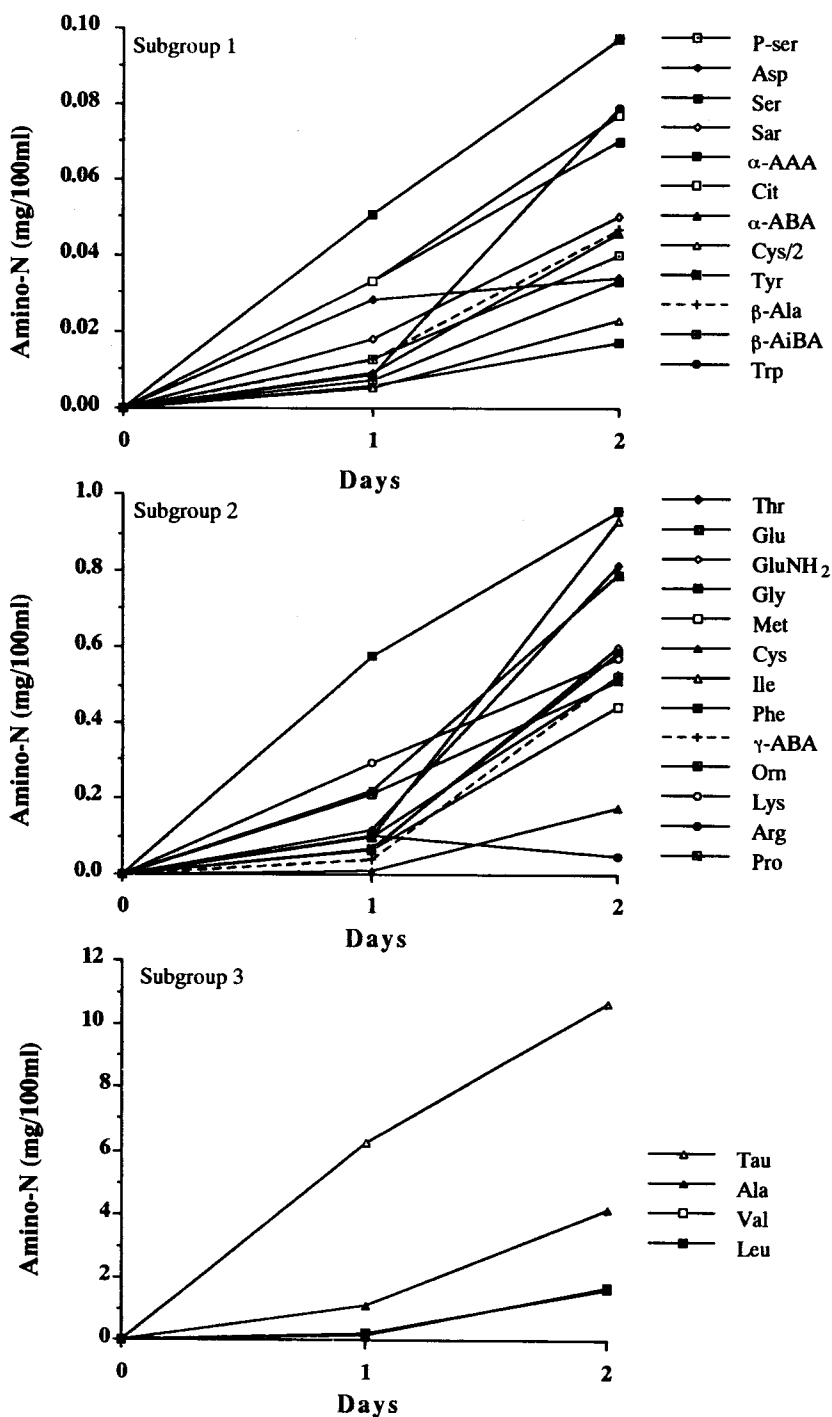


Fig. 48. Changes in amino nitrogen in control brine during sardine immersion.

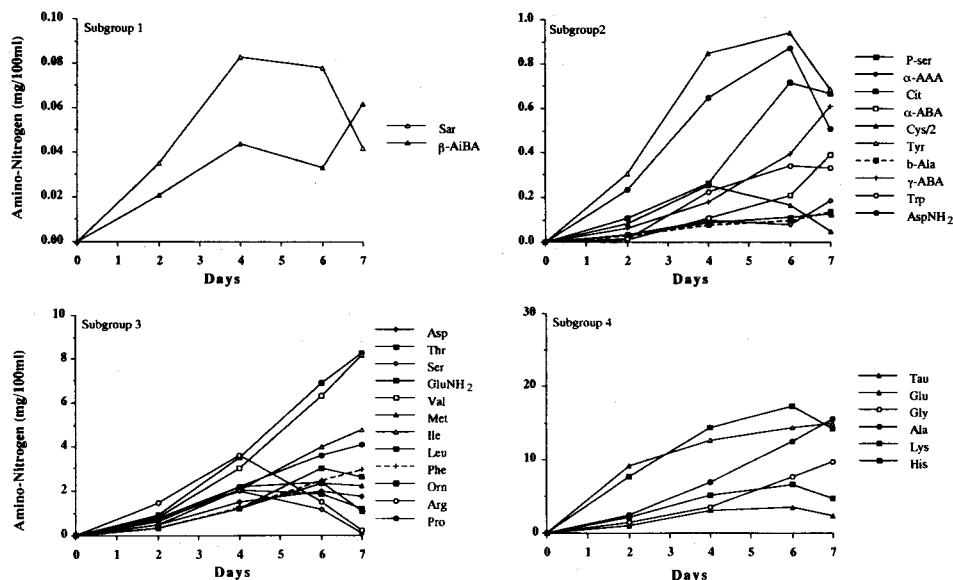


Fig. 49. Changes in amino nitrogen in brine containing sodium benzoate during sardine immersion.

On the other hand the slime, consisting as it does of mucopolysaccharide components, free amino acids, TMAO and other extractive materials representing another rich source of nutrients<sup>36</sup>.

The amounts of free amino acid coming out from immersed fish and the changes they suffer once in the brine, in order to better understand, are presented in Table 13 and in Figs. 46 and 47 for the untreated and the treated brines, respectively. In these figures amino acids are divided into four subgroups according to the recorded concentrations, facilitating in this way observation of the changes taking place.

In the untreated sample taurine (Tau) increased to reach almost 95 mg/100 ml on the 2nd day and was recognized as to reach the highest percentage recorded in comparison with all the other amino acids. Throughout the storage the amino acids changed following two clear tendencies, the first one showed a continuous increase of the coming out amino acids and the other one, an initial increase dropping later to lower values. Histidine (His) and arginine (Arg) were seen to belong to the second group and their values decreased between the first and the 2nd days. These changes can be better observed in Fig. 46, within the first and the third subdivision respectively. In the group of the lowest concentration ethanolamine (EthNH<sub>2</sub>) also showed a slight decrease. Thus, initial increase followed by a steady decrease and at the same time increases in the possible breakdown of products, such as ornitine (Orn), citrulline (Cit) and others, showed in a clear manner, degradation of the above mentioned amino acids.

When the treated samples were observed, Tau was also the amino acid which reached the highest concentration. Other important increases were recorded for glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu) and



phenylalanine (Phe). Decrease of His was not observed until the 6th day.

From the 33 evaluated amino acids 16 were observed to decrease after the 4th or 6th day. These results can be better observed in the four subdivisions arranged for this samples in Fig. 47 according to the obtained concentrations during the storage.

In the first subgroup sarcosine (Sar) dropped markedly on the 6th day. The same day this tendency was observed for Cit and triptophan (Trp) and on the 4th day for cystine/2 (Cys/2) on the 2nd subgroup. On the 3rd one aspartic acid (Asp), threonine (Thr), glutamine (GluNH<sub>2</sub>), methionine (Met), tyrosine (Tyr), asparagine (AspNH<sub>2</sub>) and Orn decreased after the 6th day and serine (Ser) and Arg from the 4th day. In the last group the most important decrease after an important increase was seen in His followed by Lys and glutamic acid (Glu). All of these decreases, the majority of which occurred from the 6th day, confirmed the statement made previously about sodium benzoate's capability of inhibition of microbial activity during this period and therefore for fish preservation.

#### 4. Inhibition or Inactivation of Some Bacteria Isolated from Spoiled Fish, by Sodium Benzoate

*Pseudomonas*, *Moraxella* and *Acinetobacter*, gram negative bacteria, are known as food spoilers. These bacteria together with *Micrococcus* were found to be the most important flora present in samples, of fish and brine, tested in section IV-3-2. Additionally, in the same section, microbial counts recorded for brine containing sodium benzoate were higher than counts obtained sections II-3 and II-6, and others published before<sup>69,70</sup>. Therefore, 2 strains each of *Pseudomonas*, *Acinetobacter*, *Moraxella* and *Micrococcus* representing the microbial flora isolated from fish immersed in brine containing sodium benzoate were tested to determine the effect of this antimicrobial on their growth and activity.

#### Materials and Methods

##### *Strain*

Strains 1459 and 1479 of *Pseudomonas* spp., 1441 and 1464 of *Acinetobacter* spp., 1448 and 1477 of *Micrococcus* spp. and 1444 and 1445 of *Moraxella* spp., isolated from fish immersed in brine containing sodium benzoate on the last day of storage (section IV-3-2), were used for this study.

##### *Sardine homogenate and nutrient broth*

Fresh sardine obtained from a local market were beheaded, eviscerated and washed thoroughly under running tap water. An adequate amount of meat from skinless fillets was homogenized in freshly made saline (4%) solution (1:9, g: ml). The homogenate was dispensed in 20 ml aliquots into 16 Erlenmeyer flasks. Another 16 Erlenmeyer flasks containing 20 ml of 4% NaCl nutrient broth (section II-4) were prepared and they together with the flasks containing the fish homogenate were sterilized at 121°C for 15 min.

*Effect of sodium benzoate on microbial growth and activity*

Sodium benzoate was added to 8 flasks containing fish homogenate and 8 containing nutrient broth to make a final concentration of 0.3% after sterilization and before adjustment of the final pH to 5 with acetic acid. Cultures of the 8 microorganisms prepared beforehand were used to inoculate all of the samples. Nutrient broth and fish homogenate without benzoate and non acidified were also inoculated and served as controls.

The 32 flasks were incubated aerobically and statically at 15°C for 8 days.

*Analyses*

The same method used in section II-4 was used to determine the antimicrobial effect of sodium benzoate on microbial growth and activity (VB-N production).

## Results and Discussion

*Microbial growth*

Microbial growth during the storage was recorded every two days for each microorganism tested. Results are presented as follows: *Pseudomonas* strains, Figs. 50 and 51; *Acinetobacter* strains, Figs. 52 and 53; *Micrococcus* strains, Figs. 54 and 55 and *Moraxella* strains in Figs. 56 and 57.

Strains of *Pseudomonas* growing in the controls increased one to two orders of magnitude, *Micrococcus* about one order and *Acinetobacter* remained almost constant.

In samples containing sodium benzoate, complete inhibition was observed on the two strains of *Pseudomonas* growing in fish homogenate (Figs. 50 and 51). When they grew in broth a decreasing tendency was observed, however the total inhibitory action of the antimicrobial agent observed in this chapter (IV-2) when tested on another *Pseudomonas* strain P-1, was not observed during this test.

The strains used in the present section were isolated from fish immersed in brine containing benzoate. Therefore, a probable adjustment of these *Pseudomonas* strains to the antimicrobial agent, which retards its inhibitory action, was thought to occur.

*Acinetobacter*-1464 (Fig. 53) and *Micrococcus*-1448 (Fig. 54) in fish homogenate containing sodium benzoate showed a rapid decrease during the incubation and in comparison with these, only small decreases were observed for the other two strains of *Acinetobacter*-1441 (Fig. 52) and *Micrococcus*-1477 (Fig. 55). Results obtained for the latter mentioned *Micrococcus* were mostly the same as presented in this chapter (IV-2) for a different strain of *Micrococcus* sp. Mi-1 (Fig. 36A).

Identification of microflora throughout this experiments, has been carried out only until the genus level, therefore different response to the action of benzoate between the two strains of *Micrococcus* (1448 and 1477) as well as those of *Acinetobacter* (1441 and 1464) suggested that the previously mentioned microorganisms belong to different species. And thus, the effect of sodium benzoate in these two cases may depend on the microorganism species.

Results of microbial counts and VB-N of *Moraxella* strains, due to a totally different behavior observed will be discussed later in this section.

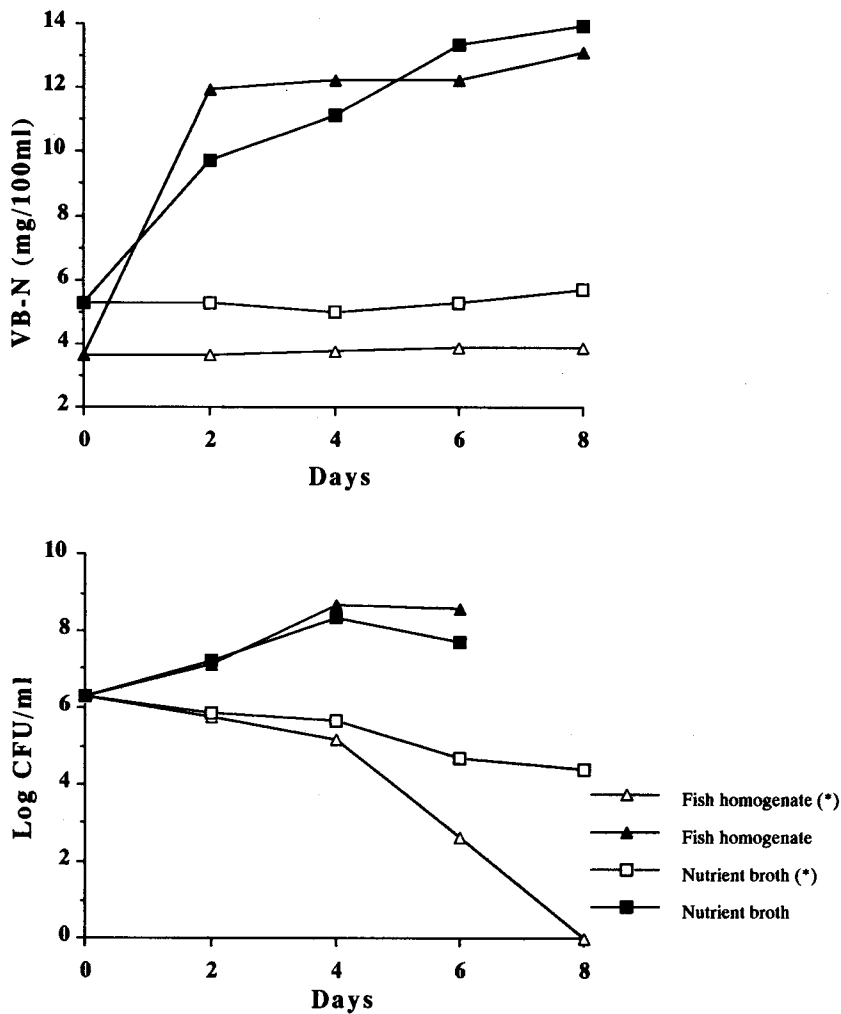


Fig. 50. Activity (VB-N) (top) and growth (bottom) of *Pseudomonas*-1459 in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

*Microbial activity*

The initial VB-N of the media of growth is that which correspond to the nutrient broth or the homogenate measured beforehand to initiate the incubation. VB-N of the homogenate was 3.61 mg/100 ml, whereas that of the broth was 5.28. Increases in VB-N for all the samples not containing sodium benzoate were observed to some extent.

The two strains of *Pseudomonas* appeared to have the highest activity. The highest VB-N values were recorded in media in which these microorganisms were grown, either broth or homogenate.

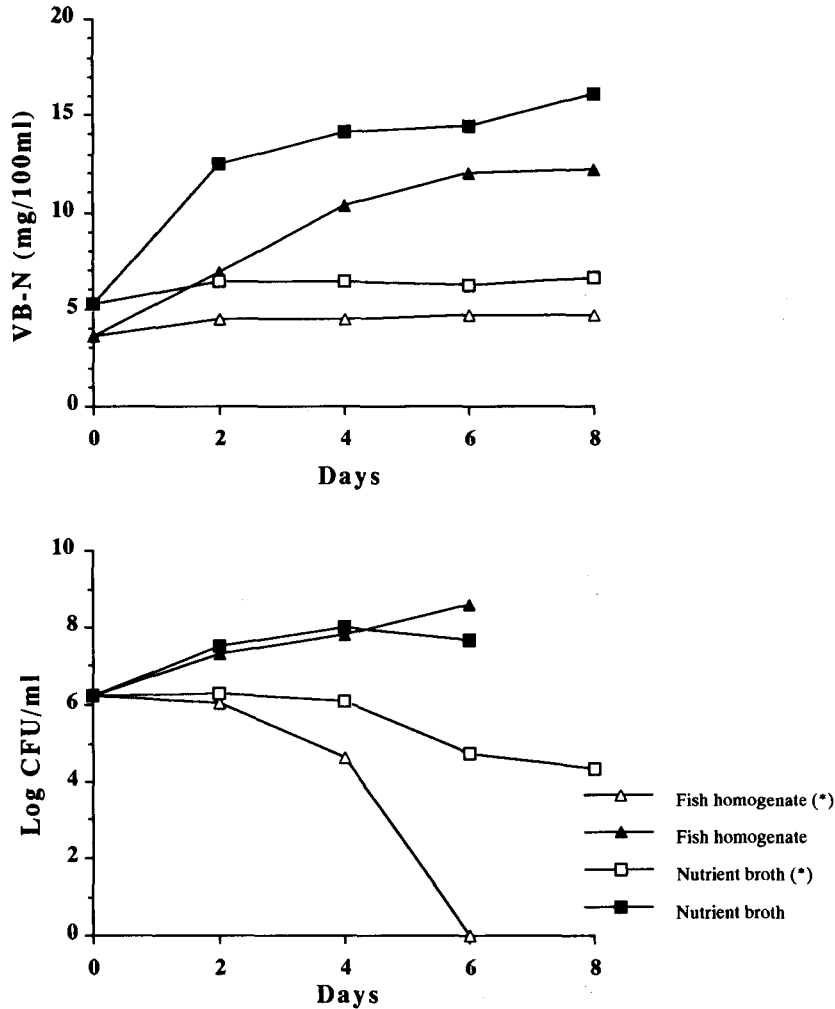


Fig. 51. Activity (VB-N) (top) and growth (bottom) of *Pseudomonas*-1479 in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

*Pseudomonas*-1459 activity appeared to be higher in the fish homogenate than in the broth (Fig. 50). While *Pseudomonas*-1479 presented the same activity in any of the growth media (Fig. 51). On the other hand, although sodium benzoate did not completely inhibit growth of both strains in nutrient broth it did inhibit their activity in this medium and in the homogenate.

After *Pseudomonas* strains, *Acinetobacter* strains gave the higher values when their activity in nutrient broth was tested. Furthermore, *Acinetobacter*-1464 showed weak activity when related to the other strain, 1441 (Figs. 52 and 53). In fish homogenate and nutrient broth, without sodium benzoate, *Acinetobacter*-1464 gave

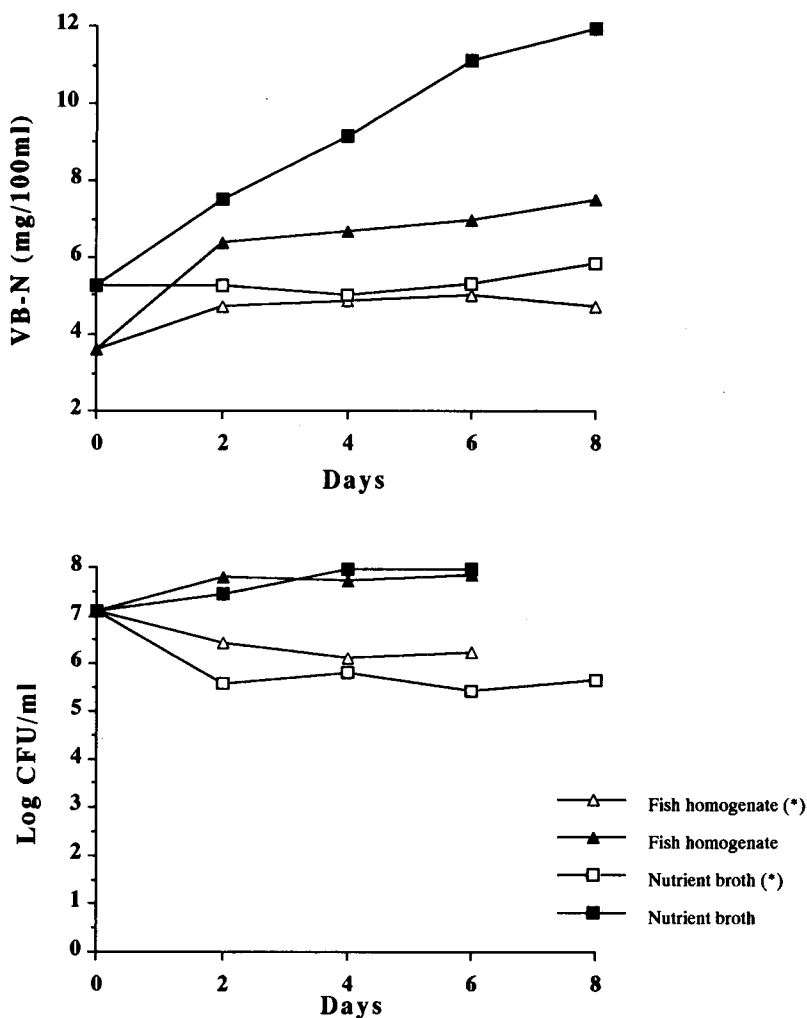


Fig. 52. Activity (VB-N) (top) and growth (bottom) of *Acinetobacter*-1441 in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

maximal VB-Ns of 5.84 and 9.74 mg/100 ml, respectively. Whereas *Acinetobacter*-1441 gave 7.51 and 11.97 mg/100 ml in the same order. Microbial numbers also decreased somewhat faster for the former when growth was performed in media containing sodium benzoate. However, activity of these microorganisms in these media was inhibited to the same degree.

When sodium benzoate was not present, as compared with both strains of *Pseudomonas* (1459 and 1479) and *Acinetobacter* (1441 and 1464), activity of *Micrococcus* strains (1448 and 1477) was the lowest observed in nutrient broth and lower than those of *Pseudomonas* strains in fish homogenate. VB-N production of

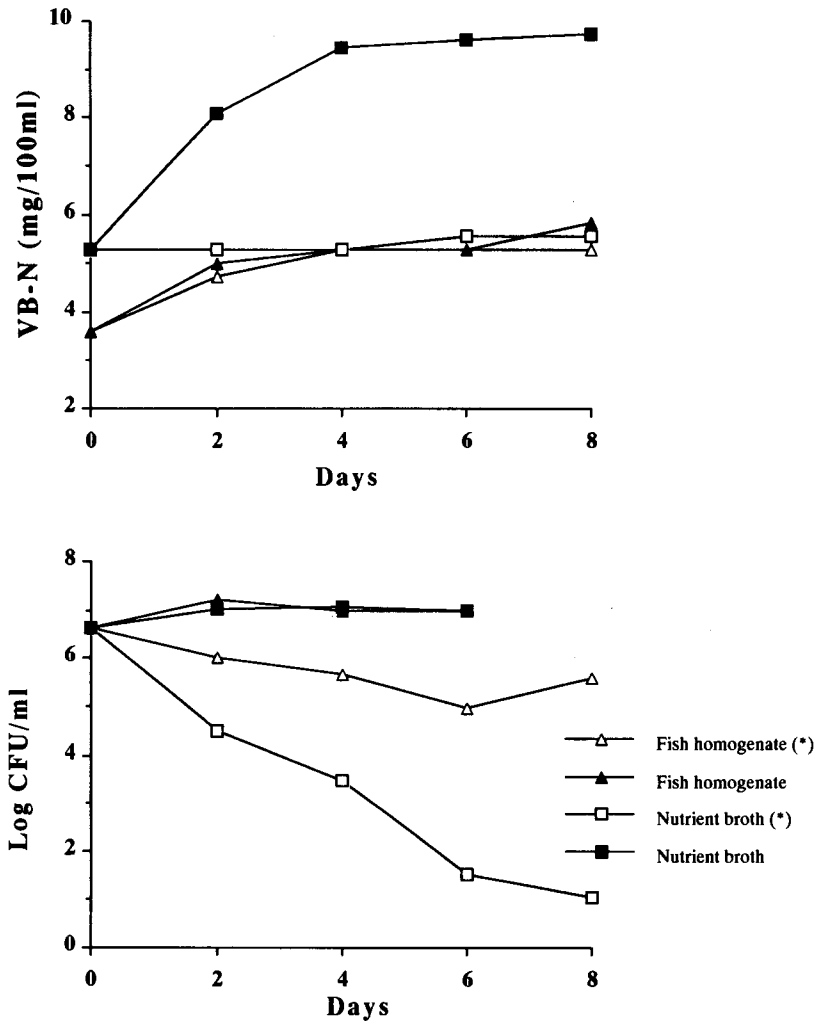


Fig. 53. Activity (VB-N) (top) and growth (bottom) of *Acinetobacter*-1464 in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

both *Micrococcus* strains, in fish homogenate followed the same pattern observed in broth. Between them, *Micrococcus*-1448 activity was strongest and caused VB-N to develop and reach higher values than those resulting from *Micrococcus*-1477 activity.

Activity of both strains growing in fish homogenate with sodium benzoate occasioned VB-N to increase in a low degree. These increases were recorded from the moment of the inoculation until the last day of storage. *Micrococcus*-1477 inoculated in nutrient broth containing benzoate showed very low activity and the other strain to be completely inhibited.

According to these results, inhibitory effect of sodium benzoate on VB-N

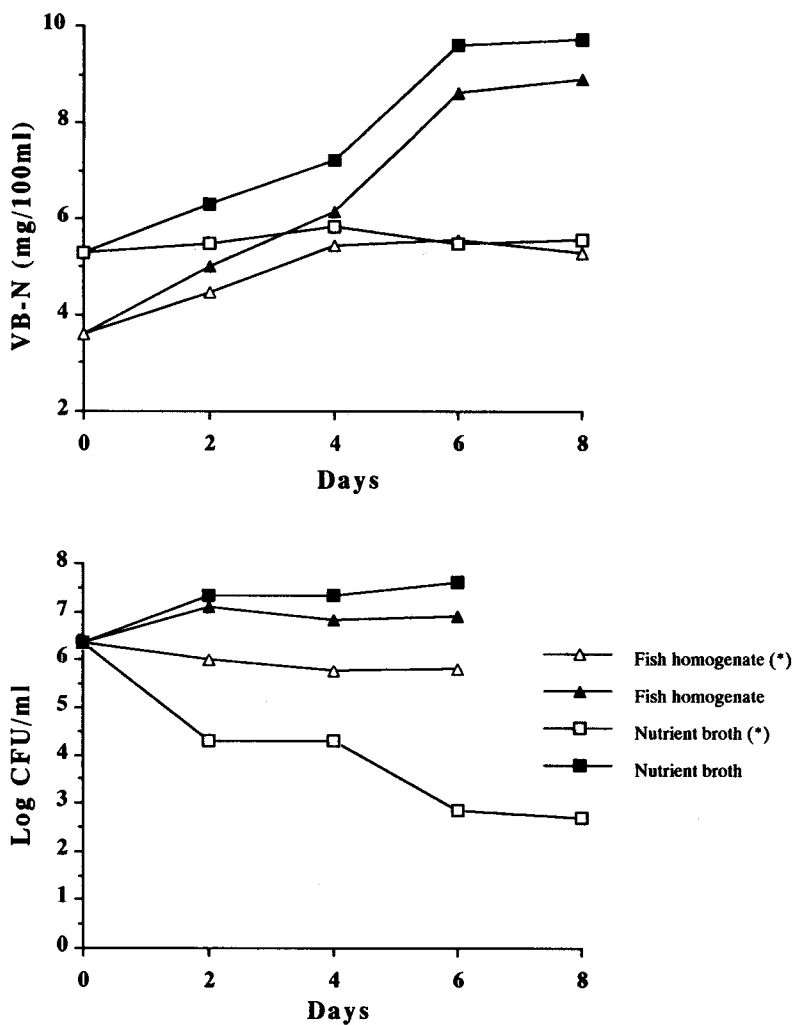


Fig. 54. Activity (VB-N) (top) and growth (bottom) of *Micrococcus-1448* in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

production of the microorganisms is clearly demonstrated.

Finally going back to results of *Moraxella* strains it would be important to refer to the initial inocula. When the effect of an antimicrobial is going to be tested, microbial counts of  $10^4$ - $10^6$  cells/ml are recommended to be use as the initial inocula<sup>56,57</sup>. Thus the effect of the tested antimicrobial could be determined without doubt.

In the case of both strains of *Moraxella*, inocula was prepared as in the case of the other microorganisms tested, and used for the determinations carried out. However the initial counts (Figs. 56 and 57) were found to hardly reach the count-

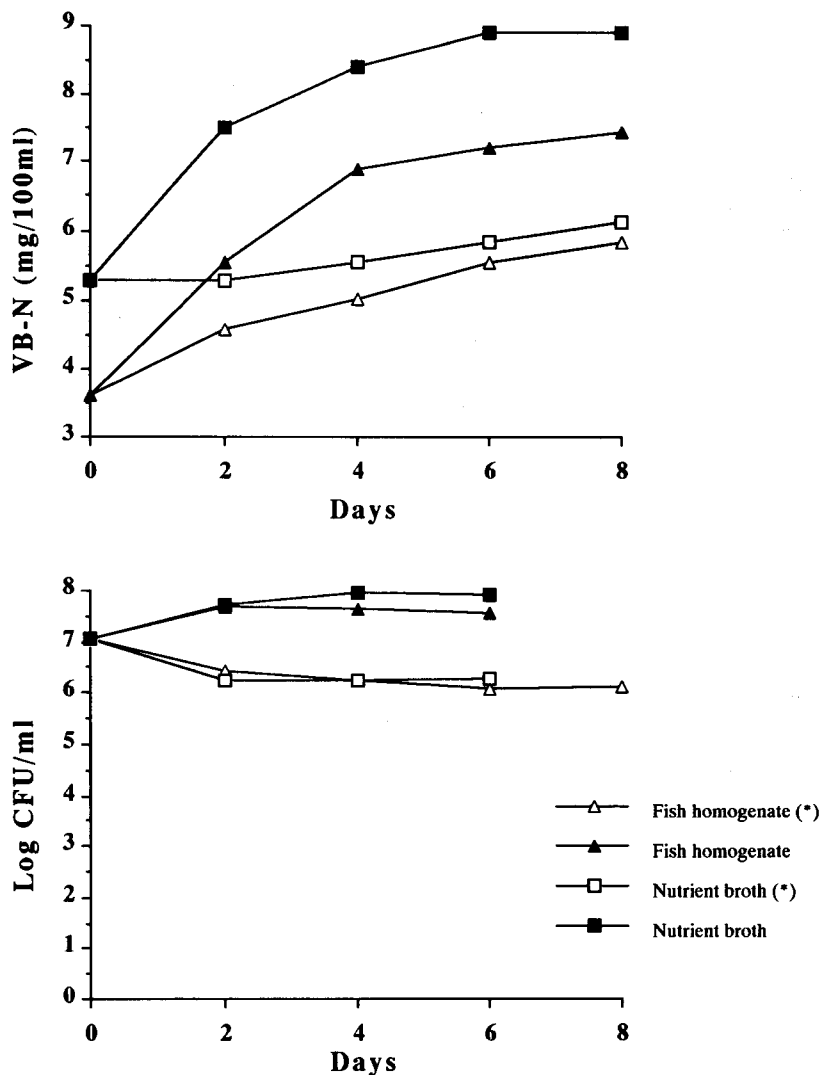


Fig. 55. Activity (VB-N) (top) and growth (bottom) of *Micrococcus-1477* in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

able range of the original media. In spite of this fact the experiment was continued and some important results coinciding with both strains were obtained. According to these, data presented here could be reliable when referred to growth and activity of both strains of *Moraxella* in fish homogenate.

Growth of both strains occurred in fish homogenate with or without sodium benzoate. Surprisingly, counts recorded in the homogenate containing sodium benzoate were higher in both cases. Total counts increased constantly until the 4th



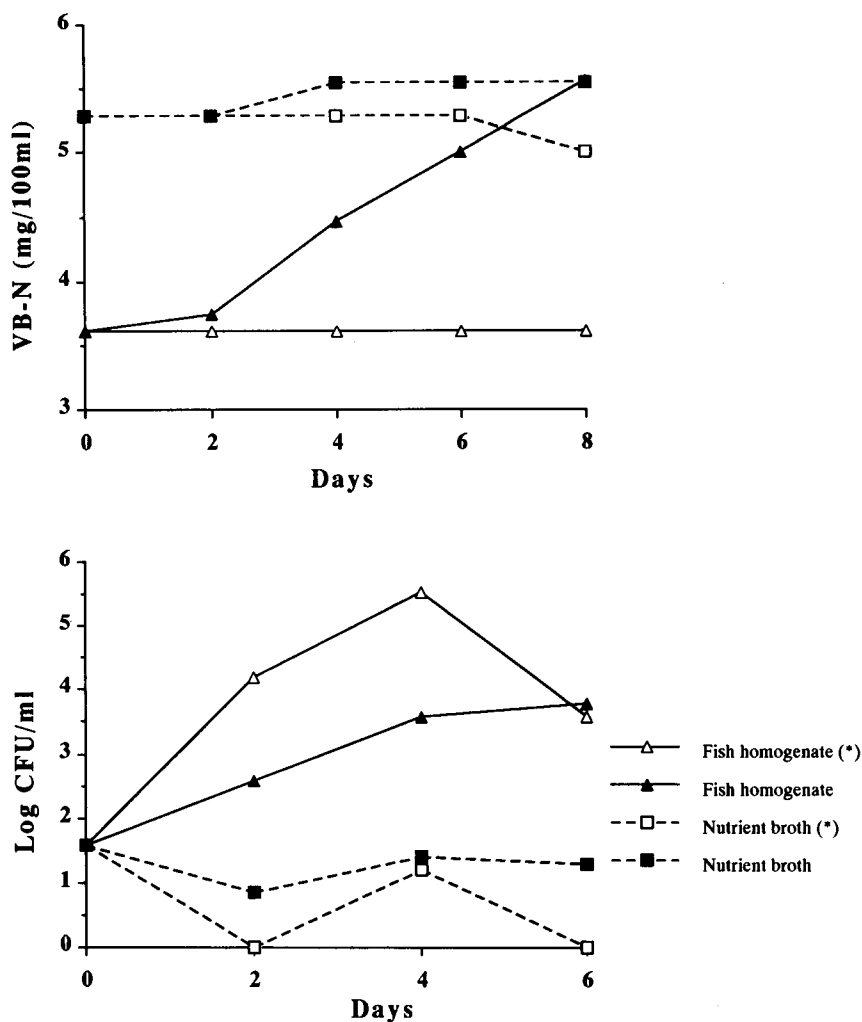


Fig. 56. Activity (VB-N) (top) and growth (bottom) of *Moraxella*-1444 in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

day of storage. Decreases, later on, were observed and assumed to be caused by depletion of nutrients in the media.

Increases in microbial numbers in the homogenate without sodium benzoate were seen throughout the storage, however at a lower rate than their counterparts containing benzoate. In these homogenates in spite of higher increases in microbial numbers increases in VB-N were of no importance or remained unchanged (Figs. 56 and 57). On the contrary they increased steadily when sodium benzoate was not contained in the homogenates.

These results could be an explanation of those obtained in this chapter (IV-3-1)

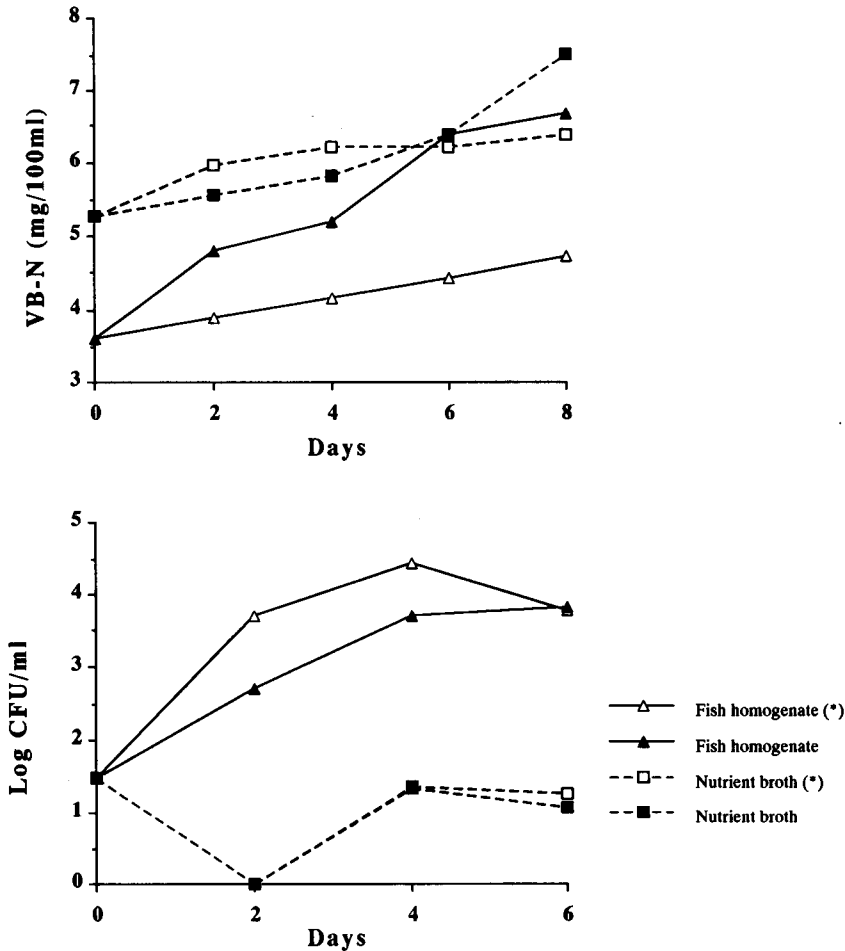


Fig. 57. Activity (VB-N) (top) and growth (bottom) of *Moraxella*-1445 in 4% NaCl nutrient broth and sardine homogenate with (\*) and without sodium benzoate. In the former case pH was adjusted to 5 with acetic acid.

in which VB-N values of fish and brine, although high microbial counts recorded, remained below of the considered limit of acceptability.

Growth in nutrient broth did not occur, and it could be believed that the microorganisms better adjusted to the fish homogenate. Conclusions for these samples in regard to the action of benzoate could not be made.

## V. General Discussion

Herring and herring-like fishes, small pelagic species such as sardines and anchovies, undoubtedly constitute the dominant group of the world fish catch. This is explained by their mass occurrence in many parts of the globe. At present

most of these catches are processed into fish meal or not caught because of lack of utilization prospects.

In a world where protein malnutrition is fairly widespread it is deplorable that the catches are not wholly converted into direct use as human food. Actions against this underutilization must be taken. However, taken into consideration the low prices paid for this kind of fish it is clear that any new methodology would not only have to extend shelf life, but it must fit easily into existing seafood industrial technology and offer an economic alternative compared with chilling or refrigeration processes.

With this aim, the present study was undertaken and 15°C, and moderately cool temperature representative of conditions when some kind of chilling is used but is inadequate, was selected as the temperature for the storage.

Factors affecting life and death of microorganisms, the most important agents of fish spoilage, are temperature, water activity, pH and oxygen availability. Taking into account these basic principles, salt in solution (brine) with or without organic acids and with or without sodium benzoate, was tested for fish preservation.

For the determination of the ratio of fish to brine, leading to an economic and accurate preservation, VB-N of samples stored at 15°C was measured and it showed, clearly, that the ratio of 1 to 1, fish to brine, offered the better alternative for the purpose of this study.

During this study VB-N values together with results of sensory evaluation and changes in microflora appeared as the better indicators of fish quality and acceptability. In most of the cases they were well correlated. Therefore they are going to be discussed here in relation to the achieved period of acceptability obtained with the different treatments carried out during this study.

When 4, 10 and 15% brine were used for fish immersed storage, the 15% brine gave the fish 5 days of acceptability, the other two, 10 and 4% brine samples showed the fish to be acceptable until the 3rd and 2nd day respectively. Results of VB-N and sensory evaluation coincided for samples in 4 and 10% brine and for 15% until the 5th day of storage, on the 7th day, in spite of a VB-N about 30 mg/100 g strong putrid odors were supposed to indicate gross spoilage, whereas the 10% sample, on the 3rd day, with a similar VB-N did not present this characteristic. This difference was related to the different salt concentrations in brine that appeared to inhibit microbial activity, but no longer inhibited autolysis at this stage of the storage.

A shift to a specific bacterial types in the microflora of brine occurred together with increases in VB-N and losses of quality of the fish. *Pseudomonas* and *Moraxella*, the species more frequently isolated from spoiling fish, being in low percentages at the initiation of the storage grew and assumed a dominant position displacing the initial ca. 65% of gram positive bacteria. These changes occurred faster in 4% brine than in 10 and finally in 15% brine samples. The time in which the highest percentage of gram negative bacteria was reached coincided with the time in which fish was considered spoiled, according to VB-N values and sensory evaluation.

The 5 days of fish acceptability attained in 15% brine were a promising period for fish preserved at 15°C. However, the 4% of salt permeated into the muscle could be a limitation for consumption of the fish thus preserved. Therefore it was thought

there was a need for improving this method.

In order to reach this objective, the addition of small quantities of an organic acid into the brine was decided as a convenient alternative. Therefore, *Pseudomonas* and *Moraxella*, the predominant species isolated from the brine, were tested to find out the acid the concentration to be used. Acetic and citric acids were used at various concentrations and acetic acid which inhibited VB-N production and growth of both microorganisms at 0.05% was selected and added to the brine at this concentration.

Addition of the acid prolonged widely fish acceptability. Samples in 10 and 15% brine were acceptable until the 7th day of storage and those in 4% until the 5th day. Compared with their counterparts immersed in brine without the acid, this means a shelf life extension of three and four days for samples in 4 and 10%, respectively, and 2 or longer for those in 15%.

Important findings with respect to brine microflora were done. Microflora in 4% brine containing the acid showed the gram negative bacteria to become almost all the percentage of the microflora and *Pseudomonas* and *Moraxella* being the predominant, this pattern of changes was the same observed in 4% brine without the acid except that the time taken in the former case for reaching similar percentages was two days longer. On the other hand gram positive bacteria increased in 10 and 15% brine containing the acid, and in 15% they made almost 95% of the total microflora. *Micrococcus* became predominant on the 7th day. These are unprecedented results for microflora related to fish or its products. Furthermore low VB-N values and good grades of sensory evaluation of these samples until the last day of storage, could be explained by the presence of these bacterial types which are not known as food spoiler agents.

Although these encouraging results to the action of the acid, another attempt at prolonging the storage time was made. With this purpose some additives were tested, among them 0.3% sodium benzoate working at pH 5 was the most effective on fish preservation under the experimental conditions.

With 4% brine containing sodium benzoate and which pH has been adjusted to 5 with acetic acid, 6 days of acceptability of the fish were achieved. The VB-N of these fish samples remained below to those to samples immersed in 4% brine alone or containing 0.05% acetic acid. It also remained below 30 mg/100 g (the considered limit of acceptability) until the 6th day of storage. Thus, it improved by 4 days fish acceptability when compared with samples in 4% brine alone and one day when compared with samples in the 4% brine containing the acid. VB-N values coincided with results of sensory evaluation.

In spite of the low VB-N values of samples in brine with sodium benzoate, different than for the samples in 4% brine alone or with acetic acid, unusual rapid microbial growth was observed during the storage. Although this rapid microbial growth in the former case changes in microflora were slower than in the latter cases. Thus, when benzoate was used the remaining 30% of gram positive bacteria observed on the last day (7th) of storage was by far larger than the almost 5% recorded in 4% brine containing 0.05% acetic acid on the 7th day and the 3% in 4% brine without the acid on the 3rd day. Together with *Pseudomonas* and *Moraxella*, *Micrococcus* was an important part of the microflora.

When sodium benzoate was used, additional studies on microflora of fish and chemical changes in brine related to the microbial flora were carried out. From them it was concluded that bacteria constituting the microflora of brine and fish was mostly the same, except for some differences in the percentages reached.

The most important findings obtained from the study on chemical changes in brine are in reference to the demonstration of the strongest effect of sodium benzoate on microbial activity rather than on microbial growth.

As a final conclusion of this study it could be said that six days of acceptability for fish during immersed storage in 4% brine at 15°C have been achieved with the addition of 0.3% sodium benzoate into the brine of immersion when the pH of the brine was adjusted to 5 with acetic acid. As a result, this preservation method could be used on board when there is a scarcity of chilling or refrigeration systems for the benefit of protein deprived populations and for the fishermen who could sell good quality catches without increasing their costs.

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