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From the Department of Food Hygiene, Royal Veterinary College, and the Department of Food Hygiene, National Institute of Public Health, Stockholm. Sweden.

EXPERIMENTAL STUDIES ON THE VOLATILE NITROGEN COMPOUNDS PRODUCED BY PSEUDOMONAS FRAGI IN FISH EXTRACTS

By

S. O. Florin

FLORIN, S. O.: *Experimental studies on the volatile nitrogen compounds produced by Pseudomonas fragi in fish extracts.* Acta vet. scand. 1972, *13*, 381—402. — The decomposition of nitrogenous compounds of extracts of cooked halibut meat due to the growth at 4°C and 17°C of Pseudomonas fragi, strain F 111, was followed with determinations of the total volatile nitrogen (TVN) and of trimethylamine (TMA). The steam-distillation method according to *Bethea & Hillig* (1965) and the Conway-microdiffusion-method according to *Farber & Ferro* (1956) were used for these determinations.

When fish extract was inoculated with the strain F 111 and stored at 4°C for 5 days or at 17°C for 3½ days an increase of TVN was started. This increase of TVN was slower at 4°C than at 17°C. It was shown that in the extract B, which was prepared from fish meat of poor but acceptable commercial quality, the initial TVN was higher, the increase of TVN caused by the action of the strain F 111 was slower, and the TVN maximum was lower than the corresponding values representing extract A. The last mentioned extract was prepared from halibut meat of good commercial quality.

The correlation between the increase of TVN and that of pH of the inoculated fish extract was poor. This indicates that the initial increase of pH was not caused by volatile basic compounds.

It was shown that the exclusion of air after 1 or more days of incubation at 17°C could delay the onset of the TVN increase but did not prevent it. The final TVN value of the sample, which was layered with paraffin oil 24 hrs. after the inoculation of the strain F 111, was approximately the same as that of the fish extract sample layered after 14 days of incubation at 17°C. In inoculated fish extract samples, which were sterile-filtered on the day when the extract was layered with paraffin oil, no further increase of TVN was observed.

It was confirmed that Pseudomonas fragi caused no increase of TMA in the extract of cooked halibut.

volatile nitrogen compounds; Pseudomonas fragi; fish.

The determination of the total amount of volatile nitrogen compounds (TVN) in fish has been used as a measure of the freshness of the raw product. The breakdown of proteins, amino acids, and other compounds that contain nitrogen is caused by microorganisms. This process ends in the liberation of ammonia and of different amines. A study on the liberation of volatile nitrogen compounds in the surface layer of pike perch has been presented (*Florin* 1971). The experiments were based on the experiences from a centralized production of precooked food for hospitals. A reinfection of precooked fish and of other dishes with Pseudomonas fragi was of importance to the shelf life of the products. This organism was found in very high numbers when off-odour was present in such dishes. The experiments on pike perch, which had been inoculated with Pseudomonas fragi, showed that off-flavour had developed in the fish meat. The amount of volatile nitrogen compounds, however, increased slightly during a 3-week storage. The experimental conditions allowed a passage of oxygen sufficient only for a strictly localized growth of the strain at the site of the inoculation. The observed increase of TVN could not, therefore, be explained by an action of the microorganisms on nitrogenous compounds of the fish product. In these experiments the fish meat used was not a suitable material for repeated sampling. In the present paper the changes in the volatile nitrogen compounds caused by Pseudomonas fragi were studied in an extract of cooked halibut meat. This homogenized material was suitable for repeated sampling. The object of the present investigation was to study:

1. The relation between the time of storage and the amount of volatile nitrogen in the fish product;
2. The significance of the total volatile nitrogen as an index of freshness for cooked fish products;
3. The influence of the quality of the raw fish on the development of volatile nitrogen compounds in cooked fish products during storage;
4. The influence of an exclusion of air on the development of volatile nitrogenous compounds in fish meat.

Investigations for similar purposes have been performed on raw fish in order to find a method to evaluate the freshness of the fish (e.g. *Lücke & Geidel* 1935). In canned fish in tomato sauce, brine, or oil studies have been presented on the correlation

between the sensory quality of the products and their content of volatile reducing substances, volatile nitrogen compounds, and trimethylamine (*Farber & Ferro* 1956). Also in some other foods Pseudomonas fragi has been shown to induce changes, e.g. ester formation in milk (*Pereira & Morgan* 1958). The latter investigation indicated that the amino-acid leucine in milk could serve as a precursor of the fruity aroma defect in dairy products.

The present studies are divided into 2 parts. The first part concerns the effect of Pseudomonas fragi on extracts of cooked halibut when air is present. The object was to find out whether the release of volatile nitrogen compounds in extracts of cooked halibut meat depends on the quality of the raw fish or on the storage temperature of the cooked fish. The second part concerns the volatile nitrogen compounds produced in precooked fish when the reinfection and some outgrowth of microorganisms had occurred before the final wrapping or vacuum packing of the product.

MATERIALS AND METHODS

A description of materials and methods used in the various experiments will be given in this section. Some additional information, especially on inoculation procedures and incubation temperatures, will be given separately for each part of the investigation.

a. *Preparation and composition of fish extracts*

The 2 fish extracts (A and B) used in these experiments were prepared from normal trade quality halibuts. The fish extract designed A was prepared from fish meat with the fresh sweet flavour and the odour of a good quality halibut. Extract B was prepared from fish meat with a lack of odour and an off-flavour bordering on the acceptable. The high level of total volatile nitrogen and of trimethylamine and the low level of total nitrogen of extract B (Table 1) in comparison with those of extract A are expressions of these quality differences.

The cooked fish was cut in 50-g pieces and treated with diluted lemon juice and salted*. This was in accordance with the normal procedure in the kitchen. About 750 g of the raw fish was vacuum packed in plastic pouches with a Cryo-Vac machine (Åkerlund & Rausing AB, Lund, Sweden). The pouches were sealed by melting the plastic outside the Cryo-Vac clip against an electrically heated metal rod. The film used was an 0.04 mm polyamide, nylon 11, Rilsan® (Aquitane-

* 1 % concentrated lemon juice in the final rinsing water; 20 g of salt per kg fish cuts.

Organico, 92-Courbevoie, France). The pouches were boiled for 20 min. and after rapid chilling in cold water they were frozen and stored at —20°C until used for the preparation of the fish extract.

The frozen fillets were ground in a meat grinder — mesh size 2 mm — and homogenized in the double amount of physiological saline solution by the use of an Ultra Turrax Tp 18/2 homogenizer, speed 24,000 r.p.m. (Janke-Kunkel). The suspension was left for 2 hrs. at room temperature. After centrifugation for 15 min. at 14,000 r.p.m. 24,000 × g) by the use of a High Speed 17,000 Refrigerator centrifuge (Measuring & Scientific Equipment LTD, London, Great Britain) the supernatant was filtered through a Seitz filter pad no. 6, and the sterility of the filtrate was tested. The filtered extract was stored at 5°C until used. Before use, the extract was diluted by adding the same volume of physiological saline solution. From this stock solution, samples — usually 20 ml — were transferred into Erlenmeyer flasks of 150 ml. The depths of the extract layer in the Erlenmeyer flasks did not exceed 5 mm.

This diluted fish extract is hereafter in the text, tables, and figures referred to as fish extract.

b. *Microorganism used in the experiments*

A strain of Pseudomonas fragi, F 111, was used in these experiments. It had been isolated from precooked fish. The strain was described in a previous paper (*Florin* 1971).

c. *Determination of the number of viable microorganisms*

To determine the number of viable microorganisms in samples of fish extract, 0.1 ml of appropriate tenfold dilutions in physiological saline solution was spread on the surface of milk-peptone-agar plates. The medium consisted of Tryptone Glucose Agar (Oxoid) to which skim milk, 1 %, was added. The plates were incubated for 48 hrs. at 17°C, after which the average diameter of the colonies of Pseudomonas fragi was 2—3 mm. Further incubation did not result in a higher total number of organisms.

In some experiments the total number of organisms that could grow in the absence of oxygen was determined in a Tatlock Anaerobic Jar (Baird Tatlock, London, Great Britain). The gas used was hydrogen (95 %) to which carbon dioxide (5 %) was added. The plates were incubated for 72 hrs. at 17°C, after which the growth of Pseudomonas fragi appeared as minute watery colonies.

d. *Inoculation of fish extract samples*

The samples of the fish extracts were inoculated with approx. 1 million cells in the logarithmic growth phase per ml of fish extract. To ensure this, the following procedure was generally adopted:

The growth curves of strain F 111 in fish extract at the selected temperatures of 4°C and 17°C were determined (Figs. 1 and 2).

From a 48-hr. fish extract culture that had been incubated at the

temperature selected for the experiment (4°C or 17°C), a tenfold dilution series in fish extract was prepared. At the same time, 0.1 ml of each dilution was spread on the surface of milk-peptone-agar plates which were incubated at 17°C. After 24 hrs. of incubation the colonies on these plates were barely visible to the naked eye. An acceptable preliminary count was obtained with a magnification of 18. The final colony count was performed after incubation for 48 hrs.

The afore-said tenfold dilution series was incubated for 24 hrs. at the selected test temperature. The tube most likely to contain 100 to 150 million cells per ml was then selected. This number was calculated from the preliminary 24-hr. colony count in relation to the above-mentioned growth curve of strain F 111.

Two ml of the culture in the selected tube was diluted with 12 ml of fish extract. After careful mixing, 0.2 ml of this final dilution was used as the inoculate, which would give a total number of organisms of 1 to 2 million cells per ml when added to a 20-ml fish extract sample.

e. *Determination of total volatile nitrogen (TVN) and trimethylamine (TMA)*

1. Determinations by the Conway microdiffusion method as described by *Farber & Ferro* (1956).

The determination of TVN was completed in Conway-Byrne's units. The clean units were treated with 0.001 N sulphuric acid and washed in glass-distilled water. They were air dried immediately before use. Two ml of the sample of fish extract was transferred to the outer well and 1 ml of boric acid containing bromocresol green and methyl red was transferred to the inner well of the unit. Vaseline was applied to the rim of the unit and the lid was almost closed. Finally, 2 ml of potassium carbonate solution was introduced with a syringe into the outer well. The carbonate solution was mixed with the sample by slow movements of the unit. After incubation for at least 1 hr. at 37°C the amount of TVN trapped in the boric acid was titrated with 0.01 N sulphuric acid to the original indicator solution colour.

The TMA was determined by the procedure outlined above after mixing the sample in the outer well with 0.7 ml of formaldehyde in order to bind the ammonia and primary amines.

The amounts of TVN and TMA present were calculated as mg of nitrogen per 100 ml of fish extract.

The method used differed from the description by *Farber & Ferro* in the following 2 respects:

Great care was taken to obtain clean glassware by rinsing the Conway-Byrne's units with a weak acid solution.

The titration of the TVN and the TMA was made with 0.01 N instead of 0.03 N sulphuric acid.

This cleaning procedure was suggested by *Spinelli* (1964), who reported on the effect of inadequately cleaned glass on the determination of TMA. Spinelli also pointed out that inaccurate titration was a major source of error in the TMA test. In a previous investigation (*Florin*) TVN and TMA were titrated with 0.03 N sulphuric acid, as

suggested by *Farber & Ferro*. In the present investigation, the 0.03 N sulphuric acid was considered to be too strong in relation to the small amount of volatile bases that was expected.

2. Steam distillation of volatile nitrogen compounds by the method of *Bethea & Hillig* (1965).

The steam-distillation assembly used in these experiments was described in a previous paper (*Florin*).

The following chemicals were used:
Calcium hydroxide, p.a., Merck
Formaldehyde (20 %), Ph. Nord.
Hydrochloric acid, puriss
Picric acid, p.a., Merck
Potassium carbonate, p.a., Merck
Toluene, p.a., Merck
Trimethylamine hydrochloride, Eastman No. 265.

To prepare a standard solution, 500 ml of formaldehyde (20 %) was shaken with 5 g of magnesium carbonate, filtered until clear, and diluted to 1,000 ml with distilled water.

The toluene was shaken with 1 N sulphuric acid (5:1), as suggested by *Bethea & Hillig*, and the separated toluene layer was distilled according to United States Pharmacopeia XVI and dried with anhydrous sodium sulphate.

Fourteen ml of the fish extract was diluted to 150 ml with glass-distilled water. The volatile compounds were steam-distilled through a calcium hydroxide trap, condensed, and collected in distilled water in a receiving flask. The distillation was continued for 45 min. and during this time 140 to 150 ml of distillate was collected. The amount of volatile bases in the distillate was titrated with 0.01 N hydrochloric acid to the yellow end-point of phenol red. The volatile nitrogen compounds were calculated as mg N per 100 ml of fish extract.

Trimethylamine is one of the volatile nitrogen compounds that are collected in the distillate and trapped by the hydrochloric acid. The content of TMA in the distillate was measured by Dyer's colorimetric method according to the description by *Bethea & Hillig*. The titrated distillate was diluted to 200 ml with distilled water. A measured amount of the diluted test solution was shaken vigorously together with formaldehyde, toluene, and saturated potassium-carbonate solution in a glass-stoppered test tube. The tube was left standing until clear (generally 10 min.). A measured volume of the toluene layer was transferred to a clean dry test tube and dried with anhydrous sodium sulphate. The dried toluene extract was mixed with an equal amount of picric acid (0.02 % picric acid in redistilled toluene). The absorbance was measured in a Unicam SP 600, spectrophotometer (Unicam Instrument, Cambridge, England) at 420 mm against a tuluene blank.

The amount of TMA was calculated from a standard curve prepared according to the description given by *Bethea & Hillig*. It was expressed as mg N per 100 ml of fish extract.

f. *Determination of total nitrogen*

The total amount of nitrogen was determined by the conventional macro-Kjeldahl method.

g. *Determination of pH*

The pH was measured with a Metrohm AG Eattery pH-meter, E 280 A, Herisau, Switzerland.

1. Volatile bases produced by Pseudomonas fragi, strain F 111, in inoculated fish extract with atmospheric oxygen present

Materials and methods

The following 4 series, which consisted of 30 inoculated and 10 uninoculated fish extract samples each, were prepared.

One series of samples of fish extract A and 1 series of samples of fish extract B were inoculated with approx. 1 million cells of strain F 111, which had been grown at 4°C. These 2 series were then incubated at 4°C. A second series of samples of fish extracts A and B, respectively, were inoculated with cells of strain F 111, which had been grown at 17°C. These 2 series were then incubated at 17°C.

On each of the 10 selected sampling days, 3 inoculated culture flasks and 1 of the controls in each series were randomly selected and tested separately. From each sample, 1 ml was transferred in order to determine the number of viable cells and 4 ml was transferred to determine TVN and TMA by *Farber & Ferro*'s (1956) technique. The remaining amounts — generally 14 ml — were used to determine the pH and the TVN and TMA by *Bethea & Hillig*'s (1965) technique.

The number of microorganisms and the TVN and TMA listed in the tables and figures represent the means of the results from the 3 samples on each sampling point.

Results and discussion

The object of these experiments was to study the changes in cooked fish during storage at temperatures above 0°C. The temperature 4°C was chosen, because this was the higher limit allowed for storage of refrigerated precooked food produced in a centralized production of hospital foods.

Table 1. Determinations of total volatile nitrogen (TVN), trimethylamine (TMA), and total nitrogen (Total N) in fish extracts A and B.

Extract	TVN mg/100 ml	TMA mg/100 ml	Total N mg/100 ml
A	2.6	0.3	119
B	4.2	2.4	82

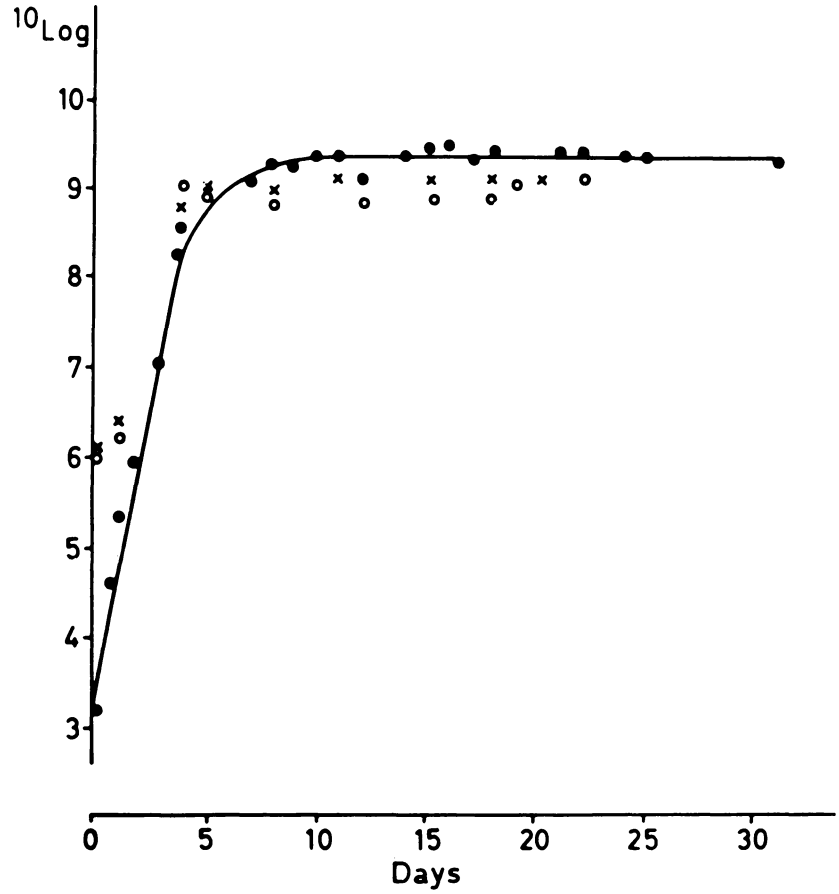

Figure 1. Growth curve of Pseudomonas fragi, strain F 111, in fish extract at 4°C (●) and total number of microorganisms at 4°C in extracts A (○) and B (×).

In order to evaluate the differences between storage at 4°C and higher temperatures, experiments were also performed using 17°C. This temperature was chosen, because Pseudomonas fragi had its optimum growth temperature at this temperature range and because changes in, e.g., pasteurized milk were studied at this temperature.

The results of these experiments, which included the use of 2 qualities of fish extract and 2 different incubation temperatures, are shown in Figs. 1 to 7 and in Tables 2 and 3.

When the inoculated fish extract was incubated at 4°C, the lag phase was 24 hrs. After 96 hrs. the maximum stationary phase

Figure 2. Growth curve of Pseudomonas fragi, strain F 111, in fish extract at 17°C (●) and total number of microorganisms at 17°C in extracts A (○) and B (×).

was reached (Fig. 1). During the following 2 to 3 weeks the number of viable cells per ml was constant — range 7×10^8 to 1×10^9. No decrease in the number of viable cells was observed during the experimental periods, which varied from 22 to 26 days.

When the incubation temperature was 17°C, the lag phase was shorter and the maximum stationary growth phase was reached within 48 hrs. (Fig. 2). The organisms were still in this growth phase at the end of the experimental period. The ranges of the viable counts per ml during this phase were 2.5×10^8 to 8×10^8 in extract A and 4×10^8 to 8×10^8 in extract B.

The amount of volatile nitrogen compounds in the inoculated fish extracts incubated at 4°C was apparently stable during the first 4 or 5 days (Figs. 3 and 4). From then on, there was an in-

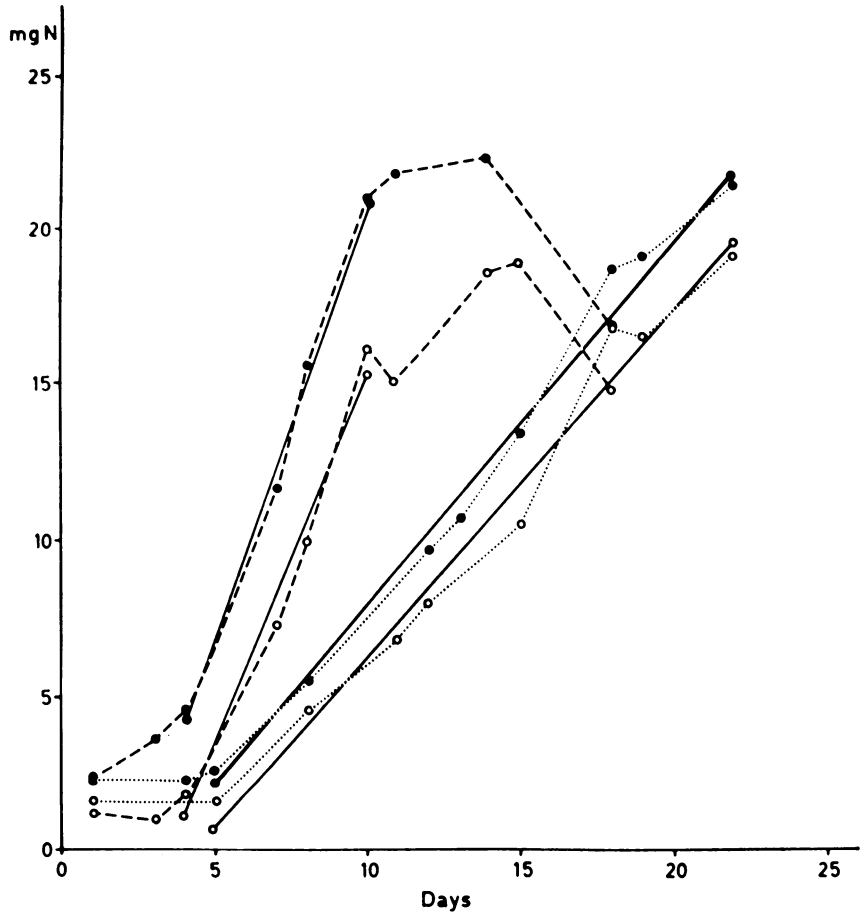

Figure 3. Total volatile nitrogen (TVN) in mg per 100 ml of fish extract A incubated at 4°C (·····) and at 17°C (-----). Determinations by the Conway method (●) and the Bethea-Hillig method (○).

crease of TVN on time in extract A until the last day of sampling, day 22. During the time between the 5th and the 15th day, the increase of TVN in extract B also seemed to follow a straight line. With one exception, the TVN values determined by the Conway method were higher than the corresponding values obtained by Bethea & Hillig's method.

When the inoculated fish extract was incubated at 17°C, the increase in TVN started on the 3rd or 4th day. A rapid, seemingly linear, increase between the 5th and the 10th day was followed by a maximum level of TVN.

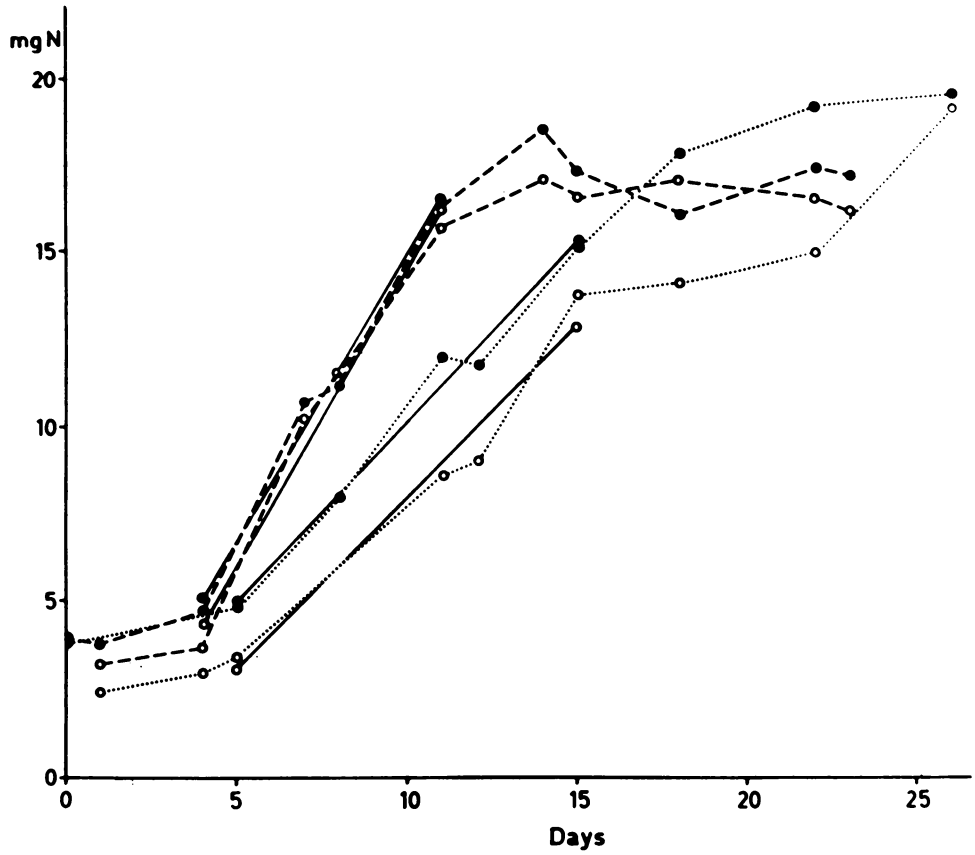

Figure 4. Total volatile nitrogen (TVN) in mg per 100 ml of fish extract B incubated at 4°C (·····) and at 17°C (------). Determinations by the Conway method (●) and by the Bethea-Hillig method (○).

The above-mentioned linear parts of the TVN curve lines were studied by the linear regression method (*Snedecor* 1956). A small difference was observed between the slopes (the b-values in Table 2), representing extract A and those representing extract B incubated at 4°C. At 17°C, however, the increase in TVN was definitely more rapid in the inoculated extract A than in extract B. No increase of TVN was observed in the non-inoculated control samples. In samples of extract A incubated at 17°C a difference was observed between the b-values of the 2 chemical methods used. The results indicated, however, that the b-value of Bethea & Hillig's method was probably inaccurate and too low. The linear parts are indicated in Figs. 3 and 4.

Table 2. The increase of total volatile nitrogen (TVN) in fish extract inoculated with Pseudomonas fragi, strain F 111. The sample regression coefficient (b) and its significance.

Temperature and method of analysis	Extract	b	t	TVN of control mg N/100 ml	Approx. number of days to the increase of TVN
4°C					
Bethea-Hillig	A	1.11	17**	0.74	5.1
„	B	0.99	10**	3.04	4.9
Conway	A	1.20	34**	2.26	5.2
„	B	1.02	10**	4.13	4.1
17°C					
Bethea-Hillig	A	2.34	9*	0.78	3.8
„	B	1.70	14**	3.84	3.7
Conway	A	2.73	14**	2.66	3.4
„	B	1.62	14**	4.32	3.5

The approximate time when TVN started to increase was calculated as the time corresponding to the TVN value of the inoculated fish-extract sample on the calculated slope of the curve. This confirmed that the increase of TVN in samples incubated at 4°C began approx. 5 days after the day of inoculation and in the sample incubated at 17°C 3½ days after the inoculation.

The TVN of the different samples of extract B inoculated with strain F 111 was studied by the method of analysis of variance (*Snedecor*). The relative standard deviation of TVN in fish extract B stored at 4°C was 7 % by the Conway method and 14 % by Bethea & Hillig's method. The determination on the samples incubated at 17°C had a standard deviation of 10 % for both methods. The results presented in Table 2 indicate that the microdiffusion method of Conway gave a higher TVN value than did the steam-distillation method of Bethea & Hillig. This difference was not significant for samples incubated at 17°C.

When plotted against time, the TMA of the inoculated samples of extracts A and B, respectively, showed a slight tendency to decrease (Table 3). This indicated that TMA was neither produced by the Pseudomonas fragi nor broken down further by the microorganism to be used for instance as a source of nitrogen.

The pH of the inoculated fish extracts changed towards alkalinity during the incubation (Figs. 5 and 6). No such change was

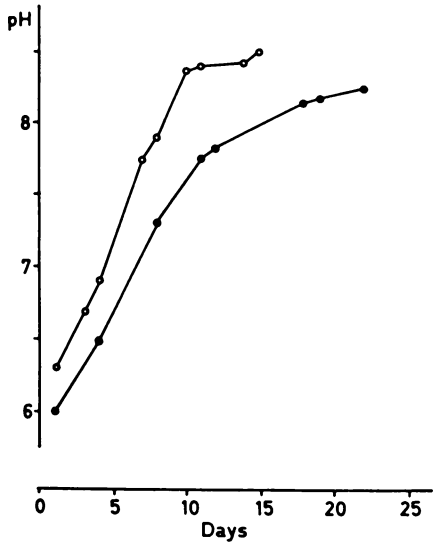

Figure 5. The pH at 4°C (●) and at 17°C (○) in inoculated fish extract A.

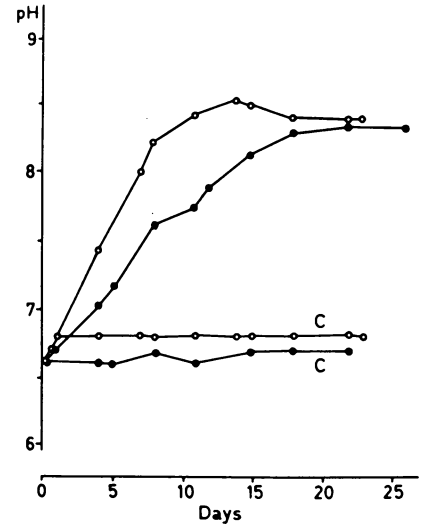

Figure 6. The pH at 4°C (●) and at 17°C (○) in inoculated fish extract B. (C = control samples).

observed in the control samples (Fig. 6). The rise in pH seemed to follow a straight line during the first 10 days. This was confirmed by the linear regression method (*Snedecor*). The incubation at 17°C caused a more rapid increase than did the incubation at 4°C. The highest pH was reached in cultures incubated at 17°C. The plot of the pH against the amount of volatile nitrogen compounds showed a poor correlation. A representative example (incubation at 4°C, the Conway method) is shown in Fig. 7. Consequently, the initial rise in pH was not caused by the volatile basic compounds.

2. Volatile bases produced by Pseudomonas fragi, strain F 111, in fish extract with atmospheric oxygen excluded

A study on the aerobic and anaerobic total number of microorganisms at 4°C in reinfected precooked food which was packed in plastic pouches, was made previously. The pouches selected for the investigation had all been rejected because of air in the pouches, which indicated the occurrence of faulty sealings or punctures of the plastic film. In that study the total number of

Table 3. The content of trimethylamine (TMA) in fish extract inoculated with Pseudomonas fragi, strain F 111, and incubated at 4°C or at 17°C.

Number of days	TMA as mg N/100 ml extract A 4°C Bethea-Hillig	extract A 4°C Conway	extract A 17°C Bethea-Hillig	extract A 17°C Conway	extract B 4°C Bethea-Hillig	extract B 4°C Conway	extract B 17°C Bethea-Hillig	extract B 17°C Conway
0	—	—	—	—	—	—	—	2.4
1	0.3	0.4	—	0.3	2.5	2.4	3.2	2.5
3	—	—	—	0.4	—	—	—	—
4	—	0.4	—	0.4	2.7	2.6	3.3	2.4
5	0.4	0.4	—	—	2.6	2.4	—	—
7	—	—	—	0.4	—	—	3.3	2.3
8	0.3	0.4	—	0.5	2.7	2.3	3.1	2.3
10	—	—	—	0.5	—	—	—	—
11	—	0.4	—	0.3	2.6	2.7	3.0	2.3
12	0.4	0.4	—	—	2.7	2.3	—	—
14	—	—	—	0.5	—	—	3.0	2.1
15	—	0.4	—	0.4	2.6	2.1	2.8	2.0
18	0.4	0.4	—	0.3	2.8	2.1	2.8	1.9
19	0.4	0.4	—	—	—	—	—	—
22	—	0.5	—	—	2.6	2.0	2.6	1.9
23	—	—	—	—	—	—	2.4	1.7
26	—	—	—	—	2.6	2.0	—	—

organisms exceeded 100 per g in 27 out of 129 samples. In 10 of the samples the infection was strictly aerobic and consisted mainly of strains of the genus Bacillus. In 15 samples, however, the total number of microorganisms growing in aerobic conditions was approximately the same as that of microorganisms growing in anaerobic conditions (Table 4). The dominating microorganisms in these samples were gram-negative rods belonging to the genus Pseudomonas.

Materials and methods

Fifty-ml samples of fish extract B were transferred to 12 Erlenmeyer flasks of 200-ml volume. Strain F 111 was grown on a milk-peptone-agar plate at 17°C for 48 hrs. The cells were harvested and washed twice by repeated centrifugation and suspension in physiological saline solution. From a final thin but visible suspension 0.5 ml was transferred to each of 10 flasks with 50 ml of fish extract. These inoculated flasks and 2 uninoculated flasks, which served as controls, were incubated at 17°C. After 24 hrs. of incubation

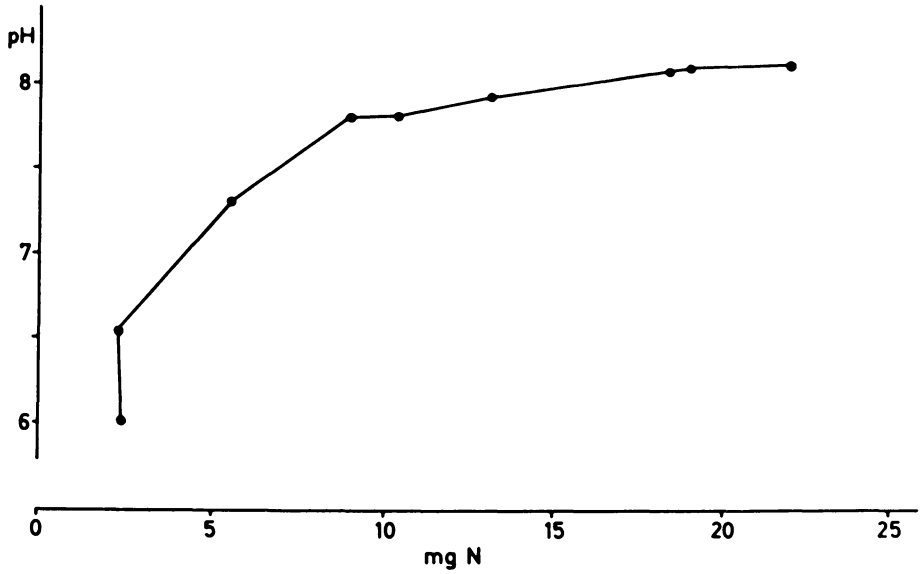

Figure 7. The pH against the total volatile nitrogen (TVN) in mg N per 100 ml of fish extract A incubated at 4°C. TVN determined by the Conway method.

the contents of 2 flasks were homogenized for 5 min. with the slow motion of a magnet rod. A first sample was withdrawn for a duplicate TVN test by the Conway method (2×1 ml) and for a determination of the total number of microorganisms per ml (1 ml). The remaining content of 1 of the 2 flasks was then sterile-filtered through a Seitz filter pad no. 6. The sterile-filtered extract and the corresponding flask with infected fish extract were both layered with 20 ml of newly heat-sterilized and rapidly chilled paraffin oil. The incubation of the layered or unlayered fish extract was then continued at 17°C, and duplicate samples to determine the TVN, TMA, and the number of microorganisms were withdrawn at intervals as indicated in Fig. 8. This procedure was repeated with duplicate flasks after 4, 7, 14, and 18 days of incubation. The TVN values listed in Figs. 8 and 9 represent the means of the duplicate tests.

Results and discussion

The 50-ml batches of extract B were inoculated with approx. 1.5×10^6 organisms per ml. The number of organisms increased to 1×10^8 in 24 hrs. and to 2.5×10^8 in 96 hrs. This was the range of the total number of microorganisms found in all later samples.

The plot curves of the number of microorganisms against the days of incubation showed that the total number of organisms de-

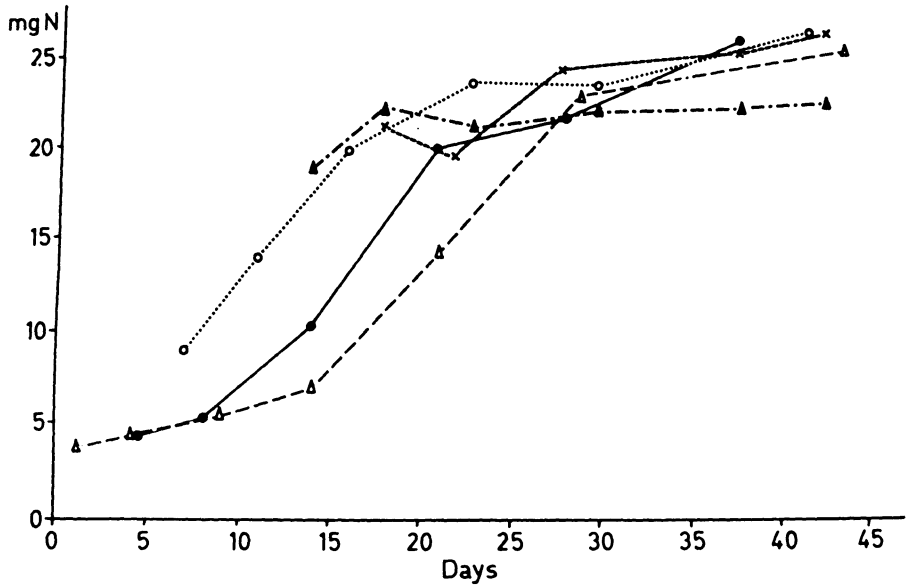

Figure 8. Total volatile nitrogen (TVN) in mg N per 100 ml of fish extract B incubated at 17°C and layered with paraffin oil after 24 hrs. (— — — —), 4 days (——), 7 days (· · · · ·), 14 days (— · — · — · —), and 18 days (- - - - - -). Incubation was continued at 17°C.

creased during the 2 days following the layering with paraffin oil. This reduction was calculated to be, on the average, 40 %. The level then reached was constant during the next few weeks.

The changes in the amount of volatile bases (TVN) in the inoculated fish extract were followed (Fig. 8). The plots representing the results of the first sampling from each flask corresponded to the results obtained after aerobic cultivation of the

Table 4. The aerobic and anaerobic total number of microorganisms at 4°C in samples of precooked food.

Anaerobic total number	Aerobic total number					
	$< 10^2$	10^2—10^3	10^3—10^4	10^4—10^5	10^5—10^6	$> 10^6$
$< 10^2$	102	2	3	4	—	1
10^2—10^3	—	—	—	1	—	—
10^3—10^4	1	—	3	—	—	2
10^4—10^5	—	—	2	2	—	—
10^5—10^6	—	—	—	—	—	—
$> 10^6$	—	—	—	—	—	6

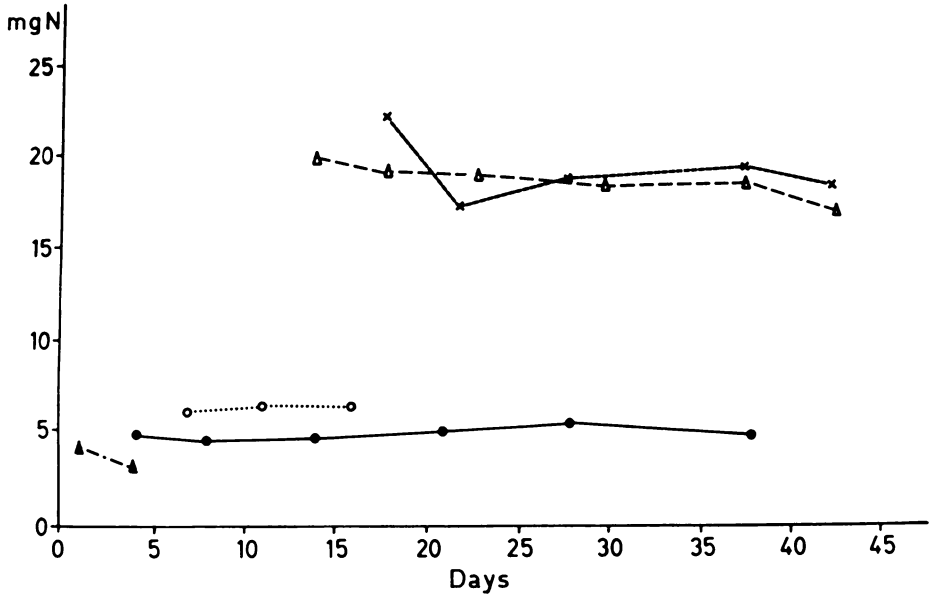

Figure 9. Total volatile nitrogen (TVN) in mg N per 100 ml of fish extract B layered with paraffin oil. The inoculated extract had been inbucated at 17°C and sterile-filtered after 24 hrs. (— . — . —), 4 days (——), 7 days (·····), 14 days (— — — —), and 18 days (------). The paraffin oil was layered and the incubation continued at 17°C.

inoculated fish extract. The curve line on these results resembled the curve line presented previously (Fig. 4). When the air was excluded by the paraffin oil 24 hrs. after the inoculation, however, the TVN changed very slowly up to the 14th day. A rapid increase followed between the 14th and the 29th day. At this time, a level of 23 to 25 mg N per 100 ml of fish extract was reached. After 24 hrs. of incubation the content of 1 of the 2 parallel flasks was sterile filtered before the paraffin oil was layered on the fish extract. In the content of this flask no increase of TVN was observed (Fig. 9).

When the paraffin oil was layered on the fish extract 7 days after the inoculation, the TVN of the first sample was 9 mg N per 100 ml. An increase in TVN had already taken place and continued in the layered extract linearly until the 16th day. Thereafter the increase diminished, and between the 23rd and the 42nd day the TVN was only 3 mg N.

When the inoculated fish extract was layered with paraffin

oil on the 14th or on the 18th day the TVN was already in the range of 20 mg N per 100 ml.

After 30 days of incubation, irrespective of when the contents of the different flasks were layered with paraffin oil, the TVN was approximately the same in all these samples of infected fish extract.

In the sterile-filtered content of the parallel flasks no increase of the TVN was observed during the period of observation (Fig. 9).

In this experiment the incubation at 17°C was chosen because of the results of preliminary experiments at both 4°C and 17°C. These had shown that when the extract sample was incubated at 17°C and was layered with paraffin oil 24 hrs. after the inoculation, a sudden increase in TVN occurred between the 15th and the 21st day of incubation. During the same period of 21 days no such change was observed in the inoculated fish extract which had been incubated at 4°C.

GENERAL DISCUSSION

In studies on the proteolytic enzymes of Pseudomonas fluorescent, *Peterson & Gunderson* (1960) showed that this microorganism produced extracellular proteolytic enzymes during the exponential growth phase. The liberation of enzymes at 5°C was undulating with the first peak close to the exponential growth phase and a second peak after 5 days of incubation at the same temperature. It was further shown that the microorganism produced more than 1 proteolytic enzyme. Expressions of such an enzymatic activity by Pseudomonas fluorescens — the casein precipitating test and the gelatin liquefaction — were also found in other microorganisms, e.g. Pseudomonas fragi (*Sandvik* 1962).

The pH of the inoculated fish extracts in the present investigations increased towards the neutral point within 24 hrs. of incubation. This showed that the growing strain F 111 in the fish extract caused reactions which were not observed in the sterile control samples. In view of the results of *Peterson & Gunderson*, these changes in the pH of the inoculated fish extract could be explained as the activity of extracellular or endocellular proteolytic enzymes which liberated basic compounds. As no increase in TVN occurred during this phase of growth, these compounds were not volatile nitrogen compounds. The results therefore indicated that the volatile bases were not produced until the

growth had reached the maximum stationary phase. It followed that the increase of TVN did not indicate the initial proteolytic reactions.

It was observed that the production of volatile bases at 4°C in extract B was slower than in extract A and that the increase of TVN in extract B was levelling off after no more than 2 weeks of incubation. Extract B had been prepared from halibut meat of poor but acceptable quality. The determination of nitrogen by the Kjeldahl method showed that the amount of extractable nitrogens was smaller than in halibut meat of good quality. The high levels of TVN and TMA of the control samples of fish extract B must have been caused by enzymatic actions of microorganisms on nitrogenous compounds of the raw fish muscle. These reactions in the raw fish probably reduced the amount of extractable compounds which could be decomposed by Pseudomonas fragi with liberation of volatile bases, e.g. ammonia. It is therefore obvious that the lower TVN maximum reached in extract B compared with that of extract A was due to a poorer quality of the raw fish meat.

The use of TVN in measuring the freshness of precooked fish could therefore be valuable, because an increase of TVN marks off the time at which contaminating proteolytic organisms reach the maximum stationary growth phase. The level of TVN in cooked fish is influenced by the quality of the raw material. To be of value as a test of freshness, the TVN should probably be constructed as a comparison between the TVN value of a sample before and after incubation. *Farber & Lerke* (1961) pointed out the possible value of such a procedure in using the volatile reducing substances or the trimethylamine for testing the freshness of raw fishery products.

It is of importance to know that if the prevailing conditions allow the start of the enzymatic reactions, which cause a liberation of ammonia or other volatile bases, these reactions will be continued even when the amount of available oxygen is reduced. The time for excluding the air influences the time at which the increase of TVN starts, but it does not prevent this increase. The delay of the liberation of volatile bases observed when the fish extract culture was layered with paraffin oil after 24 hrs. further strengthens the opinion that this enzyme reaction does not occur during the exponential growth phase. The final concentration of volatile bases was approximately the same in all flasks, regard-

less of when the layering of the inoculated fish extract was done. It had been shown by *Peterson & Gunderson* that Pseudomonas fluorescens produced extracellular enzymes during the exponential growth phase, which caused a decomposition of proteins. The present investigation shows that TVN was not increasing during this phase. It follows that the extracellular enzymes do not cause a complete decomposition of the nitrogenous compounds, as this should have included a liberation of ammonia and lower amines. Mechanisms other than the extracellular reactions discussed by *Peterson & Gunderson* must be involved. The last phases of the breakdown must depend on enzyme reactions within the cell bodies. The volatile nitrogen compounds, which are liberated in the growth matrix, must be end products of the normal metabolic reactions on proteins and other nitrogenous compounds.

The results of this investigation therefore indicate that the liberation of volatile nitrogen compounds in cooked fish is probably restricted to the area on which microorganisms are growing. Only to a limited extent would an increase of TVN in cooked fish be due to enzymes liberated from the cell bodies. The presence of volatile compounds in parts of a dish other than the surfaces available for active bacterial growth must be explained by diffusion of such volatile waste products. This emphasizes the importance of proper food handling.

The results of the present investigations indicate that further knowledge about the mechanism of the TVN reaction in cooked fish would be valuable. Further studies, e.g. on the liberation of volatile bases by the action of microorganisms on precursor substances, are therefore necessary.

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SAMMANFATTNING

Experimentella studier över flyktiga kväveföreningar, som frigjorts av Pseudomonas fragi i extrakt av fisk.

Nedbrytningen av kvävehaltiga komponenter i extrakt av kokt hälleflundra förorsakad av tillväxten vid 4°C och vid 17°C av Pseudomonas fragi följdes genom bestämningar av flyktigt kväve (TVN) och av halten trimethylamin (TMA). Dessa nedbrytningsprodukter bestämdes enligt en metod beskriven av *Bethea & Hillig* (1965) och med en Conway-mikrodiffusionsmetod som har beskrivits av *Farber & Ferro* (1956).

En ökning av halten TVN i det inokulerade fiskextraktet började efter 5 dagars inkubering vid 4°C respektive efter 3½ dagars inkubering vid 17°C. Denna ökning av TVN var långsammare vid 4°C än vid 17°C. Det visades att i ett extrakt av fiskkött av dålig kommersiell kvalitet var initialmängden TVN större, ökningen av TVN förorsakad av tillväxten av Pseudomonas fragi var långsammere, och det uppnådda maximala värdet för TVN var lägre än i ett extrakt av fisk av god kommersiell kvalitet.

Korrelationen mellan ökningen av TVN och av pH i det inokulerade fiskextraktet var dålig. Detta innebär att den initiala pH-stegringen icke förorsakades av bildningen av flyktiga basiska ämnen.

Genom att pipettera ett lager av paraffinolja över det inokulerade fiskextraktet efter en viss tids inkubation bröts kontakten med omgivande luft. Detta medförde en försening av ökningen av TVN men förhindrade icke denna. Det slutliga TVN-värdet var ungefär lika högt i det prov som hade täckts efter 24 timmars inkubering som i det prov

som täcktes först efter 14 dagars inkubering vid 17°C. Ingen ökning av TVN observerades i de infekterade prov av fiskextrakt, vilka sterilfiltrerades samma dag som paraffinolja skiktades över extraktet.

Det konfirmerades att Pseudomonas fragi icke förorsakade någon ökning av TMA i extrakt av kokt hälleflundra.

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Reprints may be requested from: Stig-Olov Florin, Board of Health and Sick Care, Stockholm County Council, Långholmsgatan 34—36, Fack Stockholm 9, Sweden.