

THE EFFECTIVENESS OF EDTA AS A FISH PRESERVATIVE¹

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SUMMARY

A concentration of 0.005% Na₄EDTA markedly inhibited the growth of the facultative psychrophiles *P. putrefaciens*, *P. fragi* and *P. fluorescens* in half strength nutrient broth at pH 7.0. *Achromobacter lipolyticum* was the only psychrophilic organism of 20 tested which was found to be insensitive to EDTA. The application of a 1.0% dip of Na₄EDTA to haddock fillets for 1 min failed to suppress the increase in bacterial numbers on fillets stored at 3 C when compared to untreated control fillets. Refrigerated storage at 3 C was, however, extended by the dip from 5 to 9-10 days based on odor and taste evaluations. The formation of trimethylamine and volatile basic nitrogen was markedly suppressed in EDTA dipped fillets.

In an effort to develop an agar growth medium for the maximum enumeration of the bacterial flora on fish (13) sodium citrate was added as one of several carbon and energy sources assessed. The presence of 0.2 to 0.5% sodium citrate at pH 7.0 in a complex nutrient agar medium was found to markedly reduce the number of developing colonies when compared to control media without sodium citrate. When fresh haddock fillets were dipped for one minute in 1.0% sodium citrate no preservative effect was noticed on storage at 3 C. The possibility that the application of a non-nutrient chelating agent such as EDTA might effectively suppress spoilage of refrigerated fillets was then investigated.

MATERIALS AND METHODS

Microorganisms. The culture of *Pseudomonas fluorescens* used was obtained from W. E. Sandine (Department of Microbiology, Oregon State University). This culture yielded true exponential growth curves at 23.5 C using OD measurements, but produced cell clumps when grown at 3 C preventing the measurement of low temperature growth from optical density determinations. *Achromobacter lipolyticum* and *Achromobacter butyricum* were obtained from the stock culture collection of this department. Other facultatively psychrophilic cultures used in this study were *Pseudomonas putrefaciens* ATCC 8071, *Pseudomonas fragi* ATCC 4973 and fifteen unidentified organisms isolated from fish and capable of growth at 3 C. These cultures were allocated to the genera *Achromobacter*, *Pseudomonas*, or *Micrococcus* according to the scheme of Shewan et al. (9).

Growth rate studies. All growth rate studies were performed using 48 ml of half strength nutrient broth in 250 ml Erlenmeyer flasks which were inoculated with 2 ml of actively growing cultures. For studies at 23.5 C flasks containing various concentrations of Na₄EDTA adjusted to pH 7.0 with 1N HCl were inoculated and incubated on an Eberbach variable speed reciprocating shaker model 75-698 set at 120 oscillations per min. Growth rates at 3 C were determined with a New Brunswick Metabolyte Water Bath Shaker model G77 set at a speed control reading of 3 and equipped with a New Brunswick refrigeration unit model XG77-R25.

Plate counts. Plate counts on fillets stored at 3 C were performed by blending 30 g fish for 2 min in 270 ml of broth consisting of 0.2% yeast extract (Difco), 0.2% trypton (Difco), 0.2% glucose, 0.25% NaCl; in distilled water at pH 7.0. Serial 1:10 dilutions were performed in broth and plated in duplicate using broth as above plus 1.5% agar.

Trimethylamine (TMA) assay. One-hundred gram samples of fish were blended with 200 ml of 7.5% trichloroacetic acid (TCA) for 2 min and the method of Dyer (2) used.

Volatile basic nitrogen. Two milliliters of the TCA extract used for the TMA assay were subjected to steam distillation after adding 1 ml of 50% NaOH to the sample chamber of the distillation vessel. The collection flask contained 5 ml of .0357 N H₂SO₄ and 200 ml of CO₂ free distilled water. Six-hundred milliliters of distillate were collected and titrated with .0357 N H₂SO₄ using 0.5% alcoholic methyl red as indicator (14). Results were reported as milligrams volatile basic nitrogen per 100 g fish tissue.

EDTA assay. A modification of the method of Darbey (1) was used as follows: 100 g fish were blended with 200 ml of 7.5% TCA for 2 min and filtered; 10 ml of extract were removed and made up to 50 ml with distilled water in a 250 ml Erlenmeyer flask; 15 ml of 1.33% (w/v) aqueous NiSO₄ was added and allowed to stand 10 min; 5 ml conc NH₄OH were added and allowed to stand 10 min; 15 ml of 1.5% (w/v) dimethylglyoxime were slowly added down the side of the flask, allowed to stand 10 min and filtered through a 0.45 micron millipore membrane filter beneath a 60 micron porosity glass fiber pre-filter disk; 60 ml were transferred to a 250 ml Erlenmeyer flask, 3.5 ml conc HCl added and allowed to stand 5 min; 0.010 g potassium dithiooxalate was then added and the optical density read against a distilled water blank after 1 min using one-half inch square glass cuvettes in a Bausch and Lomb Spectronic 20 colorimeter at 510 millimicrons. Milligrams of Na₄EDTA per 50 ml sample solution were read from a standard curve (Figure 1) and converted to milligrams Na₄EDTA per 100 g of fish.

Sensory evaluation. All fillets studied had the head and tail sections removed before treatment and storage to obtain center sections of uniform quality (7). A trained panel of six to ten judges was used throughout. Odor evaluation was performed with the fillet tissue upward and skin down using a 9 point scale similar to that used by Shewan et al. (10). Each panelist was asked to place a check beside the appropriate odor description for each sample fillet evaluated and to also

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TABLE 1. EFFECT OF 0.1% Na₄EDTA ON THE GROWTH RATES^a OF FACULTATIVELY PSYCHROPHILIC BACTERIA AT 3 C

Culture	Genus	Growth rate in absence of EDTA	Growth rate in presence of 0.1% Na ₄ EDTA
A57	<i>Achromobacter</i>	55.7	D ^b
3C	"	21.2	D
A18S	"	44.1	D
A13	"	11.0	24.8
A30	<i>Pseudomonas</i>	21.1	38.4
A37LG	"	25.2	41.0
A35	"	34.5	D
6	"	13.2	34.8 ^c
9A	"	14.9	25.1 ^c
A51	"	15.6	44.8 ^c
A47	"	14.2	39.2
A1	"	8.7	21.2
A20	<i>Micrococcus</i>	19.3	D
A45	"	16.4	D
A18LG	"	11.5	23.2
<i>P. fragi</i>		15.7	— ^d
<i>A. butyricum</i>		9.2	49.7
<i>P. putrefaciens</i>		18.8	71.8
<i>A. lipolyticum</i>		26.9	26.9

^aGrowth rates expressed as generation time in hours.

^bD signifies an immediate decrease in OD without growth.

^cIndicates slight increase in OD followed by cessation of growth at low population density followed by a decrease in OD.

^d—indicates no change in OD.

TABLE 2. MEAN ODOR SCORES ASSOCIATED WITH CATEGORIES OF ODOR ACCEPTANCE

Fillets	Acceptable		Questionable		Not acceptable	
	mean	SD ^a	mean	SD	mean	SD
EDTA treated	6.9	1.5	5.4	1.8	2.3	1.6
Untreated	6.7	1.9	4.6	1.5	2.3	1.4

^aOne standard deviation from the mean.

TABLE 3. MEAN FLAVOR SCORES ASSOCIATED WITH CATEGORIES OF FLAVOR ACCEPTANCE

Fillets	Acceptable		Questionable		Not acceptable	
	mean	SD ^a	mean	SD	mean	SD
EDTA	7.7	1.4	5.3	1.3	3.8	1.5
Untreated	8.1	1.4	4.6	0.5	2.8	1.7

^aOne standard deviation from the mean.

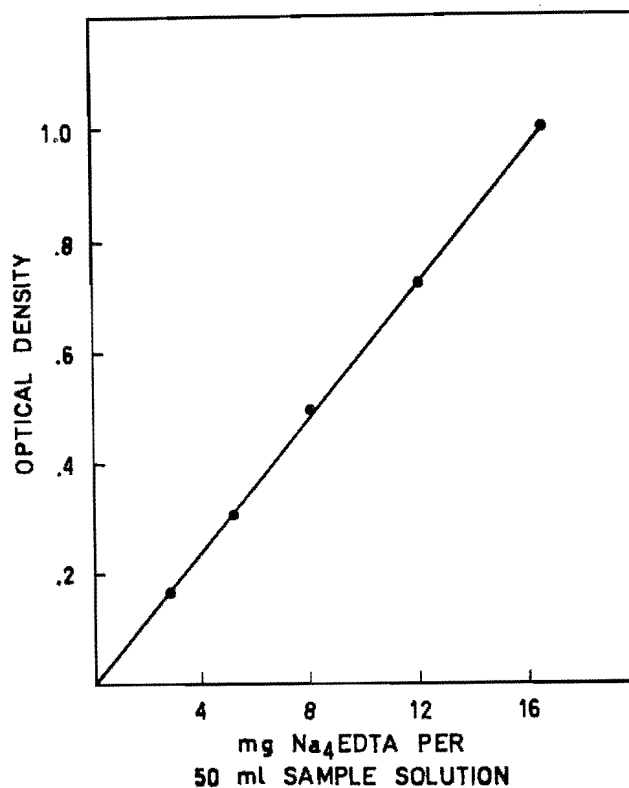


Figure 1. Standard curve for EDTA assay.

designate the degree of acceptance for each fillet (acceptable, questionable, or not acceptable).

Flavor evaluations were performed, using a ten point scale similar to that used by Shewan et al. (10) on fillets steamed for 25 min in a double boiler and served at 145 F. Flavor acceptability was also designated for each sample. The mean values for all odor and flavor scores respectively were calculated for the three categories of acceptance (acceptable, questionable, not acceptable) and the standard deviation from the mean for each degree of acceptability determined (Tables 2, 3).

RESULTS

Inhibition of bacterial growth by EDTA. The influence of EDTA on the growth of facultatively psychrophilic fish spoilage bacteria at 23.5 C is illustrated in Figure 2, 3, and 4. With all three pseudomonas cultures the growth rates were markedly suppressed by 0.005% Na₄EDTA. In contrast to the three pseudomonas cultures *Achromobacter lipolyticum* was notably insensitive to the presence of EDTA. The growth rate of this organism was completely unaffected by 0.1% Na₄EDTA and failed to be suppressed by as much as 0.5% EDTA until after 4.5 hr of growth. The effect of 0.1% Na₄EDTA on the growth rates at 3 C of 19 facultatively psychrophilic cultures showed *A. lipolyticum* to be insensitive (Table 1); six cultures experienced an immediate and continuing decrease in OD upon inoculation; three cultures showed only slight growth followed by a decrease in OD;

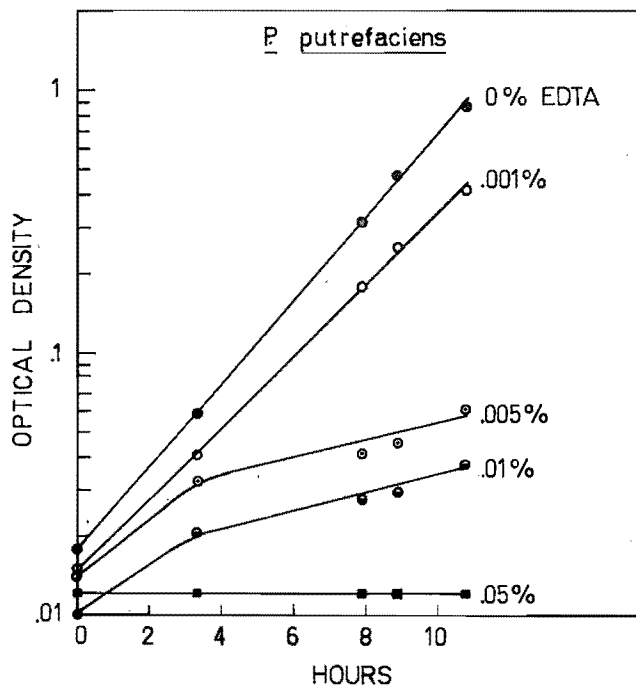


Figure 2. Growth of *Pseudomonas putrefaciens* at 23.5 C in half strength nutrient broth plus various concentrations of Na_4EDTA at pH 7.0.

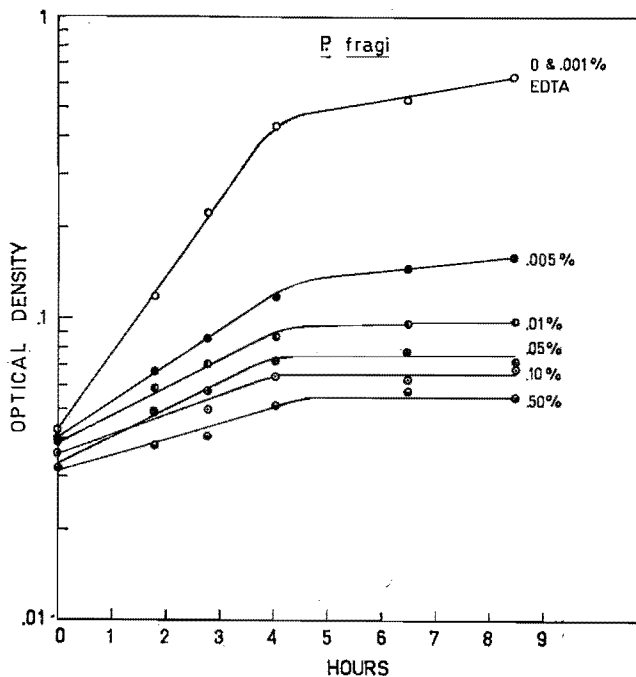


Figure 3. Growth of *Pseudomonas fragi*. See Figure 2 for experimental details.

the OD of one culture remained constant from the time of inoculation, and the growth rates of nine cultures were significantly retarded.

Preservative effect of Na_4EDTA on haddock fillets. Fillets of recognized high quality were dipped in 1%

Na_4EDTA for 1 min. wrapped and sealed individually in polyethylene bags and stored at 3 C. Untreated control fillets were dipped in distilled water and similarly wrapped and stored at 3 C. Fillets were periodically removed from cold storage at two day intervals unless otherwise noted and evaluated by the panel for odor and flavor, and subjected to chemical and bacteriological analysis. The mean value for "not acceptable" odor scores was 2.3 for both EDTA treated and untreated controls with a standard deviation of 1.6 for EDTA treated fillets and 1.4 for control fillets (Table 2). A score of 3.9 for EDTA treated and 3.7 for control fillets representing one standard deviation from the respective means was used to indicate rejection by the panel. The untreated control fillets (Figure 6) reached an odor rejection score of 3.7 after 6 days storage at 3 C while the EDTA treated fillets did not reach the odor rejection score of 3.9 until the tenth day of storage. The data in Figure 7 were obtained using a batch of fish of slightly higher quality as evidenced from the zero time odor scores. Untreated fillets were rejected after five days refrigerated storage while EDTA treated fillets were not rejected until the tenth day of storage.

The rate of decrease of flavor scores (Figure 8) for EDTA treated and untreated fillets differed considerably after the first three days of storage. A score of 5.3 (3.8 + 1.5) for EDTA treated fillets and 4.5 (2.8 + 1.7) for the untreated controls, representing one standard deviation from the respective means was used to indicate rejection by the panel (Table 3). The untreated fillets were rejected on the fifth day of storage at 3 C while the EDTA treated fillets were not rejected until the ninth day of storage.

Influence of EDTA on chemical and bacterial in-

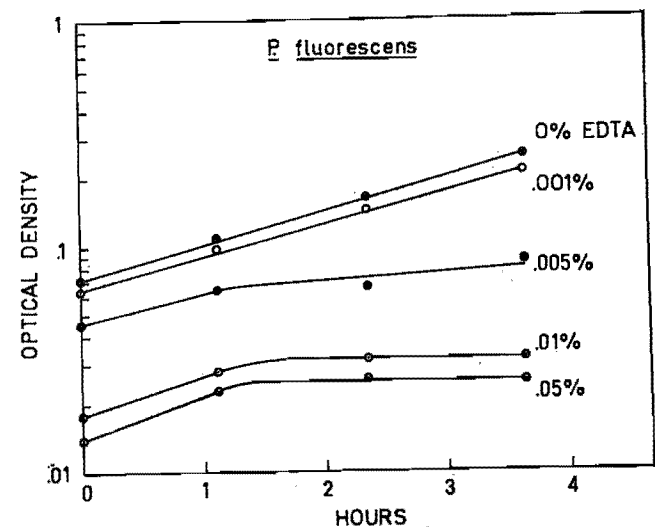


Figure 4. Growth of *Pseudomonas fluorescens*. See Figure 2 for experimental details.

dices of spoilage. Dipping fillets for 1 min in 1% Na_4EDTA completely prevented TMA production during the first seven days of storage at 3 C (Figure 9). After the seventh day a gradual increase in TMA occurred. The untreated fillets in contrast, rapidly reached a maximum TMA value on the sixth day.

The formation of volatile basic nitrogen in treated fillets was completely suppressed, during the first five days of refrigerated storage, followed thereafter by a gradual increase (Figure 10). The untreated fillets differed by rapidly increasing in volatile basic nitrogen, reaching a maximum value on the eighth day.

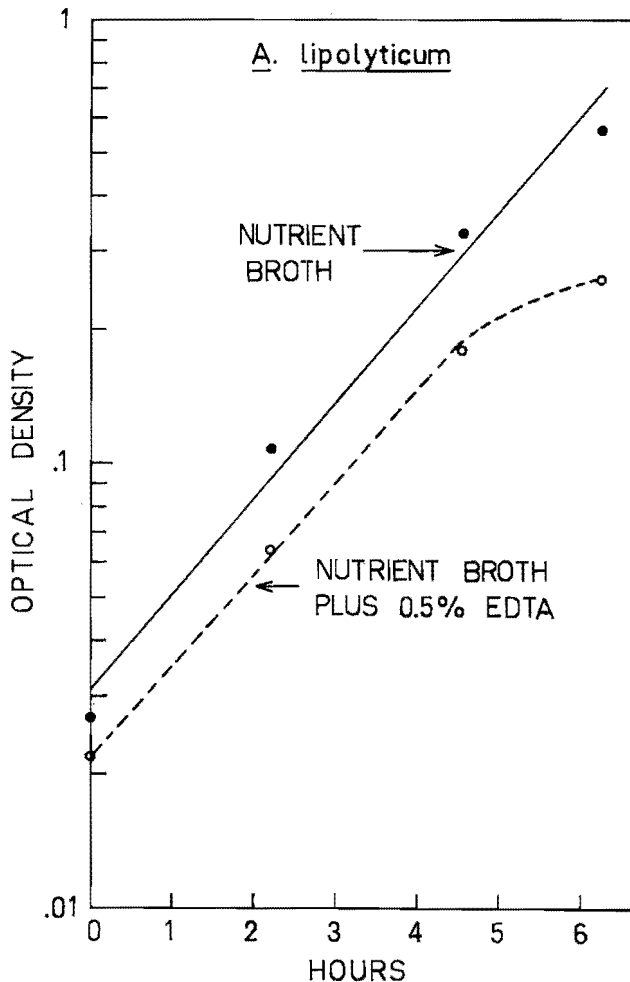


Figure 5. Growth of *Achromobacter lipolyticum*. See Figure 2 for experimental details.

From the observations on growth rate inhibition (Table 1 and Figure 2, 3, and 4) and the pronounced retardation of TMA and volatile basic nitrogen formation in EDTA treated fillets (Figure 9, 10) one is led to assume that an increase in the bacterial count on treated fillets should be notably retarded during refrigerated storage. The rate of increase of the total bacterial population on fillets was found, however,

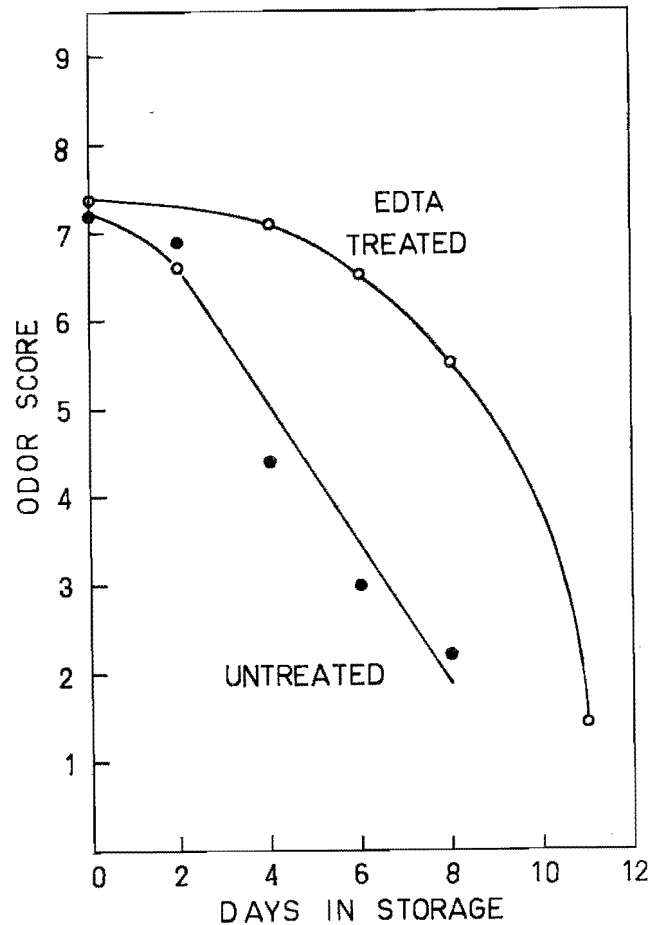


Figure 6. Decrease in odor scores of haddock fillets dipped in 1.0% Na_4EDTA for 1 min. then stored at 3 C and of untreated fillets dipped for 1 min. in distilled water and stored at 3 C.

to be unaffected by the presence of EDTA (Figure 11), with no obvious shift in predominating spoilage species on treated fillets. The initial residual EDTA on dipped fillets was 0.28% (Figure 12) and as succeeding days of storage followed this decreased to a relatively constant value of 0.18% due most accountably to initial drop off during the first four days of storage.

DISCUSSION

The destruction and inhibition of bacterial cells by chelating agents is well recognized (2, 8, 12). Gray and Wilkinson (5) observed that EDTA at an alkaline pH selectively solubilized a high proportion of the carbohydrate and phosphorous present in the cell walls of a number of gram negative bacteria resulting in a decrease in turbidity of cell wall suspensions. EDTA is known to be bactericidal for *Pseudomonas aeruginosa*, 0.0001M destroying over 99.99% of the cells in suspension (5) and 250 ppm destroying 99.999% (6). Repaske (8) treated cells with Dowex-50, a ca-

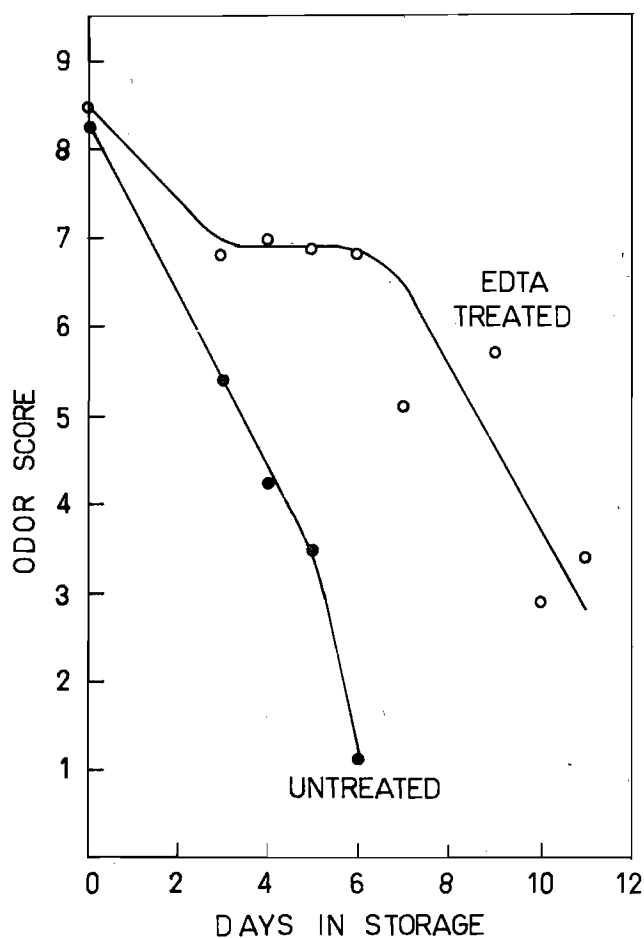


Figure 7. Decrease in odor scores of haddock fillets. See Figure 6 for experimental details.

tion exchange resin, which resulted in lysis of *Azotobacter vinelandii* and *Escherichia coli* B. and concluded that lysis by Na_4EDTA results from removal or chelation of a metal. Goldschmidt and Wyss (4) found that vegetative cells and cysts of *A. vinelandii* were insensitive to EDTA unless tris buffer was present. These authors also found that the presence of any one of 11 nitrogen compounds including NH_4Cl , urea, and cystine-HCl potentiated destruction of azotobacter cysts by EDTA. They attributed the toxicity of EDTA in the presence of such chemical agents to complexes formed between EDTA and nitrogen compounds. Such chelation complexes between EDTA and nitrogen compounds in fish tissue may possibly retard their enzymatic degradation and also prevent transport of such substrates into spoilage bacteria. Bacterial growth without rapid spoilage might then occur as a result of direct inhibition of exocellular bacterial spoilage enzymes by the binding of required cations or by the binding of specific tissue substrates of spoilage enzymes by EDTA. The percent of *P. putrefaciens* and *P. fluorescens* of the total spoilage flora on EDTA

dipped and non-dipped fillets was not notably different. The exocellular enzymes of *P. putrefaciens* have been found markedly sensitive to EDTA (in progress) accounting in part for the retarded spoilage of EDTA treated fillets even in the presence of large numbers of the organism. Although the major portion of the contaminating population on EDTA treated fillets grew unabated it is to be expected that at least a minor population of the flora was eliminated or retarded. The pronounced insensitivity of *A. lipolyticum* to EDTA in contrast to the rapid decrease in OD experienced by some cultures indicates a fundamental difference in cell wall composition and metabolism and lends credence to the possibility suggested by Shively and Hartsell (11) that sensitivity to EDTA may possibly be used as a taxonomic tool.

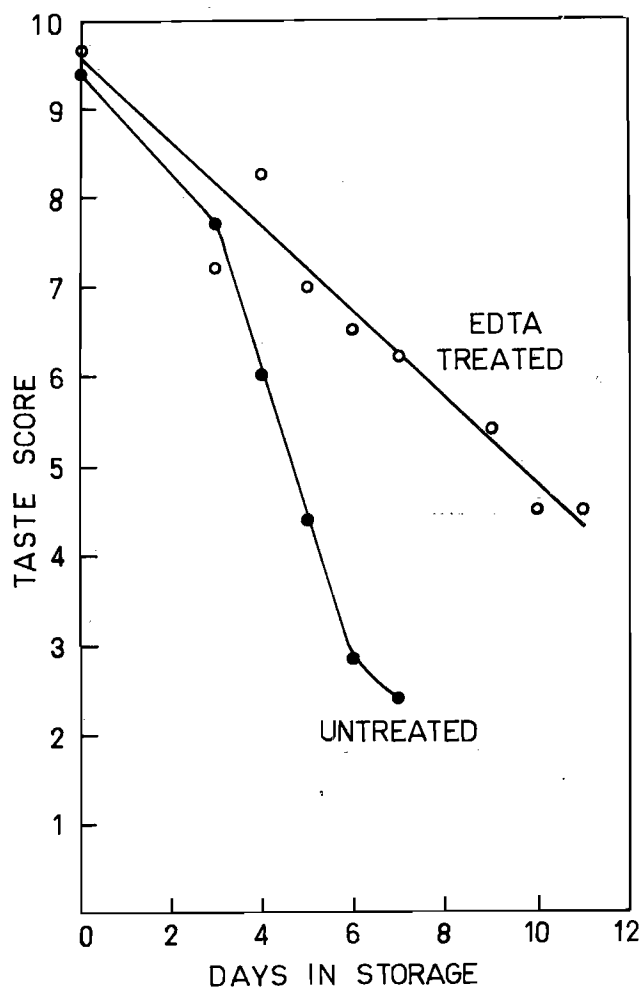


Figure 8. Decrease in taste scores of haddock fillets. See Figure 6 for experimental details.

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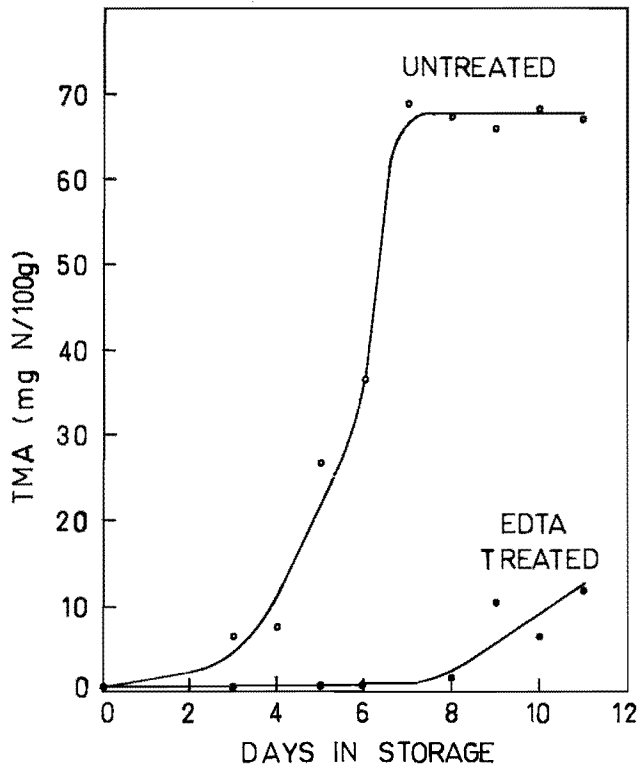


Figure 9. Increase in TMA in EDTA treated and untreated haddock fillets. See Figure 6 for experimental details.

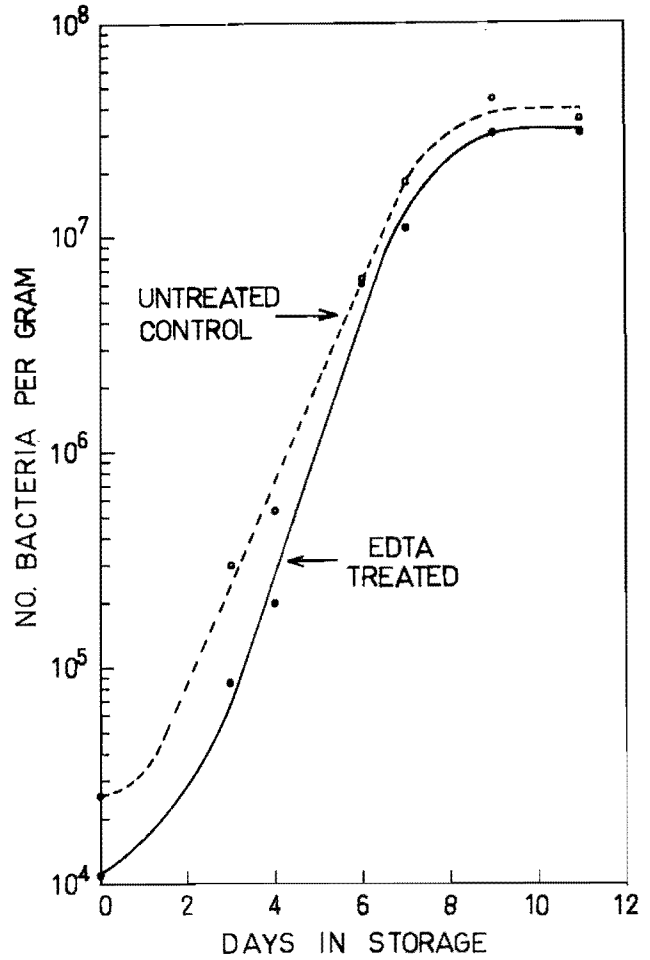


Figure 11. Increase in bacterial numbers on a single EDTA treated and a single untreated fillet. See Figure 6 for experimental details.

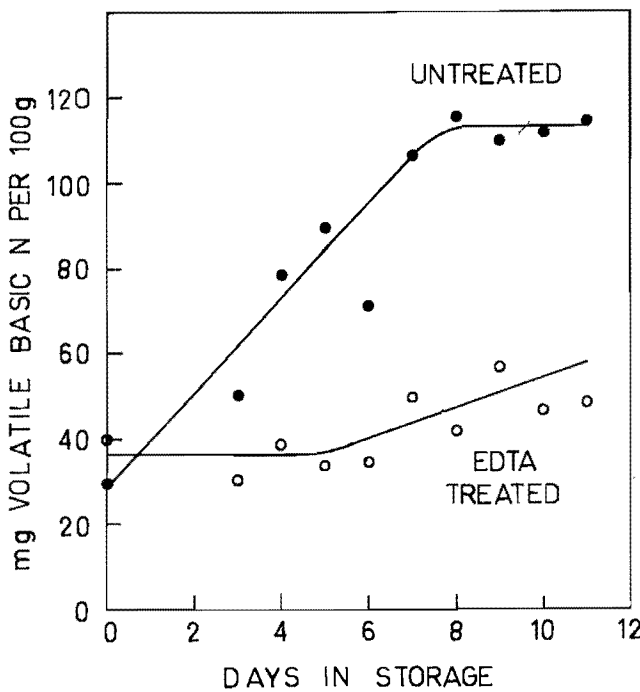


Figure 10. Increase in volatile basic nitrogen in EDTA treated and untreated haddock fillets.

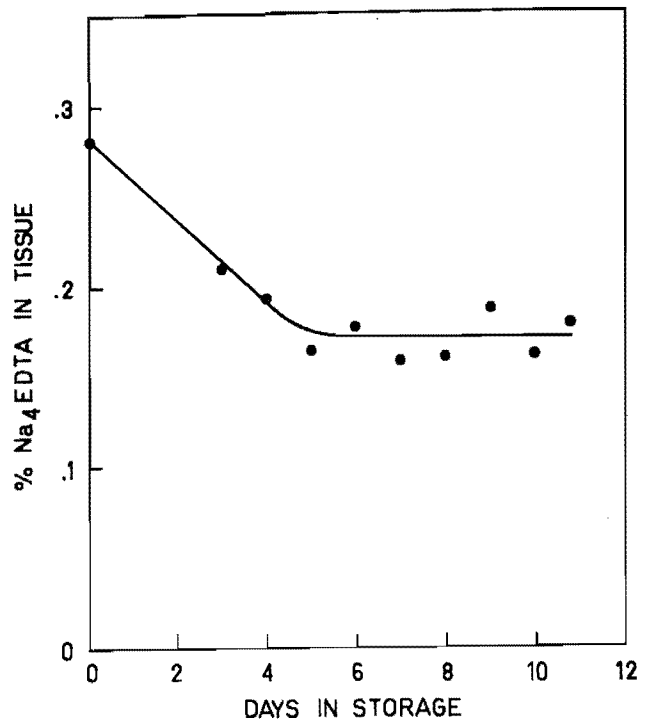


Figure 12. Residual Na₄EDTA remaining on dipped fillets during storage. See Figure 6 for experimental details.

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MILK ADULTERATION 75 YEARS AGO

The following item first appeared under the heading "Milk Adulteration" in the June, 1892, issue of the *Journal of the American Medical Association*. It was reproduced in the 75 Years Ago column in the June 26, 1967, issue.

At its last meeting the Chicago Medical Society discussed the subject of milk inspection. There is no official supervision of the Chicago milk supply, and in consequence much of the milk delivered to consumers is either watered, or skimmed or both. When Chicago is making strenuous efforts to improve its water supply, and the people are constantly urged to boil the lake water before using it, it seems strange, almost criminal, that so important a loophole as the milk supply is left open. It is not to be supposed that dealers who are sufficiently dishonest as to water milk will take the trouble to boil the water before they put it into the milk. At least none of them have as yet claimed this redeeming feature. It is well known that typhoid fever has been transmitted by means of water milk. Watered milk is therefore a direct menace to the health of the community, and the most careful family, in the absence of a thorough system of

milk inspection, is unable to guard itself fully from danger from this source.

A specimen of a milk-expanding compound was exhibited to the members of the Society. This substance, when added to watered milk, makes it resemble pure milk quite closely, in both taste and appearance. It therefore permits a greater dilution of the milk with water than would otherwise be possible without easy detection of the fraud. In fact, milk which has been diluted with four or five times its bulk of water, may be made to pass inspection by the addition of a small quantity of this substance. Last fall at the instance of the State Board of Health of Michigan, Prof. F. C. Novy, of the University of Michigan, analyzed this substance, that is presumably this same substance, as it was a mixture sold for the purpose of making milk, and found it to consist of a thick solution of cane and invert sugars, with a little salicylic acid, and salts, of which common salt made up the bulk. This substance is sold openly in Chicago as a milk preservative. "Milk expansion" is the most dangerous form of milk fraud that has yet been detected, and there is reason to believe that it is quite extensively employed in the city of Chicago, and probably also in other large cities of the country.