NITRILOTRIACETIC ACID IN SEAWATER: DETERMINATION AND BIODEGRADABILITY

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Determination and Biodegradability

by

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Abstract of the Thesis

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Nitrilotriacetic acid (NTA), $N(CH_2CO_2H)_3$, has been the subject of considerable research and public interest over the past few years. The need to predict the environmental impact of NTA became necessary when this chemical was prepared as a replacement for polyphosphates in household detergents and this instigated the bulk of recent research on this compound. A summary of this research, given in the Introduction Section of this thesis, shows that little work had been done in studying the effect NTA might have on the marine environment.

Our contribution to this area of research includes the development of a gas chromatographic method of analysis capable of determining NTA below the ppm level in seawater. This method should facilitate further studies of NTA in seawater. The analytical procedure developed was then put to use in a study of the biodegradability of NTA in seawater which is also described in this thesis.

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Abbreviations Used Throughout This Thesis

NTA	nitrilotriacetic acid
Na_3NTA	trisodium salt of nitrilotriacetic acid
$(propyl)_3NTA$	tripropyl ester of nitrilotriacetic acid
HDA	heptadecanoic acid
propyl HDA	propyl heptadecanoate
EDTA	ethylenediaminetetra-acetic acid
IDA	imidodiacetic acid
DTPA	diethylenetriaminepenta-acetic acid
CDTA	cyclohexandiamine-(1,2)-tetra-acetic acid
ppm	parts per million
ppb	parts per billion
LD50	dosage of material that results in the deaths of one half the organisms tested
g/kg	dosage in grams per kilogram of body weight
G.C.	gas chromatography, gas chromatograph
O.D.	outside diameter
N.M.R.	nuclear magnetic resonance
TMS	tetramethyl silane
S.D.	standard deviation

I N T R O D U C T I O N

Nitrilotriacetic acid (NTA), $N(CH_2CO_2H)_3$, has been the subject of considerable research and public interest over the past few years. Before this time the chief use of NTA was as a titrant in chelametric titrations[4] where it ran a poor second in popularity to the well known ethylenediamine-tetra-acetic acid (EDTA). The need to predict the environmental impact of NTA, if it were to replace phosphates in household detergents, instigated the bulk of recent research on this compound. The proposal to use NTA as a detergent builder grew out of two factors. (1) Algae blooms resulting in eutrophication of lakes were blamed on high phosphate levels - as much as 70%[14,16] of this phosphate was said to originate as detergent phosphate. Acting on this information the Canadian government limited[13] detergent phosphate levels to 20% as of August 1, 1970 and 5% as of January 1, 1972. (2) The development of commercially feasible methods of producing NTA from the relatively inexpensive starting materials, ammonia, formaldehyde and hydrogen cyanide, combined with successful testing[15] of NTA in actual detergent formulations, made NTA the choice of the large detergent manufacturers should they be forced to remove or reduce the phosphates in their products.

In this section a summary will be made of environmentally related research on NTA under three headings: (1) General Environmental Studies - including biodegradability and toxicity; (2) NTA - Seawater Studies; and (3) Analytical Methods for Determination of NTA.

Our own contribution to this area of research represents the beginning of a study of the impact of NTA on a cold seawater environment. It includes development of a G.C. method for determination of NTA below the parts per million level in seawater and a study of the biodegradability of this compound in "polluted" and "non-polluted" seawater.

I. General Environmental Studies

Before 1970 NTA was finding significant use in detergents in Sweden and also limited use in the United States. At the end of that year, however, manufacturers in the U.S.A. discontinued the use of NTA in response to a statement that, in the presence of cadmium and methylmercury, NTA had caused a significant increase in embryo abnormalities in experimental animals[17]. Since that time use of NTA in detergents has also been discontinued in Sweden. In Canada, where the 5% phosphate limit is in effect, there is no legal impediment to NTA use[18]. The current Environmental Protection Agency (USA) policy[19] is to attack the phosphate-eutrophication problem by phosphate elimination through waste treatment rather than replacement

of phosphate by a compound of still questionable safety.

The two chief causes of concern in the safety evaluation of NTA are direct toxicity of NTA (actually Na_3NTA) and the ability of NTA, as very strong chelating agent, to transport or mobilize toxic heavy metals.

The Proctor and Gamble Company has carried out many toxicity evaluation studies on NTA using experimental laboratory animals. Over a 90 day test period using rats, dogs, rabbits and monkeys, Nixon[20] reports LD50 values (1.10 - 2.33 g/kg) showing NTA to be only moderately toxic when ingested. In sub-acute oral toxicity tests he reported kidney lesions of varying severity only at very high (7500 ppm or greater) NTA levels and no other significant abnormalities. These 90 day tests were followed by a two year rat-feeding study with similar results[21], along with the additional observation that increased levels of zinc were deposited in the bones of test animals. Apparently this had no adverse effect on the animals. Nolen, et. al.[22], conducted a reproduction and teratology study involving oral ingestion of high levels (0.1 or 0.5% of diet) of NTA over two generations of rats. They reported that NTA was neither embryotoxic nor teratogenic in the rat or in similar tests conducted with rabbits. Another study for the Proctor and Gamble Company was carried out by Michael and Wakim[23] who studied the metabolism of NTA in rats and also in a rabbit, dog and monkey.

Their results showed that NTA was absorbed readily by the gastrointestinal tract of the rats and dogs but poorly from the tract of the rabbit and monkey. These workers reported that no more than three percent of the NTA administered remained in the body after 72 hours, most appearing in the bone as the calcium chelate. Since this represented only 0.007% of the amount of calcium turned over by the body in 24 hours, they believed it has no significant effect on bone development.

Similar toxicological data have been collected by other workers and summarized by reviews on the subject[17,24,25]; however, the interpretation of these data is not always consistant. Ashforth and Calvin[24] stated that the anticipated level of intake by humans would be no more than 1/20,000 of the lowest level that had produced adverse effects in rats, while Epstein[25] gave an estimated adult safety factor of 115.

It appears that less work has been done in evaluating the effect of NTA on aquatic organisms which may be exposed to NTA entering their environment from domestic sewage outflows. The published results of Pollard[15], who stated that concentrations of 100 mg/l NTA were safe to fathead minnows, were criticized by Thom[17] for containing a lack of experimental parameters. Thom had similar criticism for the work of Jancovic and Mann[26], who reported lethal concentrations

from 260 - 340 mg/l NTA for trout, eels and guppies, and for that of Gudernatsch[27], who reported 500 mg/l Na_3NTA had no effect on guppies over an 80 hour test period. Ninety-six hour tolerance tests by Flannagan[28] on 17 species of aquatic invertebrates and 2 species of amphibians were carried out with parameters other than concentration of Na_3NTA being monitored. He concluded that NTA was not directly toxic, at least to 500 mg/l, and that the deaths of test animals resulted in nearly all cases from an increase in pH caused by the hydrolysis of Na_3NTA. Animals could tolerate much higher concentrations of Na_3NTA in highly buffered hard waters than in soft waters and mortality always occured at pH 9.7 regardless of the level of Na_3NTA.

Epstein[25,16] expressed concern that NTA might prove dangerous, if not as the free acid or trisodium salt, then as a result of its degradation products or in the form of heavy metal chelates. The possibility of increased nitrate levels in water due to NTA biodegradation as well as the chance of imidodiacetic acid (IDA) production, which, in the presence of nitrite could produce a carcenogenic nitrosamine [29], were his chief causes of concern here. Reports by other workers indicated that such problems are unlikely to occur in the environment for, while Trott, Henwood and Langford[30] report small yields of IDA in photodecomposition of iron(III) complexes of NTA in sunlight, biodegradability studies, to be

discussed later, show that IDA and other possible degradation products of NTA degrade faster than NTA itself. Thus, no accumulation of NTA breakdown products is likely. Many biodegradability studies also show that most NTA nitrogen is converted to ammonia rather than nitrate, although the presence of ammonia oxidizing bacteria may result in the conversion of this ammonia to nitrate or nitrite.

While little work has been done on toxicity evaluation of NTA complexes of heavy metals[25], there are good indications that the presence of NTA will not increase the toxicity of heavy metals by the formation of such complexes. Indeed, some studies show that NTA causes a marked decrease in toxicity of copper and zinc[31] ions to fish, and of lead[32], mercury[33], and cadmium[33] ions when ingested by experimental animals. Sprague[31] has pointed out that NTA could be used as an anti-pollutant to protect fish in the event of toxic metal spills in rivers. NTA may, however, by forming soluble chelates, mobilize heavy metals which may have otherwise remained in harmless, insoluble compounds in sediments, thus increasing heavy metal concentrations of natural waters. There is little information in this area. Taylor, et. al.[34], reported little increase in metals extracted from sediments into water when NTA was present at 20 ppm in the water. Gregor[35] stated that lead in lake sediments, resulting from automobile exhausts, can be rapidly solublized if NTA is present at 20 or even 2 ppm.

A brief summary of toxicity data on NTA is given in tabular form on the following page.

Another potential problem that might arise indirectly from large-scale use of NTA is that of algal blooms. In some water nitrogen rather than phosphorus is the element which limits algal growth. Since ammonia is produced in the degradation of NTA, algal blooms in these waters might well be stimulated by the replacement of phosphate by NTA in detergents. This would, indeed, be a paradoxical state of affairs since NTA was originally proposed as a phosphate replacement to prevent eutrophication caused by algal blooms!

Any dangers caused by accumulation of NTA in the environment would not occur if the compound was rapidly and completely degraded to harmless products. A considerable amount of research has been done on the biodegradability of NTA in sewage, both in laboratory tests and in actual sewage treatment plants, and in natural freshwater systems. Pfeil and Lee[36] carried out biodegradability tests in raw sewage and activated sludge. They reported that, after an acclimatization period of about one week, rapid degradation of NTA occured at room temperature with NTA loads above 100 mg/l. Reacclimatization of the organisms was necessary if they were left in the absence of NTA for more than two days. In the review by Thom[17] the results of several other workers using activated sludge are summarized. They all showed similar acclimatization and degradation periods when incubation

Summary of Toxicity Data on NTA

Ref.	Type of test	Organisms tested	Results
20	acute oral toxicity	rats, dogs, rabbits, monkeys	LD50 values 1.10-2.33g/kg
20	sub-acute oral toxicity 90 days and 2 years	rats	kidney lesions at doses over 7500 ppm; increased zinc level in the bones
20	skin and eye irritation	rabbits, humans	NTA products similar to other current market detergents
22	reproduction and teratology	rats, rabbits	NTA not embryotoxic or teratogenic
23	metabolism	rats, dogs, rabbits, monkeys	some retention of NTA in bones of rats as calcium chelate
25	freshwater environment	fathead minnows	100mg/l NTA "safe" to minnows
26	freshwater environment	trout, eels, guppies	lethal concentrations 260-340 mg/l
27	freshwater environment (80 hour test)	guppies	500mg/l Na_3NTA had no effect
28	freshwater environment (acute toxicity)	17 species of aquatic invertebrates; 2 species of amphibians	not directly toxic at least at levels below 500mg/l; deaths of organisms appear pH dependant

(continued)

Summary of Toxicity Data on NTA (cont.)

Ref.	Type of test	Organisms tested	Results
45	seawater environment	estuarine phytoplankton	low toxicity
42	seawater environment (acute toxicity)	11 species of marine fishes and invertebrates	LD50 values 5.5-10g/l
31	freshwater environment	brook trout	presence of NTA lowers toxicity of copper(II) and zinc(II) ions
32	sub-acute oral toxicity of lead acetate	rats	presence of NTA lowers toxicity of lead(II) ion
33	teratology of methylmercury hydroxide	rats	presence of NTA lowers maternal toxicity and has no effect on the foetal toxicity or mercurial teratogenicity

temperatures were near 25°C. Warren and Malec[38] reported degradation of NTA, when spiked in levels up to 20 ppm, in Detroit and Meramec river water - incubation temperatures were not given. Chau and Shiomi[37] showed that NTA was degraded in Lake Erie water (Hamilton Harbour) in actual "in situ" studies conducted in polyethylene tubes suspended in the lake by a stationary raft. The temperature of the lake

water at the time of this study was not reported.

Several studies[25,37,16,39,41] have been done on the biodegradability of NTA present as heavy metal chelates. It is the general opinion of workers conducting these tests that the heavy metal chelates are degradable and that the stability of some (the Cu, Ni, and Hg complexes) in higher concentrations is due to the biostatic effect of the metals.

However, NTA is not biodegraded in all environments. For example the degradation of NTA under anaerobic conditions has not been reported and studies have revealed much retarded degradation as the temperature is decreased. Eden, et. al.[40], recorded a drop from 98% NTA removal, at 20°C to only 3% at 5°C, in a sewage treatment plant. Rudd and Hamilton[14] added NTA (14 ppm) to the inflow to a model sewage lagoon and reported degradation of 94, 47 and 22% at temperatures of 15, 5 and 0.5°C respectively.

Most workers made no effort to isolate and identify the bacteria responsible for NTA degradation. Focht and Joseph[41], however, isolated a Pseudomonas sp. from sewage effluent by elective culture with NTA as the sole carbon and nitrogen source for growth. This isolate has been deposited in the American Type Culture Collection and designated as Pseudomonas sp. ATCC 27109. A bacterial mutant was isolated from sewage by Wong, Lui, and Dutka[11]. They used the method of selective culture in an NTA medium after U.V. mutagenization and penicillin selection. Their mutant was able to degrade NTA much more rapidly than any natural bacterial flora reported (73%

of the NTA in a 1% solution was degraded in 4 days at 20°C) and it was able to grow in solutions containing up to 2.5% NTA. However, like the natural bacteria studied, growth of the mutant was "drastically retarded" at 4°C. We were fortunate to receive samples of this bacterial mutant for our seawater studies.

II. NTA and Seawater

Since our research has involved NTA in seawater, previous work directly concerning seawater has been kept separate from the other environmental studies despite the small amount of available information.

Several studies like those of Johnston[43], have shown that in many areas marine phytoplankton growth is limited by the availability of trace metals. Addition of EDTA to such seawaters has resulted in increased plankton growth, showing that the trace metals were actually present in the water, most likely as colloidal hydroxides, and were made available to the organisms in the soluble EDTA chelated form. It is likely that NTA would cause similar results under the same conditions. Ryther and Dunstan[44] reported that in coastal surface waters off Long Island nitrogen, not phosphorus, is the limiting nutrient for algal growth. Jenkins, et. al.[19], listed several authors who have recorded this condition in both the Atlantic and the Pacific Oceans. Thus, in seawater, NTA, either by solublizing trace metals or by adding nitrogen (as NTA or its

degradation products - NH_3, NO_3^-, etc.) seems much more likely to cause algal blooms than are phosphates.

A paper by Erickson, et. al.[45], reported that NTA is of low toxicity to estuarine phtyoplankton and in fact lowered the toxicity of copper ion to these organisms. The only report of biodegradability of NTA in seawater is contained in this same paper. NTA utilizing bacteria were isolated from a seawater sample containing surface water and sediments, collected at Narrow River, Rhode Island. These bacteria, mostly gram negative bacilli, were able to degrade NTA in synthetic, enriched and natural seawater. No NTA analysis of these samples was carried out but degradation was assumed since growth occured in all mediums and NTA was present in the synthetic seawater as the only carbon and nitrogen source. Incubation temperatures were not reported, nor was the salinity of the estuarine sample from which the original bacteria was isolated.

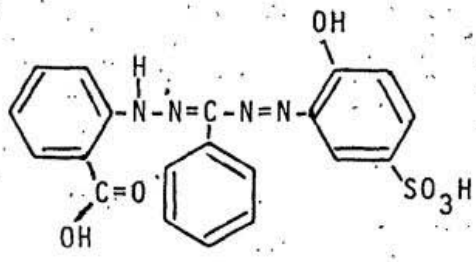
Apart from the above information on estuarine phytoplankton, the only other toxicity data available on NTA in the marine environment have been compiled by Eisler, et. al.[42]. They conducted 168 hour acute toxicity tests on eleven species of fishes and invertebrates. LD50 values in the range of 5.5 - 10 grams NTA/l. were reported. These tests showed a lower toxicity of NTA in the higher salinity water, which would be more strongly buffered, than samples of lower salinity. Flannagan[28] noted a similar situation in freshwater - that

NTA was less toxic in more highly buffered hard water than in soft water - and went on to show that deaths of his test organisms were dependant on pH rather than NTA levels. In view of the high concentrations of NTA used by Eisler, et. al.[42] and these results of Flannagan, it is unfortunate that pH levels of the NTA-seawater solutions are not reported for the marine toxicity tests. As in freshwater studies, inhibition of biocidal properties of cadmium and mercury salts was noted. Two NTA containing detergents were shown to be ca. 1000 times as toxic as NTA itself but since the surfactant (a linear alkylsulfonate) contained in these detergents had a reported LD50 value ca. 2000 times that of NTA, these reports were not surprising.

III. Analytical Methods for Determination of NTA

The most widely used method for NTA determination in water samples has been a colorimetric method described by Thompson and Duthie[46] and also by Tabatabai, et. al.[47], who used the method for determining NTA in soils. It is based on the ability of NTA to replace the Zincon (2-carboxy-2'-hydroxy-5'-sulfoformazylbenzene) ligand from the blue zinc-Zincon complex producing the colorless zinc-NTA complex. The resulting loss in absorbance at 620nm. is directly proportional to the NTA concentration. Aside from the fact that this method is only sensitive to NTA concentrations above 0.5ppm, it has other disadvantages which make its results on environmental samples questionable. The

presence of natural or synthetic chelators other than NTA,



Zincon

as well as heavy metal ions, will interfere. The procedure calls for the use of cation exchange resin (Na form of Dowex 50) to remove heavy metals before the zinc-Zincon reagent is added to the water sample. Chau and Shiomi[37] reported, however, that "the zinc-Zincon method failed in the presence of moderate concentrations of metallic ions and such interferences could not be eliminated even by increasing the amount of cation exchange resin used".

Afgan and Goulden[48] developed polarographic methods involving the reduction of either the lead-NTA or bismuth--NTA complexes. They reported that their methods are capable of detecting as little as 10ppb NTA without any pre--concentration of the sample. Various heavy metals and aminopolycarboxylic acids related to NTA, as well as detergent tripolyphosphates, are reported to interfere. The authors describe masking agents and other sample pre-treatments which, along with the option of using either the lead or bismuth complex, allow for elimination of these interferences. A further paper[49] describes an automated method capable of polarographic NTA determination of 15 samples

per hour. The latter paper contains the only report of successful determination of NTA in seawater. Another polarographic method is recorded by Asplund and Wänninen[50]. They were able to measure NTA in lake water in concentrations of 1 to 10 ppm by reduction of the cadmium-NTA complex.

NTA was determined by Longbotton[51] by high speed anion exchange chromatography using a liquid chromatograph equipped with a U.V. detector. All metal ion interferences were reported to be eliminated by the addition of iron(III) ion to the samples, resulting in the conversion of all NTA to the analyzable iron(III) chelate. No other pretreatment of the samples was necessary and a sensitivity of 1 ppm NTA was claimed.

Rechnitz and Kenny[52] describe a complexametric titration of NTA, with copper(II) ion, utilizing a solid membrane copper ion sensitive electrode. This method is useful in determining NTA in detergent formulations when no other chelating agent is present, but lacks both the sensitivity and specificity necessary for environmental samples.

The oxidation of Malachite Green by periodate ion is catalyzed by low concentrations of manganese(II) ions. In the presence of NTA, the catalytic effect is modified resulting in an increase in the rate of the oxidation reaction. Mottola and Heath[53] noted that the stimulating effect of NTA was directly proportional to the NTA concentration and utilized this system to determine ppm, and fractions of ppm,

levels of NTA. A flow-photometer system was used to collect the kinetic data. Little information was given regarding metal ion interferences although iron(III) ion present in concentrations less than that of NTA did not interfere. Chelating agents other than NTA did interfere.

Another kinetic method for determination of NTA, using a stopped-flow spectrophotometer, was described by Coombs, et. al.[54]. They reported detection and determination of NTA and other aminopolycarboxylic acids, alone or in mixtures, at levels as low as 10 ppb. The procedure is based on the large difference in rate of production of tetracyanonickelate ion from nickel(II) complexes of these chelating agents. Metal ion interferences are reported but these can be eliminated by hydroxide precipitation (not in the case of environmental samples) or anion exchange. The mixed cyano complex, $NiEDTA(CN)^{3-}$, interferes with NTA analysis as it absorbs at the same wavelength as the $NiNTA^-$ complex.

A paper chromatographic method for separation of NTA and its iron(III) chelate from other aminopolycarboxylic acids and their iron(III) chelates was reported by Hill - Cottingham[55]. No information was given regarding development of this method for quantitative analysis.

Several workers have developed gas chromatographic methods for NTA determination. All involve the preparation of a volatile derivative, the triester of a small molecular weight alcohol, and most compare the G.C. peak area of the

NTA derivative to that of an internal standard. The methods vary in the alcohol used for esterification, the internal standard used, the G.C. column and parameters utilized and in the method of extracting NTA from the original samples.

Murray and Povoledo[6] used a methanol-HCl reagent for esterification and methyl heptadecanoate as the internal standard. NTA was not separated from the original water sample: the entire residue resulting from freeze-drying a 10 ml water sample was subjected to the esterification step. The G.C. column was packed with 2% ethylene glycol adipate on Chromosorb W.

Rudling has published two papers on methods of NTA determination by G.C. The first[2] involved separation of NTA from the water sample by anion exchange (chloride form of Dowex 1 - X 8 resin) followed by esterification using a solution of boron trifluoride in 2-chloroethanol and G.C. determination utilizing a column packed with 2% QF-1 on Varaport 30 - no internal standard was used. The later method[7] had no anion exchange step as the sample is simply dried and esterified by boron trifluoride in methanol, along with CDTA as internal standard. Using a G.C. column packed with 5% OV-17 on Aeropak, the resulting methyl ester of NTA was determined along with the methyl esters of EDTA and DTPA.

The method of Aue, et. al.[9], begins with the separation of NTA by anion exchange resin (Bio-Rad AGI-X2 changed to formate form) followed by the formation of the tributyl ester in n-butanol - HCl reagent. G.C. involves the use of a column packed with 0.3% Carbowax 20M on Celite 545. This

method involves no internal standard and allows for simultaneous determination of citrate.

No anion exchange step was used in the method of Warren and Malec[8]. The samples were freeze-dried and esterified using n-butanol - HCl reagent. Glutamic acid was most often used as the internal standard and G.C. determination was carried out on a column packed with 0.65% ethylene glycol adipate on acid washed Chromosorb W. This method allows for determination of several other aminopolycarboxylic acids simultaneously with NTA.

The method of Chau and Fox[1] uses n-propanol - HCl reagent to form the tripropylester of NTA following NTA extraction from the water sample using anion exchange resin (formate form of Dowex 1 - X 8). The internal standard is heptadecanoic acid and G.C. determination utilizes a column packed with 3% OV - 1 on Chromosorb WHP. After publication of this method these workers eliminated the anion exchange step as they discovered it was unnecessary[56].

The G.C. methods for NTA determination have been summarized in a table on the following page.

Detection limits as low as 20 ppb NTA in the original water sample have been reported for the above G.C. methods. These methods have the advantage of being free from metal interferences. According to the work done by Warren and Malec[8] many related aminopolycarboxylic acids do not interfere, at least not with their particular method. Alner, et. al.[57], recorded that acid-catalyzed esterifications of pure

Summary of G.C. Methods

Ref.	Sample Pretreatment	Esterification Reagent	Internal Standard	G.C. Column Packing	Simultaneous Determination of
6	Freeze-drying	methanol-HCl	methyl heptadecanoate	2% ethylene glycol adipate on Chromosorb W	———
2	anion-exchange (Dowex 1-X8 resin in chloride form)	boron trifluoride in 2-chloroethanol	none	2% QF-1 on Varaport 30	———
7	extraction with chloroform, evaporation	boron trifluoride in methanol	CDTA	5% OV-17 on Aeropak	EDTA DTPA
9	anion-exchange (Bio-Rad AGI-X2 resin in formate form)	n-butanol-HCl	none	0.3% Carbowax 20M on Celite 545	citrate
8	freeze-drying	n-butanol-HCl	glutamic acid	0.65% ethylent glycol adipate on Chromosorb W	glycine, sarcosine, IDA, N-methyl-IDA, N-nitroso-IDA, N-oxalyl-IDA
1	anion exchange (Dowex-1-X8 resin in formate form)	n-propanol-HCl	HDA	3% OV-1 on Chromosorb WHP	———

EDTA ion resulted in the formation of small quantities of the corresponding NTA esters, however, as this would result in less than 1% NTA ester relative to EDTA ester, it is unlikely to present difficulty in NTA analysis of environmental samples. The chief disadvantage of the G.C. methods of NTA determination is the time required for evaporation, esterification and extraction procedures.

None of the authors reports the use of his method to determine NTA in seawater.

As previously mentioned, most workers attempting to predict the environmental impact of NTA have stated the need for further testing before its use in detergents becomes wide spread. If more work is necessary in studying the effect of NTA in fresh water systems, this is even more true of the marine environment where much less has been done to date. It seemed that a sensitive method of NTA determination in seawater would facilitate further NTA-seawater studies and for this reason we proposed to develope such a method.

One of the first questions when considering the impact of a potential pollutant is will it degrade or accumulate in the environment? If a compound is shown to rapidly degrade the need for toxicity studies is greatly decreased, thus, following development of the method for NTA analysis in seawater, we carried out tests to determine if NTA was likely to degrade if it entered a cold seawater environment.

EXPERIMENTAL

I. Determination of Nitrilotriacetic Acid (NTA) in Seawater

A. Instrumentation

An Aerograph 1520 dual column gas chromatograph with a flame ionization detector was used, in the single column mode, throughout this study. The chromatograms were recorded on a Honeywell Electronik 15 recorder equipped with a Disc Chart Recorded - Model 201-B. All chromatograms were run using an 8 2/3 ft. stainless steel column (O.D. 1/8 in) packed with 3% OV-1 on Chromosorb WHP (80/100 mesh) unless otherwise specified. Some preliminary work was done using a 10 ft. glass column (1/8 in O.D.) with the same packing - it is noted in such cases that the glass column was used. The following chromatographic parameters were used throughout: carrier gas (helium) - 30 ml/min, hydrogen flow - 25 ml/min, air flow - 500 ml/min, oven temperature - 200°C, injector temperature - 225°C, detector temperature - 225°C.

N.M.R. spectra were run on a Varian A-60.

Reagents

Technical grade nitrilotriacetic acid (NTA), reagent grade trisodium NTA salt (Na_3NTA), and Baker grade heptadecanoic acid (HDA) were all received from Baker Company and used without further purification.

The n-propanol used in almost all esterification reactions was reagent grade obtained from MacArthur Company. This propanol was used without further purification for NTA analysis. The reagent grade propanol received from British Drug House could not be used for NTA analysis without purification as it contained ca. 1.25 ppm NTA as well as other impurities which gave large peaks on the chromatogram used for NTA determination. While simple distillation removed the NTA from this propanol, other impurities remained which resulted in a more complex chromatogram than was desirable.

Reagent grade dichloromethane obtained from Matheson, Coleman and Bell had to be distilled before use as impurities in this reagent appeared on the chromatograms used for NTA analysis.

Hydrogen chloride gas cylinders were obtained from Matheson Company.

Dowex 1 - X 8 (50 - 100 mesh) anion-exchange resin in the chloride form was received from Bio Rad Company.

The precoated packing for gas chromatography columns, 3% OV-1 on Chromosorb WHP (80/100 mesh), was obtained from Chromatographic Specialties, Brockville, Ontario.

B. Preparation and Some Properties of the Tripropyl Ester of NTA and the Propyl Ester of HDA

1. Tripropyl nitrilotriacetate [$(propyl)_3$NTA]

To a 250 ml round-bottomed flask 125 ml of n-propanol was added. Hydrogen chloride gas, dried by passage through

H_2SO_4, was bubbled through the n-propanol until the liquid was saturated. One gram of NTA, most of which did not dissolve in the n-propanol, was then added to the flask. The mixture was refluxed with stirring for three hours. At the end of this time there remained considerable solid - unreacted, undissolved NTA - so the mixture was saturated again with HCl gas and allowed to reflux with stirring for 24 hours. The n-propanol was then removed from the reaction mixture by means of a rotary evaporator, leaving a very viscous, transparent liquid and also some white solid, presumably unreacted NTA.

A micro-distilling apparatus, consisting of a small cup (capacity ca. 1 ml) attached to the bottom of a cold finger, was fitted to the 250 ml reaction flask containing the liquid and solid residues. The flask was placed in an oil bath and attached to a vacuum pump. At room temperature, when the pressure in the flask had been reduced to 6 mm of Hg, some material (assumed to be residual propanol) was vaporized. When this material was pumped from the mixture, water was allowed to flow through the cold finger and the pressure was reduced to 0.5 mm of Hg. The temperature in the oil bath was raised to 140°C before the clear liquid was vaporized. Approximately 0.75 ml of this liquid was collected in the cup at the bottom of the cold finger. It remained a viscous, transparent liquid at room temperature.

Forty mg of the distilled liquid was dissolved in 1 ml of benzene. A 1 ul sample of the resulting solution was

injected on the glass column of the G.C. (conditions given in Sec. A). The benzene was eluted at 0.5 minutes and the other peak noted at 4.5 minutes was assigned to our clear liquid - presumably the tripropyl ester of NTA. When the stainless column was used this compound had a retention time of 5.3 minutes.

A 60 MHz N.M.R. spectrum was run on the distilled transparent liquid dissolved in $CDCl_3$ using a tetramethyl silane (TMS) as internal standard. A summary of the N.M.R. data follows:

Absorption (τ)	Integration
(a) triplet - 9.0	9
(b) sextet - 8.3	6
(c) singlet - 6.2	4
(d) triplet - 5.9	8
(e) singlet - -0.6	1

Since the above data indicated an impurity containing a -COOH group, the product was washed with sodium bicarbonate solution and extracted into chloroform. After the removal of the $CHCl_3$ by rotary evaporator another N.M.R. spectrum was run, under the same conditions as the previous spectrum, with the following results: (spectrum shown as Fig. #9)

Absorption (τ)	Integration
(a) triplet - 9.0	9
(b) sextet - 8.3	6
(c) singlet - 6.3	6
(d) triplet - 5.9	6
(e) no acid proton	-
(f) singlet - 2.6	-

2. Propyl heptadecanoate (propyl HDA)

Except for the replacement of the 1 g of NTA by 1 g of HDA, the esterification of HDA was carried out in the same manner as for NTA. It was noted that HDA was more soluble in the n-propanol-HCl reagent than was NTA. However, after 3 hours of refluxing some solid, presumably undissolved, unreacted HDA, remained. After the full reflux time of 27 hours, the n-propanol was stripped off using a rotary evaporator. The residue was a yellow, viscous liquid with small amounts of white and yellow - brown solid.

The residue was distilled in the same manner as the residue of the NTA esterification reaction and again 0.75 ml of a transparent, viscous liquid was collected at 140°C and 0.5 mm of Hg. This compound, presumably the propylester of HDA, has a melting point around room temperature changing from a transparent liquid to a white solid and back again with fluctuations in room temperature.

Thirty mg of the distilled liquid was dissolved in 1 ml of benzene. A 1 ul sample of the resulting solution was injected on the glass column of the G.C. as was done with the NTA ester. Large peaks observed at 0.5 minutes and 9.5 minutes were assigned to benzene and the HDA propyl ester respectively. The retention time of propyl HDA was later found to be 11.7 minutes on the stainless steel column.

A 60 MHz N.M.R. spectrum was run on the distilled product in $CDCl_3$ with TMS as the internal standard. The

results follow: (spectrum is shown as Fig. #10)

Absorption (τ)	Integration
(a) distorted triplet - 8.9	6
(b) complex multiplet - ca. 8.5	28
(c) triplet - 7.7	2
(d) triplet - 5.9	2
(e) no acid proton	-

C. Determination of NTA Below the ppm Level in Demineralized Water Solution

1. Esterification and G.C.

Determination of NTA in small quantities was attempted using the esterification method of Chau and Fox[1].

One ml demineralized water containing 20 ug of NTA (as Na_3NTA) was placed in each of six 10 ml round-bottomed ampoules. The water was removed by placing the ampoules in a sand bath (90 - 100°C) and flushing with a stream of air. A sample of n-propanol was saturated with HCl gas which had been dried by passing through conc. H_2SO_4. To each of the six ampoules 2 ml of this propanol-HCl reagent was added along with 100 ul of n-propanol containing 20 ug HDA. The ampoules were sealed by flame and placed in a water bath (100°C) for 1 hour. During the reaction time the necks of the ampoules acted as reflux condensors. The ampoules were then opened and the propanol-HCl removed by placing the ampoules in the sand bath and flushing with air. An oily, yellow residue remained in the ampoules.

The residue in each ampoule was dissolved in 50 ul of benzene and 1 ul of each resulting solution was injected on the glass column of the G.C. The resulting chromatograms showed a solvent (benzene) peak at 0.5 minutes, the $(propyl)_3$ NTA peak at 4.5 minutes, the propyl HDA peak at 9.5 minutes but also large impurity peaks at 2.5 minutes and 8 minutes and small impurity peaks at 2 minutes and 7 minutes.

Another six samples were run following the same procedure except that the reflux time was increased to 2 hours. There was no noticable difference in the chromatograms resulting from samples refluxed for 2 hours compared with those refluxed for only 1 hour. The ratio of the NTA ester peak area to the HDA peak area for each of the 12 samples was measured by both disc integrator and calculation of peak height x peak width at ½ height. There was little difference in the two methods of measuring relative peak areas. The resulting values of NTA/HDA were rather inconsistent with the most consistent 7 of the 12 samples showing a S.D. of ca. 10%.

Attempts were made to determine the source of the impurities noted in the chromatograms with the hope that their elimination would improve the consistency of the NTA/HDA values. Chromatograms were run on the following: (1) 1 ul of benzene (chromatographic grade); 1 ul of a 50 ul benzene solution of the residues of 2 ml of - (2) distilled propanol,

(3) reagent grade propanol, (4) distilled propanol after refluxing in a sealed ampoule for 1 hour, (5) reagent grade propanol after refluxing in a sealed ampoule for 1 hour, (6) distilled propanol after saturation with HCl, (7) reagent grade propanol after saturation with HCl, (8) distilled propanol after saturation with HCl and refluxing in a sealed ampoule for 1 hour, (9) reagent grade propanol after saturation with HCl and refluxing in a sealed ampoule for 1 hour.

Chromatograms of samples (1) through (7) showed the solvent (benzene) peak at 0.5 minutes and no detectable impurities with retention times under 12 minutes. Samples (8) and (9) showed the same impurities noted in the chromatograms of the NTA and HDA esterification mixtures mentioned above. These had retention times of 2, 2.5, 7, and 8 minutes.

The true source of these impurities was later discovered (see Sec. E). Since there was no difference in G.C. detectable impurities when distilled propanol was used in place of reagent grade propanol further esterifications were done using undistilled propanol (see Sec. A - Reagents).

2. Separation of NTA from water samples by anion-exchange

Six anion-exchange columns[1] were prepared by placing 75 mm of Dowex-1 - X 8 resin (80/100 mesh) in the chloride form above glass wool plugs in each of six 50 ml burets. The top of the resin in each column was covered with ca. 0.75

in of silica sand to keep the resin in place. The resin was converted to the formate form by eluting with 100 ml of 2M sodium formate followed by 50 ml of 25M formic acid. The columns were then washed with demineralized water until the washings had a pH between 5 and 6.

The pH of 200 ml samples of demineralized water, each containing 20 ug NTA (as Na_3NTA) was adjusted to 3 - 4 with HNO_3. One of these samples was passed through each of the six anion-exchange columns at the rate of about 2 ml per minute. The columns were then washed with 250 ml demineralized water and eluted with 100 ml of 3M formic acid. The eluate was collected in 250 ml beakers and concentrated to 1 - 2 ml on a steam bath with the aid of a stream of air. The remaining 1 - 2 ml of solution was transfered to 10 ml ampoules and evaporated to dryness in a sand bath (90 - 100°C) with the aid of a stream of air. The resulting residue was esterified according to the procedure previously described (Sec. C 1.). Resulting chromatograms showed inconsistent quantities of NTA which resulted in NTA/HDA values not suitable for quantitative analysis.

The above anion-exchange and esterification procedures were repeated except that the 200 ml demineralized water samples were each spiked with 1.00 mg of NTA (as Na_3NTA) rather than 20 ug and 1.00 mg of HDA rather than 20 ug was added in the esterification step. Small NTA peaks relative

to HDA peaks on the resulting chromatograms indicated poor recovery of NTA in the anion exchange step.

A similar procedure was attempted using the Dowex 1 - X 8 resin in its original chloride form[2]. The six columns were packed in the same manner as described above then washed with 50 ml of 1.2M HCl followed by 75 ml of demineralized water. (The washings then gave a negative chloride test with silver nitrate.) The pH of 100 ml samples of demineralized water containing 100 ug of NTA (as Na_3NTA) was adjusted to between 4.5 - 5.0 by addition of a few drops of NaAc/HAc buffer (equal amounts of 1M sodium acetate and 1M acetic acid). One of these samples was run through each of the anion-exchange columns at ca. 3 ml per minute. The columns were then each washed with three 20 ml portions of demineralized water and eluted with 30 ml of 1.2M HCl. The eluate residue was esterified in the manner previously described using 100 ug HDA as the internal standard. The NTA/HDA values obtained from G.C. analysis were lower than expected and very inconsistent.

It was thought that some NTA may have remained on the anion-exchange column so the entire procedure was repeated except that 75 ml rather than 30 ml of eluate were collected. This resulted in an improvement in NTA/HDA consistency over the formate data, however, there still remained a factor of 2 between the lowest and highest NTA/HDA value in this set of five values (one sample of the original six had been

destroyed).

Six anion-exchange columns were packed with Dowex 1 - X 8 resin as previously described. The resin was eluted with 500 ml of 4M sodium carbonate and then 300 ml of 3M ammonium carbonate to change it to the carbonate form. The columns were washed with 200 ml of demineralized water and a 100 ml sample of deionized water containing 100 ug of NTA (as Na_3NTA) was passed through each column at ca. 1 ml per minute. After washing again with demineralized water the columns were eluted with 100 ml 3M ammonium carbonate. The eluate was concentrated, dried and esterified as previously described, using 100 ug HDA as the internal standard for esterification and G.C.

If 100 ug of both NTA and HDA were used we would expect NTA/HDA values of close to unity from chromatograms of the esterified mixture when using a flame ionization G.C. detector. The chromatograms of the six samples gave an NTA/HDA average value of 0.94 with an S.D. of 0.04.

To check NTA recovery by the anion-exchange columns 100 ml samples of demineralized water containing 100 ppm NTA (as Na_3NTA) were simply dried and esterified in the same manner as the 100 ml samples eluted from the columns. Chromatograms of the resulting esterified mixtures gave an NTA/HDA average value of 0.89 with an S.D. of 0.04. The carbonate anion-exchange columns thus gave acceptable recovery of NTA from the demineralized water samples and

much better consistency than either the chloride or formate forms.

D. Attempts to Separate NTA from Seawater Using Anion-exchange

Seawater samples, collected from the running seawater system of the Marine Sciences Laboratory at Logy Bay, were filtered using 0.45 mu Millipore membrane filters and then spiked with 1.00 ppm NTA (as Na_3NTA). Using the anion-exchange techniques, followed by esterification and G.C. analysis, determination of NTA in 100 ml portions of the seawater solutions (100 ug of NTA) was attempted. The resulting chromatograms showed poor recovery of NTA when anion-exchange resin was used in each of the three (chloride, carbonate and formate) forms. As with the demineralized water samples the carbonate column gave the most consistent NTA recovery although this was only between 5 and 10 percent.

E. Development of a Workable Method for Determination of NTA Below the ppm Level in Seawater

Filtered 100 ml seawater samples, previously spiked with 1.00 ppm (100 ug) NTA (as Na_3NTA), were placed in each of two 250 ml round-bottomed flasks. The water was removed

by rotary evaporator and the samples then placed in an oven (ca. 110°C) overnight to dry the residue completely. A 200 ml portion of n-propanol was saturated with HCl by bubbling the gas through conc. H_2SO_4 then into the propanol. The propanol - HCl reagent was then divided between the two flasks containing the seawater residues. By use of a 100 ul syringe, 100 ul of n-propanol containing 100 ug of HDA was then added to each flask. The flasks were then fitted with reflux condensors and the mixtures refluxed for two hours. The n-propanol-HCl was removed by rotary evaporator and 50 ml of distilled water followed by 50 ml of dichloromethane were added to each flask. After shaking to dissolve the residue the liquids were transfered to separatory funnels, and the 50 ml of CH_2Cl_2 was run off into 250 ml beakers. The aqueous layer of each sample was then further extracted with 2 - 25 ml portions of CH_2Cl_2 which were combined with the original 50 ml of CH_2Cl_2. The CH_2Cl_2 was evaporated to 2 - 3 ml by placing the beakers on a steam bath and flushing them with a stream of air. The remaining liquid was transfered to 10 ml ampoules and evaporated to dryness with a stream of air. The resulting residue, a yellowish oil, was dissolved in 50 ul of benzene and 1 ul injected on the G.C. Chromatograms indicated that good recovery of NTA had been effected, however, a great many impurities were present resulting in a complex chromatogram which would not allow

accurate calculation of the NTA peak area (see Fig. #1).

A search for the source of impurities was again carried out by running G.C.'s of benzene solutions of solvent residues as previously described in Sec. C. This time, however, the residues of 100 ml of the solvent rather than 2 ml samples were run and samples which were refluxed were heated for 2 hours while open to the atmosphere rather than 1 hour in sealed ampoules. Chromatograms showed the n-propanol and refluxed n-propanol contained no significant amount of impurities to interfere with NTA analysis. The residue of n-propanol saturated with HCl, whether refluxed for 2 hours or simply let stand at room temperature for 2 hours gave many peaks on the chromatograms (Fig. #2).

The procedure described in this section adds the residue of 100 ml of CH_2Cl_2 to the solution injected in on the G.C. (CH_2Cl_2 was not used in the procedure described in Sec. C). This residue was also subject to G.C. analysis. Several impurities appeared on the resulting chromatogram but it was found that they could be kept to a non-interfering level by distilling the CH_2Cl_2 before use.

In an attempt to remove the impurities present in the n-propanol-HCl reagent, the HCl drying train, consisting of two H_2SO_4 traps separated by an empty trap and joined to one another by a piece of tygon tubing, was by-passed. The

HCl passed only through an empty trap attached to the HCl cylinder by Plaster of Paris. Solvent residues were again checked for impurities with the HCl added in this manner. The resulting chromatograms showed:

#1 - n-propanol after 2 hours refluxing - a shoulder on the solvent (benzene) peak plus a small peak at 2.5 minutes.

#2 - n-propanol-HCl - peaks of #1 plus a slight bend in the baseline at 5.2 and 5.6 minutes.

#3 - n-propanol-HCl after 2 hours refluxing - same peaks as #2 except that the 5.6 minute peak has increased to a significant, although still small, size (Fig. #3)

It was now evident that most of the impurities had been originating in the HCl drying train most likely from the tygon tubing. With this drying train by-passed, 100 ml samples of seawater (no NTA added) were carried through the entire drying, esterification and G.C. procedure. Resulting chromatograms were similar to #3 above, with no interfering impurities added from the seawater (Fig. #4). Unfortunately no way of eliminating the small peaks at 5.2 and 5.6 minutes was found. The result is that our calibration curves (Sec. F) do not pass through the origin.

During the G.C. analysis of samples containing the

same quantity of $(propyl)_3NTA$ it was noted that the $(propyl)_3$ NTA peaks consistently increased over the first 3 samples injected each day. This problem was eliminated by making up a benzene solution containing ca. 2 ug/ul of the $(propyl)_3$ NTA prepared and distilled as described in Sec. A and injecting two I ul samples of this solution on the column each day before samples for analysis were run. This procedure also served as a daily check on $(propyl)_3NTA$ retention time.

Three samples of seawater containing 100 ug NTA (as Na_3NTA) were analyzed for NTA by the method described above. Three identical samples were analyzed by the same procedure cutting the reflux time from 2 hours to 1 hour. No significant difference was noted in the NTA/HDA values of the two sets of data. It was thus shown that 1 hour was sufficient reflux time to quantitatively convert both NTA and HDA to their propyl esters and thus a 1 hour reflux time was used for all further analysis.

F. Calibration Curves

A calibration curve of NTA/HDA (ratio of area of chromatogram peak for $(propyl)_3NTA$ to the area for propyl HDA, as per disc integrator) versus ppm. NTA added to seawater samples, was made in the following manner. Seawater samples of 100 ml containing 0.10, 0.20, 0.50, 0.70 and 1.00 ppm

NTA (as Na_3NTA) were analyzed as described in Sec. E. Two samples at each NTA level were run and two chromatograms run for each sample. The reflux time was 1 hour and the HDA added was 100 ug.

Sample	ug NTA in sample*	NTA/HDA
#1 - 1.00 ppm		0.81
- 2nd injection	100	0.81
#2 - 1.00 ppm		0.81
- 2nd injection	100	0.85
#1 - 0.70 ppm		0.60
- 2nd injection	70	0.56
#2 - 0.70 ppm		0.58
- 2nd injection	70	0.56
#1 - 0.50 ppm		0.41
- 2nd injection	50	0.41
#2 - 0.50 ppm		sample lost
- 2nd injection	50	broken ampoule
#1 - 0.20 ppm		0.17
- 2nd injection	20	0.17
#2 - 0.20 ppm		0.16
- 2nd injection	20	0.17
#1 - 0.10 ppm		0.11
- 2nd injection	10	0.11
#2 - 0.10 ppm		0.10
- 2nd injection	10	0.11

* Note that the number of micrograms injected on the G.C. column is ca. 1/50 that contained in the sample.

A chromatogram of a sample containing 1.00 ppm NTA is shown as Fig. #5. The linear calibration curve of NTA/HDA versus ppm NTA (Curve #1) derived from the above data is

shown as Fig. #6. A linear least squares analysis of the above data gives a slope of 0.80 ppm^{-1} with S.D. 0.0001 ppm^{-1} and an intercept of 0.02 with an S.D. of 0.01.

In the biodegradability studies described in experimental section II solutions spiked with 100 ppm NTA were used. A second calibration curve was constructed, to cover the range of 0 - 100 ppm, in the same manner as Curve #1. (As only 1 ml samples were analyzed this curve actually covers the same range as Curve #1 in micrograms of NTA.). One ml samples of seawater spiked with 0, 17, 33, 50, 67 and 100 ppm NTA (as Na_3NTA) were analyzed. Again a 1 hour reflux time was used and 100 ug of HDA added. The 1 ml samples were measured using sterile, disposable plastic pipets.

Sample	ug NTA	NTA/HDA
#1 - 100 ppm	100	0.95
- 2nd injection		0.93
#2 - 100 ppm	100	1.03
- 2nd injection		0.95
#1 - 67 ppm	67	0.63
- 2nd injection		0.73
#2 - 67 ppm	67	0.69
- 2nd injection		0.63
#1 - 50 ppm	50	0.50
- 2nd injection		0.49
#2 - 50 ppm	50	0.50
- 2nd injection		0.55
#1 - 33 ppm	33	0.32
- 2nd injection		0.34
#2 - 33 ppm	33	0.36
- 2nd injection		0.34

(continued)

Sample	ug NTA	NTA/HDA
#1 - 17 ppm	17	0.17
- 2nd injection		0.17
#2 - 17 ppm	17	sample lost
#1 - 0 ppm	0	0.05
- 2nd injection		0.04
#2 - 0 ppm	0	0.02
- 2nd injection		0.03

A plot of the above data as NTA/HDA versus ppm is shown as Fig. #7.

A linear least squares analysis of the same data gave a slope of 0.0094 ppm^{-1} with an S.D. of 0.0002 ppm^{-1} and an intercept of 0.03 with an S.D. of 0.01.

G. Precedure for Determination of NTA in Seawater (as developed in Sections A - E)

The following procedure was used successfully to determine NTA in seawater samples containing 1 - 100 ug NTA in volumes up to 100 ml. Thus, with a 100 ml sample the lower limit of determination is ca. 0.1 ppm NTA although smaller amounts may be detected.

Place the seawater sample in a 250 ml round-bottomed flask and remove the water by rotary evaporator. Then place the flask in an oven (ca. 110°C) overnight to dry the residue thoroughly. Samples may be stored in the oven for periods of at least 4 days with no change in NTA content.

Saturate 100 ml of n-propanol with HCl by bubbling the

gas into the liquid through a glass trap or delivery tube attached to the HCl cylinder by Plaster of Paris (no tygon tubing). Remove the seawater residue sample from the oven and add 100 ul of n-propanol containing 100 ug of HDA to the flask, followed by the 100 ml of HCl saturated n-propanol. Attach a reflux condensor to the flask and reflux the mixture for 1 hour. Remove the propanol-HCl reagent by rotary evaporator. Add 50 ml distilled water to the flask and shake to dissolve the residue. Then add 50 ml freshly distilled dichloromethane to the flask, to extract the NTA and HDA propyl esters, shake and transfer the mixture to a separatory funnel. Draw off the CH_2Cl_2 into a 250 ml beaker and extract the aqueous layer two more times with 25 ml portions of CH_2Cl_2. Concentrate the dichloromethane extract to ca. 2 ml on a steam bath with the aid of a stream of air and transfer to a small (5 - 10 ml) ampoule. Remove the remaining CH_2Cl_2 by flushing the ampoules with a stream of air.

Inject on the G.C. two 1 ul samples of benzene containing ca. 2 ug $(propyl)_3NTA$ per ul to "condition" (see Sec. E) the G.C. column. (G.C. parameters described in Sec. A.) Then dissolve the CH_2Cl_2 extracted residue in 50 ul of benzene and inject 1 ul of the resulting solution on the G.C. Record the area under the $(propyl)_3NTA$ and the propyl HDA peaks and calculate NTA/HDA. The NTA/HDA values can be changed to ppm or ug NTA by consulting an appropriate calibration curve.

II. Biodegradability of NTA in Seawater

A. "Non-polluted" Seawater (Run #1)

A 1 l sample of seawater was collected from the running seawater system of the Marine Sciences Laboratory at Logy Bay, Nfld. On October 31, 1972. This sample was filtered through Whatman #4 to remove any large particulate matter and spiked with ca. 100 ppm NTA (as Na_3NTA). The seawater was divided into four 250 ml portions and each placed in a 500 ml conical flask. The samples were treated as follows: Flask A - covered loosely with aluminum foil; Flask B - O_2 removed by bubbling N_2 through the solution, then tightly stoppered (each time the stopper was removed to collect a sample the flask was flushed out with N_2); Flask C - continuously aerated by bubbling air through the solution; Flask D - the sample was filtered through a Millipore (0.45 mu) membrane filter, then the flask covered loosely with aluminum foil. All four flasks were placed in a constant temperature water bath ($20^\circ \pm 1^\circ C$). Aliquots of 1 ml were removed from each flask, at the beginning of the experiment and on every second day for four weeks, for NTA analysis. The results of these analyses (Analytical Data Table #1) show that there was no detectable degradation of NTA over the 4 week period. There was also no visually detected increase in turbidity over this time.

B. "Polluted" Seawater (Runs #2, #3, and #4)

Three samples of seawater were collected from St. John's Harbour (see Fig. #8 for exact locations). The sample for Run #2 was taken at Beck's Cove, ca. 10 m out from the wharf at a depth of 3 m on December 13, 1972. The Run #3 sample was collected at the same depth and distance from the wharf on January 20, 1973 at the foot of Prescott Street. One of the two main outlets for raw sewage for the St. John's - Mount Pearl area is located at the foot of Prescott Street. The second raw sewage outlet for the city is located near the Military Wharf at the east end of the harbour. The sample for Run #4 was collected at this point at a depth of 2 m, also on January 20, 1973.

The same procedure was applied to the Run #2, #3, and #4 samples. They were first filtered through Whatman #4 to remove large particulate matter, then 100 ml samples removed for NTA analysis. The resulting chromatograms showed no detectable concentration of NTA nor any impurities that would interfere with NTA analysis.

Two 250 ml samples were placed in 500 ml conical flasks and spiked with ca. 100 ppm NTA (as Na_3NTA). The flasks were plugged with cotton wool. One flask from each run was marked A and placed in an ice bath ($0^{\circ}C$) while the other was marked B and placed in an incubator ($20^{\circ}C$). Each flask was shaken manually for a brief period daily to aid aeration. Aliquots of 1 ml were removed every second day for 1 month and analyzed for NTA. In all three runs no detectable

degradation of NTA was noted (Analytical Data Table #1) and no increase in turbidity observed.

C. Seawater Samples Concentrated in Bacteria (Run #5)

As seawater off the east coast of Nfld. has a relatively low bacteria count[10], a method of concentrating bacteria is sometimes necessary before culturing. The microbiology group under Dr. G. Moskovits at the Marine Sciences Laboratory at Logy Bay, use a plankton net (bolting silk #20) to collect samples and then culture bacteria from the concentrated plankton sample. Free living bacteria would pass through such a net, however, they probably represent a fairly low percentage[10] of the marine bacteria.

We received such a concentrated plankton sample which had been collected March 22, 1973, 2 - 3 miles east of the entrance to St. John's Harbour. The sample was a combination of plankton from surface, bottom and mid-water hauls and represented a concentration factor of ca. 10^4. (The concentrate from filtering ca. 2×10^4 l of seawater had a volume of ca. 2 l).

To each of two 500 ml conical flasks containing 250 ml of sterilized (autoclave) seawater spiked with ca. 100 ppm NTA (as Na_3NTA), 2.5 ml of the concentrated plankton sample was added. The plankton sample was thus diluted by 100 giving an overall plankton concentration factor of ca. 100 and thus a bacteria concentration factor of ca. 100 (minus free living bacteria lost in original sampling).

Flask A was maintained at 0°C and Flask B at 20°C with brief manual shaking daily.

Aliquots of 1 ml were removed as soon as samples were prepared and on alternate days for 1 month. Sterile 1 ml pipets were used to collect the aliquots for NTA analysis. The results of the analysis (Analytical Data Table #1) show no significant degradation of NTA over the period of 1 month and there was also no visible increase in turbidity.

D. Marine Pseudomonas sp. (Run #6)

A very concentrated (ca. 10^9 cells/ml) 10 ml saline suspension of Pseudomonas sp. IN3FL[10] was received from Dr. Moskovits of the Biology Department of Memorial University. This species had been isolated from the ink sac of Illex illecebrosus illecebrosus, the common bait squid, in 1966. The sample we received had been stored in a maintenance medium, grown in a nutrient broth, the cells separated by centrifugation, washed and resuspended in saline solution.

The saline suspension was divided between two 500 ml conical flasks each containing 250 ml of previously sterilized (autoclave) seawater spiked with ca. 100 ppm NTA (as Na_3NTA). The flasks were plugged with cotton wool and shaken for a brief period daily. As with previous runs,

Flask A was maintained at 0°C, Flask B at 20°C and aliquots were removed every second day for NTA analysis. The analytical data (Analytical Data Table #2) showed no significant degradation of NTA over the 1 month period. Since the original samples were very turbid no increase in turbidity could be noted visually. Since the cells remained in suspension it was assumed that they remained viable throughout the experiment.

E. NTA Mutant

1. Sample #1 (Runs #7 and #8)

A lyophilized sample of an NTA degrading bacterial mutant[11] was received from Dr. Wong, Department of Environment, Canada Center for Inland Waters, Burlington, Ontario.

Small portions of this bacterium sample were transfered, by means of a sterile wire, to two 500 ml flasks containing 250 ml of sterilized seawater previously spiked with ca. 100 ppm NTA (as Na_3NTA). Flask A was maintained at 0°C and Flask B at 20°C and 1 ml aliquots removed for NTA analysis on alternate days. The flasks, plugged with cotton wool, were shaken manually for a brief period each day. The analysis (Analytical Data Table #2 - Run #7) showed that no NTA degraded over the 1 month period. While Flask A showed no increase in turbidity, Flask B developed turbidity in a few days and remained turbid until the end of the 1 month period.

Microscopic observation showed that a motile, short rod bacterium was responsible for the increasing turbidity. At the end of the 4 week period, short rods were still observed, however, a coccus (perhaps the same species as the rod in a different growth phase[12]), a small percentage of which had formed 2 - 6 membered chains, considerably outnumbered the rods. Both the rods and cocci gave a positive Gram test.

The remainder of the lyophilized bacterium was grown in a nutrient broth containing no NTA. After 2 days growth the culture was centrifuged and the pellet washed and resuspended in 10 ml of sterile seawater. One ml of the resulting suspension was placed in each of two 500 ml flasks containing 250 ml of sterilized seawater spiked with ca. 100 ppm NTA (as Na_3NTA). Flask A was maintained at $0^{\circ}C$ and Flask B at $20^{\circ}C$. As in previous experiments the flasks were plugged with cotton wool, shaken daily for brief periods, and aliquots removed every 2 days for NTA analysis. Analytical Data Table #2 (Run #8) shows that no significant degradation of NTA occured over the 2 week period. No increase in turbidity was noted in either flask.

2. Sample #2 (Run #9)

A 20% stock solution of Na_3NTA was made up by dissolving 50 g of Na_3NTA in 150 ml of distilled water, adjusting the pH to 7.0 with conc. HCl, and making up to 250 ml with distilled water.

Two liters of mineral salt solution, as used by Wong,

Lui and Dutka[11], were prepared. The solution contained (in grams): NaCl, 0.5; $(NH_4)_2SO_4$, 0.2; $CuSO_4.5H_2O$, 0.05; $ZnSO_4$. $.7H_2O$, 0.01; H_3BO_3, 0.01; $Al_2(SO_4)_3.18H_2O$, 0.01; $CoSO_4.7H_2O$, 0.01; $MnCl_2.4H_2O$, 0.06; $MgSO_4.7H_2O$, 0.02; $FeSO_4.7H_2O$, 0.01; KH_2PO_4, 1.6; K_2HPO_4, 2.6; per 1 distilled water.

One liter of NTA broth[11] was prepared by diluting 25 ml of 20% Na_2NTA stock solution with 975 ml of mineral salt solution.

A second sample of the NTA degrading bacteria mutant was received from Dr. Wong. This sample was in the form of a suspension in NTA culture rather than lyophilized as in the case of sample #1.

One ml of the suspension was placed in each of six 500 ml flasks. Flask A and B each contained 250 ml of sterilized (autoclave) NTA broth. (ca. 3500 ppm NTA in mineral salt solution.) Flask A was aerated by constant shaking on a mechanical shaker at room temperature, while Flask B was placed in an incubator (20°C) and aerated only by brief manual shaking each day. Flasks C and D each contained 250 ml mineral salt solution spiked with ca. 100 ppm NTA (as Na_3NTA). Flask C was maintained at 0°C, Flask D at 20°C and each was aerated by brief manual shaking each day. Flasks E and F each contained 250 ml sterilized (autoclave) seawater spiked with ca. 100 ppm NTA (as Na_3NTA). Flask E was maintained at 0°C and Flask F at 20°C - each was aerated by brief daily manual shaking. All flasks were plugged with non-absorbant cotton wool.

From Flasks C, D, E, and F, aliquots of 1 ml were collected for NTA analysis as in previous experiments. In the case of Flasks A and B, 1 ml aliquots were taken, diluted x 50 in volumetric flasks, and then 1 ml of the diluted NTA solution was analyzed. These samples are recorded in Analytical Data Table #3 (Run #9) as samples of volume 0.020 ml. On day 10 a full 1 ml from Flask A was analyzed and on day 12 a 50 ml sample from Flask A was analyzed. In Analytical Data Table #3 the NTA/HDA values for Run #9 have been changed to ppm values with the use of calibration curve data.

After innoculation the sample in Flask A became highly turbid overnight and remained so until discarded after the twelfth day. The culture was observed under the microscope on the 3rd and 12th day - each time short rod bacteria which gave a negative Gram test were observed. NTA analysis showed that there was insignificant degradation during the first 4 days followed by rapid degradation leaving only 1 ppm NTA in solution after the 12th day.

The sample in Flask B required 2 days to reach the high state of turbidity acquired by Flask A in 1 day. Microscopic observation showed short rods (Gram negative) a small percentage of which had formed 2 - 6 membered chains. NTA analysis showed ca. 10% degradation over a 14 day period.

Both Flasks C and E, maintained at $0^{\circ}C$, showed no

increase in turbidity and no degradation of NTA over 14 days.

Flask D showed rapid NTA degradation between day 4 and day 7 reducing the NTA content by ca. 75%. After 7 more days, however, ca. 20% of the original NTA remained. An increase in turbidity was noted visually but this was much less than in Flasks A and B.

The sample in Flask F also increased in turbidity but even less than sample D. Degradation of NTA progressed slowly over the analysis period of 18 days at the rate of ca. 2 mg/l/day.

Summary of Biodegradability Data

Run	Medium	Bacteria	Temp.	NTA spiked in ppm	Bacterial growth	NTA degrad.
#1A,B C,D	"non-polluted seawater, Logy Bay	natural	20°C	100	no	no
#2A,B	"polluted" seawater, St. John's Harbour	natural	0°C & 20°C	100	no	no
#3A,B	"polluted" seawater, St. John's Harbour	natural	0°C & 20°C	100	no	no
#4A,B	"polluted" seawater, St. John's Harbour	natural	0°C & 20°C	100	no	no
#5A,B	off shore Nfld. seawater	natural x 100	0°C & 20°C	100	no	no
#6A,B	sterilized seawater	Pseudomonas sp.	0°C & 20°C	100	no	no
#7A,B	sterilized seawater	NTA mutant #1	0°C & 20°C	100	in 20°C sample only	no
#8A,B	sterilized seawater	NTA mutant #1	0°C & 20°C	100	no	no
#9A	mineral salt solution (well aerated)	NTA mutant #2	room temp.	3500	yes	yes
#9B	mineral salt solution	NTA mutant #2	20°C	3500	yes	yes

(continued)

Summary of Biodegradability Data (cont.)

Run	Medium	Bacteria	Temp.	NTA spiked in ppm	Bacterial growth	NTA degrad.
#9C	mineral salt solution	NTA mutant #2	0°C	100	no	no
#9D	mineral salt solution	NTA mutant #2	20°C	100	yes	yes
#9E	sterilized seawater	NTA mutant #2	0°C	100	no	no
#9F	sterilized seawater	NTA mutant #2	20°C	100	yes	yes

Analytical Data Table # 1 - NTA Analysis

Sample Day	Run #1 A	Run #1 B	Run #1 C	Run #1 D	Run #2 A	Run #2 B	Run #3 A	Run #3 B	Run #4 A	Run #4 B	Run #5 A	Run #5 B
0	106	99	107	104	98	96	93	92	92	94	102	102
2	107	109	102	109	94	98	98	85	97	89	107	102
4	110	104	110	109	89	92	93	100	98	79	97	102
6	105	109	115	112	88	97	90	106	96	91	106	100
8	109	110	110	*	91	97	100	88	98	94	102	100
10	112	104	111	112	89	98	87	96	89	100	102	98
12	107	101	111	112	96	100	98	100	94	104	*	*
14	115	111	112	102	93	94	98	102	96	100	102	*
16	99	99	101	106	94	106	100	100	96	100	105	102
18	104	99	107	105	91	95	100	95	100	102	100	106
20	111	106	110	117	92	94	98	97	107	102	109	105
22	107	99	110	110	96	104	110	105	102	108	103	107
24	105	107	114	100	102	102	107	108	112	103	89	100
26	107	114	107	110	*	*	106	102	108	91	100	98
28	105	101	111	105	*	100	100	94	94	105	96	106
29					89							

Data are reported as NTA/HDA values x 100.

*Datum lost because of technical difficulties.

Analytical Data Table # 2 - NTA Analysis

Sample Day	Run #6 A	Run #6 B	Run #7 A	Run #7 B	Run #8 A	Run #8 B
0	98	105	100	100	98	95
2	103	104	107	102	102	104
4	100	100	102	104	110	100
6	100	97	95	98	100	98
8	104	96	98	100	107	100
10	105	98	98	104	100	100
12	97	98	*	*	98	98
14	93	100	96	104	105	100
16	102	95	100	97	-	-
18	102	100	100	106	-	-
20	98	102	96	110	-	-
22	110	96	105	105	-	-
24	102	96	97	98	-	-
26	104	102	105	100	-	-
28	93	95	98	100	-	-

Data reported in NTA/HDA values x 100.

*Datum lost due to technical difficulties.

-No sample taken.

Analytical Data Table # 3 - NTA Analysis - Run #9

Sample Day	A NTA/HDA	A ppm	B NTA/HDA	B ppm	C NTA/HDA	C ppm	D NTA/HDA	D ppm	E NTA/HDA	E ppm	F NTA/HDA	F ppm
0	0.62	3200	0.67	3500	0.90	94	0.90	94	1.02	106	1.08	113
2	0.62	3200	0.68	3500	0.91	97	0.86	89	1.00	104	1.04	108
4	0.65	3400	0.66	3400	1.07	114	0.86	89	1.00	104	1.00	104
6	0.45	2300	0.65	3400	-	-	-	-	-	-	-	-
7	-	-	-	-	0.83	88	0.23	22	1.04	108	0.97	101
10	level of blank		0.64	3300	*	*	0.21	20	1.07	114	0.92	96
12	0.48	1	0.60	3100	1.00	104	0.18	17	1.02	106	0.84	87
14	-	-	0.62	3200	1.10	117	0.19	18	1.00	104	0.80	83
16	-	-	-	-	-	-	-	-	-	-	0.65	67

* Datum lost due to technical difficulties.

\- No sample taken.

Aliquot volumes for Samples A and B were 0.020 ml <u>except</u>: A - 10 = 1 ml and A - 12 = 50 ml.

Aliquot volumes for Samples C, D, E, and F were all 1 ml.

FIG #1

Chromatogram of seawater sample containing 1.00 ppm NTA (and HDA as internal standard) - before reagent clean-up steps.

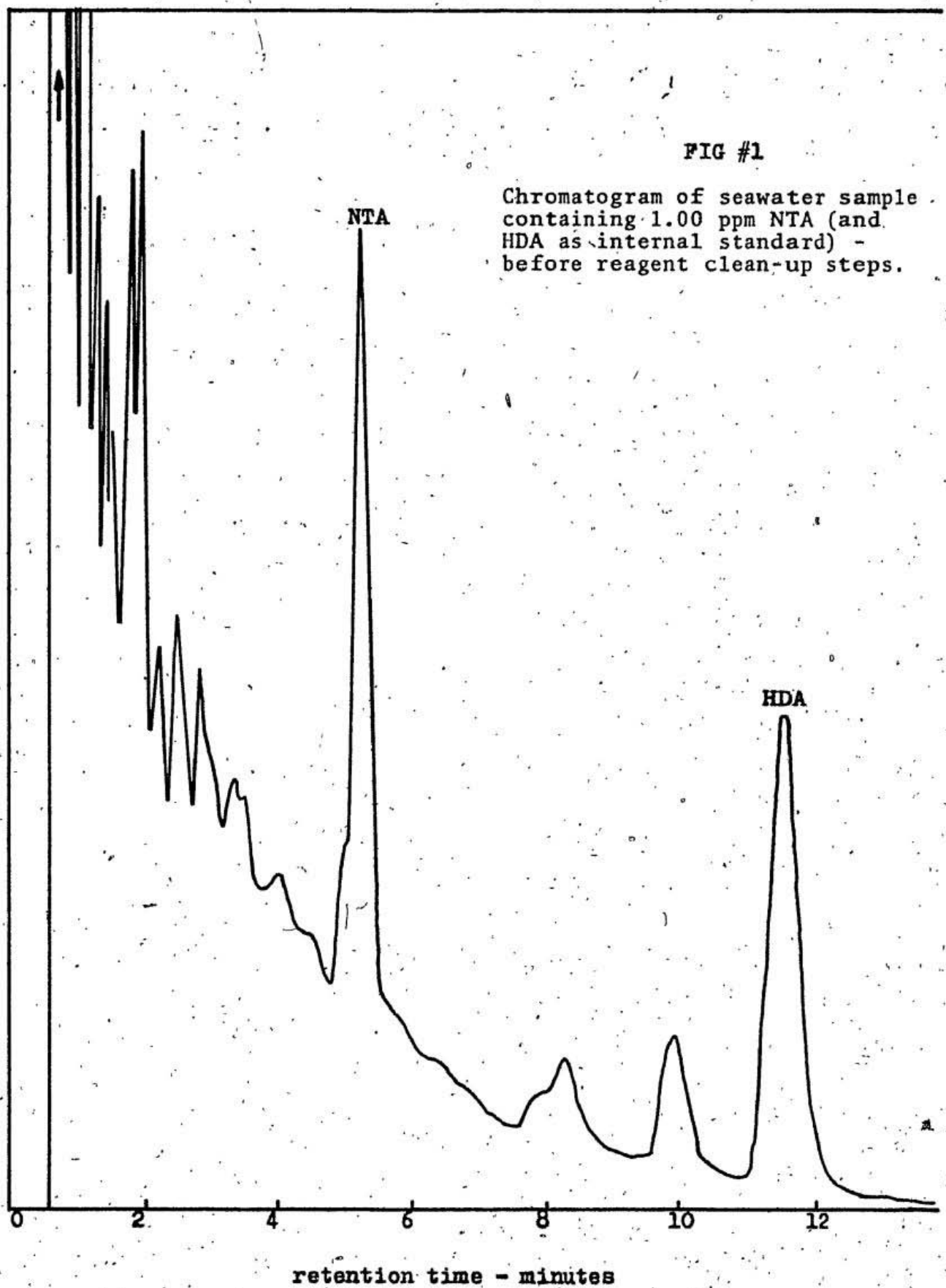
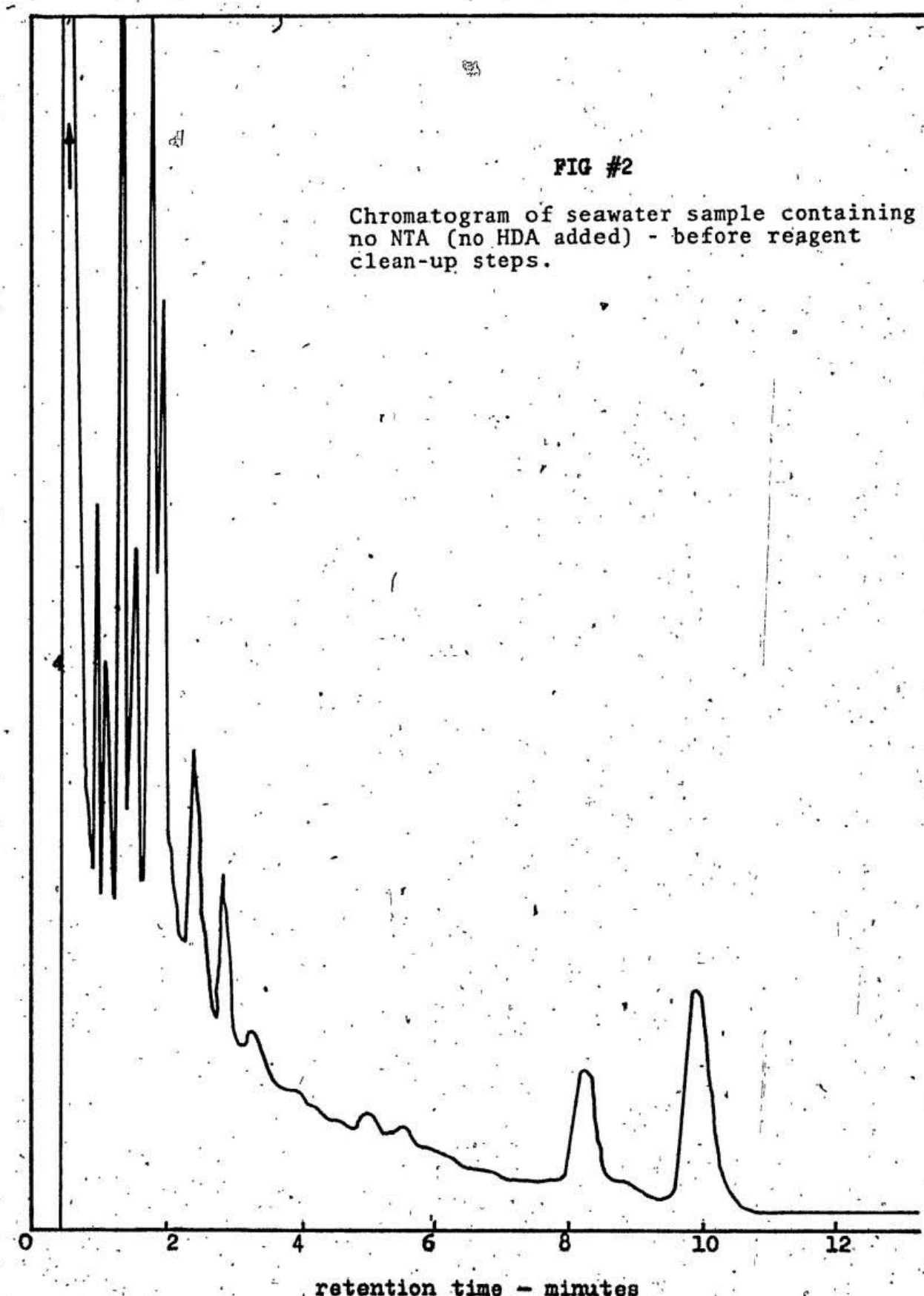


FIG #2

Chromatogram of seawater sample containing no NTA (no HDA added) - before reagent clean-up steps.



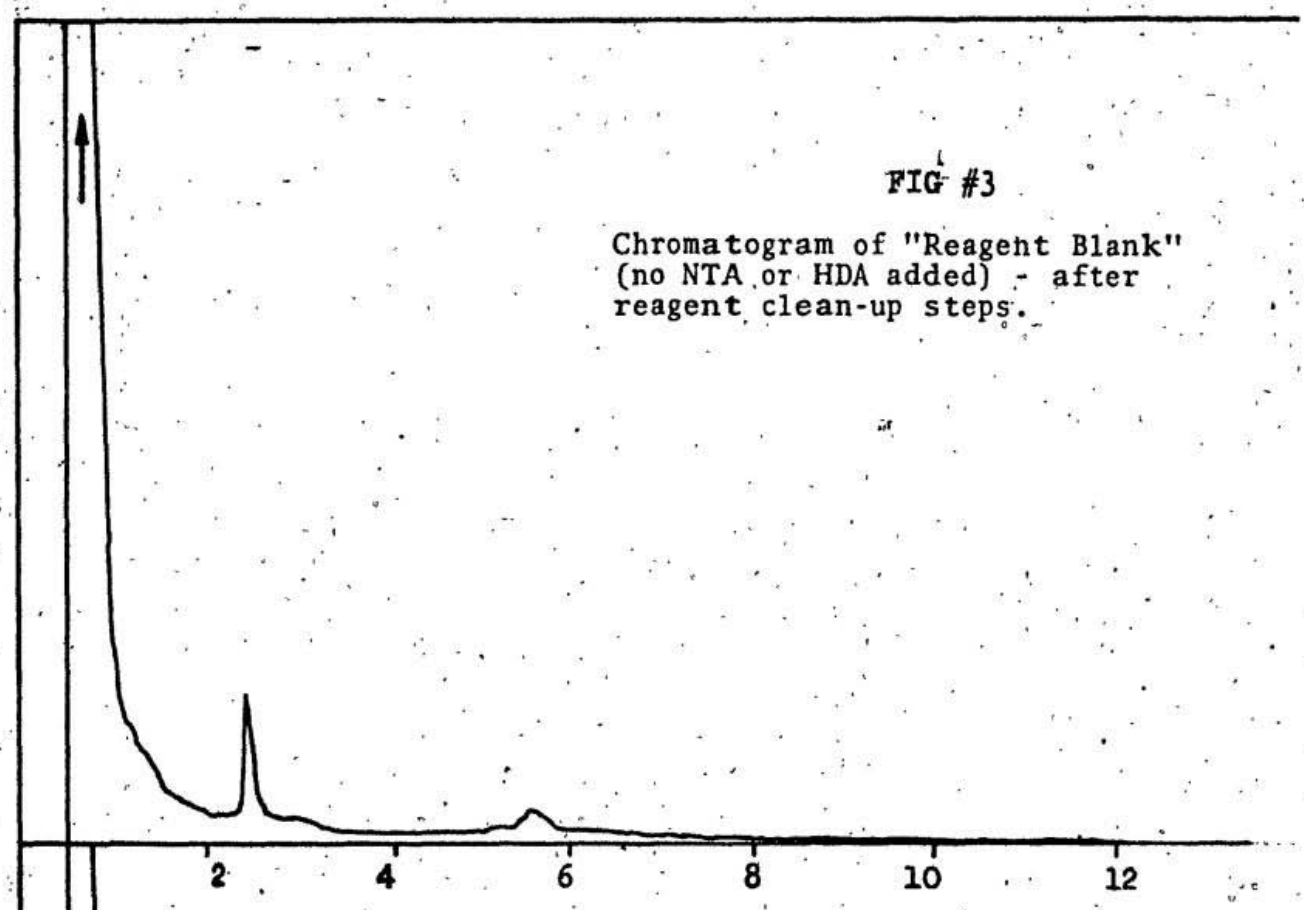


FIG #3

Chromatogram of "Reagent Blank" (no NTA or HDA added) - after reagent clean-up steps.

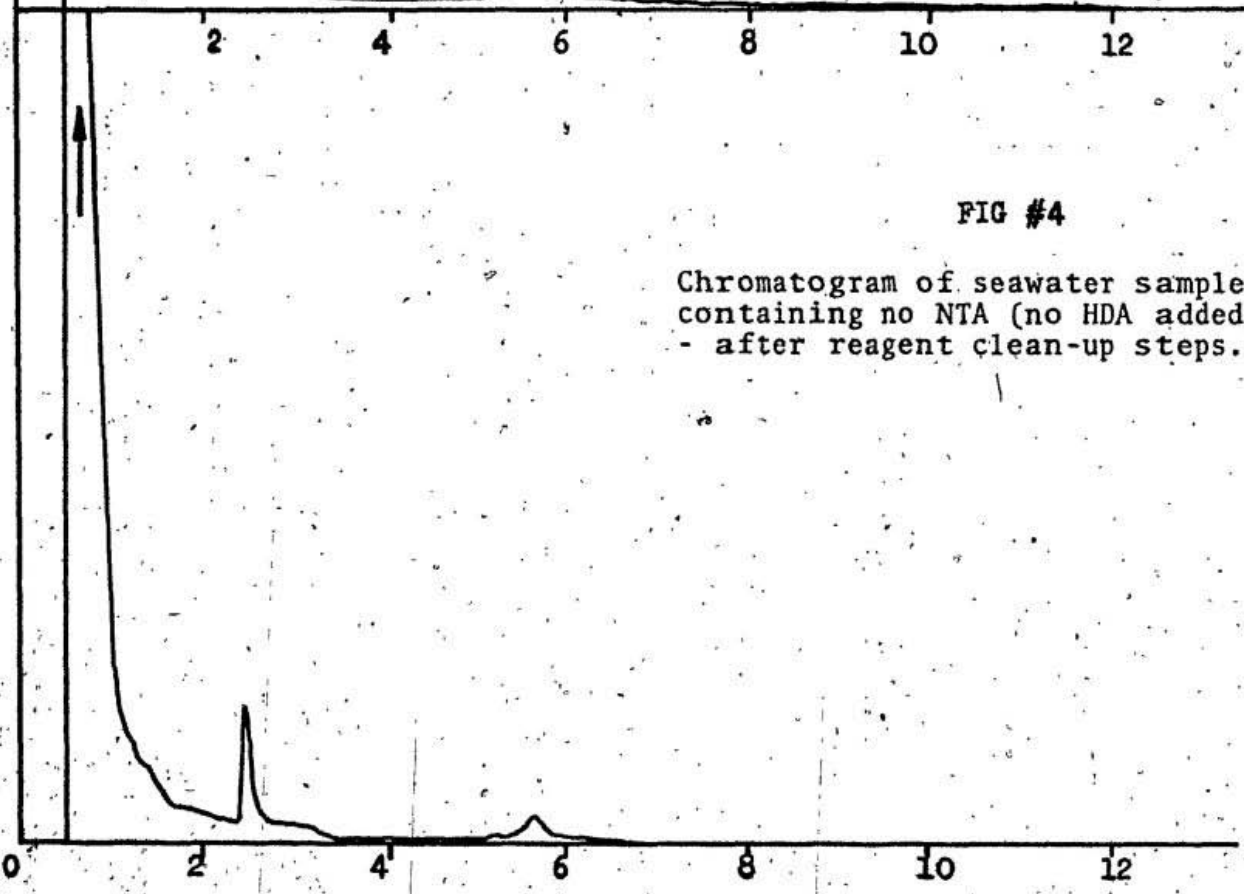


FIG #4

Chromatogram of seawater sample containing no NTA (no HDA added) - after reagent clean-up steps.

FIG. #5

Chromatogram of seawater sample containing 1.00 ppm NTA (and HDA as internal standard) - after reagent clean-up steps.

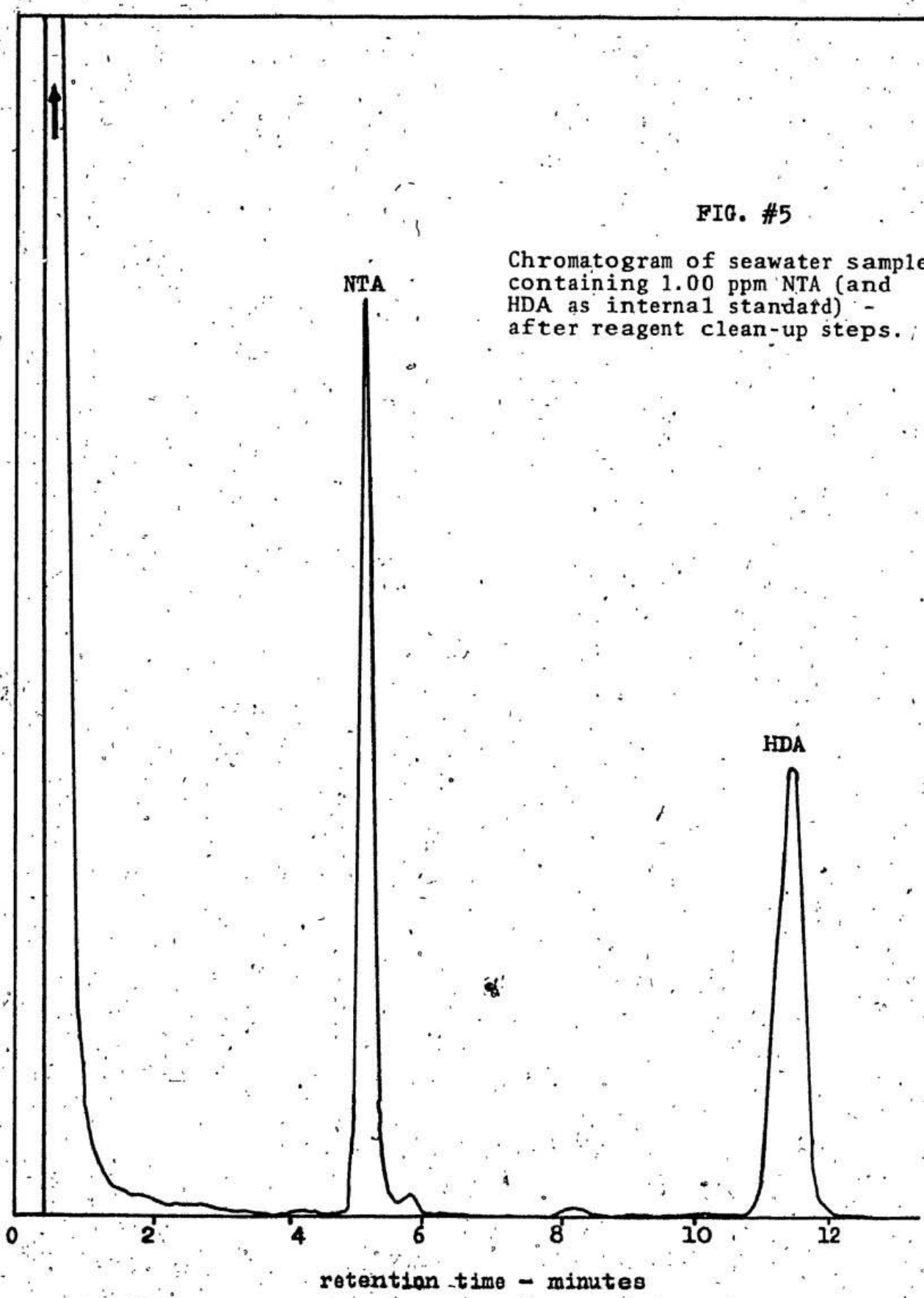


FIG. #6

Calibration Curve #1

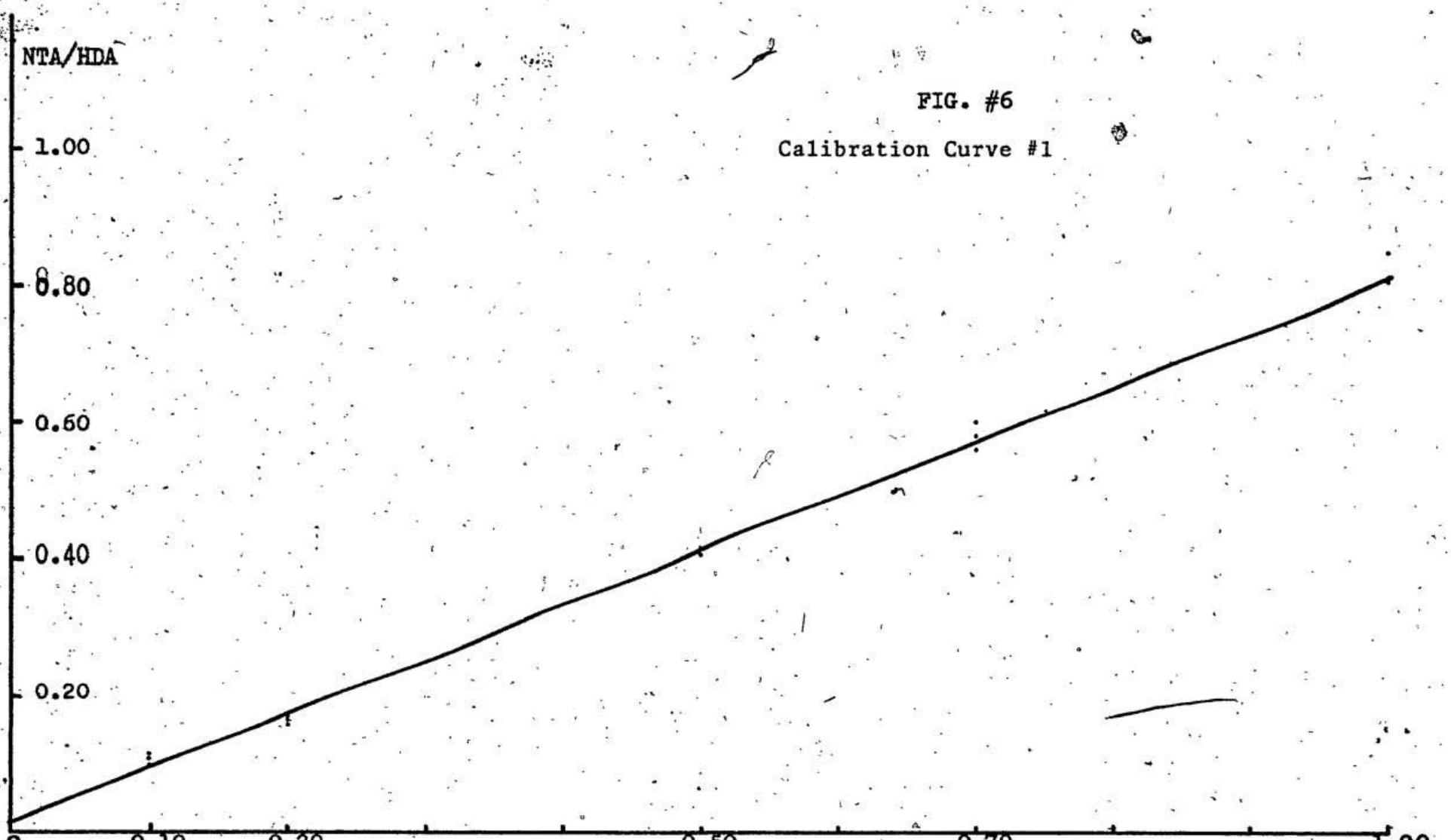
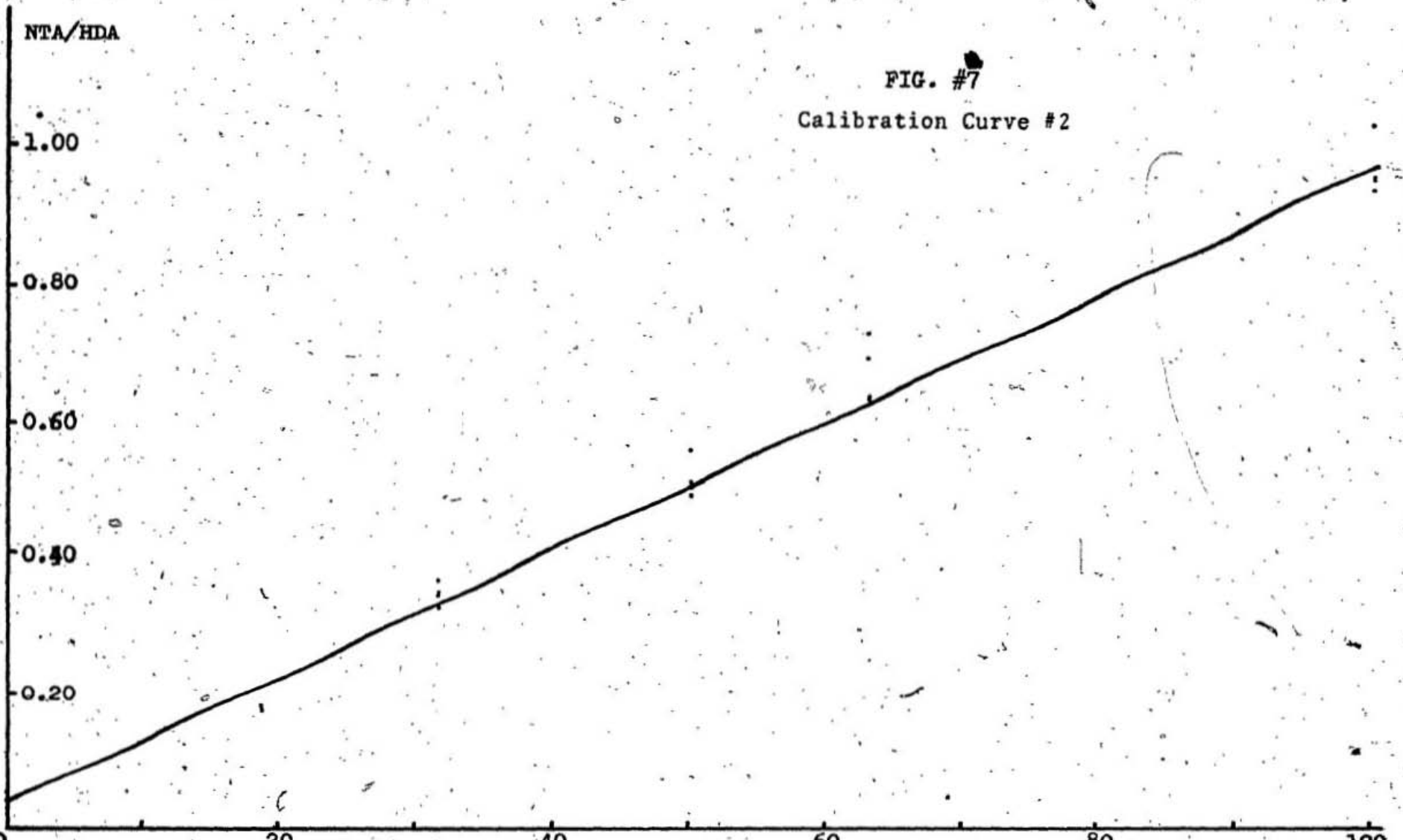


FIG. #7

Calibration Curve #2



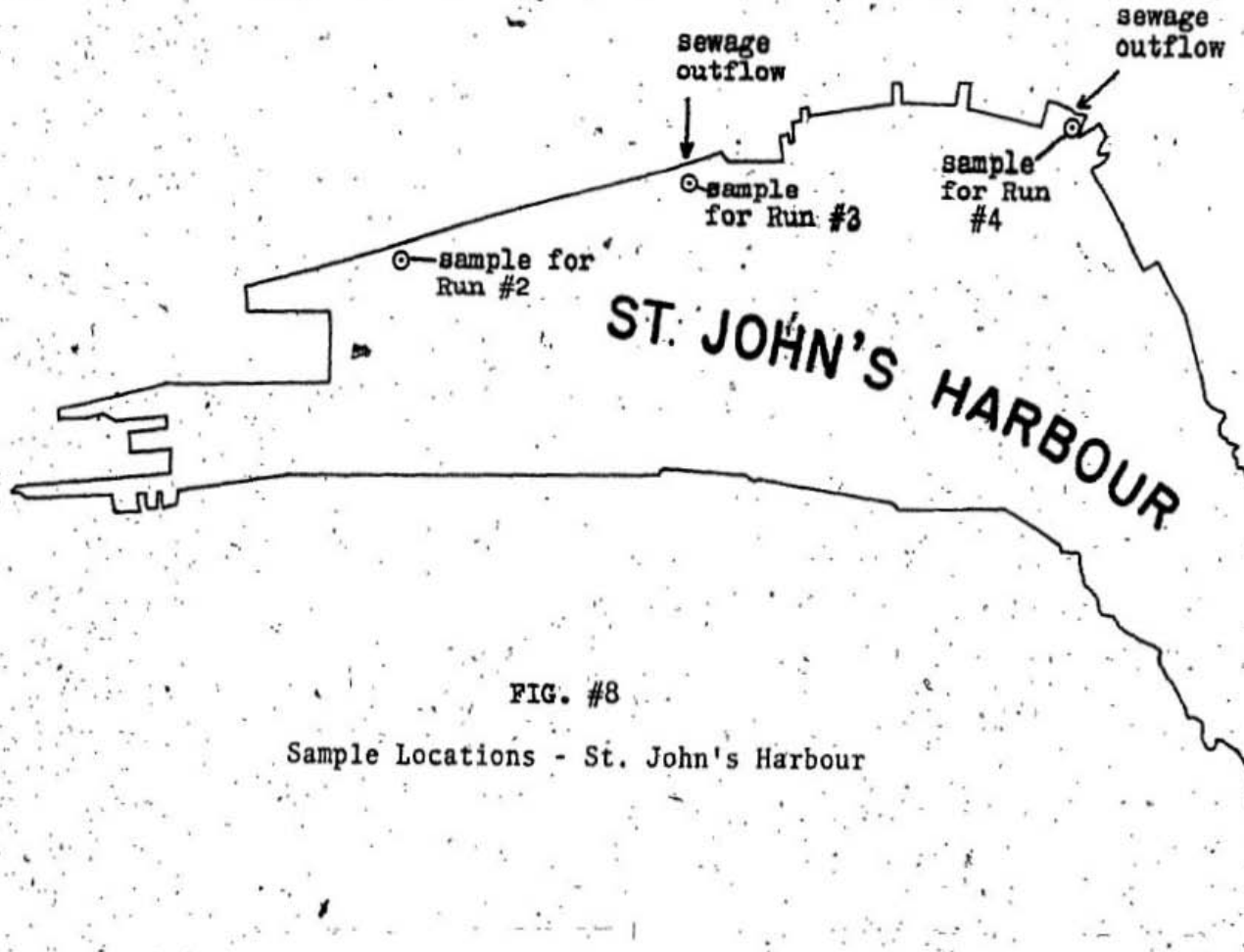
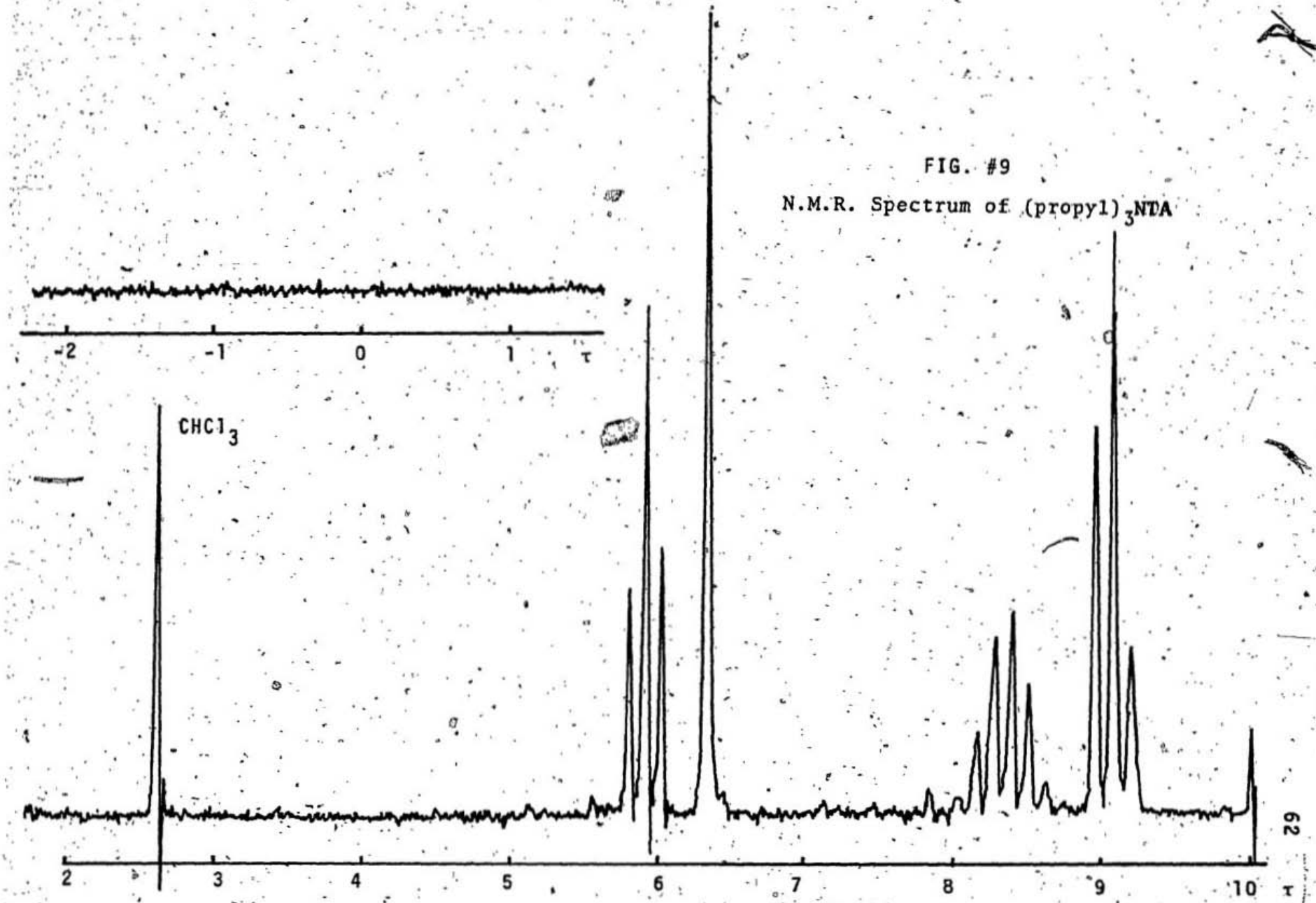


FIG. #8

Sample Locations - St. John's Harbour

FIG. #9

N.M.R. Spectrum of (propyl)$_3$NTA



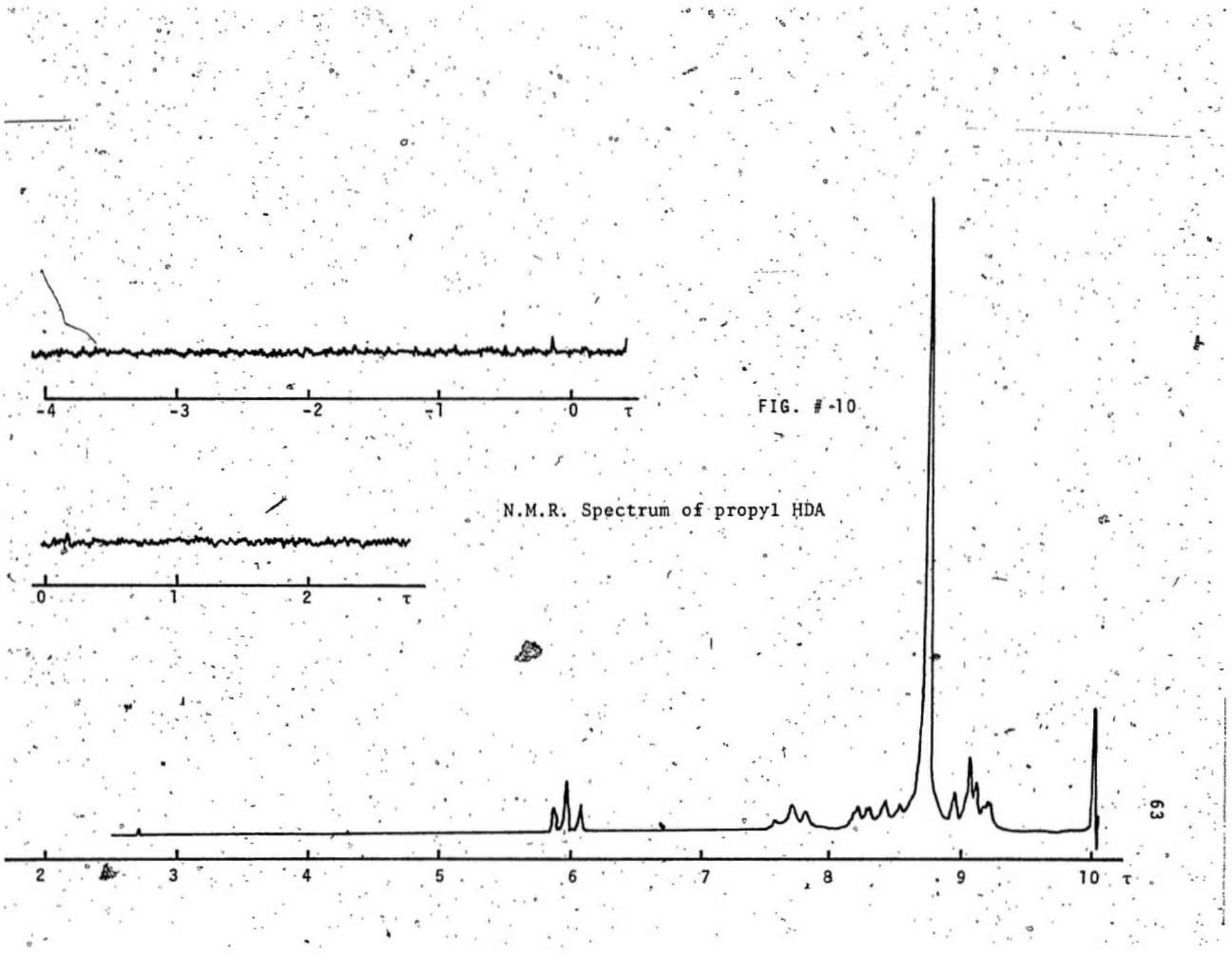


FIG. # -10

N.M.R. Spectrum of propyl HDA

D I S C U S S I O N

I. Analytical Method

Many methods for determination of NTA, based on several different analytical techniques, have been briefly discussed in Sec. 3 of the Introduction. In view of the high ionic content of seawater, along with the possibility of the presence of natural chelators, a G.C. method seemed most likely to be successful for NTA determination in seawater. The G.C. methods previously published for NTA determination in sewage and natural fresh waters are reported to be free from interferences due to metal ions and to chelators other than NTA - this is not true of the other methods discussed.

Chau and Shiomi[37] reported difficulty with the cation-exchange step in the zinc-Zincon method with a "moderate concentration of metallic ions" and we found (Sec. I, D of Experimental) that anion-exchange methods which were successful for freshwater samples did not allow for NTA recovery from seawater samples. It is thus likely that seawater samples would present difficulty in all procedures requiring ion-exchange steps. Some G.C. methods[1,2,9] contain an anion-exchange step, but it has been shown that this is not usually necessary.

Besides being the most free from interferences the G.C. methods have a further advantage over several of the other methods discussed should NTA determination become a routine

procedure in water analysis laboratories. This is simply that gas chromatographs are more often available in water quality laboratories than are dual differential cathode-ray oscilloscope polarographs, stop-flow spectrophotometers or even high speed liquid chromatographs. This instrumentation also makes these other methods more expensive than G.C. The chief disadvantage, as previously mentioned, of a G.C. method is the time required for sample preparation.

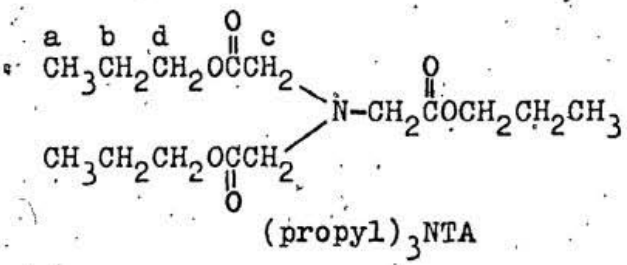
A. Gas Chromatography

At the time we began work on our analytical method only two of the publications on NTA determination by G.C. were available. Murray and Povoledo[6] used a column packed with 2% ethylene glycol adipate on Chromosorb W while Chau and Fox[1] used 3% OV-1 on Chromosorb WHP. Since the former liquid phase has a maximum usable temperature of 200°C compared with 350°C for the latter, it was assumed that the use of the latter would result in longer column life due to a slower bleed of liquid phase. We thus chose to use 3% OV-1 on Chromosorb WHP as our column packing.

Chau and Fox investigated the chromatographic properties of the methyl, n-propyl and n-butyl esters of NTA and chose the n-propyl ester as having "the most suitable volatility on the G.C., well isolated from interferences of the n-propyl esters of several fatty acids and amino acids". Hence we chose to use the n-propyl derivative of NTA,

formed by the reaction of NTA with HCl saturated n-propanol, and also the same internal standard, HDA, as these workers. Our method of obtaining chromatograms of the benzene solutions of the esterification products differed from that of Chau and Fox in two ways: (1) their ¼" O.D. column permitted injection of 5 ul samples, while our 1/8" O.D. column allowed only 1 ul injection as larger amounts resulted in column flooding and solvent tailing; (2) as our temperature programmer was not functioning, chromatograms were run isothermally at 200°C (oven) rather than programmed (185-225°C at 3°C/min).

One gram each of NTA and HDA were treated with n-propanol-HCl reagent in separate reactions and the products isolated by distillation. These products showed relative retention times on our G.C. columns similar to those for $(propyl)_3NTA$ and propyl HDA as reported by Chau and Fox[1] (a wider relative spread between peaks in our data since our samples were run isothermally) and were assumed to be these compounds. N.M.R. spectra of these products were consistent with this assumption:



$(propyl)_3NTA$

We would expect an N.M.R. spectrum of the above compound to contain: a methyl absorption in the form of a triplet and integrating for 9 protons due to the 'a' protons coupled with the 'b' protons; a methylene sextet for the 'b' protons coupled with the 'a' and 'd' protons and integrating for six protons; a methylene triplet for the 'd' protons coupled with the 'b' protons (at lower field than the 'b' protons because of the deshielding of the neighboring oxygen atom) and integrating for six protons; also integrating for six protons would be a methylene singlet (no first order coupling) due to the 'c' protons (at lower field than the 'b' protons because of the deshielding of the adjacent nitrogen and carbonyl group).

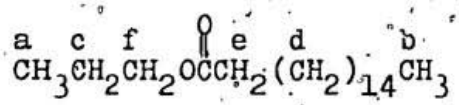
The 60 MHz N.M.R. spectrum of our distilled product of the n-propyl esterification of NTA, run in $CDCl_3$ using TMS as internal standard, gave the following results: (as noted in Experimental Sec. I. B.)

Absorption (τ)	Assignment	Expected Integration	Integration Obtained
triplet - 9.0	a	9	9
sextet - 8.3	b	6	6
singlet - 6.2	c	6	4
triplet - 5.9	d	6	8
singlet - -0.6	acid proton (impurity)	0	1

Since the above data indicated the presence of an impurity containing a -COOH group, the product was washed in bicarbonate solution, extracted in chloroform and another

N.M.R. spectrum run under the same conditions with the following results:

Absorption (τ)	Assignment	Expected Integration	Integration Obtained
triplet - 9.0	a	9	9
sextet - 8.3	b	6	6
singlet - 6.3	c	6	6
triplet - 5.9	d	6	6
no acid proton	-	-	-
singlet - 2.6	residual chloroform	-	-



propyl HDA

An N.M.R. spectrum of the above compound would be expected to contain: triplets due to two non-equivalent methyl groups ('a' and 'b'), each coupled with a methylene group and integrating for 3 protons each; a complex multiplet, due to the 'c' and 'd' protons integrating for 30 protons; a triplet integrating for 2 protons for the 'e' protons (coupled with a methylene) at lower field than the 'c' and 'd' protons due to the neighboring carbonyl group; and a triplet close to the 'd' protons of the NTA ester due to the 'f' protons (coupled with a methylene) and integrating for 2 protons.

The 60 MHz spectrum obtained for the distilled product of the n-propyl esterification of HDA, run in $CDCl_3$ using TMS as the internal standard, was consistant with the above prediction:

Absorption (τ)	Assignment	Expected Integration	Integration Observed
distorted triplet - 8.9	a & b	3 + 3	6
complex multiplet - 8.5	c & d	30	28
triplet - 7.7	e	2	2
triplet - 5.9	f	2	2
no acid proton	-	-	-

The two methyl absorptions appeared as a distorted triplet integrating for 6 protons rather than as separate triplets integrating for 3 protons each. No acid impurity was noted as in the case of the NTA ester. The obtained integration of 28 is within the experimental error of the predicted value of 30 for the 'c' and 'd' protons.

Our G.C. columns were thus standardized with respect to the retention times of the propyl esters of NTA and HDA.

B. Separation of NTA from Seawater

It was with regard to separation of NTA from our seawater samples that we were unable to follow the procedures of workers whose methods were successful for fresh water and/or sewage samples. As previously mentioned, some of these workers separated NTA from their water samples by anion-exchange. This, as well as eliminating possible interfering compounds, resulted, following evaporation of the solution used to elute the NTA from the ion-exchange resin, in the NTA being contained in a much smaller amount of residue than would be the case if the original water sample were evaporated. Thus the NTA could be esterified by a small volume (1 - 2 ml) of the esterification reagent in a

small (5 - 10 ml) ampoule or screw-cap vial. Other workers found that simply evaporating the water sample left a small enough quantity of residue for esterification in the above manner and omitted the anion-exchange step although often filtering to remove particulates before evaporation.

Our problem stemmed from the fact that we required 100 ml of seawater to detect NTA below the 1 ppm level and evaporation of these samples resulted in ca. 3 g of salt residues. It was impossible to place this quantity of residue in a 10 ml ampoule and obtain quantitative conversion of NTA to its ester using 2 ml of esterification reagent. Since all previous procedures carried out NTA esterifications in sealed tubes it was assumed that this was necessary to receive quantitative ester yields. As we had experienced a few explosions of 10 ml sealed ampoules during esterification of standard NTA samples, we wished to avoid using larger sealed reaction vessels with larger volumes of esterification reagent. For these reasons we attempted the use of anion-exchange resin to separate NTA from the bulk of dissolved solids in seawater.

As noted in Experimental Sec. I. C., anion-exchange procedures were first attempted using distilled water solutions of Na_3NTA. Dowex 1 - X 8 anion-exchange resin was used in the formate form following Chau and Fox[1], the chloride form after Rudling[2] and also in the carbonate form as we noted use of this resin by Resnik, et. al.[3], to remove polybasic organic acids from aqueous tobacco extracts.

Best recovery of NTA from the distilled water samples was accomplished using the carbonate form of the resin. When seawater samples were used the carbonate form again showed the best NTA recovery, however, this amounted to only 5 - 10% of the NTA added to the sample. This was undoubtedly due to the high chloride level of seawater - a seawater sample containing 1 ppm NTA would have a molar ratio of chloride to NTA of ca. 10^5:1.

Having thus abandoned the idea of NTA removal from seawater using anion-exchange resin, we questioned the necessity of carrying out the esterification reaction in sealed vessels. If sealed tubes were not necessary the use of large enough volumes of esterification reagent to react with the NTA in the large seawater residues would be feasible. Experimental Sec. I. E. describes the use of 100 ml of n-propanol-HCl reagent to esterify 100 ug of NTA and 100 ug of HDA present in the residue of 100 ml of seawater in a 250 ml round-bottomed flask open to the atmosphere via a reflux condensor. Following esterification the sea salts were dissolved in distilled water and the NTA and HDA propyl esters were extracted by dichloromethane. The CH_2Cl_2 was then evaporated leaving the esters in a small amount of residue, which, in benzene solution, was injected on the G.C. Results, including the calibration curves (Sec. I. F. of Experimental), showed that quantitative recovery of NTA can be accomplished by this method.

C. Interferences Due to Impurities

It is significant to note that no interferences, in the form of extra peaks on the gas chromatograms, appeared to result from compounds present in any of the water samples analyzed. Many troublesome impurities were added to the sample, however, along with the reagents and solvents. Tests carried out in Sec. I. C. of the Experimental indicated that most impurities were originating in the n-propanol-HCl reagent but not present in the n-propanol alone. At this point in our work we were using only 2 ml of esterification reagent per sample and it was found that these impurities could be ignored, however, in Experimental Sec. I. E., we began to use 100 ml of n-propanol-HCl and these impurities resulted in a very complex chromatogram (Fig. 2). Investigations proved that most of these impurities originated in the HCl drying train which consisted of conc. H_2SO_4 traps joined with tygon tubing. As by-passing this apparatus by passing the HCl through an empty glass trap, attached directly to the HCl cylinder by Plaster of Paris, eliminated most extra peaks from the chromatograms, it was concluded that the impurities were either degradation products of the tygon tubing, resulting from the reaction with HCl gas and/or conc. H_2SO_4, or plasticisers from the tubing.

Some further impurities were removed by distilling before use the dichloromethane used for extracting the NTA and HDA esters. A "solvent blank" following these two

"reagent clean-up" steps is shown as Fig. 3. The small peaks, present at 5.2 and 5.6 min retention time, interfered with the NTA ester peak at 5.3 min. These impurities increased the NTA values by an average of 2 - 3 ug per sample, judging by the intercepts (zero NTA) of Curves #1 and #2. In chromatograms run of samples containing no NTA for Curve #2, these peaks show values of 2 - 6 ug. It is these impurities rather than G.C. sensitivity that limit our method of NTA determination to samples containing at least 10 ug of NTA (0.1 ppm for a 100 ml sample and 10 ppm for a 1 ml sample). If smaller quantities of NTA are to be determined using this method these impurities could perhaps be removed by further reagent purification.

D. Calibration Curves

The increase in S.D. of the slope of Curve #2 relative to Curve #1 is probably due to the original measuring of the volumes of the NTA containing solution. For Curve #2 all samples were measured by using 1 ml sterilized, disposable, plastic pipets while for Curve #1 portions of the stock solution containing NTA were measured by 1 - 10 ml glass pipets and made up to 100 ml with seawater. One would expect some loss of precision when using the smaller volume disposable pipets.

The difference in the slopes of Curves #1 and #2 cannot be attributed completely to a lower recovery of NTA from the 100 ml samples than from the 1 ml samples since different HDA solutions were used as the internal standard.

Three pairs of identical samples were analyzed to compare the two HDA solutions and the solution used for Curve #2 gave higher NTA/HDA values in each case. While only three samples were not enough to give a precise value of the difference between the two solutions we can estimate from these values (0.93, 0.87 and 0.92 for solution #1 and 1.04, 0.95 and 0.93 for solution #2) that ca. ½ the difference in the slopes of Curve #1 and #2 is due to HDA solutions of different concentrations. Since the first solution was made up by weighing out 10 mg of HDA for 50 ml of solution while the second solution was made up by weighing 50 mg HDA and making up to 250 ml with n-propanol, it was assumed that the second solution was the more accurate. A contribution to this error may have resulted from impurities in the HDA since it was used as purchased without further purification. If HDA is used as an internal standard the same solution must be used for analysis as was used for the calibration curve.

In Sec. I. E. of the Experimental it was noted that the $(propyl)_3NTA$ peaks on the chromatograms consistently increased over the first three samples injected each day. It was decided that some of this ester must be adsorbed on certain sites in the G.C. column and that later samples were consistant as these sites had been filled during the first two injections. This problem was eliminated by injecting two samples of a benzene solution containing ca.

2 ug/ul $(propyl)_3$NTA before running samples for analysis. This also served as a daily retention time check on $(propyl)_3$NTA.

The appearance of a peak on the chromatogram at the same retention time as that of the NTA ester does not necessarily mean that NTA was present in an environmental sample containing unknown impurities. The chance of another compound having the same retention time as $(propyl)_3$ NTA cannot be overlooked. Unless the NTA ester can be identified by a G.C. - mass spectroscopic instrument, the probability of coincident G.C. peaks must be decreased by running both the unknown sample and NTA standards on G.C.'s with columns containing different packing. As our environmental samples contained no NTA and gave no peaks at 5.3 min retention time, unless spiked with NTA, we required only the one type of G.C. column.

II. Biodegradability of NTA in Seawater

The procedures described in Experimental Sections II. A., B. and C. (Runs #1 - 5) were experiments to determine if NTA would be degraded if it should enter seawater in the Newfoundland area. The samples in Sec. A. represent what can be considered "unpolluted" Nfld. seawater while the samples of Sec. B., taken from St. John's Harbour, represent some of the most "polluted" seawater in the area. Two of the St. John's Harbour samples (Runs #3 and #4) were collected

in the immediate vacinity of the two major raw sewage outlets from the city. The collection of the sample used in Run #5 is described in Experimental Sec. II. C. and represents off-shore North Atlantic water with the bacteria concentrated ca. 100 fold.

With the exception of Run #1 where all samples were maintained at 20°C, we ran duplicate samples at 0°C and 20°C. The temperature of 0°C was easily maintained in an ice bath and is typical Nfld. seawater, except for a few meters of surface water, during much of the year. It was assumed that the temperature of 20°C would promote more rapid bacterial growth. All environmental samples were spiked with 100 ppm NTA as the trisodium salt.

The samples of Run #1 were kept under different conditions: Sample A was aerated only by allowing it to stand at equilibrium with the atmosphere; Sample B was maintained in the absence of oxygen in a nitrogen atmosphere; Sample C was aerated by constantly bubbling air through it to simulate the air saturation effect of waves; Sample D was sterilized by filtering through a 0.45 mu Millipore membrane filter and thus served as a control. The samples in Runs #2 - 5 were aerated only by allowing them to stand at equilibrium with atmosphere (cotton wool plugs in the flasks) and by a brief period of manual shaking daily.

Under these conditions none of the environmental

samples spiked with NTA showed signs (no visually detectable increase in turbidity) of bacterial growth, nor of NTA degradation as noted by direct NTA analysis of aliquots of these samples over a four week period.

Since the degradation of NTA in samples of river water and lake water, as well as sewage, had been shown by several studies discussed in our Introduction, it appeared that bacteria capable of NTA degradation were quite wide-spread in the environment. In view of these publications it seemed exceptional that NTA did not degrade in our aerobic natural water samples so we wished to show that such degradation was possible under the conditions of our experiments and thus did not occur simply because there was no bacteria present capable of effecting this reaction. The remainder of our work, described in Experimental Sections II. D., E. and F., involved a search for a bacterium capable of degrading NTA under the conditions of our previous experiments and to thus serve as a positive control.

Focht and Joseph[41] had identified a bacterium, isolated from sewage and capable of NTA degradation, as a Pseudomonas sp. It thus seemed possible that a marine pseudomonad might be capable of NTA degradation in seawater. We ere able to obtain a marine pseudomonad (described in Experimental Sec. II.D.) from Dr. Moskovits, Department of Biology, Memorial University. Run #6 involves two samples of sterilized seawater innoculated with the marine pseudomonad and maintained

under the same conditions as the samples of Runs #2 - 5. Again no degradation of NTA was noted over a period of 1 month, so this bacterium was not able to serve as our positive control.

Runs #7 and #8 continued our search for a positive control with the use of a sample of a bacterial mutant supplied by Wong, Lui and Dutka[11]. These runs were carried out under the same conditions as Run #6 except that the examples were innoculated with the mutant rather than the marine pseudomonad. In the case of Run #7 a portion of the lyophilized skim milk culture of the mutant, received from the above workers, was added directly to the samples, while in Run #8 a suspension of the bacterium, after growing in a nutrient broth (containing no NTA), was used. No NTA degradation was observed in either of these runs even though bacterial growth was noted in the 20°C sample of Run #7 by both visual observation of increased turbidity and microscopic observation. The organism gave a positive Gram test.

In view of the paper by Wong, Lui and Dutka[11], where rapid degradation of NTA was noted by a Gram-negative organism and the results of our Run #9 which also showed NTA degradation by a Gram-negative organism, it appears that as a result of further mutation, or contamination of either the original lyophilized material or our cultures,

the bacterium used in Run #7 and #8 was not the NTA degrading mutant.

A second bacterium sample was received from Wong, Lui and Dutka - this sample was a suspension in NTA culture which was used to innoculate all six samples of Run #9. Sample A was NTA broth as described by Wong, et. al.[11], and was constantly aerated on a shaker at room temperature. Good growth was observed overnight by a large increase in turbidity of the sample and the NTA was degraded from ca. 3500 ppm to a level of 1 ppm in twelve days. Sample B contained the same material as A but was not placed on the shaker but rather in the 20°C incubator and treated in the same manner as samples of previous runs. Good growth was noticable by the great increase in turbidity but NTA degradation moved at a much slower rate. It was thus apparent that if rapid degradation of large quantities of NTA was to occur, oxygen must be efficiently supplied to the sample.

The remaining samples of Run #9 - C, D, E and F - were treated in the same manner as the samples in Runs #2 - 8. Samples C and D contained the mineral salt solution of Wong, et. al.[11], plus 100 ppm NTA and were maintained at 0°C and 20°C respectively. Samples E and F were sterilized seawater with 100 ppm NTA and were also maintained at 0°C and 20°C respectively. The 20°C samples both showed bacterial growth (noted by an increase in turbidity and microscopic observation) and NTA degradation (see Analytical Data Table #3).

The 0°C samples showed no increase in turbidity and no NTA degradation over a two week period. The latter result is not surprising since Wong, et. al.[11], reported that growth of the mutant was "drastically retarded" at 4°C.

It was thus shown that degradation of NTA was possible under the conditions we were using for our environmental samples (Run #9, Samples D and F) and indeed in seawater under these conditions (Run #9, Sample F) if bacteria capable of NTA degradation are present. Since we have thus established a positive control we can state that in our environmental samples (Runs #1 - 5) there was present no bacterium capable of degrading a significant quantity of NTA under these conditions.

III. Environmental Implications of Results

Toxicity studies reviewed in the Introduction have not shown NTA to be dangerous in concentrations expected in natural waters should it be widely used as a detergent builder. As we have noted, however, most researchers caution that it is possible problems may arise in areas not yet studied and point out the need for further testing. Except for the phytoplankton study of Erickson, et. al.[45], the only toxicity evaluation of NTA on marine organisms is that of Eisler, et. al.[42], who carried out 168 hour acute toxicity tests. In view of the small amount of work done on toxicity to marine organisms and the complete lack of long term studies, it is certainly not possible to state

that NTA will prove non-toxic in the marine environment.

The fact that NTA was shown by several workers to be biologically degraded in aerobic freshwater systems and thus should not accumulate to dangerous levels in the environment, has been a strong point in favor of NTA use. Although our biodegradability tests on natural seawater samples were carried out on an extremely small number of samples and under limited conditions, we can state that biodegradation of NTA in cold North Atlantic seawater appears unlikely and at least cannot be taken for granted, as now seems to be the case when considering natural, aerobic freshwater systems.

NTA accumulation in seawater is thus a possibility. Although accumulation in the open sea will most certainly remain insignificant because of the great dilution factor, a build-up of NTA in sheltered bays and harbours, with small tidal effects and significant sewage effluent, could occur. The argument that such bodies of water are already highly polluted no longer appears to be morally or even pragmatically acceptable as an excuse to ignore the possibility of further pollution.

Certain heavy metals present in only trace amounts in seawater are necessary for the survival of marine life. If such metals are chelated by NTA, they may not be easily utilized by some of these organisms. Copper, for example, which is contained in hemocyanin, the oxygen carrier in the

blood of many organisms, is present in seawater only at levels around 3 ppb[58] and its NTA chelate has a very high stability constant (ca. 10^{13})[59]. In view of the ability of NTA to protect fish[31] from the toxic effects of copper II ion, it seems that copper is not easily removed from its NTA chelate by some organisms. It is thus conceivable that small quantities of NTA may prevent the pick up of copper by organisms that require that element.

The studies on the metabolism of NTA in rats by Michael and Wakim[23] indicated that some NTA was accumulated in the bones as the calcium chelate but not in quantities large enough to cause significant effects on the bond. This accumulation in the bones could possibly lead to NTA being concentrated in the biosphere to levels high enough to have toxic effects on organisms high in the complex marine food web. This should be an area for further research on NTA metabolism.

It is possible that the method of analysis, that has been developed and successfully used for determining NTA in seawater in our work, may facilitate studies on the effects of NTA on the marine environment. This method may also be of value in toxicity work as it could prove successful in analysis for NTA in body fluids and tissues whose constituents are more likely to interfere with other methods of NTA determination.

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