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Inhibitors for Marine Sulfate-Reducing Bacteria in Shipboard Fuel Storage Tanks

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Problems of corrosive fuels, as well as corrosion of metals, from marine sulfate-reducing bacteria in shipboard fuel tanks continue to plague the Navy. We have sought a solution through finding a toxicant which can be added to the ballast or displacement water and thus suppress the growth of the organisms. Despite their apparent hardiness in fuel tanks, the bacteria have proven difficult to maintain in vigorous culture in the laboratory. So as a first step in bioassay, a system for maintaining an active inoculum was worked out, along with a test-tube procedure for estimating inhibitive dose. Fuels used in the assays to date are aviation gasoline and Navy Distillate. As candidate toxicants, seven materials were selected for laboratory trial on the basis of prior toxicity information and presumed compatibility with the fuel. From the tube studies three thiopyridine derivatives were among the best; experiments on a larger scale are suggested. Since the Navy foresees a wide use of Navy Distillate, we should anticipate our problems by looking into its apparent microbial susceptibility - and likewise susceptibility of Marine Diesel - to other classes of organisms.

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Navy Distillate fuel						
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Inhibitors for microbes in hydrocarbon fuels						

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ABSTRACT

Problems of corrosive fuels, as well as corrosion of metals, from marine sulfate-reducing bacteria in shipboard fuel tanks continue to plague the Navy. We have sought a solution through finding a toxicant which can be added to the ballast or displacement water and thus suppress the growth of the organisms. Despite their apparent hardiness in fuel tanks, the bacteria have proven difficult to maintain in vigorous culture in the laboratory. So as a first step in bioassay, a system for maintaining an active inoculum was worked out, along with a test-tube procedure for estimating inhibitive dose. Fuels used in the assays to date are aviation gasoline and Navy Distillate. As candidate toxicants, seven materials were selected for laboratory trial on the basis of prior toxicity information and presumed compatibility with the fuel. From the tube studies three thiopyridine derivatives were among the best; experiments on a larger scale are suggested. Since the Navy foresees a wide use of Navy Distillate, we should anticipate our problems by looking into its apparent microbial susceptibility - and likewise the susceptibility of Marine Diesel - to other classes of organisms.

PROBLEM STATUS

This is an interim report; work on the problem continues.

AUTHORIZATION

NRL Problem G04-01
Project RR 104-03-41-5503

INTRODUCTION

Maintaining supplies of hydrocarbon fuels clean and free of contamination presents difficulties for any fuel-handling agency. This operation poses special problems for the Navy because its fuels are very often in contact with sea water. Not only is sea water corrosive in itself but it is also a source of microorganisms, some of which add to corrosion problems. One such group of organisms, referred to as "sulfate-reducing" bacteria since they reduce sulfates to sulfides, have at times created serious problems in fuel systems, especially in storage tanks aboard ship (1).

In brief review, the most severe trouble from sulfate reducers the past few years has been in tanks of aviation gasoline (Avgas) on aircraft carriers (2,3,4,5). The sea water used during fueling operations to displace the Avgas from the tanks offers a favorable growing environment for these anaerobic sulfate-reducing bacteria, especially when fuel turnover is slow. Hydrogen sulfide and sulfur, both being soluble in Avgas, turn it corrosive so that it fails the copper strip corrosion test (ASTM Method D 130) (3). Fuel thus "off specification" is detrimental to storage tanks and to fuel systems in which it is used. Accumulations of growth and corrosion products form a sludge on the bottoms and sides of tanks which is very difficult to remove. Unfortunately, the one accepted treatment for inhibiting sulfate reducers aboard ship has proved unsatisfactory when applied to tanks containing such sludge (3,4,5). This treatment is the addition of sodium chromate at a concentration of 0.10 to 0.15% to displacement or ballast water, where it effectively prevents growth, but it does not inhibit these anaerobic bacteria when they are occluded in the oily sludge. Also, the chromate oxidizes sulfides in the sludge to elemental sulfur. Hence the necessity for costly, time-consuming removal of the sludge if sodium chromate is to be used. Because of this shortcoming of chromate and because of its toxicity to marine life when it is offloaded during refueling, there is definite need for a more suitable method of control. The goal of the study reported here has been to find a more acceptable chemical means of inhibiting the sulfate-reducing bacteria.

While the Navy's main problem with sulfate-reducing bacteria has involved tanks of Avgas, growth of these bacteria is by no means restricted to this one fuel. They have been isolated from contaminations of jet- and diesel-fuel tanks. They have also been cultured in the laboratory under not only these three fuels but Navy Distillate as well. Results of several experiments with Navy Distillate fuels are included in this report.

In work reported previously (1) the evaluation of inhibitors was complicated by the capricious behavior of cultures of sulfate-reducing bacteria used for inoculum in the test system. These bacteria, isolated from contaminated tanks, gradually lost activity over 2 or 3 months of serial transfers in API vials.[1/] Alternate transfers between API and Sisler's (6) medium, triple-strength (S3x)[1/], retained good viability for a longer time but this procedure too eventually failed to keep active cultures for inoculations. Aboard ship, these anaerobes develop under what appear to be inauspicious conditions. Something, however, obviously prevails in the shipboard situation to favor them. For maintaining cultures of suitable viability, the approach seeming to offer the most promise was to simulate tank conditions on a laboratory scale in which volumes of several liters would be used.

A mixed microbial population of sulfate reducers and other bacteria associated with them in a contaminated shipboard tank was considered more realistic for evaluating inhibitors than pure cultures of such bacteria as Desulfovibrio desulfuricans would be. Moreover, Postgate (7) has indicated where mixed cultures of Pseudomonas aeruginosa and the well known sulfate reducer Desulfovibrio desulfuricans showed an inhibition pattern differing from that of either one alone. This type of mixed microbial population is what actually developed when vials of API broth and tubes of S3x medium were inoculated with infected displacement water from the USS YORKTOWN. So it was hoped that the "water bottom" of our laboratory-simulated tank might be used directly as inoculum.

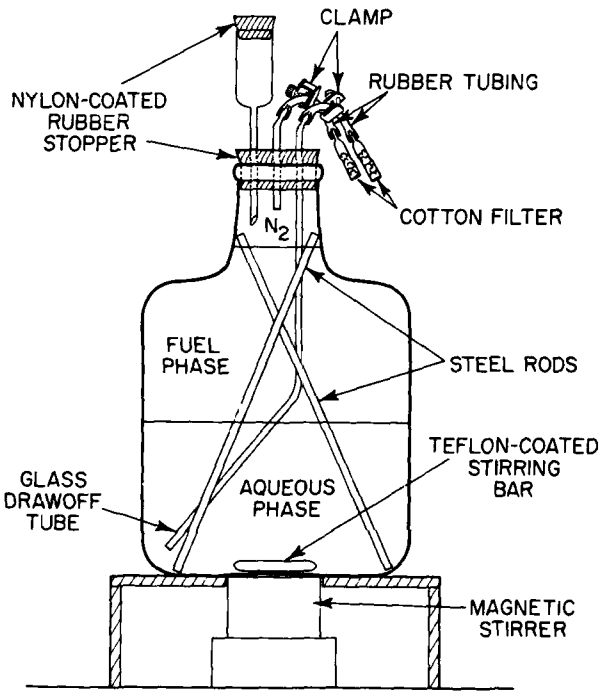
[1/] See Appendix for formulas of media.

For the past year the work has been concentrated (a) on finding means of culturing the bacteria so as to maintain a consistently reliable source of inoculum and (b) on developing a better test system for evaluating inhibitors. Selected compounds have been evaluated by improved procedures and results are reported here.

EXPERIMENTAL

(a) Maintenance of Inoculum

The mixed cultures secured from ships' water bottoms have been maintained in the laboratory by transfer in S3x medium. Basically the shipboard tank systems consist of a layer of fuel over an aqueous phase of sea water normally containing low concentrations of organic nutrients and a source of iron. In simple simulation thereof, a "tank" was assembled as follows. Ten liters of aged sea water was filtered through a 0.45-μ membrane filter into a 20-liter carboy and then enough Avgas filtered in to to fill the container to the neck. Two rods of 1020 steel, 1/4 inch in in diameter and long enough to extend from the neck to the side bottom, simulated the steel in a ship's tank and served also as source of iron for the bacteria. A magnetic stirrer was centered in the bottom and timed to operate for a minute once every hour to provide gentle agitation (Figure 1).



Since the filtered sea water would supply very little in the way of organic nutrients, it was supplemented with a small amount (0.3%) of trypticase soy broth (TSB)[2/]. Desulfovibrio species have been shown to grow well on this medium (8). Because this medium forms a slight precipitate with sea water, it was prepared separately in distilled water, 30 grams in 200 ml, sterilized and added to the carboy, giving 0.3% TSB in the aqueous phase. Finally, residual oxygen was removed by bubbling filtered nitrogen rapidly through the aqueous and fuel layers by way of the draw-off tube. In the assembly process, each constituent was sterilized by appropriate means.

The culture "tank" was then inoculated. Five ml of an actively growing culture of sulfate-reducing bacteria in S3x was pipetted into the carboy and nitrogen was again bubbled through the system. The culture was a composite of sulfate reducers obtained from samples of displacement water drawn from contaminated tanks of the USS YORKTOWN (1). Incubation has been at room temperature ($25^{o}C \pm 3^{o}$) in the dark. Over a period of months the small amount of TSB supplied in the carboy is depleted and the sulfate-reducing activity of the culture diminishes, as indicated by slow development of subcultures from the carboy into API and S3x. To keep the carboy culture sufficiently active the TSB and sea water are replenished about every six months. Two liters of the aqueous phase is siphoned off and replaced with 1800 ml of freshly membrane-filtered sea water plus 30 grams of TSB in 200 ml of distilled water which had been sterilized in an autoclave.

A similar 20-liter bottle was prepared in which the TSB was omitted. Growth was negative. Adding TSB in small increments and reinoculating gave no growth until a level of 0.15% TSB was attained. This carboy, designated "Carboy 2," has been maintained and replenished like Carboy 1.

By the third day of incubation turbidity from bacterial growth in Carboy 1 was increasing fairly rapidly but it remained light colored for about eight days. At two and a half weeks the development of sulfate-reducing bacteria was evident from the blackening of the aqueous phase and from the formation of hydrogen sulfide as indicated by its odor and

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[2/] See Appendix

darkening of lead acetate paper. It was interesting to note that the sections of the steel rods in the fuel corroded within a day after they were placed in the carboy but that the portions in the aqueous phase remained bright - at least as long as clarity of the liquid permitted observation. By four weeks the rods could no longer be seen in the aqueous phase because of its increasing turbidity. After more than a year the corrosion remains heavy on the portions of the rods in the fuel. On the other hand, in Carboy 2 (made up at first with only fuel, sea water and inoculum) the rods corroded promptly in both the fuel and the aqueous phases. Nevertheless, after addition of 0.15% TSB to Carboy 2, the growth pattern of sulfate reducers was similar to that in Carboy 1.

(b) Assay Method

Even though by this time the cultures from the carboys were actively growing, they would not develop when used as inocula for the test system: five ml of basic TSB medium plus 0.5 ml of inoculum overlaid with enough fuel to completely fill a 50-ml (25 x 150 mm.) screw-cap test tube. But positive growth was obtained when API vials were inoculated with the carboy culture. So the problem was not a lack of live organisms. As Grossman and Postgate (9), Postgate (10) and Zobell (11) have pointed out, the vagaries of these bacteria are many. We then undertook a long series of experiments, too long to detail here, dealing with volume of inoculum, surface/volume ratios, the continued anaerobicity of the inoculum and the composition of the assay medium, including its pH. Regarding the composition of the assay medium, the following four items - sodium lactate, ascorbic acid, sodium thioglycollate and magnesium sulfate - were added to the basic TSB formulation with favorable results. These additions are based on work reported by Allred (12), Butlin et al (13), Iverson (8), Grossman and Postgate (9) and Postgate (14). Ferrous ammonium sulfate was also included in the assay medium as indicator of growth of the bacteria as well as a source of required iron. The presence of ferrous ions in the medium, on the other hand, enhances resistance of sulfate

reducers to inhibitors (10) thus making the assay of the biocides more rigorous.

We established that a relatively large volume (4 ml) of our inoculum was desirable; this quantity of inoculum however, would give a false positive test immediately, because of residual hydrogen sulfide in the inoculum. So the following procedure was worked out to minimize H_2S. The required amount of culture from Carboy 1 was siphoned in beneath a protective layer of fuel or pure hydrocarbon; n-heptane was found to work very well. Excess H_2S was washed out by bubbling nitrogen through the liquid for about 15 minutes, and then letting it stand undisturbed under the hydrocarbon for 3 to 4 hours. Longer outgassing inhibited subsequent growth, perhaps by excessive depletion of hydrogen sulfide which would otherwise tend to maintain a favorable reducing environment (7,9,11,12). But on the other hand, Iverson (8) and Postgate (7) indicated that sulfide can be truly toxic to sulfate reducers. Perhaps the seeming contradiction is a matter of difference in concentrations.

We have noted above that TSB medium gives a precipitate when added to sea water. So for the supplemented TSB, a 30% solution was made up in distilled water; the inhibitor under test was also made up as a concentrated aqueous solution. All three liquids were appropriately sterilized. Suitable volumes of each were combined in an assay tube to make a total aqueous volume of 6 ml under a layer of fuel; 4 ml of the nitrogen-washed inoculum was then added beneath the fuel layer. When the total aqueous volume was thus brought to 10 ml, the desired concentrations of inhibitor and nutrients were attained. In no case did the addition of distilled-water solutions dilute the sea-water medium more than 10%. The final pH of this aqueous phase was 7.3 ± 0.1. The assay tube was filled to the top with fuel and closed with a Teflon-lined screwcap. The tubes were incubated in the dark at $26^{\circ} \pm 1^{\circ}C$ for three weeks; during this time they were inspected frequently for growth as evidenced by formation of black ferrous sulfide from the reaction of sulfide with ferrous ion in the assay medium. At the end of the

incubation period, tubes which were negative or dubiously positive were tested for the presence of viable organisms by inoculating tubes of S3x medium with a portion of the aqueous phase.

For the hydrocarbons used in these assays, the Avgas was from the same lot as that used previously (1). The sample of Navy Distillate Fuel (NDF), described as a "reference fuel", was furnished by courtesy of Naval Ship Engineering Center, Philadelphia Division. As stated earlier, this fuel is replacing certain other fuels in the Navy; it was included here in anticipation of microbiological problems that may develop with its use. Before sufficient quantity of NDF was available for assays of all the inhibitors, two small samples of distillate fuels, furnished by the Chemistry Division, NRL, were assayed against one inhibitor only (Table 1). One of these fuels, DF-N, was of Nigerian origin; the other, DF-V, was of Venezuelan origin. The DF-V was like and possibly from the same procurement lot as the NDF used in most of the assays under distillate fuel.

Regarding inhibitors, many substances have been reported (15,16,17,18) as inhibiting sulfate reducers, but most of these can be rejected on the basis of cost, the dosage required, or some other practical consideration. Two compounds were selected from Saleh's compilation (15), and five more from commercial information, on the basis of effectiveness at low concentrations and likely compatibility with hydrocarbon fuel. The two compounds chosen from the Saleh list are chlorhexidine diacetate, and the sodium salt of dichlorophene (2,2'-dihydroxy-5,5'-dichlorodiphenylmethane). The other five included three thiopyridine derivatives, an N-substituted s-triazine compound, and methylene bisthiocyanate in a solvent system containing dispersing agents.

DISCUSSION

A glance at the data of Tables 2 and 3 suffices to indicate the higher toxicity in our bioassay of the three pyridine derivatives in comparison to the other four compounds. The sodium compound (A), it must be pointed

out, performed poorly under one of the distillate fuels (Table 1) but we have no data to help decide whether the test was anomalous, or whether any of the other inhibitors would have done better. The butylamine derivative (B) is described by its supplier as a "jet-fuel biocide", inhibitory to Desulfovibrio desulfuricans at 2.4 ppm, as well as to other bacteria and fungi. In our test under Avgas, it was not so effective - the inhibitive dose was about 10 ppm. However, under Navy Distillate (which more nearly resembles jet fuel) it was apparently effective even at 5 ppm. Regarding practical application of (B) we are informed that it has an appreciable solubility in kerosene, over 400 ppm. One may suggest therefore, that (B) in a water bottom may dissolve appreciably in the oily sludge and attack the sulfate reducers protected therein. The disulfide compound (C) shows a toxicity comparable to those of (A) and (B). From observation of stock solutions of (C), we believe that it may be much less stable than the other two. This observation is consonant with the fact that (C) was advocated as having a minimal pollutant effect when it would be dumped back to the ocean because it would oxidize rather readily to innocuous products.

Our advance information on compounds (D) and (E) (15,19) indicated them to be more inhibitive than we found them to be, but differences in the assay methods can be invoked to explain the differences in apparent toxicity (7): ours was a mixed microflora under a layer of hydrocarbon, the others were pure-culture tests in single-phase aqueous systems. And, of course, there are differences in the composition of the nutrient media. Saleh (19) found the inhibitory action of the chlorhexidine compound (D) varied greatly among forty-five strains of sulfate-reducing bacteria - as much as ten-thousandfold; moreover, his salt-water strains had very high resistance to this substance. On the other hand, he found the substituted phenylmethane (E) to be a broad-spectrum inhibitor within a much narrower concentration range.

The inhibitors discussed above, (A) to (E) inclusive, were all highly purified compounds. The other two were proprietary formulations. The

substituted triazine (F) has been prescribed as an effective inhibitor of sulfate reducers in cutting-oil emulsions at 1500 ppm. These emulsions are so notoriously vulnerable to bacterial attack, that it is scarcely surprising that we found the compound to be effective at a much lower concentration - around 50 ppm of active toxicant. The second formulation (G) was inhibitory at 30 ppm of the toxic ingredient, methylene bisthiocyanate, which is 6% of the material as furnished. Hence the 30 ppm corresponds to 500 ppm of the product supplied.

The reader will note that many of the assays showed an initial (3-day) darkening which later fades. Some of it is doubtless due to traces of H_2S in the inoculum. However, as noted above, transfers from the tubes showing no or little blackening (0 to 1 rating) after 21 days tested negative in S3x medium thus verifying biocidal potency.

The limitations of the test-tube type of bioassay are well known, but we believe that at least the three heterocyclics (A), (B), and (F), and perhaps (E) and (G) d serve further study. The next step would be trials in fuel/water systems of larger capacities, with conditions thus more nearly resembling those in a ship's tank. Our experience as described above indicates that the smaller the test system, the more nutrients and growth promoters the mixed cultures of sulfate reducers require. Systems of about 5-gallon capacity could be handled in the laboratory, and from our work in developing carboy cultures for inoculum, no unrealistic supplements of nutrient would be entailed. We have noted above that the problem in the Fleet is aggravated by the bacteria's being occluded within protective lumps of debris. For this reason, the pilot test of selected inhibitors should be performed in heavily contaminated "tanks" as well as clean ones. In suggesting, purely on the grounds of bioassay data, compounds for additional study, we are not adverting to the obviously important matter of cost. But we have no data which would exclude any of them from consideration. And somewhere in the course of R & D development the pollutant possibilities of any good inhibitor must be evaluated. At NRL we could assess effects upon marine phytoplankton and small zooplankton when the tank tests warrant such inquiries.

SUMMARY

1. Sulfate-reducing bacteria, already found extensively in storage tanks of Avgas and in at least some diesel and JP-5 contaminations, will also grow under the proposed Navy Distillate Fuel. Possibilities of a need for corrective action should be considered.

2. Procedures for the long-term maintenance of active sulfate reducers in mixed culture and for the bioassay of inhibitors have been worked out.

3. Water soluble chemicals for inhibiting the bacteria have been reviewed and the most promising seven have been assayed in the laboratory. Of these, three - possibly five - appear to warrant trials that more nearly simulate conditions of use. A two-phase system of perhaps 5-gallon total volume is being considered.

4. Though most of the experiments to date have been performed with Avgas as the supernatant, the data on bio-inhibitors should be equally applicable to other types of hydrocarbon fuel, including Navy Distillate and Marine Diesel. However, each inhibitor should be evaluated with the fuel concerned, since there are variations in effective concentrations among the different hydrocarbons.

5. In view of the expected widespread use of Navy Distillate, its susceptibility as well as that of Marine Diesel, to organisms other than sulfate reducers - such as fungi, yeasts and aerobic bacteria - will be examined.

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Table 1. Toxicant Effects Upon Sulfate-Reducing Bacteria Under Two Distillate Fuels

Fuel	Inhibitor	Conc. ppm	Growth Response[1] 3 days	10 days	21 days
DF-N[2]	None	--	4	4	4
	(A) Sodium pyridyl-N-oxide-2-thiolate	5	3	4	4
		10	1	<1	0
DF-V[2]	None	--	4	4	4
	(A) Sodium pyridyl-N-oxide-2-thiolate	5	2	4	4
		10	2	4	4

[1] Arbitrary scale based on visible blackening when tube is shaken, viz. 1 = slight gray, 4 = intense opaque black. All assays were run in duplicate and since duplicate responses were always the same, a single numerical entry is shown in the table.

[2] DF-N was of Nigerian origin, DF-V of Venezuelan origin.

Table 2. Toxicant Effects Upon Sulfate-Reducing Bacteria Under Avgas

Inhibitor	Conc. ppm	Growth Response[1] 3 days	10 days	21 days
None	--*	<1	3	4
	--	4	4	4
(A) Sodium pyridyl-N-oxide-2-thiolate	5	1	0	0
	10	1	0	0
(B) 2-(t-Butylamine)thio-pyridine-N-oxide	1	4	4	4
	1	3	4	4
	5	4	3	4
	5	2	2	2,1
	10	2	0	0
	20	3	0	0
(C) Bis(2-pyridyl-N-oxide) disulfide	1	3	4	4
	5	3	2	3,4
	10	2,3	0	0
	20	3	0	0
(D) Diacetate of chlorhexidine	1*	1	3	4
	5*	1	3	4
	10	4	4	4
	20	4	4	4
(E) Sodium salt of dichlorophene	1*	1	4	4
	20*	<1	1	1
(F) Hexahydro-1,3,5-tris-(2-hydroxyethyl)-s-triazine[2]	5	4	4	4
	25	4	4	4
	25	4	4	4
	50	1	0	0
	100	2	0	0
	100	0	0	0
	400	0	0	0
(G) Methylene bisthiocyanate[3]	6	3	4	4
	30	<1	0	0
	60	0	0	0

* Data were gathered in several experiments, each having growth controls containing no inhibitor. These controls were intense black throughout except in the first experiment, designated by an asterick(*).

[1] Arbitrary scale based on visible blackening when tube is shaken, viz. 1 = slight gray, 4 = intense opaque black. All assays were run in duplicate and whenever the responses were the same, a single numerical rating is shown in the table; when the responses were different, both ratings are given.

[2] Concentrations given are based on active ingredient; material is 78.5% pure.

[3] Concentrations given are based on active toxicant, which is 6% of the product supplied.

Table 3. Toxicant Effects Upon Sulfate-Reducing Bacteria Under Navy Distillate Fuel

Inhibitor	Conc. ppm	Growth Response[1] 3 days	10 days	21 days
None	--*	2	4	4
	--	4	4	4
(A) Sodium pyridyl-N-oxide-2-thiolate	5	2	0	0
	10	2	0	0
(B) 2-(t-Butylamine)thio-pyridine-N-oxide	1	3	3	1
	1	4	4	4
	5	3	0	0
	5	1	0	0
	10	<1	0	0
	20	2	0	0
(C) Bis(2-pyridyl-N-oxide) disulfide	1	3	4	4
	5	1,2	0,3	0,3
	10	1	0	0
	20	<1	0	0
(D) Diacetate of chlorhexidine	1*	2	4	4
	5*	<1	3	4
	10	4	4	4
	20	4	4	4
(E) Sodium salt of dichlorophene	1*	2	4	4
	20*	1	1	1
(F) Hexahydro-1,3,5-tris-(2-hydroxyethyl)-s-triazine[2]	5	4	4	4
	25	4	4	4
	25	2	4	4
	50	1	0	0
	100	2	0	0
	100	0	0	0
	400	0	0	0
(G) Methylene bisthiocyanate[3]	6	3	4	4
	30	<1	0	0
	60	0	0	0

*Data were gathered in several experiments, each having growth controls containing no inhibitor. These controls were intense black throughout except in the first experiment, designated by an asterisk(*).

[1] Arbitrary scale based on visible blackening when tube is shaken, viz. 1 = slight gray, 4 = intense opaque black. All assays were run in duplicate and whenever the responses were the same, a single numerical rating is shown in the table; when the responses were different, both ratings are given.

[2] Concentrations given are based on active ingredient; material is 78.5% pure.

[3] Concentrations given are based on active toxicant, which is 6% of the product supplied.

APPENDIX

Formulas of Media

(1) API Vials: Difco Laboratories, Bacto-Sulfate API Broth, 9 ml per vial, prepared according to formulation given in the American Petroleum Institute Recommended Practice 38, First Edition, 1959, cited in Difco Supplementary Literature, Nov. 1966, page 302.

This API broth contains in 100 ml of medium:

Bacto - Yeast Extract	0.10	g
Ascorbic acid	0.01	g
Sodium lactate	0.52	g
Magnesium sulfate	0.02	g
Dipotassium phosphate	0.001	g
Ferrous ammonium sulfate*	0.01	g
Sodium chloride	1.00	g

(2) Sisler's Triple-Strength Medium (S3x):

Magnesium sulfate	0.06	g	Medium is dispensed into
Ammonium sulfate	0.30	g	20x125 mm screw-capped
Sodium sulfite	0.03	g	tubes, 20 ml per tube,
Dipotassium phosphate	0.06	g	and autoclaved at 15 lb
Ferrous ammonium sulfate*	0.03	g	for 15 min.; cooled to
Ascorbic acid	0.03	g	about 50°C, then in-
Calcium lactate	1.05	g	verted repeatedly until
Difco neopeptone	0.30	g	medium gels.
Difco agar	0.30	g	
Sea water, filtered	100.	ml	

(3) Trypticase Soy Broth (TSB); dehydrated product, BioQuest, BBL #11768:

The customary 3% solution contains in 100 ml:

Trypticase	1.7	g	Note - TSB without the
Phytone	0.3	g	dextrose, (BBL #11774)
Sodium chloride	0.5	g	is equally satisfactory.
Dipotassium phosphate	0.25	g	
Dextrose	0.25	g	

For all TSB media in sea water, a 30% "stock" solution is prepared in distilled water and autoclaved at 15 lb for 15 minutes. The required volume of this concentrate to give a 0.3% TSB can be added to membrane filtered sea water.

APPENDIX (cont'd)

TSB media as used in experiments:

(a) Basic TSB:

TSB, 30% stock solution	1.0	ml	A 2.5% solution of $Fe(NH_4)_2(SO_4)_2.6H_2O$ is prepared in distilled water and 2 ml of this membrane filtered (MF'd) into the sea water.
Ferrous ammonium sulfate*	0.05	g	
Sea water, membrane filtered	97.	ml	

(b) Supplemented TSB:

TSB, 30% stock solution	1.0	ml	
Ferrous ammonium sulfate*	0.05	g	in 2 ml dist. H_2O, MF'd
Magnesium sulfate	0.2	g	} in 5 ml dist. H_2O, autoclaved
Sodium lactate, 60%	0.4	ml	
Ascorbic acid	0.01	g	} in 2 ml dist. H_2O, MF'd
Sodium thioglycollate	0.01	g	
Sea water, membrane filtered	90.	ml	

*The $Fe(NH_4)_2(SO_4)_2.6H_2O$ serves as indicator of production of H_2S by the bacteria and hence as means of evaluating their growth.