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Topic: Comments Regarding U.S. Coast Guard Proposed Goals and Standards
for Ballast Water Treatment; **Docket Number USCG-2001-10486**

Comments made on behalf of Baker Petrolite Corporation by:

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Comments on possible goals:

G1. Should read "No discharge of **viable** zooplankton and . . ." If the organisms have been killed or deactivated, their presence in the discharge should not be perceived as noncompliance.

"For bacteria, *Enterococci* and *Escherichia coli* will not exceed . . ." Bacteria chosen as markers of successful ballast water treatment should be of a genus typically found in seawater, rather than those of concern in drinking water. Only by monitoring for control of varieties of bacteria expected to be present in marine environments can the efficacy of the treatment regime on bacteria be determined. Such control can be an excellent indicator of control of potential pathogens as well. Examples of bacteria that would be naturally present in significant quantities, and therefore more useful indicators of a treatment's success are *Pseudomonas sp.* and *Bacillus sp.* Also, it should specify **viable** bacteria only, for the same reasoning provided earlier.

G2. Treat for living organisms at least to the same extent as drinking water. This is an arbitrary and irrelevant goal. Standards for drinking water are related to

organisms that may be present in drinking water and that could impact public health or appeal of the water for drinking purposes (i.e., taste, odor). These standards and the named organisms are not necessarily relevant to the issue of controlling noninvasive aquatic species.

G3. "Ballast water treatment technologies would demonstrate, through direct comparison with ballast water exchange, that they are at least as effective as ballast water exchange. . ." When it has been clearly acknowledged in earlier comments and summarized in this docket that the efficacy of ballast water exchange has been difficult to establish, is not well documented, and is prohibitively expensive to validate, this goal may not be achievable.

Comments on possible standards:

S1. This would be a viable standard. It would be advisable to include a representative variety of bacteria as one of the organisms to be monitored.

S2. Limiting a standard to organisms only of a given size would not achieve adequate protection against introduction of all invasive aquatic species.

S3. "Remove 99% of all . . ." No standard should be limited to the removal of organisms. By not specifically including wording to include removal, kill and/or deactivation, the allowable treatment methodologies become limited to only filtration methods. Chemical treatments and other methods of control could no longer be considered, even when such methods are clearly superior to filtration.

Also, bacteria such as *Enterococci* and *Escherichia coli* are not typical in marine environments, and therefore are not a good technical fit as markers of efficacy. *Pseudomonas sp.* and/or *Bacillus sp.* would be better choices.

S4. "Discharge no organisms . . ." As stated above, any standard must not be limited to physical removal of organisms. To allow evaluation and use of all possible treatment methodologies, it must be broadened to "discharge of no viable organisms."

Response to Questions:

Q1. Neither G1, G2, nor G3 are satisfactory goals as currently stated. G1 should include a bacteria commonly found in marine environments. G2 is arbitrary. G3 can only be viable if the efficacy of ballast water exchange can be clearly and consistently demonstrated.

Q2. Of the four standards proposed, S1 is the closest to acceptable as an interim standard. It would be advisable to include a variety of bacteria commonly found in marine environments as one of the organisms monitored.

Q3. Baker Petrolite has demonstrated in laboratory studies the efficacy of acrolein in controlling organisms potentially of concern in ballast water discharge. Our technical report is enclosed with these comments. Additionally

we are including a summary of other aquatic toxicology studies which indicate acrolein's efficacy in this venue.

Q4. No comments.

Q5. No comments.

Q6. The potential environmental impacts of the goals or standards would be directly related to the strictness and viability of such goals or standards. Having a numeric standard, 95% or higher kill, removal or deactivation of organisms, would have the most measurable impact in preventing introduction of unwanted invasive species.

Other comments:

Baker Petrolite will shortly be conducting a vessel trial of our chemical treatment technology, utilizing acrolein. We anticipate this will provide more favorable data regarding the cost and efficacy of this technology versus the majority of those referenced in Table 1. We will be happy to share these results once compiled.

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RESEARCH AND TECHNICAL OPERATIONS REPORT

DATE: June 19, 2000

REPORT TITLE: Procedures and results for planktonic kill studies to determine the efficacy of acrolein for control of bacteria and dinoflagellates in Galveston sea water

REPORT NUMBER: FCT-549-200

PROJECT NUMBER: FC2507-651

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PURPOSE:

This work was performed to determine the efficacy of low concentrations of acrolein against microorganisms commonly found in seawater environments.

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Background

The introduction of foreign marine life, especially non-indigenous species, into U.S. waters is a growing concern nationwide. Therefore, new regulations are being put into place in an attempt to control the organisms in ballast water discharge from ocean vessels. These regulations prove costly for the shipping companies and the efficacy of current technology (ballast water exchange) is in question. In an effort to determine whether acrolein is a viable alternative to current treatment options, a kill study was performed to determine the potential for low concentrations of acrolein to provide effective control of a variety of different prokaryotic (bacteria) and eukaryotic (dinoflagellates) microorganisms in sea water. The organisms selected for this study included mixed cultures of general aerobic and facultative anaerobic bacteria that are always present in sea water, pure cultures of spore forming bacteria which are very difficult to kill due to resistance of the spores to chemical or physical treatment, pure cultures of gram negative and gram positive bacteria which are typically used as benchmarks for comparisons of microbiocidal efficacy, and dinoflagellates which have been reported to be very difficult to control by other treatment methods. This study was designed to simulate the conditions of an ocean vessel's ballast water during a voyage.

Methodology

Bacterial Cultures

Sea water was collected from Galveston Bay in the Gulf of Mexico. Two liters of the sea water were filtered sterilized by passing the raw sea water through a 0.2 μm membrane into a sterile filtration flask and stored in the refrigerator for the kill study experiments. One mL aliquots of the sea water were transferred into a series of culture bottles containing 9 mL each of phenol red dextrose medium supplemented with 3.5% Instant Ocean sea salts to isolate general aerobic and facultative anaerobic bacteria (GAB). The sea water was also transferred into a series of culture bottles containing Baker Petrolite's proprietary formulation (West Texas formulation) of sulfate reducing bacteria (SRB) medium supplemented with 3.5% Instant Ocean sea salts for isolation of SRB. These cultures were incubated at ambient temperatures (approximately 17-23°C) until the day of the kill study.

The goal of this experiment was to test acrolein against representatives of the main classes of bacteria encountered in marine environments. For this purpose, GAB, diverse group of aerobic and facultative anaerobic bacteria, and SRB were isolated from Galveston sea water as described above. In addition, the specific bacterial strains, *Pseudomonas fluorescens* (ATCC #6972), *Bacillus cereus* (ATCC #2), *Bacillus subtilis* (ATCC #465), and *Staphylococcus epidermidis* (ATCC #155), were obtained from American Type Culture Collection (ATCC, Manassas, VA). *Pseudomonas fluorescens* is a Gram negative, non-sporulating bacterium. *Bacillus cereus* and *Bacillus subtilis* are Gram positive, spore-forming bacteria. *Staphylococcus epidermidis* is a Gram positive, non-sporulating bacterium. These strains of bacteria were revived on nutrient agar plates and incubated at 30°C. Each isolate was then streaked for isolation onto nutrient agar amended with 3.5% Instant Ocean sea salts to obtain mutated bacteria from each strain capable of growing in sea water salt concentrations. Isolated bacterial colonies were transferred from the 3.5% nutrient agar plates into a series of culture bottles containing 9 mL of 3.5% nutrient broth and incubated at 30°C until the day of the kill study.

On the day of the kill study, the *Bacillus subtilis* cultures were combined into a common sterilized glass jar, mixed thoroughly, and split into two separate sterile containers. One of the splits was heat shocked to isolate those bacterial cells that had formed endospores. The heat shock procedure involved placing the liquid inoculum into a water bath containing 100°C water for 30 minutes. This procedure should kill all vegetative bacterial cells in the culture and select for those bacterial cells that had formed endospores prior to the heat shock treatment.

Dinoflagellate Culture

Three different sources were contacted to obtain dinoflagellate cultures. *Gymnodinium breve* (CCMP #718) was obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) in West Boothbay Harbor, Maine. *Gymnodinium sanguineum* was obtained from Roscoff Culture Collection of Marine Phytoplankton (RCC) in France. *Cryptocodinium cohnii* (ATCC #30334) was obtained from American Type Culture Collection in Manassas, VA. L1 culture medium (CCMP formulation) was utilized for growing both *Gymnodinium* species, while ATCC culture medium 460 A2E6 was utilized for growth of the *Cryptocodinium* species. Cultures were grown using a 30 watt, 10,000 Kelvin high intensity purified super daylight lamp with a 14 hour light/10 hour dark cycle. They were incubated at ambient temperatures (17-23°C). Good growth was obtained with the *Gymnodinium sanguineum* culture, while poor to marginal growth was obtained for the other two cultures. Therefore *Gymnodinium sanguineum* was the dinoflagellate culture chosen for the kill study.

Planktonic Kill Study

Bacterial Study

A planktonic time kill study was performed to determine what concentration of acrolein would be required to provide the best kill of all bacterial types described previously and whether the kill could be maintained for a 72 hour time period. This time frame was chosen to mimic the amount of time the ballast water would be maintained on a ship. The filter-sterilized Galveston sea water was distributed into sterilized 8 ounce glass prescription bottles in 99 mL aliquots. One mL aliquots of each type of bacterial culture were inoculated into four of the prescription bottles. Acrolein was added to each set of four bottles for each bacterial type at a concentration of 0 ppm (control, bacteria only), 1 ppm, 3 ppm, and 10 ppm. All prescription bottles were incubated at room temperature in the dark. The surviving bacteria in each of the acrolein-treated and control samples were enumerated into the previously described culture media for each of the samples following 24 and 72 hours contact with the acrolein using the serial dilution method. Serial dilutions were performed according to BPCI test method TMM 3.36 (based on the NACE Standard Test Method 0194-94 "Field Monitoring of Bacterial Growth in Oilfield Systems"). A six vial serial dilution string was run for the acrolein-treated samples and a 12-vial string was utilized for all controls, allowing for detection of a maximum bacterial reduction of 12 orders of magnitude. The GAB and SRB culture bottles were incubated at ambient temperatures (17-23°C) and the pure culture isolates were incubated at 30°C. Culture bottles were visually inspected daily to monitor bacterial growth. Results were reported when no additional bottles turned positive for three consecutive days (10 days incubation period). The results of the kill study are presented in Table 1.

Dinoflagellate Study

A planktonic time kill study was performed to determine what concentration of acrolein would be required to provide kill of *Gymnodinium sanguineum* and whether control could be maintained for a 72 hour time period. *Gymnodinium sanguineum* was cultured in L1 medium under the

conditions described previously. In preparation for the kill study, four 100-mL aliquots of the culture were dispensed into 125 mL glass centrifuge tubes and centrifuged using a GCA/Precision Scientific Universal Centrifuge (Chicago, IL) for 10 minutes at 1000 rpm. The supernatant was decanted and the pellets resuspended in 50 mL of filter-sterilized Galveston sea water in a wash step to remove remnants of the L1 culture medium, which could interfere with the acrolein efficacy. This mixture was centrifuged for 10 minutes at 1000 rpm and the supernatant decanted. The pellets were resuspended in 3 mL of Galveston sea water and the concentrated cell suspensions were combined into a single culture. Cell viability was confirmed by looking for motile and structurally intact cells in an aliquot of the concentrated cell mixture by visible light microscopy. Ninety-eight mL of filter-sterilized Galveston sea water was distributed into eight sterilized 8-ounce glass prescription bottles. Two mL aliquots of the concentrated *Gymnodinium sanguineum* suspension were added into each of the prescription bottles. Acrolein was added in duplicate to the prescription bottles at a concentration of 0 ppm (control, *Gymnodinium sanguineum* only), 1 ppm, 3 ppm, and 10 ppm. All prescription bottles were incubated at room temperature under the lighting conditions described previously. The surviving dinoflagellates were enumerated by serially diluting each of the acrolein-treated and control samples into a series of 6 glass test tubes containing 9 mL each of L1 culture media following 24 and 72 hours contact with the acrolein. The inoculated L1 media tubes were incubated at ambient temperatures (17-23°C) and appropriate lighting conditions. At the start of the experiment just prior to acrolein addition and at the time of sampling (24 and 72 hours), an aliquot of fluid from each of the prescription bottles was analyzed via light microscopy at 100X magnification to ensure that motile, structurally intact dinoflagellates were present. The inoculated culture tubes from the serial dilutions were also visually inspected daily to monitor dinoflagellate growth. A tube was marked as positive for growth if viable organisms were observed. Adverse effects on the dinoflagellates following acrolein addition were also noted and are recorded and photographed (Table 2, Photographs 3 and 4). The results of the kill study and visual observations are presented in Table 2.

Table 1. Acrolein Ballast Water Bacterial Kill Study Results

Organism	Acrolein Concentration (ppm)	Log ₁₀ Bacteria / mL (24 hours contact time)	% Reduction from Control (0 ppm) (24 hours contact time)	Log ₁₀ Bacteria / mL (72 hours contact time)	% Reduction from Control (0 ppm) (72 hours contact time)
<i>Staphylococcus epidermidis</i>	0 ppm	9	-	7	-
	1 ppm	2	99.99999	2	99.999
	3 ppm	1	99.999999	1	99.9999
	10 ppm	1	99.999999	1	99.9999
<i>Bacillus subtilis</i>	0 ppm	6	-	6	-
	1 ppm	2	99.99	2	99.99
	3 ppm	2	99.99	2	99.99
	10 ppm	1	99.999	1	99.999
<i>Bacillus subtilis</i> (heat shocked ¹)	0 ppm	6	-	6	-
	1 ppm	2	99.99	3	99.9
	3 ppm	2	99.99	ND ²	≥99.99999
	10 ppm	1	99.999	ND	≥99.99999
<i>Pseudomonas fluorescens</i>	0 ppm	6	-	≥ 12	-
	1 ppm	2	99.99	2	≥99.99999999
	3 ppm	1	99.999	1	≥99.999999999
	10 ppm	1	99.999	1	≥99.9999999999
<i>Bacillus cereus</i>	0 ppm	7	-	6	-
	1 ppm	2	99.999	3	99.9
	3 ppm	1	99.9999	2	99.99
	10 ppm	1	99.9999	1	99.999
Galveston GAB ³	0 ppm	11	-	≥ 12	-
	1 ppm	5	99.9999	4	≥99.999999
	3 ppm	3	99.999999	1	≥99.999999999
	10 ppm	ND	≥99.999999999	ND	≥99.9999999999
Galveston SRB ⁴	0 ppm	11	-	7	-
	1 ppm	3	99.999999	5	99
	3 ppm	2	99.9999999	ND	≥99.99999
	10 ppm	ND	≥99.999999999	ND	≥99.99999

¹ The *Bacillus subtilis* culture was split and half of the culture was heat shocked by boiling at 100°C for 30 minutes to select for those bacteria that had produced endospores.

² ND = none detected.

³ Log₁₀ general aerobic and facultative anaerobic bacteria (GAB) isolated in 3.5% NaCl Phenol Red Dextrose culture bottles from sea water collected from Galveston Bay in the Gulf of Mexico.

⁴ Log₁₀ Sulfate Reducing Bacteria (SRB) isolated in 3.5% NaCl West Texas SRB culture bottles from sea water collected from Galveston Bay in the Gulf of Mexico.

Table 2. Acrolein Ballast Water Dinoflagellate Kill Study Results

Acrolein Conc. (ppm)	24 Hour Contact Time			72 Hour Contact Time		
	Mean Log ₁₀ Dinoflagellates / mL	% Reduction from Control (0 ppm)	Structurally Intact Motile Cells? ²	Mean Log ₁₀ Dinoflagellates / mL	% Reduction from Control (0 ppm)	Structurally Intact Motile Cells? ²
0 ppm	5	-	yes	4	-	yes
1 ppm	BD ³	≥99.999	no	BD	≥99.99	no
3 ppm	BD	≥99.999	no	BD	≥99.99	no
10 ppm	BD	≥99.999	no	BD	≥99.99	no

¹ Mean log₁₀ number of viable *Gymnodinium sanguineum* dinoflagellates recovered via serial dilution into duplicate series of L1 media culture tubes.

² Viability determined by analyzing an aliquot of each replicate sample from each treatment regime for motile and structurally intact *Gymnodinium sanguineum* dinoflagellates by light microscopy at 100 X magnification.

³ BD = below detection limit of assay.

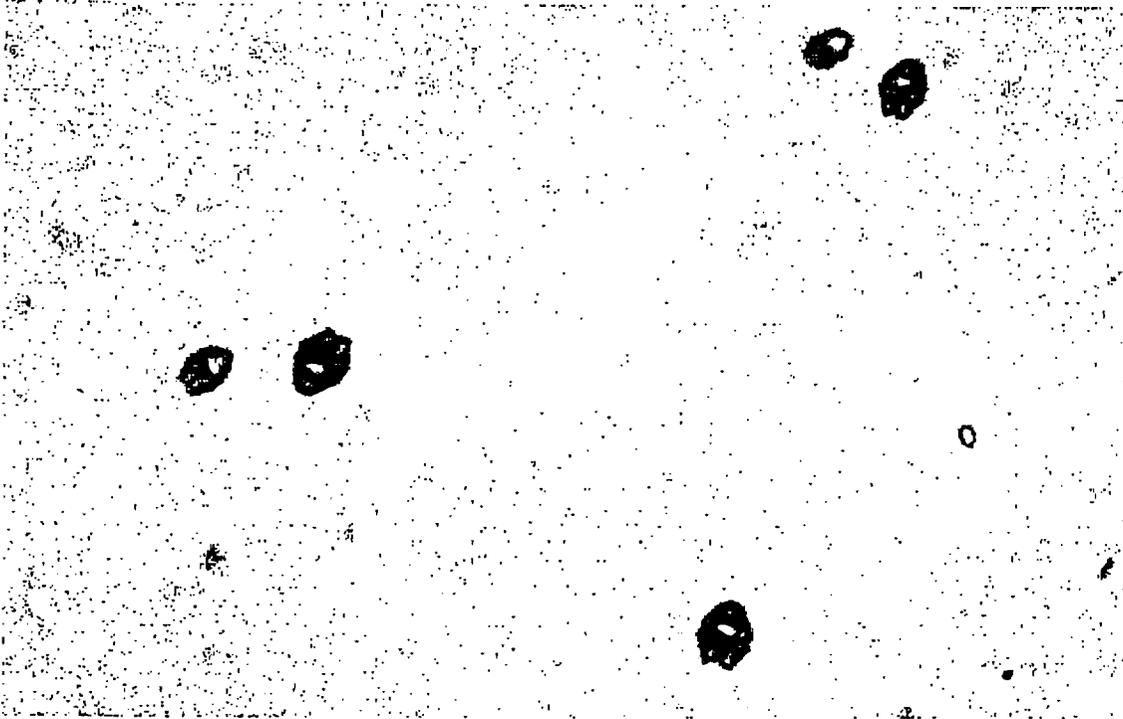
Results

Bacterial Kill Study

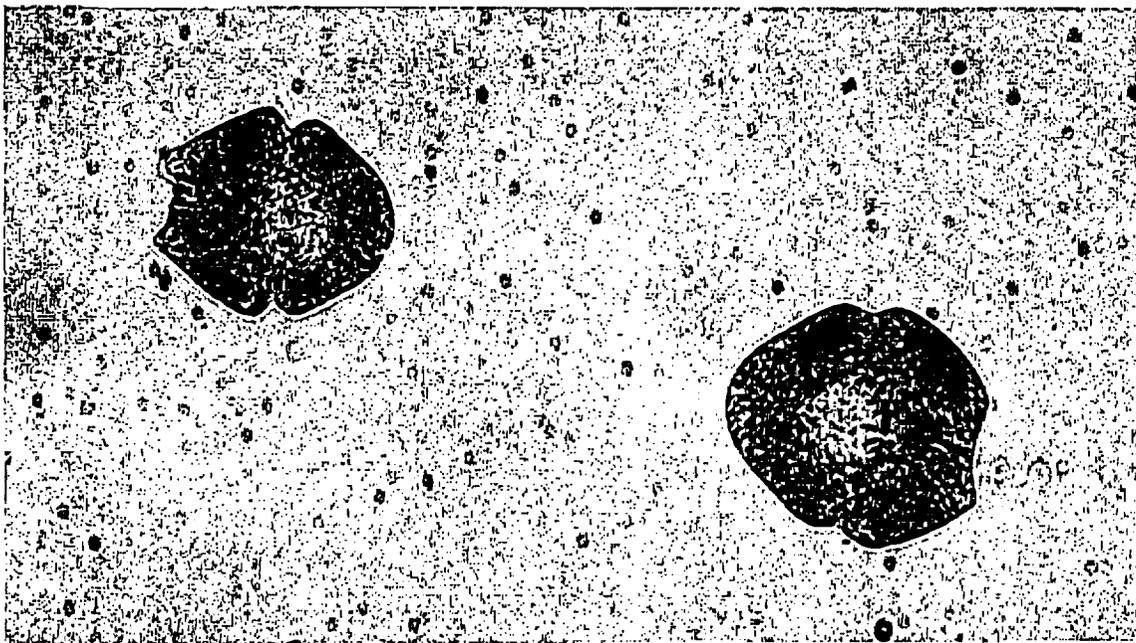
- Significant reductions in all types of bacteria tested were achieved with the lowest concentration of acrolein used in the study (1 ppm). It is anticipated that 3 ppm will be used during initial field trials of acrolein. At this concentration, reductions in bacteria numbers ranged from 4 orders of magnitude (99.99% reduction) with the 3 spore forming organism *Bacillus subtilis* to eleven orders of magnitude (99.99999999 % reduction) with both general aerobic bacteria isolated from Galveston, Texas sea water and the pure culture of *Pseudomonas fluorescens*.

Dinoflagellate Kill Study

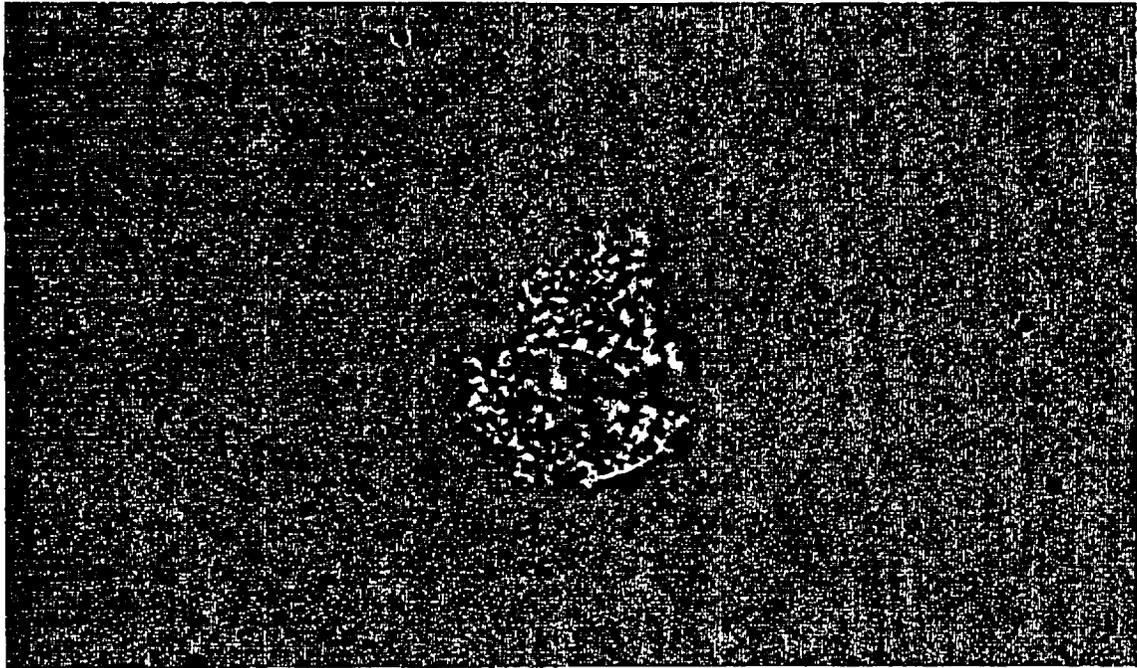
- All concentrations of acrolein (1, 3, and 10 ppm) were capable of reducing the dinoflagellate concentration below the detectable limit of the assay.
- No viable, motile dinoflagellates were observed in any of the acrolein treated samples.
- The integrity of the dinoflagellate cell was completely destroyed by the acrolein application. Photographs of the dinoflagellate cells before and after acrolein treatments are shown in Photographs 1 - 4.



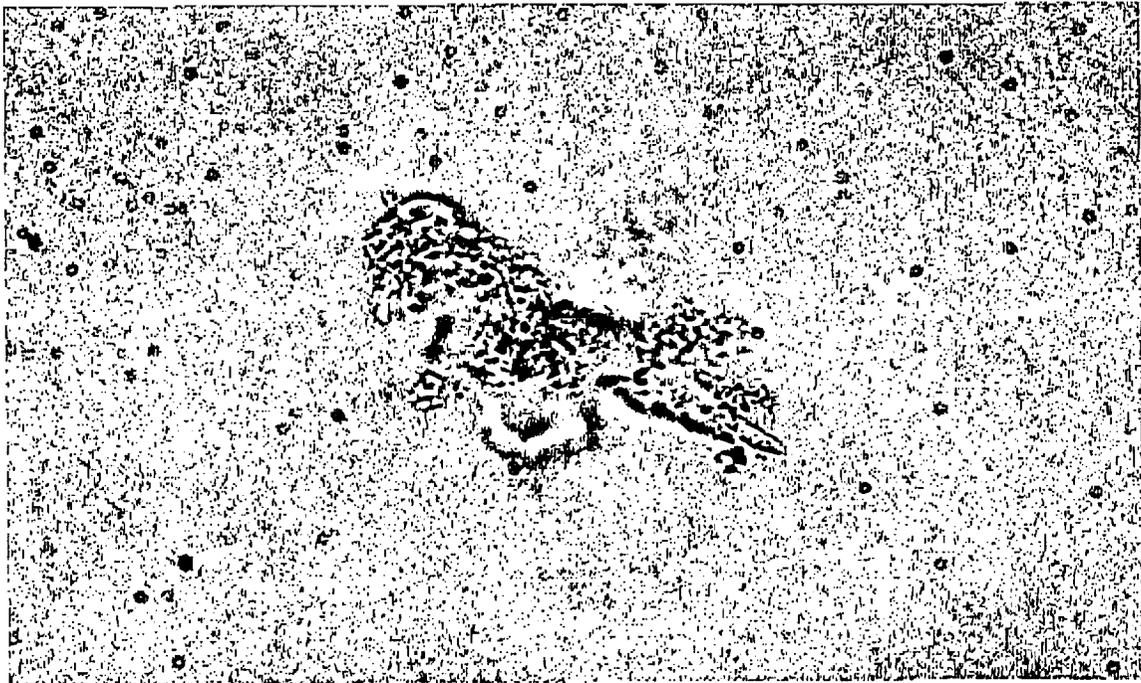
Photograph 1
Viable *Gymnodinium sanguineum* Dinoflagellates
Bright Field Microscopy, 100X Magnification



Photograph 2
Viable *Gymnodinium sanguineum* Dinoflagellates
Bright Field Microscopy, 400X Magnification



Photograph 3
***Gymnodinium sanguineum* Dinoflagellates Following Exposure to Acrolein**
Bright Field Microscopy, 400X Magnification



Photograph 4
***Gymnodinium sanguineum* Dinoflagellates Following Exposure to Acrolein**
Bright Field Microscopy, 400X Magnification

Acrolein Studies Summary (2)

Study Title	Laboratory	Completion Date	Results	Citations
Shwater Fish Toxicity (LC50 at 96 Hours) in a mwater Species (Bluegill Sunfish) and a dwater Species (Rainbow Trout)	ABC Laboratories (Columbia, MO)	1980	In acute flow-through tests, the 96 Hour LC50 for both species was approximately 24 ppb.	
Shwater Invertebrate Toxicity (LC50 at 48 Hours) <i>Daphnia magna</i>	ABC Laboratories (Columbia, MO)	1980	In an acute flow-through test, the 48 HR LC50 for <i>Daphnia magna</i> was 22 ppb.	
ile Toxicity to Eastern Oysters Under Flow- ough Conditions with Acrolein	Springdom Laboratories, Inc. (Wareham, MA)	1984	LC50 = .18 ppm; NOEC = .092 ppm.	
ile Toxicity to Mysid Shrimp Under Flow-Through iditions with Acrolein	Springdom Laboratories, Inc. (Wareham, MA)	1984	LC50 = .60 ppm; NOEC = .036 ppm.	
ile Toxicity to Sheepshead Minnow Under Flow- ough Conditions with Acrolein	Springdom Laboratories, Inc. (Wareham, MA)	1984	LC50 = .57 ppm; NOEC = .19 ppm.	