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Fuel-soluble biocides for control of *Cladosporium resinae* in hydrocarbon fuels

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SUMMARY

Five fuel-soluble biocides – a benzimidazole fungicide, an organoboron, a pyridinethione and two isothiazolone products – were evaluated for inhibition of a typical hydrocarbon fuel contaminant, *Cladosporium resinae,* in fuel/water systems. The biocides exhibited marked differences in anti-fungal activity with storage and in the presence of sludge. A methylchloro/methyl-isothiazolone mixture prevented growth of the fungus at a concentration of one part per million and, in contrast to other biocides tested, showed no tendency to be inactivated by storage or the presence of sludge.

INTRODUCTION

Microbial contamination of hydrocarbon fuels can cause operational problems ranging from corrosion to filter plugging wherever the presence of water offers the opportunity for growth [3]. On naval ships, water is invariably present in fuel storage tanks as a result of condensation or deliberate addition as ballast [5,14]. Because microbial contamination occasionally causes problems which are time-consuming and expensive to correct, it has been considered advisable to have effective methods on hand for coping with acute situations which may arise in the future.

Biocides offer an obvious method of preventing microbial growth. Earlier work in this laboratory emphasized biocides which could be added directly to the water phase, because it appeared unlikely that fuel-soluble biocides at acceptable concentrations could partition sufficiently into a relatively large volume of water to reach a biocidal concentration there [6–8]. The present study was undertaken because fuel-soluble biocides which are inhibitory at very low concentrations are now available, and it appeared worthwhile to evaluate certain of these in systems relevant to the situation in ship storage tanks. An important practical advantage in the use of fuel-soluble biocides would be that fuel could be pretreated with biocide at the necessary concentration, and the handling and injection of toxic materials on ships could be avoided.

Choices of biocides for this study were made on the basis of the work of other investigators

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[2,3,11,15,17,18], previous studies in this laboratory [6–8] and preliminary experiments. For comparison, a widely used fuel biocide was included. The anticipated applications required that emphasis be placed on determining the stability of the biocides with storage in fuel/water systems and their susceptibility to inactivation by fuel tank sludge. The fungus, *Cladosporium resinae,* was chosen as the test organism because it is ubiquitous in fuel systems and is recognized as a significant source of particulate contamination [3,14]. Additional screening studies are in progress with mixed bacterial inocula containing sulfate-reducers, a well-known cause of sulfur contamination in stored fuels.

MATERIALS AND METHODS

Biocides

Table 1 shows the sources, letter designations, and active ingredients of the biocides used; Fig. 1 shows the chemical structures. In the test systems described below, biocides were added to autoclave-sterilized naval distillate fuel. Two different concentrations of each biocide were selected to give border-line control of microbial growth at the lower concentration and complete control at the higher concentration. These concentrations were calculated on a weight basis (w/w).

Test organism

Cladosporium resinae DK, originally isolated from a contaminated ship tank, was grown on Potato Dextrose Agar (Difco, Detroit, MI) supplemented with 0.5% yeast extract (Difco). Inocula were prepared by washing the surface of actively growing 1-week-old slant cultures with a small amount of 0.05% Tween 80 in Bushnell-Haas mineral salts medium [1]. The resulting suspension was diluted with additional medium to give viable counts of about 10^7 colony-forming units per ml.

Test systems

The general procedure was to challenge biocide-treated fuel/water test units which had been stored for periods of up to 6 months in the presence and absence of sludge with inoculations of fungi and to monitor growth thereafter.

To test the effect of storage, 4 ml of sterile Bushnell-Haas mineral salts medium [1] were added to 25 × 145 mm test tubes and overlaid with 40 ml of sterile naval distillate fuel with or without dissolved biocide. At zero time and after 2, 4, 8, 16 and 24 weeks of storage at 26°C, the water bottoms of du-

Table 1

Biocides tested

Biocide designation	Active ingredients	Stock solution	Source
A	1-(Butylamino)carbonyl-^{1}H-benzimidazol-2-yl carbamic acid methyl ester	5% in dimethyl formamide + 2.5% butyl isocyanate	DuPont
B	Mixture: 2,2′-oxybis-(4,4,6-trimethyl-1,3,2-dioxaborinane) (27.4%) and 2,2′(1-methyltrimethylene dioxy)bis-(4-methyl-1,3,2-dioxaborinane) (67.6%)	95% in petroleum naphtha	U.S. Borax
C	Mixture: 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one	1.5% in dipropylene glycol	Rohm and Haas
D	2-*N*-octyl-4-isothiazolin-3-one	45% in propylene glycol	Rohm and Haas
E	Tertiary butylamine pyridinethione	1.69% in propylene glycol	Olin

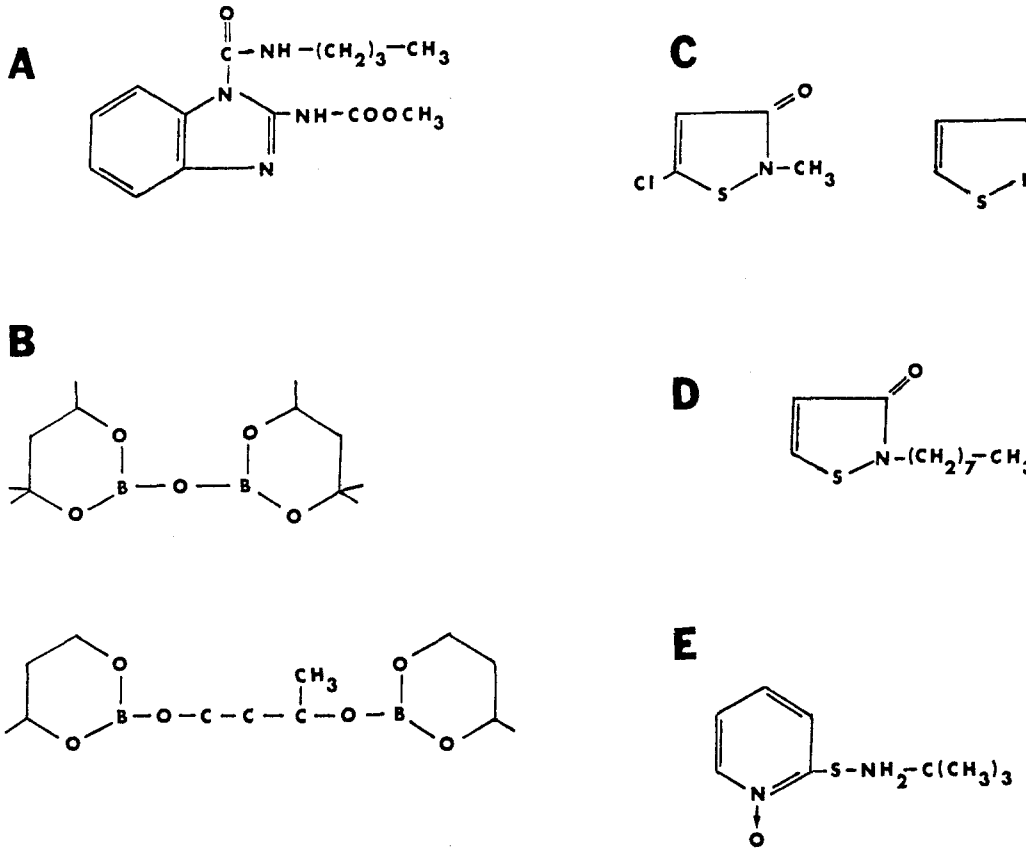

Fig. 1. Structural formulae of biocides tested.

plicate test units in each treatment category were inoculated with 10^7 colony-forming units of *C. resinae*.

Tests to determine the effects of sludge were carried out at the same time in similar test units and on the same time schedule. Forty milliliters of fuel were dispensed over 3 ml of mineral salts medium and 1 ml of sludge containing solid matter which distributed itself partly at the bottom of the test units and partly at the fuel/water interface, leaving a clear intermediate aqueous layer. The sludge was prepared by pooling 15 different sludge samples from storage tanks of four ships, and consisted of 33% oil, 46% water (estimated from refractive index to contain 25 ppt sea salt) and 21% sediment. It was sterilized by autoclaving prior to use. Inoculation, duplication, incubation and storage were as described above for non-sludge-containing units.

The test units were examined each week and estimates of fungal growth were made visually using a numerical rating from one to six as given in Fig. 2. In previous investigations using a similar system, it was shown that reproducible results could be obtained when ratings were made in a consistent manner by the same person; biomass approximately doubled for each unit increase in rating [12]. Occasionally, to confirm doubtful cases, small samples of aqueous phase were removed and examined by phase contrast microscopy.

RESULTS AND DISCUSSION

Growth of *C. resinae* was rapid and prolific in control test units without biocide whether sludge was present or not (Fig. 2). When biocide-containing test units were inoculated without prior storage, only biocides A and C showed complete control of

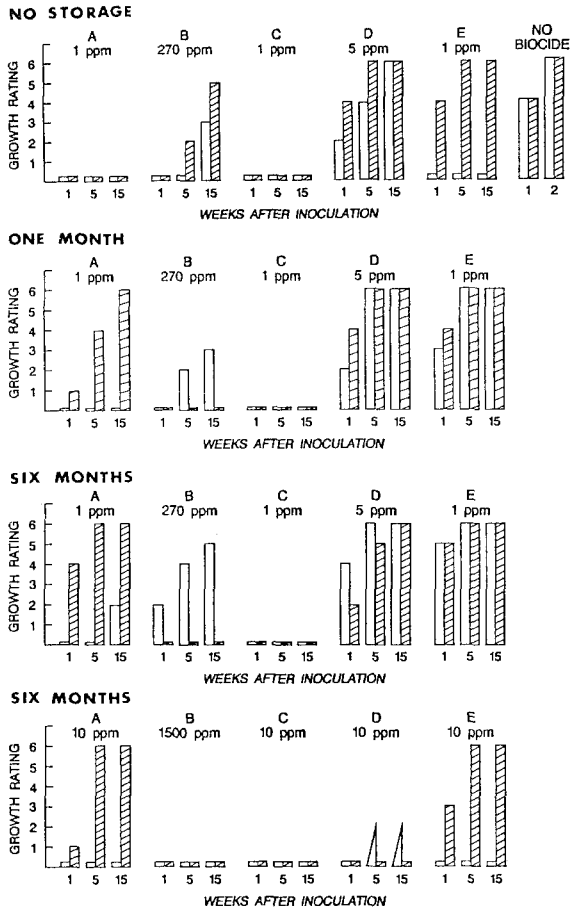

Fig. 2. Growth of *C. resinae* inoculated at zero time and after 1-month and 6-month storage of fuel/water systems treated with biocides A–E. ☐, without sludge; ▨, with sludge. Growth ratings: 0 = no growth, 1 = slight mycelial sediment, 2 = formation of substantial mycelial floc, 3 = floc plus some interfacial growth, 4 = substantial interfacial growth, 5 = fungal mat at interface, 6 = thick mat at interface.

fungal growth at the lower concentration levels. Biocide B allowed retarded growth, which is not unexpected since it is not intended for situations where the water/fuel ratio is so high. Biocide D showed only a slight inhibition. Biocide E was inhibitory in clean systems, but completely inactivated in systems containing sludge. At the higher concentrations with no storage (data not shown in Fig. 2), fungal growth was completely controlled by all biocides except D which allowed a growth rating of three in clean systems only 5 weeks after inoculation.

After 1 month of storage, biocide A allowed growth in the presence of sludge, while uninhibited growth occurred with biocides D and E. Biocide B allowed growth in clean systems but, in contrast with unstored systems, completely inhibited growth with sludge. At the higher concentration levels (data not shown in Fig. 2), no growth occurred with any biocide with the exception of D which allowed the same growth rating of three in 5 weeks' time after inoculation as in the case with no storage.

After 6 months of storage at the lower concentration levels, biocide C alone retained complete control of fungal growth. Biocide A was uninhibitory with sludge but allowed only slow growth without sludge. The loss of anti-fungal activity of biocide B in clean systems seen after 1 month of storage was accentuated after 6 months, but control with sludge was retained. With biocide D there was also an indication of better control in systems with sludge. Biocide E was no longer inhibitory after 6 months.

After 6 months at the higher concentrations, biocides A and E showed the same inactivation by sludge seen at the lower concentration but inhibition in clean systems was complete. Biocide D continued to show better control with sludge than without, as seen at the shorter storage times. The tapered bar graph (Fig. 2) in this case is meant to show that only one of the duplicate test units showed growth, an exception to the otherwise general agreement of duplicates.

Considerable differences clearly exist in the responses of the different biocides to storage and sludge. A number of factors may be responsible. Most biocides are subject to hydrolysis or oxidation and therefore become inactive with time [4,9,10,13,17,19]. The oil/water partition ratios of the biocides differ and the rates at which partition equilibrium is reached may also differ. Sludge provides particulate surfaces on which adsorption and/or inactivation of biocides can occur. As an interfacial barrier, sludge may retard the rate of biocide diffusion from the oil to the aqueous phase and thus, as may be the case with biocides B and D, prolong a biocidal concentration at the interface where fungal growth is most likely to occur. It

should also be pointed out that those test units to which sludge had been added contained about 12% less water than those without. Other explanations for our observations are likely to exist as well, but there are insufficient data to speculate more specifically.

Our results with biocides A and E generally agree with those of Smith and Crook [17] using a different test method. However, biocide A appeared to be more resistant to sludge inactivation in their experiments. These authors also observed that low concentrations of mixtures of biocides A and E were very effective against microbial fuel contaminants.

A major finding of this study is that biocide C, the methylchloro/methyl-isothiazalone mixture, completely inhibited fungal growth at a concentration of 1 ppm; effectiveness at this low concentration was retained with storage and in the presence of sludge. There are few reports of the use of this biocide to treat distillate fuels [11,18], and none that detail the effects of storage and sludge stress on its efficacy. While the results of laboratory investigations should only be extrapolated to 'real world' systems with great caution, we feel that the favorable results observed here with biocide C justify further evaluations of its application limits.

These screening evaluations were intended to select those biocides which were effective against a major fungal biodeteriogen under conditions generally favorable to the growth of fungi (nutrients, pH and temperature) and unfavorable to the biocides (aging and sludge presence). Similar screening evaluations are being carried out with mixed bacterial cultures containing sulfate-reducing bacteria. These combined results should serve to define conditions for further work using more realistic test systems.

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