

Biocide Efficacy vs. Acid Producing and Iron Oxidizing Bacteria

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ABSTRACT

Five water treatment biocides were evaluated for efficacy against four bacteria rarely isolated but frequently implicated in cooling water biofouling. The organisms tested were *Leptothrix discophora* (ATCC #43182), *Thiobacillus novellus* (ATCC #8093), *Hyphomicrobium indicum* (ATCC #19614), and *Sphaerotilus natans* (ATCC #15291). The evaluations were conducted at pH 7.5 and ambient temperature. Each biocide was tested at concentrations which represent recommended treatment dosages for industrial water systems. The biocidal efficacy was evaluated after contact times of 1, 4, and 7 hours. All of the biocides proved to be highly effective against *L. discophora*, whereas efficacy against *T. novellus*, *H. indicum*, and *S. natans* varied.

Introduction

Microbially induced corrosion (MIC) of metal surfaces is a well-known phenomenon which can severely disrupt the integrity of industrial water systems. The corrosion that occurs is a result of microbial activity in a biofilm, which is usually inhabited by a variety of microorganisms (1). While sulfate reducing bacteria (e.g. *Desulfovibrio*) have been historically singled out as being a major cause of MIC, other types of bacteria are thought to play either a direct or an indirect role in this phenomenon (2).

One group of organisms which have been recently implicated as contributing to MIC is the so-called iron bacteria, which include organisms in the genera *Gallionella*, *Leptothrix*, *Crenothrix*, and *Sphaerotilus* (3,4). Other species thought to contribute to MIC include the sulfuric acid producing *Thiobacillus* as well as organisms with complex surface structures such as the bacteria *Caulobacter* and *Hyphomicrobium*.

Because of the importance of acid producing bacteria and iron bacteria in the corrosion of aqueous industrial systems, the efficacy of some common water treating biocides was evaluated against the following selected species: *Thiobacillus novellus*, *Leptothrix discophora*, *Hyphomicrobium indicum*, and *Sphaerotilus natans*.

Thiobacillus novellus is a Gram-negative, obligate aerobic bacterium. Although this organism does not typically produce sulfuric acid (as do other species of *Thiobacillus*), it is able to oxidize some sulfur compounds to sulfate. The production of sulfates is important in an ecosystem containing sulfate reducing bacteria, where the sulfate is converted to sulfide, resulting in odor and corrosion problems.

Leptothrix discophora and *Sphaerotilus natans* are bacteria which surround themselves with sheaths of metal oxides. The oxides from *Leptothrix* can contain either iron or manganese while those from *Sphaerotilus* contain only iron (5). These bacteria have been reported in connection with the formation of raised growths on the surface of iron water pipes and water mains. The metal oxide deposits can restrict flow and reduce heat transfer efficiency in once-through and recirculating cooling systems. (6).

Hyphomicrobium indicum is also a Gram-negative obligate aerobe which oxidizes manganese and produces scale-like deposits. It is a budding or appendaged organism and its complex surface structure is thought to play a role in biofilm formation. *H. indicum* is considered a "secondary periphyte," which means that it may colonize a surface after a primary biofilm has been established (7).

Although these organisms are often cited in the literature as causing problems in industrial cooling systems, they are rarely studied in controlled laboratory experiments.

One reason for this may be the difficulty in isolating and identifying these bacteria. The purpose of this study was thus twofold: first, to develop methodology that would enable the routine culturing of these organisms; and second, with this ability in hand, to measure biocide efficacy against them using some common cooling tower microbiocides. The biocides tested were as follows: glutaraldehyde (45% active ingredient); alkyldimethyl benzyl ammonium chloride (ADBAC, a 50% active mixture); a glutaraldehyde/ADBAC blend (50% total active ingredient); isothiazolones (5-chloro-2-methyl-4-isothiazolin-3-one, 1.15% + 2-methyl-4-isothiazolin-3-one, 0.35%, 1.5% total active ingredient); and 2, 2-dibromo-3-nitropropionamide (DBNPA, 5% active ingredient).

Experimental (Materials and Methods)

All organisms involved in this study were obtained from the American Type Culture Collection (ATCC). These bacteria are all relatively sensitive to laboratory conditions and were therefore handled with extreme care. Culturing techniques, including preparation of specific growth media, were carefully adhered to. For all of these organisms, subculturing from the original ATCC source was carried out in several steps. The original culture was first transferred to 5-10 mL of appropriate medium and allowed to grow and adapt to that environment for 2-3 days before another transfer was made. At that time approximately 1 mL of the growing culture was transferred to a larger volume, approximately 25 mL, and again allowed to acclimate over time. These successive transfers were made until the desired culture volume had been achieved. All cultural procedures described below utilized this incremental transfer procedure.

A 24-hour culture of *T. novellus* was inoculated into 1100 mL of nutrient broth which had been diluted to one-tenth normal strength. This was allowed to grow overnight at 26°C. The fresh culture was then split into 100 mL samples where one was retained as a control and the others were treated with the various biocides at the levels indicated in Table 3. Levels of *T. novellus* were monitored at 1, 4, and 7 hours via plate counts on 0.1X nutrient agar.

L. discophora was grown in *Leptothrix* 2xPYG Medium. 10 mL of a 72 hour active culture was inoculated into eleven flasks, each containing 90 mL of the *Leptothrix* medium. One flask was kept as a control and the others were treated with biocides as shown in Table 4. The samples were maintained at room temperature, as were the samples for all of the biocide time kill studies mentioned here. Survival of *L. discophora* was determined via plate counts on the *Leptothrix* medium (supplemented with 1.5% agar) at 1, 4, and 7 hours.

For *H. indicum*, a 24 hour culture was also used. The day-old culture was inoculated into a solution of Marine Broth to make 1100 mL total. The seeded broth

was allowed to grow overnight at room temperature. The fresh culture was then split into 11 100 mL samples where one was retained as a control and the other ten were dosed with biocide as indicated in Table 5. Levels of *H. indicum* were monitored at 1, 4, and 7 hours via plate counts on Marine Agar.

S. natans was grown in *Sphaerotilus* CGYA Medium. 10 mL of a 72-hour active culture was inoculated into eleven flasks, each containing 90 mL of the *Sphaerotilus* medium. One flask was kept as a control and the others were treated with the various biocides at the levels indicated in Table 6. The samples were retained at room temperature and plated on *Sphaerotilus* medium (supplemented with 1.5% agar) at 1, 4, and 7 hours.

Results and Discussion

The results are shown in Tables 1-6 and Figures 1-4. The choice of biocide levels to be tested was primarily based on doses typically used for cooling water treatment. Two different concentrations were tested for each biocide. Preliminary data were obtained on glutaraldehyde and the glutaraldehyde/quat blend (Tables 1 and 2), and the dose levels of those biocides were raised or lowered in some of the subsequent comparative studies (Tables 3-6) in order to obtain additional information on the efficacy range of these products. Figures 1-4 are included to better illustrate the efficacy of each biocide at one level against all four organisms.

It was noted during the course of the experiments that glutaraldehyde was partially inactivated by the different media that were used in these experiments. For this reason, the chemical stability of glutaraldehyde, as well as the other biocides was examined in each of the media. The results for glutaraldehyde are shown in figure 5. The loss of glutaraldehyde due to reaction with the media ranged from 80% in one hour in the *Sphaerotilus* CGYA Medium and marine broth to approximately 50% in seven hours in the *Leptothrix* 2xPYG Medium and diluted nutrient broth. The media utilized in the preliminary data for *Thiobacillus novellus* was undiluted nutrient broth which also resulted in an 80% loss of glutaraldehyde in one hour. By contrast, glutaraldehyde loss in a pH 7.5 aqueous solution is negligible over this time frame.

The impact of glutaraldehyde inactivation on biocidal performance was further examined by comparing the efficacy of this biocide versus *Pseudomonas aeruginosa* in the presence and absence of the various media (Table 7). For example, glutaraldehyde at 110 ppm will result in complete kill of this organism in seven hours in a 0.1 M phosphate buffer as well as in the *Leptothrix* 2xPYG Medium and diluted nutrient broth. In the *Sphaerotilus* CGYA Medium and marine broth, however, efficacy is completely inhibited. Unfortunately, the sensitivity of the microorganisms to laboratory conditions prevented the use of testing solutions that were less reactive with glutaraldehyde.

Similar experiments were carried out on the other biocides tested. The various media employed in these experiments were found to have no adverse affect on the stability or efficacy the other biocides.

Table 3 illustrates the efficacy of each biocide against *Thiobacillus novellus* at 1, 4, and 7 hour contact times. The ADBAC was the most effective of all five compounds tested yielding complete kill of the microorganisms at 50 ppm after a 1 hour contact time. The glutaraldehyde/quat blend was the next most effective of the compounds tested, showing complete kill at 200 ppm after only one hour. Glutaraldehyde at 220 ppm showed the same effect at the four hour time point. Isothiazolone at 200 ppm and DBNPA at 100 ppm needed 7 hours in order to bring about significant reductions. The lower level doses of isothiazolone and DBNPA showed minimal efficacy.

Efficacy versus *Leptothrix discophora* is shown in Table 4 . This organism was the most susceptible to treatment of all the bacteria tested. All of the biocides were very effective against *L. discophora*. Because isothiazolone is a slower acting product compared to the others in this study, it did take longer to demonstrate complete kill against this organism.

Hyphomicrobium indicum proved to be the most difficult organism to control with DBNPA and isothiazolone, which were only able to achieve a 90% reduction at either concentration for any of the time points. (Table 5). Complete kill was achieved with 50 ppm ADBAC at 1 hour. Glutaraldehyde, at 110 ppm was able to bring about complete kill at the seven hour time point. The glutaraldehyde/quat blend at 100 ppm was able to bring about > 99% reduction at all three time points.

The results of biocide efficacy tests versus *Sphaerotilus natans* are shown in Table 6. ADBAC once again resulted in complete kill at every time point. Both concentrations of DBNPA were effective, yielding > 99.99% reduction at all three time points. The slower acting isothiazolone required a longer time period to bring about a similar level of efficacy. The glutaraldehyde/quat blend was also effective yielding complete kill at the 100 ppm level at all three time points. The glutaraldehyde/quat blend showed greatly enhanced performance against this bacteria, as compared to the glutaraldehyde alone. However, it should be remembered that much less glutaraldehyde was actually present due to inactivation by the media. In fact, glutaraldehyde performance appears to be related to the amount of inactivation caused by each of the media. For example, the largest amount of inactivation occurred in the *Sphaerotilus* CGYA Media, where glutaraldehyde efficacy was the poorest. It is likely that in an environment more stable to glutaraldehyde lower levels could be used with similar results. Work is currently underway to find conditions that will maintain the viability of these organisms without interfering with any of the biocides.

Figures 1-4 are included to better illustrate the comparative efficacy of all four biocides against the four organisms. The biocide concentrations in each graph are expressed as ppm of product and are as follows: glutaraldehyde, 110 ppm(220 ppm versus *T. novellus*); glutaraldehyde/quat, 100 ppm; isothiazolone, 200 ppm; DBNPA, 100 ppm and ADBAC, 50 ppm.

The results in these figures show that 50 ppm of ADBAC is sufficient to bring about complete kill of all the organisms in 1 hour. The glutaraldehyde/quat blend at 100 ppm was also very effective, resulting in reductions of at least 99.99% in the microorganism population in 1 hour. Rapid kill is important in cooling towers because the contact time and turnover rate can be such that if a biocide does not demonstrate rapid efficacy, the concentration will soon drop to ineffective levels. The efficacy of glutaraldehyde at 110 ppm, isothiazolone at 200 ppm and DBNPA at 100 ppm varied. All were able to completely kill *L. discophora*, but results against the remaining organisms varied.

While the ADBAC was the most effective of the products tested, problems are often encountered when using quaternary ammonium compounds. Foaming, inactivation by hard water and incompatibility with anionic chemicals such as corrosion and scale inhibitors could prevent its use in many systems. The glutaraldehyde/quat blend was also effective and could minimize these potential problems because of the lower amount of quaternary compound introduced into the system.

The results in general indicate that biocide efficacy is species specific. It is very important to consider this fact when planning for treatment of cooling waters in the field. Typically microbial populations in industrial fluids are monitored with agar dip slides or occasionally with laboratory plate counts. It is, however, very unlikely that any of the four bacteria in this study will be detected by these methods. Indications of no growth of common aerobic bacteria such as *Pseudomonas* or *Flavobacterium* can be misleading. The results of this study indicate that biocide levels normally considered effective may actually be unable to control certain organisms thought to play a key role in biofouling. The results in this paper point to the need for occasional testing of these more unusual bacteria in order to determine appropriate biocide treatment levels.

Table 1 • Glutaraldehyde Efficacy

Microorganism	ppm product	Microorganism population (cfu/ml)	
		1 hours	7 hours
<i>Thiobacillus novellus</i>	0	1.0×10^8	1.6×10^8
	55	1.2×10^8	8.0×10^7
	110	1.4×10^8	7.4×10^7
<i>Leptothrix discophora</i>	0	1.5×10^5	6.6×10^4
	55	< 10	< 10
	110	< 10	< 10
<i>Hyphomicrobium indicum</i>	0	3.0×10^7	5.0×10^7
	55	1.9×10^5	2.0×10^5
	110	< 100	< 100
<i>Sphaerotilus natans</i>	0	1.3×10^6	1.9×10^6
	55	3.3×10^4	5.0×10^5
	110	1.4×10^4	2.4×10^2

Table 2 • Glutaraldehyde/Quat Efficacy

Microorganism	ppm product	Microorganism population (cfu/ml)	
		1 hours	7 hours
<i>Thiobacillus novellus</i>	0	1.0×10^8	1.6×10^8
	50	4.8×10^5	3.1×10^6
	100	1.9×10^6	1.6×10^1
<i>Leptothrix discophora</i>	0	1.5×10^5	6.6×10^4
	50	< 10	< 10
	100	< 10	< 10
<i>Hyphomicrobium indicum</i>	0	3.0×10^7	5.0×10^7
	50	5.0×10^3	3.0×10^3
	100	< 100	< 100
<i>Sphaerotilus natans</i>	0	1.3×10^6	1.9×10^6
	50	< 1	< 1
	100	< 1	< 1

Table 3 • Efficacy versus *Thiobacillus novellus*

Biocide	ppm product	Microorganism population (cfu/ml)		
		1 hours	4 hours	7 hours
Glutaraldehyde	220	3.0×10^3	<1	<1
Glutaraldehyde/Quat	100	1.0×10^2	4.0×10^1	<1
	200	<1	<1	<1
2,2-dibromo-3-nitropropionamide	40	4.1×10^6	2.0×10^6	1.3×10^6
	100	1.3×10^6	4.0×10^5	<1
Isothiazolone	66	4.4×10^6	3.4×10^6	4.0×10^5
	200	3.6×10^6	2.3×10^5	1.5×10^2
Alkyldimethyl benzyl ammonium chloride	50	<1	<1	<1
	100	<1	<1	<1
Control	0	1.0×10^7	1.7×10^7	3.5×10^7

Table 4 • Efficacy versus *Leptothrix discophora*

Biocide	ppm product	Microorganism population (cfu/ml)		
		1 hours	4 hours	7 hours
Glutaraldehyde	55	<1	<1	<1
	110	<1	<1	<1
Glutaraldehyde/Quat	50	<1	<1	<1
	100	<1	<1	<1
2,2-dibromo-3-nitropropionamide	40	1.8×10^7	<1	<1
	100	<1	<1	<1
Isothiazolone	66	4.0×10^4	5.0×10^2	<1
	200	<1	<1	<1
Alkyldimethyl benzyl ammonium chloride	50	<1	<1	<1
	100	<1	<1	<1
Control	0	1.0×10^5	1.4×10^5	2.0×10^5

Table 5 • Efficacy versus *Hyphomicrobium Indicum*

Biocide	ppm product	Microorganism population (cfu/ml)		
		1 hours	4 hours	7 hours
Glutaraldehyde	55	5.4×10^5	2.3×10^5	1.2×10^5
	110	1.3×10^4	2.7×10^2	<1
Glutaraldehyde/Quat	50	1.1×10^6	4.1×10^5	1.6×10^6
	100	1.4×10^5	5.9×10^3	1.5×10^2
2,2-dibromo-3-nitropropionamide	40	1.7×10^6	3.4×10^6	1.1×10^6
	100	1.6×10^6	5.8×10^5	7.8×10^5
Isothiazolone	66	8.9×10^5	4.8×10^5	1.1×10^5
	200	8.7×10^6	6.5×10^5	1.5×10^6
Alkyldimethyl benzyl ammonium chloride	50	<1	<1	<1
	100	<1	<1	<1
Control	0	1.1×10^6	5.9×10^5	1.1×10^6

Table 6 • Efficacy versus *Sphaerotilus natans*

Biocide	ppm product	Microorganism population (cfu/ml)		
		1 hours	4 hours	7 hours
Glutaraldehyde	110	4.8×10^5	1.7×10^5	4.1×10^5
	220	6.7×10^5	2.6×10^5	5.7×10^4
Glutaraldehyde/Quat	50	<1	<1	<1
	100	<1	<1	<1
2,2-dibromo-3-nitropropionamide	40	3.8×10^2	5.0×10^2	3.0×10^2
	100	1.1×10^2	8.0×10^1	4.5×10^2
Isothiazolone	66	4.5×10^5	2.9×10^4	2.2×10^4
	200	4.4×10^5	2.0×10^4	2.9×10^2
Alkyldimethyl benzyl ammonium chloride	50	<1	<1	<1
	100	<1	<1	<1
Control	0	7.5×10^7	1.0×10^6	1.2×10^6

Table 7 • Glutaraldehyde Efficacy versus *Pseudomonas aeruginosa*

Media	ppm product	Microorganism population (cfu/ml)	
		1 hours	7 hours
Diluted nutrient broth	0	5.4×10^6	7.0×10^6
	110	1.4×10^3	< 100
<i>Leptothrix</i> Media	0	5.3×10^6	6.4×10^6
	110	4.4×10^3	< 100
Marine Broth	0	4.7×10^6	7.2×10^6
	110	7.2×10^6	7.1×10^5
<i>Sphaerotilus</i> Media	0	4.2×10^6	7.0×10^6
	110	5.7×10^6	4.8×10^5
Control (No media)	0	5.2×10^6	6.2×10^6
	110	5.7×10^1	< 100

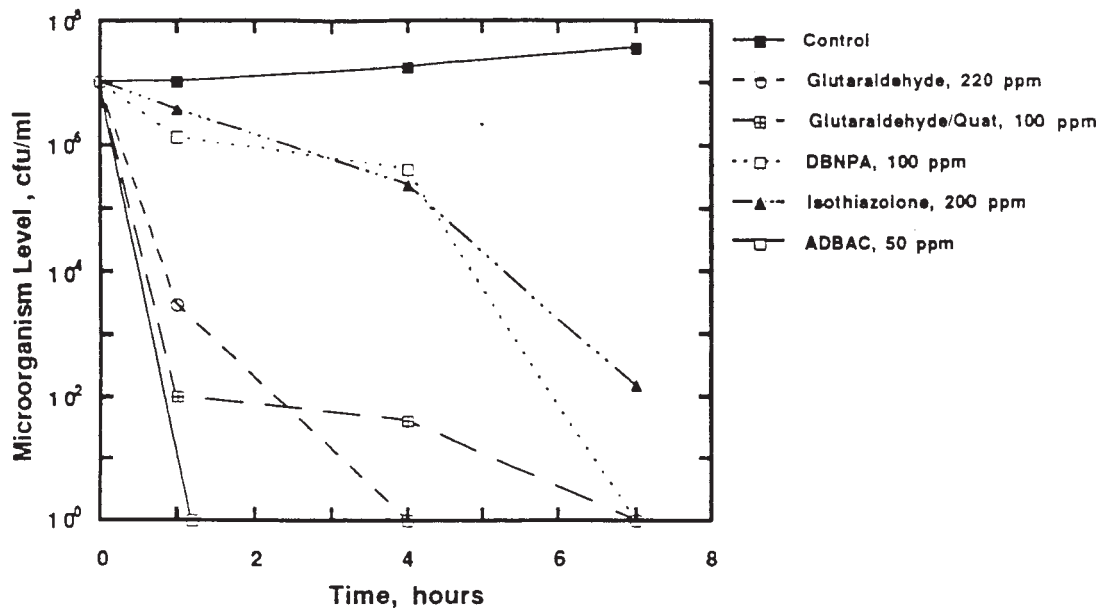


Figure 1 Efficacy versus *Thiobacillus novellus*

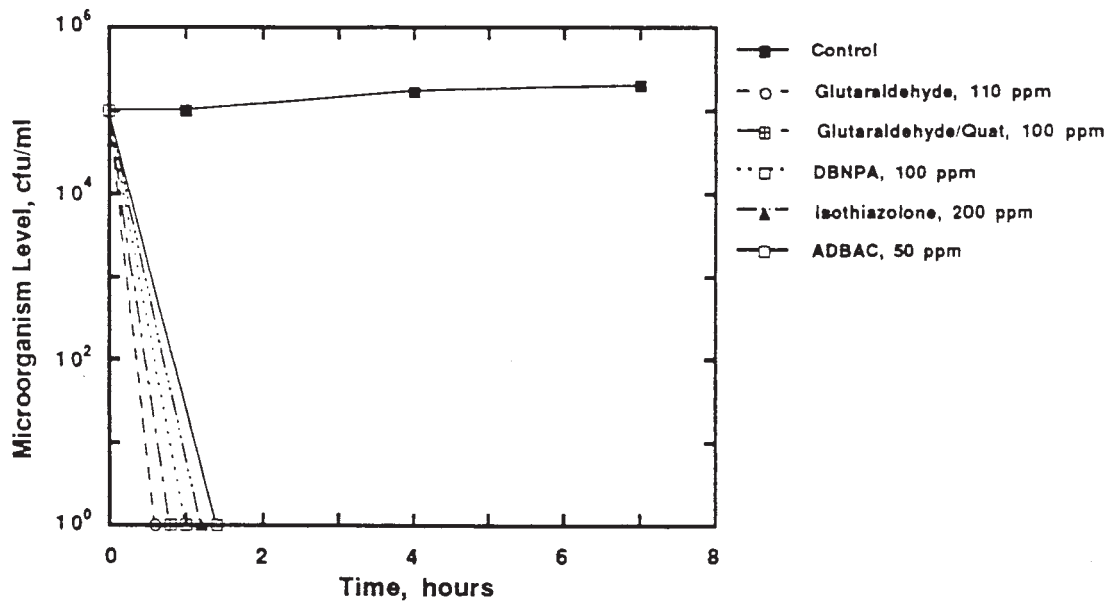


Figure 2 Efficacy versus *Leptothrix discophora*

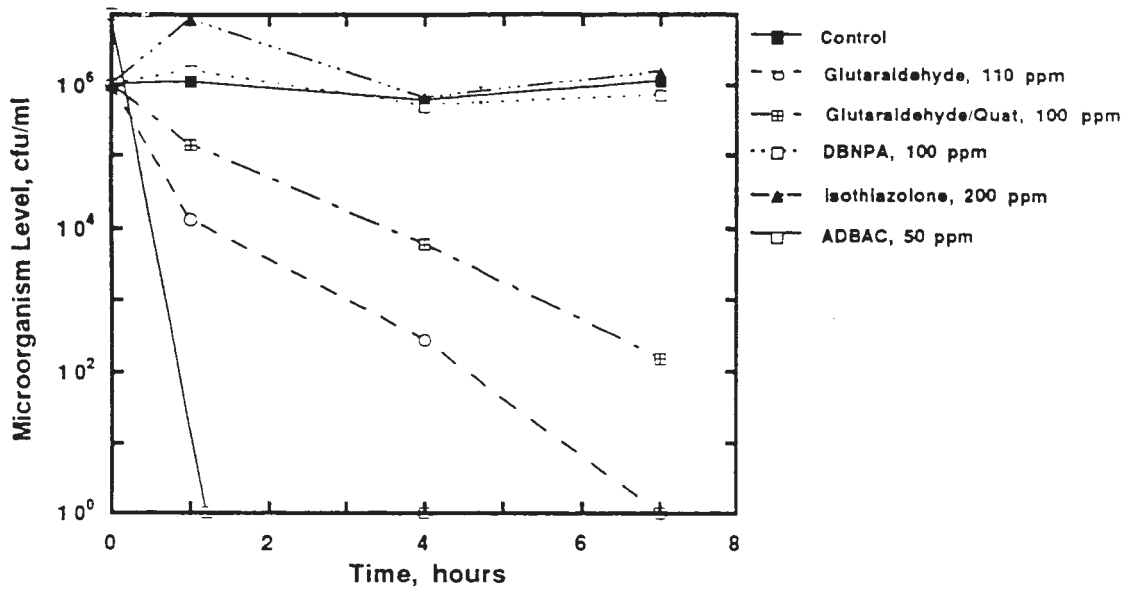


Figure 3 Efficacy versus *Hyphomicrobium indicum*

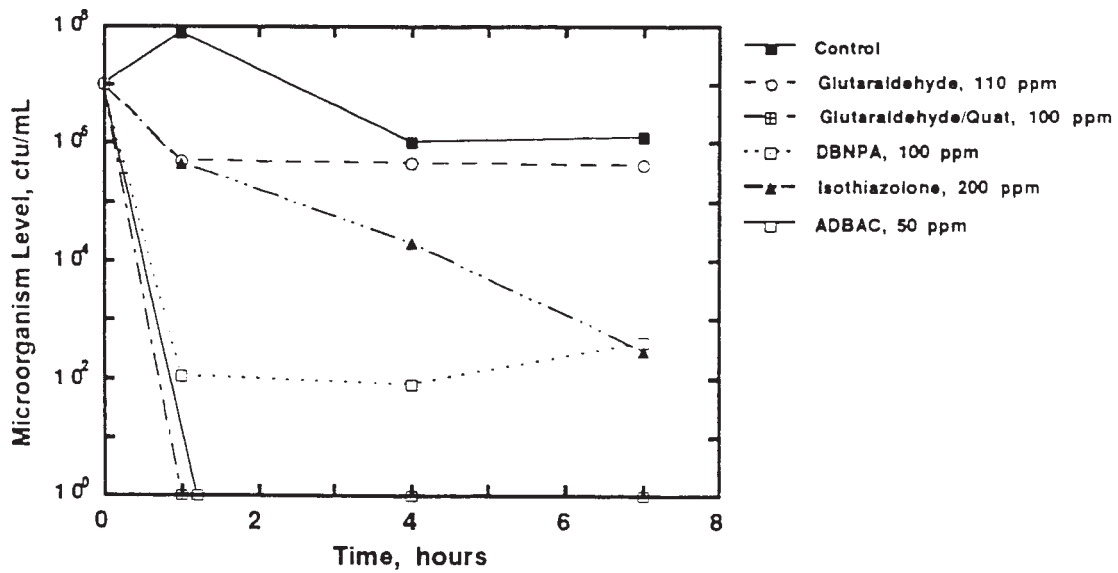


Figure 4 Efficacy versus *Sphaerotilus natans*

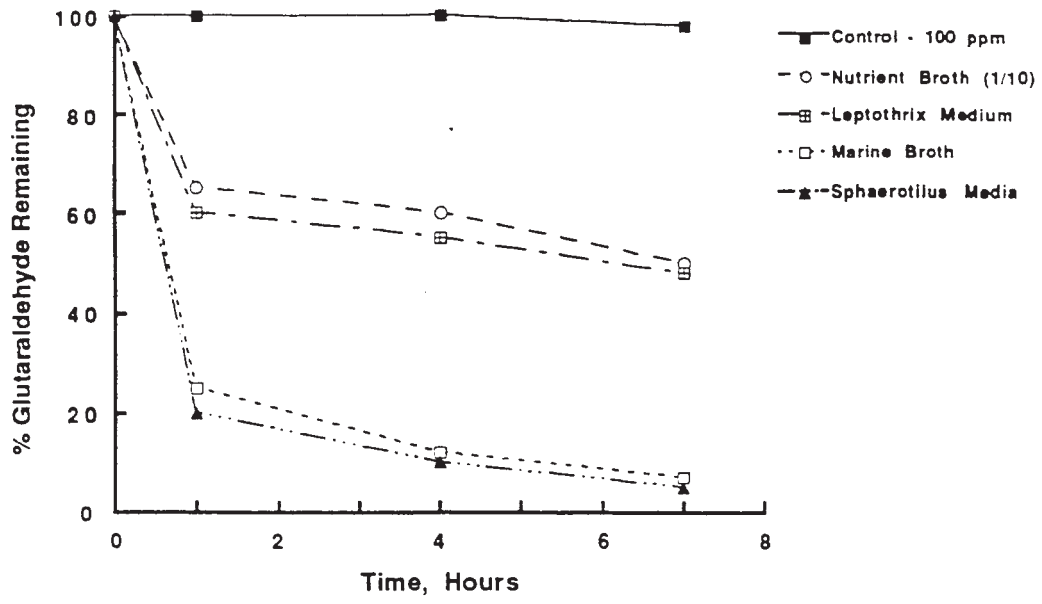


Figure 5 Stability of Glutaraldehyde in Media

MEDIA FORMULATIONS/Liter

Nutrient Agar (IX)

Beef Extract	0.3g
Peptone	0.5g
Agar	15g

Marine Agar

Peptone	5.0g
Yeast Extract	1.0g
Ferric Citrate	0.1g
Sodium Chloride	19.459g
Magnesium Chloride (dried)	5.9g
Sodium Sulfate	3.24g
Calcium Chloride	1.8g
Potassium Chloride	0.55g
Sodium Bicarbonate	0.16g
Potassium Bromide	0.08g
Strontium Chloride	0.034g
Boric Acid	0.022g
Sodium Silicate	0.004g
Sodium Fluoride	0.0024g
Ammonium Nitrate	0.0016g
Disodium Phosphate	0.008g
Agar	15g

Leptothrix 2xPYG Medium

Peptone	0.5g
Yeast Extract	0.5g
Glucose	0.5g
Magnesium Sulfate.7H ₂ O	0.6g
Calcium Chloride.2H ₂ O	0.07g
HEPES	3.57g
Manganese Sulfate.H ₂ O	17.0mg
Agar	15g

Sphaerotilus CGYA Medium

Casitone	5g
Glycerol	10g
Yeast Autolysate	1g
Agar	15g

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