

FROM: Walters, A.H. and A.E.
Hueck-Van Der Plas (Eds.), Bio-
detn. of Materials 2: 286-293.
London: Appl. Science Publ. Ltd.

**ANTI- AND PRO-MICROBIAL ACTIVITY OF HEXAHYDRO
1,3,5 TRIS (2-HYDROXYETHYL)-S-TRIAZINE IN CUTTING
FLUID EMULSION**

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Summary

Hexahydro 1,3,5 tris (2-hydroxyethyl) triazine [G] in o/w cutting fluid emulsions effectively controlled bacteria but resulted in fungal growth. The addition of each of four compounds, tribromosalicylanilide, dimethyldithiocarbamic acid plus mercaptobenzothiazole, tetrachloromethylsulfonyl pyridine, and hydroxypyridine thione completely or partially prevented fungal growth. However, when used against pure cultures of several fungi, only the latter compound was compatible with [G]. Attempts to demonstrate utilization of [G] as sole carbon source were inconclusive.

INTRODUCTION

During the past ten years, hexahydro 1,3,5 tris (2-hydroxyethyl)-s-triazine [G] (Grotan^R, Lehn and Fink Industrial Products Div.) has been used successfully to control bacterial growth in a number of industrial situations, more specifically, cutting fluid. However, one of the problems associated with continual use or misuse has been the growth of nuisance fungi which themselves were difficult to control. We have found previously (Rossmoore, De Mare and Smith 1971) that the action of [G] was as much stimulatory to fungal growth as it was a selective biocide for Gram-negative bacteria, since the appearance of fungus seemed to be concentration dependent.

Hueck (1968) pointed to the resemblances between problems in biodeterioration and pathology, and the post-biocide appearance of fungi in cutting fluid is a good example of these parallelisms. Perhaps the major difference in cutting fluid is that the 'disease' is caused by a heterogeneous population and inefficient biocides permit the survival of resistant species rather than strains. The appearance of fungi after use of [G] for varying periods of time, usually longer than four weeks, follows the destruction of the bacterial populations. This sudden onset of mycotic growth could result from resistance to the biocide coupled with the disappearance of competing bacterial species or from the utilization of the biocide by resisting fungal species. Indeed, the former situation is a well-known occurrence in 'broad-spectrum' antibiotic treatment for bacterial infections which sometimes leave the poor patient with a debilitating moniliasis.

The present study is directed toward two areas: one, to make use of the bactericidal properties of [G], combining it with synergistic companions to prevent the growth of fungi and two, to determine, if possible, whether [G] is selective or stimulatory for fungal growth.

MATERIALS AND METHODS

Synergism in cutting fluid

Four compounds, known to be compatible with cutting fluids, were used in combination with 250, 500 and 1000 mg/litre of [G]. They were 2,3,5,6-tetrachloro-4-methylsulfonyl pyridine [D] (Dowicil S13^R, Dow Chem. Co.), 3,4',5-tribromosalicylanide [T] (TBS, Maumee Chem. Co.), dimethyldithiocarbamic acid (90%) plus 2-mercaptobenzothiazole (8%) [V] (Vancide 51Z^R, R. T. Vanderbilt Co.), and 1-hydroxypyridine-2-thione [O] (Omadine^R, Olin Corp.), all at a final concentration of 50 mg/litre. Evaluation in 5% o/w emulsions (Sunseco^R, Sun Oil Co.) simulated industrial conditions (Heinrichs and Rossmoore, 1970). Air was bubbled through 1-litre samples of cutting fluid containing 10 g of cast iron chips plus 10% spoiled cutting fluids as a source of mixed flora. After five days, the air was shut off for two days to encourage anaerobic growth. This corresponded to a weekend shut-down period. Approximately 10% of each sample was withdrawn at the end of the quiescent period and replaced with fresh fluid prior to restarting the bubbling. Aliquots from portions removed were used for bacterial plate counts on trypticase soy agar (TSA-Baltimore Biological Lab.) and fungal plate counts on Sabouraud Dextrose Agar (SDA-Difco Corp.). Incubation of the former was 48 hours at 30°C and the latter five days at 25°C.

Growth and maintenance of fungal cultures

We used six fungal strains for the studies reported here, *Geotrichum candidum*, *Penicillium notatum*, *Rhizopus stolonifer*, *Mucor pusillus*, *Mucor humicolus*, from the Wayne State culture collection, and a *Fusarium* sp. isolated from a spoiled cutting fluid emulsion. The cultures were maintained on SDA slants, incubated at 25°C and transferred every five days until ready for use. The spore yield was washed from the slants with 1 ml sterile H₂O and added to 100 ml of Sabouraud Broth (Difco Corp.) in a 500 ml Erlenmyer flask, and incubated two days at 25°C without shaking. The resultant growth was subsequently used for evaluation of the combined biocides.

Biocide evaluation

We evaluated the compounds in SDA plates inoculated with 1 ml of the previously described broth cultures; the various inhibitor concentrations, mentioned earlier, were added to ¼ inch diameter wells, four to each plate; incubation was at 25°C and zones of inhibition were read at two days and again at five days before discarding.

Utilization of [G] as carbon source

Mildew test agar (MTA-Difco Corp.) which lacks a source of utilizable carbon was used to determine if [G] could serve as the sole source of that element. The same technique as described for biocide evaluation was applied here, except that we looked for zones of growth rather than inhibition around the wells.

RESULTS AND DISCUSSION

Our previous experience in which fungi appear prominently in 0.05% [G] (Rossmoore, De Mare, Smith, 1971) is repeated here (Table I). However,

Table I
THE EFFECT OF
HEXAHYDRO 1,3,5 TRIS (2-HYDROXYETHYL)-S-TRIAZINE [G]
ON CUTTING FLUID* FLORA†

| Biocide % | Week 1 | | Week 2 | | Week 3 | |
|-----------|----------------------|---------------------|---------------------|---------------------|---------------------|-----------------|
| | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml |
| [G]0.1 | 0 | 10 ² | 6 × 10 ³ | 10 ³ | 0 | 10 ⁴ |
| [G]0.05 | >3 × 10 ⁶ | 2 × 10 ⁵ | 8 × 10 ⁵ | 2 × 10 ⁵ | 2 × 10 ⁷ | 10 ⁴ |
| [G]0.025 | >3 × 10 ⁶ | 10 ⁶ | 5 × 10 ⁶ | 2 × 10 ⁶ | 9 × 10 ⁶ | 10 ⁵ |
| Control | 2 × 10 ⁶ | 0 | 6 × 10 ⁵ | 0 | 2 × 10 ⁶ | 0 |

* In 5% o/w emulsion.

† Inoculum for both sets consisted of 10% v/v of a spoiled mixed cutting fluid with 3 × 10⁷/ml aerobic bacteria and no recoverable fungi.

one distinction should be pointed out. In contrast to our earlier report, 0.025% [G] also resulted in even more extensive fungal growth. The major difference between the two protocols was the source of the emulsion, a fact of importance to bear in mind when any biocide evaluations are being done. At the level of 0.1% [G], which is 50%, less than recommended as optimal, the bacterial count is zero. However, the reduction of [G] to 0.05% brings disaster, the bacterial counts equalling and surpassing the unprotected control. This critical concentration coefficient effect is strong evidence against diluting biocide concentration before knowing its behaviour. Certainly, in practice in cutting fluids, the strong possibility exists that the same emulsion fluid concentrate may be used at a dilution ratio of from 1:10 to 1:50. The fluid compounder who includes a biocide in his formulation must take into consideration the potential danger of its over-dilution. Adding excess biocide to compensate for this probability is unwise and economically unsound. It is for this reason (and others) that we strongly recommend that the addition of biocide be a responsibility of the user so that the optimal amount of the most compatible compound can be added at the most appropriate time.

In Tables II, III, IV and V can be seen the results of attempts to control [G]-induced fungi with four reputed fungicides, [T], [V], [O] and [D]. It will be noted (Table II) that [T] is very effective combined with higher levels of

Table II
THE EFFECT OF
HEXAHYDRO 1,3,5 TRIS (2-HYDROXYETHYL)-S-TRIAZINE [G]
AND 3,4,5 TRIBROMOSALICYLANILIDE [T] ON
CUTTING FLUID* FLORA†

| Biocide % | Week 1 | | Week 2 | | Week 3 | |
|----------------|---------------------|-----------------|---------------------|---------------------|---------------------|---------------------|
| | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml |
| [G]0.1 + [T] | 0 | 0 | 0 | 30 | 0 | 9 |
| [G]0.05 + [T] | 0 | 10 ² | 0 | 10 ³ | 0 | 5 × 10 ² |
| [G]0.025 + [T] | 3 × 10 ³ | 10 ⁴ | 0 | 2 × 10 ⁵ | 0 | 10 ⁵ |
| [T]0.005 | 2 × 10 ⁵ | 0 | 6 × 10 ⁵ | 0 | 9 × 10 ⁵ | 0 |
| Control | 2 × 10 ⁶ | 0 | 6 × 10 ⁵ | 0 | 2 × 10 ⁶ | 0 |

* In 5% o/w emulsion.

† Inoculum for both sets consisted of 10% v/v of a spoiled mixed cutting fluid with 3 × 10⁷/ml aerobic bacteria and no recoverable fungi.

Table III
THE EFFECT OF
HEXAHYDRO 1,3,5 TRIS (2-HYDROXYETHYL)-S-TRIAZINE [G]
AND
DIMETHYLDITHIOCARBAMIC ACID + 2-MERCAPTOBENZOTHIAZOLE [V]
ON CUTTING FLUID* FLORA†

| Biocide % | Week 1 | | Week 2 | | Week 3 | |
|----------------|---------------------|-----------------|---------------------|-----------------|---------------------|-----------------|
| | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml |
| [G]0.1 + [V] | 0 | 0 | 0 | 0 | 0 | 0 |
| [G]0.05 + [V] | 0 | 0 | 0 | 10 ³ | 0 | 10 ³ |
| [G]0.025 + [V] | 0 | 10 ³ | 2 × 10 ⁶ | 10 ⁶ | 10 ⁶ | 10 ⁶ |
| [V]0.005 | 2 × 10 ⁵ | 0 | 5 × 10 ⁵ | 0 | 6 × 10 ⁵ | 0 |
| Control | 2 × 10 ⁶ | 0 | 6 × 10 ⁵ | 0 | 2 × 10 ⁶ | 0 |

* In 5% o/w emulsion.

† Inoculum for both sets consisted of 10% v/v of a spoiled mixed cutting fluid with 3 × 10⁷/ml aerobic bacteria and no recoverable fungi.

Table IV
THE EFFECT OF
HEXAHYDRO 1,3,5 TRIS (2-HYDROXYETHYL)-S-TRIAZINE [G] AND
1-HYDROXYPYRIDINE-2-THIONE [O] ON CUTTING FLUID* FLORA†

| Biocide % | Week 1 | | Week 2 | | Week 3 | |
|----------------|---------------------|----------|---------------------|----------|---------------------|----------|
| | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml |
| [G]0.1 + [O] | 0 | 0 | 0 | 0 | 0 | 0 |
| [G]0.05 + [O] | 0 | 0 | 0 | 0 | 0 | 0 |
| [G]0.025 + [O] | 0 | 0 | 33 | 0 | 40 | 0 |
| [O]0.005 | 7 × 10 ⁵ | 0 | 10 ⁶ | 0 | 2 × 10 ⁶ | 0 |
| Control | 2 × 10 ⁶ | 0 | 6 × 10 ⁵ | 0 | 2 × 10 ⁶ | 0 |

* In 5% o/w emulsion.

† Inoculum for both sets consisted of 10% v/v of a spoiled mixed cutting fluid with 3 × 10⁷/ml aerobic bacteria and no recoverable fungi.

Table V
THE EFFECT OF
HEXAHYDRO 1,3,5 TRIS (2-HYDROXYETHYL)-S-TRIAZINE AND
2,3,5,6 TETRACHLORO-4-METHYL SULFONYLPYRIDINE [D] ON
CUTTING FLUID* FLORA†

| Biocide % | Week 1 | | Week 2 | | Week 3 | |
|----------------|---------------------|---------------------|---------------------|---------------------|---------------------|----------|
| | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml | Bact/ml | Fungi/ml |
| [G]0.1 + [D] | 0 | 0 | 0 | 0 | 0 | 0 |
| [G]0.05 + [D] | 0 | 0 | 0 | 0 | 0 | 0 |
| [G]0.025 + [D] | 300 | 0 | 0 | 0 | 0 | 0 |
| [D]0.005 | 10 ⁶ | 5 × 10 ³ | 10 ⁶ | 2 × 10 ⁴ | 1 × 10 ⁴ | 0 |
| Control | 2 × 10 ⁶ | 0 | 6 × 10 ⁵ | 0 | 2 × 10 ⁶ | 0 |

* In 5% o/w emulsion.

† Inoculum for both sets consisted of 10% v/v of a spoiled mixed cutting fluid with 3 × 10⁷/ml aerobic bacteria and no recoverable fungi.

[G] for bacteria and fungi. This pattern is also true for [V] (Table III), but to a lesser extent. In contrast, [O] and [D] (Tables IV and V) appear to be synergistic with [G] at all concentrations. The only one of the four fungicides evaluated by us in another emulsion earlier, [D], actually was antagonistic to [G]. Thus, before proceeding to further screening in additional fluids, we examined the combinations against pure cultures of selected fungi.

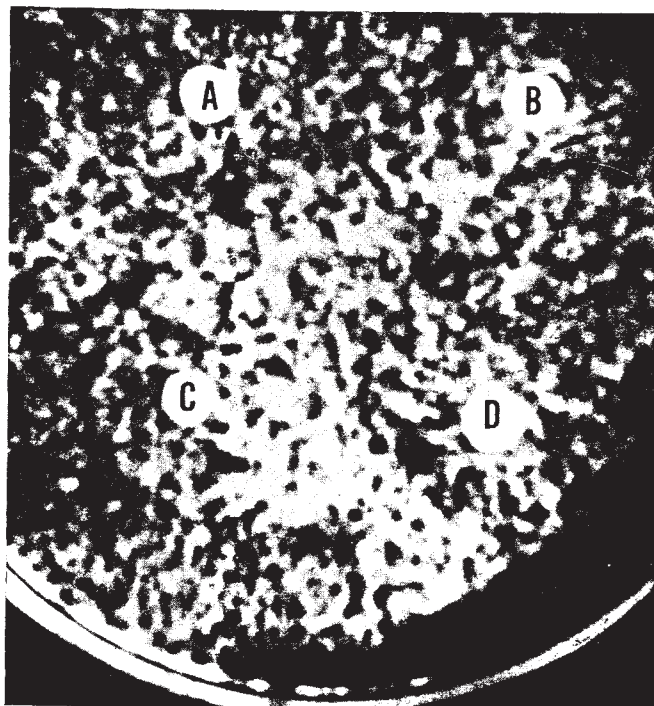


Fig. 1. Growth of *Fusarium* sp. in the presence of hexahydro 1,3,5 tris (2-hydroxyethyl)-s-triazine [G].

- A 50 mg/litre of [G].
- B 250 mg/litre of [G].
- C 500 mg/litre of [G].
- D 1000 mg/litre of [G].

Biocide effect on isolated fungi

The strain of primary interest to us was isolated from an industrial sample containing [G] and identified as a *Fusarium*. In Figs. 1-3 the results are obvious. Only [O] and [D] inhibited fungal growth and only the former did in the presence of [G]. The picture was no different with *G. candidum*, *P. notatum*, *M. pusillus*, *M. humicolus* and *R. stolonifer*. Thus, only [O] produced the same qualitative effect as observed in the emulsion. The antagonism between [D] and [G] poses an interesting problem. At present we have no answers with regard to [T] and [V]. We would suggest that they not be used with [G] without prior evaluation in the specific cutting fluid.

Utilization of [G] as growth substance

Although the appearance of fungi has been more evident after drastic reduction (oftentimes to zero) of viable bacterial populations by [G], the loss of ecological competition might not be the sole reason for fungal ascendancy. The possibility exists that [G] (or some derivative) could serve as carbon source for fungal growth. Indeed, Kaufman, Kearney and Sheets

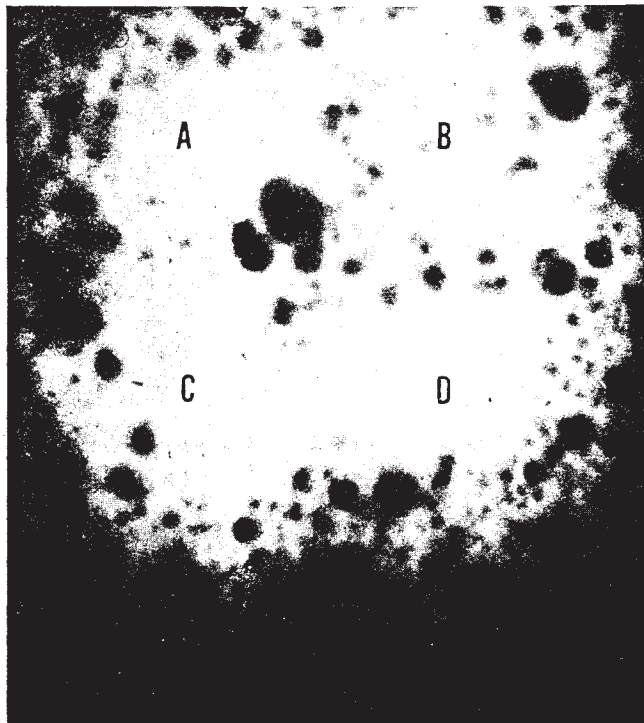


Figure 2. Growth of *Fusarium* sp. in the presence of hexahydro 1,3,5 tris (2-hydroxyethyl)-s-triazine [G] and 1-hydroxypyridine-2-thione [O]

- A 50 mg/litre of [O]
- B 50 mg/litre of [O] plus 250 mg/litre of [G].
- C 50 mg/litre of [O] plus 500 mg/litre of [G].
- D 50 mg/litre of [O] plus 1000 mg/litre of [G].

(1963, 1964) not only reported that a variety of fungal species (including *Fusarium* and *R. stolonifer*) not only degraded the herbicide, simazine (2-chloro-4,6-bis (ethylamino)-1,3,5-triazine, but also grew on it in the absence of other carbon sources. Also, Sikka *et al.* (1965) found that another herbicide, atrazine (2-chloro-4-ethyl-amino-6-isopropylamino 1,3,5 triazine), even at 10 ppm, stimulated growth of several fungal species. Unfortunately, our results were inconclusive in attempting to demonstrate utilization of [G]. Fungal inocula grew on MTA with and without the addition of [G]. The system we used was not sensitive enough to detect anything but qualitative differences.

Accordingly, nitrilite carry-over with the inoculum probably was responsible at least for the growth in MTA without [G]. Before ruling out the utilization of [G] for fungal growth, we are currently repeating these studies with severely washed inocula.

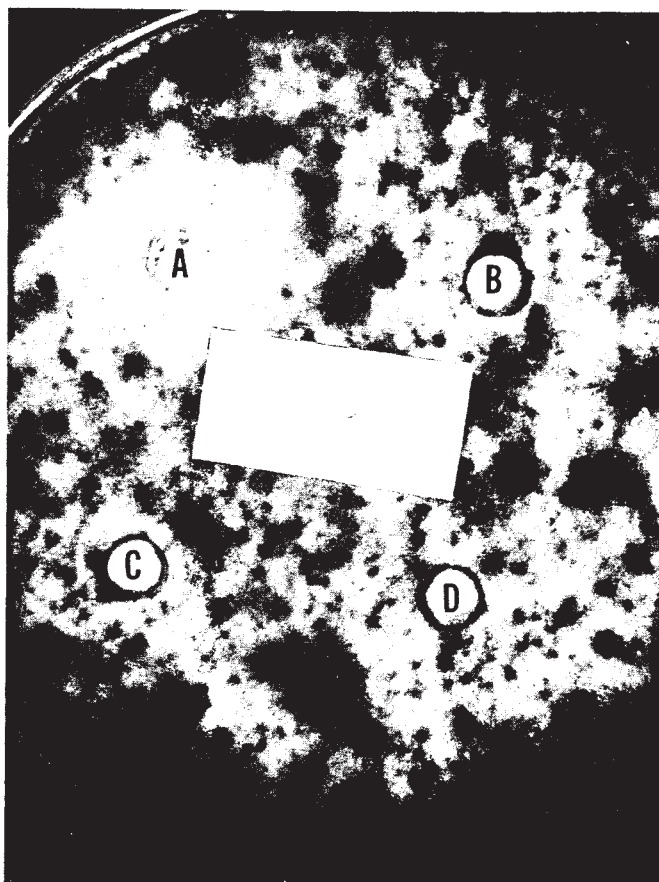


Fig. 3. Growth of *Fusarium* sp. in the presence of hexahydro 1,3,5 tris (2-hydroxyethyl)-s-triazine [G] and 2,3,5,6 tetrachloro-4-methyl sulfonylpyridine [D].

- A 50 mg/litre of [D].
- B 50 mg/litre of [D] plus 250 mg/litre of [G].
- C 50 mg/litre of [D] plus 500 mg/litre of [G].
- D 50 mg/litre of [D] plus 1000 mg/litre of [G].

However intriguing is the problem of fungal growth with [G], it is distressing from a pragmatic point of view. Two recent studies (Druskeit and Eggenperger, 1971, and Rossmoore and Williams, 1972) have shown that bacterial resistance does not develop to [G] and that in coolant systems where fluid replacement rate is rapid, *i.e.* 100% in two weeks, there was no fungal problem accompanying bacterial control. Thus, [G] still has potential as a

bactericide in the cutting fluid milieu, but it should be recommended judiciously with the *caveat* to watch concentration levels and replacement rates. This should be taken as a general warning for all biocides and not restricted to [G] alone.

Acknowledgement

We would like to thank Mr G. H. Holtzman for his technical assistance.

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