

## The Potentiation of Industrial Biocide Activity With $\text{Cu}^{2+}$ . II. Synergistic Effects With 5-Chloro-2-Methyl-4-Isothiazolin-3-One

V. F. Riha<sup>a</sup>, M. Sondossi<sup>b</sup> & H. W. Rossmoore<sup>a</sup>

<sup>a</sup>Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202, USA

<sup>b</sup>Université du Québec, Institut National de la Recherche Scientifique, 245 Boulevard Hymus, Pointe-Claire, Québec, Canada H9R 1G6

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### ABSTRACT

*The role of  $\text{Cu}^{2+}$  in enhancing 5-chloro-2-methyl-4-isothiazolin-3-one (IT) activity was investigated. This study was carried out in two directions. Firstly, the level of enhanced activity from a  $\text{Cu}^{2+}$  and IT mixture where environmental destruction of IT is minimal was examined. Secondly, the level of protection from  $\text{Cu}^{2+}$  in environments known to irreversibly reduce IT activity was studied. The bactericidal activities were determined in tryptic soy broth medium, mineral salt base-glucose medium and 0.9% NaCl solution. Under certain conditions,  $\text{Cu}^{2+}$  also stabilizes and/or protects the sensitive chlorinated IT molecule. Both synergistic activity and stabilization or protection by  $\text{Cu}^{2+}$  enhance the antimicrobial activity of IT under test conditions. Alternative sequential treatment with IT and  $\text{Cu}^{2+}$  was used to further characterize enhanced activity. The results suggest synergism. The utility of all the findings was investigated in metalworking fluid.*

### INTRODUCTION

One of the biocides widely used in an industrial setting is an isothiazoline mixture (Allsopp & Allsopp, 1983; Rossmoore, 1983) in which 5-chloro-2-methyl-4-isothiazolin-3-one (IT) is the active molecular

species. It is highly susceptible to irreversible destruction by nucleophiles in the environment. The commercial IT molecule requires the addition of large amounts of nitrates to maximize stability (Miller & Weiler, 1978), although this fact may be unrelated to nucleophilic reactions. Specifically, the nitrate ion is required for aqueous stability, whereas the cation is of no consequence (for example, copper nitrate and magnesium nitrate protect, whereas the  $\text{CuSO}_4$  does not). Metal salts of IT, including  $\text{Cu}^{2+}$ , were shown to have antimicrobial activity, although appropriate controls were lacking for the free IT and dosage levels were fairly high (Miller & Weiler, 1971).

The first published report of the apparent extended activity of IT with soluble  $\text{Cu}^{2+}$  involved monocopper citrate as the copper source (Rossmoore, 1986). Copper citrate has gained some acceptance as a metalworking fluid (MWF) additive due to the stability of this copper complex at the alkaline pH values of MWFs and its ability to sequester metabolic products producing obnoxious odors (e.g. sulfides and amines). This paper is based on the differentiation of the environmental and cellular effects of  $\text{Cu}^{2+}$  on IT.

## MATERIALS AND METHODS

### Media and culture condition

Unless stated otherwise, media and culture conditions were essentially the same as previously described (Sondossi *et al.*, 1990).

### Biocide and copper sources

A commercial mixture of 5-chloro-2-methyl-4-isothiazolin-3-one (IT active ingredient 10.87% w/w), and the non-chlorinated form (3.63% w/w) was used. Fresh dilutions of the biocide were prepared each time prior to use in concentrations required throughout the experiments. Concentrations indicated are based on the active ingredient.

Disodium monocopper (II) citrate (MCC) was available as a commercial concentrated aqueous solution, 1 M as copper. Other copper sources ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ ) were reagent grade chemicals.

### Determination of Cu-IT interactions

Unless stated otherwise, the procedure for determining enhanced activities was as previously described (Sondossi *et al.*, 1990).

The stability of the biocide is even more critical in concentrate when storage time is indefinite. The following two procedures were constructed to simulate the two kinds of biocide dosing. Firstly, the activities of IT and  $\text{Cu}^{2+}$  in soluble oil were evaluated for up to 28 days. Two 5% soluble oil emulsions were employed: one in which destruction of IT was previously shown to be negligible (emulsion A), and the other which had been known to inactivate IT (emulsion B). In these experiments, IT, IT- $\text{Cu}^{2+}$  mixture, and  $\text{Cu}^{2+}$  were added to the emulsions seeded with *Pseudomonas aeruginosa*. All permutations were in 120-ml bottles with a final volume of 100 ml, and each was plated periodically for up to 28 days.

The above experiments were not designed to differentiate between extracellular protection of the IT molecule or potentiation of IT antimicrobial activity. In order to determine the level of protection afforded IT by the  $\text{Cu}^{2+}$  in incompatible and compatible concentrates, a second series of experiments was performed. Concentrations of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$  added to the MWF concentrates were 20 mM which, upon preparation of a seeded 5% emulsion, yielded 1 mM. The concentration of IT added was 0.91 mM which yielded 0.045 mM on dilution. After 28 days, the concentrates were diluted to 5% and inoculated with *Ps. aeruginosa* as described previously (Sondossi *et al.*, 1990). Control 5% emulsions were also made from both concentrates in which IT and IT- $\text{Cu}^{2+}$  were added just prior to dilution. All experiments were carried out in 120-ml bottles with a final volume of 100 ml.

### Sequential treatment

Sequential treatment with  $\text{Cu}^{2+}$  and IT was as previously described (Sondossi *et al.*, 1990).

## RESULTS AND DISCUSSION

Increased activity of the IT and  $\text{Cu}^{2+}$  is clearly not an additive dose response effect. Addition of 5 mM  $\text{CuSO}_4$  to various levels of IT in tryptic soy broth medium (TSB) resulted in a considerable increase in antimicrobial activity (Fig. 1).

Copper toxicity is affected substantially by different media due to complex formation, with and without precipitation, and variation in pH. Thus, the selection of the copper compound (MCC) plays an important role in the quantitative aspects of the  $\text{Cu}^{2+}$  interactions (Jardim & Pearson, 1985; Sondossi *et al.*, 1990). Enhanced biocidal activity is

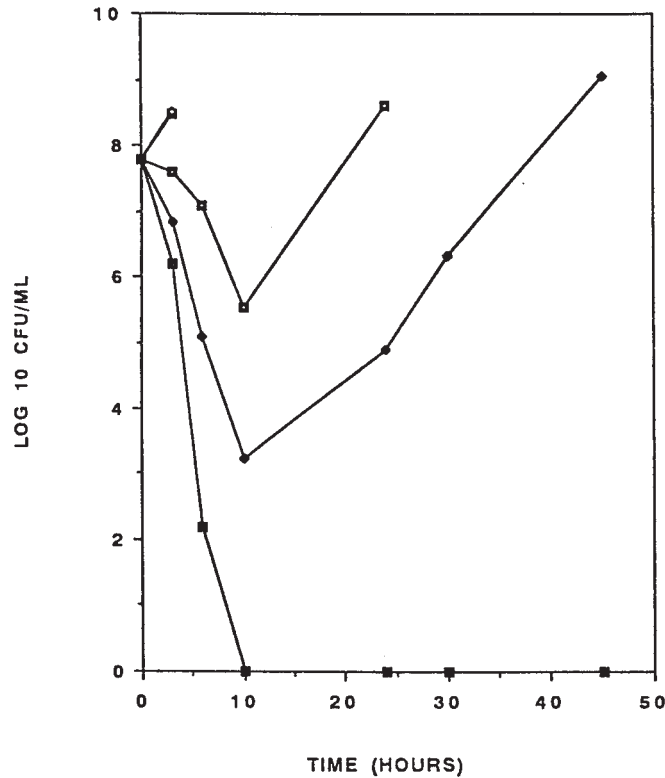


Fig. 1. *Pseudomonas aeruginosa* grown in TSB and treated with various concentrations of IT plus 5 mM CuSO<sub>4</sub>. Controls were 14 μM IT and 5 mM CuSO<sub>4</sub>; □, 5 mM CuSO<sub>4</sub>; ◆, 4.5 μM IT + CuSO<sub>4</sub>; ■, 2.3 μM IT + CuSO<sub>4</sub>; ◇, 14 μM IT; ■, 14 μM IT + CuSO<sub>4</sub>.

evident when combinations of IT and non-toxic levels of MCC are used in mineral salt base-glucose medium (MSB-G) and soluble oil medium (Fig. 2).

In a non-supportive medium (NaCl), regrowth of inhibited populations can be ruled out with the combination. In this unbuffered medium, CuSO<sub>4</sub> (15 μM) has an apparent activity greater than that of MCC (1 mM) when combined with IT (Fig. 3). In contrast, results with CuSO<sub>4</sub> in TSB (Fig. 1) demonstrate that higher concentrations of copper and IT were needed to give comparable results.

The results this far do not indicate whether the role of Cu<sup>2+</sup> is to protect the molecule in the extracellular environment, enhance activity at the level of the cell, or both. Two MWF systems known to be compatible (emulsion A) and incompatible (emulsion B), based on high-pressure liquid chromatography (HPLC) data (Law & Lashen, 1990), were

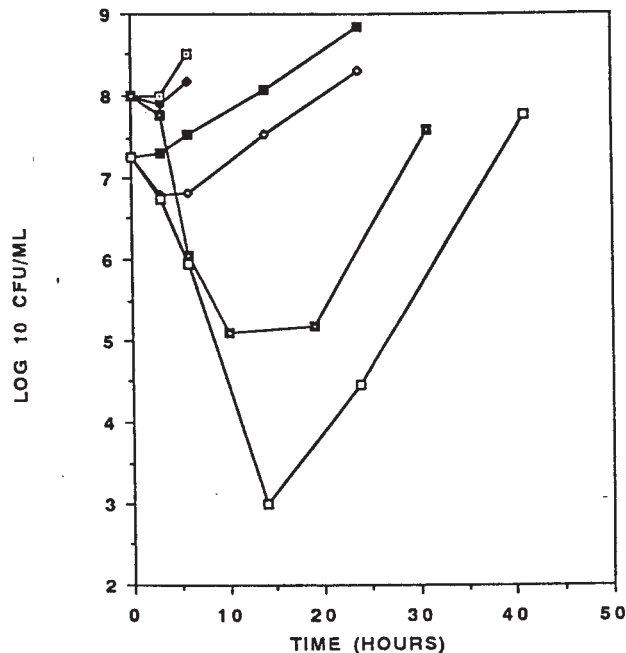


Fig. 2. *Pseudomonas aeruginosa* grown in MSB-G or 5% soluble oil emulsion and treated with various MCC (1 mM as Cu), 2.3  $\mu\text{M}$  IT, and MCC + IT.  $\square$ , MCC (MSB-G);  $\blacklozenge$ , 2.3  $\mu\text{M}$  IT (MSB-G);  $\blacksquare$ , MCC + IT (MSB-G);  $\diamond$ , 1 mM MCC (SO);  $\blacksquare$ , 2.3  $\mu\text{M}$  IT (SO);  $\square$ , MCC + IT (SO).

evaluated microbiologically with and without three different  $\text{Cu}^{2+}$  salts. The inoculum and copper were added to the emulsions at the same time. Thus, cell nucleophiles were competing with MWF nucleophiles in the same time frame. The results (Table 1) indicate that the incompatibility, in this short time-frame, is minimal. In addition, the anion used with the copper played no significant role, although special claims for nitrate anions have been made previously (Miller & Weiler, 1978). These authors stated that, among copper salts tested, only copper nitrate was affected. When levels of reactants ( $\text{Cu}^{2+}$ , IT) were increased in MWF concentrate and time of contact before inoculation was extended to 28 days, IT lost considerable activity in the absence of  $\text{Cu}^{2+}$  in emulsion B (Table 2).

Results obtained here, especially in the medium which does not inactivate IT (minimum extracellular interference) suggest synergism. On the other hand, the protection of IT in an incompatible environment (concentrate B) also indicates that it is possible for  $\text{Cu}^{2+}$  to stabilize the sensitive chlorinated IT molecule and/or competitively react with the

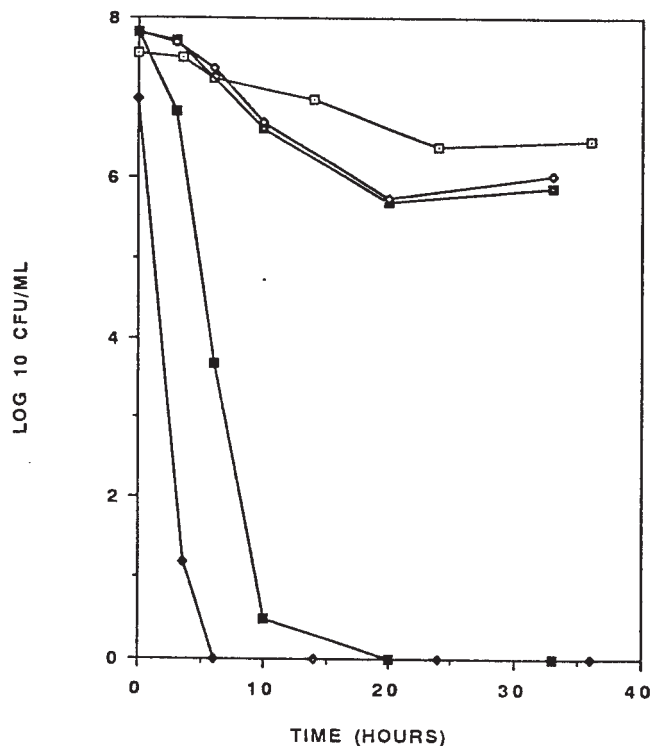


Fig. 3. *Pseudomonas aeruginosa* grown in TSB, resuspended in saline, treated with 15  $\mu\text{M}$   $\text{CuSO}_4$ , MCC (1 mM as Cu), 2.3  $\mu\text{M}$  IT, IT + MCC, and IT +  $\text{CuSO}_4$ . —□—, 15  $\mu\text{M}$   $\text{CuSO}_4$ ; —◇—,  $\text{CuSO}_4$  + 2.3  $\mu\text{M}$  IT; —■—, 1 mM MCC; —■—, MCC + IT.

nucleophiles that destroy IT. To further differentiate protection of IT in an extracellular environment from synergism at the cellular level, the bactericidal activities of IT and  $\text{Cu}^{2+}$  were studied in sequence. Sequential treatment of populations with IT and  $\text{CuSO}_4$  in either order in TSB apparently did not produce any increase in activity, while pretreatment with Cu-IT mixture followed by Cu-IT produced an effect greater than that shown in Fig. 1. Similarly, sequential pretreatment offered no advantages in MSB-G with MCC as the copper source over IT alone. However, sequential treatment in saline did produce synergistic effects (Fig. 4). Although pretreatment with both MCC and  $\text{CuSO}_4$  sensitized the cells to sequential treatment with IT, only  $\text{CuSO}_4$  had post-treatment effect after initial IT exposure. The reasons for this difference are not readily explained at this time.

The synergistic activity undoubtedly is related to both the mode of action of  $\text{Cu}^{2+}$  and IT. The combined antimicrobial activities of mixtures

**TABLE 1**  
Enhanced Activity of IT<sup>a</sup> with copper Salts in 5% Soluble Oil Emulsion A (compatible) and 5% Soluble Oil Emulsion B (incompatible) on *Pseudomonas aeruginosa*

ppm of biocide	cfu ml <sup>-1</sup>					
	24 h		72 h		17 days	
	Emulsion A	Emulsion B	Emulsion A	Emulsion B	Emulsion A	Emulsion B
Control	3 × 10 <sup>8</sup>	~10 <sup>9</sup>	2 × 10 <sup>8</sup>	1.5 × 10 <sup>8</sup>	2 × 10 <sup>8</sup>	1 × 10 <sup>7</sup>
17 μM IT <sup>a</sup>	1.5 × 10 <sup>4</sup>	3 × 10 <sup>6</sup>	3 × 10 <sup>7</sup>	2.5 × 10 <sup>7</sup>	4.6 × 10 <sup>7</sup>	2.1 × 10 <sup>6</sup>
1 mM CuSO <sub>4</sub>	1.2 × 10 <sup>5</sup>	3 × 10 <sup>6</sup>	6 × 10 <sup>5</sup>	2.3 × 10 <sup>7</sup>	7 × 10 <sup>4</sup>	3.0 × 10 <sup>4</sup>
1 mM Copper citrate	1.5 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>	3.5 × 10 <sup>5</sup>	3.5 × 10 <sup>5</sup>	2 × 10 <sup>5</sup>	—
1 mM Cu(NO <sub>3</sub> ) <sub>2</sub>	1.5 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>	1.2 × 10 <sup>7</sup>	1.4 × 10 <sup>7</sup>	5 × 10 <sup>6</sup>	1 × 10 <sup>3</sup>
17 μM IT <sup>a</sup> + CuSO <sub>4</sub>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
17 μM IT <sup>a</sup> + Copper citrate	2 × 10 <sup>2</sup>	4.5 × 10 <sup>3</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>
17 μM IT <sup>a</sup> + Cu(NO <sub>3</sub> ) <sub>2</sub>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>	<10 <sup>2</sup>

<sup>a</sup>5-chloro-2-methyl-4-isothiazolin-3-one.

TABLE 2  
Bactericidal Efficacy of  $\Gamma^v$  in a Compatible and an Incompatible Metalworking Fluid Emulsion

	<i>IT added 28 days before zero time; Cu<sup>2+</sup> added at zero time</i>			<i>IT and Cu<sup>2+</sup> added 28 days before zero time</i>		
	Time 0	24 h	7 days	Time 0	24 h	7 days
Emulsion A (compatible)						
45 $\mu\text{M}$ $\Gamma^v$ <sup>a</sup>	$2 \times 10^5$	$< 10^2$	$< 10^2$	—	—	—
1 mM Cu(NO <sub>3</sub> ) <sub>2</sub> + 45 $\mu\text{M}$ $\Gamma^v$ <sup>a</sup>	$2 \times 10^5$	$< 10^2$	$< 10^2$	$2 \times 10^5$	$< 10^2$	$< 10^2$
Emulsion B (incompatible)						
45 $\mu\text{M}$ $\Gamma^v$ <sup>a</sup>	$4 \times 10^4$	$1.1 \times 10^7$	$3 \times 10^7$	—	—	—
1 mM (CuNO <sub>3</sub> ) <sub>2</sub> + 45 $\mu\text{M}$ $\Gamma^v$ <sup>a</sup>	$4 \times 10^4$	$6 \times 10^6$	$7 \times 10^6$	$4 \times 10^4$	$< 10^2$	$< 10^2$

<sup>a</sup> 5-chloro-2-methyl-4-isothiazolin-3-one.

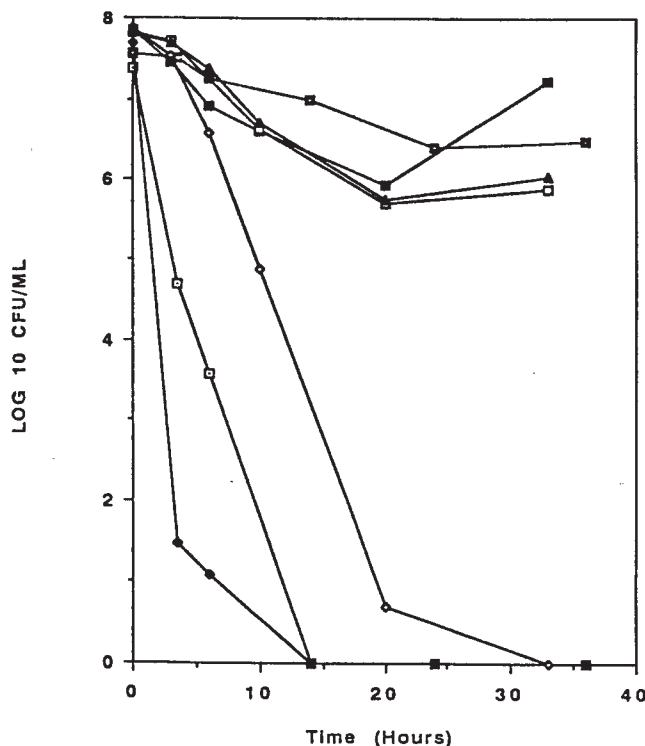


Fig. 4. *Pseudomonas aeruginosa* grown in TSB, resuspended in saline, treated sequentially (see Materials and Methods) with  $15 \mu\text{M}$   $\text{CuSO}_4$ ,  $2.3 \mu\text{M}$  IT. The cells were treated sequentially with MCC (1 mM as Cu),  $2.3 \mu\text{M}$  IT. ◻,  $\text{CuSO}_4$ -to-IT; ◆, IT-to- $\text{CuSO}_4$ ; ◼, -to- $\text{CuSO}_4$ ; ◊, MCC to IT; ■, IT to MCC; ◻, -to MCC; ▲, -to IT.

can be estimated from the dose effect or dose response curves of the mixture and each compound alone (Nordberg *et al.*, 1978). Synergism (when the effect of the combined exposure is greater than the additive effect) and antagonism (when the dose response to the mixture is less than additive), and other factors that may influence the responses, have been discussed in other reports (Veldstra, 1956; Murphy, 1980; Sondossi *et al.*, 1990).

No reports have been published on the mode of action of IT, although one report exists on a benzisothiazolin derivative (Fuller *et al.*, 1985) which suggests that this compound reacts with thiol-containing enzymes and glutathione. This may be the major mode of action. Based on the reactivity of IT with reduced sulfur compounds, i.e. neutralization, the same could be said for the mode of action of IT. With respect to  $\text{Cu}^{2+}$ , it has been suggested (Salhany *et al.*, 1978) that the cupric ion can directly oxidize the membrane protein sulfhydryls. Therefore, when sites of

interaction are considered (Veldstra, 1956; Murphy, 1980; Sondossi *et al.*, 1990), it is possible that  $\text{Cu}^{2+}$  could oxidize thiol groups of the cell membrane, preventing their interaction with IT and therefore enhancing the activity of IT by directing its toxicity to more vital targets. This suggestion does not exclude manifestation of synergism based on overall reduced capacity of the cells exposed to both compounds (Murphy, 1980; Sondossi *et al.*, 1990).

Some of the above suggestions may be used in the interpretation of results obtained with sequential treatment. Such treatment offers a new approach in the study of mode of action of synergistic mixtures.

These data may have important practical significance. Isothiazoline is the most dose-effective preservative currently in use in industry. It has a broad spectrum of activity; in-use levels have an acceptable toxicological profile; and it is readily degraded in the environment (Krezminski *et al.*, 1975). However, its reactivity with nucleophilic groups shortens half-life and results in excessive dosing and the premature selection of another biocide.

The use of  $\text{Cu}^{2+}$  with IT may effectively reduce the dose of IT and minimize its toxicological potential. Biocides are applied in two ways in MWFs: in concentrated MWF which is then diluted prior to use, or directly into the diluted MWF at the recommended dose. The former practice, although it has certain technical drawbacks (inability to regulate time and size of dose, for example), has logistical and marketing advantages for the user (e.g. eliminating the need for specialized management and record keeping). The reactivity of IT for nucleophiles has made it impractical and even impossible to add to a number of MWFs rich in nucleophilic molecules, e.g. amines.

Copper has been used successfully in making IT additions at appropriately high levels in MWF concentrates without subsequent loss of activity (Law & Lashen, 1990) under some environmental conditions. The use of Cu-IT mixture appears to satisfy two separate goals: protection of IT from nucleophilic attack in the extracellular environment, and synergistic enhancement of activity at the cellular level.

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