

# The Mechanism of Action of Isothiazolone Biocides

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## ABSTRACT

**Isothiazolone biocides have proven efficacy and performance for microbial control in a variety of industrial water treatment applications. Understanding the mechanism of action of industrial biocides is important in optimizing their use and combating resistance if encountered. Isothiazolones utilize a two-step mechanism involving rapid inhibition (minutes) of growth and metabolism, followed by irreversible cell damage resulting in loss of viability (hours). Cells are inhibited by disruption of the metabolic pathways involving dehydrogenase enzymes. Critical physiological functions are rapidly inhibited in microbes, including growth, respiration (oxygen consumption), and energy generation (adenosine triphosphate synthesis). Cell death results from the destruction of protein thiols and production of free radicals. The rate and extent of killing may be enhanced by various adjuvants including surfactants. This unique mechanism results in a broad spectrum of activity, low use levels, and difficulty in attaining resistance.**

## INTRODUCTION

Isothiazolone biocides are widely used in a variety of industrial water treatment applications for control of microbial growth and biofouling [1]. The most frequently used product is a 3:1 ratio of 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT) and 2-methyl-4-isothiazolin-3-one (MIT). CMIT/MIT has broad spectrum efficacy versus bacteria, algae, and fungi. 1,2-benzisothiazolin-3-one (BIT) products have been used in a limited range of industrial applications requiring long-term preservation for bacterial control, including mineral processing and closed loop cooling systems. Recently, a new microemulsion technology was introduced using 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) as an algicide for cooling water treatment and a fungicide for paper mill applications [2]. The most recent isothiazolone biocide for industrial water systems is based on the MIT active ingredient alone and is targeted at long-term preservative applications with higher pH and temperature ranges.

Understanding the mode of action of industrial biocides is important in optimizing their use and combating resistance if encountered [3]. The antimicrobial effects of isothiazolone biocides have been broadly defined for CMIT and

BIT [1,3–11]. These biocides function as electrophilic agents, reacting with critical enzymes to inhibit growth and metabolism, with cell death occurring after several hours contact. Less is known about the mechanism of MIT and DCOIT. Oxidizers also function as electrophiles, with rapid speed of killing, whereas quats and other membrane active agents tend to directly affect cell membranes (*Figure 1*) [3].

The intent of this paper is to review the mechanism of action of isothiazolone biocides across a range of microorganisms, including cell binding, inhibition of growth and respiration, interaction with proteins and enzymes, and subsequent loss of viability (cell death). The impact of the mechanism will be discussed relative to monitoring efficacy, resistance and ways to improve their performance.

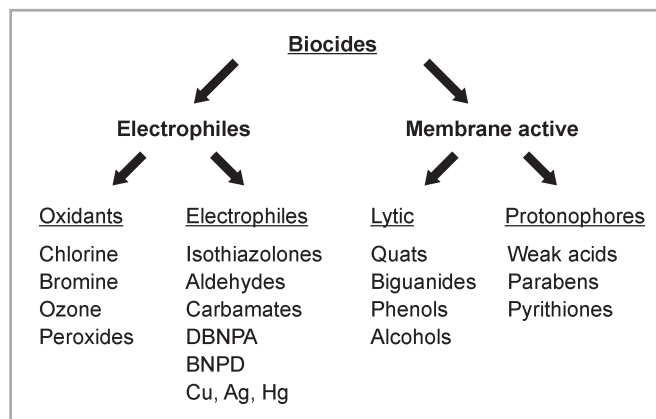


Figure 1: Mechanism of action of industrial biocides.

DBNPA 2,2-dibromo-2-cyanoacetamide  
BNPD 2-bromo-2-nitropropane-1,3-diol

## MATERIALS AND METHODS

### Biocides Evaluated

The chemical structures of the isothiazolone biocides described in this paper are shown in *Figure 2*. CMIT/MIT is composed of a 3:1 ratio of CMIT:MIT at a final concentration of 1.5 % total active ingredient (ai).<sup>(1)</sup> DCOIT is formulated at a concentration of 4.25 % active ingredient in a water-based microemulsion.<sup>(2)</sup> MIT biocide is a 9.5 % active solution in water.<sup>(3)</sup> OIT biocide is a 5 % active solu-

<sup>(1)</sup> Kathon™ WT 1.5% Microbicide (Rohm and Haas Company; Philadelphia, PA)

<sup>(2)</sup> Klarix™ 4000 Microbicide (Rohm and Haas Company; Philadelphia, PA)

<sup>(3)</sup> Kordek™ MLX Microbicide (Rohm and Haas Company; Philadelphia, PA)

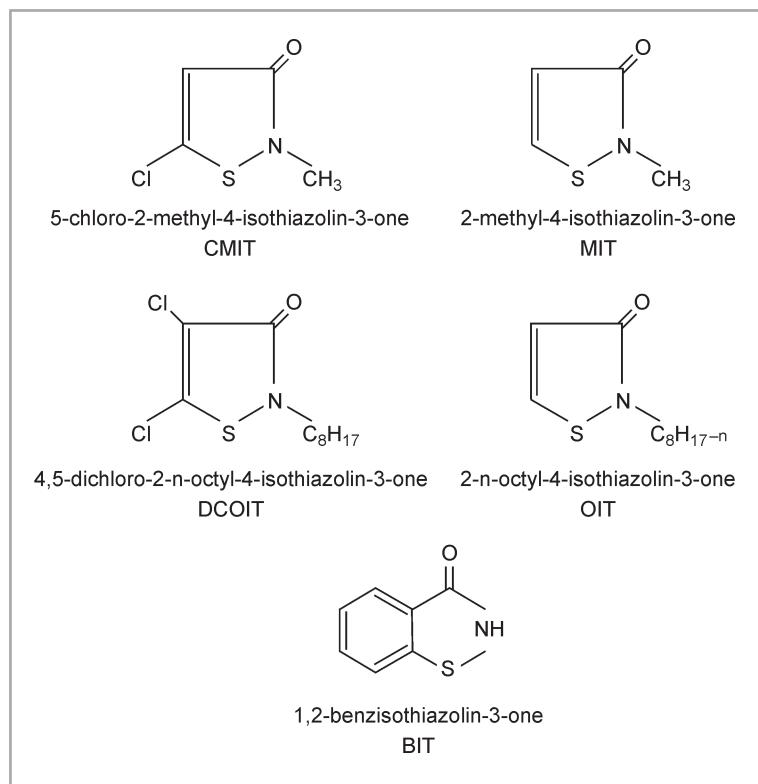


Figure 2: Chemical structure of isothiazolone biocides.

tion in propylene glycol and was included for comparison. All biocide concentrations in this paper are reported on an active ingredient basis. The isothiazolone biocides described in this paper are manufactured by Rohm and Haas Company. Solutions of CMIT and MIT were prepared in distilled water and kept frozen until use. DCOIT and OIT were diluted in 50:50 (v:v) ethanol:water. All other chemicals used were reagent grade or the highest available purity, and most were obtained from Sigma Chemical Company, St. Louis, MO, unless stated otherwise.

### Organisms and Culture Conditions

Pure cultures were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). *Saccharomyces cerevisiae* ATCC 4921, *Aspergillus niger* ATCC 6275, and *Pseudomonas aeruginosa* ATCC 13388 were grown in Sabouraud's dextrose broth (SDB), potato dextrose broth (PDB), and M9G minimal salts media with glucose at 30 °C, respectively [4]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 15442 were also obtained from ATCC and were routinely grown in M9G media. Cultures were incubated at 37 °C (*E. coli*) or 30 °C (*P. aeruginosa*).

### Transport and Binding of Isothiazolone Biocides with *S. Cerevisiae* Cells

Cell binding studies were conducted to determine the association of the isothiazolone biocide with microbial

cells. Chemostat-grown cells of *S. cerevisiae* were used in these studies in the presence of CMIT, MIT, and DCOIT biocides. Cells were treated with radio-labeled biocide and radioactivity levels were used to determine the uptake of the biocide into the cells.

Fungal cultures were grown in New Brunswick Scientific BioFlow chemostats at 30 °C and an agitation rate of 100 revolutions per minute. *S. cerevisiae* was grown in SDB + 0.4 % glucose at a generation rate of 5.6 h. Cells were harvested in sterile 50 mL Oak Ridge tubes, washed, and resuspended to an absorbance (660 nm) of 0.8 in 10 mM PO<sub>4</sub> buffer (pH 7.2). Samples were frozen and protein content was determined.

Protein was determined using the Bio-Rad automated microassay in 96-well microplates using the Biomek 2000 Workstation. The plates were incubated at room temperature for 5 to 60 minutes. Optical density (OD) at 650 nm was measured using a Molecular Devices Thermomax MicroPlate Reader. Data was analyzed using the Molecular Devices SoftMax program.

### Determination of Growth Inhibition and Susceptibility

The lowest concentration of biocide required to inhibit growth was determined by a high resolution minimum inhibitory concentration (MIC) test. Varying amounts of the test compound were added to media in a 96-well microtiter plate. Ten-fold serial dilutions were performed on a Biomek 2000 Workstation to obtain a range of closely spaced concentrations. A cell suspension, adjusted to provide 10<sup>6</sup> colony forming units (cfu) per ml in each well, was added to the microtiter plate. Microtiter plates were incubated at 30 °C for 48 h and then were checked for the presence or absence of growth in each well. The concentration of compound in the first microtiter well demonstrating no growth was the MIC for the test compound.

Minimum biocidal concentrations (MBCs) were determined by transferring an aliquot from the MIC plates to trypticase soy broth (TSB) media containing 0.5 mg · mL<sup>-1</sup> sodium thioglycolate to neutralize the remaining biocide. The lowest concentration of biocide in which no survivors were recovered was defined as the MBC. Data reported are the average of multiple determinations ± the standard error.

The response of *Escherichia coli* (ATCC 25922) to CMIT inhibition, over the course of the growth cycle, was determined by following increases in absorbance (turbidity) of the culture at 660 nm at 37 °C in M9G media. Biocide was added to the culture and growth measured in the presence of 1.0 and 2.5 mg · kg<sup>-1</sup> CMIT. Control samples contained no biocide.

### Effects on Microbial Respiration (Oxygen Uptake)

Respiration in *E. coli* was measured using a polarographic electrode. Cells were grown overnight in M9G medium, diluted 1:50 into fresh M9G and allowed to grow to an absorbance of 0.2 at 660 nm. The cultures were washed in glucose-free M9G, resuspended to the same density in glucose-free M9G, and incubated 2 h at 37 °C to deplete the cells of endogenous carbon reserves. The absorbance was readjusted to 0.2 absorbance units at 660 nm. The assay was performed on 2 mL aliquots placed in a water jacketed steel chamber equipped with a Clark oxygen electrode (Hansatech LTD, Norfolk, UK). Substrate (glucose) was added to the sealed chamber to a final concentration of 10 mM, and biocide (134 µM) was added after a steady rate of oxygen consumption was reached (generally within 2 min).

### Effects on ATP Synthesis

ATP (adenosine triphosphate) was determined by the luciferin-luciferase reaction using a Turner 20DE Lumino-meter and the Sigma Luciferase Kit (Sigma Chemicals). A mid-log phase culture of *E. coli* grown in M9G media was diluted 1:2 into fresh M9G media pre-warmed to 37 °C. The culture was divided into 25 mL subcultures and placed in a shaking water bath at 200 revolutions per minute and 37 °C. The cultures were allowed to equilibrate, with 500 µL samples removed every 5 min. One culture received 5 µg · mL<sup>-1</sup> final concentration CMIT and the other received an equal volume of sterile water. Over time, 500 µL aliquots were removed, added to 100 µL 1.5 M HNO<sub>3</sub>, vortexed, and allowed to sit on ice for 5 min. The samples were then diluted into 4.4 mL of 0.25 M Tris (hydroxymethyl)-aminomethane-HCl (Tris-HCl), pH 7.8, and mixed. A 50 µL sample was placed in the ATP assay tube along with 50 µL of assay mix. The assays were initiated by the injection of 5 µL aliquots of a 1:10 dilution of the luciferase enzyme. Peak luminosity values were recorded, and compared to a standard curve constructed in spiked cell extracts. Control experiments demonstrated that high levels of CMIT did not inhibit the luciferase reaction.

### Isothiazolone-Induced Inhibition of Yeast Alcohol Dehydrogenase

Alcohol dehydrogenase (ADH) was assayed according to Vallee and Hoch [12]. In this method, the reduction of nicotinamide adenine dinucleotide (NAD) to NADH was followed by measuring absorbance of the reaction mixture at 340 nm. The reaction mix was added to a 3 mL cuvette and contained the following: 0.3 mL of a 12 mg · mL<sup>-1</sup> NAD solution; 0.3 mL of a 19.4 % ethanol solution; 1 mL of a 0.1 M phosphate buffer (pH 7.8); 50 µL of 25 µg · mL<sup>-1</sup> ADH solution, isothiazolone solution, and deionized water. The reaction mixture was initiated by the addition of the ADH enzyme.

### Effect of Isothiazolone on Cell Viability and Intracellular Thiols

An overnight culture of *S. cerevisiae* was diluted 1:20 into 250 mL of fresh media and incubated for 3 h. The culture was divided into 40 mL aliquots and dosed with various concentrations of DCOIT or with other isothiazolones. After a 30 min incubation, viable counts were determined by the most probable number (MPN) method in media containing 500 mg · kg<sup>-1</sup> sodium thioglycollate. 40 mL of the sample was centrifuged at 5 000 revolutions per minute for 5 min and resuspended in ice-cold media supplemented with 500 mg · kg<sup>-1</sup> sodium thioglycollate. The samples were washed twice by centrifugation, each time being resuspended in 10 mL of ice-cold supplemented media. After the final wash, cell pellets were resuspended in 1 mL of ice-cold 10 % trichloroacetic acid and incubated on ice for 30 min. At this time, the samples were centrifuged at 14 000 revolutions per minute in a microcentrifuge for 2 min. The supernatant was decanted and saved. The pellet was resuspended in 1 mL of 0.4 M Tris-EDTA (pH 8.9). Samples (200 µL) of both the acid soluble extract and insoluble material were added to 1 mL of Tris-EDTA buffer, then 20 µL of dithiobisnitrobenzoic acid (DTNB)/methanol (99 mg/25 mL) solution was added. After 5 min incubation of this mixture, the absorbance at 412 nm was measured. Thiol concentrations in the samples were determined from a linear equation derived from a standard curve of glutathione spiked into both the acid soluble and insoluble material samples. Acid soluble and insoluble thiols were determined using Ellman's reagent (dithiobisnitrobenzoic acid, DTNB) to react with available thiol.

### Free Radical Studies

Electron spin resonance (ESR) spectroscopy was used to detect radicals (unpaired electrons) by their resonance characteristics in a changing magnetic field. Spin trapping is used to capture highly reactive radicals with reporter molecules (usually nitroso or nitrones) to create less reactive adducts (nitroxides). ESR scans were conducted on bacterial cells in the presence and absence of biocide.

The organism used in these studies was *Escherichia coli* ATCC 25922. *E. coli* was grown in M9G medium in a chemostat (Bioflo I, New Brunswick Scientific, New Brunswick, NJ) with a generation time of 3 h. Cells were prepared by harvesting 320 mL of chemostat culture into eight 40 mL Oak Ridge centrifuge tubes, and pelleted at 6 000 revolutions per minute for 5 min in a J20 rotor. The cell pellets were resuspended in an equal volume of 10 mM sodium phosphate buffer, pH 7.0, vortexed, and pelleted. The cells were resuspended again in phosphate buffer and placed on an orbital shaker until use.

Experiments were performed by placing 1.5 mL of concentrated culture in a microcentrifuge tube and pelleting the cells in a microcentrifuge at 14 000 revolutions per minute for 45–60 sec. The pellet was resuspended to approximately 175 µL with 10 mM phosphate buffer. 60 µL of 1 M DMPO (5,5-dimethylpyrroline-N-oxide) solution was added

and mixed, and then the reaction was initiated by the addition of 60  $\mu\text{L}$  of biocide stock solution. For CMIT and MIT the stock solution was 0.5 M; for NEM (N-ethyl maleimide) the stock solution was 0.25 M. The cells were resuspended to 115  $\mu\text{L}$  and the amount of biocide addition was 120  $\mu\text{L}$ . Biocide stock solutions were made in deionized water. The mixture was placed in an ESR flat cell using a syringe and 20 gauge needle, and incubated at room temperature for 20 min before ESR measurements were begun.

ESR measurements were made using a Bruker ESP300 spectrometer operated at 9.5 GHz with 100 kHz modulation frequency. ESR spectra were collected from samples placed in a standard variable temperature flat cell, 0.3 mm thick and 13 mm wide. Typical conditions for acquisition of spectral data were as follows: temperature, ambient; microwave power, 20 mW; modulation amplitude, 0.2837 mT; conversion time, 10.24 ms; time constant, 1.28 ms; sweep time, 10.486 s; accumulations averaged, fifty scans; sweep width, 20 mT with 1024-point resolution. Data collection conditions were chosen to optimize signal-to-noise ratio as much as possible while avoiding power saturation or significant line broadening of the spectrum. The time of the experiment is started at the point at which the biocide is added to the cells or solution being studied. The time indicated (e.g., 20 min) is the time at which data collection was initiated.

## RESULTS AND DISCUSSION

### Transport and Binding of Isothiazolone Biocides with Cells

Results of binding studies with yeast cells showed that DCOIT exhibited the greatest affinity for microbial cells with rapid uptake over the 10 minute contact period (Figure 3). CMIT showed a slow but steady increase in cells but the extent was approximately one-fifth that of DCOIT (the more hydrophobic molecule). MIT showed the lowest rate and extent of binding with values slightly above

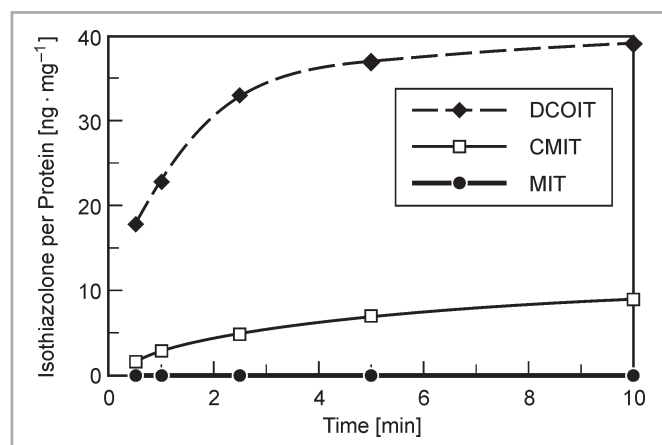


Figure 3: Binding of isothiazolone biocides (10  $\mu\text{M}$ ) with the yeast *S. cerevisiae*.

baseline controls. It should be noted that MIT is the most hydrophilic and water soluble of all the isothiazolones tested.

Biocide transport studies conducted by Diehl and Chapman with CMIT and MIT biocides showed that two processes are distinguished: one active at use levels (1–15  $\text{mg} \cdot \text{kg}^{-1}$ ) and the other active at very high levels [5]. Low level biocide transport is an active process requiring an energized membrane (highest uptake rate). Higher levels of biocide (> 15  $\text{mg} \cdot \text{kg}^{-1}$ ) inhibit the uptake process. Collier et al. observed a greater uptake with CMIT versus MIT in both bacteria and yeast cells [10]. These results indicate that CMIT and MIT are likely analogues of a natural substrate, which would be actively transported into the cells to fill specific metabolic needs. Additionally, MIT is a poor inhibitor of CMIT uptake.

### Inhibition of Microbial Growth

Studies with five isothiazolone biocides versus two fungi were conducted to compare the lowest biocide level required for both inhibition (MIC) and cell death (MBC) (Table 1). With the exception of MIT, all isothiazolones

Biocide	<i>Aspergillus niger</i> <sup>2</sup>		<i>Saccharomyces cerevisiae</i> <sup>3</sup>	
	MIC	MBC	MIC	MBC
CMIT	0.35 ± 0.05	0.42 ± 0.04	0.58 ± 0.1	0.63 ± 0.1
CMIT/MIT	0.40 ± 0.07	0.47 ± 0.05	0.71 ± 0.1	0.76 ± 0.2
MIT	166 ± 52	300 ± 0	60.0 ± 0	87 ± 10.3
DCOIT	0.12 ± 0.07	0.23 ± 0.14	0.55 ± 0.1	0.79 ± 0.5
OIT	0.05 ± 0.01	0.05 ± 0.01	0.56 ± 0.1	0.65 ± 0.2

Table 1: Minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) values for isothiazolone biocides<sup>1</sup>.

<sup>1</sup> Values reported as  $\text{mg} \cdot \text{L}^{-1}$  active  $\pm$  standard deviation of 3–5 replicates.

<sup>2</sup> The inoculum was approximately  $10^4$  spores per ml and the medium was potato dextrose broth.

<sup>3</sup> The inoculum was approximately  $1\text{--}2 \cdot 10^5$  cfu per ml and the medium was Sabouraud's dextrose broth.

showed extremely high efficacy at levels less than  $1 \text{ mg} \cdot \text{kg}^{-1}$  active. In most cases, the inhibitory and cidal levels were nearly identical. No major differences were seen between CMIT, CMIT+MIT, DCOIT and OIT. MIT showed the weakest efficacy versus the two fungi. Previous studies evaluating both bacteria and fungi confirmed MIT as less effective than CMIT [1,5].

The effect of CMIT biocide on the growth of bacteria was evaluated versus *E. coli*. Biocide was added to an actively growing culture and growth (cell density absorbance) was monitored for 90 minutes. Addition of  $1 \text{ mg} \cdot \text{kg}^{-1}$  CMIT slowed the initial rate of growth of the bacteria within 30 min of addition, but cells continued to grow (Figure 4). Addition of  $2.5 \text{ mg} \cdot \text{kg}^{-1}$  CMIT showed an immediate cessation of growth in less than 30 min and no further increase in cells was observed.

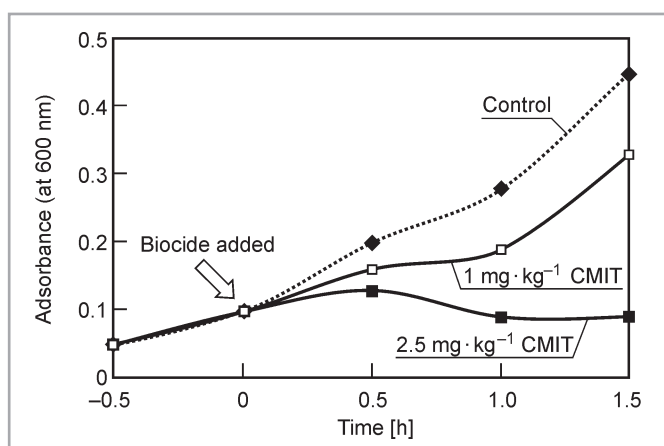


Figure 4: Inhibition of bacterial growth (*E. coli* ATCC 25922) by CMIT.

Previous studies with CMIT/MIT biocide showed both inhibition and killing of a mixed population of gram negative bacteria in synthetic cooling water [1]. Higher doses of biocide increased the rate and extent of killing with  $5 \text{ mg} \cdot \text{kg}^{-1}$  producing greater than 99.9 % kill within four hours. Similar use levels also were shown to be effective versus several strains of *Legionella* [1]. Addition of various non-ionic surfactants increased both the rate and extent of killing for CMIT/MIT. Studies with DCOIT showed  $< 2 \text{ mg} \cdot \text{kg}^{-1}$  active destroyed algal chlorophyll in studies with green algae [1,2].

### Inhibition of Respiration (Oxygen Uptake)

The effects of biocides on microbial respiration (oxygen uptake) by *E. coli* demonstrated that all of the isothiazolone biocides rapidly shut down consumption of oxygen using glucose within 6 min of contact (Figure 5). Biocides were added at equal molar amounts (134 M). CMIT ( $20 \text{ mg} \cdot \text{kg}^{-1}$ ) and OIT ( $29 \text{ mg} \cdot \text{kg}^{-1}$ ) showed the most rapid rate of inhibition, followed by MIT ( $15 \text{ mg} \cdot \text{kg}^{-1}$ ), with DCOIT ( $38 \text{ mg} \cdot \text{kg}^{-1}$ ) the slowest of the four tested. These data are in good agreement with previous studies showing  $2 \text{ mg} \cdot \text{kg}^{-1}$  of CMIT/MIT producing immediate inhibition of

respiration within 5 min of addition to a mixed culture of bacteria in synthetic cooling water [1]. Additional studies confirmed the same effect was observed in cooling tower water with native bacteria. Similar results have been observed for DCOIT (data not shown).

The effect of shutting down respiration on microorganisms means that no oxygen is consumed to produce energy for growth and metabolism. Thus all aerobic processes cease to function. Anaerobic processes do not rely on oxygen, yet isothiazolone affects growth, energy (ATP), and enzymes, all of which are critically disabled.

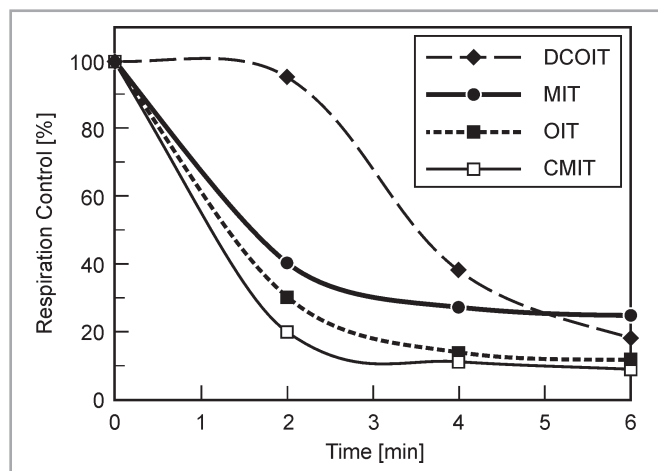


Figure 5: Rapid inhibition of bacterial respiration (oxygen uptake) by isothiazolones ( $134 \mu\text{M}$ ) with *E. coli* (ATCC 25922).

### Inhibition of ATP Synthesis

A critical function in microbial cells is the production of energy. This is accomplished via the high energy carrier, ATP. All living functions rely on ATP to drive their biochemical reactions. Without ATP, cells cannot grow, divide, or repair damaged functions.

Isothiazolone biocides rapidly inhibit the production of ATP in bacterial cells within a short contact period. Studies with

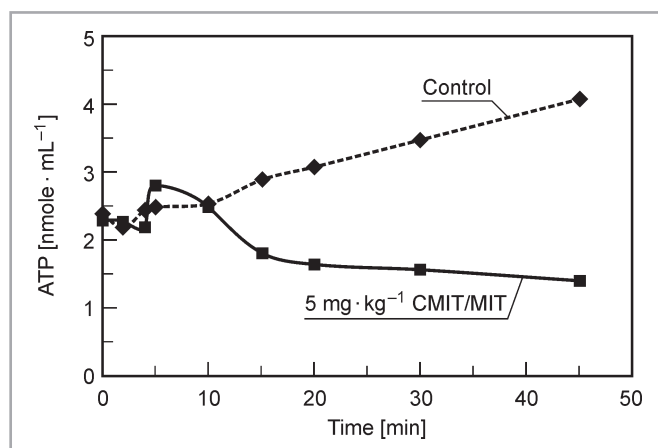


Figure 6: Inhibition of bacterial (*E. coli*) ATP synthesis by CMIT.

*E. coli* (Figure 6) demonstrate that low levels of CMIT/MIT biocide effectively disable the ATP generation potential in microorganisms. Within 10 minutes of contact, no further ATP is produced by the cells. Coupled with the loss of respiration, loss of ATP production will cripple cells, preventing both growth and repair of damaged components.

**Enzyme Inhibition**

In this study, the effect of four isothiazolone biocides on purified alcohol dehydrogenase was evaluated (Figure 7). The results showed that DCOIT and OIT displayed the most rapid inhibition, with near-complete reduction in activity within 15 min. CMIT and MIT showed similar effects with approximately 70 % of the activity remaining after 15 min. In this case, the more hydrophobic molecules provided the most rapid inhibitory effects on the enzyme *in vitro*.

Previous studies with isothiazolone biocides have shown that certain sulfur-containing dehydrogenase enzymes are the specific enzymes inhibited among a wide variety tested [4,6,7,9]. The specific enzymes which isothiazolones inhibit include pyruvate dehydrogenase, alpha-ketoglu-

tarate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase, lactate dehydrogenase, and alcohol dehydrogenase. The only non-dehydrogenase enzyme inhibited by isothiazolones was adenosine-triphosphatase. Enzyme inhibition studies have confirmed that isothiazolones inhibit critical dehydrogenase enzymes on both purified enzyme extracts (*in vitro*) and whole cell or cell extracts (*in vivo*). This high degree of specificity results in low use levels for the isothiazolone family of biocides and also means that the biocide is not "used up" reacting with non-essential proteins or enzymes (those not containing thiols).

Disabling these dehydrogenase enzymes can result in complete inhibition of critical metabolic functions, the same as mentioned previously for ATP and respiration inhibition. Inhibition of dehydrogenase enzymes also plays a critical role in energy generation and cell growth, involving the Krebs (tricarboxylic acid) cycle. As illustrated in Figure 8, the isothiazolones block or control sites entering, leaving and within the hub of the Krebs cycle. Therefore, cells cannot process required intermediate compounds for growth or generate energy by electron transport (with oxygen). Crippling this key pathway is central to the growth-inhibiting mechanism of isothiazolone biocides.

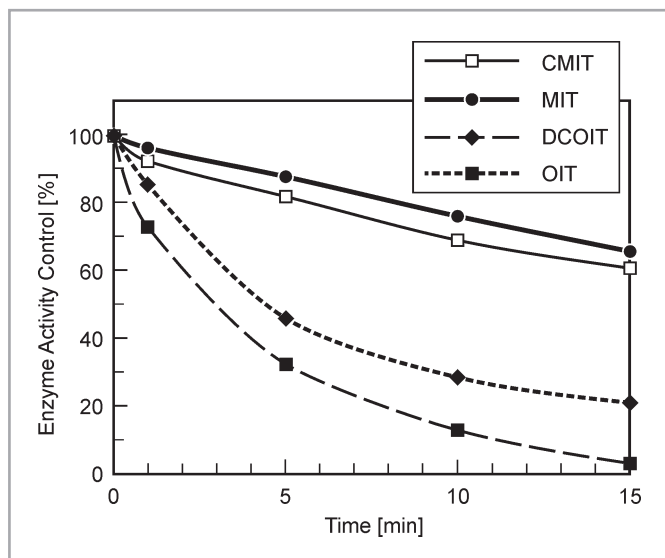


Figure 7: Rapid inhibition of alcohol dehydrogenase enzyme by 500 μM CMIT, MIT, DCOIT and OIT.

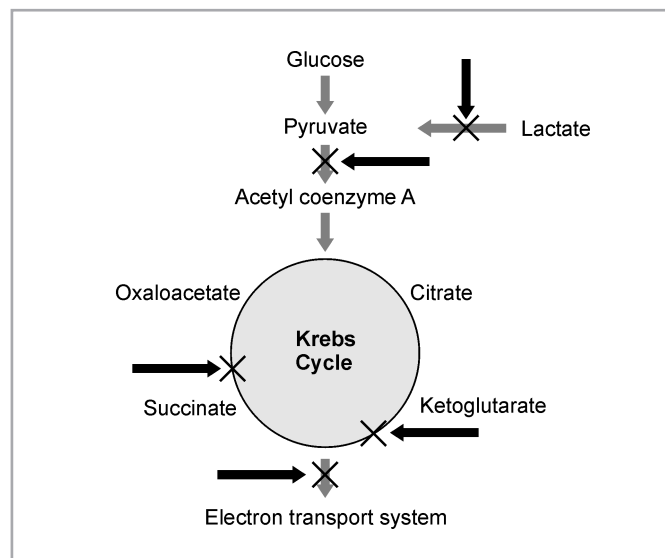


Figure 8: Sites of isothiazolone-induced enzyme inhibition involving the central metabolic pathway of the Krebs cycle.

Isothiazolone	Survivors Remaining [%]	Soluble Thiols Remaining [%]	Insoluble Thiols Remaining [%]
DCOIT (5 μM)	2.5 · 10 <sup>-5</sup>	35.5	58.5
CMIT (50 μM)	2.5 · 10 <sup>-5</sup>	52.6	68.2
OIT (50 μM)	1.5 · 10 <sup>-5</sup>	44.4	70.7
MIT(500 μM)	52	70.8	74.9

Table 2: Effect of 30 min exposure on viability and acid-soluble and acid-insoluble thiol content in the yeast *Saccharomyces cerevisiae*.

### Reaction with Nucleophiles (Protein Thiols)

Isothiazolone biocides are known to react with nucleophilic materials. This in part affects their stability in the presence of reducing agents as well as defines their mechanism of action for critical cell reaction sites [1]. Thiols are key active sites on many proteins and enzymes. This study demonstrated that all isothiazolones react with protein thiols, destroying both soluble and insoluble types (Table 2). This reactivity may also be linked to the killing effect of the biocides such that few survivors remain after contact with the biocides and loss of thiols. Once the biocide reacts with a thiol, the ring opens, and the isothiazolone is no longer active. Many different reactions are possible and are illustrated in Figure 9. The relationship between loss of viability and loss of thiol content is shown in Figure 10. A strong correlation is associated between CMIT killing and thiol destruction.

Thiol-active sites are common to dehydrogenase enzymes and other proteins as well. Thus, the strong reactivity of isothiazolones to thiols is linked to cell death and inhibition of critical cell functions. Various reduced-sulfur molecules exist in microbial cells including cysteine, cystine, thioglycollate, and glutathione. These have all been implicated in the mechanism of action of the different isothiazolone biocides [1,4–11].

### Production of Intracellular Radicals

Thus far, the results and discussion have focused on inhibition of growth and metabolic functions. The cidal or killing mechanism of isothiazolone is linked to the inhibitory actions of the biocide, but inhibition alone does not account for the killing observed within a several hour contact period. Disruption of metabolism and reactivity with thiols will create disorder within the very complex processes of microbial regulation, growth and repair. If cells cannot repair damage, then accumulated damage will ultimately overpower the cells and death will occur. Microbial cells have various repair systems, all of which require energy (ATP) and functioning metabolic processes for efficiency.

Free radicals are produced by microbial cells as a normal function of metabolism, including growth, reproduction, energy generation, and oxygen consumption or detoxification. Healthy cells normally scavenge or detoxify radicals within the cells, reducing their toxicity. Excess production of radicals or lack of their control will disable cells, resulting in death. Biocides may also produce unwanted radicals upon contact with microorganisms.

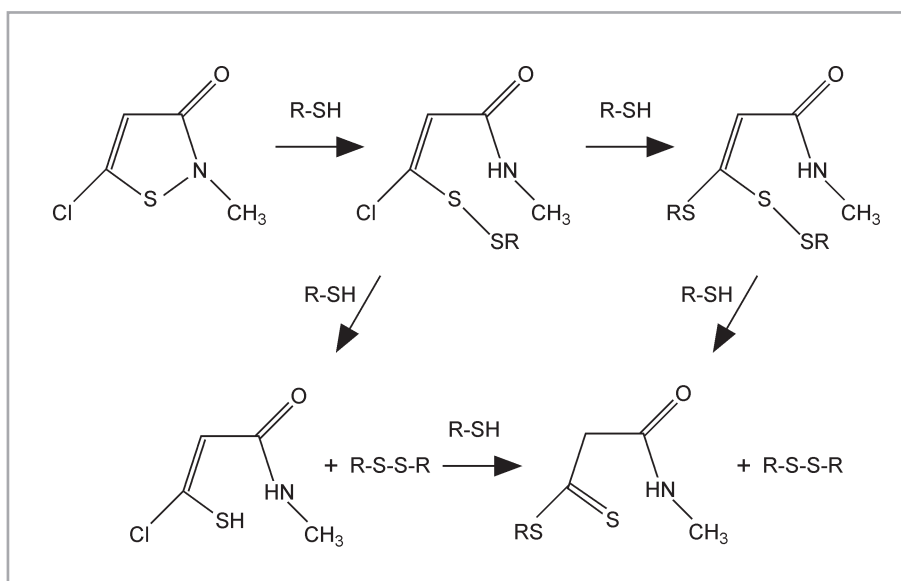


Figure 9: Interaction of nucleophiles with isothiazolones.

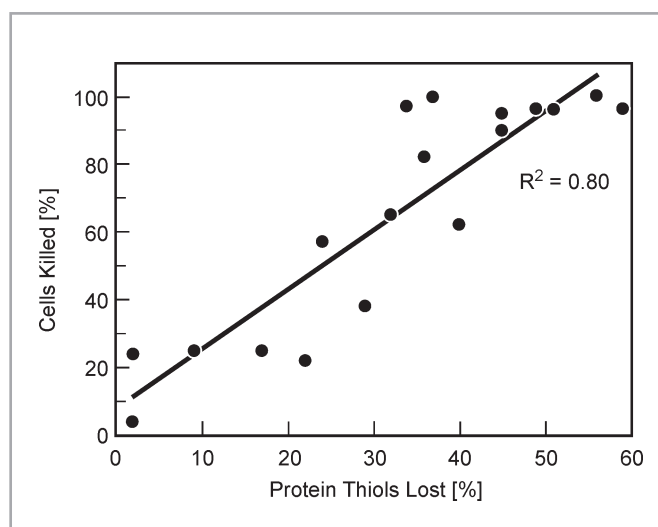


Figure 10: Relationship of cell death (viability) to loss of protein thiols.

In this study we evaluated the presence of free radicals using electron spin resonance spectroscopy. Figures 11 and 12 show ESR data demonstrating the production of radicals after contact with both CMIT and MIT biocides. Compared to the control (no biocide) sample, the effect of CMIT addition on the generation of a radical spectrum is very evident. A similar result is shown when comparing CMIT with MIT. Both isothiazolones produced a similar effect.

Based on these studies, the production of free radicals by the isothiazolone biocides is likely the critical event in the cidal reaction of the molecules contributing to cell death. Similar results with other isothiazolones and biocides have been conducted supporting this mechanism of killing [11].

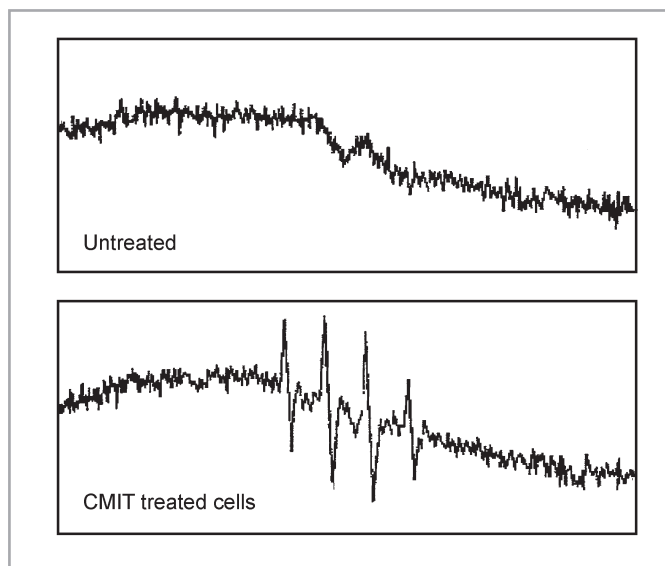


Figure 11: CMIT-induced radicals in bacterial cells (*E. coli*) versus control.

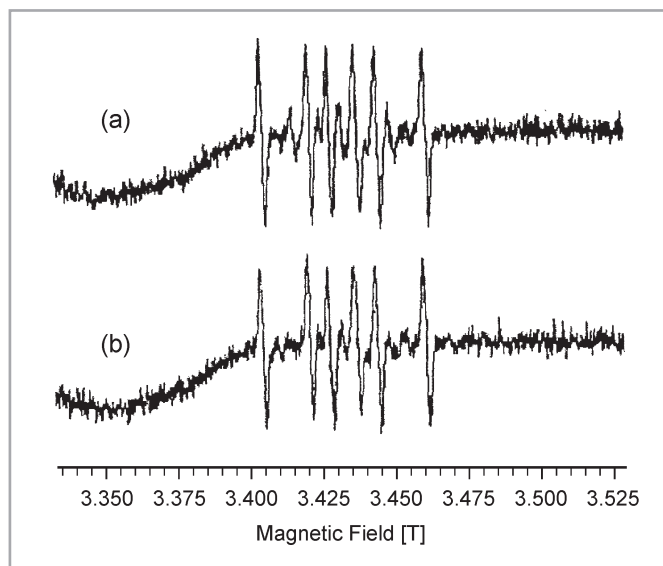


Figure 12: ESR radical spectra for CMIT (a) and MIT (b) in bacterial cells (*E. coli*) at 20 min contact time (80 mM biocide concentration).

### CONCLUSIONS

The mechanism of action of isothiazolone biocides is complex and considered a two-step process involving rapid growth inhibition leading to a loss of viability. Growth inhibition is the result of rapid disruption of the central metabolic pathways of the cell by inhibition of several specific enzymes, including dehydrogenases. The specific dehydrogenase enzymes which react with isothiazolones include those involved in the tricarboxylic acid (Krebs) cycle (alpha-ketoglutarate, pyruvate dehydrogenase, succinate dehydrogenase, and lactate dehydrogenase) and energy generation (NADH dehydrogenase). Key physiological activities that are rapidly inhibited in microbial cells are respiration (oxygen consumption) and energy generation (ATP synthesis). Many of these key enzymes are present in both aerobic and anaerobic microorganisms, which explains why CMIT/MIT is such a broad spectrum biocide. Biocide transport studies showed DCOIT and CMIT were rapidly transported inside the cell via a process which requires energy, whereas the MIT component enters the cell more slowly, through a more typical diffusion process.

Inhibition of cellular activity by isothiazolones is rapid and occurs within minutes, whereas cell death (cidal activity) is observed after several hours contact. In general, the higher the concentration of biocide, the shorter the contact time required for more complete kill. Cell death results from the progressive loss of protein thiols in the cell from one of multiple pathways (Figure 13). As critical pathways in cell metabolism are disrupted, free radicals are produced, which also results in cell death. Isothiazolones also inhibit ATP synthesis and utilization, which affects the potential of the cells to maintain the energy balance for growth and cell repair. This unique mechanism results in the broad spectrum of activity, low use levels for microbial control, and difficulty in attaining resistance.

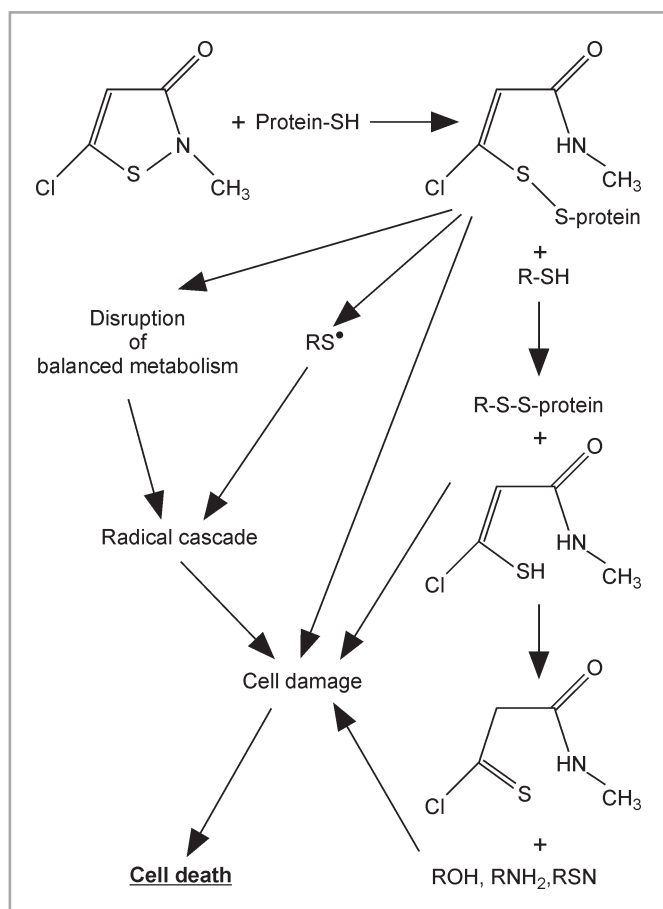


Figure 13: Proposed pathways of isothiazolone biocide lethality using CMIT as the model biocide.

Microorganisms have shown resistance to most commonly used industrial biocides, including quats, glutaraldehyde, halogens, isothiazolone, and others [13–17]. Various mechanisms of biocide resistance have been proposed for

antibiotics and industrial biocides and cross-resistance has been reported. Although microbial resistance to isothiazolones has been reported, it is infrequently encountered in industrial systems and easily remedied by rotating biocides, using combinations with other actives, or addition of surfactants or adjuvants to enhance efficacy. These practices have been used successfully by the water treatment industry in controlling microbial growth and biofouling in various applications.

The results of these studies showed that the five different isothiazolone biocides have strong similarity in their basic antimicrobial mechanism of action. Some variations are observed in rate of inhibition, cell binding, transport, and chemical properties which may collectively influence the activity of certain molecules versus certain groups of bacteria, fungi, and algae.

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