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Izothiazolone based biocides: efficiency evaluation on *Staphylococcus warneri* isolated from e-coat process rinse water

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Highlights: The paper addresses the inactivation kinetics of Staphylococcus warneri, isolated from e-coat painting process tanks, by commercial biocides. It has a practical relevance, since it can assist companies, that use e-coat process, in the choice of biocide and in the definition of application conditions.

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ABSTRACT - The e-coat process is the first step in automotive painting and consists of immersing the part in a conductive water-based paint bath, followed by several rinse tanks and preceded by a rinse tank with demineralized water after pre-treatment. In this process, microbiological contamination is common, which is prevented or eliminated by adding isothiazolone based biocides combined with other active components. The objective of this study was to evaluate the efficiency of two commercial biocides containing the active components, 5-Chloro-2-methyl-2H-isothiazol-3-one (CIT) and 2-Methyl-2H-isothiazol-3-one (MIT), in the inactivation of two *Staphylococcus warneri* strains, isolated from contaminated water from tanks of an e-coat paint applicator company. The Minimum Inhibitory Concentration (MIC) was 5 mg/L and the Minimum Bactericide Concentration (MBC) was 10 mg/L for both biocides. Survival curves were obtained and the Weibull model was adjusted. The models obtained indicated lower resistance of *S. warneri* strain isolated from the demineralized water tank and higher inactivation rate of the biocide containing the association of isothiazolinone and ethylenedioxy (dimethanol).

Keywords: Painting; Electrodeposition; Coatings. Weibull model; MIC.

INTRODUCTION

The first coat of conventional automotive painting is e-coat, which consists of the immersion of the part in a conductive paint bath composed of resin, pigments, and fillers, dispersed in demineralized water. This type of painting is part of the corrosion protection process of metallic components, consisting of several tanks: pre-rinse; cleaning with hot alkaline degreasers; pre-treatment (phosphatization or conversion coating); painting itself; and post-painting rinses (Wang, Yang, Antonelli & Yasad, 1996).

Over the years, this technology has evolved greatly, becoming less harmful to the environment. On the other hand, the stages of the process became more prone to the development of microbiological contaminations, present in the liquids of the tanks, as well as in biofilms adhered to the pipes or the walls of the process tanks, causing defects in the final painting of the parts (La Rosa & Giese, 2008).

To prevent or eliminate the microbiological contamination that occurs in the e-coat process tanks, biocides with different compositions are used, however, isothiazolone based compounds are widely used, due to their known efficiency, and also for their good compatibility with resin systems. The main active components used in commercial products, domestic and imported, are 5-Chloro-2-methyl-2H-isothiazol-3-one (CIT) and 2-Methyl-2H-isothiazol-3-one (MIT).

Isothiazolones act in two stages: initially they promote inhibition of growth and metabolism of microorganisms (minutes) and later they cause irreversible damage to the cell, blocking respiration and ATP synthesis (Williams, 2007). According to Morley et al. (1998), the efficiency of these compounds is explained by the ability to quickly cross the cell wall of bacteria and fungi and react with important sulfur-containing proteins or with simpler compounds such as glutathione, causing damage to cell metabolism. Some practical studies on painting lines demonstrate that the action of these biocides occurs within 72 hours after their addition (Orr, 2008)

Other compounds used in commercial biocide formulations are Bronopol (2-Bromo-2-nitropropane-1,3-diol) and Ethylenedioxy (dimethanol). Bronopol blocks the action of enzymes necessary for microbial metabolism, in aqueous solutions, rapidly degrades in several products, mainly 2-Bromo-2-nitroethanol and bromonitromethane, but its antimicrobial activity remains acceptable. The decomposition compounds present toxicity to the aquatic environment equivalent to or higher than Bronopol (Cui et al, 2011).

Hemiacetals, such as ethylenedioxy(dimethanol), compounds derived from aldehydes, release formaldehyde, which destroys the protein nucleus of bacteria (Williams, 2007). Ethylenedioxy(dimethanol) is a widely used option to replace pure formaldehyde, which is very volatile and reactive, but has the disadvantage that formaldehyde is metabolized by some types of microorganisms (Maiuta et al, 2009)

Rossmore (1990) conducted a study to evaluate the synergistic effect of using biocide mixtures with different chemical characteristics (and consequently mechanism of action), demonstrating that isothiazolones present synergisms with formaldehyde. A possible explanation for this is the ability of formaldehyde to react with lipopolysaccharide (LPS) present in the external membrane of gram-negative bacteria, thus allowing the entry of isothiazolones inside the cytoplasm.

According to Ribeiro e Stelato (2011), the action of biocides is relatively fast, but not immediate. The antimicrobial action is a function of several factors, such as type of microorganism and growth phase, young cells of bacteria and fungi are more vulnerable than organisms in stationary or declining phase or the process of sporulation; the size of the microbial population; concentration and time of exposure to the biocide.

The objective of this work was to compare the effect of two commercial isothiazolone-based biocides on the kinetics of inactivation of microorganisms isolated from contaminated water from rinsing tanks before *e-coat* painting.

MATERIAL AND METHODS

a) Biocides

Two commercial biocides, commonly used in e-coat processes, were kindly supplied by a common supplier company (Biocide A) and by user company (Biocide B).

Biocide A, is of national origin and is generally used in small and medium-sized companies.

It is composed of isothiazolones (chloromethyl-/ methylisothiazolone - CIT/ MIT) and contains as an additional component Ethylenedioxy(dimethanol) (which is a hemiacetal).

The active component of biocide "B" is an aqueous combination of chloromethyl-/methylisothiazolone (CIT/MIT) also containing 2-Bromo-2-nitropropane-1,3-diol (Bronopol). It is a product imported from Germany and used mainly by large automakers or auto parts manufacturers. Both biocides have content of the main active component around 1%.

Isolation and Identification of the Bacteria

Water samples were collected from the "pre-paint" rinse tank and the demineralized water storage tank (storage tank) of a *job coater* company, from ABC Paulista, on three different dates.

For the isolation of bacteria, 0.1 ml of water samples were spread plate on the surface of PCA (Plate Count Agar, Oxoid) and incubated at 36 ± 1°C for 48 h. Isolated colonies were characterized for morphology and Gram and stored in TSA (Tryptic Soy Agar, Oxoid) under refrigeration.

Two bacterial isolates, called I2 and I9, were identified by molecular analysis. From the growth in TSA at 35°C, DNA extraction was performed using the commercial ZR Fungal/Bacterial DNA Miniprep kit (ZymoResearch). The amplification of the target DNA, through the Polymerase Chain Reaction (PCR), was obtained through *primers* 27F (5' - AGAGTTGATCMTGCTCAG -3') and 1492R (5' - TACGGYTACCTTGTACGACTT -3') (WEISBURG et al., 1991).

After the TBE-agarose 1.5 % (w/v) gel electrophoretic run, the product was purified through the EasyPure PCR Purification Kit (TransGen Biotech) and added the respective oligonucleotides for sequencing, performed on the ABI 3500 Genetic Analyzer (Life Technologies) platform.

The consensus sequence was generated (BioEdit) from the sequences obtained by the *primers*. The taxonomic classification was performed by comparison with GenBank data (www.ncbi.nlm.nih.gov/) via MEGABLAST.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (CBM)

The minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) were determined according to Gadea et al. (2017), with modifications.

The isolates I2 and I9 were transferred to TSB (Tryptic Soy Broth, Oxoid), incubated at 36°C for 24 h, and the absorbance determined in a spectrophotometer (Hach, China). From the growth in TSB, 0.05 mL was transferred to 4.75 mL of the same medium and 0.20 mL of the biocide in concentrations 10, 5, 2.5 and 1.25 mg/L of the main active compound (considering 1 %, as indicated by the manufacturers). These concentrations are equivalent to the following concentrations of commercial biocide, 1, 0.5, 0.25, and 0.125 g/L of water to be treated. The tubes were incubated at 36°C for 72 h and the absorbance was determined using the culture medium without the bacteria as blank. The growth of the microorganism without the biocide was also determined as control.

The value of MIC (minimum inhibitory concentration) was defined as the lowest concentration at which there was no microbial growth (the result of absorbance close to zero). In parallel, the bacteria count in the broth without biocide was determined, after 48 h of incubation at 36°C, using the TSA medium by *pour plate*.

The tubes that did not show growth in the MIC test were used to determine the minimum bactericide concentration (MBC). For this purpose, 1 mL of the non-growth TSB was transferred to a petri dish, the TSA (Oxoid) medium was added and the number of colonies determined after incubation at 37°C for 48h (SALFINGER and TORTORELLO, 2015).

The value of MBC (minimum bactericide concentration) was defined as the lowest concentration at which there was no microbial growth in TSA medium.

Determination of bacterial inactivation kinetics

The kinetic inactivation of bacterial isolates by biocides A and B was performed according to the methodology described by Raikos et al. (2012), with modifications. Isolated microorganisms stored in TSA under refrigeration were first activated in the TSB medium. After incubation at 35-37°C for 19-24 h, aliquots of 50 µL were transferred to 4.75 mL of TSB and incubated again under the same conditions of time and temperature. The absorbance was determined at 610 nm using sterile TSB broth as the blank. After incubation, 0.20 mL of the biocide was added to each tube to obtain the previously determined minimum bactericide concentration (MBC) and 0.20 mL of sterile water was added to the control instead of the biocide. The tubes were maintained at 35 to 37°C and determined intervals, the number of surviving bacteria was quantified by *pour plate* technique on

PCA medium, using samples from different tubes for each time. All experiments were repeated at least three times.

Weibull's frequency distribution model (Mafart et al, 2002) was used for the adjustment of survival curves of microorganisms, and consequently resistance to biocidal agents. The Integrated Pathogen Modeling Program (HUANG, 2013) was used to adjust the Weibull equation (Equation 1) and to determine the kinetic parameters.

$$\mathrm{Log\,N} = \mathrm{Log\,N_0} - \mathrm{k} \cdot \mathrm{t}^{\alpha} \qquad \text{Eq.1}$$

Where: N - number of surviving bacteria in CFU/mL, at a given time of biocide exposure; N0 - initial number of bacteria in CFU/mL; α and k, kinetic parameters; t - the time of exposure to the biocide.

RESULTS AND DISCUSSION

The bacterial isolates from the rinse water stage of the e-coat pre-treatment (I2) and the demineralized water storage tank (I9) were identified as *Staphylococcus warneri*, based on the 16S rRNA subunit.

Staphylococcus sp. is a Gram-positive, catalase-positive, oxidase-negative bacterium; present in the microbiota of human and animal skin, but also frequently found in hospital environments (Baptista, 2015). According to Kloos and Schleifer (1975), as well as other coagulase-negative *staphylococci*, *Staphylococcus warneri* rarely causes disease, but occasionally can cause infection in patients whose immune system is compromised. Despite this merely opportunistic characteristic, cases of urinary tract infections, meningitis and orthopedic infections caused by *S. warneri have* been reported in the literature (Leighton & Little, 1986). On the other hand, there is no report in the literature on the presence of this microorganism in the *e-coat* painting process.

Table 1 presents the average results of the MIC test for biocides A and B, for isolates I2 and I9. The MIC tests were replicated at least 3 times for each combination of bacterial isolate and biocide used. The results showed that the MIC for both biocides was 5 mg/L of active compound, which represents 0.5 g/L of biocide.

Table 1. Absorbance Results - MIC Test

Isolates	I2		I9	
Biocide	A	B	A	B
Control	0,938	0,899	0,874	0,916
10 mg/L	0,003	0,001	0,003	0,000
5 mg/L	0,014	0,003	0,007	0,005
2,5 mg/L	0,870	1,061	0,900	1,057
1.25 mg/L	0,903	0,865	0,914	0,964

The results obtained for MIC are similar to the values found by Orr (2008), 2 to 3 mg/L of active component for a bactericide containing the mixtures of isothiazolones on bacteria from a sample of pre-treatment water of *e-coat* process.

The minimum bactericide concentration (MBC) for the two biocides evaluated was 10 mg/L of active compound, which is equivalent to 1 g/L of the commercial product for both bacterial isolates (Table 2). These data indicate that the biocides presented the ability to eliminate *S. warneri at the* same use concentration.

Table 2. Growth in TSA - MBC Test

Biocide	Isolates	A				B			
10 mg/L	I2	No	No	No	No	No	No	No	No
5 mg/L		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
10 mg/L	I9	No	No	No	No	No	No	No	No
5 mg/L		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

In the work of Orr (2008), the minimum bactericide concentration (MBC) was also determined after 72 hours of the addition of the isothiazolone biocide. The result showed that concentrations of 6 to 7 mg/L were necessary for the complete elimination of bacteria, values close to those obtained in this study for both commercial biocides.

The inactivation kinetics of isolates I2 and I9 exposed to biocides A and B and the curves representing the adjustments of the Weibull model are shown in Figures 1 and 2 respectively. The values of the coefficients of determination (R^2) indicate a good fit for the Weibull model. The lowest value (0.972) was obtained for biocide A with the isolate I2 (Table 4).

The value of α is a function of the concavity of the curve. Values smaller than 1 are obtained for curves with concave concavity, larger than 1 for convex, and when α is equal to 1, the adjustment is linear. The survival curve is normally concave (α < 1) when the microorganism is exposed to a lethal agent in lesser intensity. This means that the most sensitive cells of the population die initially, remaining those of higher resistance. For α > 1 when the intensity of the lethal agent is greater, reflecting in the accumulation of damage to the cell and therefore greater sensitivity among survivors (Peleg, 1999).

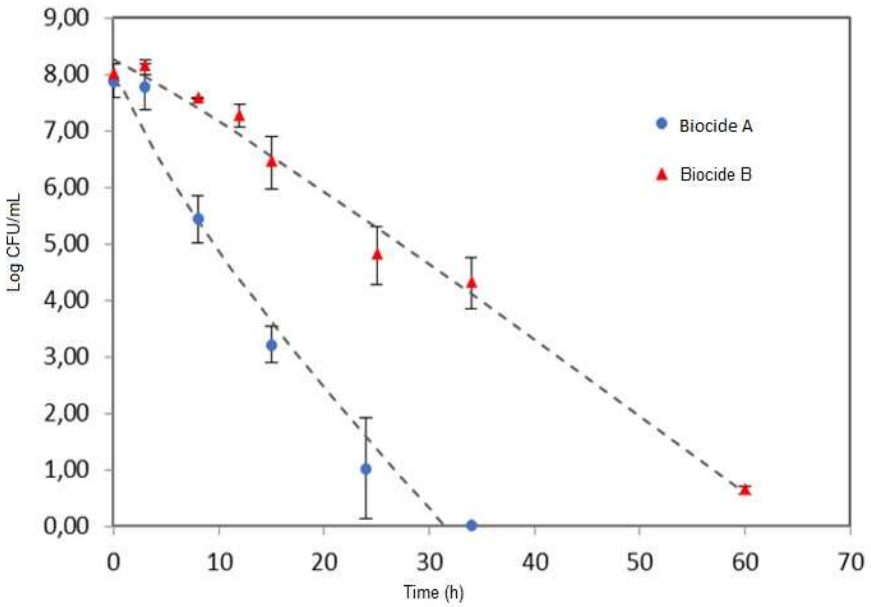

Figure 1. Survival curves of Isolate 2 (I2) exposed to biocides A and B

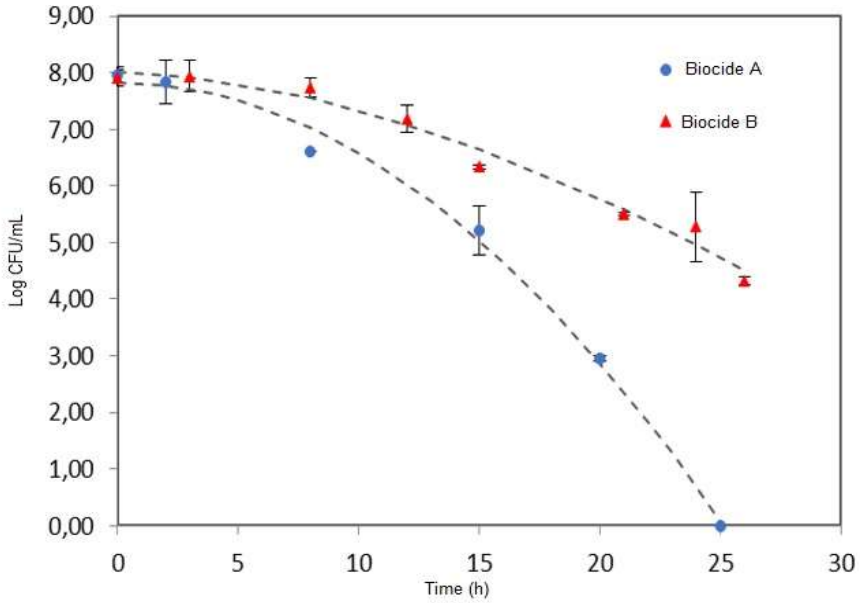

Figure 2. Survival curves of Isolate 9 (I9) exposed to biocides A and B

Table 3. Kinetic parameters of the Weibull model and coefficient of determination R^2

Parameters	I2		I9	
	Biocide A	Biocide B	Biocide A	Biocide B
Y0 (Log CFU/mL)	8,29	8,27	7,82	8,01
k	0,578	0,090	0,012	0,013
α	0,77	1,08	2,00	1,72
R^2	0,972	0,992	0,995	0,989

It is observed in Table 4 that the values of α for I2 Isolate are lower or close to 1 (0.77 and 1.08 for biocides A and B respectively), while for I9 Isolate the values are much higher than 1 (2.0 and 1.72 for biocides A and B respectively) indicating that initially cells of I9 isolate were more resistant to the biocides but the surviving cells showed less resistance than I2. Both isolates were identified as *Staphylococcus warneri*, however, I2 was isolated from the pre-paint rinse tank and I9 from the demineralized water storage tank. The presence of some nutrients in the rinse tank, such as phosphate, iron, sodium, potassium, calcium, and carbonates, may have increased cell resistance to biocides.

The survival curves of isolates I2 and I9 (Figures 1 and 2, respectively), prove greater bactericidal action of biocide A compared to biocide B, as discussed in the model presented by Peleg (1999). For biocide A, after 34 and 25 h of exposure of isolates I2 and I9 respectively, it was not possible to detect the bacteria in the culture medium, whereas for biocide B, the presence of I2, about 1 log CFU/mL log, was still verified after 60 h of exposure to the product. These results justify the same Minimum Bactericide Concentration (MBC) values obtained for the two biocides since the determinations were performed with only 72 h of exposure to the products.

As the main active component of both biocides is the same (mixture of CIT: MIT), it is understood that the most efficient action of biocide A on *S. warneri* is due to Ethylenomethoxy(dimethanol), a compound absent in biocide B.

The action of isothiazolones on *Staphylococcus warneri* has not been published in the literature so far, however, Raikos et al. (2012) studied the action of commercial biocide (Kathon Fp 1.5), composed of the mixture of 5-chloro-2-methyl-4-isothiazol-3-one and 2-methyl-4-isothiazol-3-one, on *Staphylococcus epidermides* in aviation fuel tanks. The results showed that after 30 days of incubation in the presence of 100 ppm of the biocide, the bacterium showed the lowest reduction (73.5%) when compared to other microorganisms evaluated. The authors also found that for higher concentrations (up to 130 ppm) the bactericidal effect was reduced, probably due to a mechanism of resistance to the biocide development by the microorganism. These results indicate the high resistance of *Staphylococcus* to isothiazolones.

CONCLUSION

Two strains, of *Staphylococcus warneri* were isolated from the pre-treatment water rinsing tank and the demineralized water lung tank from the e-coat painting process of automotive parts. The inactivation kinetics of the microorganisms exposed to two commercial isothiazolone-based CIT/MIT biocides indicated greater resistance of the rinse tank isolate. The biocide containing Etilenedioxy(dimethanol), in addition to isothiazolones, showed a higher destruction rate of S. warneri compared to the biocide with Bronopol addition.

The synergistic combination of CIT/MIT mixture with Etilenedioxy(dimethanol) seems to be more efficient in eliminating contamination in water and rinsing stages tanks of the e-coat process.

CONTRIBUTIONS of AUTHORS

In this study, both authors contributed effectively; Cynthia Jurkiewikz and Leo Kunigk supervised the study.

COMPETING INTERESTS

The authors have declared that no competing interests exist

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