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# Influence of a triazine derivative-based biocide on microbial biofilms of cutting fluids in contact with different substrates

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**Abstract** Although biofilms are often associated with hospital infection problems owing to their high resistance to antimicrobial agents, in recent years biofilms have also been studied in the industrial sector, mainly because they are a major cause of contamination outbreaks in facilities and products. The aim of this study was to investigate whether different materials commonly found in the metalworking industries have different biofilm formation characteristics when in contact with contaminated cutting fluid as well as to establish an optimal concentration of a triazine-based antimicrobial agent to protect the oil/water emulsion and also to delay or interrupt the development of biofilms. Biofilms grown on the surface of carbon steel, stainless steel, aluminum, polyvinyl chloride, and glass were analyzed in terms of cell growth and susceptibility to the tested biocide. The results showed that the type of material used had little influence on cell adhesion or on the microbicide concentration required to control and eradicate microorganisms suspended in the emulsion and in the biofilms.

**Keywords** Biofilms · Metalworking fluid · Cutting fluid · Biocides · Triazine · Minimum inhibitory concentration

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## Introduction

Bacteria have been traditionally studied as single-celled organisms and analyzed mainly on the basis of the concept of pure cultures by diluting and culturing in liquid or solid culture media. Some of these bacteria have the ability to adhere to surfaces, forming biofilms stabilized by a complex extracellular polymeric substance (EPS).

The EPS not only provides protection to the biofilm-forming cells but also plays an important role in the morphology, structure, operational integrity, and cohesion of biofilms [7]. The EPS matrix is heterogeneous and may contain proteins, nucleic acids, and polysaccharides as well as environmental components, debris, organic materials, and other living beings [8, 24].

Microbial adhesion is a phenomenon that occurs naturally in the environment and is a strategy developed to protect the cells. When in biofilms, microorganisms are able to establish cultures on different types of material and under extreme nutritional conditions with facilitated intercellular communication through signaling molecules [15]. For these reasons, bacteria preferentially grow in the form of biofilms, and only around 10% of the bacterial cells are found in the planktonic form [3], meaning that ca. 90% of the microbial population is attached to biofilms (in sessile form).

Biofilms constitute a problem in human health and are associated with the failure of several conventional treatment procedures [23], but they are also found in many different types of industry, especially those with circulating fluids [10] where they are the main cause of contaminations that are difficult to control. Biofilms are more resistant to antimicrobial agents than planktonic cells and are more difficult to remove from the contaminated system. Examples of industrial problems caused by biofilm formation are reduced heat transfer in heat exchangers, pipe blockage, and product

contamination. Deterioration of industrial equipment attributed to microbial biofilms is also common, resulting in increased replacement, cleaning, and maintenance costs in addition to deleterious effects on the final product [4].

An industrial segment that suffers the effects of biofilm formation is metalworking. Frequently these industries use metalworking fluids (or cutting fluids) to cool and/or lubricate metal workpieces when they are machined, ground, and milled. The metalworking fluids help to reduce the heat and friction between the cutting tool and the workpiece and to prevent burning and smoking. In addition, the use of metalworking fluids may improve the quality of the workpiece by continuously removing the fines, chips, and swarf from the tool being used and the surface of the workpiece.

Cutting fluids commonly used in metalworking frequently are of the semisynthetic class. These fluids contain 5–30% refined petroleum oils, 30–50% water, and emulsifiers, and they may be formulated with fatty acids, sulfur, chlorine, and phosphorus derivatives to provide lubrication for higher speeds and feed rates [14]. All these components make the semisynthetic cutting fluids very suitable for sustaining microbial cell growth, and contaminants frequently found are *Pseudomonas aeruginosa*, *Enterobacter* spp., *Escherichia coli*, *Candida albicans, Klebsiella pneumoniae*, *Proteus mirabilis*, *Desulfovibrio* spp., *Hormoconis resinae* (previously *Cladosporium resinae*), *Paecilomyces variotii, Fusarium* sp., *Candida* sp., and *Cephalosporium* sp. [1, 16, 20].

Since water-based metalworking fluids may support microbial growth, serious problems regarding worker exposure, equipment biocorrosion and biodeterioration, and product contamination as well as environmental issues and costs related to premature fluid disposal may arise from the development of biological contaminants in industrial processes.

Given the susceptibility of the oil/water emulsions to microbial contamination, one possible solution for preserving the fluid is the use of antimicrobial agents or biocides. Cutting fluid biocides are normally nonoxidizing compounds, since the oxidant antimicrobial agents are frequently highly reactive, having no residual action and often causing changes in the characteristics of the emulsion in addition to corrosion in pipes and equipment [12].

The minimum inhibitory concentration (MIC) and minimum concentration for cell death (MCD) are regularly used as indicators of antimicrobial activity [21]. Nevertheless, these approaches are directly applicable to planktonic cells, but not to cells in biofilms, for which the determination methods are not yet fully standardized. When comparing planktonic to sessile cells in terms of their susceptibility to biocides, the concentrations of antimicrobial agents required to control and eliminate them can be from 100 to 1,000 times lower than those used for cells in biofilms [3]. Several factors may account for this behavior, such as the EPS protection effect, dormancy of cells, changes in cell membranes when in a biofilm, production by cells of compounds capable of inhibiting or inactivating biocides, limitation of mass transfer of the biocide to the cells, and xenobiotic metabolism responses such as biocide efflux pumping, among others. Thus, the use of biocides for controlling biofilms based solely on data obtained for planktonic cells should be cautious, given that low dosages can provide a false sense of safety, in addition to inducing selection of resistant microorganisms and causing contamination outbreaks [5].

For metalworking fluid applications, one of the most frequently used classes of biocides is formaldehyde condensates [20], such as triazine derivatives, which are able to generate formaldehyde in situ. Nucleophilic groups of cell molecules such as amino acids and proteins can interact with the formaldehyde, causing an effect that is frequently lethal to cells.

Two main strategies for adding microbicide can be employed in these systems, the continuous feeding approach and the shock approach. The latter, which consists of adding a high dose of biocide, is used when the formation of biofilms is intense and microbial contamination in the fluid is also quite high [12]. Continuous feeding of biocide, on the other hand, is used in microbiologically controlled systems, and the lowest antimicrobial agent concentration to maintain the conditions of biostatic systems is usually employed in this situation [22].

Biofilm formation in different types of materials has been studied focusing on many different areas, such as dentistry, medicine, food industry, water distribution piping, and even during space flights by NASA. Frequently, biofilm development strategies on different media are based on traditional immersion of coupons produced with the materials to be tested in the contaminated fluid. Many different approaches are used to study biofilm development and control [11], such as the Calgary biofilm formation device [3], carrier cylinders, hydrogels, chemostats, simple flow reactors, and the Robbin's device. However, only a few works cover detailed aspects of biofilm prevention, control, and removal in industrial metal machining [1, 6, 16–20]. Given that contamination occurs at a high frequency in this industrial segment, the susceptibility of biofilms formed on the surface of different materials commonly used (carbon steel, stainless steel, aluminum, polyvinyl chloride, and glass) in contact with contaminated cutting fluid to a triazine-based biocide was evaluated in a traditional Brazilian metalworking company in the present work.

## Materials and methods

### Materials

Test samples (2.5 × 7.5 cm coupons with an average thickness of 1–1.2 mm) of aluminum, 1020 carbon steel, 304L

stainless steel (from Nacional Esferas Ltda., São Paulo, Brazil), glass (Só Esferas Comércio de Esferas Ltda., São Paulo, Brazil), and polyvinyl chloride (PVC, TRM Resinas Termoplásticas Indústria e Comércio Ltda. São Paulo, Brazil) were used as supports for the formation of biofilms and the analysis of their susceptibility to the antimicrobial agent tested.

The cutting fluid used consisted of a 5% (v/v) mineral oil in water emulsion and was kindly donated by a metalworking company located in São Paulo, Brazil. The contaminated cutting fluid employed as inoculum in biofilm formation tests had been used by the industry for 2 years and had a contaminant concentration of 9.7 x $10^6$ CFU/mL in terms of total heterotrophic bacteria.

The BP-180 biocide used in the study consisted of a triazine derivative in monoethanolamine at a volume ratio of 8:2 and was kindly provided by the company IPEL Itibanyl Produtos Especiais Ltda., Jarinu, SP, Brazil.

Quantification of the microbial populations recovered from both the contaminated fluid and the tested supports was performed with tryptic soy agar (TSA) medium (Difco, USA). Sterile distilled water and/or NaCl 0.9% (w/v) in water was used as diluent and wash solution, as suggested by Capelletti et al. [2]. Samples containing the biocide had been previously treated with D/E neutralizing broth (Difco).

### Selection of materials to be tested: type and formation of biofilm on their surfaces

The materials to be tested were selected on the basis of a survey conducted of 70 metalworking companies in Brazil to determine which materials were the most commonly used as components of equipment and facilities in the metal machining industries. Other aspects assessed in this survey were knowledge of points prone to contamination as well as operating procedures for cleaning and disinfection.

In order to reproduce the conditions found in metalworking industry, multiple thin coupons composed of the selected materials were hung and submerged in a glass tank (30 cm in height × 45 cm in length × 24 cm in depth) containing 15 L of contaminated cutting oil with continuous recirculation of the fluid at a flow rate of 5 L/h using a pump (model SB 50, Sarlo Better). In all experiments the initial contaminant concentration was in the order of magnitude of $10^6$ CFU/mL in terms of total heterotrophic bacteria.

Samples were periodically collected during up to 15 days from the liquid phase for cell counts. Simultaneously, coupons of the different materials tested were removed from the system, washed with saline solution, and transferred to flasks containing 10 mL of diluents. In the case of samples obtained in the presence of the BP-180 biocide, the first diluent used was the D/E neutralizing broth. The supports were sonicated for 30 min in a bench sonicator (model USC1450, Thornton) operating at 25 kHz, a condition previously found not to affect cell viability in control experiments [11], and then a 1-mL aliquot was collected, properly diluted, and pour plated in TSA medium. The samples from the liquid phase were directly diluted and plated. After incubation for 48 h at 35–37°C, the microbial colonies were counted using the plates containing from 30 to 250 colonies and cell concentrations in the original samples were then calculated. All experiments were performed in triplicate and the results are expressed as average values.

### Analysis of minimum residual concentration of biocide required to retard or prevent biofilm formation in different materials

These tests aimed to determine the residual concentration of biocide needed to maintain control of microbial population growth in an industrial system. The experiments were performed essentially as described in the previous section, with the difference that every 24 h an aliquot of 1.5% fresh emulsion (not contaminated) was mixed with 15 or 30 mL of the BP-180 biocide (to give concentrations of triazine derivative of 0.1 or 0.2% in volume, respectively) to keep a constant fluid level in the tank and to provide a residual level of antimicrobial agent equivalent to 800 or 1,600 ppm, respectively. In these experiments, samples were collected from the liquid phase and the tested supports up to 16 days for cell counts. All results are expressed as average values of three independent experiments.

Volume replacement ratio, biocide type, and concentration were selected on the basis of previous contamination control procedures used in the company that supplied the cutting fluid. According to the company, adequate daily rates of fresh fluid replacement varied from 1 to 2% with the addition of microbicide in the proportions of 0.1–0.2% being required, depending on the pH of the contaminated emulsion.

### Determination of residual concentration of triazine

The residual concentration of triazine in the BP-180 biocide was measured every 2 days by colorimetry using the commercially available RQflex® system (Merck), which allows the determination of the concentration of formaldehyde released by the triazine derivative in each sample by comparison to a previously obtained standardized calibration curve.

## Results and discussion

### Distribution of types of materials used in metalworking manufacturing facilities and equipment

The following materials are found in the metalworking industry segment in Brazil and are given together with their

frequency of use: 73% carbon steel, 5% aluminum, 2% stainless steel, 2% PVC, 1% glass, and 17% of other material types. The metalworking companies surveyed were from different regions in Brazil, comprising the states of Paraná, São Paulo, Goiás, Bahia, Mato Grosso, and Rio Grande do Sul. Of the companies surveyed, 20% were in the beverages packaging segment (soft drinks and beer), 16% belonged to the area related to production of containers of canned goods, 28% were in the automobile parts production segment, and 36% worked on the production of heavy industrial equipment parts.

Clearly the material most commonly used by the metal-mechanics industry in Brazil is carbon steel. Aluminum, stainless steel, PVC, and glass are also used, but in much lower proportions. Despite being more prone to corrosion, carbon steel is less expensive and easier to replace. By analyzing the data on knowledge of critical contamination points and of cleaning and sanitizing procedures, we observed that the personnel consulted are generally unaware of the significance of biocorrosion and of problems arising from microbiological contamination. With respect to the major hot spots of microbial contamination in industry, the majority of the respondents mentioned equipment, pipes, and dead ends. However, this supposed knowledge is not fully put into practice because less than 20% of the surveyed companies have a plan for cleaning and sanitizing to prevent biofilm development.

Since metalworking is one of the segments most frequently affected by microbial biofilm formation and carbon steel, aluminum, stainless steel, PVC, and glass are regularly used by Brazilian companies, these materials were selected for this study.

## Biofilm formation on the surface of the selected materials

The results of the experiments performed by immersion of test samples produced from the selected materials in the tank containing contaminated cutting fluid circulating at a flow rate of 5 L/h are shown in Fig. 1.

The number of microorganisms recovered from the surface of each material in the given sampling period was high in all cases. The microbial population in the liquid varied from $6.2 \times 10^6$ to $8.3 \times 10^9$ CFU/mL during the 15-day test, reaching $8.7 \times 10^8$ CFU/mL on the last day. The adherence of microorganisms is very fast, and it is possible to consider that all biofilms were already mature by the end of the first week, i.e., with a microbial population above $10^6$ CFU/cm$^2$ [13]. The maximum population on the surfaces fluctuated from around $10^9$ CFU/cm$^2$ (on stainless steel, PVC, and aluminum) to $10^{10}$ CFU/cm$^2$ (on glass and carbon steel), which showed the latter to be the materials that promoted higher cell adherence.

These results clearly show the need to control the microbial population in contaminated cutting fluids. However, it

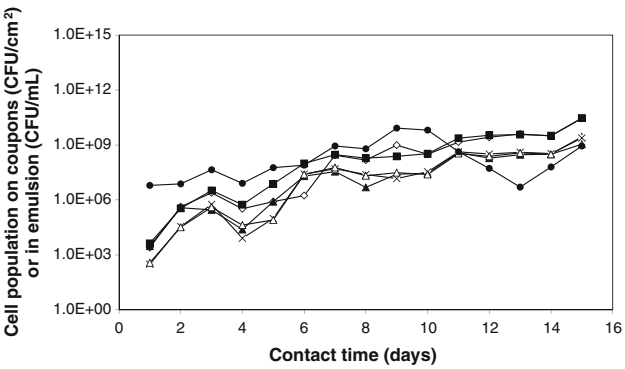


**Fig. 1** Recovery of microbial biofilms from the cutting fluid emulsion (*circles*) and from the surface of glass (*diamonds*), carbon steel (*squares*), stainless steel (*filled triangles*), PVC (*crosses*), and aluminum (*open triangles*) samples

is necessary to determine the minimum concentration of residual biocide required to retard or prevent the establishment and proliferation of microbial biofilms in continuous processing systems. Several aspects should be considered, since they may directly affect the rate of water replacement in the oil emulsion system and consequently the rate of addition of antimicrobial agent to the system. Aspects such as evaporation rate, production demand, number of shifts worked, workpiece geometry, operation type, chip formation, type of chip formed, and occurrence of leaks, among others, are relevant in this case. These factors make it difficult to establish a general calculation procedure for the emulsion replacement rate because of the particularities of each company. For these reasons, the replacement rate selected in the present study was defined on the basis of the procedure traditionally adopted by the company supplying the emulsion employed.

## Determination of minimal residual concentration of biocide to retard or prevent formation of biofilms

The second step of this study was to determine the minimum residual concentration of the triazine-based biocide necessary to delay or prevent the formation and proliferation of biofilms on the surface of the five materials tested. Since antimicrobial agents are inherently toxic and undergo slow natural degradation, they can remain in the environment and cause chemical contamination of areas far from the treated site [9]. Therefore, the selection of biocide type, concentration, and application strategy is of the utmost importance.

BP-180, the microbide selected, contains hydroxyethyltriazine [hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine], one of the most frequently used formaldehyde-condensate compounds for metalworking fluids, which has an excellent cost-benefit ratio and is commonly employed at a dose rate

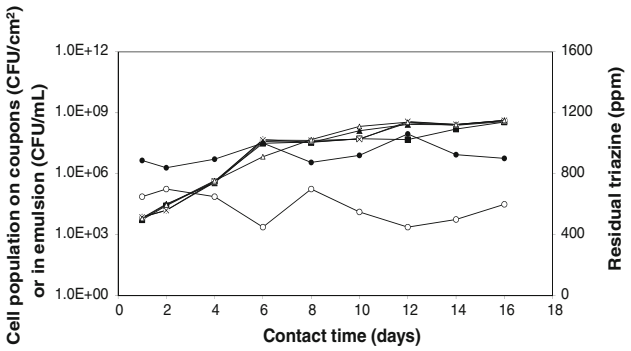


**Fig. 2** Residual triazine (*open circles*) content and cell recovery from the cutting fluid emulsion (*filled circles*) and from the biofilms developed on the surface of glass (*diamonds*), carbon steel (*squares*), stainless steel (*filled triangles*), PVC (*crosses*), and aluminum (*open triangles*) samples in the system with 0.1% of microbicide replenishment

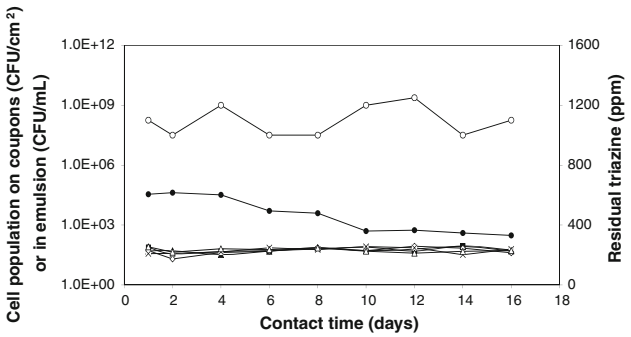


**Fig. 3** Residual triazine (*open circles*) content and cell recovery from the cutting fluid emulsion (*filled circles*) and from the biofilms developed on the surface of glass (*diamonds*), carbon steel (*squares*), stainless steel (*filled triangles*), PVC (*crosses*), and aluminum (*open triangles*) samples in the system with 0.2% of microbicide replenishment

of 1,500 ppm (0.15%) in a use-diluted fluid [20]. Therefore, the effects of BP-180 on biofilm formation were analyzed at dose rates of 0.1 and 0.2%.

The 0.1% dose of the BP-180 biocide resulted in an initial concentration of microbicide in the emulsion of around 800 ppm, whereas in the second trial, performed independently of the first with a 0.2% dose of BP-180, the initial microbicide concentration in the emulsion was approximately 1,600 ppm. In both trials, the residual concentrations of triazine were monitored, and the results are shown in Figs. 2 and 3.

It can be clearly observed in Fig. 2 that the 0.1% dose of BP-180 dosage was not sufficient to prevent biofilm formation. Despite the fact that the residual concentration of triazine in these samples varied from 450 to 700 ppm, probably because of partial degradation and evaporation, the microbial population in the liquid phase ranged from $2.0 \times 10^6$ to $9.1 \times 10^7$ CFU/mL, i.e., lower than the values achieved without the biocide. In this experiment, the time required for biofilm maturation was longer than that in the situation without biocide, which indicates that the lower population in the bulk emulsion and the action of the microbicide in the cells in the outer layer of the biofilms (which are theoretically more sensitive to the biocide) probably delayed or made cell adhesion more difficult. However, the lower cell population and the presence of the biocide at the 0.1% dose rate were not able to fully prevent the adhesion and proliferation of the biofilms. On day 16, the microbial counts on the surfaces of all materials tested were around $4.0 \times 10^8$ CFU/cm$^2$, indicating that the residual concentration of triazine required to prevent biofilm formation is higher than 700 ppm.

In the experiments in which the dose of BP-180 was 0.2%, it is possible to observe that the residual concentration of triazine was between 1,000 and 1,250 ppm during all the period evaluated (Fig. 3). Lower cell proliferation occurred in the emulsion, but biofilm formation was still observed on the surfaces of all materials tested, suggesting that the minimum concentration of triazine to prevent the rapid development of biofilms should be higher than 0.2% of the BP-180 biocide, regardless of the volume of replenished emulsion. Over the 16-day period, while the microbial population in the bulk emulsion ranged from $3.0 \times 10^2$ to $4.2 \times 10^4$ CFU/mL, the total microbial populations recovered from the biofilms were at concentrations less than 100 CFU/cm$^2$, quite low compared with those in cases previously evaluated.

In the metalworking industry, contamination levels in the emulsion up to $10^4$ CFU/mL are considered acceptable, causing no major harm to the cutting fluid in the short to medium term. Considering this scenario, the results achieved therefore showed that for the system tested, the commonly employed initial dose rate of 1,500 ppm of hydroxyethyltriazine in the emulsion [20] was not sufficient to fully eliminate biofilm formation, but was very effective in controlling it. In the particular case of the emulsion tested, total biofilm eradication was attained by increasing the biocide concentration to 0.25% (results not shown).

## Conclusions

The results obtained showed that for cutting fluid naturally contaminated with mixed wild microorganisms there was no significant difference between cell adherence and biofilm establishment, maturation, and elimination observed on cell counts in the different surfaces tested.

The addition of 0.1% BP-180 to the replenishing cutting fluid resulted in residual concentrations of triazine from 450 to 700 ppm. Under this condition, the microbial populations in the biofilms were maintained below $4.5 \times 10^8$ CFU/cm$^2$ in

the different materials evaluated throughout the testing period.

The use of 0.2% BP-180, in turn, gave a residual activity of between 1,000 and 1,250 ppm of triazine, allowing adequate control of the microbial population in the biofilms (below 100 CFU/cm$^2$), but not preventing their formation. Concentrations above that could be used; however, levels above 0.5% are not recommended because of potential skin, eye, and respiratory system irritation.

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