Factors Affecting Regrowth of *Pseudomonas Aeruginosa* Following Biocide Treatment©

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Treatment of cultures of Pseudomonas aeruginosa *with a formaldehyde condensate biocide, 1,3,5 tris hexahydro ethyl-s-triazine (ET), resulted in bacterial population regrowth. Regrowth is a sudden increase in colony-forming units following a period of undetectable viability (eclipse period). A number of factors are described that affected survival in minimal inhibitory concentrations of biocide. Siliconized glass surfaces shortened the eclipse time, and increasing the surface area resulted in considerable differences in biocidal activity. Larger system volumes showed slower initial kill by ET than smaller volumes. The eclipse period and regrowth were also affected by volume. Complex medium reduced ET activity in the absence of bacterial cells.*

The studies reported here all are relevant to comparable microbiological problems in metalworking fluids.

INTRODUCTION

There are many factors that contribute to biodegradation of metalworking fluids (MWF). Early detection of alarming microbial levels is necessary for successful treatment of systems with biocides. Waste metal and grinding swarf are produced during most operations. These small particles increase surface area for microbial attachment, subsequent growth, and biofilm formation. They can also react with fluids and biocides (*10*). Concentrations of Ca^{++} and Mg^{++} ions found in water used for emulsions could also have different effects on the fluid itself and microorganisms (*4*), (*1*). Prediction of a microbiological problem, detection, and treatment of contaminated systems are undoubtedly very important tasks. Steps to eliminate microbial contamination in industrial systems include detection of the degree of contamination, microbial population levels, evaluation of biocide effectiveness for the system, and dosage schedule.

Population succession in MWF has also attracted the interest of many investigators (*11*). *Pseudomonads* have been called, in most cases, the biological pioneers in metalworking ecosystems. Their extracellular polysaccharides enable them to initiate colonization of surfaces (*6*), (*2*) and produce aggregates. Biofilm formation follows attachment of bacteria to surfaces. The biofilm later can become the home of many different microbial populations. Establishment of aerobic bacteria in metalworking systems opens the doors for fungal contaminants. Fungi have never been reported from fluids where neither the fluid nor the system had previous aerobic bacterial growth (*10*).

Formaldehyde and alkylamine condensates are an important group of industrial antimicrobial compounds. They have found applications in the metalworking industry as biocides to prolong cutting fluid longevity. Cost effectiveness, compatibility, and relative lack of toxicity have given these compounds good recognition in the market (*9*). There is great chemical variety among these compounds (*8*) and not much is known about their mode of action.

Formaldehyde condensates have been reported to be very effective against bacterial contaminants but they have the same ultimate shortcoming also common to formaldehyde itself, differential quantitative inhibition between bacteria and fungi (*11*), (*7*), (*3*).

In an earlier study (*12*), regrowth of *Pseudomonas aeruginosa* in minimal inhibitory concentrations (MICs) of a formaldehyde condensate, 1,3,5 tris hexahydro ethyl-*s*-triazine (ET), was described. Regrowth is a sudden increase in colony-forming units following a period of undetected viability (eclipse period). The initial experiments suggest regrowth also occurs when other formaldehyde condensates are used. Regrowth was dependent upon the initial cell density, cell aggregates, type of media, the manner in which the culture was prepared, and the concentration of biocide used (Fig. 1).

This report describes the results of further studies determining the factors affecting survival of *Ps. aeruginosa* in MICs of ET, a formaldehyde condensate compound. Regrowth of *Ps. aeruginosa* and its relationship to surface area, surface properties, volume of suspension in use, and inactivation of biocide by media will be discussed.

MATERIALS AND METHODS

Media and Culture Preparation

An isolate of *Ps. aeruginosa* obtained from contaminated

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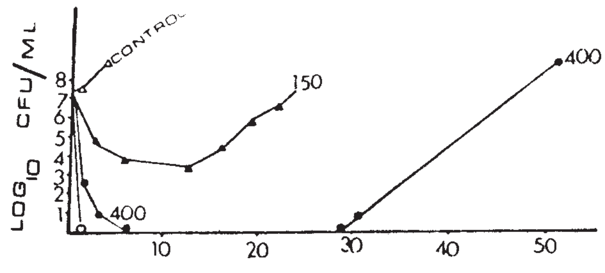

Fig. 1—Regrowth of *Pseudomonas aeruginosa* in MICs of hexahydro, 1,3,5-tris ethyl-s-triazine. Numbers indicate ppm of biocide in final volume. Suspension with 400 ppm shows regrowth, (*12*).

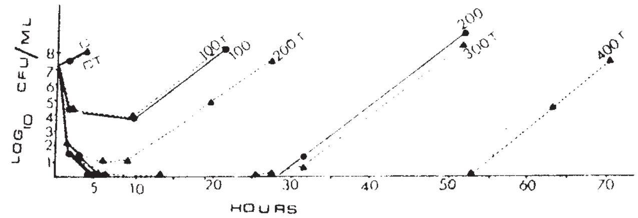

Fig. 2—Effect of surface properties on regrowth. Inhibition and regrowth of *Pseudomonas aeruginosa* in different concentrations of hexahydro-1,3,5 tris ethyl-s-triazine in silicon treated (T) and untreated flasks. The numbers indicate ppm of biocide in final volume of suspensions.

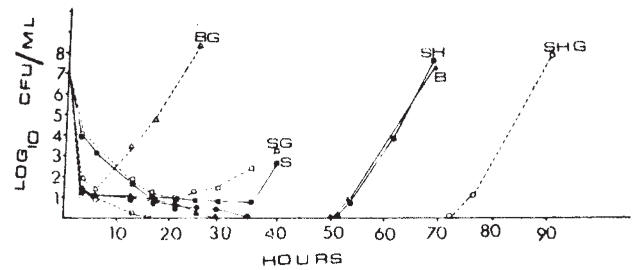

Fig. 3—Effect of surface area and incubation conditions on biocide activity and regrowth. Bacteriocidal activity of hexahydro tris ethyl-s-triazine (300 ppm) on *Pseudomonas aeruginosa*.

KEY:

△-----△ = bubbling with glass beads (BG)

▲——▲ = bubbling (B)

●——● = shaking (SH)

○-----○ = shaking with glass beads (SHG)

■——■ = stationary (S)

□-----□ = stationary with glass beads (SG)

MWF was used for these experiments. Cultures were maintained on soybean casein digest agar (USP) and grown in soybean casein digest medium (USP) at 30°C overnight. To insure that cultures were growing exponentially, overnight cultures were transferred to fresh media for 3–5 hours prior to use. Fresh, exponential cultures at $1-5 \times 10^7$ colony forming units/ml were used in all experiments.

Biocide Treatment

A 10 percent stock solution of ET in distilled water was used (10 percent active ingredient, w/v). The biocide was added to flasks containing fresh culture to obtain desired concentrations in the final volume. The effect of biocide was evaluated by standard plate count, and plates were observed for colony formation for up to seven days to detect delayed growth.

Incubation Conditions

All the experiments were carried out at 30°C in 250-ml flasks with a total suspension volume of 100 ml, with the exception of experiments done to determine the effects of suspension volume on biocide activity. The suspensions were incubated in either a controlled environment incubator/shaker or stationary with or without aeration (bubbling).

Silicon-Treated Surfaces

Silicon liquid was used for silicon treatment of glass surfaces. Flasks were rinsed with the silicon liquid to obtain a neutral surface after air drying.

Surface Areas Increase

Glass beads, 150–200 uM diameter, were washed with acid, rinsed repeatedly, and added to flasks (10 g/flask) to increase surface area for growth.

Suspension Volume Effects

Four sets of flasks containing 50, 100, 200, and 400 ml of *Ps. aeruginosa* suspensions were used. Concentrations of biocide and cells were the same in all suspensions.

RESULTS

Effect of Surface Properties on Biocide Activity and Regrowth

Providing a siliconized surface resulted in a shorter eclipse time and faster inactivation of biocide by *Ps. aeruginosa* in suspension (Fig. 2). After initiation of the regrowth process, microcolonies attached to the glass at the medium-air interface were abundant.

Surface Area Effect on Biocide Activity

This experiment is shown in Fig. 3. Increasing the surface area in stationary incubated flasks resulted in an earlier regrowth and faster inactivation of biocide in the presence of glass beads when compared with the control flask without glass beads. Aeration by bubbling helps *Ps. aeruginosa* regrowth probably by shortening the generation time with optimal oxygen levels. Aeration by shaking permits the glass beads to disrupt cell aggregates formed in the medium and results in a longer eclipse period than the control. This difference is not due to destruction of *Ps. aeruginosa* cells by glass beads since there was no evidence for greater initial kill rate with the glass beads.

Effect of Suspension Volume on Biocide Activity and Regrowth

Initial inhibition rates (Fig. 4) were greater in smaller suspension volumes. Concentration of ET was 300 ppm for all suspensions. Eclipse period and regrowth were also affected by suspension volume and initial inhibition rate. That is to say, in the larger volume onset of inhibition is slower and eclipse period is shorter.

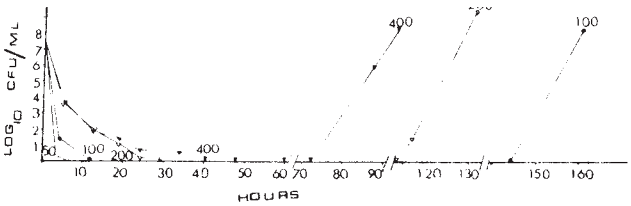

Fig. 4—Suspension volume effect on initial inhibition rate and regrowth. Bacteriocidal activity of hexahydro-1,3,5-tris ethyl-s-triazine, 300 ppm, on *Pseudomonas aeruginosa* suspensions of different final volume. Numbers indicate total final volume (milliliters). The 50-ml suspension did not have regrowth up to eight days.

Inactivation of Formaldehyde Condensate by Baterial Growth Medium

Medium and biocide, ET, were mixed 24 h prior to addition of cells to flasks. Results of the biocide activity of different final concentrations are shown in Fig. 5 when compared to controls. In the control flask biocide, cells, and medium were mixed at zero time. These results clearly indicate that biocide reacts with the medium in the absence of bacterial cells. This is not a simple dissociation of the biocide molecule since it does not happen in distilled water.

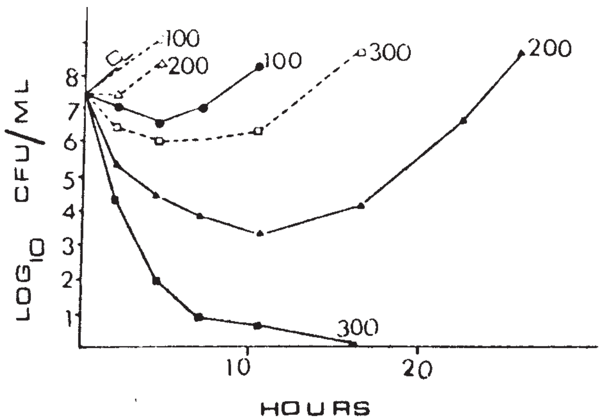

Fig. 5—Inactivation of hexahydro 1,3,5-tris ethyl-s-triazine by soybean casein digest medium.

- - - - - - - indicate addition of biocide to media 24 h prior to addition of *Pseudomonas aeruginosa*.

———— (solid lines) indicate mixing of biocide, medium, and cells at zero time. Numbers indicate ppm of biocide in final volume.

DISCUSSION

Treatment of contaminated MWF is not a new problem. The proper application of biocide to MWF is a factor which, unfortunately, is sometimes neglected in practice. However, it is obvious that even the most effective industrial biocides cannot perform their functions unless they reach the contaminating organisms in all parts of the system. Many factors, in addition to the choice of proper biocide, affect the success of biocide treatment in metalworking systems.

Our recent report (*12*) indicates the presence of undetectable, possibly injured, populations of *Ps. aeruginosa* after treatment with ET in MICs. This population could begin exponential growth after a longer period of eclipse. Possible involvement of cell aggregates and extracellular polysaccharides were also suggested.

Marshall (*5*) indicated that siliconized germanium prisms immersed in bacterial cultures for 10 minutes had protein films adsorbed to the surface. Adsorption of bacteria was higher and distribution more uniform on the hydrophobic siliconized surfaces than on normal prism surfaces. Results shown in Fig. 2 suggest involvement of *Pseudomonas* adsorption to the surface in inactivating the biocide.

Another and sometimes neglected problem is expeditious removal of chips and fines that increase the surface area for microbial attachment. Figure 3 shows the results of increasing surface area and aeration on biocide activity. Incubation with shaking resulted in delayed regrowth in the presence of glass beads, suggesting again the importance of cell aggregates in protecting the cells against biocide.

It is known that treatment of very large contaminated metalworking systems is much more difficult than small systems. Problems involved in determining levels of contamination in these systems may be overlooked. Many methods and techniques have been suggested that include a variety of approaches. Currently the plate count method or a facsimile is used most frequently. It must be remembered that the counts will only determine the floating population; therefore, a larger population that exists in biofilms formed in the system will be missed. On the other hand, in the systems already treated with biocides, a different problem could result. Underdosing the system could leave injured cells hidden in aggregates (*12*) and biofilms that will not be detected by currently used methods.

The size of the delivery system plays a major role in the prevention, detection, and treatment of microbial problems in MWF. The results shown in Fig. 4 suggest that the rate of kill in larger volumes is slower when compared to the smaller volumes. Preliminary experiments suggest that it is possible that these differences result from faster elimination of free-floating individual cells than cells in aggregates and biofilms. This difference in kill rate will be more pronounced in larger systems than smaller ones when the rate of biocide neutralization is also taken into consideration. Rate of neutralization of ET in growth medium in the absence of *Ps. aeruginosa* within 24 hours is shown in Fig. 5. It is clear that the biocide is reacting with organic molecules in the medium. This neutralization process may also explain how injured cells could regrow after long incubation. In heavily contaminated systems, there are large quantities of cellular-derived organics present, and the initial kill by biocide will release more reactive cellular material by lysis into the fluid that will react with biocide.

CONCLUSIONS

There are two lessons to be learned from the preliminary results presented here. One is intrinsic to the development of test methods for evaluation of biocides and stresses the importance of several variables on biocide performance. These include not only aeration but the method of inducing aeration (i.e. bubbling vs shaking), surface area, system volume, and the reactivity of the biocide with the uninoculated system. The second lesson relates to the extrapolatibility of test data to the field. The factors affecting biocide activity described here are all operable in metalworking systems.

Perhaps lack of attention to these factors in the past has been partially responsible for unexpected biocide failures in use situations.

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