



Fact and Fantasy Regarding Resistance to Microbicides

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Resistance to animal and human therapeutic antibiotics is well known and understood among both the scientific and lay community. Performance failures with industrial microbicides are commonly discussed as if the same phenomenon occurs here also. Much of this is indeed fantasy, as the mode of action of most, but not all, industrial microbicides are based on a fundamentally different principle than are therapeutic antibiotics. Industrial microbicides are designed to be broad spectrum and achieve this by attacking some fundamental principle that is present in most microorganisms and without which the organism cannot survive. Antibiotics, on the other hand, are designed to be as narrow spectrum as possible, so that side effects in the animal or human host are minimized. A clear understanding of why a broad spectrum microbicide fails in a particular situation is necessary in order to have a successful program. Application errors, such as using an inappropriate biocide and calling it resistance, will ultimately be economically detrimental. Using different modes of action products in sequence is understood in antibiotic science to encourage true resistance. This may very well also promote resistance in broad spectrum microbicides. A thorough understanding of microbicides and the particular industrial situation in which they are used is necessary to have a successful, cost-effective program, while minimizing promotion of resistance.

Introduction

Occasionally a previously successful industrial biocide program gets into trouble. The material intended to be preserved is deteriorating or a microbial problem solved is now reappearing. Because of our knowledge of the resistance of microorganisms to therapeutic antibiotics, we expect the same thing to occur with the use of our common industrial microbicides used in leather manufacturing. It is very important to ascertain whether there is true resistance or not, so that a proper and lasting solution can be applied. A misunderstanding as to the causes of the failed program would produce a temporary solution at best.

Many physical and chemical factors impact the efficacy of a microbicide, and therefore change the product itself. However, this is different from resistance. True resistance is carried on a stable genetic element that is easily transferred to other organisms (bacteria, yeasts), or if chromosomal as in filamentous fungi, transmitted to

progeny thereby spreading the problem. The current research into the nature of resistance to industrial microbicides is reviewed

Therapeutic Antibiotics and Resistance

Although the chemistries of therapeutic antibiotics are typically very different from industrial microbicides, the intended result is the same: namely destruction of the problem causing microorganism. Unfortunately, after more than 50 years of antibiotic therapy many organisms have developed resistance to the better antibiotics. This is becoming a life-threatening problem in hospitals.

Staphylococcus aureus causes many hospital acquired infections. This organism is resistant to almost all antibiotics with vancomycin being the remaining effective one. Moderate resistance has now been reported even to vancomycin. Drug researchers have attempted to improve the spectrum of activity of many antibiotics so that resistance won't develop as readily. A good example is the change from the narrow spectrum penicillin to the more broad spectrum ampicillin.

Most resistant prokaryotic microorganisms carry the genes for resistance on a small genetic piece called a plasmid. These plasmids can be transferred to other organisms through narrow pipelike structures called pili. The genetic transfer of resistance allows for a rapid spread of this characteristic throughout a similar population. Some transfer to dissimilar populations has been demonstrated. The genes for resistance to several different antibiotics in bacteria frequently travel together as a unit, conferring resistance in one step to a number of different chemistries. The filamentous fungi appear to carry the resistance genes in the normal chromosomal material. This is not as effective a competitive advantage as the bacterial resistance genes. (*Lawrence, J.G., 2000*)

The main problem here is with the specificity of targeting of antibiotics. If the target is an enzyme, other enzymes are often present that can function in the same activity. In other cases, enzymes are available in the organism that change or destroy the offending molecule. Specificity is necessary for therapeutics so that the beneficial microflora is left reasonably intact. But, specificity is also the main reason that the targeted organisms can find a method to avoid harm.

Bacterial Antibiotics

Resistance Mechanism

Penicillin	B-Lactam ringbreaking enzymes
Streptomycin, erythromycin	Alteration in binding site (ribosome)
Aminoglycoside-aminocyclitol antibiotics	Phosphorylation (changes in solubility)
B-Lactam antibiotics	Tolerance (autolysin-defective organisms)
Mercury	Vaporization
Monoquats	Outer membrane alterations

Monoquats, tetracycline

Efflux mechanism

Table 1. Therapeutic/industrial bacterial antibiotics and resistance mechanisms.

Resistance factors have been known to be transferred between species and even genera of bacteria. However, the more similar an organism is, the higher the potential for transfer. This is an important factor in favor of industrial microbicides as the microbial ecology of current industrial systems is quite varied. The variety of the microbial ecology may be an important factor in minimizing resistance. Such transfer has not been reported between fungal organisms, although the ability to transfer genetic material is present in some. Therefore, the situation is very different as regards fungi compared to bacteria. It is still unknown if archaea have similar systems for genetic transfer as the bacteria have. It is quite possible as the archaea have about 50% similarity to the prokaryotic bacteria and 50% similarity to the eukaryotic fungi.

Most perceived resistance is intrinsic/natural

When a biocide program gets into trouble, it is usually because of one or more of several factors which are called intrinsic resistance (Table 3).

Types of intrinsic factors	Organism type
Endospores	Bacteria (some)
Pigmented reproductive spores	Bacteria, fungi
Capsule/ biofilm production	Bacteria, fungi, algae
Pigments (cell wall)	Bacteria, fungi
Insensitivity to compound	Lack of process microbicide attacks: any organism
Cysts	amoebae, protozoa and nematodes
Genetic variation	All organisms

Table 3. Intrinsic resistance factors and the organisms they apply to.

Such factors can be related to survival mechanisms from the microorganisms such as endospores or capsules. Modern microbicides don't have a total microbial efficacy spectrum (broad spectrum) such as was obtained with the now mostly outlawed mercurial microbicides. Therefore, some microbicides are more effective against bacteria, and others against fungi. One can say, then, that fungi have intrinsic resistance to bactericides, while bacteria have intrinsic resistance to fungicides. Choosing a bactericide to be used against a fungus does not mean that some new dangerous resistance factor has developed, but just that an inappropriate product was selected.

Some intrinsic resistance factors are especially important. Bacterial endospores are tremendously resistant to all adverse conditions. Endospore formation is a survival mechanism, not a reproductive one. Only one endospore is produced by each cell, and could be described as a capsule form of the most important structures of the cell. The

many spore coats that cover the cell make it almost impervious to any microbicide. The original cell, however, is typically quite easy to kill. The million year old bacteria that have been isolated from amber survived as such endospore structures.

Efflux mechanisms in both bacteria and fungi have been under serious study for the last ten years. It is clear that many organisms have methods for ejecting the toxicant from the cell, thereby rendering it harmless. Such mechanisms appear to be almost universal.

Fungi typically produce reproductive spores, many of which are pigmented. These spores are usually less sensitive than the fungal hyphae to a fungicide. The most important intrinsic resistance factor in water industrial environments is the production of a capsule and subsequent biofilm, while dry surfaces usually cause organisms to produce either survival or reproductive spores.

One of the first reports on microbial resistance (Chaplin, C.E., 1952) determined that the bacterial resistance to a monoquat occurred because of cell surface changes. This was postulated to be due to selection and adaptation, with some crisis intervention. In the 1980's, research demonstrated resistance to formaldehyde releasing microbicides. Some fifteen different microbicides were tested (Candal, F.J. et al, 1984). Later this resistance was determined to be placed on a plasmid (Sandossi, M. et al, 1986), thereby increasing the potential for transfer to other organisms. Resistance to formaldehyde releasing preservatives has the potential to be a costly problem to the industry. Most cost effective preservatives for coatings and fillers are based on releasing formaldehyde from a larger molecule. As resistance to this chemistry becomes more widespread, fewer and fewer cost effective preservatives will be available. The final cost to our industry could be considerable. Fortunately, there are still some non-formaldehyde releasing preservatives available, some of which have FDA dry end allowances.

Although some studies appear questionable, lessons learned from them include the use of subinhibitory concentrations to encourage "adaptation". It also appears likely that higher level organisms (protozoa) may react very differently to microbicides than the target organisms (Srikanth S. et al, 1993).

Acquired resistance to bactericides

Formaldehyde releasing microbicides	Formaldehyde dehydrogenase enzyme
Quaternary ammonium compounds	Membrane structure changes – lipids
Mercury	Mercury volatilizing enzyme
Hydrogen peroxide	Catalase enzymes

Table 4. Demonstrated resistance and the chemistry that it relates to.

Fungicide resistance to agricultural fungicides is widespread among the pathogens that invade plant hosts. There are a number of different reasons for this, many of which relate to the methodology and application technologies. There is a lot we can learn about resistance to leather fungicides from the resistance to agricultural fungicides. However, there are a number of conclusions that do not transfer. The best example of this is the relationship between resistance development from sequential treatments versus combination treatments. What we have to remember is that the agricultural treatment is against surviving fungi that now will be exposed to the same or a new fungicide. In leather treatments, you usually only expose the organisms once to the treatment. Each treatment is against a new population, not one surviving from previous treatments. Of course, there is a certain small amount of exposure of airborne spores to the fungicides used in wet blue treatment, but this is very minimal and unlikely to have any impact. Another source of concern in the tannery relates to the inadequate degreasing of hides.

Following the application of a fungicide, subsequent removal of residual grease later in processing may strip away a significant portion of the lipophilic antifungal chemical. The appearance of a fungal contaminant may be wrongly interpreted as a "resistant" mold.

Developing resistance to an antimicrobial compound

There is only minimal research available in the literature on how to increase resistance to industrial microbicides. Most of our information comes from the lessons in antibiotic resistance. There are indications that underfeeding (using concentrations below effective use concentrations) would cause resistance to appear. Secondly, shockfeeding, - which would kill of all sensitive organisms - followed by underfeeding, would favor growth of any surviving (resistant) organism. The overall process is accentuated when there are large populations of few species of microorganisms. Some agricultural researchers have tried to elucidate which is the better: alternate or sequential treatments. The agricultural data is from the field and leans towards sequential treatment as the better alternative. However, the fact that in agriculture you are treating surviving organisms is very different from leather uses. All of this gives us cues on avoiding resistance.

Minimizing resistance

The first cue here is to reduce the population level. This can be done by maintaining good housekeeping practices, using effective broad spectrum microbicides and appropriate treatment strategies. A well designed program will keep population levels at acceptable levels for the system and minimize the statistical chance for effective mutations or resistance transfer/ development. The second cue is to study the system well for the applicability of the proposed program. Resistance should not be used as an excuse for a poorly studied and designed program.

Strategies when resistance is perceived

The two most common resistance mechanisms are (endo)spores and biofilms. Bacterial endospores are very resistant structures, but the cells from which they are derived are not resistant. As the cells go from vegetative cell to endospore it becomes more and more resistant. Fungal spores can be more resistant than the fungal mycelium. It becomes evident then, that the biocide should be used on the cells and not the spores, for effective control.

Biofilm formation - slime deposits - is probably the most common resistance mechanism in water containing systems such as race ways or process materials. Here we have a number of strategies available, but the basic idea is to divide and conquer. When the biofilm is dispersed, the organisms become sensitive again. Good housekeeping and strategic use of dispersants and enzymatic dispersants can easily take care of biofilms and make the program effective.

For fungicides, the most important aspect is to use broad spectrum fungicides. In this context, broad spectrum is in relationship to the mode of action, not the variety of species that are sensitive to the product. "Pathogen populations that develop resistance to one fungicide automatically and simultaneously become resistant to other fungicides that are affected by the same gene mutation and the same resistance mechanism. Generally, these have proved to be fungicides that bear an obvious chemical relationship to the first fungicide.... This is the phenomenon known as cross-resistance." (Brent, K.J., 1995). Brent also discusses the reason that some of the well-known older fungicides have not produced resistance. This is due to being broad spectrum, while newer ones are narrow spectrum as to their target or mode of action (many azoles and carbendazim).

Degradation of microbicides

Deactivation of microbicides can be due to physical conditions in the use environment. Many microbicides break down rapidly under alkaline pH, while others are sensitive to acid pH. Other conditions also impact the efficacy, but physical deactivation has nothing to do with microbial resistance. Again, an inappropriate compound selection or addition sequence is the problem.

Future trends in resistance

Resistance to industrial microbicides so far is mainly limited to one commonly used type of bactericides (formaldehyde releasers), and to the narrow spectrum fungicides. The reason for this limitation is partly the broad action of typical industrial microbicides and the varied microflora present in industrial situations. The varied microflora part of the equation could be changing in the future. With less genetic variability and higher numbers may follow an increase in resistance, as this was the pattern for antibiotic resistance.

The current scarcity of companies that perform research into new industrial antimicrobial actives ensures that there will be few new actives available should resistance become wide spread. The potential loss of usable materials and lower quality of the finished product could become very costly to a competitive market. While there does not appear to be a reason for concern currently, this could change if the profound misunderstanding of how resistance patterns evolve in the leather processing industry is not changed.

What does all this mean to the industrial user?

It is easy to think that the difference between intrinsic and acquired resistance is irrelevant to the industrial user of microbicides. The opposite is true. Intrinsic resistance occurs when the inappropriate compound or treatment regiment has been used. It also occurs when housekeeping is poor. The good news is that this is easily remedied, and does not follow us forever. It should never be an excuse for not determining the real problem. If there isn't real resistance, we should take care not to get it started by misunderstanding what is happening when there is a lack of control with the program. Changing microbicide has a role to play when real resistance has occurred. It could be a dangerous game to play if the program is poorly conceived.

The topic of resistance to industrial microbicides is a complicated issue. This paper covers only the most major issues. Very little research regarding the industrial products is being published for us all to learn from. The great need for further published research is, so we can determine if the situation is changing. Rigorous investigations into "resistance" problems would help us all determine what really is going on in a particular situation. There is little support for such investigations in our industry, however, as other work is typically more pressing.

Acquired resistance however, would be a real and serious problem to us all. There is a limited number of active ingredients registered by the US EPA or international regulatory agencies. We must first make sure we understand any failures so that appropriate measures can be applied. Secondly, we must apply products appropriately to minimize the risk for true resistance to appear. In addition, there is some evidence from antibiotic resistance and other studies, that cross-resistance to previously unused antibiotics does occur. In bacteria, this cross resistance occurs even with different chemistries, but is only infrequently happening in filamentous fungi. It follows then, that

to change microbicide because of a perceived resistance problem is detrimental to the industry.

References

- Armstrong, J. L., D. S. Shigeno, J. J. Calomiris, and R S Seidler. 1981. Antibiotic-Resistant Bacteria in Drinking Water. *Appl. Envir. Microbiology*: 277-283.
- Block, S. S. 1991. *Fundamental Principles of Activity In Disinfection, Sterilization, and Preservation*, 4th Ed. pp. 47-58.
- Brotzel, V S and T E Cloete. 1991. Resistance of bacteria from cooling waters to bactericides. *J Ind. Microbiology* 8: 273-276..
- Brozel, V S, B Pietersen and T E Cloete. 1993. Adaptation of bacterial cultures to non-oxidising water treatment bactericides. *Water SA*. Vol 19: 259-260.
- Candal, F J and R G Eagon. 1984. Evidence for plasmid-mediated bacterial resistance to industrial biocides. *Int. Biodeterioration*. Vol. 20, No. 4: 221-224.
- Chaplin, C. E. 1952. Bacterial resistance to quaternary ammonium disinfectants. *J. Bacteriol.* 63: 453-458.
- Collier, P J, P Austin, and P Gilbert, 1991. Isothiazolone biocides: enzyme-inhibiting prodrugs. *Int. J Pharmaceutics* 74: 195-201
- Eagon, R.G., and C P Barnes. 1986. The mechanism of microbial resistance to hexahydro-1,3,5-triethyl-s-triazine. *J Ind. Microbiology* 1: 113-118.
- Gillespie, M T, J W May, and R A Skurray. 1986. Plasmid-encoded resistance to acriflavine and quaternary ammonium compounds in the methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiology Letters* 34: 47-51.
- Gorman, S P, and E. M Scott. 1986. Antimicrobial Activity, Uses and Mechanism of Action of Glutaraldehyde. *J of Appl. Bacteriology* 48: 161-190.
- Grace, R D, N E Dewar, W G Barnes, and G R Hodges. 1981. Susceptibility of *Legionella pneumophila* to Three Cooling Tower Microbicides. *Appl. Envir. Microbiology*: 233-236.
- Heinzel, M. 1986. On the influence of different growth conditions to the resistance of some methylotrophic bacteria to Aldehydes. *Zbl. Bakt. Hyg. B* 182: 299-309.
- Hugo, W B. 1991. The Degradation of Preservatives by Microorganisms. *Int. Biodeterioration* 27: 185-194.
- Kaulfers, P-M, H. Karch, and R. Laufs. 1987. Plasmid-mediated Formaldehyde Resistance in *Serratia marcescens* and *Escherichia coli*: Alterations in the Cell Surface. *Zbl. Bakt. Hug. A* 266, 239-248.
- LeChevallier, M W, C D Cawthon, and R G Lee. 1988. Inactivation of Biofilm Bacteria *Appl. Environ. Microbiology*:
- Lawrence, J G. 2000. Clustering of antibiotic resistance genes: Beyond the selfish Operon. Vol. 66, No. 5. *ASM News*, 281-286.

Murray, G E, R S Tobin, B. Junkins, and D J Kushner. 1984. Effect of Chlorination of Antibiotic Resistance Profiles of Sewage-Related Bacteria. Appl. Envir. Microbiol.: 73-77.

Russell, AD and G W Gould . 1988. Resistance of Enterobacteriaceae to preservatives and disinfectants. J Appl. Bacteriology Symposium Suppl.: 167S-195S.

Russell, A D. 1991. Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. J. Appl. Bacteriology 71: 191-201.

Sondossi, M, H. W. Rossmoore, and J W Wireman. 1986. The Effect of fifteen biocides on formaldehyde-resistant strains of Pseudomonas aeruginosa. J. Ind. Microbiology 1:87-96.

Srikanth, S and S. Berk . 1993. Stimulatory Effect of Cooling Tower Biocides on Amoebae. Appl. Environ. Microbiology: 3245-3249.



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