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SELECTED ANTIMICROBIAL APPLICATIONS OF OXIDISED COAL PRODUCTS

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SELECTED ANTIMICROBIAL APPLICATIONS OF OXIDISED COAL PRODUCTS

by

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DECLARATION

I the undesigned hereby certify that the work contained in this thesis is my own original work and has not previously, in its entirety or in part, been submitted at any university for a degree

Signature: .....

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SELECTED ANTIMICROBIAL APPLICATIONS OF OXIDISED COAL PRODUCTS

by

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Promoter: Prof. T.E. Cloete

Department: Microbiology and Plant Pathology

Degree: PhD (Microbiology)

SUMMARY

South African bituminous coal was converted by controlled wet oxidation to a highly oxidized water-insoluble product, oxicoal, and a water soluble product, oxifulvic acid. Oxihumic acid was produced by the extraction of the oxicoal with base and precipitation of the extract with acid. Due to the reported antimicrobial activity of humic substances, antimicrobial applications for the coal-derived products were investigated since a market exists for the production of low cost and effective antimicrobial compounds in South Africa.

Oxifulvic acid was the most bactericidal of all the coal-derived products evaluated since 150.0 mg/l was required for bactericidal activity against seventeen bacterial test isolates. The antibacterial activity of oxifulvic acid was pH dependant, with the optimum

pH for bactericidal activity between pH 3.0 and pH 4.0. A linear relationship was exhibited between the calcium concentration and bactericidal concentration of oxifulvic acid. An eight fold increase in oxifulvic acid was required for bactericidal activity in the presence of organic matter.

Oxifulvic acid was bactericidal at 100 mg/l, in *in vitro* studies, against a range of twelve bacteria isolated from South African water-cooling systems, but was unable to control the growth (oxifulvic added at 200.0 and 400.0 mg/l) of sessile and planktonic bacteria during *in situ* studies. The concentrations of oxifulvic acid required for fungicidal (> 4000.0 mg/l) and algicidal (400.0 mg/l) activity indicated that oxifulvic acid does not have potential for use as a fungicidal or algicidal compound.

Oxifulvic acid (800.0 mg/l) proved to be more effective than a commercially available coal-tar disinfectant (48 000.0 mg/l). A concentration of 96.0 mg/l oxifulvic acid was virucidal against coliphage V_1 within 10 min. Oxifulvic acid did not exhibit any sporicidal activity. Oxifulvic acid (25.0 mg/l) in combination with sodium dodecyl sulphate (250.0 mg/l) exhibited synergistic activity. This combination has potential for use as a surface disinfectant on hard non-porous surfaces.

Oxifulvic acid showed potential for use in the control of acid mine drainage formation by iron-oxidizing bacteria, since it inhibited iron-oxidation by these bacteria at 100.0 mg/l in *in vitro* studies.

The antibacterial action of oxifulvic acid against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* strain K12 was ascribed to the failure of the pH homeostasis mechanism of the bacteria, to maintain a constant intracellular pH. *Pseudomonas aeruginosa, S. aureus* and *E. coli* strain K12 were able to tolerate 125.0, 250.0 and 250.0 mg/l higher concentrations of oxifulvic acid after 10 subcultures in the

presence of oxifulvic acid. This increased tolerance to oxifulvic acid was ascribed to the habituation of the bacteria to a sub-lethal pH.

Oxicoal removed 100% of the coliphages of *E. coli* strain K12 present in sterile distilled water and raw sewage in *in vitro* studies. The results indicated that oxicoal could be used as a substitute for coal in a dual-media sand/coal filter for the removal of enteric viruses from treated and untreated water.

GESELEKTEERDE ANTIMIKROBIESE TOEPASSINGS VAN STEENKOOLOKSIDASIEPRODUKTE

deur

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OPSOMMING

'n Suid Afrikaanse steenkool kan deur middel van 'n nat oksidasie proses omgesit word na 'n water onoplosbare produk oksikool en 'n water oplosbare produk oksifulviensuur. Oksihumiensuur kan berei word deur geoksideerede steenkool te ekstraeer met 'n alkali en die ekstrak te presipiteer met 'n suur. Na aanleiding van die beweerde antimikrobiese aktiwiteit van humus, is die antimikrobiese aktiwiteit van die steenkoolafgeleide produkte geëvalueer, aangesien daar tans in Suid Afrika 'n behoefte bestaan vir die vervaardiging van goedkoop en effektiewe antimikrobiese middels.

Oksifulviensuur was die produk met die hoogste antimikrobiese aktiwiteit, aangesien 150.0 mg/l benodig was vir kiemdodende aktiwiteit teen sewentien bakterieële toets organismes. Die kiemdodende aktiwiteit van oksiefulviensuur was afhanklik van pH,

aangesien die optimum pH vir kiemdodende aktiwiteit tussen pH 3.0 en pH 4.0 geleë was. Daar was 'n liniêre verband tussen kalsium konsentrasie en kiemdodende aktiwiteit van oksiefulviensuur. Die konsentrasie oksiefulvensuur benodig in die teenwordigheid van organiese materiaal was agt maal hoër as die konsentrasie in die afwesigheid van organiese materiaal.

'n Konsentrasie van 150.0 mg/l oksiefulviensuur was nodig vir kiemdodende aktiwiteit teen twaalf bakterieë wat uit Suid Afrikaanse verkoelingswatersisteme geisoleer was, maar oksifulviensuur was nogtans nie instaat om die groei (oksiefulviensuur teen 200.0 en 400.0 mg/l bygevoeg) van aangehegte en planktoniese bakterieë in verkoelingswatersisteme te beheer nie. Die konsentrasies oksifulviensuur benodig om swamgroei (> 4000.0 mg/l) en algegroei (400.0 mg/l) te inhibeer, toon dat die produk geen potensiaal het om as 'n swamdoder of algedoder aangewend te word nie.

Oksiefulviensuur (800.0 mg/l) was meer effektief as 'n kommersieel verkrygbare steenkool-teer (48 000.0 mg/l) afgeleide ontsmettings middel. 'n Konsentrasie van 96.0 mg/l oksiefulviensuur van nodig vir kiemdodende aktiwiteit teen bakteriofaag V_1 binne 10 minute. Oksiefulviensuur het geen aktiwiteit teen bakteriese spore getoon nie. 'n Mengsel van oksifulviensuur (25.0 mg/l) en natriumlaurielsulfaat (250.0 mg/l) het potensiaal om as 'n oppervlak onsmettingsmiddel vir harde nie-porieuse oppervlaktes gebruik te word.

Aangesien oksifulviensuur die bakterieë wat verantwoordelik is vir die vorming van suurafloop vanaf steenkool myne, geïnhibeer het (100.0 mg/l oksifulviensuur), kan dit moontlik gebruik word om die vorming van suurafloop te beheer.

Die kiemdodende aktiwiteit van oksiefulviensuur teen *S. aureus*, *P. aeruginosa* en *E. coli* ras K12 was toegeskryf aan die onvermoë van die bakterieë om 'n konstante pH binne die sel te handhaaf. *Pseudomonas aeruginosa, S. aureus* en *E. coli* ras K12 was

instaat om konsentrasies van oksiefulviensuur wat 125.0, 250.0 en 250.0 mg/l hoër was te weerstaan, na voortplanting vir 10 keer in die teenwoordigheid van oksiefulviensuur. Die verhoogde verdraagsaamheid vir oksiefulviensuur is toegeskryf aan 'n aanpassing van die bakterieë om by 'n nie optimale pH te kan groei.

Oksikool was instaat om 100% van die bakteriofage, van *E. coli* ras K12, vanuit 'n suspensie en rou riool verwyder. Die resultate dui aan dat oksikool, in plaas van steenkool, aangewend kan word in 'n steenkool/sand filter om enteriese virusse uit onbehandelde en behandelde water te verwyder.

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CHAPTER 1

INTRODUCTION

The editorial style of the Journal of Applied Bacteriology was followed in this chapter.

South Africa is a country rich in many natural resources. One of the most abundant of these is coal (Department of Water Affairs and Forestry, 1986). The production of value added products using low rank (low grade) bituminous coal is a possible way of increasing the value of raw coal. A controlled wet oxidation process was therefore developed by Cronje (1990) for the production of value added products from South African bituminous coal. The coal can be converted via oxidation and extraction to oxicoal, oxifulvic acid and oxihumic acid. The coal-derived products differ from natural humic substances with regards to the following: (1) The ratio of phenolic to carboxyl groups and total acidity are somewhat higher, in the coal-derived oxifulvic and oxihumic acids, than the corresponding values reported for the natural acids, (2) The coal-derived products contain more aromatic and phenolic compounds than natural humic or fulvic acids, (I.J. Cronje pers. comm.)*.

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Uncontrolled growth of microorganisms can lead to numerous problems. Disease, food spoilage, fouling of surfaces and biodeterioration of materials are but a few problems associated with uncontrolled microbial growth (Parr, 1990; Russel and Chopra, 1990). Use of antibacterial agents are one of the most common methods for the control of uncontrolled microbial growth (Russel, 1982). Currently more than 90 % of the raw materials used in the manufacture of antimicrobial compounds, in South Africa, are imported (Cloete *et al.*, 1989). A market therefore exists in South Africa for the production of a low cost and effective biocide. Due to the reported antimicrobial activity of humic substances (Hasset *et al.,* 1987; Kai *et al.*, 1990) and the need for the production of a low cost biocide, the biocidal applications of the coal-derived products were investigated.

Evaluation of the antimicrobial activity of biocides can be divided into three stages (Russel, 1982). First stage testing includes determination of the bactericidal activity of the biocide and the effect of environmental factors on the bactericidal activity of the biocide (Russel, 1982). There are many factors that affect the concentration required for antimicrobial activity of biocides. The most important are pH, water hardness and presence of organic material (Russel, 1982). The activity of antimicrobial compounds are influenced by changes in pH, due to dissociation of the carboxyl (COOH) functional group *i.e.* phenols and organic acids (Russel, 1982). Water hardness (Ca^{2+} and Mg^{2+} concentration) reduces the activity of certain antimicrobial compounds by binding to the antimicrobial compound. The interference of organic matter with the antimicrobial activity of compounds normally takes the form of a reaction between the antimicrobial compound and the organic matter (Russel, 1982). The effect of pH, Ca^{2+} concentration and organic matter on the bactericidal activity of the most bactericidal coal-derived product (oxifulvic

acid) was therefore studied.

The uncontrolled growth of bacteria in water-cooling systems may lead to biocorrosion and biofouling of surfaces (McCoy, 1979). Fungi may cause problems in water-cooling systems, with the degradation of wooden structures and the clogging of filters the main consequences of fungal growth (Iverson, 1987). The problems associated either directly or indirectly with algal growth in water-cooling systems are foul odours, clogging of filters, thereby shortening filter life, colouration of the water due to the presence of planktonic algae and the production of algal slimes which could lead to under deposit corrosion (McCoy, 1979). Studies on the possible use of oxifulvic acid to control bacterial, fungal and algal growth in water-cooling systems were therefore undertaken.

Disinfectants have been described as bactericidal agents that are used on inanimate objects and includes antibacterial agents that are too toxic, irritant or corrosive to be applied to body surfaces or tissues, but are suitable for disinfection of equipment or the inanimate environment (Russel and Chopra, 1990). The activity of disinfectants against mycobacteria, bacterial spores, fungi and viruses is not as important as their activity against bacteria, and depends on the area of application (Reybrouck, 1982). The activity of oxifulvic acid against bacteria, bacterial spores and viruses was therefore studied.

Acid mine drainage is a pollution problem resulting from the oxidation of pyritic material by iron-oxidizing bacteria. The bacteria accelerate the oxidation of the pyrite (FeS_2) present in gold and coal mine dumps, with the formation of acid drainage (Kleinmann, 1979). Pollution of water systems with acid drainage renders them unfit for use (Walsh, 1978). Due to the failure of sodium lauryl sulphate and sodium benzoate to inhibit iron-oxidizing bacteria *in situ* (Bosch, 1990) the inhibition of iron-oxidizing bacteria by oxifulvic acid was therefore studied *in vitro*.

Synergism between bactericides has been reported and used to enhance activity or increase the spectrum of activity of bactericides (Lehmann, 1988). Denyer (1990) described two possible mechanisms of synergism between two antimicrobial compounds, namely biochemical and permeabilisation synergism. Biochemical synergism involves the selection of biocides to maximise the spectrum of damage and minimise cell recovery. Permeabilisation synergism involves the selection of biocides which enhance activity by increasing the permeability of the microbial cell. Possible synergistic combinations with other antimicrobial compounds were therefore studied.

Eklund (1983) has shown that the undissociated form of an organic acid is the more bactericidal. The mode of action of organic acids has not been clearly identified although acidification of the cell cytoplasm (Salmond *et al.*, 1984), inhibition of nutrient uptake (Freese *et al.*, 1973; Eklund, 1980) and inhibition of cellular synthesis (Cherrington *et al.*, 1991) have been reported. Resistance of bacteria to organic acids has not been reported (Cherrington *et al.*, 1991). There is evidence, however, that exposure of bacteria (*e.g. Escherichia coli*) to sublethal acidic conditions increases their tolerance to exposure to organic acids (Goodson and Rowbury, 1989) and this also favours their subsequent survival in environments at lethal pH values. Studies of the mode of antibacterial action of oxifulvic acid were undertaken to determine whether the mode of action was similar to that of organic acids and phenols. The development of resistance of bacteria to oxifulvic acid was investigated due to the reported resistance of bacteria to organic acids (Goodson and Rowbury, 1989).

Viruses are naturally found in domestic and industrial wastewater, and their fate once they enter the environment is of concern due to their possible pathogenicity (Grabow *et al.,* 1978; Bixby and O'Brien, 1979). Pathogenic viruses (*e.g.* hepatitis B) are capable

of surviving in the environment (Lewis and Metcalf, 1988). Their removal from wastewater is therefore of great importance. Activated carbon adsorption or sand filtration followed by a chlorination are currently used to produce water free of pathogenic organisms (Cairncross and Feacham, 1983; Lewis and Metcalf, 1988). Chlorination, however, only removes up to *ca.* 84% of the enteric viruses present in sewage effluent (Lewis and Loutit, 1989). A more feasible alternative would be to enhance the effectivity of existing treatment procedures. Since coal and activated carbon have been used to remove viruses from wastewater (Orza and Chaudhuri, 1977; Bitton, 1980), oxicoal was investigated as an alternative to coal or activated carbon for virus removal from potable and non-potable (wastewater) water.

The aims of this study have been divided into their various areas of application for convenience, as listed below.

Biocidal Applications

Evaluate coal-derived products for use in the control of biofouling and biocorrosion by:

- Determination of the bactericidal activity of the coal-derived products
- Determination of the effect of pH, calcium concentration and organic matter on the bactericidal activity of oxifulvic acid (most bactericidal coal-derived product)
- Determination of the bactericidal activity of oxifulvic acid against bacteria dominant in water-cooling systems
- Field trial evaluations in water-cooling systems to determine the efficiency of oxifulvic acid to control bacterial growth under field conditions

Fungicidal Applications

- Determine the fungicidal activity of oxifulvic acid against a range of fungi

Algicidal Applications

- Determine the algicidal efficacy of oxifulvic acid

Disinfectant Applications

- Determination of the disinfectant activity of oxifulvic acid against vegetative bacteria, spores and viruses
- Comparison of the bactericidal activity of oxifulvic acid to that of a commercial coal-tar disinfectant
- Determination of synergism between oxifulvic acid and ethylenediaminetetraacetic acid (EDTA), a quaternary ammonium compound (QAC), hydrogen peroxide, ethanol, sodium hypochlorite, copper and sodium dodecyl sulphate
- Determination of bacterial resistance to oxifulvic acid
- Studies on the mode of bactericidal action of oxifulvic acid on selected bacterial isolates

Control of Acid Mine Drainage Bacteria

- Determination whether oxifulvic acid could be used to control the formation of acid mine drainage

Water Purification Application

- Determine whether oxicoal could be used as an alternative to coal or activated carbon for virus removal from treated and untreated effluent

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CHAPTER 2

LITERATURE REVIEW

The editorial style of the Journal of Applied Bacteriology was followed in this chapter

2.1 Introduction

Coal tar, coke, aqueous liquor, light oil and gas are amongst the primary products produced by the destructive distillation or carbonization of bituminous coal (Rhodes, 1945; Hugo and Russel, 1982). Three variables, namely: type of coal, carbonization equipment and carbonization temperature determine the yields and physical properties of the primary products produced (Rhodes, 1945). The most important phenolic constituents of coal tar are phenols, cresols, high-boiling tar acids and xylenols. Coal tar disinfectants are formulated either by the emulsification or solubilisation of the coal tar acid fractions in a soap (Hugo and Russel, 1982).

Humic substances are defined as that portion of the soil organic matter that has undergone sufficient transformation to render the parent material unrecognizable (Atlas and Bartha, 1981). Humic compounds are divided according to their solubility characteristics into humin, humic acid and fulvic acid (Atlas and Bartha, 1981). The solubilities of the fractions are as follows, humin is the alkaline insoluble fraction of soil

organic matter, humic acid the alkaline soluble and acid insoluble fraction and fulvic acid the water soluble fraction (Stevenson, 1982). Fulvic acid has been described by Bixby and O'Brien (1979) as being a mixture of aliphatic and aromatic macro-molecules substituted with hydrophilic carboxylic, phenolic and carbonyl acid functional groups. Humic acid, isolated from domestic sewage, was shown by Hasset *et al.* (1987) to have bactericidal activity against *Serratia marcescens* and *Staphylococcus aureus*. No direct reference to the antimicrobial activity of fulvic acid has been reported in the literature. However, water soluble extracts, possibly fulvic acid, of bark compost were shown by Kai *et al.* (1990) and Hardy and Sivasithamparan (1991) to have antifungal activity.

The uncontrolled growth of bacteria can lead to numerous problems. Disease, food spoilage, fouling of surfaces and biodeterioration of materials are but a few problems associated with uncontrolled bacterial growth (Parr, 1990; Russel and Chopra, 1990). The use of antibacterial agents is one of the most common methods used to control the detrimental activity of bacteria. There are currently many antibacterial compounds available to control bacterial growth and the search continues for more effective and environmentally safe compounds. The evaluation of a biocide or disinfectant can be divided into three phases (Reybrouck, 1982). The first phase involves preliminary screening in the laboratory, to determine whether a compound has antimicrobial activity. The second and third stages involve the determination of the use-dilution and field trial testing (Reybrouck, 1982). Combination of biocides can lead to three possible interactions, namely: (1) synergism, (2) additive/indifferent or (3) antagonism (Beale and Sutherland, 1983). Synergy is exhibited between a mixture of two biocides when the effect of both biocides is greater than that expected from the addition of the effects of the two biocides individually (Hodges and Hanlon, 1991). Synergism between bactericides

has been reported and used to enhance activity or increase the spectrum of activity of bactericides (Lehmann, 1988).

The activity of biocides are influenced by three main factors, namely: the physical environment, the specie of microorganism and the ability of the organism to degrade, inactivate or change the biocide (Russel, 1982). The factors affecting the activity of biocides during *in vitro* studies can be divided into three main areas, namely: pre-treatment, in-treatment and post-treatment factors (Russel, 1982).

Knowledge of the mechanism of action of a biocide can assist in the design of new compounds, combinations of compounds and the understanding of resistance mechanisms. Mechanism of action studies indicate that antimicrobial compounds can no longer be considered as general cell poisons (Russel and Chopra, 1990). The target regions for antibacterial agents can be classified as cell wall, cytoplasmic membrane or cytoplasm (Russel and Chopra, 1990). Two major mechanisms of resistance have been identified, namely: (1) intrinsic resistance and (2) stable or unstable acquired resistance (Heinzel, 1988; Russel and Chopra, 1990; Brözel and Cloete, 1991; Russel, 1991a; Brözel *et al.*, 1993).

Domestic wastewater may contain more than 120 types of enteric viruses (Bitton, 1980). The infective dose (ID) of many viruses is low ($ID_{50} < 10^2$) and since they are able to survive in the environment they pose a serious health risk (Lewis and Metcalf, 1988). The activated sludge process, activated carbon adsorption or sand filtration followed by chlorination is currently used to produce water free of pathogenic viruses (Cairncross and Feacham, 1983; Lewis and Metcalf, 1988). Chlorination and the activated sludge process, however, only remove *ca.* 84% and 99% of the enteric viruses present in sewage effluent, respectively (Lewis and Loutit, 1989). Complete removal of enteric viruses from

sewage can be accomplished by tertiary treatment procedures that involve coagulation, flocculation, sedimentation and disinfection (Lewis and Metcalf, 1988).

2.2 Production of Antimicrobial Compounds from Coal

Numerous products are produced by either the destructive distillation or carbonization of bituminous coal (Hugo and Russel, 1982). Coal tar, coke, aqueous liquor, light oil and gas are amongst the primary products produced (Rhodes, 1945). According to Rhodes (1945) three variables, namely: (1) kind of coal, (2) carbonization equipment and (3) carbonization temperature determine the yields and physical properties of the primary products produced.

2.2.1 Production of phenolic fractions from coal

The term tar acid is a general term which includes the phenolic constituents of coal tar (Rhodes, 1945). The most important constituent of the tar acids are phenols, cresols, high-boiling tar acids and xylenols. The preparation of phenol from coal tar involves several steps, namely: (1) distillation of the coal tar, (2) extraction of the fraction with caustic soda, (3) purification of the extract, (4) liberation of the tar acids, (5) dehydration and distillation of the tar acids (Rhodes, 1945). The cresols consist of a mixture of 2-,3- and 4-cresol. The xylenols consist of the six isomeric dimethylphenols plus ethylphenols. A commercial product, crecylic acid, is made of a mixture of cresols and xylenols. The high-boiling tar acids consist of a higher alkyl homologue of phenol, *i.e.* the diethylphenols, tetramethylphenols, methylphenols, together with ethylindanols, naphtols and methylresorcinols (Hugo and Russel, 1982).

2.2.2 Formulation of coal tar disinfectants

Most of the phenols used for the manufacture of disinfectants are obtained by the destructive distillation of coal (Hugo and Russel, 1982). Phenol, together with iodine and chlorine, are some of the earliest disinfectants used (Hugo and Russel, 1982). Coal tar disinfectants are formulated either by the emulsification or solubilisation of the coal tar fractions in a soap (Hugo and Russel, 1982). Coal tar fractions that are solubilized in a soap are referred to as black fluids. The black fluids include cresols, xylenol-rich cresylic acid and crude phenol fractions solubilized in a wide variety of soap formulations. The activity of the black fluids against bacteria, bacterial spores and mycobacteria, depends on the coal tar fraction used in the formulation (Hugo and Russel, 1982).

White fluids are produced by the emulsification of coal tar fractions in animal glue, casein or a carbohydrate extracted from the seaweed, Irish moss (Hugo and Russel, 1982). As is the case with the black fluids, the antimicrobial activity of the white fluids varies depending on the coal tar fraction emulsified. White fluids have the advantage over black fluids in that their activity is not reduced upon dilution and they are more stable in the presence of electrolytes. They are, however, less stable than black fluids during storage.

2.3 Formation and Structure of Humic Compounds

Humic compounds are defined by Atlas and Bartha (1981) as that portion of the soil organic matter that has undergone sufficient transformation to render the parent material unrecognizable.

Humic compounds are formed via several pathways (Stevenson, 1982) by the decomposition of plant or animal remains in soil, with the four main pathways illustrated

in Fig. 1.

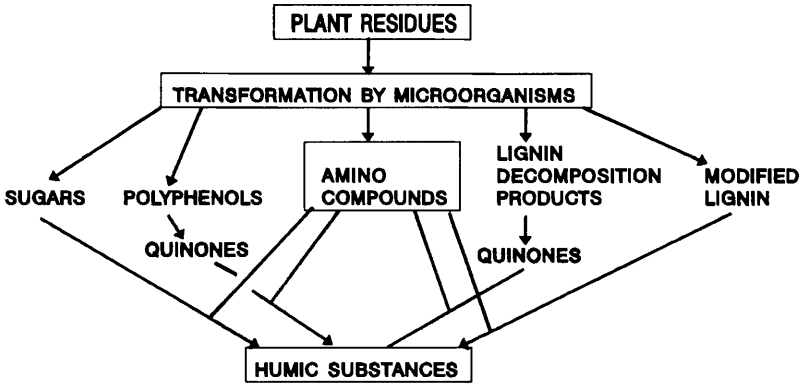


Fig. 1 Mechanisms for the formation of humic substances in soil (After Stevenson, 1982).

The genesis of humic material may proceed in two stages. The first stage involves microbial degradation of organic polymers to monomeric constituents such as phenols, quinones, amino acids and sugars. The second stage involves the polymerization of the monomers due to spontaneous chemical reactions (autoxidation) and oxidation catalysed by microbial enzymes such as laccases, polyphenoloxidases and peroxidases (Stevenson, 1982). The aromatic ring structures that serve as building blocks of the humic compounds may originate from the microbial degradation of lignin or other plant phenolic compounds. These structures may also be synthesised by various microorganisms from other carbon substrates (Stevenson, 1982).

Humic substances are divided according to their solubility characteristics into, humic acid, fulvic acid and humin. Humic acids are defined as that portion of the humic substances that can be extracted from soil by various alkaline reagents and which are precipitated by weak acid (Stevenson, 1982). Fulvic acids on the other hand are defined

as that portion of the humic substances that are alkaline soluble but not precipitated by weak acid (Stevenson, 1982).

Many structures for humic and fulvic acid have been proposed (Atlas and Bartha, 1981; Stevenson, 1982; Paul and Clarke, 1989). All the proposed structures agree that the compounds consist of a mixture of heterogeneous compounds for which no single structural formula will suffice (Stevenson, 1982). Paul and Clarke (1989) proposed that humic acids are composed of high molecular weight materials containing aromatic rings and nitrogen in cyclic forms and in peptide chains. The main differences between humic acids and fulvic acids are that fulvic acids have higher oxygen but lower carbon content than humic acids, and they contain considerably more functional groups of an acidic nature, particularly COOH (Stevenson, 1982). The oxygen content of fulvic acid can be accounted for by the known functional groups (*i.e.* COOH, OH, C=O). A high portion of the oxygen in humic acids, however, seems to occur as a structural component of the core of the molecule. The hypothetical structure of humic acid proposed by Stevenson (1982) is shown in Fig. 2.

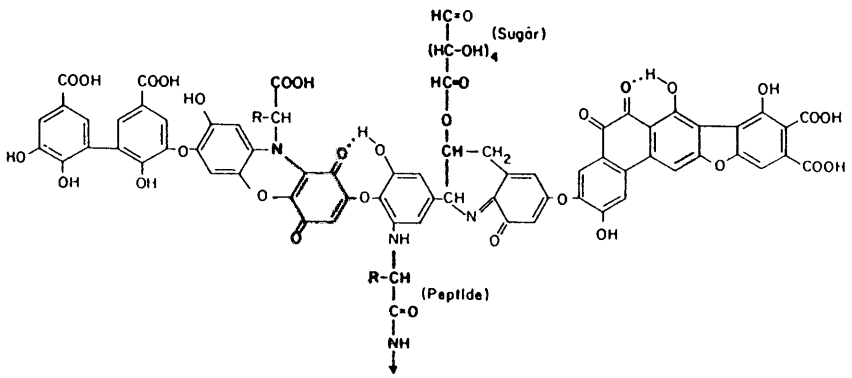


Fig. 2 Hypothetical structure of humic acid (After Stevenson, 1982).

The structure for fulvic acid (Fig. 3) proposed by Schnitzer and Khan (1972) consists of phenolic and benzenecarboxylic acids held together by hydrogen bonds to form a polymeric structure of considerable stability.

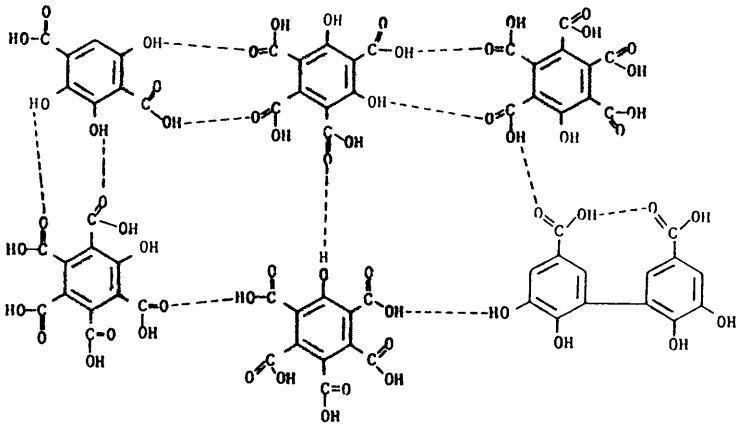


Fig. 3 Proposed structure of fulvic acid (After Schnitzer and Khan, 1972).

A further distinguishing characteristic of the fulvic acid molecules are the large voids in the molecule which are capable of entrapping low-molecular-weight organic or inorganic compounds, such as pesticides and metal ions, provided the charges are complementary (Paul and Clark, 1989).

2.3.1 Antimicrobial activity of humic compounds

Since no exact structure can be ascribed to either fulvic or humic acid the mechanism of their inhibitory action could be due to a number of factors. The relatively large molecular weight of the humic and fulvic acids could prevent their movement through the cell wall and cytoplasmic membrane of microorganisms. Therefore inhibition of internal cell functions seems unlikely. However, the presence of many carboxyl or free and bound phenolic hydroxyl groups would give the compounds similar characteristics to many other

organic acids. Their mode of inhibition could thus be similar. The compounds could line up on the surface of the cell blocking any transfer of nutrients or ions across the cell wall (Dugan and Lundgren, 1965). Disruption of the cell wall could result due to the reaction between the organic acids and the cations which contribute to the structural integrity of the cell envelope of microorganisms. Disruption of the permeability barriers could result in blocking either of nutrient transport through the envelope or electron transport in the membrane (Tuttle *et al.*, 1977)

2.4 Evaluation of the Antimicrobial Activity of Biocides

Many tests, to evaluate the activity of biocides, have been described and can be subdivided in many different ways as illustrated in Table 1.

Tests determining the activity of biocides can be divided into three categories, namely: *in vitro* tests, practical tests and in-use tests (Spooner and Sykes, 1975; Russel, 1982; Eigner, 1988).

2.4.1 *In vitro* tests

In vitro tests, can be classified as suspension tests, capacity tests and carrier tests (Russel, 1982).

2.4.1.1 Suspension tests

Suspension tests determine either the concentration of biocide that inhibits the growth of microorganisms suspended in a medium (minimum inhibitory concentration, MIC) or kills all the microorganisms suspended in a diluent (Russel, 1982).

The MIC is determined by mixing the biocide with a nutrient broth in decreasing concentrations as illustrated in Fig. 5. The tubes are then inoculated with a bacterial culture, and after a suitable period of incubation the lowest concentration which inhibits growth is the MIC concentration. According to Russel (1982) these tests are rarely used in the evaluation of disinfectants, since disinfectants are required to kill microorganisms and not inhibit growth. They are however used in the Kelsey-Sykes test (Kelsey and Maurer, 1974) to select the microorganism most resistant to a biocide. The concentration of biocide that kills all the microorganisms suspended in a diluent, can be determined either qualitatively or quantitatively.

Table 1 Classification of antimicrobial evaluation tests (After Russel, 1982)

A. Classification according to test organism

Antibacterial activity: bactericidal tests, tuberculocidal and sporicidal tests

Antifungal activity tests

Virucidal activity tests

Algicidal activity tests

B. Classification according to type of action

-static tests - microorganisms are inhibited but not killed.

-cidal test - microorganisms are killed upon exposure to the compound.

C. Classification according to test structure

In vitro tests:

suspension tests

capacity tests

carrier tests

Practical tests

In-use tests

D. Classification according to aim of test

First stage testing:

Tests determining whether a compound possesses antibacterial activity

Tests determining the relationship between exposure periods and dilution of the compound

Tests determining the effect of environmental factors on the bactericidal activity of the compound

Second stage tests:

Tests to determine the use-dilution of a compound for a specific application

Third stage testing:

Tests to determine the activity of the compound in the field

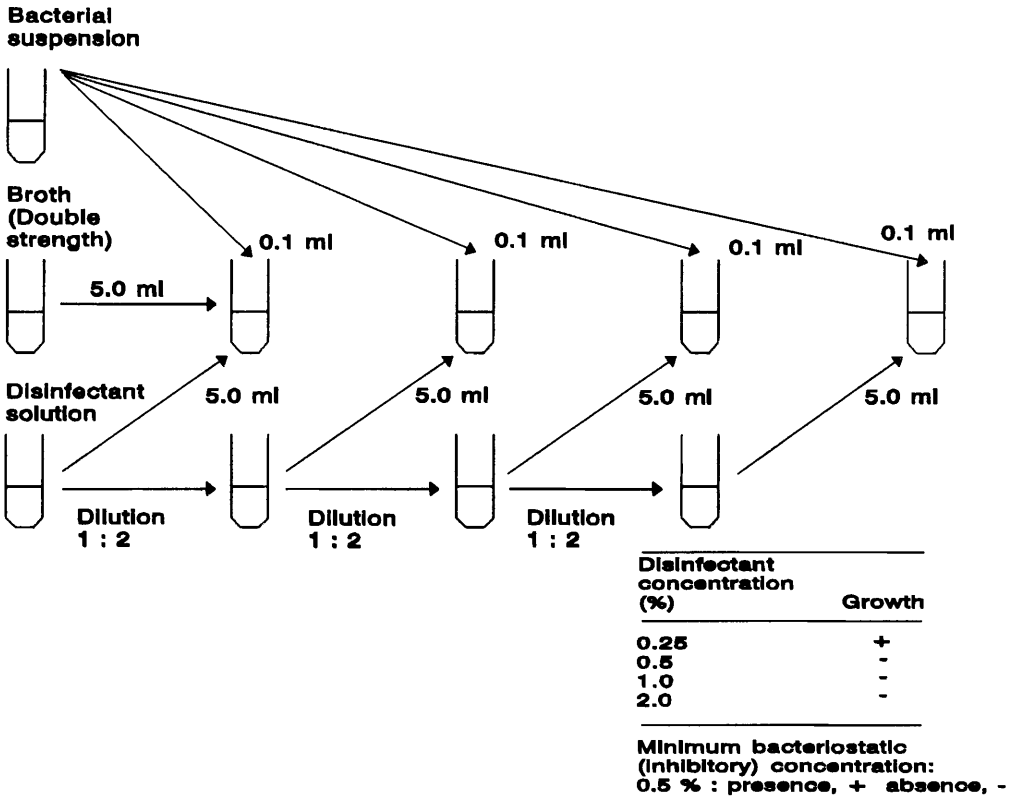
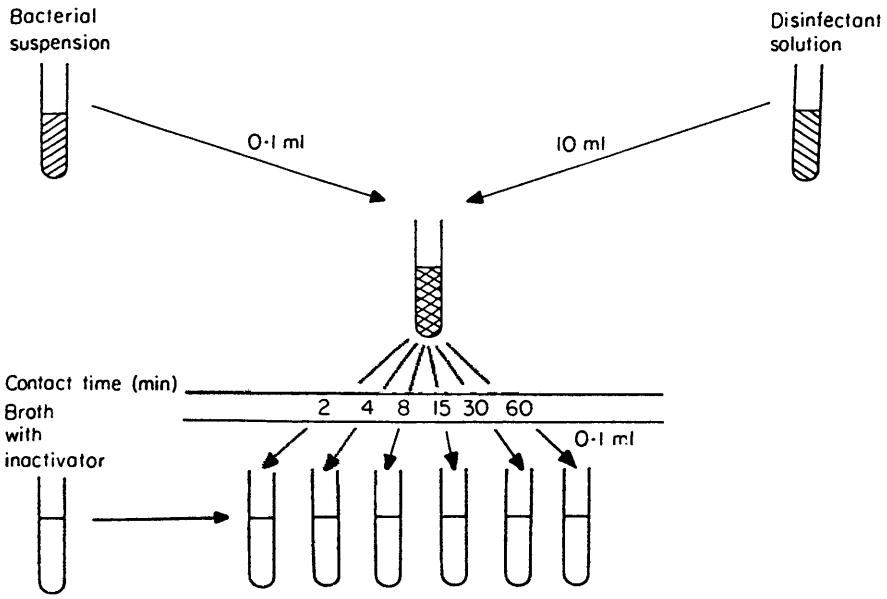


Fig. 4 Diagram of test to determine MIC of biocide (After Reybrouck, 1982).

Qualitative suspension tests (Fig. 5) determine the concentration of biocide required to kill all the cells, in suspension, by the presence or absence of growth in a recovery medium. The main disadvantage of the qualitative suspension test as stated by Russel (1982), is that survival of a single cell would give the same result as the survival of the whole cell inoculum. In the critical concentration ranges (concentrations close to the effective concentration) both negative and positive results can be recorded. The latter problem could be overcome by using multiple subcultures, since if only small numbers of cells survive, not all the subcultures will show positive growth.



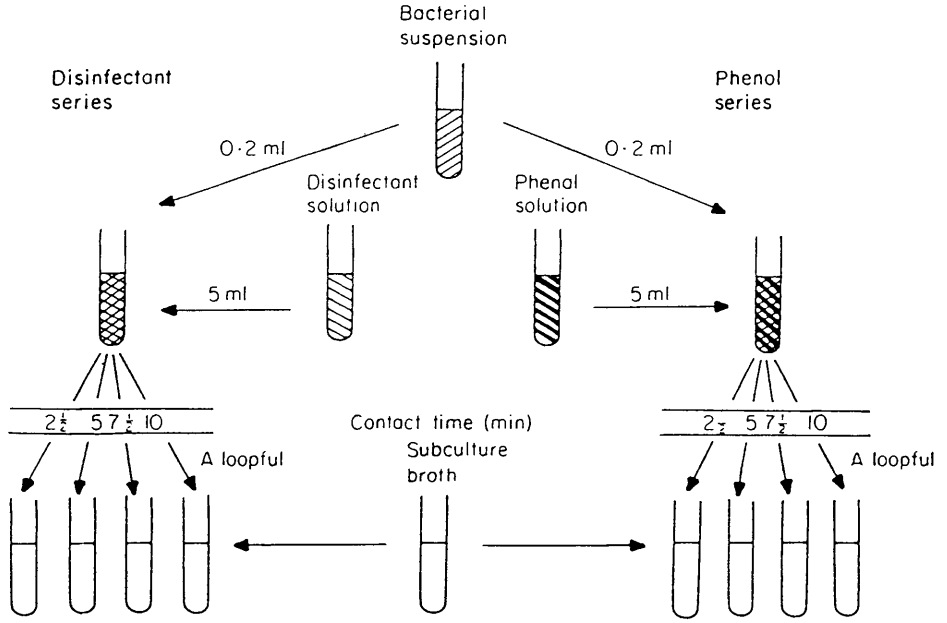
Disinfectant concentration (%)	Growth in subculture after					
	2 min	4 min	8 min	15 min	30 min	60 min
1.0	+	−	−	−	−	−
0.5	+	+	−	−	−	−
0.25	+	+	−	+	+	−

Presence, +; absence, −.

Fig. 5 Diagram of qualitative suspension test (After Reybrouck, 1982).

The proportion of negative cultures would therefore give a semi-quantitative indication of the activity of the biocide (Russel, 1982). The reproducibility of the qualitative suspension test can be increased by the comparison of a biocide to another well known biocide, *e.g.* phenol. This principle is used in the Rideal-Walker phenol coefficient test (Russel, 1982). Comparison with a well known biocide would eliminate all unforeseen factors that could influence the resistance of organisms to a particular biocide.

The phenol coefficient test (Fig. 6) is limited since like has to be compared with like (*i.e.* phenolic biocides with phenol) to obtain any degree of accuracy (Russel, 1982).



Disinfectant concentration	Growth in subculture after			
	2½ min	5 min	7½ min	10 min
1/1000	−	−	−	−
1/1100	+	−	−	−
1/1200	+	+	−	−
1/1300	+	+	+	−
Phenol control				
1/100	+	+	−	−

Phenol coefficient $= \frac{1200}{100} = 12.0.$

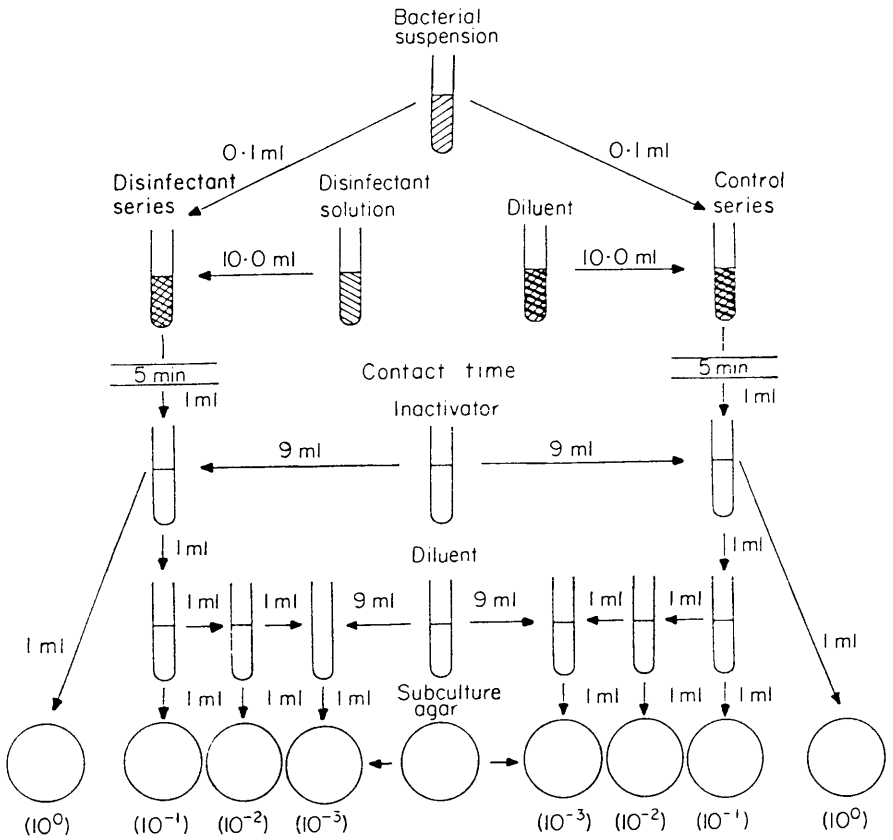
Presence, +: absence, −.

Fig. 6 Diagram of phenol coefficient test (After Reybrouck, 1982).

Quantitative suspension tests (Fig. 7) are a more accurate and reliable test for the determination of the antimicrobial activity of a biocide (Russel, 1982). These tests determine the concentration of a biocide required to kill a cell suspension by determining the amount of cells surviving after exposure (Russel, 1982). The decimal reduction rate, or germicidal effect (GE) of the biocide can be calculated using the following formula:

$$GE = \text{Log } N_c - \text{Log } N_o$$

where N_c is the number of colony forming units in the control and N_o the number of colony forming units in the exposed suspension, after a given period of exposure (Russel, 1982).



Dilution of the subculture	Number of colony-forming units (cfu)			
	in control series		in disinfectant series	
	number	log	number	log
10^{0}	tntc*	—	tntc	—
10^{-1}	tntc	—	88	1.94
10^{-2}	tntc	—	6	0.78
10^{-3}	tntc	—	0	—
10^{-4}	110	2.04	0	—

*Too numerous to count
Germicidal effect = $\log N_C - \log N_D$
= (4 + 2.04) − (1 + 1.94)
= 3.10 (after 5 min)
where N_C is number of cfu in control series, and N_D is number in disinfectant series.

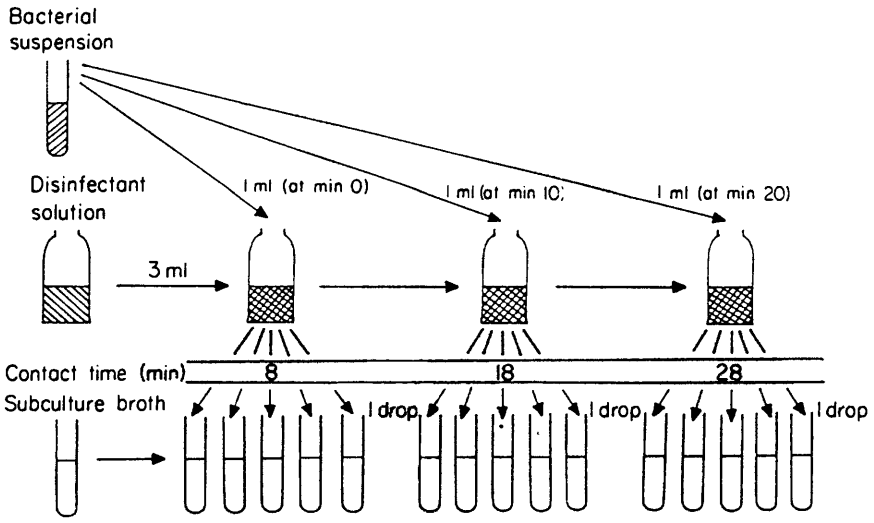
Fig. 7 Diagram of quantitative suspension test (After Reybrouck, 1982).

Numerous versions of the quantitative suspension test are used for the evaluation of biocides either routinely or for research purposes (Russel, 1982). One of the most widely used quantitative suspension tests for the evaluation of disinfectants is the 5-5-5 test, which uses 5 test organisms, an exposure time of 5 min and a 5 log reduction cell count is needed for the biocide to be considered effective (Croshaw, 1981).

2.4.1.2 Capacity tests

Capacity tests simulate the practical aspects of housekeeping and instrument disinfection by determining the ability of a biocide to retain activity in the presence of increasing loads of microorganisms and organic matter (Russel, 1982). The most widely used capacity test used is the modified Kelsey-Sykes test as described by Kelsey and Maurer

(1974) and illustrated in Fig. 8.



Disinfectant concentration (%)	Number of subculture broths (out of 5) showing growth after		
	8 min	18 min	28 min
2.0	0	0	3
1.0	0	2	5
0.5	1	4	5
0.25	5	5	5

Use-dilution, 1.0%.

Fig. 8 Diagram of modified Kelsey-Sykes capacity test (After Kelsey and Maurer, 1974).

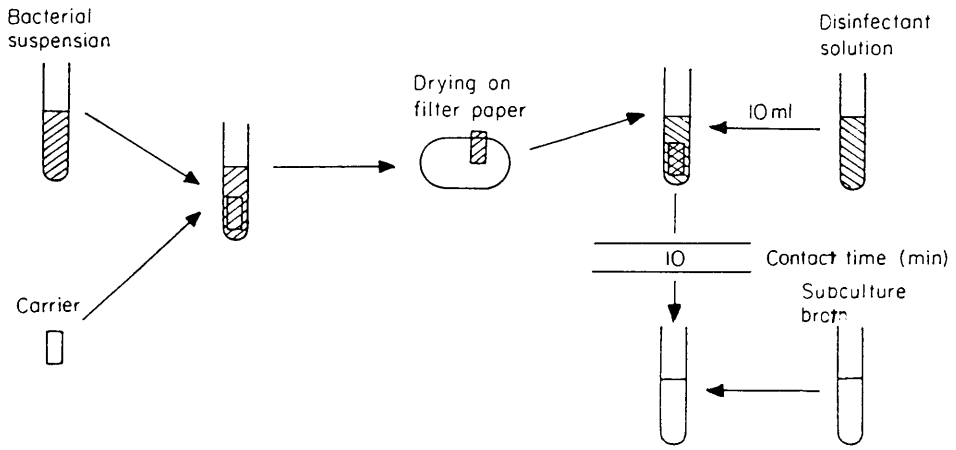
The Kelsey-Sykes test is more stringent than the previously described suspension tests, since it not only takes into account the effect of increased loading, with microorganisms and organic matter, but includes the effect of water hardness as well. This test is normally used to determine the use-dilution of a biocide and forms part of the second stage of evaluation of a biocide.

2.4.1.3 Carrier tests

Carrier tests (Fig. 9) are used as practical tests to evaluate biocides for use as surface disinfectants. These test are regarded as *in-vitro* tests since the carrier surfaces are standardised into non-realistic objects (*e.g.* porcelain cylinder or metal plate) and the conclusions are applied to other fields of application (Russel, 1982).

The accuracy of the tests are increased by including at least 10 carrier pieces per

biocide dilution. This eliminates, as in the case for the qualitative suspension tests, the negative and positive cultures that would be obtained at the critical concentration, since the proportion of negative tubes would give a semi-quantitative indication of the activity of the biocide. This type of test is normally used to determine the use-dilution of a biocide and forms part of the second stage evaluation of a biocide (Table 1).



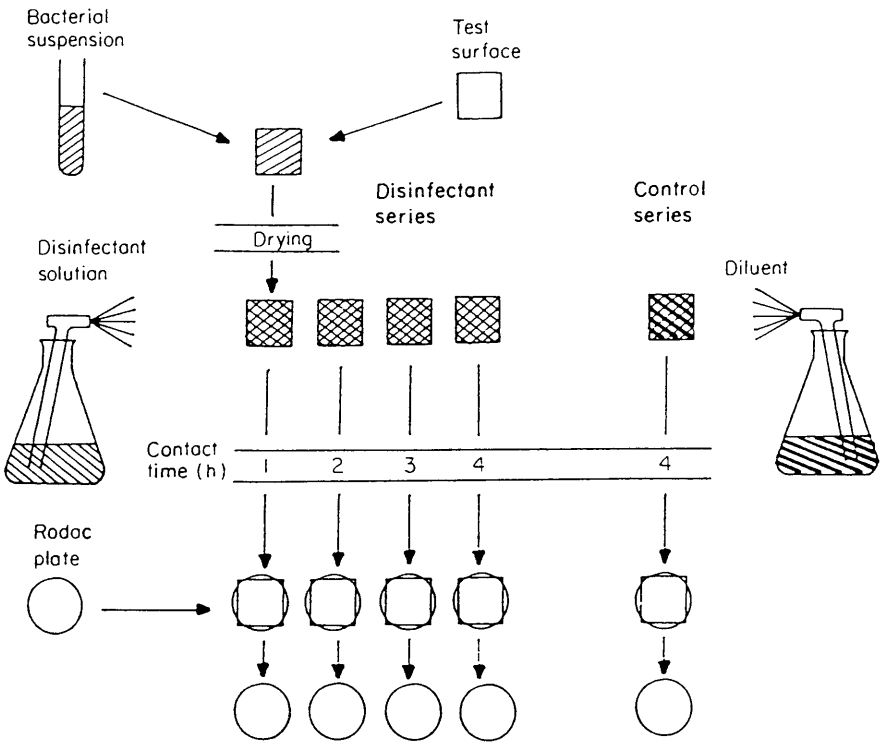
Disinfectant concentration (%)	Number of subculture broths (out of 10) showing growth
0.25	10
0.5	2
1.0	0
2.0	0

Use-dilution: 1.0%.

Fig. 9 Diagram of carrier test (After Reybrouck, 1982).

2.4.2 Practical tests

Practical tests are used to determine whether the use-dilution calculated, in the *in-vitro* test (i.e. Kelsey-Sykes test), is still adequate under conditions in which the biocide would be used practically (Russel, 1982). These tests are in most cases not very reproducible since the conditions that affect the activity of the biocide and the resistance of the test organism cannot be standardised (Russel, 1982). In spite of these constraints many types of practical tests have been described and are currently in use in many countries (Russel, 1982). The various tests differ depending on the application. An example of a test for the evaluation of a surface disinfectant is shown in Fig. 10.



*Too numerous to count.

Germicidal effect = $\log N_C - \log N_D$
= (log 10^5 + log 250) − log 350
= (5 + 2.40) − 2.54
= 4.86 (after 2 h) [or 7.40 − 1.30
= 6.10 (after 3 h)].

	Number of colony-forming units on Rodac plate after			
	1 h	2 h	3 h	4 h
disinfectant	tntc*	350	20	0
control series (dilution 10^{-5})				250

Fig. 10 Diagram of a practical test for the evaluation of surface disinfectants (After Reybrouck, 1982).

2.4.3 In-use tests

These tests are part of the third and final stage of evaluation of a biocide under field conditions. The principles that apply to the formulation of in-use tests are the following: (1) the biocide must be added to the environment at the use dilution determined in stage two and (2) the effect of the biocide on the microbial population must be monitored to determine whether any reduction in the population occurs.

2.4.4 Determination of combined biocide action

Biocides in combination lead to one of three possible interactions, namely: (1) synergistic, (2) additive/indifferent or (3) antagonistic (Beale and Sutherland, 1983). Synergy is

exhibited between a mixture of two biocides when the effect of both biocides is greater than that expected from the addition of the effects of the two biocides individually (Hodges and Hanlon, 1991). Synergism between biocides has been reported and used to enhance activity or increase the spectrum of activity of biocides (Lehmann, 1988). Denyer (1990) described two types of synergism, namely biochemical and permeabilisation synergy (Table 2). Biochemical synergism involves the selection of biocides that minimise cellular recovery and maximise spectrum of damage by exploiting the interrelationship between the biochemical and biophysical functions of the cell.

Permeabilisation synergism involves the selection of biocides which increase cellular permeability together with intracellularly-acting biocides or to couple intracellularly-acting biocides to actively transported substrates in order to exploit the natural concentrating potential of the cell transport system.

Combined action can be determined using various methods either qualitatively or quantitatively (Hodges and Hanlon, 1991). Qualitative methods have the advantage that they are easy to perform and less time consuming than quantitative methods. Quantitative methods are more accurate since they quantify the degree of synergy, unlike qualitative methods which simply demonstrate whether the phenomenon occurs.

The concept of fractional inhibitory concentration (FIC) is most commonly used to quantify the degree of synergy (Hodges and Hanlon, 1991). The FIC for each compound, in a combination, is calculated as the ratio of the MIC for the substance alone and in combination as follows;

$$\text{MIC } A_1/\text{MIC } A_2 = \text{FIC A}$$

where, A_1 is the MIC of the compound in combination and A_2 the MIC of the compound alone. The sum of the FIC values for each compound (FIC index) is indicative of the nature of the interaction (Beale and Sutherland, 1983).

$$\text{FIC index} = \text{FIC A} + \text{FIC B}$$

The following criteria have been proposed by Hodges and Hanlon (1991) to interpret the nature of the interaction based on the FIC index. When the FIC index is ≤ 0.5, the combination is considered synergistic, although a value of 0.7 was proposed by Kerry *et al.* (1975). A combination is considered antagonistic when the FIC index is ≥ 2.0 and

additive if the FIC index approximates 1.0.

Table 2 Known synergistic combinations of biocides and proposed mechanism of synergy (modified from Denyer, 1990)

Synergistic combination	Proposed mechanism
Phenylmercuric acetate and 3-cresol or benzalkonium chloride	Permeabilisation
Lipophilic weak acids and fatty alcohols	Permeabilisation and biochemical enhancement
Chlorhexidine and Bronopol	Permeabilisation
Acetate and lipophilic weak acids	Biochemical enhancement
N-chloramines and diazolidinyl urea	Permeabilisation and biochemical enhancement
Chlorocresol and 2-phenylethanol	Biochemical enhancement

2.4.4.1 Agar diffusion tests

This test procedure is based on the principle that biocides are made to diffuse through an inoculated agar to give a continuous spectrum of concentration ratios (Beale and Sutherland, 1983). The nature of the interaction is determined by the interpretation of the pattern of resulting growth as illustrated in Fig. 11.

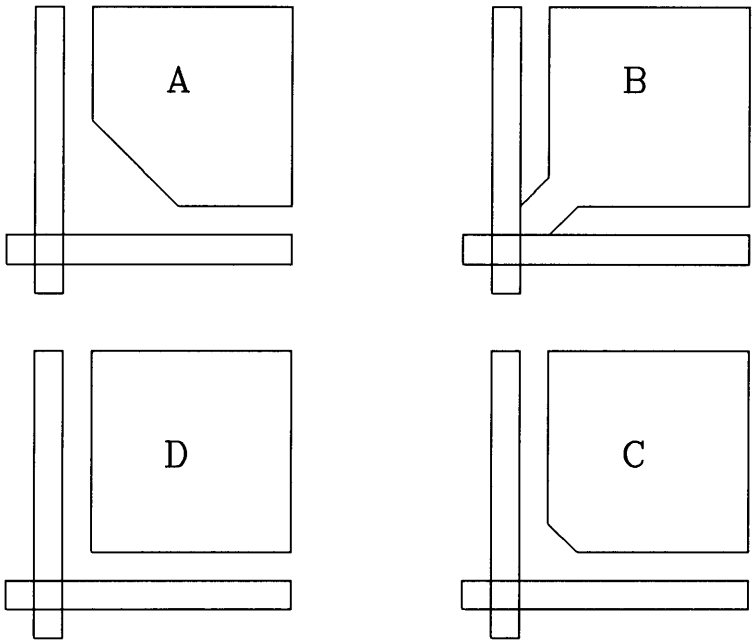


Fig. 11 Patterns of growth inhibition by agar diffusion method, (a) synergism, (b) antagonism, (c) indifference and (d) additive (After Hodges and Hanlon, 1991).

A major limitation of this technique is that the concentration of biocides which exhibit synergy in the agar are not known (Hodges and Hanlon, 1991). Due to this limitation this test is most commonly employed as an initial screen to determine whether synergy exists between biocides (Hodges and Hanlon, 1991).

2.4.4.2 Spiral plating

This is an improved agar diffusion test, since it permits the concentration in the synergistic combination to be calculated. Briefly, this technique involves the application of biocide solutions in a spiral pattern on three seeded petri dishes. Two of the petri dishes are used to determine the inhibitory concentration of each biocide. The remaining petri dish is seeded with the two biocides, in combination, in proportion to their

inhibitory concentrations (Hodges and Hanlon, 1991).

2.4.4.3 Chessboard method

This method is most commonly used to quantify combined action (Beale and Sutherland, 1983). Organisms are exposed to between six and ten doubling-dilutions of each biocide from concentrations slightly higher than the MIC values. The results may be presented by either plotting the MIC or FIC values in an isobologram, with the shape of the plots indicating the nature of the interaction (Fig. 12). Alternatively, the FIC values can be used to calculate the FIC index to determine the nature of the interaction. This method has the advantage that is more sensitive than the agar diffusion test (Hodges and Hanlon, 1991).

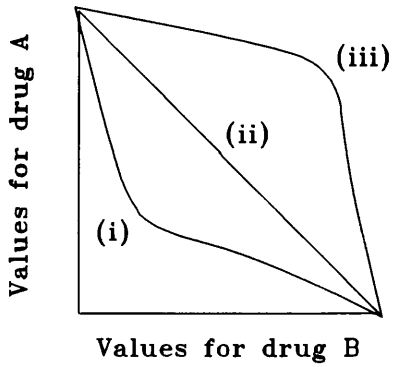


Fig. 12 Isobologram representing the three possible interactions (i) synergism, (ii) indifference and (iii) antagonism (After Beale and Sutherland, 1983).

2.4.4.5 Growth inhibition tests

Growth inhibition by a combination of biocides can be determined using viable count tests and turbimetric studies (Beale and Sutherland, 1983; Lehmann, 1988; Hodges and Hanlon, 1991).

2.4.4.5.1 Viable count tests

These tests determine the interaction between biocides quantitatively either by analyses of cell number or end-point analyses (Lehmann, 1988). Analyses of cell number, involves the determination of the viable cell count over a period of time, in tubes containing selected concentration of each biocide alone and in combination. The resulting kill-curves (Fig. 13) are used to determine the nature of the interaction. According to Beale and Sutherland (1983) this is the best method available for the assessment of combined action. The nature of the interaction as described by the viable count tests has been defined by Lehmann (1988) as follows:

- **Synergy**: The combination of the two biocides produces a greater increase in the rate of kill than that produced by twice the concentration of either agent alone.
- **Addition**: The rate of killing of the agents in combination is approximately that expected from simple algebraic summation of a single agent alone.
- **Antagonism**: The rate of killing of the combination is lower than that of one or both of its components.

End-point analysis tests determine the time needed for complete killing of cells exposed to a combination of biocides, since samples are transferred into liquid medium after exposure to a biocide combination. The nature of the interaction has been defined by Lehmann (1988) as follows:

- **Synergism**: The killing time of the combination is shorter by either one-half or less, than that needed by twice the concentration of either agent alone.
- **Addition:** The killing time of the combination is nearly the same as that needed by twice the concentration of either agent alone, *i.e.* a mean value of the two killing

times.

- **Antagonism:** The killing time of the combination is longer than that needed by twice the concentration of either agent alone.

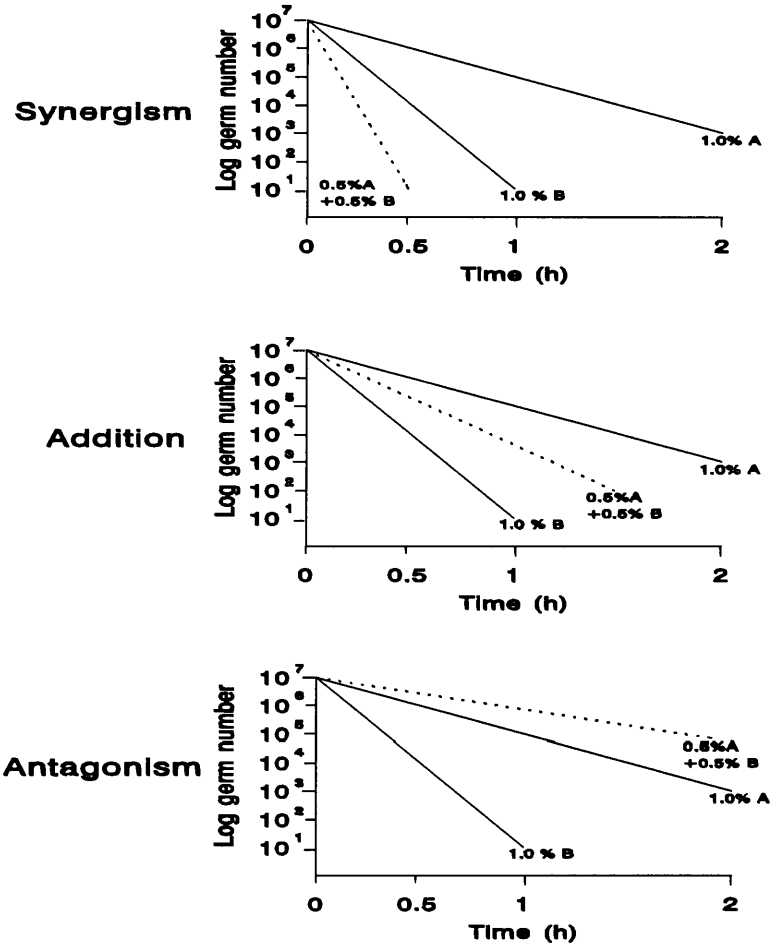


Fig. 13 Kill curves resulting from the interaction of two biocides in combination (After Lehmann, 1988).

2.4.4.5.2 Turbimetric tests

Turbimetric methods assess the interaction of biocide combinations and cells by continuous monitoring of growth curves and are ideal if the interaction leads to cell lysis

(Beale and Sutherland, 1983). These methods also provide information, on the kinetics of the interaction, which are not obtained with the agar diffusion or chessboard tests (Hodges and Hanlon, 1991). Synergy can be assessed on the basis of the minimum concentration required to produce a deviation in the growth curve (Fig. 14) from that obtained in the absence of the biocide combination and either biocide alone (Beale and Sutherland, 1983).

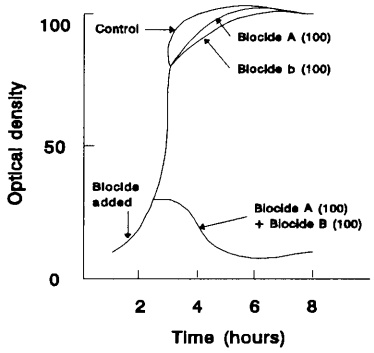


Fig. 14 Prediction of synergy based on turbimetric measurement of growth (After Beale and Sutherland, 1983).

2.5 Determination of the Antimicrobial Activity of Biocides

The first step in the evaluation of an antimicrobial compound would be to determine the bactericidal activity of the compound *in vitro*, since Russel (1982) stated that for a chemical to be regarded as an effective biocide it has to be effective against vegetative bacteria. Thereafter, the activity of the compound against bacterial spores, fungi and even algae can be determined depending on the area of application.

2.5.1 Determination of the bactericidal activity

There are numerous tests to determine the bactericidal activity of a biocide (Russel,

1982). The suspension, capacity and carrier tests described in the previous section are those tests that are mostly used to determine the bactericidal activity of a biocide (Russel, 1982). Thereafter the use-dilution can be determined using a number of tests, as described in the previous section, depending on the eventual application of the biocide.

2.5.2 Determination of the sporicidal activity

The determination of the sporicidal activity of a biocide is very important since bacterial spores are more resistant to the action of biocides than any other living organism (Russel, 1982). The extensive use of sterile objects in the medical, pharmaceutical and food industries dictates that a sporicidal agent would have to be a sterilant (kill all bacterial spores present). The tests used to determine the sporicidal activity are therefore stringent. Numerous tests have been described to determine the sporicidal activity of biocides (Russel, 1982). These are mostly modifications of suspension or carrier tests, with the carrier tests being the most effective in determining the sporicidal activity of a biocide (Russel, 1982). These tests make use of the most resistant spores and optimum resuscitation conditions, after exposure to the biocide (Russel, 1982).

2.5.3 Determination of fungicidal activity

The procedure used to determine the fungicidal activity of a biocide would depend on the application of the biocide. For the evaluation of a biocide as a surface, textile or instrument disinfectant, modified bacterial suspension or carrier tests are used (Russel, 1982). Other fungicidal tests have been published and different varieties of testing methods are followed in the preservation of wood (Hilditch, 1992) and paints (Springle and Briggs, 1992).

2.5.4 Determination of virucidal activity

Viruses are obligate intracellular parasites, which can survive in the environment, but not multiply. Tissue culture, used to enumerate viruses, requires appropriate facilities and skilled personnel (Wright, 1970). This presents a problem since biocides are toxic to living cells, and thus to cells in tissue culture. Due to this limitation not many tests have been described to determine the virucidal activity of a biocide. Those tests that have been described are based mostly on *in vitro* suspension test techniques (Wright, 1970).

The abovementioned limitations can be overcome with the use of bacteriophages to determine the virucidal activity of biocides. Bacteriophages could be used since they are commonly used as indicators of the presence of enteric viruses in the environment (Kfir, 1989) or as models to determine the effect of various factors on the survival or removal of viruses from the environment (Bitton, 1980). Phages have the added advantage that they can be enumerated more rapidly and with the use of less specialised equipment (Kfir, 1989).

2.5.5 Determination of algicidal activity

Numerous methods are available for the evaluation of algicides (Grant, 1982; Morton, 1986). In most cases the criterion for assessing the effectivity of a product is the persistence or otherwise of pigmentation which is used to determine the MIC of a biocide. In most cases no quantitative assessment is made of the degree of growth inhibition, thus reducing the results to a simple positive or negative system for recording results. To obtain a more quantitative determination of algicidal activity chlorophyll *a* concentration can be used. Chlorophyll *a* concentrations are used to determine algal biomass in polluted water (Pieterse and Toerien, 1978), and have been shown by Goysich and McCoy (1989)

to give an accurate indication of algal growth on surfaces in water cooling towers treated with algicides. Therefore by measuring the reduction in chlorophyll *a* concentration over time, a more quantitative measurement of algicidal activity can be obtained (Bosch *et al.*, 1993).

2.6 Factors Influencing the Efficacy of Biocides

The activity of biocides is influenced by a wide range of factors. Russel (1982) stated that the physical environment, the specie of microorganism and the ability of the organism to degrade, inactivate or change the biocide are the three main factors affecting the efficacy of a biocide. The factors affecting the activity of biocides during *in vitro* studies can be divided into three main areas, namely: pre-treatment, in-treatment and post-treatment factors (Russel, 1982).

2.6.1 Pre-treatment factors influencing the efficacy of biocides

Pre-treatment factors can be described as all those factors that influence the physiological state of the microorganism prior to exposure to an antimicrobial compound. These factors include growth conditions, physiological condition of organism and pre-treatment with chemicals (Russel, 1982).

2.6.1.1 Growth conditions

Cells are usually grown in batch culture, and in certain cases in continuous culture, prior to exposure to an antimicrobial compound. The main criticism against the use of batch grown cultures for the evaluation of antimicrobial compounds is that cells of different physiological ages will be present. This is important, since it is well documented that

actively growing cells are normally more sensitive to antimicrobial compounds than cells in the stationary phase of growth (Russel and Chopra, 1990). Cells grown in continuous culture on the other hand are all of the same physiological age and will all respond similarly to the antimicrobial compound.

Regardless of whether the cells are grown in batch or continuous culture the medium composition, medium pH and temperature of incubation, also influence the sensitivity of the cells to an antimicrobial compound. The composition of the medium can influence the composition of the cell wall of the microorganism, and since the cell wall is the initial barrier to an antimicrobial compound it influences the activity of the compound against the microorganism. Gram positive cells grown in medium containing glycerol have an increased cell wall lipid content, which increases their resistance to phenols and esters of p-hyroxybenzoic acid (Russel, 1982). Cells of *Escherichia coli* grown in a medium containing L-alanine, have a structural deformity which renders the cell wall more permeable, and thus more sensitive to antibacterial agents (Russel and Chopra, 1990). *Pseudomonas aeruginosa* grown in broth containing Tween 80 was more sensitive to certain antimicrobial compounds *e.g.* QAC (Russel and Chopra, 1990).

The pH of the medium also influences the response of cells to antibacterial agents. This is due mainly to a change in the cell wall composition or habituation to growth at a low pH which makes cells more resistant (Russel, 1982; Goodson and Rowbury, 1989a,b). Goodson and Rowbury (1989b) reported that *E. coli* exposed to a sub-lethal pH (pH 5.0) was more resistant to organic acids than cells not exposed to the sub-lethal pH. This phenomenon was also reported by Foster (1992) for *Salmonella typhimurium* cells exposed to sub-lethal pH (pH 5.8) .

Due to the differences in cell wall composition between psycrophilic, mesophilic

and thermophilic bacteria it can be said that the temperature of incubation would influence the cell wall composition of microorganisms particularly the phospholipid composition, and thus their response to antibacterial agents (Russel, 1982). This would be true in cases where cells of the same species are incubated at different temperatures and their response to antibacterial agents determined.

2.6.1.2 Condition of microorganism

Russel (1982) stated that the state of hydration of the microorganisms influences their response to antimicrobial compounds. Cells that were dried to a relative humidity of 1% were more resistant to ethylene oxide and glutaraldehyde than unhydrated cells. An important factor that influences the resistance of dehydrated cells to antimicrobial compounds is the medium from which the cells are dried (Russel, 1982). The richer the medium the greater the protective effect. This is possibly due to entrapment of the cells within crystals or adsorption of organic matter present in the rich broths (Russel, 1982).

2.6.1.3 Influence of pre-treatment with chemicals on the efficacy of biocides

Pre-treatment involves the exposure of cells to agents that alter cell wall permeability, induce mutations or induce the formation of osmotically fragile forms of the microorganisms, which alter the response of the cells to antimicrobial compounds. Compounds that are able to alter the cell wall permeability include Tween 80, benzylakonium chloride and Ethylenediaminetetraacetic acid (EDTA) , all of which increase the sensitivity of the cells to biocides (Russel, 1982).

Mutation of microorganisms can be induced by exposure to various mutating agents *e.g.* acridine orange. In most cases the mutations change the cell wall composition,

thus increasing the sensitivity of the mutant to a biocide (Russel, 1982).

Osmotically fragile forms of bacteria *i.e.* spheroplasts, protoplasts and mureinoplasts can be induced by treating bacterial cells with agents that remove the outer cell membrane (spheroplasts) or the lipopolysaccharide and lipoprotein layers in the case of gram positive bacteria (mureinoplasts) or the complete cell wall (protoplasts). Treatment of these structures may be of value in assessing the influence of the various layers of the cell wall in the penetration of antimicrobial compounds (Russel, 1982).

2.6.2 In-treatment factors affecting the efficacy of biocides

Various factors influence the efficacy of biocides during exposure. These include concentration of the agent, number and type of organism, temperature, pH and the presence of interfering matter (Russel, 1982).

2.6.2.1 Concentration of biocide

The concentration of biocide used determines the time necessary to achieve a given degree of kill on a bacterial suspension. Russel (1982) stated that knowledge of the effect of concentration on the antimicrobial activity is essential in the following situations: (1) evaluation of disinfectants, (2) sterility testing of pharmaceutical and medical products and (3) ensuring adequate levels of preservatives in pharmaceutical products. Therefore, depending on the criteria used, *i.e.* time or concentration, to achieve a required degree of kill, the concentration of biocide may be varied. The correlation between time and concentration is referred to as the concentration exponent (*n*) (Russel, 1982). The concentration exponent can be determined by measuring the time taken to achieve a comparable degree of kill of a bacterial suspension at two different concentrations of

biocide (Russel, 1982). The concentration exponent can then be expressed as follows:

$$n = (\log t_2 - \log t_1)/(\log C_2 - \log C_1)$$

Where C_1 and C_2 represent the two concentrations and t_1 and t_2 represent the respective times to achieve an equivalent degree of kill. From the equation it can be seen that *n* is a measure of the effect of changes in biocide concentration on cell death rate. Therefore, compounds with a high *n* value will be less effective upon dilution that those with a low *n* value (Russel, 1982).

2.6.2.2 Number of microorganisms

This factor follows on from the concentration exponent, since *n* is a measure of the rate of action of an antimicrobial compound. Therefore, the higher the initial concentration of microorganisms the longer the time required to achieve the required degree of kill.

2.6.2.3 Temperature

The activity of an antimicrobial compound is usually increased by an increase in temperature. The phenomenon can be expressed as the temperature coefficient (Θ) in the following formulae:

$$\Theta\ (T_2 - T_1) = k_2/k_1 \quad \text{or} \quad \Theta\ (T_2 - T_1) = t_2/t_1$$

where k_2 and k_1 are the rate constants at temperatures T_2 and T_1 respectively, or t_2 and t_1 are the respective times to bring about a complete kill at T_1 and T_2. The temperature coefficient refers to the effect of a rise in temperature of 1 °C on the antimicrobial activity of a compound, and is usually expressed as the Θ_{10} value which is the change in activity per 10 °C rise in temperature (Russel, 1982).

2.6.2.4 Environmental pH

Environmental pH can either increase or decrease the activity of biocides (Russel, 1982) The activity of biocides can be influenced as follows:

- **Changes may occur in the molecule**. Substances such as phenol and various organic acids are effective only in the undissociated form. Thus as the pH rises the molecules become more dissociated, consequently reducing their antimicrobial effectivity.
- **Changes may occur in the cell surface.** As the pH of the environment increases the number of negatively charged sites in the cell surface increases, with the result that positively charged molecules are attached more strongly to the cell surface.
- **Partitioning of a compound**. The partitioning of a compound, present in an antimicrobial formulation, between the product in which it is present and the microbial cell may be influenced by pH.

2.6.2.5 Interfering substances

Interfering substances can take the form of organic matter, surface-active agents or metal ions (Russel, 1982). Organic matter may occur in various forms *e.g.* serum, blood, faecal material. The interference by organic matter normally takes the form of a reaction between the antimicrobial compound and the organic matter. An alternative would be that the organic matter protects the microorganisms from attack (Russel, 1982). The degree of interference of organic matter with a biocide is related, in most cases, to the chemical reactivity of the compound, *e.g.* the antimicrobial activity of oxidizing biocides are affected more by the presence of organic matter than non-oxidizing biocides (Russel, 1982; Russel and Chopra, 1990). Macromolecular polymers such as sodium dodecyl

sulphate and non-ionic detergents are surface active agents that greatly reduce the activity of certain antimicrobial compounds (*e.g.* methyl and propyl *p*-hydroxybenzoates) by reacting with these compounds.

The presence of metal ions can either reduce, enhance or not affect the activity of antimicrobial compounds (Russel, 1982). For instance, the bactericidal activity of anionic surfactants increases in the presence of low concentrations of divalent ions, whereas the bactericidal activity of long-chain fatty acids was reduced in their presence (Galbrath and Miller, 1973). The activity of biocide formulations containing EDTA is decreased in the presence of divalent metal cations, since EDTA binds to the metal ions.

2.6.3 Post-treatment factors affecting the efficacy of biocides

Post-treatment factors concentrate on those factors that influence the recovery of microorganisms exposed to antimicrobial compounds. The following factors influence the recovery of microorganisms namely; recovery medium composition, inactivation of the biocide, temperature of incubation and the diluent used in enumeration of the exposed cells (Russel, 1982).

2.6.3.1 Recovery medium composition

The composition of the recovery medium influences the survival of cells after exposure to a biocide (Russel, 1982). Bacteria injured by exposure to a biocide may be unable to produce colonies on a medium on which undamaged cells can grow (Russel, 1991b). A resuscitation medium should therefore be chosen that does not place the injured cells under any stress (Russel, 1991b). Optimum recovery of injured cells after exposed to a biocide would then be achieved. Stress can be caused by the presence of selective agents

in the enumeration medium as illustrated Przybylski and Witter (1979). Przybylski and Witter (1979) determined the number of acid injured *E. coli* cells by comparing counts obtained on Trypticase Soy Agar (TSA) and Violet Red Bile agar (VRB). Injured cells were those able to grow on TSA but unable to grow on VRB.

2.6.3.2 Inactivation of the biocide

Inactivation of the biocide present in the recovery medium is essential to accurately determine its effect on a microorganism (Hugo and Russel, 1982). Inactivating agents may be added either to the recovery medium or to the first diluent tube or both (Russel, 1982). Inactivation can be achieved by adding an inactivating agent to the recovery medium, by diluting the agent to a sub-inhibitory concentration in the recovery medium or by membrane filtration (Russel, 1982). The inactivating agent should according to Russel (1982) satisfy the following criteria; (1) it should be non-toxic to the microorganism and (2) any product resulting from the neutralisation must be non-toxic. A number of agents used in inactivating antimicrobial compounds are listed in Table 3. Dilution can be used as a means of neutralization with antimicrobial compounds that have a high dilution coefficient, since these compounds rapidly lose their activity on dilution. It must, however, be verified that the dilution has reduced the level of the compound to a sub-inhibitory concentration. Inactivation using membrane filters involves the filtration of the antimicrobial compound and microorganism mixture through a membrane. The filtered cells are then washed, by *in situ* filtration, to remove all traces of the compound. The filter can then be transferred to a suitable recovery medium to enumerate the surviving cells (Russel and Chopra, 1990).

2.6.3.3 Diluent used in enumeration

Cells exposed to a biocide are already under stress, therefore, a diluent that increases the stress load, would exacerbate this condition. The increase in stress on the injured cells would lead to an inaccurate reflection of the effectivity of a biocide (Russel, 1982). Sterile distilled water, quarter strength Ringers solution, 0.9% ($^{m}/_{v}$) saline and nutrient broth are commonly used as diluents (Russel, 1982). Before use a diluent should be screened to determine which has the least toxic effect on the microorganism being evaluated (Hugo and Russel, 1982).

Table 3 Possible inactivating agents of certain antibacterial agents (After Russel, 1982)

Antimicrobial agent	Possible inactivating agent (s)
Phenols and cresols	Dilution Tween (polysorbates)
Parabens	Dilution Tween (polysorbates)
Iodine and related compounds	Sodium thiosulphate
Chlorine and hypochlorites	Nutrient broth Sodium thiosulphate
Glutaraldehyde	Sodium thiosulphate Glycine Dilution
Mercury compounds Organic arsenicals Bronopol	-SH compounds -SH compounds -SH compounds
Quaternary ammonium compounds and chlorhexidine	Lubrol + lecithin Lecithin + Tween

2.6.3.4 Temperature of incubation

Bacteria that have been exposed to a biocide may recover better if incubated at a temperature lower than their optimum growth temperature (Russel, 1982). Reduction of

incubation temperature reduces the stress on the injured cells, thereby increasing the recovery of injured cells. A more accurate reflection of biocide activity would then be obtained.

2.7 Mode of Antibacterial Action of Organic Acids and Phenol

There is a growing awareness of the need to determine the mechanism of action of antibacterial agents. Knowledge of the mechanism of action can assist in the design of new compounds, combinations of compounds and the understanding of resistance mechanisms. Studies relating to the mechanism of action of antimicrobial compounds indicate that they antimicrobial compounds can no longer be considered as general cell poisons (Russel and Chopra, 1990). The target regions for antibacterial agents can be classified as cell wall, cytoplasmic membrane or cytoplasm (Russel and Chopra, 1990). It should be remembered that antibacterial agents will have more than one target within the bacterial cell. It is therefore important that the vital interdependence of cellular functions should not be ignored.

Due to the high carboxyl and phenolic functional group content of oxifulvic acid (Cloete *et al.*, 1990), it can be assumed that the antibacterial action of oxifulvic acid would be similar to that of compounds containing carboxyl and phenolic functional groups, *i.e.* organic acids and phenols. The mode of action and factors affecting the application of phenols and organic acids will therefore be discussed in the following section.

2.7.1 Organic acids

Organic acids are distinguished from other acids by the carboxyl (COOH) functional

group which is attached to a hydrocarbon chain of varying length (Dillard and Goldberg, 1978). Common names used to describe this group of organic compounds include fatty acids, volatile fatty acids, lipophilic acids, weak or carboxylic acids. The straight chain organic acids may be further subdivided, based on the number of carbon atoms in the hydrocarbon chain, into short-chain fatty acid (SCFA), medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA), which contain 1-6, 7-10 and >11 carbon atoms, respectively. The two oxygen atoms in the carboxyl group make the O-H bond polar (Dillard and Goldberg, 1978), so that the hydrogen atom in the OH group is readily donated to bases such as water, giving the organic acid a negative charge. In the solid and liquid phase, organic acids exist predominantly in the dimeric form. As the chain length increases, the water solubility decreases. However, MCFA readily form acid salts which are soluble in water (Cherrington *et al.,* 1991).

Organic acids are weakly acidic, since they do not readily donate the hydrogen atom in aqueous solution. The relative strength of an organic acid is reflected in the ionization constant (K_a) value. The acid (RCOOH) dissociates in the water to the proton (H^+) and the anion ($RCOO^-$), such that at equilibrium $[H^+][RCOO^-]/[RCOOH] = K_a$

$$RCOOH + H_2O \text{ ---> } RCOO^- + H_2O + H^+$$

The dissociation constant is independent of the acid concentration. Dissociation of organic acids is pH dependant and increases as the pH value approaches neutrality. The proportion of the undissociated acid present at any given pH value can be calculated from the formula:

$$RCOOH = [H^+] \div ([H^+] + [H^+][RCOO^-]/[RCOOH])$$

By analogy with the formula used to define pH ($pH = -\log [H^+]$), if a negative logarithm of K_a is taken a number, from 0-14 is obtained. This is called the pK_a value. Inspection of

the formula defining K_a (K_a = [$RCOO^-$] + [H^+]/[RCOOH]), indicates that the ratio of $RCOO^-$: RCOOH is dependent on the pH of the solution in which the acid is dissolved. The Henderson and Hasselbach equation explains this relationship as follows:

$$\log RCOO^-/RCOOH = pH - pK_a$$

At a pH value equal to the pK_a, 50% of the organic acid would be in the $RCOO^-$ and 50 % would be in the RCOOH form. Lactic acid (pK_a 3.8), is therefore a stronger acid than butyric acid (pK_a 4.2), since at all pH values a larger percentage of the lactic acid will be in the undissociated (RCOOH) form.

Organic acids are more effective than mineral acids as biocides. Although organic acids exhibit a broad spectrum of activity, the antibacterial activity varies between individual acids (Freese *et al.*, 1973; Sheu *et al.*, 1975). The antimicrobial activity of organic acids has been ascribed to the undissociated molecule (Jay, 1978). The optimum pH for antimicrobial activity of an organic acid would, therefore, be at a pH equal to or less than a pH value equivalent to the pK_a of the organic acid, since 50% of the acid would be undissociated (Fig. 15). Lowering of the pH, below a pH value equivalent to the pK_a of the organic acid, would increase the toxicity of the environment. This toxicity of the environment is due to the activity of the H^+ ions on the transport and metabolic enzymes of the cell. The degree of ionization of the cell surface also affects the adsorption of the undissociated organic acids on the cell. Too simplistic a view of the effect of pH on the activity of weak acids should therefore not be assumed. The antimicrobial activity of organic acids increases with decreasing pH (Freese *et al.*, 1973). Since a greater percentage of the undissociated molecule is present at decreasing pH, it has been assumed that the antimicrobial activity of organic acids is due to the undissociated molecule (Jay, 1978).

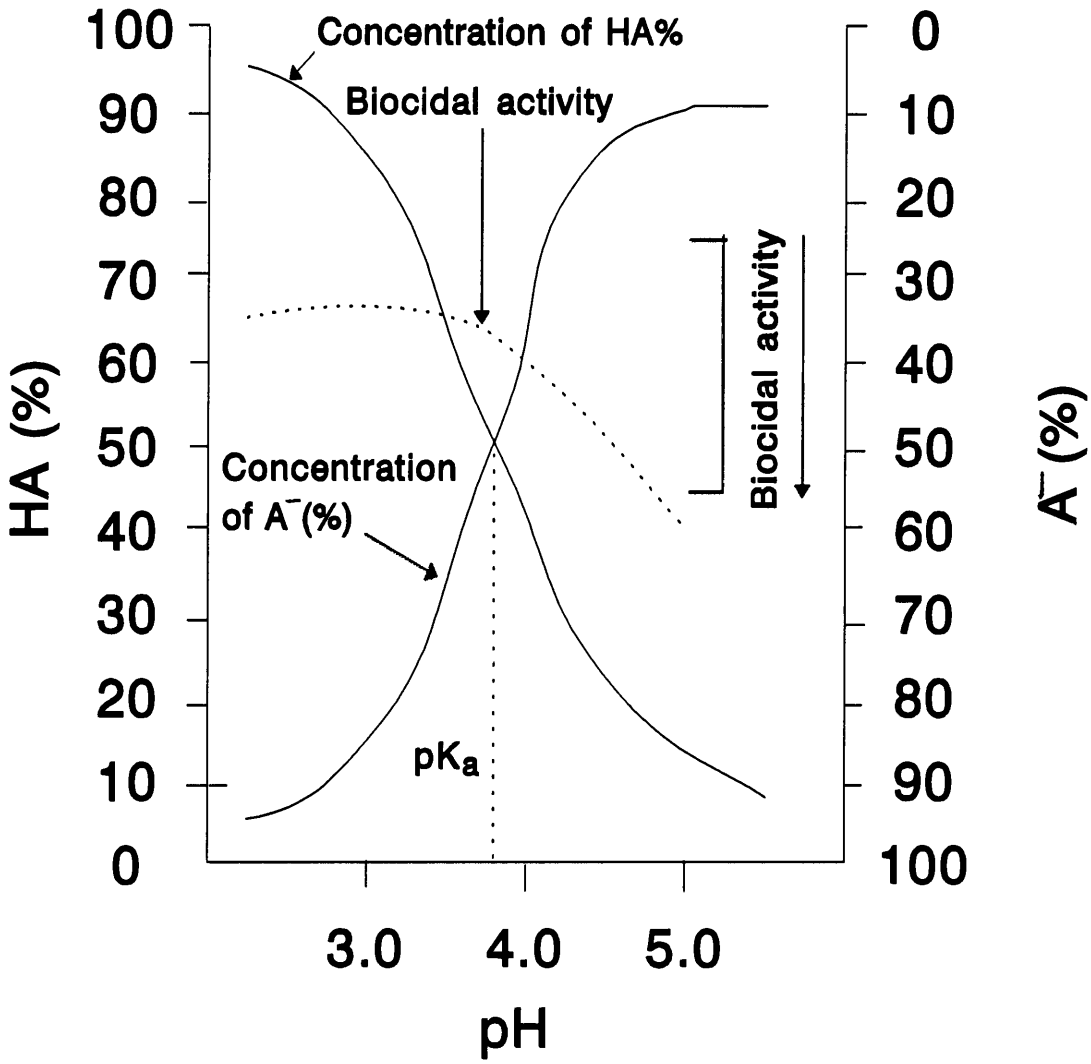


Fig. 15 Diagram of the effect of pH on the ionization and biocidal activity of organic acids (After Hugo and Russel, 1982).

This does not take into account the activity of the acid inside the cell. The pH of the cytoplasm is strictly regulated (Foster, 1992) with estimates ranging from pH 7.4 - 7.6 (Padan *et al.*, 1981) and pH 8.2 - 8.7 (Booth, 1985) over an external pH range of 5.5 - 9.0. At the pH of the cytoplasm, an organic acid would dissociate, assuming that the dissociation of the acid in the cytoplasm is the same as in aqueous solutions. It is

probable that both the proton (H^+) and the acid anion ($RCOO^-$) would contribute to the inhibition of bacterial growth (Eklund, 1980; Eklund, 1983; Salmond *et al.,* 1984; Cherrington *et al.,* 1990). Eklund (1985) using a mathematical model calculated that the anions of benzoic, propionic and sorbic acids contributed to more that 50% of the growth inhibition of *E. coli* in a medium of pH 6.0 or higher.

The cell membrane, enzymes, macromolecule synthesis and DNA have been identified as potential targets for the action of organic acids (Freese *et al.*, 1973; Tuttle and Dugan, 1976; Eklund, 1980; Cherrington *et al.*, 1990). The cell membrane may either be disrupted (Sheu and Freese, 1972) or cell membrane functions may be disrupted (Salmond *et al.*, 1984). Membrane disruption is believed to be caused by the disruption of the phospholipid bilayer in the cell membrane (Cherrington *et al.*, 1991). Cell lysis has only been reported for protoplasts or membrane vesicles but not whole cells (Freese *et al.*, 1973; Eklund, 1980). Membrane functions are affected due to the disruption of the proton-motive force (PMF) in the cytoplasmic membrane (Cherrington *et al.*, 1991). The PMF comprises of an electrical and proton gradient across the cell membrane (Cherrington *et al.*, 1991). Therefore, as the pH of the cytoplasm decreases due to the release of protons by an organic acid, the electrical gradient would have to be increased to maintain the PMF. Acidification of the cytoplasm by organic acids would lead to a reduction in the proton gradient in the PMF (Cherrington *et al.*, 1991). Organic acids have therefore been classified as uncoupling agents since they rapidly shuttle protons across the cell membrane, thereby interfering with the PMF and consequently oxidative phosphorylation (Cherrington *et al.,* 1991).

Acidification of the cytoplasm would inhibit enzyme activity and macromolecule synthesis in the cytoplasm since the activity of biosynthetic enzymes decreases with a

reduction in pH (Cherrington *et al.*, 1991).

There are conflicting reports regarding the mechanism of action of organic acids on DNA. Sinha (1986) reported that the physical conformation of DNA molecules in DNA-repair-deficient mutants of *E. coli* were disrupted by acetic and lactic acid. In contrast, Cherrington *et al.* (1991) were unable to detect any damage of DNA molecules in DNA-repair-deficient mutants of *E. coli*. They believe that the acid anion interfered with the conformation of the DNA molecule by interacting with the ion charges around it. This observed effect of organic acids would explain the differences in activity of organic acids with the same or similar pK_a value (Sheu *et al.*, 1972; Salmond *et al.*, 1984).

2.7.2 Phenols

The chemical structure of the phenols, cresols and their derivatives have been described in section 2.2.1 and will therefore not be discussed further. The relationships that exist between the structure and antimicrobial activity of phenols, cresols and their derivatives (Hugo and Russel, 1982) can be listed as follows:

- *para* (4)-Substitution of an alkyl chain up to six carbon atoms in length, with a straight chain substitution, increases antibacterial activity more than substitution with a branched chain containing the same amount of carbon atoms.
- Halogenation increases activity if it occurs at the *para*(4) position and is coupled with the introduction of an alkyl group at the *ortho* (2) site (Russel *et al.*, 1987).
- Nitration increases activity, but has the disadvantage that it also increases the systemic toxicity of the compound.
- Activity of the bis-phenol series of compounds increases with a direct bond between the C_6H_5- groups or if the groups (C_6H_5-) are separated by -CH_2-, -S-, or

-O-, bonds.

Phenols, cresols and their chlorinated derivatives are described as membrane active agents since they cause damage to the cytoplasmic membrane of bacteria. They could also cause coagulation of cytoplasmic proteins (Russel and Chopra, 1990). Chlorinated phenolics were the most widely used biocides initially (Strauss and Puckorius, 1984). Recently certain of the compounds were found to be toxic to man and this has restricted their use.

Phenolics induce leakage of intracellular materials from bacteria due to the disruption of the cell wall, possibly due to the coagulation of membrane proteins (Russel and Chopra, 1990). Cells may, however, be able to recover rapidly following the removal of the phenolics (Russel and Chopra, 1990). Phenolic compounds are sometimes referred to as protein precipitants. This term masks the more subtle effects they have on the bacterial cell at low concentrations. At low concentrations certain chlorinated phenols (hexachlorophene and tetrachlorosalicylanide) inhibit the energy-dependant uptake of amino acids and glucose in cellular material. Tetrachlorosalicylanide is also capable of discharging the membrane potential component of the proton motive force in *Streptococcus faecalis* (Russel and Chopra, 1990).

Chlorinated phenols adsorb to the cell surface, probably by hydrogen bonding, rather than any chemical reaction of the phenolic compound with the cell wall. After adsorption, the phenolic diffuses into the cytoplasm where it forms a colloidal solution. This solution is responsible for the precipitation of proteins in the cytoplasm, which leads to cell death. At low concentrations, chlorinated phenolics are not affected by the presence of excessive amounts of organic material, unlike chlorine, and are insensitive to

pH in the range of pH 5.5 to 8.5.

2.8 Resistance Mechanisms of Bacteria to Biocides

Several types of resistance to biocides can occur in bacteria (Table 4). However, basically two major mechanisms of resistance have been identified, namely: (1) intrinsic resistance and (2) stable or unstable acquired resistance (Heinzel, 1988; Russel and Chopra, 1990; Brözel and Cloete, 1991; Russel, 1991a; Brözel *et al.*, 1993).

2.8.1 Mechanisms of intrinsic resistance to biocides

The intrinsic resistance of a microorganism is determined by the naturally occurring chromosomally controlled genes within the microorganism (Russel and Chopra, 1990; Russel, 1991a). Intrinsic resistance is therefore normally due to the nature of the cell envelope (Gilbert and Wright, 1987) which acts as a penetration barrier or by enzyme action (Heinzel, 1988; Brözel and Cloete, 1991).

2.8.1.1 Penetration barriers

Low intrinsic resistance is encountered amongst the Gram-positive bacteria due to the following factors; (1) no specific receptor molecules or permease exists to prevent biocide penetration and (2) the exclusion limit of the Gram-positive cell wall (Fig. 16) is too high to prevent the entry of low molecular weight biocides (Russel, 1991a). Therefore, most biocides readily enter the Gram-positive cell rendering these bacteria more sensitive to biocides, than Gram-negative cells, due to their cell wall structure.

Table 4 Mechanisms of bacterial resistance to biocides (After Russel and Chopra, 1990)

Microorganism	Intrinsic resistance	Acquired resistance
Spore	Spore coat	Not known
Mycobacteria	Waxy cell wall	Not known
Staphylococci	Unusual	Plasmids and methicillin resistant *Staphylococcus aureus* (MRSA) strains: Usually efflux mechanisms or increased wall lipid content
Gram negative bacteria	Outer membrane	Mutation (training): Unstable resistance. Plasmids (metals and formaldehyde)

In contrast, the outer membrane of Gram-negative bacteria (Fig. 16) limits the entry of many biocides into the cell (Nikaido and Vaara, 1985). The entry of biocides are limited, since the initial action of a biocide involves binding to the surface layers of the cell followed by passage through the outer membrane into the cytoplasmic membrane or deeper sites within the cell (Russel and Chopra, 1990). Thus, any interference with this process would increase the resistance of bacteria to biocides.

The cell surface of wild-type Gram-negative bacteria is hydrophilic in nature, due to the presence of lipopolysaccharide (LPS) molecules on the cell surface (Nikaido and Vaara, 1985). Therefore, in wild-type bacteria, low molecular weight (<*ca.* 600 to 650 Dalton) hydrophilic molecules cross the outer membrane via aqueous porins and the entry of hydrophobic molecules is prevented by the presence of the LPS as illustrated in Fig. 17 (Russel, 1991a). Wild-type strains are thus more resistant to hydrophobic biocides

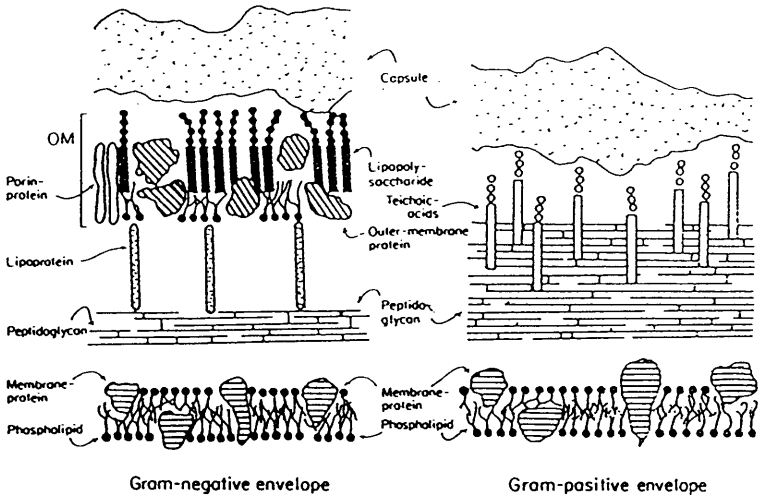


Fig. 16 Diagrammatic representation of the Gram-positive and Gram-negative cell envelopes (After Gilbert and Wright, 1987).

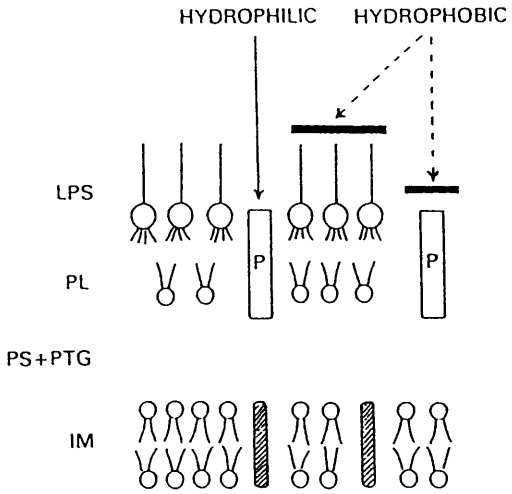


Fig. 17 Comparison of intracellular penetration of hydrophobic and hydrophillic molecules into wild-type Gram-negative bacteria, (LPS) Lipopolysaccharide layer, (PL) Phospholipid layer, (PS) Polysaccharide ,(PTG) Peptidoglycan, (P) porin and (IM) Inner membrane (After Russel and Chopra, 1990).

Russel and Chopra (1990) stated that bacteria within a biofilm are more resistant to biocides than are cells in batch type culture. The two reasons given for this phenomenon are; (1) physiological changes in the cells and (2) the expopolysaccharide matrix acting as a penetration barrier to the biocide. The latter mechanism is considered the most important, since subculture of cells *in-vitro* results in loss of biocide resistance as illustrated in Fig. 18.

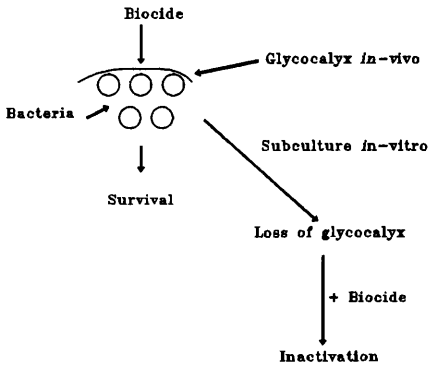


Fig. 18 Effect of glycocalyx on intrinsic resistance against a biocide (BIO) (After Russel and Chopra, 1990)

The formation of a slime capsule on the outside of a cell, particularly in the case of Gram-positive cells, can lead to increased resistance to certain biocides (Russel and Chopra, 1990). The slime could either act as a penetration barrier, similar to the LPS in Gram-negative cells, or as a loose layer interacting with or adsorbing the biocide reducing the biocidal effect (Russel and Chopra, 1990).

2.8.1.2 Enzymes

The presence of enzymes that are able to either transport the biocide out of the cell or degrade the biocide increases the intrinsic resistance of a microorganism (Heinzel, 1988).

Permease enzymes enable microorganisms to actively excrete biocides from the cell, thereby reducing their toxic effect on the cell (Heinzel, 1988).

Degradation of biocides by excreted enzymes is a more common mechanism of protection (Heinzel, 1988). This form of protection does have the disadvantage that it can be overcome by increases in the concentration of the biocide (Heinzel, 1988). Notwithstanding this, many enzyme degradation systems against a variety of biocides have been identified (Heinzel, 1988; Russel and Chopra, 1990). The most notable of these are the catalases, peroxidases, aldehyde dehydrogenases and enzymes capable of detoxifying metal compounds. Catalase and peroxidase enzymes increase the intrinsic resistance of bacteria towards hydrogen peroxide. Aldehyde dehydrogenase enzymes isolated from *Pseudomonas* spp. have been linked to formaldehyde resistance (Eagon and Barnes, 1986; Sondossi *et al.*, 1986). Detoxification of metals is normally achieved by the reduction of the metal anion to the elemental metal by enzyme catalysed reduction or by a non-specific reaction with a reducing agent *e.g.* thiols produced by the bacteria (Heinzel, 1988).

2.8.2 Mechanisms of acquired resistance to biocides

Acquired resistance is the resistance that results from the exposure of a microorganism to specific conditions, *e.g.* biocides (Heinzel, 1988). The resistance of the microorganism can result from mutation of the genome, acquisition of genetic material (usually due to plasmid transfer or transformation), induction of an enzyme or the formation of a penetration barrier (Heinzel, 1988). Acquired resistance can be either stable or unstable. Unstable acquired resistance is also referred to as adaptation, since resistance can be lost if the microorganism is no longer exposed to the selective pressure (*e.g.* biocide)

(Heinzel, 1988; Russel, 1990)

2.8.2.1 Resistance determined by chromosome gene mutation

With a few exceptions resistance due to chromosome gene mutation is normally unstable (Russel and Chopra, 1990). Chromosome gene mutations do not directly lead to increased resistance, but initiate the formation of cell constituents or transport systems that increase the resistance of the cell. Increased cell wall lipid content, a decrease in the ratio of inner membrane phosphatadylethanolamine to anionic phospholipids and mutant periplasmic proteins which bind or trap a biocide are a few of the examples stated by Russel and Chopra (1990) for the formation of additional or modified cell constituents that increase cell resistance.

2.8.2.2 Acquisition of genetic material

Genetic material can be carried over either due to plasmids, transposons or by transformation (Russel and Chopra, 1990). Plasmids carry the genetic determinants for resistance to silver compounds, inorganic and organic mercurials and certain biocides (*e.g.* formaldehyde) in Gram-negative bacteria (Russel and Chopra, 1990). Plasmid encoded cell envelope changes and degradation of the biocide have been cited as possible mechanisms of plasmid-mediated biocide resistance (Russel and Chopra, 1990). In the case of Gram-positive bacteria the best documented case of plasmid-mediated resistance is that of the methicillin-resistant *Staphylococcus aureus* strains, which are also resistant to QAC (Masaudi *et al.*, 1991). Transformation has been implicated in the transfer of chlorhexidine resistance in *Streptococcus sanguis* (Russel and Chopra, 1990).

2.8.2.3 Induction of enzymes capable of metabolizing the biocide

The only difference between plasmid-mediated metabolic enzyme induction and intrinsic enzyme-mediated resistance is that the former does not occur naturally in the cell but is induced by the presence of a plasmid. In most cases the induction of metabolic enzymes is plasmid mediated (Heinzel, 1988).

2.8.2.4 Formation of penetration barriers

This can be either plasmid-encoded or non-plasmid-encoded (Russel, 1990). Plasmid-encoded formation of penetration barriers has been discussed previously (2.8.2.2) and this section will deal only with non-plasmid-encoded formation of penetration barriers. Penetration barriers can form due to the growth of bacteria in a rich medium (Hugo and Russel, 1982) or exposure to a biocide (Russel, 1990). This form of acquired resistance is unstable (Brözel *et al.*, 1993).

2.8.3 Strategies for counteracting resistance to biocides

Two methods for counteracting resistance to biocides and involving combined systems, have been identified by Russel and Chopra (1990).

2.8.3.1 Inclusion of an agent to enhance the biocidal activity

Biocidal activity can be increased the inclusion of agents that enhance the penetration of antibiotics into bacterial cells *in-vitro*. This can be achieved by using EDTA, polylysine, lactoferin and transferin in combination with antibiotics (Hugo and Russel, 1982). EDTA is also used in combination with many biocides to increase their activity. The use of a combination of compounds to enhance the activity of the formulation is known as

synergism (Lehmann, 1988; Russel and Chopra, 1990)

2.8.3.2 Inclusion of a physical stress factor to enhance activity

The use of a biocide in combination with physical stress factors such as sub-optimal pH, temperature and water activity increase the activity of the biocide. This is achieved by placing additional energy demands on the cell through interference with energy-requiring homeostatic mechanisms (Russel and Chopra, 1990).

2.9 Removal of Pathogenic Viruses from Potable and Domestic Wastewater

Water is the pre-eminent vector for life and human activity (Degremont, 1992). At present the use of water (domestic, industrial and agricultural use) in the world has been calculated by Degremont (1992) to be *ca.* 250 m^3 per person per year. Since the demand is certain to grow with the increase in world population the available water resources will therefore have to be protected, treated and preserved to prevent pollution to cope with the increasing demand for water.

Potable water is obtained mainly from ground and surface sources (Degremont, 1992). Untreated surface water is rarely potable since it is usually polluted. Three main sources of pollutants have been identified by Degremont (1992), namely; (1) municipal, (2) industrial and (3) agricultural activities. The pollutants originating from these sources can be classified as (1) biological, (2) mineral, (3) organic and (4) radioactive impurities (Degremont, 1992). Biological impurities occurring in water include pathogenic microorganisms (*i.e.* bacteria, viruses, protozoa and helminths).

Domestic wastewater may contain more than 120 types of enteric viruses (Bitton, 1980). Their fate once they enter the environment is therefore of concern due to their possible

pathogenicity (Grabow *et al.*, 1978; Bixby and O'Brien, 1979). As the infective dose of many viruses is low ($ID_{50} < 10^2$) and they are able to survive in the environment, their release of into the environment poses a serious health risk (Lewis and Metcalf, 1988). There is ample evidence to indicate that human enteric viruses may appear in the environment due to the release of inadequately treated wastewater from treatment plants (Lewis and Metcalf, 1988; Thurman and Gerba, 1988). Hepatitis A, Norwalk and Rota viruses have been associated with waterborne outbreaks of disease due to release of treated sewage effluent (Lewis and Metcalf, 1988).

2.9.1 Virus removal from potable water

Two categories of water treatment plants are used for the production of potable water, namely; conventional filter plants and softening plants (Bitton, 1980). Conventional filter plants are the most common and include the following steps; mixing (addition of coagulant), flocculating (floc formation) followed by clarification (settling), filtration and disinfection. Softening plants are used to treat water with a high calcium or magnesium content (hard water) and include a softening process (removal of Ca^{2+} and Mg^{2+}), clarification (settling), filtration and disinfection.

Viruses are removed from surface and ground water during the production of potable water either physically or by inactivation (Bitton, 1980). Physical removal of viruses occurs by adsorption, during the clarification and filtration steps included in water treatment. Inactivation of the virus is due to protein denaturation by high pH (> pH 11) during water softening and disinfection (Bitton, 1980).

The physical removal of viruses during clarification and filtration, is due to the removal of floc-associated viruses (Bitton, 1980). During coagulation the viruses are

adsorbed by the coagulant and coagulant aids used and are thus removed as part of the settled floc in the clarifier. This process can remove up to 99% of the viruses present in the water when optimal conditions are met. The percentage reduction is lower in practice, since optimal conditions are rarely attained (Bitton, 1980). Filtration, using diatomaceous earth and rapid sand filtration, of the clarifier effluent removes those floc-associated (coagulated) viruses that did not settle out during clarification. Sand filtration is the most effective filter medium for the removal of viruses provided the viruses are floc-associated. The effectivity of diatomaceous earth filters can be increased by prior coating of the diatomaceous earth with a polyelectrolyte (Bitton, 1980).

Viruses are also removed by adsorption to magnesium hydroxide flocs or inactivated by the high pH generated during water softening in the lime-soda process (Bitton, 1980). Bitton (1980) suggested that coagulation and filtration contributed indirectly to the inactivation of viruses by the removal of organic material, thus increasing the effectivity of the disinfection process.

2.9.2 Virus removal from domestic wastewater

Domestic wastewater may contain enteric viruses and their fate once they enter the environment is of concern due to their pathogenicity (Grabow *et al.*, 1978; Bixby and O'Brien, 1979; Bitton, 1980). Domestic wastewater must therefore be treated before release into surface water sources. Sewage treatment plants are used to remove or reduce undesirable biological components (*e.g.* viruses and bacteria), suspended solids, organic materials, recalcitrant organic compounds and heavy metals from wastewater (Bitton, 1980). Sewage treatment comprises three stages namely primary, secondary and tertiary treatment (Bitton, 1980).

Primary treatment involves the physical removal of coarse debris followed by sedimentation. Secondary treatment involves the biological treatment of the primary effluent utilizing either activated sludge, trickling filters and oxidation ponds or a combination of processes, followed by a disinfection step. Tertiary treatment involves the physio-chemical removal of nitrogen, phosphorus, some dissolved organic matter, heavy metals and pathogenic organisms (Bitton, 1980).

The efficacy of removal of enteric viruses from wastewater increases as treatment proceeds from primary to tertiary treatment (Lewis and Metcalf, 1988). Since primary treatment is essentially the physical removal of solid particles, virus removal during this step is achieved largely due to the removal of solid associated viruses (Bitton, 1980; Lewis and Metcalf, 1988). Primary treatment therefore only plays a minor role in the removal of enteric viruses from domestic wastewater since only *ca.* 10% of the viruses present in the water will be removed (Lewis and Metcalf, 1988).

The largest reduction in viruses is achieved during secondary treatment of domestic wastewater. During this stage a 50 to 95% reduction in viruses numbers can be achieved depending on the secondary treatment process applied (Bitton, 1980). The activated sludge process is the most effective with virus removal rates of up to 95% being achieved. The more effective removal is due to a number of factors identified by Lewis and Metcalf (1988) and Bitton (1980), namely: (1) microbial aerobic enzyme-induced bio-oxidation of organic matter, with a loss of virus structural integrity, (2) adsorption or encapsulation of viruses within the sludge flocs, (3) inactivation by sewage bacteria and (4) ingestion by protozoa and small metazoa.

Removals rates of *ca.* 50 to 60 % are obtained during the trickling filter treatment of domestic wastewater (Lewis and Metcalf, 1988). This reduced rate of removal, in

comparison to the activated sludge treatment, has been ascribed to the fact that the bio-oxidative action upon the viruses is less intense in the trickling filter system. Virus removal is due to the adsorption or encapsulation of the virus in the zoogleal film present in the trickling filters (Bitton, 1980).

The effectivity of oxidation ponds for the removal of viruses depends on the retention time used, since the longer the retention time, the higher the degree of virus removal (Bitton, 1980; Lewis and Loutit, 1989). Bitton (1980) identified various physical factors that lead to the inactivation and/or removal of viruses from oxidation ponds, namely; (1) inactivation by temperature, (2) inactivation by solar radiation, (3) inactivation by high water pH (> pH 11), (4) adsorption to suspended solids (5) inactivation by bacteria and (6) ingestion by protozoa and small metazoa.

Tertiary treatment removes up to 99.9% of the viruses remaining after secondary treatment (Lewis and Metcalf, 1988). The virus-reducing effectivity of the various steps included in tertiary treatment (*i.e.* coagulation-flocculation or sedimentation, sand filtration, activated carbon adsorption and disinfection) are attributed to the removal of turbidity and finely suspended solids with which the viruses are associated (Lewis and Metcalf, 1988). The complete removal of particulate material prior to disinfection increases the removal rate to at least 99.99% (Lewis and Metcalf, 1988).
According to Lewis and Metcalf (1988) a virus-removal capacity of 8 to 9 log is theoretically achievable when a 2 log removal in secondary treatment is combined with a 3 log removal during tertiary treatment plus another 3 to 4 log removal during disinfection. This total can be increased by a further 2 log if activated carbon adsorption treatment is used. An additional 5 to 6 log removal can be achieved with the use of reverse osmosis treatment. Therefore, if all the above mentioned treatments were applied,

a virus-removal capability of 15 log would theoretically be achievable. Complete removal of enteric viruses from sewage is therefore technically feasible provided appropriate advanced waste-treatment procedures are included (Lewis and Metcalf, 1988). The removal of viruses is thus a function of the technical and economic factors, influenced by the societal factors. Any one, or a combination of the factors can be used to decide on the choice of a particular treatment strategy (Lewis and Metcalf, 1988).

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CHAPTER 3

(Submitted for publication to the Journal of Applied Bacteriology*)

BACTERICIDAL ACTIVITY OF COAL-DERIVED HUMIC SUBSTANCES

* The language and style used in this chapter are in accordance with the requirements of the Journal of Applied Bacteriology)

ABSTRACT

Due to the reported antimicrobial activity of humic substance the antibacterial activity of oxicoal, oxihumic acid and oxifulvic acid, obtained by the controlled wet oxidation of South African bituminous coal were evaluated. Oxifulvic acid was the most bactericidal of all the coal-derived products evaluated. The influence of pH, calcium concentration and organic matter on the bactericidal activity of oxifulvic acid was determined as part of the screening of the biocidal activity oxifulvic acid. The antibacterial activity of oxifulvic acid was pH dependant, with the optimum pH for bactericidal activity between pH 3.0 to pH 4.0. A linear relationship was exhibited between calcium concentration and the concentration of oxifulvic acid required for bactericidal action. An eight fold increase in oxifulvic acid was required for bactericidal activity in the presence of organic matter.

INTRODUCTION

Disease, food spoilage, fouling of surfaces and biodeterioration of materials are but a few problems associated with uncontrolled bacterial growth (Parr, 1990; Russel & Chopra, 1990). Application of antimicrobial agents (biocides) are one of the most common methods used to control the detrimental activity of bacteria. There are currently many antibacterial compounds available to control bacterial growth and the search continues for more effective and environmentally safe compounds. Currently more than 90 % of the raw materials for the manufacturing of antimicrobial compounds in South Africa are imported (Cloete *et al.*, 1989). Therefore a market exists in South Africa for the production of low cost and effective biocides.

South Africa is a country rich in many natural resources with coal being one of the most abundant (Department of Water Affairs and Forestry, 1986). The production of value added products using low rank (low grade) bituminous coal is a possible way of increasing the value of raw coal. A controlled wet oxidation process was developed by Cronje (1990) for the production of value added products from South African bituminous coal. The coal can be converted via oxidation and extraction to oxicoal, oxifulvic acid and oxihumic acid. The coal-derived oxifulvic and oxihumic acids differ from natural humic and fulvic acids since they contain more aromatic and phenolic compounds. The ratio of phenolic to carboxyl groups and total acidity are also somewhat higher than the corresponding values reported for the humic and fulvic natural acids (I.J. Cronje pers. comm.)*.

Humic substances are defined as that portion of the soil organic matter that has undergone sufficient transformation to render the parent material unrecognizable (Atlas and Bartha, 1981). Humic compounds are divided according to their solubility characteristics into humin, humic acid and fulvic acid (Atlas and Bartha, 1981).
The solubilities of the fractions are as follows, humin is the alkali insoluble fraction of soil organic matter, humic acid the alkali soluble and acid insoluble fraction and fulvic acid the water soluble fraction (Stevenson, 1982). Fulvic acid has been described by Bixby and O'Brien (1979) as a mixture of aliphatic and aromatic macro-molecules substituted with hydrophilic carboxylic, phenolic and carbonyl acid functional groups.

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Humic acid, isolated from domestic sewage was bactericidal against *Serratia marcescens* and *Staphylococcus aureus* (Hasset *et al.*, 1987). There has been no direct reference to the antimicrobial activity of fulvic acid reported in the literature. Water soluble extracts of bark compost, possibly fulvic acid, were shown by Kai *et al.* (1990) to have antifungal activity. Due to the reported antimicrobial activity of humic substances and the need for the production of a low cost and effective biocide in South Africa, the bactericidal activity of the complete range of coal-derived products was determined.

The evaluation of antimicrobial compounds can be divided into three stages namely; preliminary screening, establishment of the use-dilution and field tests (Reybrouck, 1982). The first stage involves the screening to determine whether a compound has antimicrobial activity. The influence of extrinsic factors on the compound, *i.e.* pH, water hardness and organic matter are also determined. No chemical compound can be regarded as antimicrobial if it is not active against vegetative bacteria since this is the most important requirement (Reybrouck, 1982). Numerous methods exist to evaluate the antibacterial activity of compounds. These can be classified into suspension tests, capacity tests or carrier tests (Spooner and Sykes, 1975; Croshaw, 1981; Reybrouck, 1982; Eigner, 1988). The choice of a method would depend largely on the type of compound being evaluated and the possible use of the compound (Croshaw, 1981). Qualitative or quantitative suspension tests are recommended for preliminary screening of compounds (Reybrouck, 1982).

There are many factors that affect the concentration required for antimicrobial activity of biocides. The most important are number of microorganisms, time of exposure, temperature, pH, water hardness and presence of organic material (Russel, 1982).

Since the number of microorganisms, time of exposure and temperature are determined by the test method used, pH, water hardness and presence of organic material can be used as variables, to determine the effect of these factors on antimicrobial activity. The activity of certain antimicrobial compounds is influenced quite markedly by changes in pH (Russel, 1982). Compounds that rely on undissociated functional groups for their antimicrobial activity are affected negatively by an increase in environmental pH (Russel and Chopra, 1990). The increase in pH decreases the concentration of undissociated functional groups, reducing the antimicrobial activity of the compound. Examples of such compounds are phenols and organic acids which are not effective as bactericides at high pH values (Russel and Chopra, 1990).

Water hardness is determined by the concentration of calcium and magnesium ions in the water. Many bactericidal tests utilize synthetic hard water formulations as part of the test, since the antibacterial activity of certain compounds are reduced by the presence of calcium or magnesium (Russel, 1982). The presence of calcium and magnesium decreases the activity of biocide formulations containing ethylenediaminetetraacetic acid (EDTA) (Russel, 1982). Other compounds capable of binding calcium or magnesium would also be affected by the presence of these minerals. Therefore, in determining the bactericidal activity of a compound the effect of calcium or magnesium on antimicrobial activity, should be included.

Organic matter may have a negative effect on the antimicrobial activity of biocides (Russel, 1982). This is particularly true for the oxidizing biocides *e.g.* chlorine disinfectants (Russel, 1982). The antimicrobial activity of certain non-oxidizing biocides *e.g.* phenols and organic acids are also inhibited by organic matter (Gelinas and Goulet, 1983).

The interference generally results from a reaction between the compound and the organic matter, leaving a reduced concentration of antimicrobial agent available for antimicrobial activity (Russel, 1982; Gelinas and Goulet, 1983). To simulate the effect of organic matter on the antimicrobial activity of a biocide under test conditions, yeast is normally added to the test suspension (Reybrouck, 1982). Tests including organic matter give an indication of the in-use concentration of an antimicrobial compound (Kelsey and Maurer, 1974; Russel, 1982) These test are performed during the preliminary screening of a compound (Russel, 1982).

This study reports on the evaluation of the bactericidal activity of the coal-derived products and the effect of pH, calcium ion concentration and organic matter on the bactericidal activity of oxifulvic acid.

MATERIALS AND METHODS

Coal-derived products

The coal-derived coal products, prepared by the controlled wet oxidation of South African bituminous coal (Cronje, 1990), were obtained from the Division for Energy Technology, CSIR, Pretoria, South Africa. Oxicoal was obtained as a dry powder. Oxifulvic, oxihumic acids and the extracts (butanone and methanol) of oxifulvic acid were obtained as solutions.

Characterization of coal-derived products

The carboxylic group content was determined by ion exchange with calcium acetate (Stevenson, 1982). The concentration of acetic acid liberated during the reaction was thereafter determined with sodium hydroxide. The content of carboxyl (COOH) groups in milliequivalents per gram (meq/g) was obtained using equation (1).

$$COOH = \{(V_s - V_b) \times N \times 10^3\} \div \text{mg of sample} \qquad (1)$$

Where V_s and V_b represent the volumes of standard base (0.5 N NaOH) used for the sample and blank respectively, and N is the normality of the base.

The total acidity was measured by barium hydroxide exchange (Stevenson, 1982). The sample was allowed to react with excess barium hydroxide, whereafter the unused base was titrated with 0.5 N HCl to pH 8.4. The total acidity (meq/g) was calculated as shown in equation (2).

$$\text{Total acidity} = \{(V_b - V_s) \times N \times 10^3\} \div \text{mg of sample} \qquad (2)$$

The phenolic group content of the oxihumic and oxifulvic acids, is defined as the difference between total acidity and carboxylic acidity (*i.e.* carboxylic group content).

Test organisms

Bacterial cultures were obtained from the culture collection of the Environmental Biotechnology Laboratory at the Department of Microbiology and Plant Pathology, University of Pretoria. All test isolates with the exception of *Escherichia coli* strain K12 and *Salmonella typhi* were isolated from water-cooling systems in South Africa (Cloete *et al.,* 1989). *Escherichia coli* strain K12 and *Salmonella typhi* (obtained from Prof W.O.K. Grabow, Head, Department of Medical Virology, University of Pretoria) were isolated from domestic wastewater and identified using the API system. Bacterial cultures were maintained on Standard One Nutrient Agar (STD1, Biolab, Merck, South Africa) slants at 4 °C.

Bactericidal assay

The bactericidal activity of oxicoal, oxihumic acid and oxifulvic acid were evaluated using the bactericidal assay described by Brözel and Cloete (1991).

The stock solutions of the coal-derived products were prepared as follows: Oxicoal powder (1000.0 mg) was suspended in 100.0 ml sterile distilled water and from this the test concentrations were made up in quarter strength Ringers solution. Oxihumic acid and oxifulvic solutions were dissolved in 100.0 ml sterile distilled water to yield concentrations of 10000.0 mg/l active ingredient. Butanone and methanol extracts of oxifulvic acid were dissolved in 100.0 ml sterile distilled water to yield a concentration of 10000.0 mg/l active ingredient. The coal-derived compounds were added from the 10000.0 mg/l stock solutions to quarter strength Ringers solution to yield solutions (9.0 ml) with the test concentrations.

An bacterial inoculum for the bactericidal assay was prepared as described by Brözel and Cloete (1991). The bactericidal assay was initiated by the addition of 1.0 ml bacterial (*ca.* 10^7 cells/ml) suspension (Brözel and Cloete, 1991) to the test solution (9.0 ml). The resulting mixture (10.0 ml) was incubated at 28°C for 6 h, when viable cell counts were determined (Brözel and Cloete, 1991). Control solutions contained 1.0 ml cell suspension in 9.0 ml quarter strength Ringers solution. The bactericidal evaluation of the butanone and methanol extract of oxifulvic acid included control solutions of 1.0 ml cell suspension in 9.0 ml 1.43 % butanone and 2.2 % methanol solutions, respectively. These methanol and butanone concentrations were equivalent to the concentrations of butanone and methanol in the oxifulvic acid extracts.

A kill percentage was calculated for each of the individual culture-coal-derived product combination using the formula of Brözel and Cloete (1991). The results were tabulated as bar charts to obtain a bactericidal fingerprint for oxifulvic acid and the butanone and methanol extracts of oxifulvic acid, respectively. A concentration yielding a percentage kill of 100% was considered to be the bactericidal concentration. Each assay was done in duplicate and duplicate dilutions were plated for determination of viable cell counts after 6 h.

Effect of pH, calcium concentration and organic matter on antibacterial efficacy of oxifulvic acid

The effect of pH, calcium concentration and organic matter on the bactericidal activity of the oxifulvic acid was determined, using a modified bactericidal assay described by Brözel and Cloete (1991). All assays were initiated by the addition of 1.0 ml bacterial suspension (*ca.* 10^7 cells /ml) to the test solution (9.0 ml). The resulting mixture was incubated at 28°C for 6 h, when viable cell counts were determined (Brözel and Cloete, 1991).

A kill percentage was calculated for each of the individual culture-oxifulvic acid combination using the formula of Brözel and Cloete (1991). A concentration yielding a percentage kill of 100% was considered to be the bactericidal concentration. Each assay was done in duplicate and duplicate dilutions were plated for determination of viable cell counts after 6 h.

pH

Due to the acidity of oxifulvic acid (pH 1.89) the effect of pH on the bactericidal activity was determined in a buffered and unbuffered system.

In the unbuffered assay oxifulvic acid stock solutions (10000.0 mg/l) were adjusted to pH 3.0, 4.0, 5.0, 7.0 and 9.0, respectively, with 1 M NaOH. The pH of quarter strength Ringers solutions were adjusted to pH 3.0, 4.0, 5.0, 7.0 and 9.0, respectively, using 1 M H_2SO_4 or 1 M NaOH. Oxifulvic acid was then added from the pH adjusted 10000.0 mg/l stock solutions to the corresponding pH adjusted quarter strength Ringers solution to yield the test concentrations, in a volume of 9.0 ml. The pH adjusted (pH 3.0, 4.0, 5.0, 7.0 and 9.0) quarter strength Ringers solutions (containing no oxifulvic acid) and oxifulvic acid stock solutions, were used as inoculated controls.

In the buffered assay oxifulvic acid stock solutions (10000.0 mg/l) were prepared in pH 3.0, 4.0, 5.0, 7.0 and 9.0 buffer solutions. A 0.1 M Citric acid/K_2HPO_4 buffer was used to prepare stock solutions at pH 3.0 and 4.0, respectively. Oxifulvic acid stock solutions were prepared at pH 5.0, 7.0 and 9.0, respectively, in a 0.1 M K_2HPO_4/KH_2PO_4 buffer. A 0.1 M Citric acid/K_2HPO_4 buffer was used to prepare quarter strength Ringers solutions at pH 3.0 and 4.0, respectively and a 0.1 M K_2HPO_4/KH_2PO_4 buffer was used to prepare quarter strength Ringers solutions at pH 5.0, 7.0 and 9.0,

respectively. Oxifulvic acid was then added from the pH buffered 10000.0 mg/l stock solutions to the corresponding pH buffered quarter strength Ringers solutions to yield the test concentrations, in a volume of 9.0 ml. The pH buffered (pH 3.0, 4.0, 5.0, 7.0 and 9.0) oxifulvic acid stock solutions and quarter strength Ringers solutions, were used as inoculated controls.

Calcium

The effect of calcium concentration on the bactericidal activity of oxifulvic acid was determined by substituting the quarter strength Ringers solution (Brözel and Cloete, 1991) with a calcium solution. Calcium solutions were made up to calcium concentrations of 125.0, 250.0 and 500.0 mg/l Ca^{2+}, respectively, using $CaCl_2.2H_2O$ (Merck, South Africa) as calcium source. Oxifulvic acid was added from a stock solution (10000.0 mg/l) to the respective calcium solutions to yield concentrations of 50.0, 100.0, 200.0, 400.0, 600.0 and 800.0 mg/l, respectively, in a volume of 9.0 ml. The assay was initiated as stipulated above. Calcium solutions (125.0, 250.0 and 500.0 mg/l, respectively) were used as inoculated controls for the assays.

Organic matter

The effect of organic matter on the bactericidal activity of oxifulvic acid was determined by adding 1.0 ml of yeast extract solution (5000.0 mg/l) (Biolab, Merck, South Africa) to 8.0 ml quarter strength Ringers solution. Oxifulvic acid was then added from a stock solution (10000.0 mg/l) to the quarter strength Ringers solution-yeast extract mixture to yield concentrations of 100.0, 200.0, 400.0, 600.0 and 800.0 mg/l oxifulvic acid, respectively, in a volume of 9.0 ml. The yeast extract solution (5000.0 mg/l) was used to ensure that each oxifulvic acid concentration was exposed to an identical concentration of yeast extract. The assay was initiated as stipulated above. Inoculated control solutions contained 1.0 ml yeast extract solution in 8.0 ml quarter strength Ringers solution.

RESULTS

Characterization of coal-derived products

The total acidity, carboxylic group and phenolic group content of selected coal-derived products are shown in Table 1. The oxifulvic acids were the most acidic products produced, with a total acid content of 11.8, 12.1 and 10.5 meq/g for oxifulvic acid, methanol extract and butanone extract, respectively (Table 1). The oxifulvic acids contained mainly carboxylic functional groups as indicated by the high carboxylic group content of 8.3, 11.8 and 10.7 meq/g for the butanone extract, methanol extract and oxifulvic acid, respectively. Oxicoal and oxihumic acid contained mostly phenolic functional groups (4.04 and 4.1 meq/g, respectively) and to a lesser degree carboxylic functional groups (1.24 and 3.4 meq/g, respectively) as shown in Table 1.

Bactericidal assay

The bactericidal concentrations of the coal-derived products are given in Table 2. Oxicoal was bactericidal against *Pseudomonas aeruginosa* and *S. aureus* at 8000.0 and 10 000.0 mg/l, respectively. Oxihumate was less effective than oxicoal, since 10 000.0 mg/l was bactericidal against *P. aeruginosa* and *S. aureus*. The oxihumic acid was more effective than oxihumate since 2000.0 and 4000.0 mg/l were bactericidal against *P. aeruginosa* and *S. aureus*, respectively. The butanone and methanol extracts of oxifulvic acid were bactericidal at 100.0 and 400.0 mg/l, respectively, against *S. aureus*. *Pseudomonas aeruginosa* was more tolerant to the butanone extract than the methanol extract of oxifulvic acid, with 300.0 and 200.0 mg/l, respectively, being bactericidal. Oxifulvic acid was the most effective with only 100.0 and 25.0 mg/l required for bactericidal action against *P. aeruginosa* and *S. aureus,* respectively.

Bactericidal fingerprints (Brözel and Cloete, 1991) obtained, using a wider range of bacteria, for the butanone and methanol extracts and oxifulvic acid are given in Figs 1 to 3. The butanone and methanol extracts both required 400.0 mg/l to be bactericidal against the isolates used. Oxifulvic acid was bactericidal at 150.0 mg/l against all the bacteria (Fig. 3). *Escherichia coli* strain K12 and *Salmonella typhi* were the most resistant to oxifulvic acid requiring a concentration of 150.0 mg/l for bactericidal action.

Effect of pH, calcium concentration and organic matter on antibacterial efficacy of oxifulvic acid

pH

Oxifulvic acid did not exhibit any bactericidal activity against *P. aeruginosa*, *S. aureus,* or *P. fluorescens* at a pH $\geq$ pH 5.0 in the buffered or unbuffered test systems, respectively (Table 3). Growth of *P. aeruginosa*, *S. aureus,* and *P. fluorescens* was inhibited at pH 3.0 in the unbuffered controls and at pH 3.0 and 4.0 in the buffered controls (Table 3). Oxifulvic acid inhibited *P. aeruginosa*, *S. aureus,* and *P. fluorescens* at 400.0, 100.0 and 200.0 mg/l, respectively, in the unbuffered test system.

Calcium

The effect of calcium on the bactericidal concentration of oxifulvic acid is shown in Table 3. The bactericidal concentration of oxifulvic acid against *S. aureus* increased from 50.0 to 200.0 mg/l with an increase in calcium concentration from 125.0 to 500.0 mg/l (Table 3). The bactericidal concentration of oxifulvic acid against *P. aeruginosa* increased from 200.0 to 800.0 mg/l with an increase in calcium concentration from 125.0 to 500.0 mg/l. Oxifulvic acid was bactericidal at 100.0 and 400.0 mg/l in the presence of 125.0 and 500.0 mg/l calcium, respectively, against *P. fluorescens*.

Organic matter

The effect of yeast extract (5000.0 mg/l) on the bactericidal concentration of oxifulvic acid against *P. aeruginosa*, *S. aureus,* and *P. fluorescens* in shown in Table 3. An eight-fold increase in oxifulvic acid concentration was required for bactericidal activity against *P. aeruginosa*, *S. aureus,* and *P. fluorescens* in the presence of organic matter.

DISCUSSION

Characterization of coal-derived products

The presence of more phenolic functional groups (4.04 meq/g) than carboxylic functional groups (1.24 meq/g) in the oxicoal fraction implies the presence of phenolic compounds and to a lesser extent carboxylic acid compounds in this fraction.

The functional group content of oxifulvic acid, butanone extract and methanol extract indicated that they consist mainly of hydrophilic carboxylic and to a lesser extent phenolic functional groups (Table 1). This functional group content is analogous to the structure of natural fulvic acids described by Bixby and O'Brien (1979). The presence of mostly carboxylic functional groups in the oxifulvic acids, indicates that the oxifulvic acids consist mostly of carboxylic acids (*e.g* formic acid, benzoic acid and sorbic acid).

Oxihumic acid, however, consists of a more equal mixture of phenolic and carboxylic acids compounds as indicated by the presence of 45% carboxylic and 55% phenolic functional groups.

Bactericidal assay

No antibacterial properties have been reported for coal in the literature. Therefore, the antibacterial activity exhibited by oxicoal (Table 2) was due to the release or exposure of antibacterial functional groups in the coal as a result of the oxidation process (Cronje, 1990). The presence of 77% phenolic and 23% carboxylic functional groups (Table 1) in the oxicoal fraction implied the presence of phenolic and carboxylic acid compounds in the oxicoal. Since the antibacterial activity of phenol is well documented (Russel and Chopra, 1990) the antibacterial activity exhibited by oxicoal was attributed to the presence of the phenolic and carboxylic compounds and functional groups in the oxicoal fraction. Similarly, the antibacterial activity of the oxihumic acid was related to the presence of the phenolic (45%) and carboxylic (55%) functional groups. The increased antibacterial activity exhibited by the oxihumic acid was attributed to the fact that the oxihumic acid contained more carboxylic functional groups (3.4 meq/g) than oxicoal (1.24 meq/g).

The oxifulvic acids contained 80% to 97 % carboxylic and 3% to 20% phenolic functional groups (Table 1). The presence of these functional groups implied the presence of carboxylic acids and phenolic compounds, both of which are known antimicrobial substances (Russel and Chopra, 1990). Therefore, the antimicrobial activity of oxifulvic acid was ascribed to the presence of the carboxylic acids and phenolic compounds.

The effectiveness of the two butanone and methanol extracts were not due to any inhibitory effect of the butanone or methanol used in the extraction process, since neither the inoculated butanone or methanol controls exhibited any bactericidal activity against *P. aeruginosa*, *S. aureus,* or *P. fluorescens*.

The Gram negative bacteria *S. typhi, P. aeruginosa* and *E. coli* strain K12 were more resistant to oxifulvic acid than the Gram positive bacteria used in the bactericidal evaluation (Fig. 1). The intrinsic resistance of Gram negative bacteria to many biocides has been reported in the literature (Russel and Chopra, 1990). The increased resistance of Gram negative bacteria is associated with the structure of the cell wall (Chopra, 1991). The outer cell membrane (OM) plays an important role in the intrinsic resistance of Gram negative bacteria (Russel, 1990). The lipopolysaccharide (LPS) layer of Gram negative bacteria often acts as an exclusion barrier for most hydrophobic biocidal compounds (Russel, 1990; Chopra, 1991). Oxifulvic acid is water soluble, and therefore hydrophilic. This mechanism would therefore not prevent diffusion of oxifulvic acid through the cell wall. Hydrophilic molecules can enter Gram negative cells via aqueous porins (Russel, 1990). The entry of hydrophilic molecules is, however, restricted to low molecular weight (< *ca.* 600 Daltons) hydrophilic molecules (Russel, 1990). The molecular weight of oxifulvic acid is not known, but given that oxifulvic acid is similar to natural fulvic acid, it can be assumed that it has a similar structure. Natural fulvic acids have been described as a mixture of aromatic and aliphatic macromolecules (Bixby and O'Brien, 1979). Oxifulvic acid molecules would therefore be too large (> *ca.* 600 Daltons) to move through the porins, restricting their entry into the Gram negative cell and their bactericidal activity against Gram negative bacteria.

Effect of pH, calcium concentration and organic matter on antibacterial efficacy of oxifulvic acid

pH

The functional group content of oxifulvic acid (Table 1) indicated that oxifulvic acid was a mixture of carboxylic acids and phenolic compounds. These compounds are adversely affected by an increase in environmental pH, with a reduction in antimicrobial activity as the pH increases. (Russel and Chopra, 1990). This is due a reduction in the concentration of the undissociated molecule, which is ascribed to be the antimicrobial fraction (Eklund, 1983; Russel and Chopra, 1990). Increasing the pH above the pK_a of an organic acid reduces the percentage of undissociated molecule and thus the antimicrobial activity.

Therefore, the decreased activity of oxifulvic acid above pH 5.0 could be due to less of the carboxylic acid and phenolic compounds being undissociated and thus unable to exert antibacterial activity. The results (Table 3) indicated that the optimum pH for bactericidal activity of oxifulvic acid is at a pH < pH 5.0 and > pH 3.0.

The difference in the results obtained at pH 4.0 in the buffered and unbuffered systems was due to the fact that no viable cells could be enumerated from the pH 4.0.1 M Citric acid/K_2HPO_4 buffer control tubes. A solution at pH 4.0, containing 0.1 M K_2HPO_4 would not be expected to exhibit any bactericidal activity against *P. aeruginosa, S. aureus* or *P. fluorescens*, due to the pH of the solution (Tanner and James, 1992). Therefore, cell death in the pH 4.0 control could be caused by the presence of the citric acid (0.1 M) in the buffered control. Citric acid is a known antimicrobial agent (Cherrington *et al.*, 1991) and is used for preservation in the food industry. With a pK_a value of 3.1 a proportion of the citric acid would be in the undissociated form at pH 4.0. The undissociated citric acid would therefore be able to cause the death of the cells in the controls, since the antibacterial activity of organic acids is ascribed to the presence of the undissociated acid molecule (Cherrington *et.al.*, 1991).

Calcium

Solutions containing calcium have been used in the evaluation of antimicrobial agents in tests such as the Kelsey-Sykes and Maff disinfection tests (Crowshaw, 1981). The calcium is added to simulate hard water conditions which in certain cases can seriously inhibit the antimicrobial action of compounds. The results (Table 3) indicated that calcium inhibited the bactericidal activity of oxifulvic acid. The linear relationship between the calcium concentration and bactericidal concentration of oxifulvic acid (Table 3) indicated that binding of the oxifulvic acid functional groups by calcium reduced the concentration of oxifulvic acid available for bactericidal action. In the case of *S. aureus* at 125.0 mg/l calcium, 50.0 mg/l oxifulvic acid was required for bactericidal action. However, as seen for the control, 25.0 mg/l oxifulvic acid was bactericidal against *S. aureus*, indicating that 25.0 mg/l of oxifulvic acid was removed by the calcium. At 250.0 mg/l calcium, a two-fold increase of oxifulvic acid (100.0 mg/l) was required for bactericidal action.

Calcium could inactivate the oxifulvic acid by binding to the carboxylic and phenolic functional groups present in the oxifulvic acid molecule, affecting the antibacterial activity of the oxifulvic acid. Binding of the calcium by the dissociated carboxylic and phenolic functional groups would thus reduce the number of functional groups available for bactericidal action. The actual mechanism of interference was not investigated since the main purpose of this investigation was to determine the effect of calcium of the bactericidal activity of the oxifulvic acid. The removal of calcium ions from solution by oxifulvic acid (Swart *et al.*, 1990) indicated that the bactericidal activity of oxifulvic acid was affected by adsorption of the calcium ions to the undissociated carboxylic and phenolic functional groups present in the oxifulvic acid. The use of oxifulvic acid as biocide in hard water would be limited due to the increase concentration of oxifulvic acid required for bactericidal activity in the presence of calcium.

Organic matter

The eight-fold (Table 3) increase in oxifulvic acid concentration required for bactericidal activity against *P. aeruginosa, P. fluorescens* and *S. aureus* indicated that organic matter (*i.e.* yeast extract) inhibited the bactericidal activity of oxifulvic acid. Interference by organic matter is due to either a reaction between the biocide and organic matter or the organic matter may protect the bacteria from the biocide (Russel and Chopra, 1990). The presence of organic matter increases the concentration of phenol and organic acids required for bactericidal action (Gelinas and Goulet, 1983; Russel and Chopra, 1990). Due to the presence of carboxylic and phenolic functional groups in oxifulvic acid, an increase in bactericidal concentration of oxifulvic acid was, therefore, expected in the presence of organic matter. The exact mechanism of interference was not investigated since the main purpose of this investigation was to determine the effect of organic matter on the bactericidal activity of oxifulvic acid. The use of oxifulvic acid as biocide under soiled (*i.e.* high concentrations of organic matter) conditions would be limited due to the increase concentration of oxifulvic acid required for bactericidal activity in the presence of organic matter. Bactericidal activity would only be obtained once the capacity of the inactivating organic matter has been satisfied to leave a residual concentration which would be bactericidal.

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Table 1 Functional group content of different coal-derived products

Product	Total acid content (meq/g)	Carboxylic group content (meq/g)	Phenolic group content (meq/g)
Oxicoal	5.28	1.24 (23%)"	4.04 (77%)"
Oxihumic acid	7.50	3.40 (45%)"	4.10 (55%)"
Butanone extract*	10.50	8.30 (80%)"	2.20 (20%)"
Methanol extract+	12.10	11.80 (97%)"	0.30 (3%)"
Oxifulvic acid#	11.80	10.70 (90%)"	1.10 (10%)"

" Functional group content as percentage of total acidity

* Butanone extract of oxifulvic acid

+ Methanol extract of oxifulvic acid

Raw oxifulvic acid (Oxifulvic acid from reactor before extraction)

Table 2 Bactericidal concentrations of different coal-derived products evaluated against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

	Bactericidal (100 % kill) concentration (mg/l)	
Product	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Oxicoal	8 000	10 000
Oxihumate	10 000	10 000
Oxihumic acid	2 000	4 000
Oxifulvic acid*	300	100
Oxifulvic acid+	200	400
Oxifulvic acid#	100	25

* Butanone extract of oxifulvic acid

\+ Methanol extract of oxifulvic acid

\# Oxifulvic acid (Oxifulvic acid from reactor before extraction)

Table 3 Effect of pH, calcium concentration and organic matter on the bactericidal concentration of oxifulvic acid

Factor	Bactericidal concentration (mg/l)					
	Staphylococcus aureus		*Pseudomonas aeruginosa*		*Pseudomonas fluorescens*	
Control*	25		100		75	
Calcium concentration (mg/l)						
125	50		200		100	
250	100		400		200	
500	200		800		400	
Organic matter#	200		800		400	
pH	Adj	Buff	Adj	Buff	Adj	Buff
3.0	NG	NG	NG	NG	NG	NG
4.0	100	NG	400	NG	200	NG
5.0	> 10 000	> 10 000	> 10 000	> 10 000	> 10 000	> 10 000
7.0	"	"	"	"	"	"
9.0	"	"	"	"	"	"

* Bactericidal concentration in unbuffered quarter strength Ringers solution

\# Bactericidal concentration in the presence of 5000.0 mg/l yeast extract

Adj Bactericidal concentration in pH-adjusted quarter strength Ringers solution

Buff Bactericidal concentration in buffered quarter strength Ringers solution

NG No growth (Bacteria inhibited by pH of inoculated buffered and unbuffered controls)

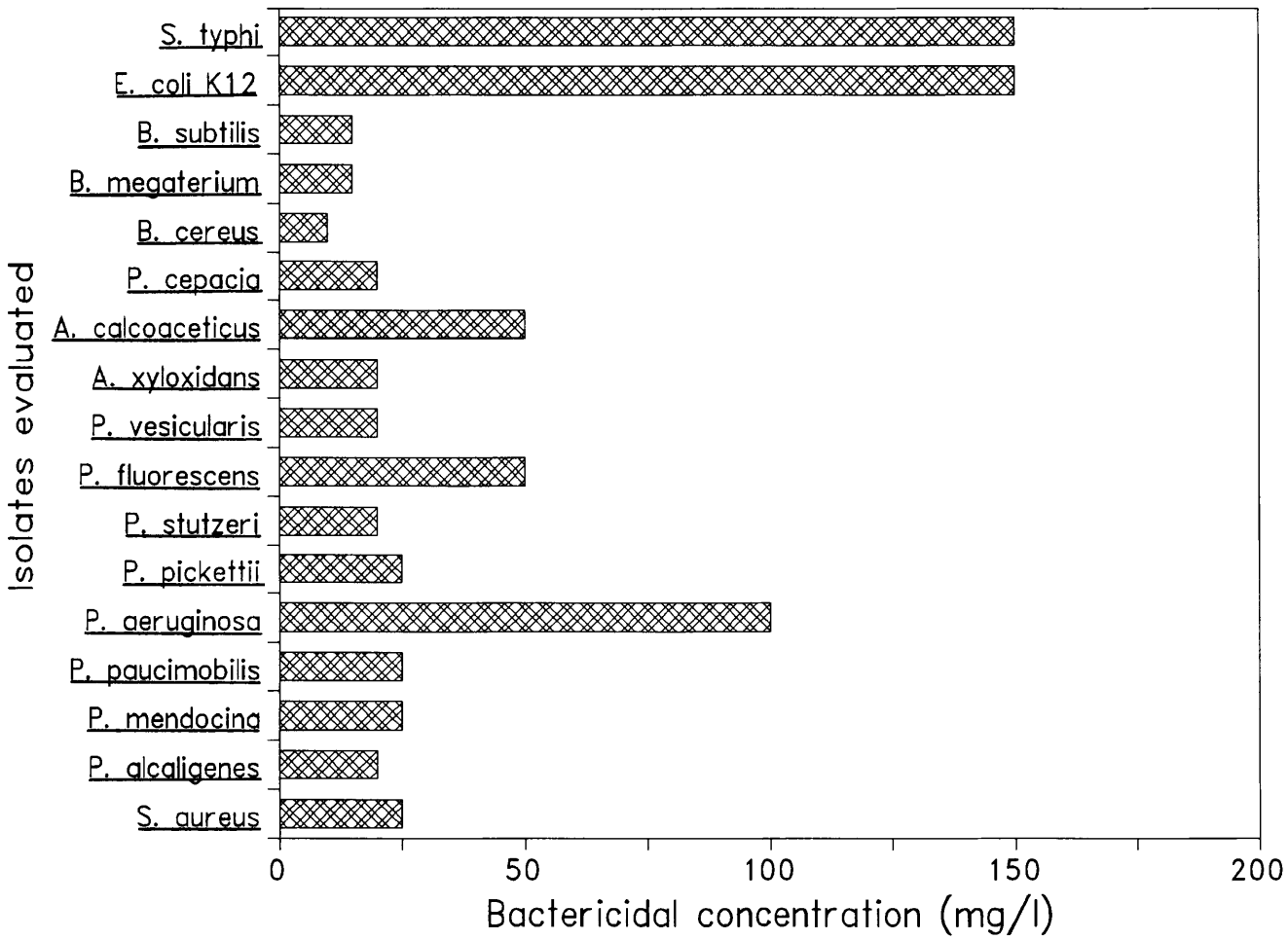

Fig. 1. Bactericidal concentration of oxifulvic acid against seventeen test strains exposed to oxifulvic acid (added at 0.0 to 200.0 mg/l) for 6 h.

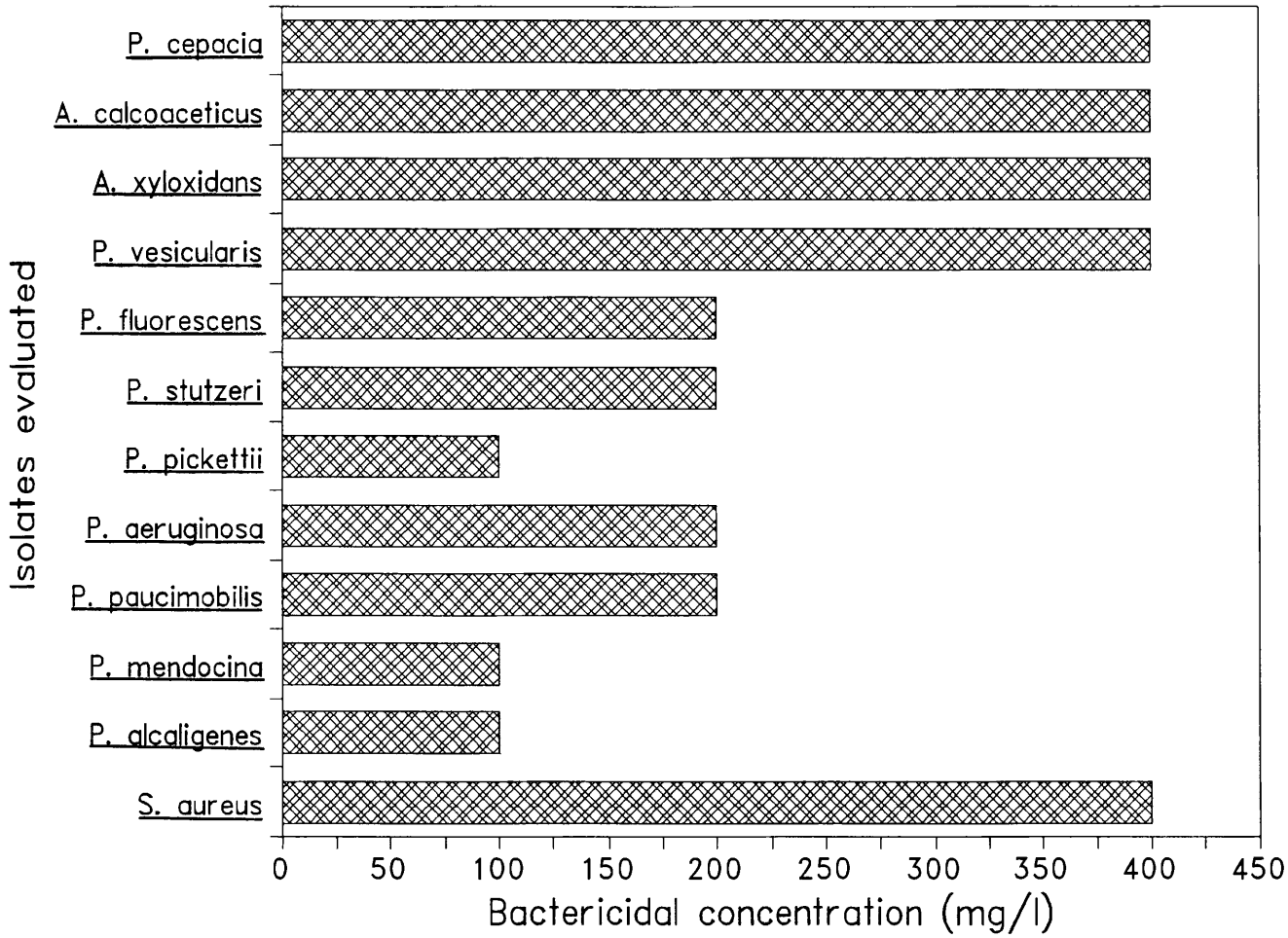

Fig. 2. Bactericidal concentration of oxifulvic acid (methanol extract) against twelve test strains exposed to oxifulvic acid (added at 0.0 to 500.0 mg/l) for 6 h.

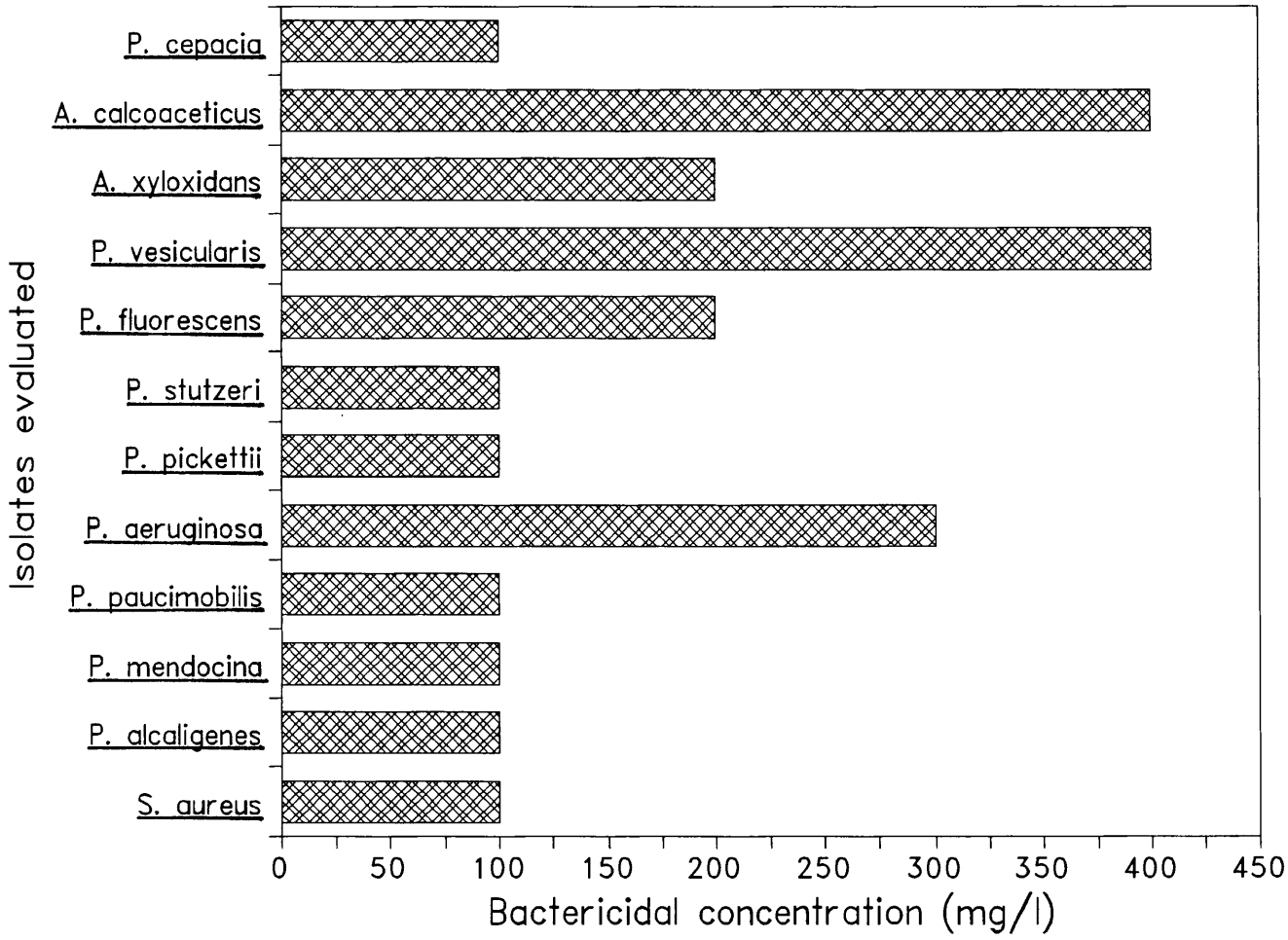

Fig. 3. Bactericidal concentration of oxifulvic acid (butanone extract) against twelve test strains exposed to oxifulvic acid (added at 0.0 to 500.0 mg/l) for 6 h.

CHAPTER 4

(Submitted for publication in the Journal of Industrial Microbiology)

BIOCIDAL AND DISINFECTANT ACTIVITY OF OXIFULVIC ACID

The language and style used in this chapter is in accordance with the requirements of the Journal of Industrial Microbiology.

SUMMARY

Oxifulvic acid was evaluated for use as a biocide or disinfectant by determining the bactericidal, fungicidal, algicidal, sporicidal and virucidal activity of the compound. Possible synergistic combinations were also investigated. The bactericidal activity of oxifulvic acid was determined against a range of bacteria including iron-oxidizing bacteria. Oxifulvic acid was bactericidal at 150 mg/l and proved to be more effective than a commercially available coal-tar disinfectant. Oxifulvic acid was virucidal within 10 min at 96.0 mg/l. The concentrations of oxifulvic acid required for fungicidal (> 4000.0 mg/l) and algicidal (400.0 mg/l) activity indicated that oxifulvic acid did not have potential for use as a fungicidal or algicidal compound. Oxifulvic acid did not exhibit any sporicidal activity. Complete inhibition of *Pseudomonas aeruginosa* was exhibited by a combination of oxifulvic acid (50.0 mg/l) and sodium lauryl sulphate (125.0 mg/l). This combination was the most synergistic combination evaluated.

INTRODUCTION

Antimicrobial agents comprise a group of substances that either inhibit growth, cause death of microorganisms, or destroy spores. Antimicrobial agents include disinfectants, antiseptics, preservatives and antibiotics. The term biocide is a general term that encompasses antiseptic, disinfectant and preservative activity and denotes a non-chemotherapeutic agent that kills microorganisms [47]. The term has also been used to describe antimicrobial substances used to control microbial growth in water-cooling systems [10].

In South Africa the biocide market is worth *ca.* R 18.3 million per annum [13].

Currently more than 90 % of the raw materials for the manufacturing of biocides in South Africa are imported [12]. A market therefore exists in South Africa for the production of a low cost and effective biocide. Oxifulvic acid was found to be the most bactericidal coal-derived product evaluated in a previous study [13]. This product was therefore evaluated as a potential low cost biocide for the South African market.

The growth and proliferation of bacteria, algae and fungi in industrial water-cooling systems may cause severe problems [7,19,20,27,29,37,43]. Two main problems are associated with microbial growth, namely: biofouling and biocorrosion. Biofouling of water-cooling systems leads to increased fluid friction, reduced heat transfer, clogging of filters, degradation of wooden structures and may cause or accelerate corrosion of the system [16,27,29]. Biocorrosion in a system reduces process efficiency and equipment service life, with major economic consequences [20,37]. The use of biocides is one of the most effective ways to prevent biofouling and biocorrosion [7,19,41]. The use of oxifulvic acid to control microbial growth in water-cooling systems was evaluated by determining antimicrobial activity of oxifulvic acid against bacteria, fungi and algae.

The bactericidal activity of oxifulvic acid was evaluated against bacteria isolated from South African water-cooling systems by Cloete *et al.* [12] using the bactericidal assay described by Brözel and Cloete [10]. Field trial evaluations were conducted in a water-cooling system to determine the efficacy of oxifulvic acid to control bacterial growth *in situ*. The efficacy of oxifulvic acid was monitored using planktonic populations since Cloete *et al.* [14] indicated that planktonic populations could be used to determine the efficacy of a biocide treatment. Monitoring of the sessile population was included since they are responsible for the biofouling and biocorrosion in water-cooling systems [37].

The antifungal activity of compounds can be evaluated using either a suspension test or by mixing the compound with an agar medium [25]. The agar medium test was selected to evaluate the fungicidal activity of oxifulvic acid since this method is used to determine the antifungal activity of compounds used for wood preservation [25].

Algal growth is controlled by chlorination [20]. Chlorination is, however, not always successful since certain algal specie are chlorine resistant [20]. The chlorine resistance is overcome with the use of algicides [41]. A number of methods are available to determine the activity of algicidal compounds [22]. These methods include the use of solid test pieces [38], agar plate tests and qualitative suspension tests [39]. The algicidal efficacy of oxifulvic acid was determined using the South African Bureau of Standards (SABS) method [48] with modifications described by Bosch *et al.* [8].

Disinfectants have been described as bactericidal agents that are used for the disinfection of equipment (*e.g.* surgical instruments) or inanimate objects [47]. Disinfectants include antibacterial agents that are too toxic, irritant or corrosive to be applied to body surfaces or tissues [47]. Since disinfection has been defined as the selective elimination of certain undesirable microorganisms, the purpose of testing disinfectants is to determine whether microorganisms are killed or eliminated by the action of the disinfectant [47]. The criteria which are used to determine whether a disinfectant is effective depends on the evaluation method used. Disinfectants do, however, have to comply with certain basic criteria as described by Ayliffe and Collins [4]. A chemical compound cannot be regarded as a disinfectant if it not active against vegetative bacteria [4,47]. The activity of a disinfectant against mycobacteria, bacterial spores, fungi and viruses depends on the area of application [46]. The activity of oxifulvic acid against bacteria, bacterial spores and viruses were, therefore, determined.

Two types of sporicidal tests have been described in the literature, namely: suspension and carrier tests [46]. Suspension tests are usually modified bactericidal suspension tests, the Dutch 5-5-5 test being an example [46]. The Dutch 5-5-5 test requires a one log reduction in spore count after a 5 min exposure to the test compound, for the compound to be considered effective. Since carrier tests are used to determine the use-dilution of a disinfectant [46] a suspension test was selected to determine the sporicidal activity of oxifulvic acid.

Tests to determine the activity of disinfectants against viruses are varied [44]. Most of the tests are based on the *in vitro* techniques described in the bactericidal suspension test of the American Association of Analytical Chemists (AOAC) [53]. The evaluation of the antiviral activity of disinfectants has the disadvantage that since disinfectants are toxic to living cells they would be toxic to the cells used in tissue culture for the detection of viruses [46]. This disadvantage creates technical problems with the use of human viruses, as test organisms, for the evaluation of the virucidal disinfectants. Coliphages can be used as an alternative to human viruses since they are used as indicators of the presence of human enteric viruses in water [32]. Furthermore, coliphages are used as model viruses in studies to determine the effect of chlorination, flocculation and adsorption on human enteric viruses [21,35]. Therefore, the effect of oxifulvic acid on a coliphage was determined as an indication of its activity against enteric viruses.

Iron-oxidizing bacteria are responsible for the accelerated oxidation of pyrite (FeS_2), present in gold and coal mine dumps, with the formation of acid drainage [33,51]. The release of acid drainage into water systems increases the acidity, water hardness, metal-ion and suspended solids concentrations. The lipophilic benzoic and sorbic acids

have been shown to inhibit iron-oxidizing bacteria *in vitro* at 10.0 mg/l [40]. The effectiveness of oxifulvic acid against the iron-oxidizing bacteria was therefore studied to determine whether oxifulvic acid could be used to inhibit the formation of acid mine drainage.

Synergism between bactericides has been reported and used to enhance activity or increase the spectrum of activity of bactericides [34]. The results of synergism studies may be interpreted by calculating the fractional inhibitory concentration (FIC) index [5]. A mixture is synergistic when the FIC is ≤ 0.5, additive when the FIC is between 0.5 and 1.0, and antagonistic if the FIC is above 1.0. Synergism studies with oxifulvic acid were initiated to determine whether the activity of the oxifulvic acid could be enhanced by the use of oxifulvic acid in combination with other antimicrobial compounds.

This study reports on the evaluation of oxifulvic acid for application as a: (1) Biocide to control biofouling and biocorrosion in industrial water systems, (2) Disinfectant for the control of bacteria, bacterial spores and viruses on inanimate objects and (3) To control the formation of acid mine drainage by iron-oxidizing bacteria. The investigation of synergistic combinations to enhance the bactericidal activity of oxifulvic acid was included in this investigation.

MATERIALS AND METHODS

Production of Oxifulvic Acid

Oxifulvic acid, prepared by the controlled wet oxidation of South African bituminous coal [17], was obtained from the Division for Energy Technology, Council for Industrial and Scientific Research (CSIR), Pretoria, South Africa. Oxifulvic acid was obtained as a solution.

Test organisms

Bacterial cultures obtained from the culture collection of the Environmental Biotechnology Laboratory in the Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa were used. All test isolates with the exception of *Escherichia coli* strain K12 and *Salmonella typhi* were isolated from water-cooling systems in South Africa by Cloete *et al.*,[12]. *Escherichia coli* strain K12 and *Salmonella typhi* (obtained from Prof W.O.K. Grabow, Head, Department of Medical Virology, University of Pretoria) were isolated from domestic wastewater and identified using the API system. Bacterial cultures were maintained on Standard One nutrient agar (STD1) (Biolab, Merck, South Africa) slants at 4 °C.

Inhibition of *Thiobacillus ferrooxidans* WLR (water leach residue) and a iron-oxidizing mixed culture was investigated using the assay described by Bosch [9]. The *T. ferrooxidans* WLR strain was obtained from a previous study [9]. The iron-oxidizing mixed culture was isolated by inoculating 100.0 ml of a ferrous iron medium [24] with 10.0 ml acid drainage from a coal discard dump at Witbank, South Africa. The inoculated medium was incubated at 28°C until growth occurred, as indicated by a change in the colour of the medium from blue to brown, due to the oxidation of ferrous iron. The oxidation of ferrous iron was monitored using the potassium dichromate titration of Vogel [50], with modifications described by Conradie [15].

Fungal cultures (Table 1) were obtained from a waste paper recycling plant (Nampak, Rosslyn, Pretoria) and the Plant Protection Research Institute (PPRI) (Pretoria, South Africa) (Table 1). Fungi from the waste paper recycling plant were isolated by plating serial dilutions onto Potato Dextrose agar (PDA) (Biolab, Merck, South Africa) and incubating at 20°C. Dominant fungi, isolated from the highest dilutions showing

growth, were purified by repeated sub-culture on PDA. The dominant fungi isolated from the waste paper recycling plant were identified by the PPRI. All fungal cultures were maintained on PDA at 4 °C.

Algae were isolated from an open recirculating water-cooling tower at the CSIR, Pretoria, South Africa. The algae were isolated by suspending growth, scraped from the sides of the sump and drift eliminators in the water-cooling tower in 100.0 ml water-cooling tower water. The sample was then homogenized for 2 min in a high speed blender. Serial dilutions of the homogenate were made in quarter strength Ringer solution and plated out on algal cultivation medium CM [48], incubated under continuous illumination at 26 °C as described by Bosch *et al.* [8]. Single colonies were purified by repeated sub-culture on CM and identified using the key of Palmer [41].

Evaluation of oxifulvic acid as biocide

Bactericidal assay. The bactericidal activity of oxifulvic acid was evaluated using the bactericidal assay described by Brözel and Cloete [10]. Oxifulvic acid was added from a 10000.0 mg/l stock solution to quarter strength Ringers solution to yield oxifulvic acid concentrations of 0.0 to 200.0 mg/l. An bacterial inoculum for the bactericidal assay was prepared as described by Brözel and Cloete [10]. The bactericidal assay was initiated by the addition of 1.0 ml bacterial suspension (*ca.* 10^7 cells /ml) to the test solution (9.0 ml). The resulting mixture (10.0 ml) was incubated at 28°C for 6 h, when viable cell counts were determined [10]. Control solutions contained 1.0 ml cell suspension in 9.0 ml quarter strength Ringers solution. The concentration yielding a percentage kill of 100% was considered to be the bactericidal concentration. Each assay was done in duplicate and duplicate dilutions were plated for the determination of viable cell counts.

Field trial evaluations. A biofouling test rig (Fig. 1) obtained from the CSIR (Dr R.E.M. Archibald, Watertek, CSIR, Pretoria, South Africa) was used for the field trial evaluation of oxifulvic acid. The biofouling test rig was connected to a water-cooling system at the Iron and Steel Corporation (ISCOR), Pretoria, South Africa. The test rig consisted of 4 tubes connected to holding drums (100 l). Water from the sump of the water-cooling system at ISCOR was pumped into the holding drums. Pumps then circulated the water through the tubes. The growth of sessile bacteria was monitored using metal coupons (corrosion coupons) placed in tubes 1 to 4, parallel to the flow of water through the tubes.

The flow rate, retention time and holding time index of each tube were determined as described by Warner [52]. The flow rate was determined at the outflow from the holding drums. The biofouling test rig was left for 11 d to allow for the growth of sessile bacteria on the metal coupons in tubes 1 to 4. This trial was run over a period of 39 d.

Tubes 1 to 4 of the biofouling test rig were used for the evaluation of oxifulvic acid and hydrogen peroxide (H_2O_2). The H_2O_2 was added to tube 1 of the biofouling test rig, at 50 mg/l. Tubes 2 and 3 were treated with oxifulvic acid at concentrations of 200.0 and 400.0 mg/l respectively. Tube 4 was left untreated as a control. The biocides, oxifulvic acid and H_2O_2, were added in slug doses at 48 h intervals beginning at 11 d until 25 d, from 25 d to 30 d the biocides were added at 24 h intervals.

Samples to determine the total aerobic planktonic and sessile populations were taken at 5 d intervals from 5 d to 39 d. The planktonic populations were determined by taking samples (*ca.* 100.0 ml) from the holding drums, using whirl-pack bags (Norton Dairy Equipment, Johannesburg, South Africa), of tubes 1 to 4, respectively. The sessile population present in the biofouling test rig was determined by removing one metal

coupon at 5 d intervals from tubes 1 to 4, respectively. The metal coupons from each tube were placed in separate sterile McCartney bottles containing 10.0 ml sterile quarter strength Ringers solution. The McCartney bottles containing the metal coupons were sonicating for 10 min at 47.5 khz in a jewellers bath prior to serial dilution in quarter strength Ringers solution. All samples were kept below 4 °C in transit to the laboratory where duplicate serial dilutions were made in quarter strength Ringers solution and plated onto R_2A agar [45]. The plates were incubated at 28 $_oC$ for 7 d. After incubation the visible colonies were counted using an illuminated counting chamber. The planktonic bacteria were plotted as colony forming units (cfu)/ml. The sessile population present per square centimetre (cfu/cm^2) of the metal coupon was determined using the following formula:

cfu/cm^2 = sessile (cfu/ml) x sample volume sonicated (ml) ÷ area of metal coupon (cm^2)

Fungicidal activity of oxifulvic acid. The antifungal activity of oxifulvic acid was determined using the agar medium test described by Hilditch and Mendes [25]. Fungal spore suspensions were prepared as follows: Agar discs (5.0 mm diameter) of actively-growing mycelia were placed on PDA plates and incubated at 28 °C. After 7 d spores were washed from the surface of the plates with sterile distilled water containing 2.0% Tween 80. Spores were harvested by centrifugation (10 000 x g for 10 min), resuspended in sterile quarter strength Ringers solution and stored at 4 °C until needed. Oxifulvic acid (10000.0 mg/l stock solution) was dissolved in 100.0 ml molten PDA, Nutrient agar (NA), STD1 agar and water agar (WA) (1.5% agar in sterile distilled water) at the test concentration (0.0, 200.0, 400.0, 600.0, 800.0, 1000.0, 2000.0, and 4000.0 mg/l) and dispensed in 20.0 ml volumes. Concentrations of oxifulvic acid >

4000.0 mg/l were not evaluated since these concentrations prevented the respective agar media (*i.e.* PDA,NA, STD1 and WA) from solidifying. The plates were then inoculated, in the centre, with 10.0 μl of spore suspension using a micro pipette. After inoculation the plates were incubated at 28 ºC. Plates of PDA, NA, STD1 and WA, respectively, containing no oxifulvic acid were used as inoculated controls. Radial growth on the treated and controls plates was measured after incubation at 3, 6 and 14 d

Fungi exhibiting no radial growth were transferred to PDA plates containing no oxifulvic acid. This step was used to determine whether oxifulvic acid was fungicidal or fungistatic. Oxifulvic acid was considered fungicidal if no growth occurred on the re-inoculated PDA plates, and fungistatic if growth occurred on the re-inoculated PDA plates, after 14 d incubation at 28 ºC .

Algicidal activity of oxifulvic acid. The algicidal activity of oxifulvic acid was evaluated using three algal species namely *Chlorella, Chlorococcum* and *Calothrix* isolated from an open recirculating water-cooling tower at the CSIR, Pretoria, South Africa. The algicidal efficacy was determined using the SABS method [48], with modifications described by Bosch *et al.* [8]. Oxifulvic acid was added to duplicate flasks containing culture medium [48] (CM) from a 10000.0 mg/l stock solution to give concentrations of 0.0, 50.0, 100.0, 200.0, 400.0 and 800.0 mg/l. The flasks were incubated at 26ºC and inspected visually for growth after 5, 10 and 28 d. Oxifulvic acid was considered effective if no visible growth was evident after 28 d in CM. In order to determine whether oxifulvic acid was algicidal or algistatic, 5.0 ml of the algal suspension exposed to oxifulvic acid was removed after 5 and 10 d and re-inoculated into fresh CM, without oxifulvic acid. If any re-growth occurred in the re-inoculated CM after 14 d

incubation, oxifulvic acid was considered algistatic. If no re-growth occurred in the re-inoculated CM after 14 d incubation, oxifulvic acid was considered to be algicidal.

Disinfectant evaluation of oxifulvic acid

Bactericidal activity. The disinfectant activity of oxifulvic acid and a commercially available coal tar disinfectant were evaluated at 26 °C using the method described by Jeffrey and Matthews [30]. *Pseudomonas aeruginosa, S. aureus* and *E. coli* strain K12 were used as test organisms.

Sporicidal activity of oxifulvic acid. Spore suspensions were prepared as described by Beeby and Whitehouse [6]. *Bacillus cereus, B. megaterium and B. subtilis* were cultured on NA containing 0.03 g/l $MgSO_4$ and 0.25 g/l KH_2PO_4 at 30 °C for 7 d to give *ca.* 95% sporulation as determined by observation of samples stained with 0.5% ($^{w}/_{v}$) Methylene blue solution (Sigma, South Africa). Spores were harvested by centrifugation (10 000 x g at 4°C for 10 min) and washed three times in sterile quarter strength Ringers solution. The washed suspension (*ca.* 10^7 spores/ml) was stored at 4°C until required. The assay was initiated by the addition of 1.0 ml spore suspension to 9.0 ml quarter strength Ringers solution, containing oxifulvic acid (10000.0 mg/l stock solution) at 0.0, 1000.0, 2000.0, 4000.0 and 8000.0 mg/l, to give a final count of *ca.* 10^6 spores/ml. Control flasks contained 1.0 ml spore suspension in 9.0 ml quarter strength Ringers solution. The treated and control flasks were incubated at 28°C for 48 h. Samples (1.0 ml) were withdrawn at 6, 12, 24 and 48 h and suspended in 9.0 ml Nutrient Broth (NB) (Biolab, Merck, South Africa), to inactivate the oxifulvic acid by dilution. Serial dilutions were made in sterile quarter strength Ringers solution and the viable spore count determined on

NA using the pour-plate method of Beeby and Whitehouse [6]. For all experiments the viable count was determined for the treated flasks and an untreated control. The oxifulvic acid stock solution (1000.0 mg/l) was included as a control. The results were expressed as percentage kill [6], with 100% kill of spores as an indication of sporicidal activity.

Virucidal activity of oxifulvic acid. The virucidal activity of oxifulvic acid was determined using coliphage V_1, (Prof. W.O.K. Grabow, Department of Medical Virology, University of Pretoria, Pretoria, South Africa). A phage titre stock of (*ca.* 10^6-10^7 pfu/ml) was prepared using *E. coli* strain C (Prof. W.O.K. Grabow, Department of Medical Virology, University of Pretoria, Pretoria, South Africa) as host according to the method described by Adams [1]. Titre stock (100.0 μl) was added to 9.9 ml quarter strength Ringers solution containing 0.0, 12.0, 24.0, 48.0, 96.0, 192.0, 348.0 and 768.0 mg/l oxifulvic acid. The inoculated flasks were incubated at 28°C for 10 min. After 10 min 1.0 ml was removed and diluted in 9.0 ml quarter strength Ringers solution. Serial dilutions were made in 9.0 ml quarter strength Ringers solution and the viable phage count determined by the agar-overlay method [1], using *E. coli* C as host.

Investigation of synergism between oxifulvic acid and other antimicrobial compounds

A possible synergistic action between oxifulvic acid (0.0 to 200.0 mg/l) and ethylenediaminetetraacetic acid (0.0 to 1000.0 mg/l) (EDTA, Unilab, Saarchem, South Africa), a quaternary ammonium compound (QAC) Tetra-amine (0.0 to 100.0 mg/l) (Merck, South Africa), hydrogen peroxide (0.0 to 24.0 mg/l) (30% solution, Saarchem, South Africa), copper (0.0 to 100.0 mg/l Cu^{2+}) as copper sulphate solution (Merck, South

Africa), ethanol (2.5 to 80% $^{v}/_{v}$) (99%$^{v}/_{v}$, Merck, South Africa), sodium dodecyl sulphate (0.0 to 8000.0 mg/l) SDS (Merck, South Africa) and sodium hypochlorite (0% to 24% $^{v}/_{v}$) (10% $^{v}/_{v}$ solution, Saarchem, South Africa) was investigated. The chess board procedure described by Hodges and Hanlon [26] was used to evaluate the combinations.

Bactericidal effect against iron-oxidizing bacterial cultures

Inhibition of *Thiobacillus ferrooxidans* WLR and the iron-oxidizing mixed bacterial culture were investigated in the liquid medium (HJJ medium) of Harrison *et al.* [24]. Oxifulvic acid was added to duplicate flasks, from a 10000.0 mg/l stock solution, to give concentrations of 0.0, 25.0, 50.0, 75.0 and 100.0 mg/l. The inhibition of the iron-oxidizing bacteria was determined using the assay described by Bosch [9]. The oxidation of ferrous iron in excess of that in an uninoculated control medium was monitored as an indication of bacterial growth, using the potassium dichromate titration of Vogel [50], with modifications described by Conradie [15].

RESULTS AND DISCUSSION

Evaluation of oxifulvic acid as biocide

Bactericidal assay. Oxifulvic acid was bactericidal at 100.0 mg/l against all the dominant bacteria isolated from South African water-cooling water systems (Fig. 2). *P. aeruginosa, P. fluorescens* and *Acinetobacter calcoaceticus* were the most resistant to oxifulvic acid (Fig. 2). These isolates were inhibited by 100.0, 50.0 and 50.0 mg/l oxifulvic acid, respectively (Fig. 2). The remaining nine isolates were all inhibited by an

oxifulvic acid concentration ≥ 25.0 mg/l (Fig. 2).

The most frequently occurring (35.5% of population) bacterium isolated from South African water-cooling systems by Cloete *et al.* [12] was *Pseudomonas fluorescens*. Oxifulvic acid would, therefore, be considered an effective biocide for the control of biofouling and biocorrosion in South Africa water-cooling systems if it was effective against *P. fluorescens*. This is, however, not true since biocide addition to water-cooling systems would result in the bacterial species most resistant to the added biocide becoming the dominant population [11]. Oxifulvic acid would thus have to be effective against a range of bacteria isolated from water-cooling towers before it could be considered an effective biocide. Furthermore, *P. aeruginosa* was more resistant to oxifulvic acid (100.0 mg/l) than *P. fluorescens* (50.0 mg/l). This result indicated that in systems where both bacteria occurred *P. aeruginosa* would become the dominant specie. A concentration of oxifulvic acid bactericidal to *P. aeruginosa* would have to added to control growth of this bacteria.

The bacterium most resistant to oxifulvic acid was a *P. aeruginosa* isolate since 100.0 mg/l oxifulvic acid was required for bactericidal activity against this *P. aeruginosa* isolate. The intrinsic resistance of *P. aeruginosa* species to biocides has been widely reported in literature [47]. The increased intrinsic resistance of the *P. aeruginosa* isolate to oxifulvic acid is therefore in agreement with the resistance of *P. aeruginosa* species to biocides reported in the literature. The increased resistance of *P. aeruginosa* species has been ascribed to the presence of the lipopolysaccharide layer in the cell wall of *P. aeruginosa* and other Gram negative bacteria [47].

Comparison of the percentage kill fingerprint obtained with the oxifulvic acid to other biocide fingerprints, obtained by Brözel and Cloete [10], indicated that oxifulvic

acid ranked amongst the top ten biocides currently available in South Africa [10].

Field trial evaluations. Oxifulvic acid was unable to inhibit the growth of planktonic and sessile bacteria in tubes 2 and 4, at 200.0 and 400.0 mg/l, respectively, in the field trial evaluation using the CSIR biofouling test rig (Fig 3, 4). The flow rate, retention time and holding time index of tubes 1 to 4 in the biofouling test rig are shown in Table 2.

The different flow rates and retention times (Table 2) in the holding drums of tubes 1 to 4 made comparison of the results obtained between tubes difficult since conditions were not uniform. The different flow rates were due to flaws in the design and malfunctions of the pumps supplying water to tubes in the biofouling test rig. Flow rates of 4.14, 15.00, 5.07 and 16.82 l/min were recorded for tubes 1 to 4, respectively (Table 2). The retention times calculated for tubes 1 to 4 were $\leq$ 60 min (Table 2). However, a retention time of at $\geq$ 6 h is required before a system can be use to effectively evaluate the effectiveness of a biocide [14]. In two open recirculating cooling water systems, of 50 and 1400 m^3, used to evaluate biocides Cloete *et al.* [14] calculated their retention times to be 6.6 and 40 h respectively. Retention times in cooling towers are dependant on various factors and may vary between 8 and $>$ 24 h [52]. The retention times for the biofouling test rig were therefore not similar to retention times found in practice.

The total number of bacteria from the planktonic and sessile samples are shown in Figs. 3 and 4. The planktonic populations (Fig. 3) in the water from all the tubes increased from 5 d to 39 d, when sampling was stopped. No correlation between biocide dosage and planktonic count could be seen since the planktonic populations in the treated as well as the control tubes were all in the same order of magnitude. Addition of the biocides at 24 h intervals from 25 d had no effect on the planktonic populations. This was

due to insufficient contact time between biocide and planktonic bacteria as a result of the short retention times in tubes 1 to 3.

The biocide dosage has no effect on the growth of the sessile bacteria during the field trial (Fig 4) since the sessile population increased from 15 d to 39 d. The sessile counts from the tubes indicated that a biofilm containing *ca.* 10^5 cells/cm^2 was formed within 10 d. The sessile counts increased to log 6 cfu/cm^2 in all tubes till 15 d, regardless of biocide treatment. After 15 d the sessile counts decreased to log 5 cfu/cm^2 and remained stable at log 5 cfu/cm^2 until 39 d, with the exception of tube 3. The decrease at 15 d could not have been due to biocide addition since the sessile population in the control tube also decreased. Biocide addition at 24 h interval from 25 d had no effect on the sessile population in tubes 1 to 3 (Fig. 4). The sessile count in tube 3 (400.0 mg/l oxifulvic acid) increased from log 5 cfu/cm^2 at 20 d to log 7 cfu/cm^2 at 35 d. This increase suggested that the addition of oxifulvic acid at 400,0 mg/l stimulated the growth of the sessile bacteria. The stimulation of growth was suspected since the sessile counts recorded in tube 3 were higher (log 7 cfu/cm^2) than the values recorded in the control tube 4 (log 5 cfu/cm^2) over the corresponding period.

The results obtained in the field trial evaluation suggested that oxifulvic acid did not have the potential to be used as a biocide to inhibited the growth of planktonic and sessile bacteria in water-cooling systems since in was unable to control bacterial growth in the biofouling test rig.

Fungicidal activity of oxifulvic acid. Oxifulvic acid was fungicidal against ten of the 12 fungal species used in the fungicidal evaluation (Table 3). Oxifulvic acid was most fungicidal in water agar since 600.0 to 2000.0 mg/l was required to completely inhibit

fungal growth in all cases except for *Aspergillus niger* and *Aspergillus foetidus* (Table 3). Fungal growth was inhibited at oxifulvic acid concentrations of 800.0 to 4000.0 mg/l in all the other media (*i.e.* PDA, NA and STD1) evaluated. *Aspergillus niger* and *Aspergillus foetidus* were the most resistant to oxifulvic acid since a concentration > 4000.0 mg/l was required to inhibit growth in all the agar media evaluated (Table 4).

Growth of the fungi was stimulated at non-fungicidal concentrations of oxifulvic acid in all the media evaluated. The stimulation of growth was particularly evident on the water agar plates, indicating that at sub-lethal concentrations oxifulvic acid could be used as a substrate by the fungi.

The inhibition of fungal growth by fulvic acids has not been reported. However, the antifungal activity of bark compost extracts and leachates from composted bark from *Eucalyptus* sp. has been reported [23,31]. Bark compost extracts (200 μg extract) inhibited fungal growth of four fungal species [31]. Leachates from composted *Eucalyptus* have been shown to inhibit growth, sporangial production and chlamydospore formation of certain *Phytophthora* species [23]. The fungicidal activity reported [23,31] could have been due to the presence of fulvic acids in the water soluble fraction.

The data (Table 3) indicated that oxifulvic acid does not have potential for use as a wood preservative since the fungicidal concentration required is too high (> 4000.0 mg/l) and stimulation of fungal growth occurred at sub-lethal concentrations of oxifulvic acid.

Algicidal activity of oxifulvic acid. No algal growth was visible after 28 d, and no growth occurred in sub-cultures made after 5 and 10 d, in the cultures exposed to 400.0 mg/l oxifulvic acid (Table 4). Oxifulvic acid was thus algicidal against all three isolates at 400.0 mg/l. An organo-sulphur and a quaternary ammonium algicide evaluated by Bosch

et al. [8] were algicidal at concentrations of 5.0 to 25.0 mg/l, respectively. In comparison oxifulvic acid is not as effective an algicide and therefore did not show potential for use as an algicide. The low effectivity of oxifulvic acid can be explained by the fact that compounds containing carboxylic acid and phenolic functional groups have not been reported in the literature to be effective algicides [3,8,20].

Disinfectant evaluation of oxifulvic acid

Bactericidal activity. Oxifulvic acid was bactericidal at 800.0 mg/l against *P. aeruginosa, E. coli* strain *K12* and *S. typhi*. *Bacillus cereus* and *S. aureus* were inhibited by 100.0 mg/l oxifulvic acid. The coal tar disinfectant required 3 000.0 to 48 000.0 mg/l to be effective against *P. aeruginosa, E. coli* strain *K12*, *S. typhi*, *B. cereus* and *S. aureus*. *Pseudomonas aeruginosa* and *B. cereus* were the most tolerant with 48000.0 and 24000.0 mg/l coal-tar disinfectant required for bactericidal activity respectively. *Salmonella typhi, S. aureus* and *E. coli* K12 were inhibited at 12000, 6000.0 and 3000.0 mg/l, respectively, by the coal-tar disinfectant.

Oxifulvic acid was effective at lower concentrations than the coal-tar disinfectant (Table 5). The results obtained with the coal-tar disinfectant are in general agreement with data reported by Jeffrey and Matthews [30]. The difference that occurred, between the reported data and our results, could be due to the fact that different species of bacteria were used in this evaluation. Coal-tar disinfectants are stated to have greater antibacterial activity against Gram negative bacteria, with the exception of *P. aeruginosa* [30]. The lower concentrations of oxifulvic acid required, in comparison to the coal-tar disinfectant, indicated that oxifulvic acid warrants further investigation for use as a disinfectant.

Sporicidal activity of oxifulvic acid. The activity of oxifulvic acid against spores of *B. cereus, B. subtilis* and *B. megaterium* are shown in Table 6. A percentage kill of >98% was achieved after 48 h exposure to oxifulvic acid stock solution (10000.0 mg/l). This percentage kill is equivalent to a *ca.* 2 log reduction in viable spore count.

As expected, spores had a higher resistance than vegetative cells to oxifulvic acid. Previous studies indicated that vegetative cells of *B. subtilis*, *B. megaterium* and *B. cereus* were inhibited by 15.0, 15.0 and 10.0 mg/l oxifulvic acid, respectively. In comparison with glutaraldehyde, which is sporicidal at 2% in 10 min against spores of *B. subtilis*, *B. megaterium* and *B. cereus* [46], it was concluded that oxifulvic acid was not an effective sporicidal agent since only a *ca.* 2 log reduction in viable spore count was observed. For oxifulvic acid to be considered an effective sporicidal agent no viable spores would have to be isolated after exposure.

Virucidal activity of oxifulvic acid. Oxifulvic acid was completely inhibitory at 96.0 mg/l within 10 min against coliphage V_1 (Table 7). The activity of disinfectants against vesicular stomatitis virus has been evaluated [53]. Of the twenty four disinfectants tested, ten did not exhibit any virucidal activity [53]. The substituted phenolics, halogens and cresylic acids were the most virucidal products, since the minimum effective concentrations were all below 2.0%. In comparison, oxifulvic acid was virucidal at 0.0096% (0.0096 g/100 ml). A direct comparison of the results is not possible since a different viruses was used in the evaluations [53]. The results, however, still indicated that oxifulvic acid has potential for use as a virucidal agent.

The virucidal activity of oxifulvic acid could be due to the presence of phenolic compounds (10%) and carboxylic acids (90%) in oxifulvic acid. The virucidal activity

was ascribed to the presence of the phenolic compounds, since these were the most virucidal compounds evaluated by Wright [53]. Furthermore, since acetic acid was only marginally effective at 5.0 % [53] and no virucidal activity has been reported for any other carboxylic acids [44].

Investigation of synergism between oxifulvic acid and other antimicrobial compounds

No synergistic affect was achieved in combinations of EDTA (0.0, 200.0, 400.0, 600.0, 800.0 and 1000.0 mg/l), QAC (0.0, 25.0, 50.0, 75.0 and 100.0 mg/l), hydrogen peroxide (0.0% 6.0%, 12.0% and 24%) and copper (0.0, 250.0, 500.0, 1000.0, 2000.0, 4000.0 and 8000.0 mg/l) with oxifulvic acid (0.0, 25.0, 50.0, 100.0 and 200.0 mg/l). The combinations were only bactericidal when one of the compounds, in the combination, was present at their respective bactericidal concentration.

An additive effect was seen with oxifulvic acid (50.0 mg/l) and ethanol (10.0 %) in combination, since the FIC value calculated for activity against *P. aeruginosa* was equal to 1.0, indicating an additive effect [26].

The FIC values calculated for *S. aureus, P. aeruginosa* and *P. fluorescens* where 0.45, 0.93 and 0.63, respectively, for oxifulvic acid in combination with sodium hypochlorite. The FIC values obtained indicated that oxifulvic acid in combination with sodium hypochlorite was synergistic against *S. aureus*, and only partially synergistic against *P. aeruginosa* and *P. fluorescens*. This suggests that either an oxifulvic acid and sodium hypochlorite mixture or possibly a chlorinated oxifulvic acid could be effective as a biocide or disinfectant.

Sodium dodecyl sulphate was not bactericidal against *P. aeruginosa* at 8000.0 mg/l. However, 125.0 and 250.0 mg/l SDS were bactericidal, against *P. aeruginosa*, in combination with 50.0 and 25.0 mg/l oxifulvic acid, respectively. This reduction in bactericidal concentration for the SDS and oxifulvic acid indicated that oxifulvic acid and SDS in combination were more bactericidal than either compound on its own. The FIC values calculated for the SDS-oxifulvic acid combination were 0.52 (125.0 mg/l SDS and 50.0 mg/l oxifulvic acid) and 0.28 (250.0 mg/l SDS and 25.0 mg/l oxifulvic acid). The interaction between oxifulvic acid and SDS could more accurately be described as potentiation [34]. Potentiation, refers to the enhancement of the activity of an antimicrobial compound by the addition of a second compound, which may have little or no antimicrobial activity [34]. Surfactants, at low concentrations, can enhance the activity of a mixture due to accumulation of the synergistic compound at the cell surface, within micelles of the surfactant which adsorb to the cell surface [34]. This mechanism would, however, not explain the synergistic effect between oxifulvic acid and SDS, since the SDS was present at a concentration lower than the critical micelle concentration (CMC) reported for SDS (CMC 660.0 mg/l) [42]. Disruption of the bacterial cell membrane and denaturisation of membrane proteins by SDS [47] would, however, enable the large (> 600 Dalton) hydrophilic fulvic acid molecules to enter the cell. This mechanism would, therefore, increase the antibacterial activity of the oxifulvic acid.

The combination of SDS and oxifulvic acid has the most potential for use as an industrial disinfectant formulation (*e.g.* abattoirs and agriculture) since the optimum pH for the antimicrobial activity of SDS is in the same range as oxifulvic acid (pH 3.0 to pH 4.0). The detergent action of the SDS makes this a useful formulation, since it might allow simultaneous cleaning and disinfectant action.

Bactericidal effect of oxifulvic acid against iron-oxidizing bacteria

Oxifulvic acid inhibited *T. ferrooxidans* WLR and the iron-oxidizing mixed bacterial culture bacterial culture at 75.0 and 100.0 mg/l, respectively (Figs. 5 and 6). The inhibitory effect of various compounds on iron-oxidizing bacteria have been reported in the literature [15,36,40,49,51]. *Thiobacillus ferrooxidans* WLR was inhibited by sodium benzoate and sorbic acid at 15.0 mg/l respectively and by sodium dodecyl sulphate (SDS) at 2.0 mg/l [15,36]. Oxifulvic acid was not as effective against *T. ferrooxidans* WLR (75.0 mg/l) in the laboratory studies, in comparison to sodium benzoate (15.0 mg/l) and SDS (2.0 mg/l).

Field trial studies with application of SDS or sodium benzoate did not reduce acidification of the coal discard [9]. This indicated that sodium benzoate and SDS were unable to the inhibit iron-oxidizing bacteria responsible for acid mine drainage under field conditions. Since oxifulvic acid inhibited the iron-oxidizing bacteria, it has potential to be a more effective alternative to either SDS or sodium benzoate for the inhibition of iron oxidizing bacteria with a concomitant reduction in acid mine drainage.

Applications of oxifulvic acid

In summary, oxifulvic acid did not have potential for use as a biocide to control biofouling and biocorrosion in water cooling systems. This was indicated by the inability of oxifulvic acid to inhibit the growth of sessile bacteria in a water-cooling system and the high concentrations required for fungicidal (2000.0 to $\geq$ 4000.0 mg/l) and algicidal ($\geq$ 400.0 mg/l) activity *in vitro*.

Oxifulvic acid had potential for use as a disinfectant, since oxifulvic acid was effective at a lower concentration (800.0 mg/l) than a commercial coal-tar disinfectant

(3000.0 to 48000.0 mg/l) and also exhibited virucidal activity at 96.0 mg/l. Furthermore, the disinfectant activity of oxifulvic acid was enhanced in combination with SDS. This oxifulvic acid-SDS combination shows potential for use as a disinfectant formulation in abattoirs and for agricultural application (*e.g.* cleaning of animal pens). The application of oxifulvic acid is limited, however, since only a 2 log reduction in spore counts was achieved.

The use of oxifulvic acid to control acid mine drainage *in situ* should be investigated due to the exhibited *in vitro* antibacterial activity of oxifulvic acid against iron-oxidizing bacteria (75.0 and 100.00 mg/l).

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Table 1

Fungi used for the screening of the fungicidal activity of oxifulvic acid.

Code	Test culture	Source
3732	*Penicillium funiculosum*	PPRI[b]
4046	*Aspergillus foetidus*	PPRI
3592	*Rhizopus stolonifer*	PPRI
3757	*Penicillium variable*	PPRI
4671	*Aurebasidium pullulans*	PPRI
3250	*Phoma herbarum*	PPRI
4066	*Chaetomium globosum*	PPRI
3253	*Aspergillus niger*	PPRI
Nam 2	*Aspergillus alutaceus*	Nampak[c]
Nam 3	*Penicillium crustosum*	Nampak
Nam 6	*Cladosporium cladosporiodes*	Nampak
Nam 7	*Penicillium minioluteum*	Nampak

[b] PPRI = Plant Protection Research Institute

[c] Nampak = Nampak waste paper recycling plant, Rosslyn, Pretoria.

Table 2

Flow rate, retention time and holding time index of tubes 1 to 4 in biofouling test rig used in the field trial evaluation of oxifulvic acid as biocide to control biofouling and biocorrosion

	Tube number			
	1	2	3	4
Flow rate (l/min)	4.14	15.00	5.07	16.82
Retention time (min)	24.15	6.60	19.72	5.94
Holding time index (min)	16.74	4.57	13.66	4.11

Table 3

Fungicidal activity of oxifulvic acid evaluated in various growth media.

Code	Test culture	Growth media used			
		PDA[a]	STD1[b]	NA[c]	WA[d]
		concentration oxifulvic acid (mg/l) inhibiting growth			
3732	*Penicillium funiculosum*	4 000	4 000	4 000	2 000
4046	*Aspergillus foetidus*	>4 000[f]	>4 000	>4 000	>4 000
3592	*Rhizopus stolonifer*	4000	4 000	4 000	2 000
3757	*Penicillium variable*	4 000	4 000	4 000	2 000
4671	*Aurebasidium pullulans*	4 000	4 000	2 000	2 000
3250	*Phoma herbarum*	2 000	4 000	4 000	2 000
4066	*Chaetomium globosum*	800	2 000	2 000	NG[e]
3253	*Aspergillus niger*	>4 000	>4 000	>4 000	>4 000
Nam 2	*Aspergillus alutaceus*	2 000	2 000	1 000	800
Nam 3	*Penicillium crustosum*	2 000	4 000	4 000	800
Nam 6	*Cladosporium cladosporiodes*	1 000	2 000	2 000	600
Nam 7	*Penicillium minioluteum*	2 000	4 000	4 000	1 000

[a]PDA = Potato dextrose agar
[b]STD1 = Standard one nutrient agar
[c]NA = Nutrient agar
[d]WA = Water agar
[e]NG = No growth on plate containing no oxifulvic acid (Control plate)
[f] = Highest concentration evaluated (solidification of agar media affected at concentrations of oxifulvic acid > 4000.0 mg/l)

Table 4

Inhibition of algal growth by oxifulvic acid and regrowth of algae after inoculation from treated samples into cultivation medium (containing no biocide) after 5 and 10 days and growth after 10 and 28 days.

Culture	Concentration oxifulvic acid (mg/l)	Growth (days)			Regrowth (days)	
		5	10	28	5	10
Chlorella sp.	0	+	+	+	+	+
	50	+	+	+	+	+
	100	+	+	+	+	+
	200	+	+	+	+	+
	400	-	-	-	-	-
	800	-	-	-	-	-
Chlorococcum sp.	0	+	+	+	+	+
	50	+	+	+	+	+
	100	+	+	+	+	+
	200	-	+	+	+	+
	400	-	-	-	-	-
	800	-	-	-	-	-
Calothrix sp.	0	+	+	+	+	+
	50	+	+	+	+	+
	100	+	+	+	+	+
	200	+	+	+	+	+
	400	-	-	-	-	-
	800	-	-	-	-	-

Table 5

Comparison of the bactericidal concentration of oxifulvic acid and a coal tar disinfectant.

	Oxifulvic acid	Coal-tar disinfectant
Culture	Bactericidal concentration (mg/l)[a]	
Staphylococcus aureus	100	6000
Escherichia coli strain K12	800	3000
Pseudomonas aeruginosa	800	48 000
Salmonella typhi	100	24 000
Bacillus cereus	800	12 000

[a] Concentration of oxifulvic acid required for bactericidal action after 10 min exposure to oxifulvic acid in quarter strength Ringers solution.

Table 6

Sporicidal activity of oxifulvic acid against spores of *B. cereus, B. megaterium and B. subtilis*.

Culture	Oxifulvic acid concentration (mg/l)	Percentage kill after exposure to oxifulvic acid over a 48 h period with samples taken at indicated time intervals			
		Exposure time (hours)			
		6	12	24	48
Bacillus cereus	1000	86.41	87.95	90.08	91.76
	2000	90.32	92.48	93.24	93.72
	4000	93.19	93.76	93.77	93.72
	8000	97.95	97.86	98.28	98.34
	Conc.[a]	98.28	98.65	98.78	98.85
Bacillus megaterium	1000	88.00	89.22	89.33	89.34
	2000	90.82	92.09	93.08	93.33
	4000	93.93	93.73	94.25	94.26
	8000	96.51	96.41	96.55	96.91
	Conc.	97.08	97.99	98.17	98.01
Bacillus subtilis	1000	89.22	90.04	90.17	90.44
	2000	91.57	92.09	93.08	93.33
	4000	93.75	94.48	94.67	94.81
	8000	96.86	96.87	96.90	96.91
	Conc.	98.86	99.11	99.23	99.26

[a] Sporicidal effect of oxifulvic acid stock solution (10000.0 mg/l)

Table 7

The effect of oxifulvic acid on the survival of coliphage V_1 after 10 min exposure to oxifulvic acid (0.0 to 768.0 mg/l) in a quarter strength Ringers solution.

Oxifulvic acid concentration % (%/v)	Viable coliphage after 10 min exposure to oxifulvic acid (Plaque forming units/ml)	Percentage kill after 10 min exposure
0	1.61×10^4	0
0.08 (12.0)[a]	6.1×10^3	72.11
0.16 (24.0)[a]	4.0×10^3	75.16
0.31 (48.0)[a]	3.0×10^3	81.37
0.63 (96.0)[a]	0	100.00
1.25 (192.0)[a]	0	100.00
2.5 (348.0)[a]	0	100.00
5.0 (768.0)[a]	0	100.00

[a] = Concentration of oxifulvic acid (mg/l)

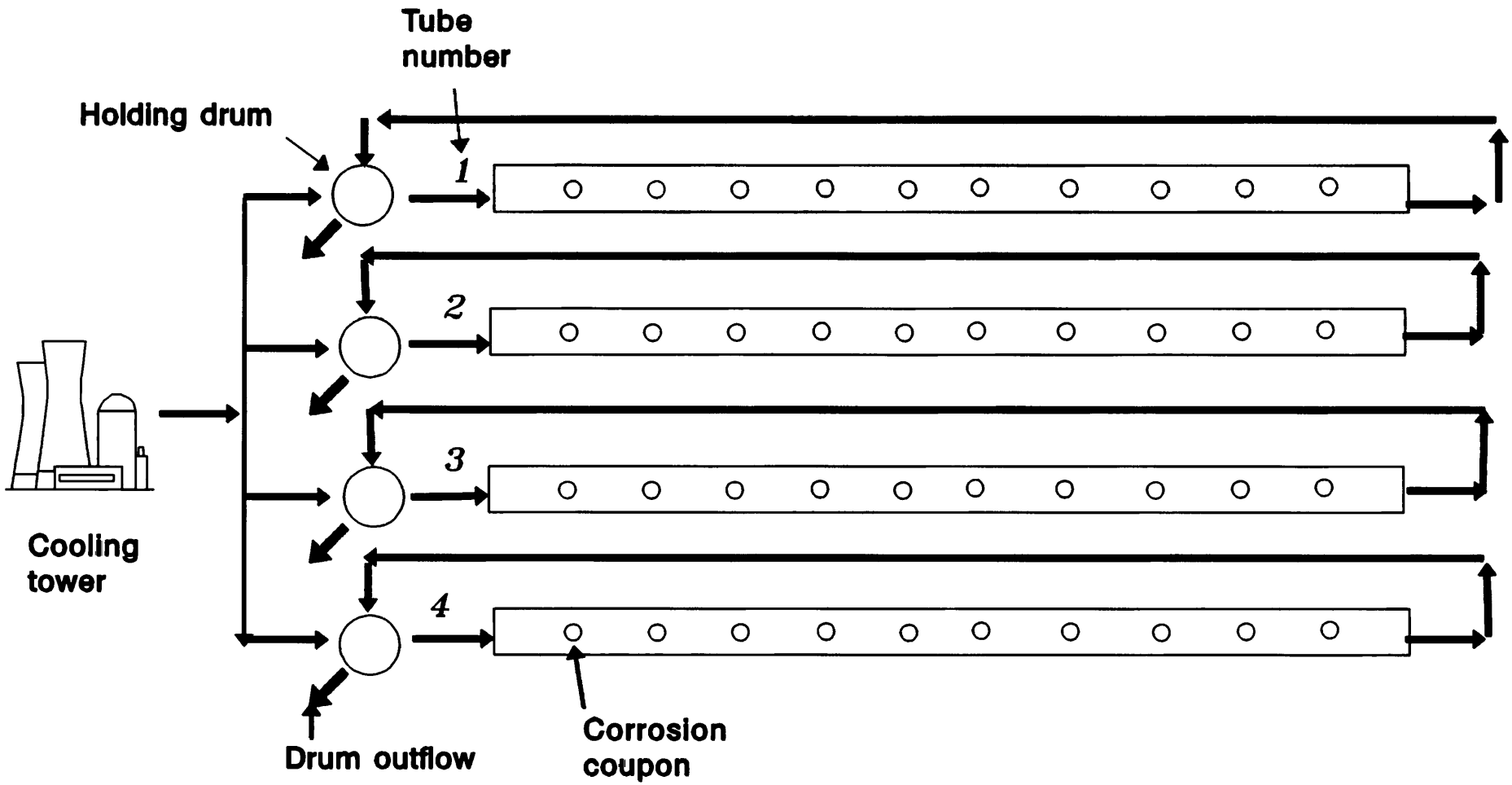

Fig. 1. Diagrammatic representation of biofouling test rig used in field trial evaluations of oxifulvic acid.

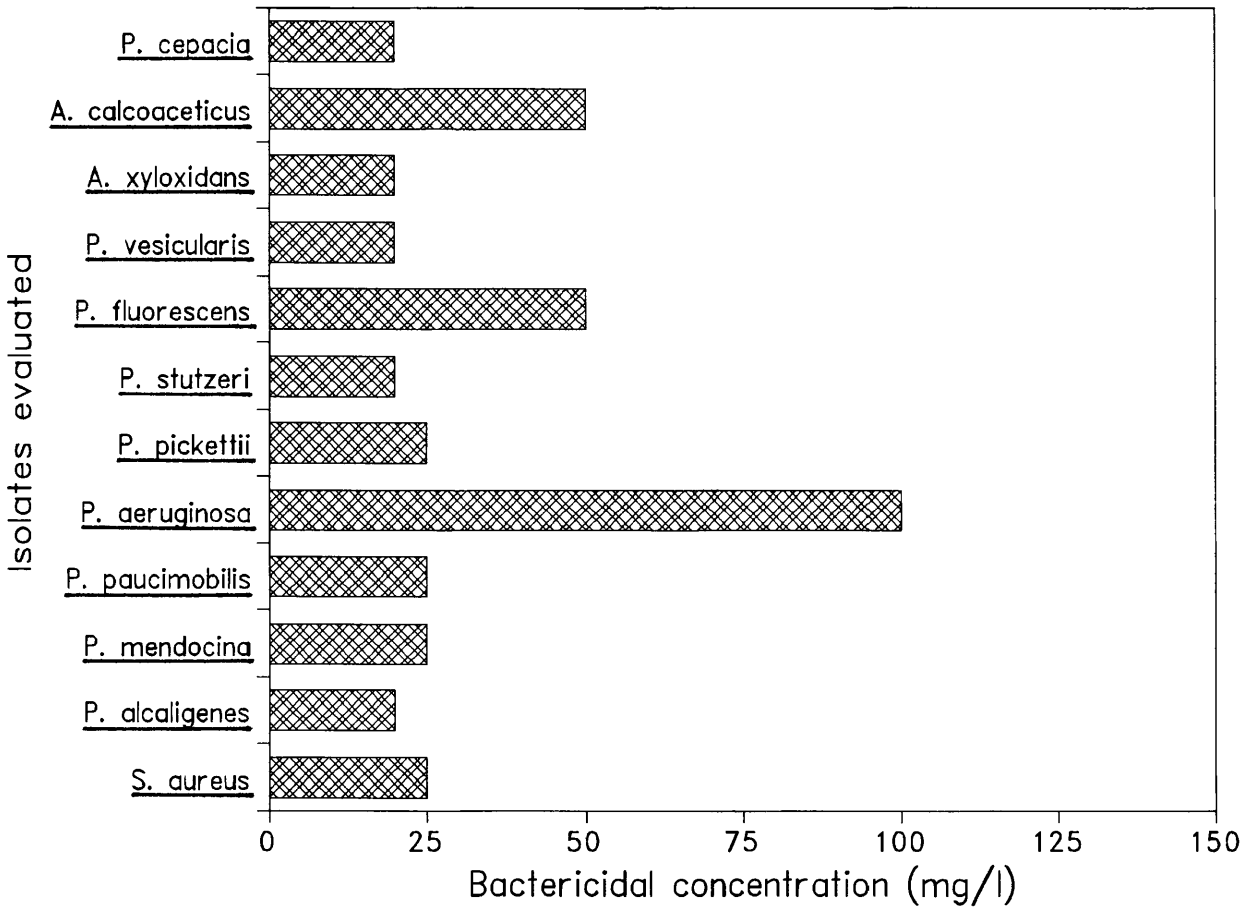

Fig. 2. Bactericidal concentration of oxifulvic acid against test strains, isolated from water-cooling systems in South Africa, exposed to oxifulvic acid (0.0 to 200.0 mg/l) for 6 h.

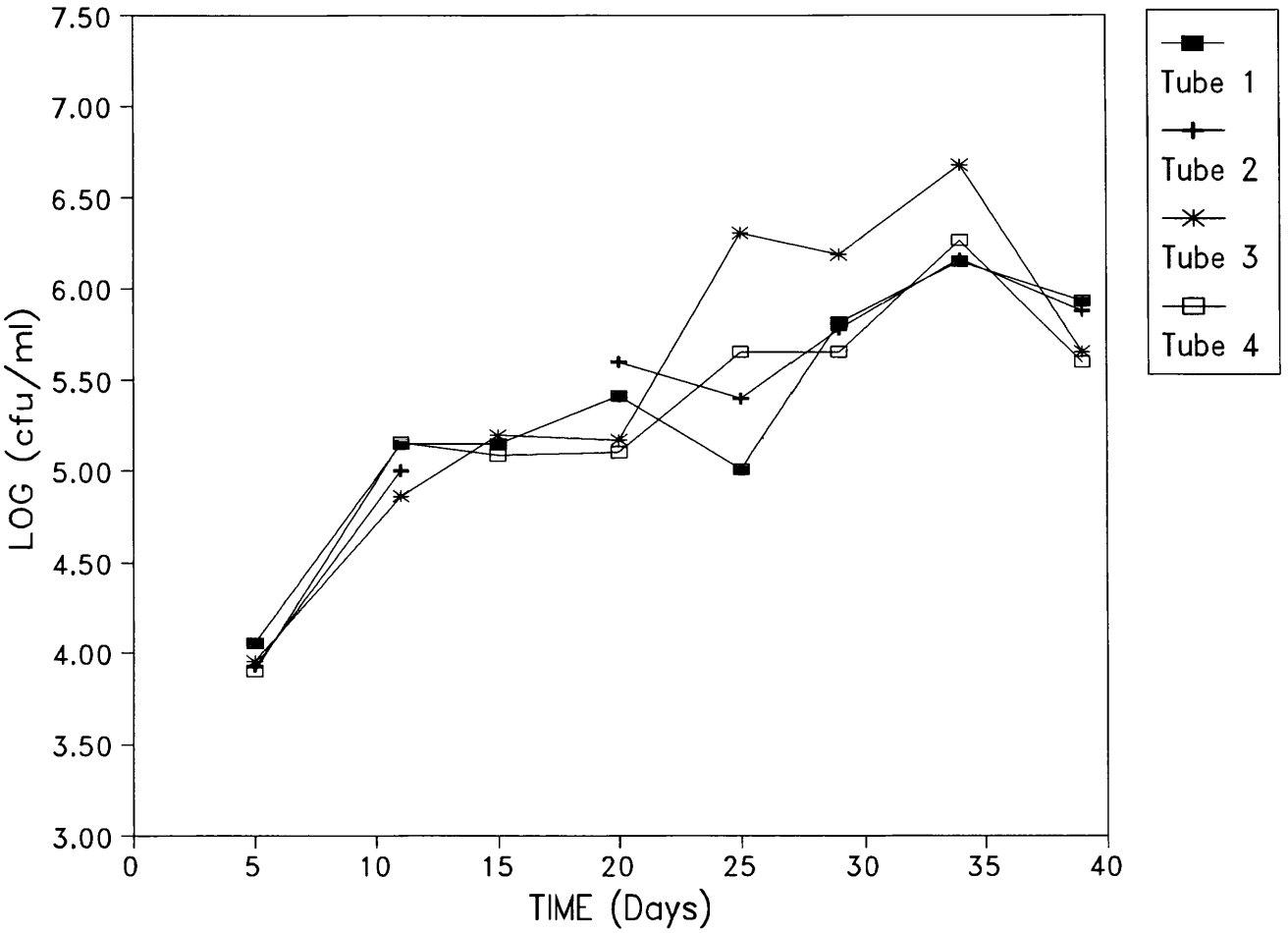

Fig. 3. Effect of oxifulvic acid added at 200.0 and 400.0 mg/l to tubes 2 and 3, respectively, and hydrogen peroxide added at 50.0 mg/l to tube 1, on planktonic aerobic count, in comparison to the untreated tube 4.

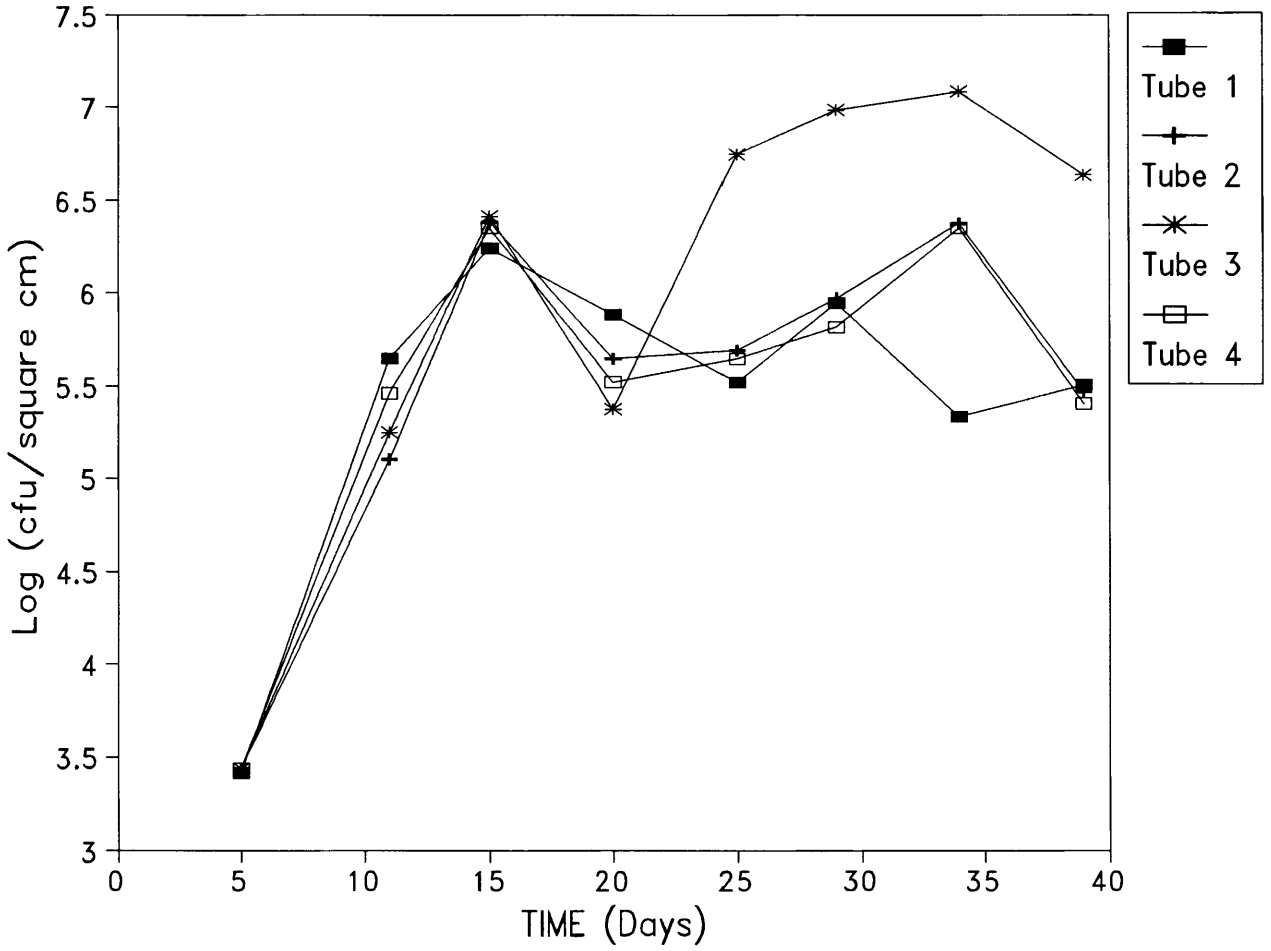

Fig. 4. Effect of oxifulvic acid added at 200.0 and 400.0 mg/l to tubes 2 and 3, respectively, and hydrogen peroxide added at 50.0 mg/l to tube 1 on sessile counts from corrosion coupons, in comparison to the untreated tube 4.

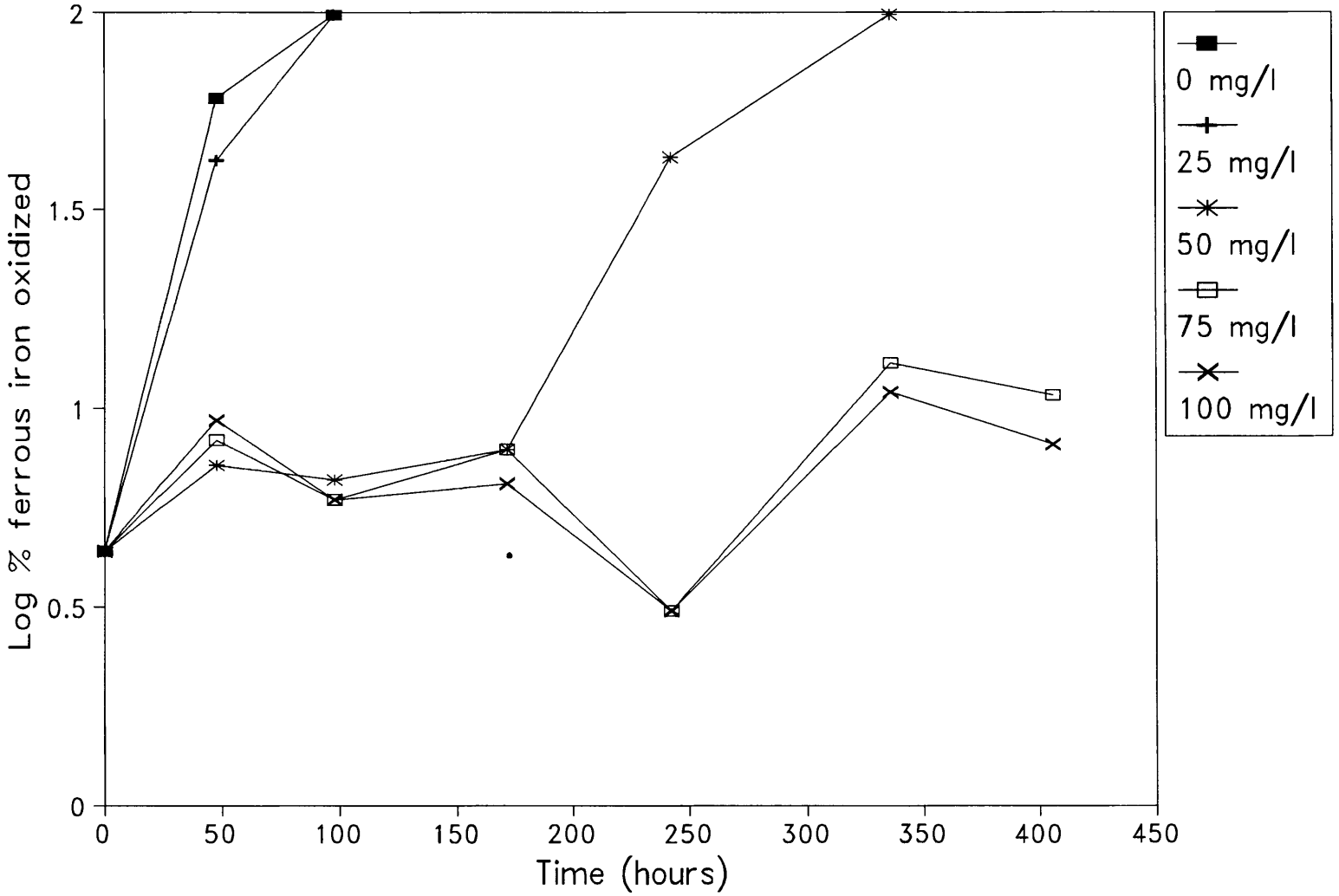

Fig. 5. Growth of *T. ferrooxidans* WLR in the presence of oxifulvic acid (from 10000.0 mg/l stock solution) added at the beginning of incubation.

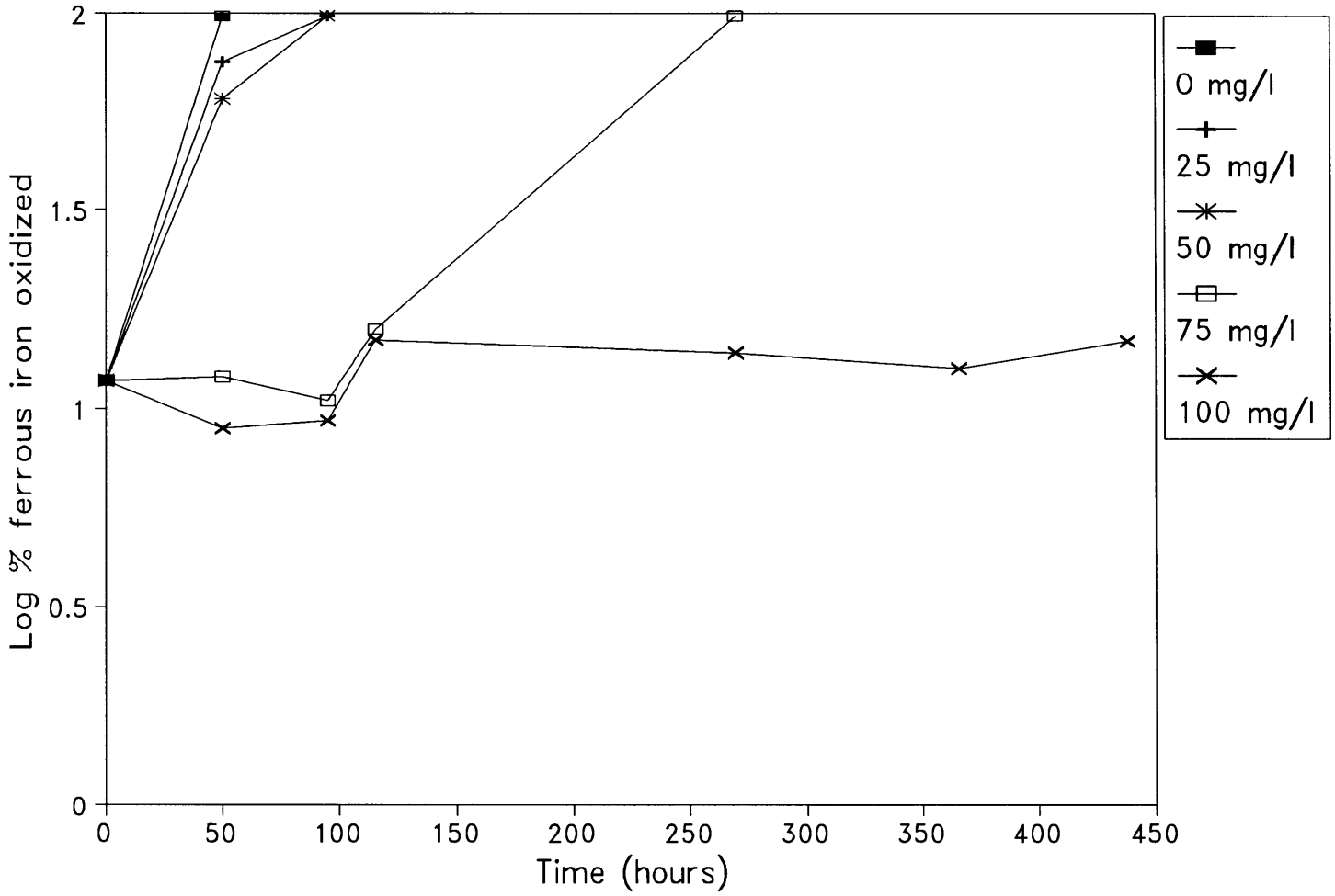

Fig. 6. Growth of iron-oxidizing mixed bacterial culture in the presence of oxifulvic acid (from a 10000.0 mg/l stock solution) added at the beginning of incubation.

CHAPTER 5

(Submitted for publication in the Journal of Applied Bacteriology)

ANTIBACTERIAL ACTION OF OXIFULVIC ACID AND DEVELOPMENT OF RESISTANCE TO OXIFULVIC ACID BY *Escherichia coli* strain K12, *Pseudomonas aeruginosa* AND *Staphylococcus aureus*

* The language and style used in this chapter are in accordance with the requirements of the Journal of Applied Bacteriology)

ABSTRACT

The mode of antibacterial action of oxifulvic acid, a coal-derived product, against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli strain* K12 was investigated in conjunction with the ability of *P. aeruginosa, S. aureus* and *E. coli* strain K12 to develop resistance to oxifulvic acid. Antibacterial action of oxifulvic acid against *S. aureus*, *P. aeruginosa* and *E. coli strain* K12 can be ascribed to the failure of the pH homeostasis mechanism of the bacteria, to maintain a constant intracellular pH. *Pseudomonas aeruginosa, S. aureus* and *E. coli* strain K12 were able to tolerate a minimum inhibitory concentration (MIC) of oxifulvic acid 125.0, 250.0 and 250.0 mg/l higher than the original MIC (MIC before exposure to oxifulvic acid) after 10 subcultures in the presence of oxifulvic acid. This increased tolerance to oxifulvic acid was ascribed to the habituation of the bacteria to a sub-lethal pH.

INTRODUCTION

South African bituminous coal can be converted via a controlled wet oxidation to oxihumic acid, oxifulvic acid and oxicoal (Cronje, 1990). The coal-derived oxifulvic and oxihumic acids differ, in certain aspects, from the naturally occurring humic and fulvic acids. Coal-derived oxifulvic and oxihumic acids contain more aromatic and phenolic compounds, and the ratio of phenolic to carboxyl groups and total acidity are also somewhat higher than the corresponding values reported for the natural humic acids (I.J. Cronje, pers. comm.)*.

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Natural fulvic acid has been described as a mixture of aliphatic and aromatic macromolecules substituted with hydrophilic carboxylic, phenolic and carbonyl acid functional groups (Bixby and O'Brien, 1979). Since oxifulvic acid contains a mixture of carboxyl and phenolic functional groups (Cloete *et al.*, 1990), their mode of antibacterial action could be similar to that of other carboxylic acids.

The mode of action of organic acids has not been clearly identified, although acidification of the cell cytoplasm (Salmond *et al.*, 1984), inhibition of nutrient uptake (Freese *et al.*, 1973; Eklund 1980) and inhibition of the synthesis of cellular material (Cherrington *et al.*, 1990) have been reported. Since the bactericidal effect of organic acids increases with decreasing pH (Eklund, 1983; Salmond *et al.*, 1984) it has been assumed that the undissociated acid, which is present in greater proportions at low pH, is the antibacterial agent. Antimicrobial action of organic acids is believed to be caused by dissociation of the acid into anions and protons in the cytoplasm. The organic acid would dissociate due to cytoplasmic pH, which is *ca.* pH 7.0 (Foster *et al.,* 1973; Cherrington *et al.*, 1990). The anion ($RCOO^-$) and protons (H^+) would exert an inhibitory effect on the bacterial cell (Eklund, 1983; Salmond *et al.*, 1984; Cherrington *et al.*, 1990).

Resistance of bacteria to organic acids has not been reported (Cherrington *et al.*, 1991). There is evidence, however, that exposure of bacteria *e.g. Escherichia coli* to sublethal acidic conditions increases their tolerance to exposure to organic acids (Goodson and Rowbury, 1989b). This exposure also favours their subsequent survival in environments at lethal pH values (pH 3.0). This phenomenon is illustrated by the increased tolerance of *E. coli* habituated at pH 5.0 to subsequent exposure to pH 3.0 (Goodson and Rowbury, 1989a). This paper reports on the investigation of the mode of inhibitory action of oxifulvic acid against selected bacterial species and the ability of those

species to develop resistance to oxifulvic acid.

MATERIALS AND METHODS

Production of oxifulvic acid

Oxifulvic acid, prepared by the controlled wet oxidation of South African bituminous coal (Cronje, 1990), was obtained from the Division of Energy Technology, CSIR, Pretoria, South Africa. Oxifulvic acid was obtained as a solution from the CSIR.

Bacteria and growth conditions

Bacterial cultures were obtained from the culture collection of the Environmental Biotechnology Laboratory at the Department of Microbiology and Plant Pathology, University of Pretoria. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were isolated from water-cooling systems in South Africa (Cloete *et al.,* 1989). *Escherichia coli* strain K12 (obtained from Prof W.O.K. Grabow, Head, Department of Medical Virology, University of Pretoria) was isolated from domestic wastewater and identified using the API system. Bacterial cultures were maintained on Standard One Nutrient Agar (STD1, Biolab, Merck, South Africa) slants at 4 °C.

Effect of oxifulvic acid on the pH of quarter strength Ringers solution and Nutrient Broth

Stock solutions containing 1000.0 mg/l and 10000.0 mg/l oxifulvic acid were made up in quarter strength Ringers solution (Merck, South Africa). The oxifulvic acid was added to quarter strength Ringers solution at 0.0, 25.0, 50.0, 75.0, 100.0, 200.0, 400.0, 600.0, 800.0 and 1000.0 mg/l. This procedure was repeated using Nutrient Broth (NB, Biolab,

Merck, South Africa) in place of quarter strength Ringers solution to determine the effect of oxifulvic acid on the pH of NB. The NB-oxifulvic acid mixture was prepared by adding 1.0 ml of a ten times strength NB solution (10x NB, NB made up to 10 times normal strength, *i.e.* 16.0 g NB/100 ml) to 8.9 ml sterile distilled water containing oxifulvic acid at 0.0, 250.0, 375.0, 500.0, 750.0, 875.0, 1000.0, 2000.0, 4000.0 and 8000.0 mg/l. The pH of the solutions containing oxifulvic acid was determined using a Beckmann 3002 pH meter. The 10 x NB was solution was used to ensure that each test tube contained the same amount of NB. This experiment was repeated in duplicate and the average pH value determined for each of the experiments.

Antibacterial action of oxifulvic acid

The mode of antibacterial action of oxifulvic acid was determined by comparing the die-off rate of cells exposed to a bactericidal concentration of oxifulvic acid and quarter strength Ringers solution adjusted to a pH equivalent to the pH of quarter strength Ringers solution containing the bactericidal concentration of oxifulvic acid. The pH of quarter strength Ringers solution was adjusted, using 1M H_2SO_4, to a pH value equivalent (Table 1) to the pH of a quarter strength Ringers solution containing oxifulvic acid (10000.0 mg/l stock solution) at bactericidal concentrations against *P. aeruginosa, S. aureus* and *E. coli* strain K12 (*i.e.* 100.0 mg/l, 25.0 mg/l and 75.0 mg/l, respectively). The pH adjusted quarter strength Ringers solution was then filter sterilised. Oxifulvic acid was added to quarter strength Ringers solution bactericidal concentrations against *P. aeruginosa, S. aureus* and *E. coli* strain K12 (*i.e.* 100.0 mg/l, 25.0 mg/l and 75.0 mg/l, respectively) from a 10000.0 mg/l stock solution. Cells (1.0 ml) from an overnight culture *ca.* 16 h, incubated at 28 °C, were harvested by centrifugation (10 000 x g at 4 °C

for 5 min) and resuspended in 1.0 ml quarter strength Ringers solution (pH 7.2). An inoculum of 100.0 μl (10^8 cfu/ml) from the cell suspension, was added to flasks (9.9 ml) containing pH adjusted quarter strength Ringers solution and the quarter strength Ringers solution-oxifulvic acid mixture, respectively. Controls contained quarter strength Ringers solution (pH 7.2).

Samples (100.0 μl) were removed after 1.5 h, 3.0 h and 6.0 h and the viable cell count determined, by plating duplicate serial dilutions onto Nutrient Agar (NA) (Biolab, Merck, South Africa). The NA plates were incubated for 48 h at 28°C. After incubation the number of visible colonies were counted on plates containing 30 to 300 visible colonies. These experiments were repeated in duplicate. The average pH value and cell count was determined for each of the experiments.

Resistance of bacteria to oxifulvic acid

The development of resistance to oxifulvic acid by *P. aeruginosa, S. aureus* and *E. coli* strain K12 was investigated by growth in NB containing oxifulvic acid. The NB was prepared by adding 1.0 ml of a 10x NB solution to 8.9 ml sterile distilled water containing the test concentration of oxifulvic acid. Oxifulvic was added to the sterile distilled water from a filter sterilized (0.2 μm filter) 10000.0 mg/l stock solution at the test concentration. The 10x NB was used to ensure that the conditions were identical in each test tube containing the NB-oxifulvic acid mixture.

The minimum inhibitory concentration (MIC) for *P. aeruginosa, S. aureus* and *E. coli* strain K12 was determined initially in NB containing oxifulvic acid at 250.0, 375.0, 500.0, 625.0, 750.0, 875.0 and 1000.0 mg/l, respectively. Cells (1.0 ml) from an overnight culture *ca.* 16 h, incubated at 28 °C, were harvested by centrifugation (10 000

x g at 4°C for 5 min) and resuspended in 1.0 ml quarter strength Ringers solution. An inoculum of 100 μl (10^8 cfu/ml) from the cell suspension, was added to test tubes containing 10.0 ml NB-oxifulvic acid mixture (prepared as indicated above). The cultures were incubated at 28 °C for 24 h. After incubation the lowest concentration showing no growth after incubation was recorded as the MIC. The cultures were then exposed to an oxifulvic acid concentration 125.0 mg/l less that the MIC, and incubated for 24 h at 28 °C. Thereupon the new MIC was determined and cultures exposed to a concentration 125.0 mg/l less than the new MIC. This procedure was repeated 10 times. During the later stages of adaption (subcultures 5 to 10) the cultures were incubated for 48 h at 28 °C.

Development of resistance was monitored by determining the minimum inhibitory bactericidal concentration (MBC) of cultures exposed to oxifulvic acid. The MBC was determined as described by Brözel and Cloete (1991). In order to determine whether resistance was due to exposure to oxifulvic acid or adaption to sub-lethal pH the MBC of cultures exposed to oxifulvic acid was compared to the MBC of cultures grown in pH adjusted NB. The pH of the NB was adjusted, using 1M H_2SO_4, to a pH equivalent to the pH of the NB containing oxifulvic acid, as indicated in Table 2.

RESULTS

Mechanism of bactericidal action of oxifulvic acids

Lowering of external pH (pH outside bacterial cell) was suspected to be the mode of inhibitory action of oxifulvic acid, since the oxifulvic acid solution was acidic (pH 1.89). The effect of exposure of *S. aureus, P. aeruginosa* and *E. coli* strain K12 to bactericidal concentrations of oxifulvic acid and a pH equivalent to the bactericidal concentration (in

quarter strength Ringers solution) is shown in Fig. 1-3. Oxifulvic acid and the equivalent pH had a similar effect on cell viability against *S. aureus* and *E. coli* strain K12 with a 6 log and 3 log reduction in cell count after 6 h, respectively. A log reduction of 4 log and 6 log was observed for *P. aeruginosa* exposed to oxifulvic acid and the pH equivalent, respectively.

Resistance of bacteria to oxifulvic acid

The MIC, MBC and concentration of oxifulvic acid tolerated by *S. aureus, P. aeruginosa* and *E. coli* strain K12 are shown in Fig. 4-6. The MIC increased from 375.0, 625.0 and 875 mg/l to 500.0, 875.0 and 1125.0 mg/l, after exposure to oxifulvic acid, for *S. aureus*, *P. aeruginosa* and *E. coli* strain K12, respectively. All the bacteria were able to adapt to growth in higher concentrations of oxifulvic acid, with a maximum concentration tolerated after 3 subcultures in the presence of oxifulvic acid for *S. aureus* and 2 subcultures in the presence of oxifulvic acid for *E. coli* strain K12 and *P. aeruginosa*.

The MBC of oxifulvic acid against *P. aeruginosa, E. coli strain* K12 and *S. aureus* habituated to sub-lethal concentrations of oxifulvic acid and a pH equivalent to the sub-lethal oxifulvic acid concentration, respectively, are shown in Table 3. *Staphylococcus aureus* cells habituated to sub-lethal concentrations of oxifulvic acid and a pH equivalent to the oxifulvic acid concentration exhibited the same MBC (150.0 mg/l) after 10 subcultures (Table 3). The MBC of *P. aeruginosa* and *E. coli* strain K12 habituated to sub-lethal concentrations of oxifulvic acid (400.0 and 450.0 mg/l, respectively) was 50.0 mg/l higher than the MBC of cultures habituated to a pH equivalent to the oxifulvic acid concentration (350.0 and 400.0 mg/l, respectively).

DISCUSSION

Antibacterial action of oxifulvic acid

The survival of *P. aeruginosa, S. aureus* and *E. coli* strain K12 in quarter strength Ringers solution acidified by the addition of oxifulvic acid or sulphuric acid was investigated (Fig. 1-3). This would determine whether pH alone or the presence of oxifulvic acid caused cell death. Bacterial cells are capable of growing over a wide range of external pH values due to the pH homeostasis mechanism (Foster, 1992). The physiologically triggered pH homeostasis mechanism maintains a relatively constant intracellular pH (pH_i) over a broad range of external pH values (pH_o) (Goodson and Rowbury, 1989a; Foster, 1992). However, with increasing acidification of the environment, the pH homeostasis system fails and the cell loses its ability to maintain a neutral internal pH resulting in eventual cell death. Acidification of the quarter strength Ringers solution (Table 1) would therefore place stress on the pH homeostasis mechanism of the bacteria (Foster, 1992). Furthermore, a pH of pH 3.0 or lower has a bactericidal effect, within 60 s against *P. aeruginosa* (Tanner and James, 1992). A pH value of pH 3.0 was bactericidal against *E. coli* strain within 60 min (Goodson and Rowbury, 1989a).

Staphylococcus aureus and *E. coli* strain K12 were exposed to pH values of pH 4.05 and 3.47, respectively (Table 1). The reported bactericidal effect of pH values between pH 3.0 to 4.0 (Goodson and Rowbury, 1989a; Tanner and James, 1992) suggested that the antimicrobial activity of oxifulvic acid was due to the lowering of the external pH (pH_o) below pH 4.0. The lowering of pH_o would as indicated by Foster (1992) would result in the failure of the pH homeostasis mechanism of the cells. This was indicated by the 6 and 4 log reduction, respectively, of *S. aureus* and *E. coli* strain K12 cells exposed to oxifulvic acid and a pH equivalent to the oxifulvic acid concentration

(Fig. 1-2). The failure of the homeostasis mechanism of *S. aureus* and *E. coli* strain K12 would lead to eventual cell death. The antibacterial activity of oxifulvic acid against *S. aureus* and *E. coli* strain K12 was therefore ascribed to the lowering of pH_o, with the subsequent failure of the pH homeostasis mechanism of the cultures.

Pseudomonas aeruginosa was exposed to a pH value of pH 3.27 (Table 1). The difference in log reduction of *P. aeruginosa* cells exposed to oxifulvic acid (4 log) and a pH equivalent (log 6) indicated that an additional mechanism, other than pH, was responsible for cell death. Since most of the oxifulvic acid would be in the undissociated form (RCOOH) at pH 3.27 (pH 3.0 to 4.0 optimum pH for bactericidal activity), most of the H^+ ions present would be bound to the carboxylic and phenolic functional groups of oxifulvic acid. A reduction the concentration of H^+ ions in solution can therefore be expected (Russel and Chopra, 1990). Mineral acids on the other hand dissociate into anions (R^-) and protons (H^+) in solution (Tanner and James, 1992). Therefore at pH 3.27 the difference in cell death noted in the oxifulvic acid solution and the acidified quarter strength Ringers solution was ascribed to a difference in the concentration of H^+ ions present in the acidified quarter strength Ringers solution.

Resistance of bacteria to oxifulvic acid

The inability of *P. aeruginosa, S. aureus* and *E. coli* strain K12 to adapt to higher concentrations of oxifulvic acid, after 2 to 3 subcultures in NB containing oxifulvic acid, indicated that no further resistance to oxifulvic acid was developed (Fig. 4-6). This phenomenon ruled out enzymatic degradation of the oxifulvic acid since this mechanism of resistance requires the induction of an enzyme capable of degrading oxifulvic acid. Induction of an enzyme capable of degrading oxifulvic acid would, however, have led to

an increase in the MIC and also an increase in resistance to oxifulvic acid, after each sub-culture. Changes in cell wall structure of the bacteria can lead to a gradual increase in the concentration of a biocide tolerated (Russel and Chopra, 1990). This was, however, not seen from the results (Fig. 4-6).

Goodson and Rowbury (1989b) reported that *E. coli* exposed to sub-lethal pH values was more resistant to organic acids than cells incubated at neutral pH. To determine whether this was the mechanism of resistance to oxifulvic acid, the MBC of cells grown in the presence of oxifulvic acid was compared to the MBC of cells grown at a pH (Table 2) equivalent to that of the oxifulvic acid concentration. The results (Table 3) indicated that resistance was due to exposure of the cells to a sub-lethal pH, particularly in the case of *S. aureus*. This was indicated by the fact that the MBC of *S. aureus* cells grown in the presence of oxifulvic acid was the same as cells grown at a pH (Table 2) equivalent to that of the oxifulvic acid concentration. Habituation of *S. aureus* at the sub-lethal pH, as suggested by Goodson and Rowbury (1989b) had therefore increased the resistance to oxifulvic acid. Since adaptation to a sub-lethal pH is a stress response (Goodson and Rowbury, 1989a; Foster, 1992) this increased resistance of *S. aureus* to oxifulvic acid was not due to an acquired resistance mechanisms (Table 3). The MBC of *P. aeruginosa* and *E. coli strain* K12 cells grown in oxifulvic acid was higher (+ 50 mg/l) than cells grown in an equivalent pH. The increase in resistance of *P. aeruginosa* and *E. coli strain* K12 cells grown in the presence of oxifulvic acid, indicated that the presence of oxifulvic acid induced resistance. The difference (± 50 mg/l) suggested however, that the main mechanism of resistance was due to habituation to a sub-lethal pH.

ACKNOWLEDGEMENTS

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Table 1 pH values of quarter strength Ringers solutions containing various concentrations of oxifulvic acid

Oxifulvic acid concentration (mg/l)	Oxifulvic acid stock solution (1000.0 mg/l)	Oxifulvic acid stock solution (10000.0 mg/l)
0	7.53	7.53
25	3.98	4.05
50	3.57	3.62
75	3.42	3.47
100	3.34	3.27
200	3.04	3.01
400	2.81	2.74
600	2.74	2.59
800	2.65	2.54
1000	ND	2.47

ND = Not determined, since 1000.0 mg/l would be equivalent to undiluted stock solution

Table 2 pH values of Nutrient Broth containing various concentrations of oxifulvic acid*

Oxifulvic acid concentration (mg/l)	pH of Nutrient Broth
0	7.72
250	6.87
375	6.32
500	5.77
750	5.25
875	5.00
1000	4.74
2000	4.04
4000	3.45
8000	2.90

* = oxifulvic acid stock solution of 10000.0 mg/l used

Table 3 The minimum bactericidal concentration of oxifulvic acid against *P. aeruginosa, S. aureus* and *E. coli* strain K12 habituated to a sub-lethal concentration of oxifulvic acid and a pH equivalent to the oxifulvic acid concentration in Nutrient Broth

Culture	Habituation condition	Number of times subcultured												
		0	1	2	3	4	5	6	7	8	9	10	11	12
S. aureus	Oxifulvic acid*	25	75	100	100	100	125	150	150	150	150	150	150	150
	pH#			50		100		150		150		150		150
P. aeruginosa	Oxifulvic acid	100	225	250	300	350	375	400	400	400	400	400	400	400
	pH			250		250		350		350		350		350
E. coli strain K12	Oxifulvic acid	150	250	350	350	400	450	450	450	450	450	450	450	450
	pH			350		350		400		400		400		400

* = The cultures were grown in NB containing an oxifulvic acid concentration 125.0 mg/l less that the MIC, and incubated for 24 h at 28 °C

\# = The cultures were grown in pH adjusted NB. The pH was adjusted, using 1M H_2SO_4, to a pH equivalent to the pH of the NB containing oxifulvic acid at 125.0 mg/l less than the MIC

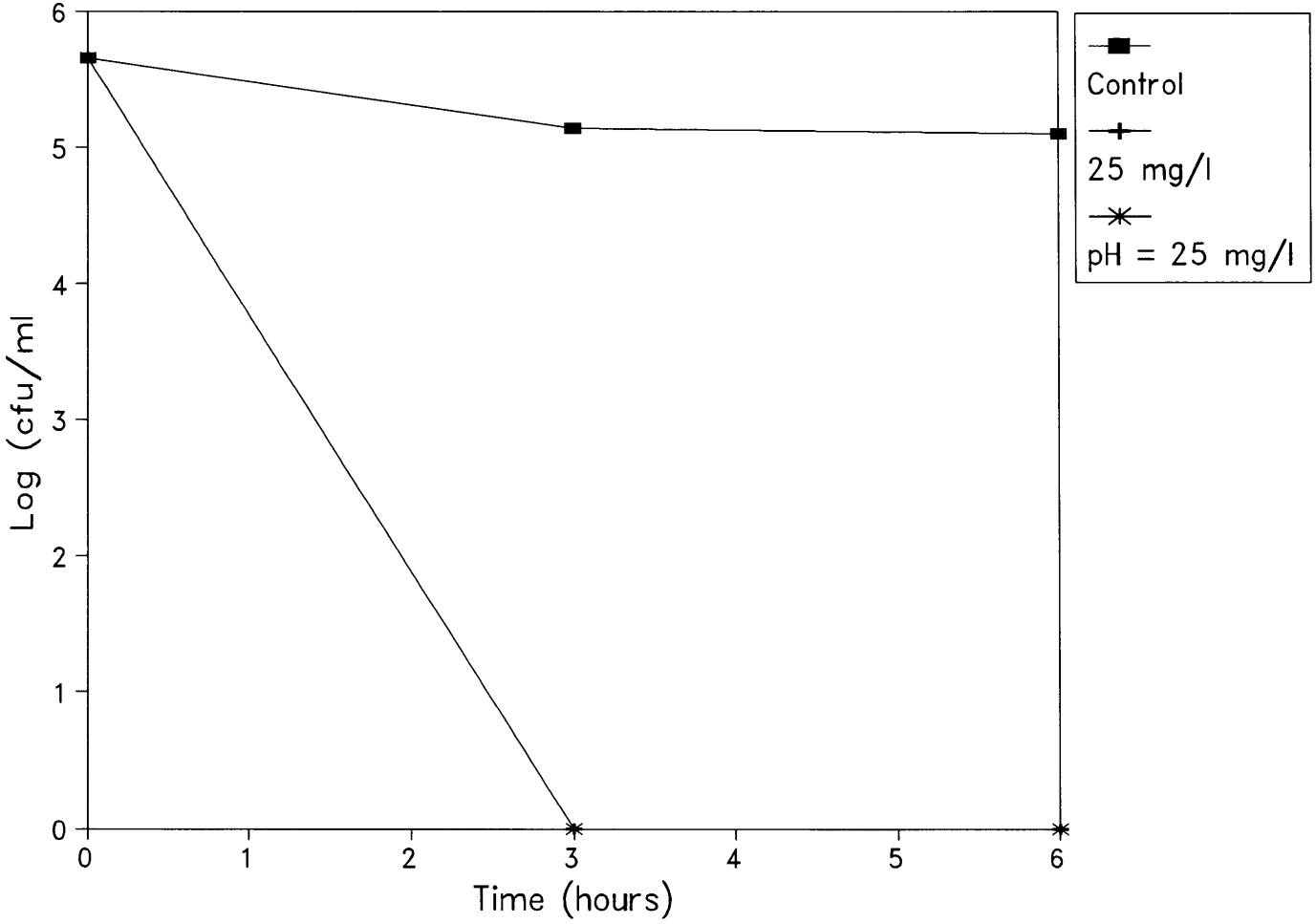

Fig. 1 Effect of oxifulvic acid and pH equivalent (pH 4.05) to oxifulvic acid concentration on *S. aureus* over 6 hours (pH = quarter strength Ringers solution adjusted to pH value equivalent to the pH of the concentration of oxifulvic acid).

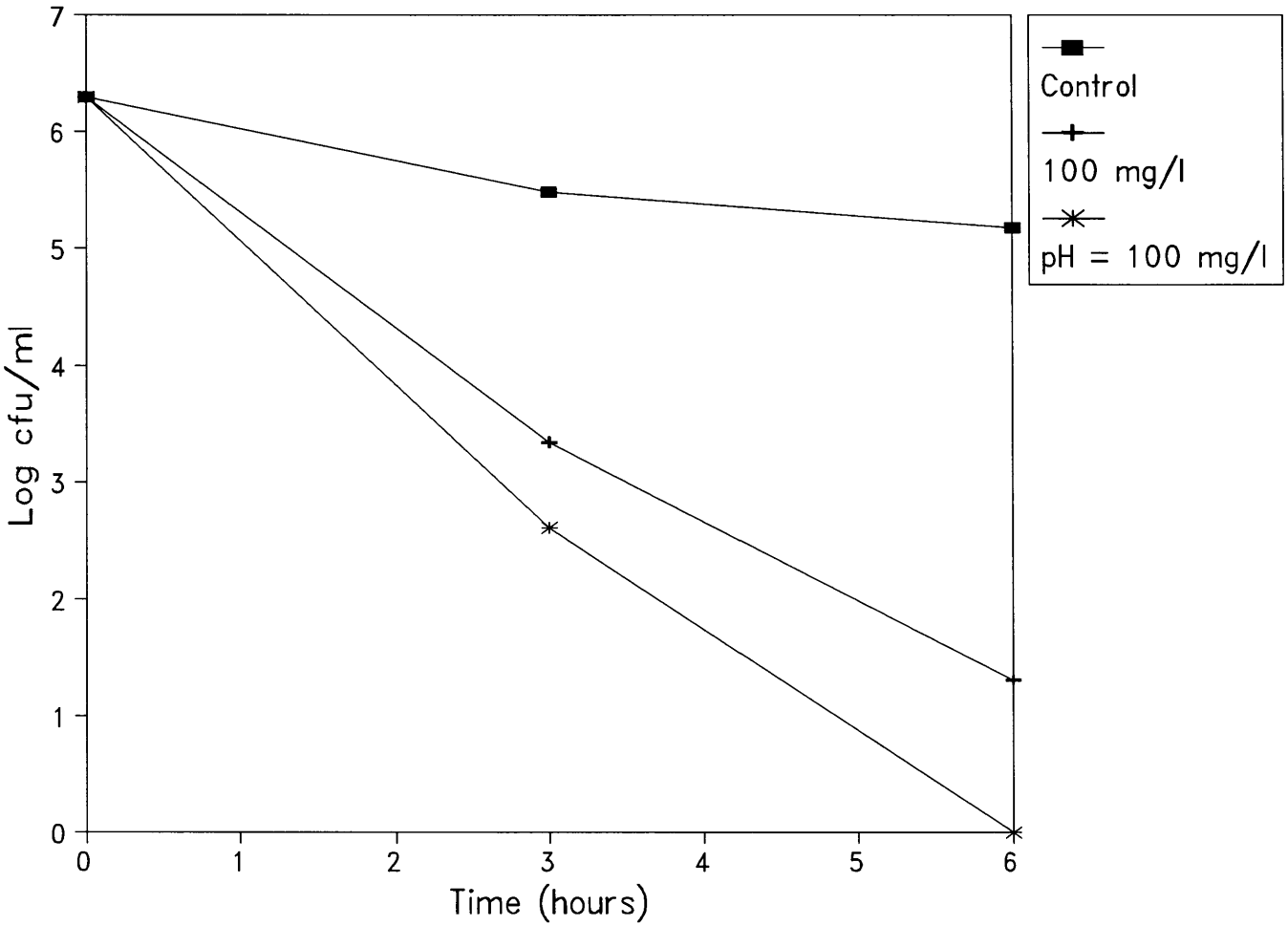

Fig. 2 Effect of oxifulvic acid and pH equivalent (pH 3.47) to oxifulvic acid concentration on *P. aeruginosa* over 6 hours (pH = quarter strength Ringers solution adjusted to pH value equivalent to the pH of the concentration of oxifulvic acid).

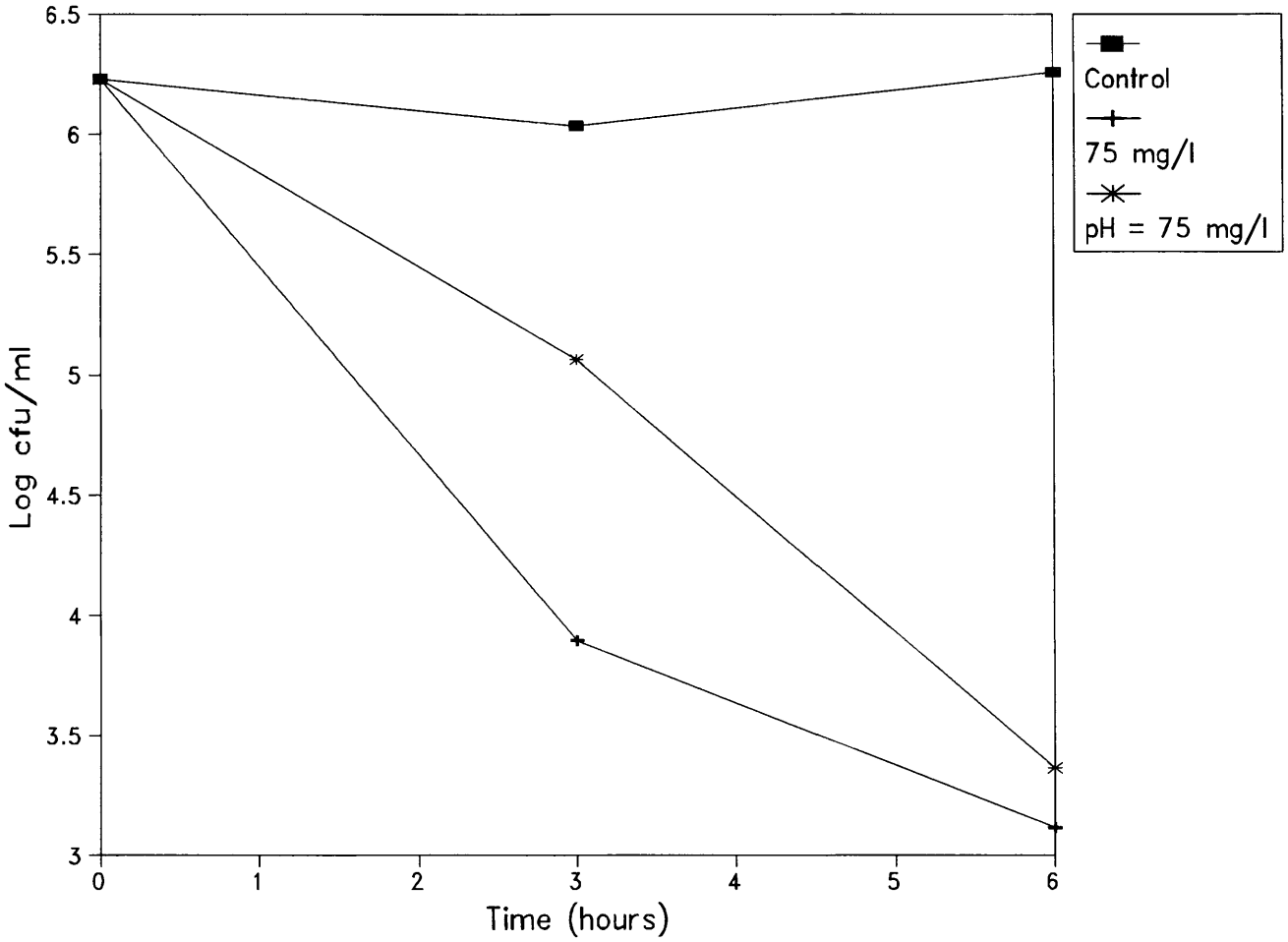

Fig. 3 Effect of oxifulvic acid and pH (pH 3.27) equivalent to oxifulvic acid concentration on *E. coli* K12 over 6 hours (pH = quarter strength Ringers solution adjusted to pH value equivalent to the pH of the concentration of oxifulvic acid).

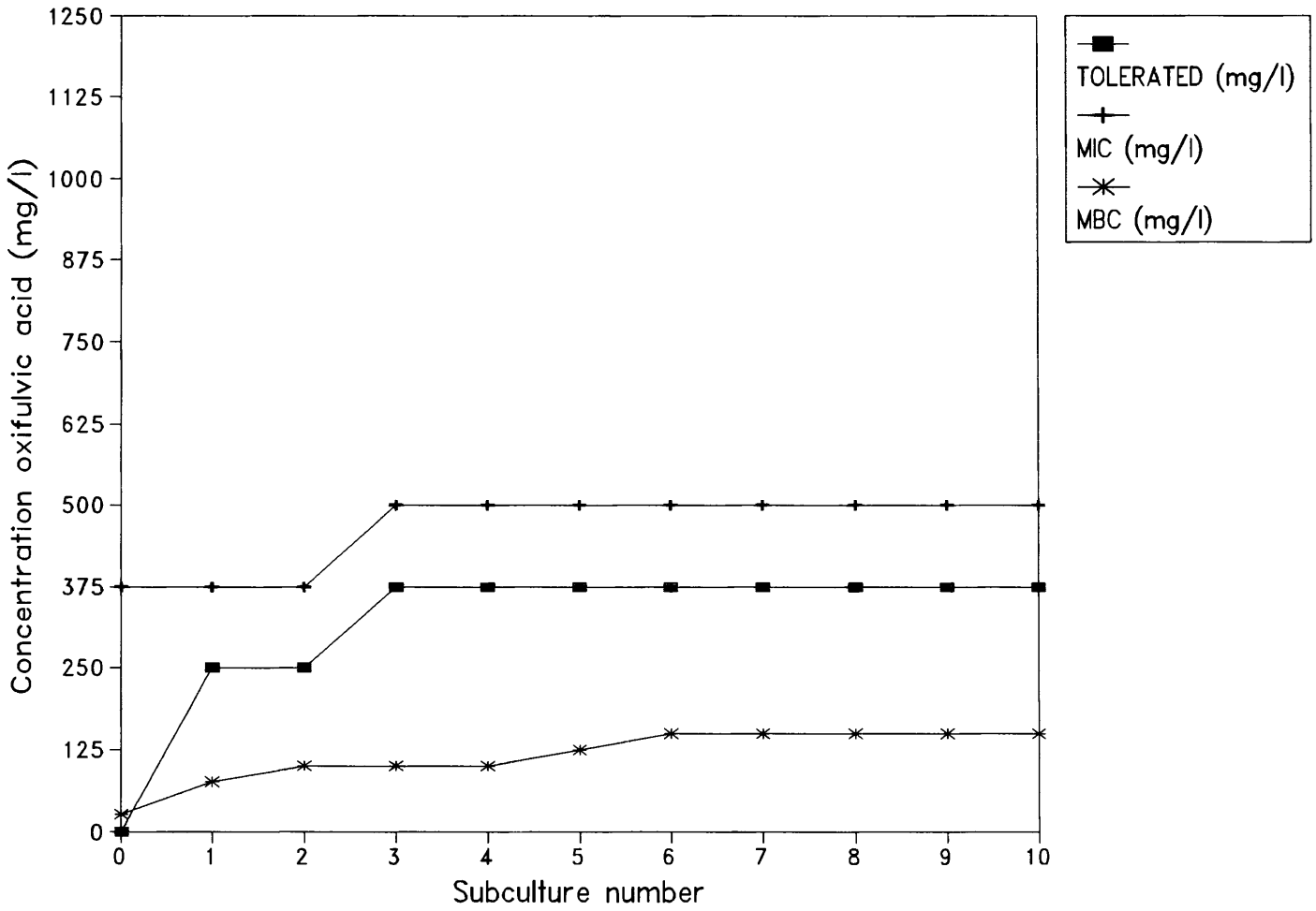

Fig. 4 Development of resistance to oxifulvic acid by *S. aureus* in NB (Tolerated = highest concentration of oxifulvic not inhibiting growth; MIC minimum inhibitory concentration; MBC minimum bactericidal concentration).

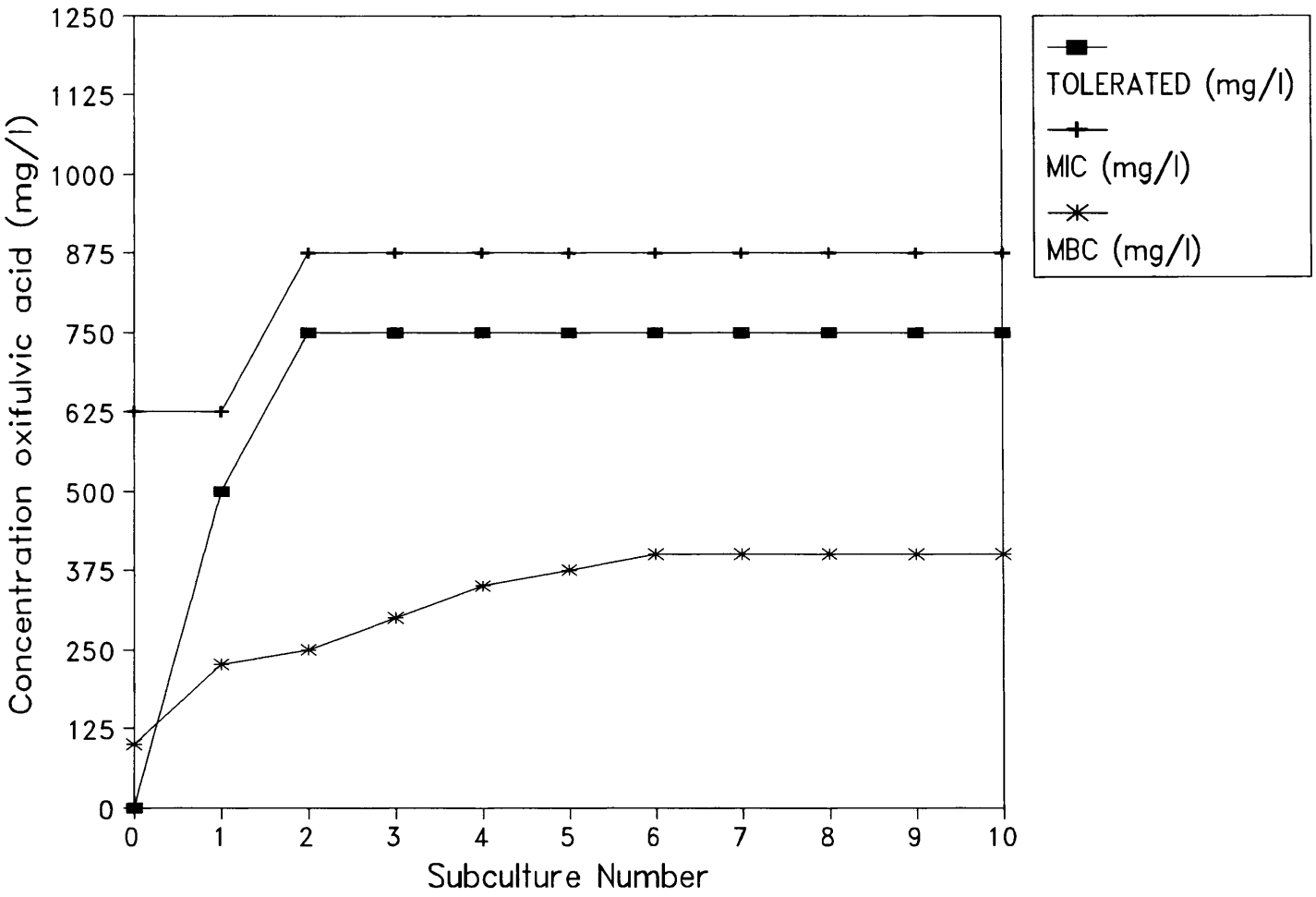

Fig. 5 Development of resistance to oxifulvic acid by *P. aeruginosa* in NB (Tolerated = highest concentration of oxifulvic not inhibiting growth; MIC minimum inhibitory concentration; MBC minimum bactericidal concentration).

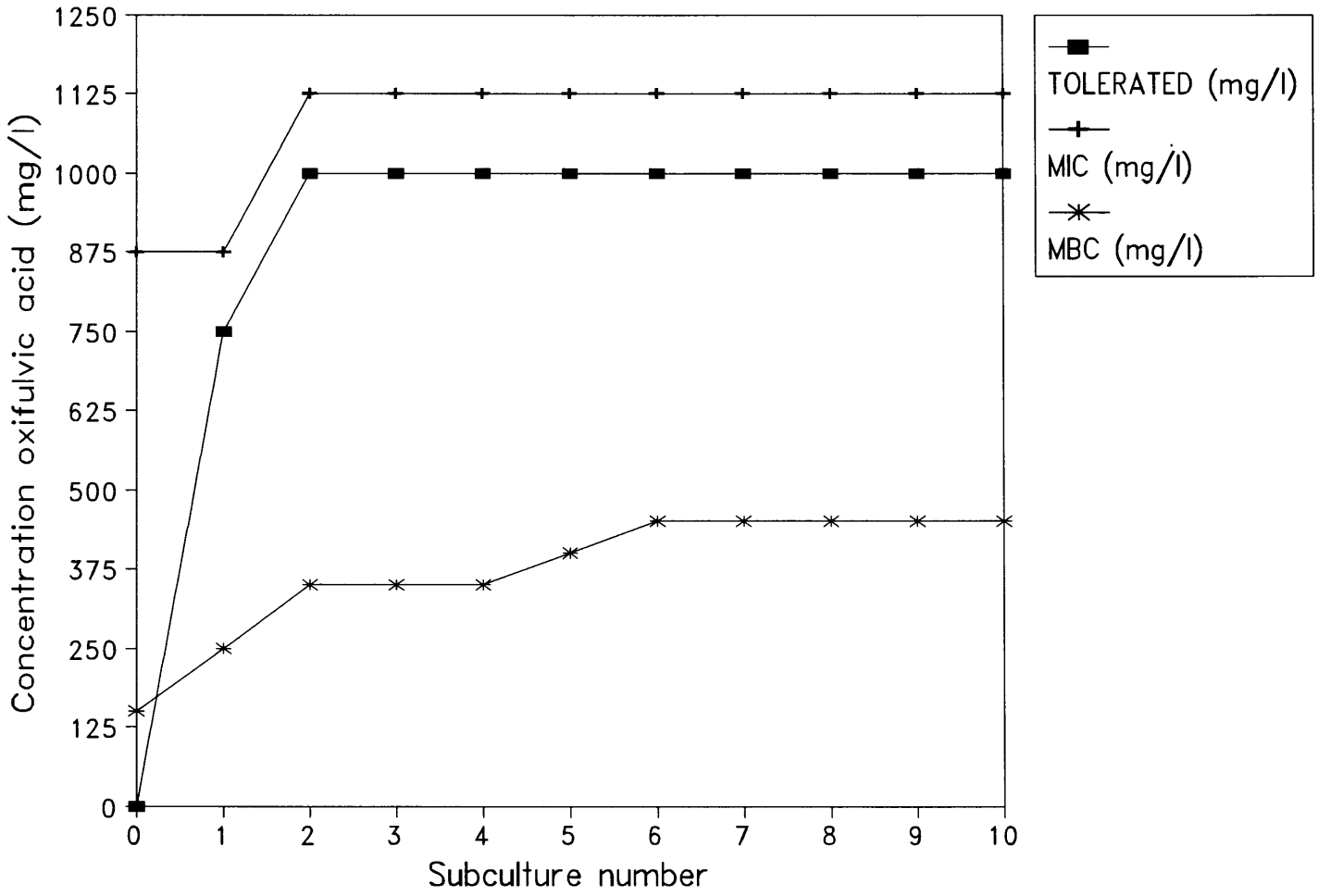

Fig. 6 Development of resistance to oxifulvic acid by *E. coli* strain K12 in NB (Tolerated = highest concentration of oxifulvic not inhibiting growth; MIC minimum inhibitory concentration; MBC minimum bactericidal concentration).

CHAPTER 6

(Submitted for publication in Water Research*)

THE EFFECT OF OXIDIZED COAL ON BACTERIA AND BACTERIOPHAGE IN RAW SEWAGE

* The language and style used in this chapter are in accordance with the requirements of Water Research.

ABSTRACT

The presence of as little as one infectious enteric virus in the environment poses a potential health hazard to water users. At present various methods are employed for the removal of enteric viruses from wastewater. Oxidised coal (oxicoal) was evaluated as an alternative to coal or activated carbon for the removal of enteric viruses from treated and untreated effluent, using an *Escherichia coli* strain K12 coliphage as model virus. The effect of time and oxicoal concentration on the removal of coliphages from a suspension and raw sewage was determined. Oxicoal removed 100% of the coliphages present in seeded sterile distilled water and raw sewage. The results indicated that oxicoal could be used as a substitute for coal in a dual-media sand/coal filter for the removal of enteric viruses from treated and untreated water.

INTRODUCTION

Viruses are found naturally in domestic and industrial wastewater and their fate once they enter the environment is of concern due to their possible pathogenicity (Grabow *et al.*, 1978; Bixby and O'Brien, 1979). These pathogenic viruses are generally referred to as enteric viruses since the viruses are released by the faecal route. Since the infective dose of many viruses is low and they are able to survive in the environment, the release of enteric viruses into the environment can pose a serious health risk to water users (Lewis and Metcalf, 1988). There is ample evidence to indicate that human enteric viruses may appear in the environment due to the release of inadequately treated wastewater from

treatment plants (Lewis and Metcalf, 1988; Thurman and Gerba, 1988). Hepatitis A, Norwalk and Rota viruses have been associated with waterborne outbreaks of disease due to the release of treated sewage effluent (Lewis and Metcalf, 1988).

The problems associated with the isolation and detection of enteric viruses has led to the use of coliphages to determine the effect of various strategies on the removal of enteric viruses from treated and untreated water (Kott *et al.*, 1974; Grabow *et al.*, 1984). Coliphages are used since they fulfil the basic criteria needed for an ideal indicator as described by Kott (1992).

Activated carbon adsorption (Cookson, 1967), sand filtration followed by chlorination or oxidation ponds (Pretorius, 1962) are currently used to produce water free of pathogens, including enteric viruses (Bitton, 1980; Lewis and Metcalf, 1988). Chlorination, however, only removes *ca.* 84% of the enteric viruses present in sewage effluent (Lewis and Loutit, 1989). The effectiveness of chlorination is further dependent on the prior removal of particulate material by filtration, sedimentation or adsorption (Lewis and Loutit, 1989). The activated sludge process, used to treat wastewater, removes *ca.* 99% of enteric viruses in wastewater (Bitton, 1980; Lewis and Metcalf, 1989). Complete removal of enteric viruses from sewage can be accomplished by tertiary treatment procedures that involve coagulation, flocculation, sedimentation and disinfection (Lewis and Metcalf, 1989). This treatment procedure is, however, very costly limiting its application. A more feasible alternative would be to enhance the effectivity of existing treatment procedures. Therefore, since coal and activated carbon have been used to remove viruses from wastewater (Orza and Chaudhuri, 1977; Bitton, 1980), oxicoal was investigated as an alternative to coal or activated carbon for virus removal.

MATERIALS AND METHODS

Production of oxidized coal

Oxidized coal, prepared by the controlled wet oxidation of a South African bituminous coal (Cronje, 1988), was obtained (as a dry powder) from the Division for Energy Technology, Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa.

Selection of coliphage as model for enteric viruses

Bacteriophages are indicators of the presence of human enteric viruses in water (Kfir, 1989). Coliphages have similar survival patterns during water treatment processes to those of enteric viruses. They are therefore often used as model viruses in studies to determine the effect of various strategies on the removal of enteric viruses from polluted water (Bitton, 1980; Ketratanakul and Ohgaki, 1989). They have the further advantage in that detection and enumeration of the coliphages is rapid and easy to perform. Various coliphage (*e.g.* MS2, T4 and KN1) have been used as model viruses to determine the effect of removal strategies on enteric viruses. *Escherichia coli* strain K12 has been used as host by Ketratanakul and Ohgaki (1989) for the isolation of phage in a study to determine the removal of phage by an activated sludge process. The choice of a particular host for the isolation of coliphage would depend on the aim of the study conducted. *Escherichia coli* strain K12 was selected as host in this study since coliphage could easily be isolated, enumerated and a high titre stock prepared using *E. coli* strain K12 as host.

Isolation of bacteriophage from sewage

Raw sewage was collected from the influent stream to an activated sludge plant at the Daspoort (Pretoria, South Africa) wastewater reclamation plant. Bacteriophage was isolated according to the enrichment technique described by Adams (1957). Briefly, a sample of raw sewage (40.0 ml) was mixed with 5.0 ml ten times strength (Nutrient Broth, made up to 10 times normal strength, *i.e.* 16.0 g NB/100 ml) Nutrient Broth (NB, Merck, South Africa) solution. An inoculum of 5.0 ml from an overnight culture (*ca.* 16 h at 37 °C in 10.0 ml NB) of *E. coli* strain K12 (obtained from Prof W.O.K. Grabow, Head, Department of Medical Virology, University of Pretoria) was added to the raw sewage-NB mixture. The mixture (raw sewage-NB-*E. coli* strain K12) was incubated for 48 h at 37 °C to allow for multiplication of the bacteriophage. After incubation the enriched coliphage suspension was centrifuged (10 000 x g for 10 min at 4 °C) in a Hermle 360K bench top centrifuge (Omni Science, Johannesburg, South Africa) to remove any unlysed cells. Cell debris and whole cells were removed by filtration though 0.2 μm filters (Millipore, South Africa). The filtered supernatant, containing the phage particles, was stored at 4°C and the phage titre determined using the agar-overlay method of Adams (1957).

Effect of time and oxicoal concentration on phage removal from distilled water

The effect of time and oxicoal concentration on the removal of phage from distilled water was determined using 0.0, 3125.0, 6250.0, 12500.0 and 25000.0 mg/l oxicoal incubated in 20 ml sterile distilled water containing 10^5 plaque forming units (pfu)/ml. The oxicoal-coliphage mixture was shaken (120 r.p.m.), to keep the oxicoal suspended, at 28 °C. Aliquots were removed after 15, 30 and 60 min and centrifuged to

remove the oxicoal. The supernatant was assayed for viable phage by the agar-overlay method (Adams, 1957), using *E. coli* strain K12 as host. Distilled water containing no oxicoal was used as a control. This experiment was done in duplicate and the average viable coliphage count determined.

An adsorption equilibrium was assumed to have been achieved when no more viable coliphage could be enumerated in the supernatant. The equilibrium adsorption capacity (EAC) for each oxicoal concentration was calculated by substituting the control (pfu/l) recorded at the time of equilibrium in the following formula:

$$EAC = \text{Control (pfu/l)} \div \text{Oxicoal (mg/l)}$$

Effect of time and oxicoal concentration on the removal of phage from raw sewage

The concentration of oxicoal capable of removing coliphage, coliforms and aerobic bacteria from raw sewage was determined. Samples of raw sewage (100.0 ml) were mixed with 0.0, 5000.0, 10000.0, 20000.0 and 30000.0 mg/l oxicoal and incubated with shaking (120 r.p.m.) at 28 °C for 24 h. At selected time intervals aliquots were removed and centrifuged to remove the oxicoal. The supernatant was assayed for viable coliphage by the agar-overlay method (Adams, 1957), using *E. coli* strain K12 as host. The aliquots were also assayed for total aerobic bacteria, on Standard One Nutrient Agar (STD1, Biolab. Merck, South Africa), and coliform bacteria on McConkey agar (Biolab, Merck, South Africa) plates incubated at 37°C for 48 h. Raw sewage without oxicoal was used as a control. This experiment was done in duplicate and the average aerobic bacterial count, coliphage and coliform count determined.

RESULTS AND DISCUSSION

Phage removal was dependent on exposure time and oxicoal concentration (Fig. 1). Oxicoal at 25000.0 mg/l removed 100% of the phage from solution, within 1 min after addition of the coliphage. A time of 15 min was required using 12500.0 mg/l oxicoal to remove the phage. Thirty minutes was needed for complete removal of the phage by 3125.0 and 6250.0 mg/l oxicoal, respectively.

The increased contact time required for the removal of coliphage from suspension indicated that contact time and available reactive surface area of the oxicoal played a role in the removal of the coliphage. This was demonstrated by the fact that the large surface area available at 25000.0 mg/l oxicoal removed the coliphage within 1 min after exposure. However, at the lowest available surface area (3125.0 mg/l oxicoal) complete removal only occurred after 30 min exposure. The removal of the coliphage by 3125.0 mg/l oxicoal in 30 min could have been due to the adsorption of the coliphages to the available surface area, as indicated by the reduction in coliphage concentration within 1 min after exposure. The more gradual decline in coliphage count that occurred, thereafter (Fig. 1) was ascribed to the release of inactivated coliphage from the surface and subsequent adsorption of viable coliphage to the surface, of the oxicoal. This mechanism has been reported as one of the possible mechanisms of virus inactivation by aluminium metal and clay (Thurman and Gerba, 1988). The presence of inactive coliphage in the supernatant was, however, not determined since the aim of this study was to determine the effect of oxicoal on coliphage in solution and not the mechanism of removal.

Work done on the removal of coliphage and enteric viruses by activated carbon has been reviewed by Bitton (1980) and Gerba (1984). Activated carbon is capable of

removing a maximum of 76% of the coliphages and enteric viruses present in a solution (Bitton, 1980: Gerba, 1984). The percentage removal is dependant on flow rate and the presence of soluble organic compounds which competed with the virus particles for adsorption sites on the activated carbon (Gerba, 1984). Therefore, when compared to activated carbon, oxicoal (100% removal) was more effective in removing coliphage from suspension.

By contrast, coal used in dual-media filtration with sand, was capable of removing 100% of the coliphages from suspension (Orza and Chaudhuri, 1977). Orza and Chaudhuri (1977) reported that the equilibrium adsorption capacity of 1000.0 mg/l Giridih, Churcha and Neyveli coals were 4.3×10^3, 3.8×10^3 and 3.6×10^3 pfu/mg, respectively. The equilibrium adsorptive capacity of oxicoal was calculated to be 1.38×10^8, 9.6×10^6, 7.2×10^6, and 3.6×10^6 pfu/mg for 3125.0, 6250.0, 12500.0 and 25000.0 mg/l oxicoal, respectively.

The equilibrium adsorption capacities of oxicoal indicate that oxicoal was capable of removing *ca.* 1×10^4 phage/mg more from suspension than the coals evaluated by Orza and Chaudhuri (1977). Since identical conditions and coliphages were not used a direct comparison was not possible. Based on the equilibrium adsorptive capacity values obtained oxicoal was more effective than coal in removing coliphages from suspension.

Effect of time and oxicoal concentration on the removal of phage from raw sewage

Oxicoal at 5000.0 mg/l was not capable of removing coliphage, aerobic bacteria and coliforms from raw sewage after 24 h exposure (Fig. 2). Only 45% of the coliphage present in raw sewage was removed by oxicoal at 5000.0 mg/l. Coliform and aerobic bacteria were removed 100% after 24 h exposure to 20000.0 and 30000.0 mg/l oxicoal

(Fig. 2).

Oxicoal at 20000.0 and 30000.0 mg/l required 90 and 180 min, respectively, to remove 100% of the coliphage from raw sewage (Fig. 3). The initial decrease (log 2.80 to log 2.30) and subsequent increase (log 2.30 to log 2.90) in control coliphage counts (Fig 3) can be ascribed to adsorption of the coliphage to the *E. coli* present in the raw sewage with subsequent lysis of infected *E. coli* and release of coliphage into the suspension.

Coliform numbers were reduced by 100% after 180 and 1440 min exposure to 30000.0 and 20000.0 mg/l oxicoal, respectively (Fig. 4). Total aerobic bacterial numbers were reduced by 100 % after exposure for 720 min to 30000.0 mg/l oxicoal (Fig. 5). A 4-log reduction in total aerobic bacterial count was achieved with exposure to 20000.0 mg/l oxicoal for 720 min (Fig. 5). The increase in aerobic count from 25 colony forming units (cfu)/ml at 720 min to 60 cfu/ml at 1440 min indicated re-growth occured of aerobic bacteria that were not adsorbed by the oxicoal.

Initial experiments (Fig. 1 and 2) indicated that, in the presence of sewage effluent, higher concentrations of oxicoal (20000.0 mg/l) were required for complete removal of coliphages in comparison to distilled water (3125.0 mg/l). Oxicoal at 20000.0 mg/l was required to remove 100% of the *E. coli* strain K12 coliphages present in sewage influent within 24 h (Fig. 2). Orza and Chaudhuri (1977) reported that in the presence of 5% settled domestic sewage sludge the equilibrium adsorptive capacity of giridih coal was reduced by 23.0 %. The complete removal of coliphage, coliform and total bacteria by 30000.0 mg/l oxicoal indicated that oxicoal has potential use in the provision of potable water. Potable water could be produced by using oxicoal as a substitute for coal in dual-media sand/coal filters for more effective removal of enteric viruses and pathogenic

bacteria in drinking and waste water reclamation plants. Alternatively, oxicoal-sand filters could be used to clean the water prior to chlorination. Oxicoal could further be added in powder form to non-potable water suspected of being contaminated by enteric viruses, prior to treatment for the supply of potable water.

ACKNOWLEDGEMENTS

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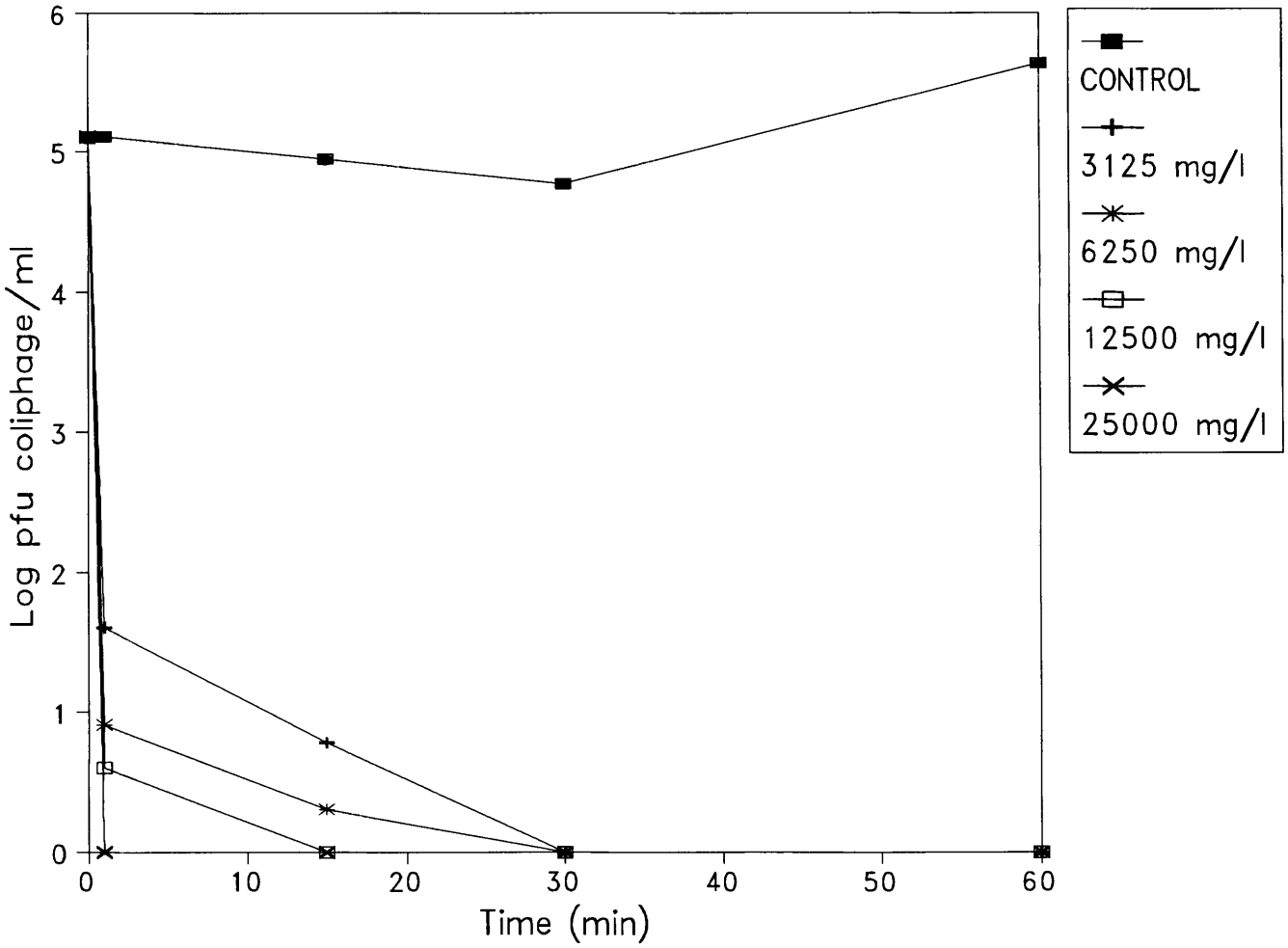

Fig. 1. Effect of exposure time on the removal of coliphages from distilled water by oxicoal (added at 0.0, 3125.0, 6250.0 12500.0 and 25000.0 mg/l).

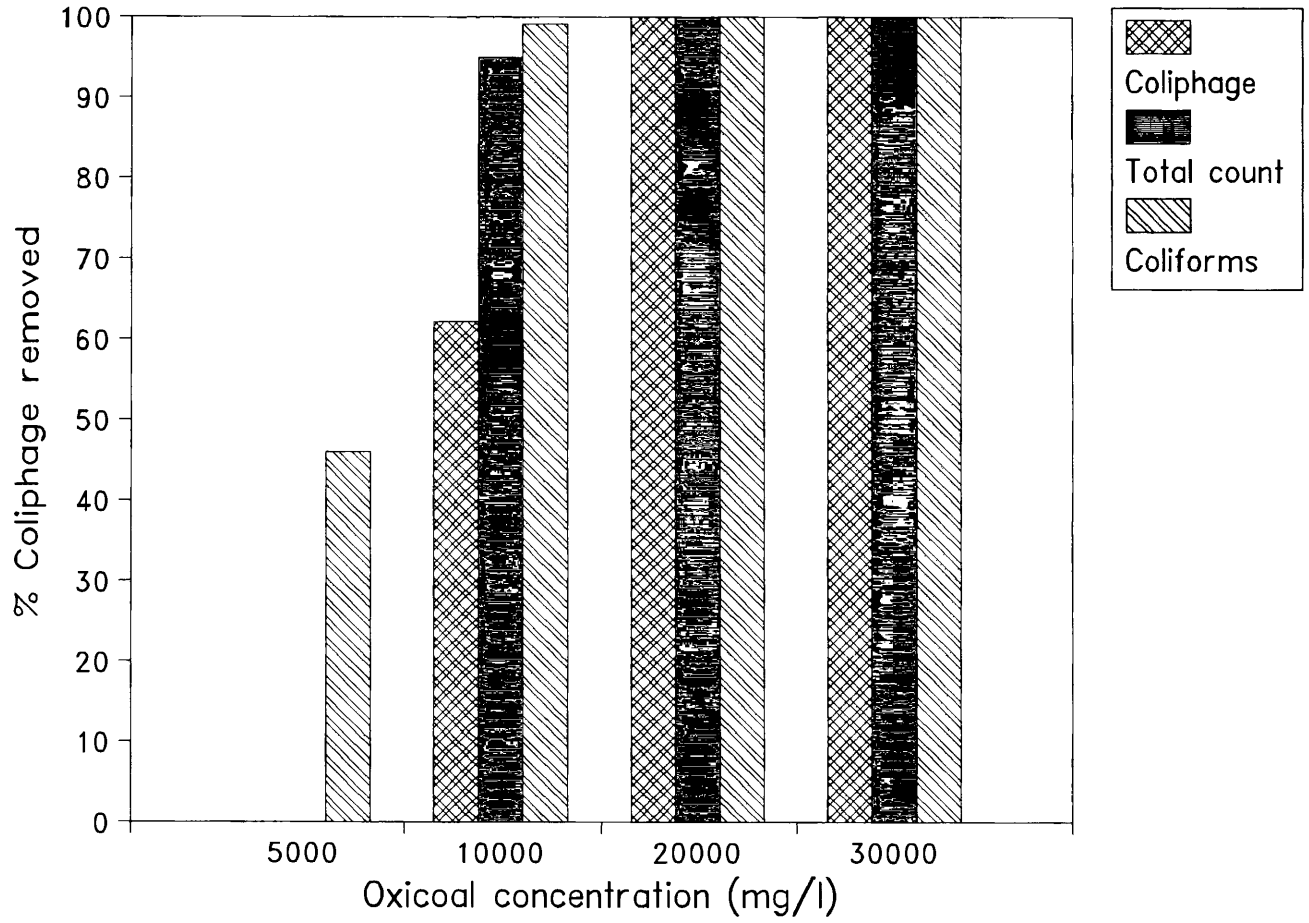

Fig. 2. Percentage removal of coliphage, aerobic bacteria and coliforms from raw sewage after exposure for 24 hours to oxicoal (added at 5000.0, 10000.0, 20000.0 and 30000.0 mg/l).

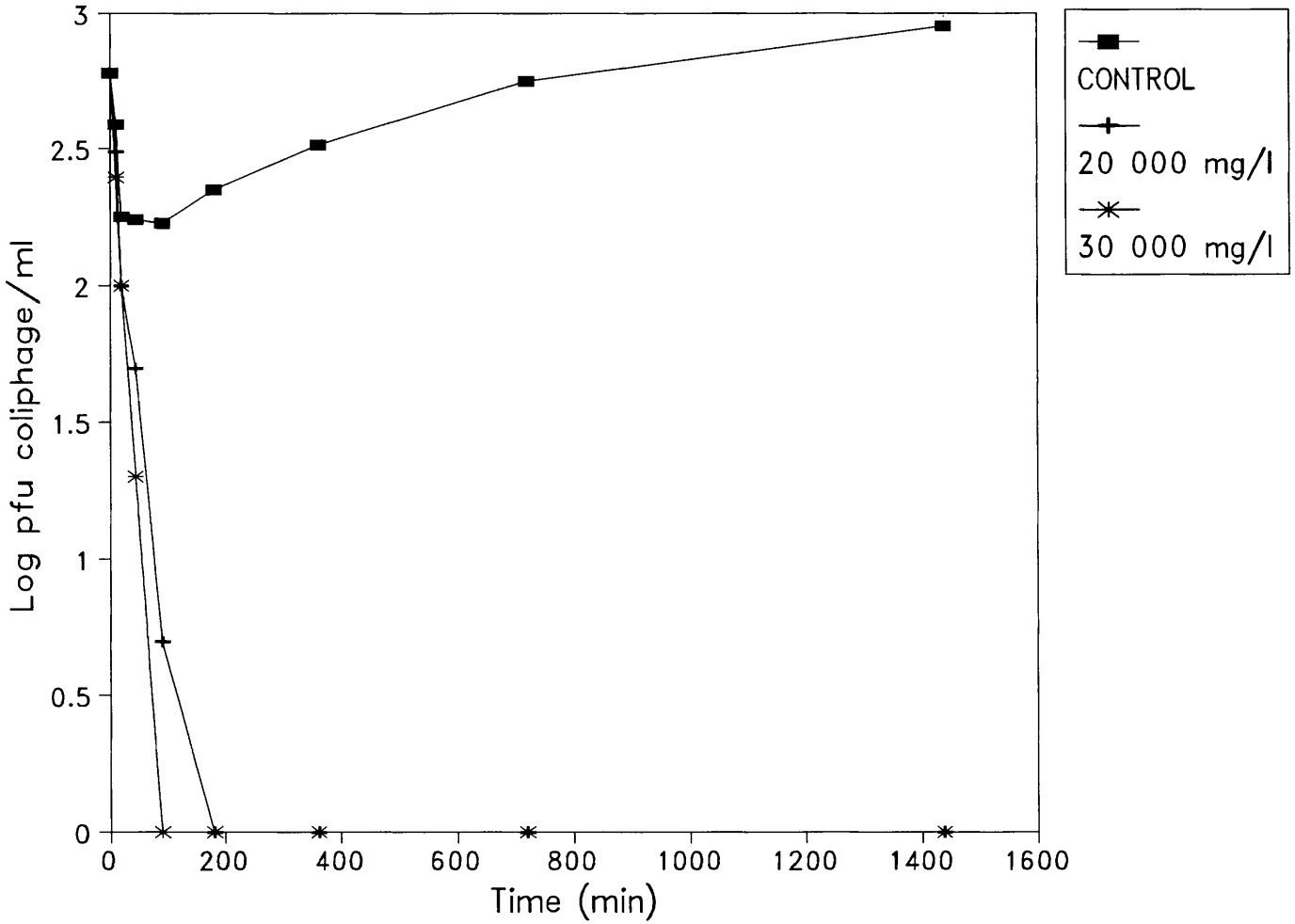

Fig. 3. Effect of exposure time on the removal of coliphages from raw sewage by oxicoal (added at 20000.0 and 30000.0 mg/l).

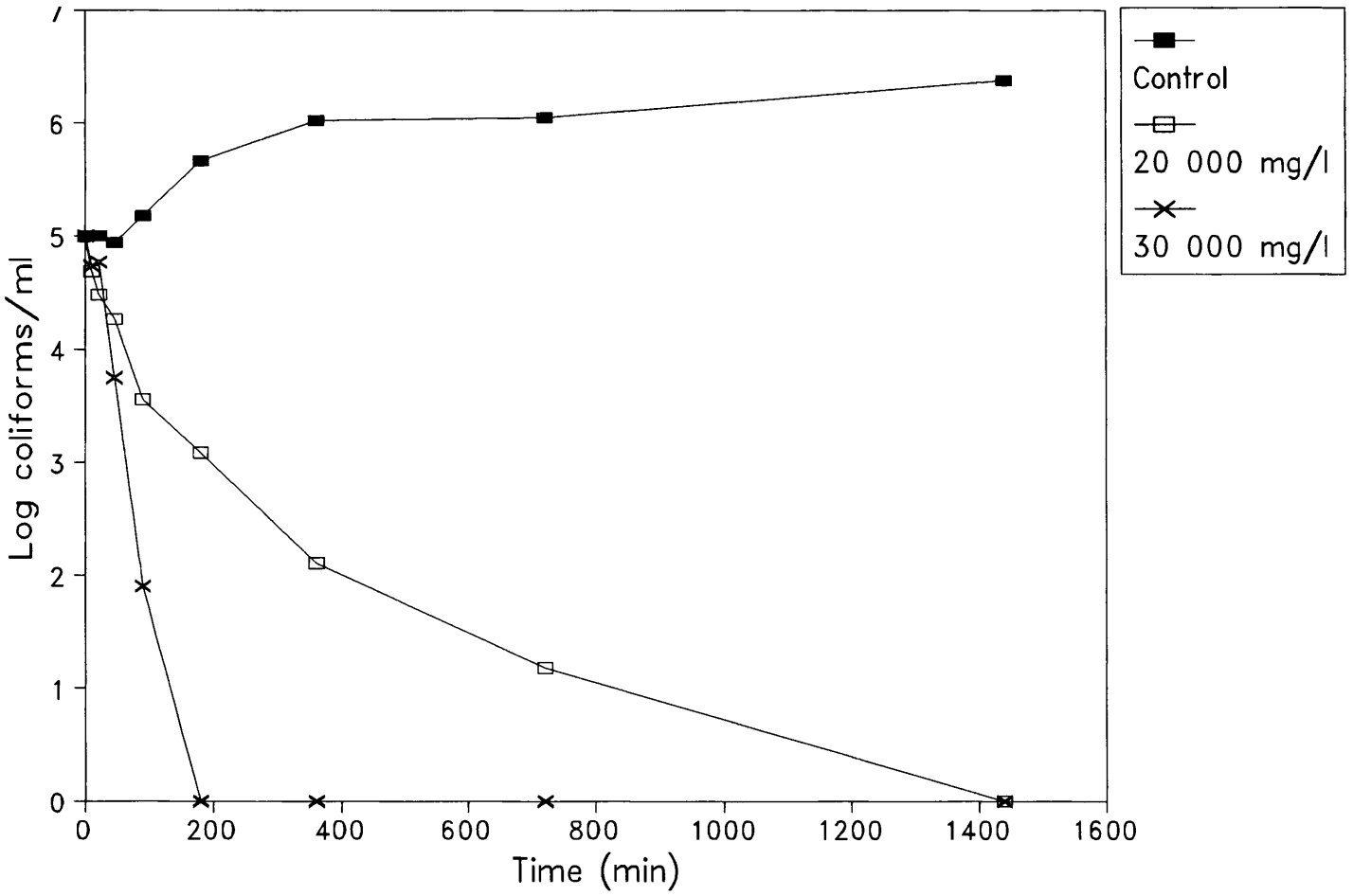

Fig. 4. Effect of exposure time on the removal of coliform bacteria from raw sewage by oxicoal (added at 20000.0 and 30000.0 mg/l).

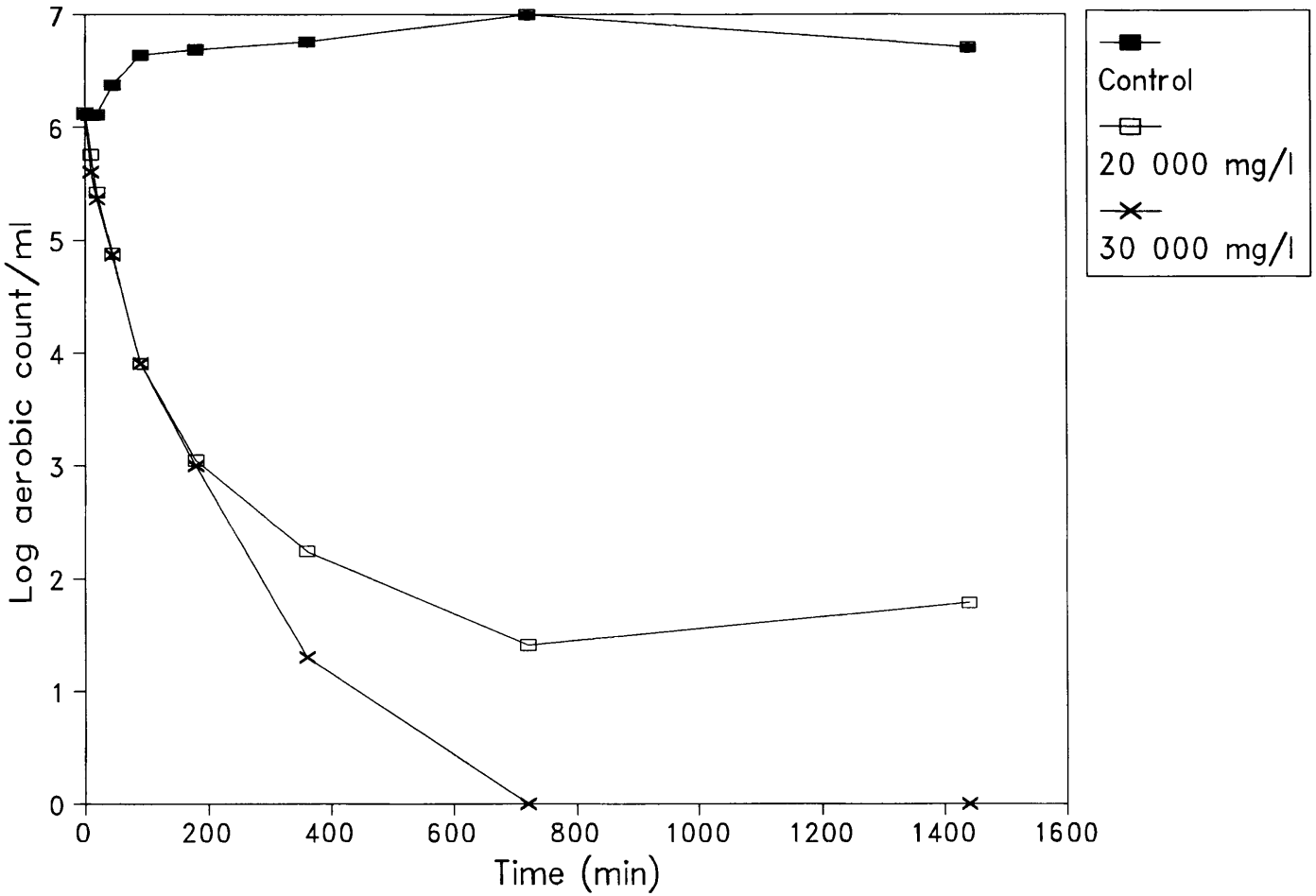

Fig. 5. Effect of exposure time on the removal of aerobic bacteria from raw sewage by oxicoal (added at 20000.0 and 30000.0 mg/l)

CHAPTER 7

CONCLUSIONS

Bactericidal applications

- Oxicoal, oxihumate, oxihumic acid and oxifulvic acid were bactericidal at 8000.0, 10000.0, 2000.0 and 100.0 mg/l, respectively, against *P. aeruginosa*.
- Oxifulvic acid was the most bactericidal coal-derived product produced, since 150.0 mg/l oxifulvic acid was required for bactericidal activity against seventeen test bacteria.
- Oxifulvic acid was bactericidal at 100.0 mg/l against twelve bacteria isolated from water-cooling systems in South Africa. Use of oxifulvic acid to control biofouling and biocorrosion in water-cooling systems is restricted since the optimum pH for bactericidal activity of oxifulvic acid (pH 3.0 to 4.0) is lower than the average pH (pH 8.0) reported for water-cooling systems. Furthermore, the bactericidal activity of oxifulvic acid was inhibited by the presence of calcium ions (125.0, 250.0 and 500.0 mg/l) and organic matter (5000.0 mg/l), both of which often occur in water-cooling systems.
- Field trial evaluations of oxifulvic acid indicated that oxifulvic acid (200.0 and 400.0 mg/l) was unable to control the growth of sessile and planktonic bacteria in a water-cooling system.

Fungicidal application

- The concentration (> 4000.0 mg/l) of oxifulvic acid required for fungicidal action indicated that it did not have potential for use as a fungicidal compound in the water-cooling or wood preservation industries.

Algicidal application

- Oxifulvic acid was effective at 400.0 mg/l against *Chlorella, Chlorococcum* and *Calothrix* species isolated from water-cooling systems. Oxifulvic acid does not show potential for use as an algicide since the concentration required for algicidal activity (400.0 mg/l) was higher than the algicidal concentrations reported for commercial algicides (5.0 to 25.0 mg/l).

Disinfectant applications

- Oxifulvic acid was bactericidal at 800.0 mg/l, in comparison to the 48000.0 mg/l coal-tar disinfectant required for bactericidal action. This indicated that oxifulvic acid warrants further investigation for use as a disinfectant in place of the coal-tar disinfectants.
- Oxifulvic acid has no potential for use as a sporicidal compound since a concentration > 10000.0 mg/l oxifulvic acid was required for sporicidal action against the spores of *B. cereus, B. subtilis* and *B. megaterium.*
- Oxifulvic acid was completely (100%) inhibitory at 96.0 mg/l within 10 min against coliphage V_1. This increased the possible applications of oxifulvic acid as a disinfectant for use in hospitals and agriculture.

- A additive effect was exhibited by oxifulvic acid combined with ethanol (oxifulvic acid 75.0 mg/l and ethanol 10.0 %). The addition of acids to ethanol to increase the antimicrobial activity of ethanol is well known since acidified ethanol (ethanol acidified with HCl) is used as a disinfectant in hospitals. This combination of oxifulvic acid does therefore have a potential use as a disinfectant formulation for the disinfection of floors in hospitals and agriculture.
- The synergistic combination of sodium hypochlorite and oxifulvic acid indicated that either an oxifulvic acid and sodium hypochlorite mixture or possibly a chlorinated oxifulvic acid could be effective as a disinfectant given that the formulation is non-corrosive.
- The combination of sodium dodecyl sulphate SDS (125.0 and 250.0 mg/l) and oxifulvic (50.0 and 25.0 mg/l) acid was the most synergistic combination evaluated. This combination has the greatest potential for use as a disinfectant formulation, since the pH optimum for antimicrobial activity of SDS (an anionic detergent) is in the same range as that of oxifulvic acid (pH 3.0 to pH 4.0). The detergent action of the SDS makes this a useful formulation, since it would allow for a simultaneous cleaning and disinfectant action. This formulation could be used for the cleaning of hard non-porous surfaces in hospitals and agriculture.
- The increase in resistance of *P. aeruginosa, S. aureus* and *E. coli* strain K12 to oxifulvic acid was ascribed to habituation of the bacteria to a sub-lethal pH.

- The bactericidal action of oxifulvic acid against *P. aeruginosa, S. aureus* and *E. coli* strain K12 was ascribed to the reduction in external pH resulting in the failure of the pH homeostasis mechanism of the bacteria to maintain a neutral (pH 7.2) internal pH.

Control of acid mine drainage bacteria

- In comparison to sodium benzoate (15.0 mg/l) and sodium lauryl sulphate (2.0 mg/l), oxifulvic acid inhibited iron oxidation by iron-oxidizing bacteria at 100.0 mg/l in laboratory studies. However, depending on the cost effectivity, oxifulvic might be a better alternative to use in practice than sodium benzoate and sodium lauryl sulphate especially since the latter has proved to be ineffective in field applications.

Biological water purification

- Increasing oxicoal concentrations, from 3125.0 mg/l to 25000.0 mg/l produced more efficient removal of bacteriophages from solution. Removal of the coliphages was due to adsorption of the viruses to the surface of the oxicoal. The viral removal capacity of oxicoal suggested that it could be used in place of coal in a dual-media sand/coal filter for more effective removal of enteric viruses and bacteria in potable and waste water reclamation plants. The oxicoal/sand filters could be used to remove viruses from potable water prior to chlorination. Oxicoal could furthermore be added in powder form to water suspected of being contaminated by enteric viruses in order to remove the viruses, before treatment for the

provision of potable water. The lowering of the pH of the oxicoal treated water might, however, be a limiting factor and needs to be investigated.

Practical feasibility

- The practical application of oxifulvic acid is limited due to the presence of phenolic compounds and the low pH (1.89) of the oxifulvic acid fraction. Application of oxifulvic acid as a biocide in industries (*e.g.* food, beverage and meat processing industries) that are sensitive to the presence of phenolic compounds and low pH would therefore not be advisable. The synergist formulation of oxifulvic acid could, however, be used for the disinfection of hard non-porous surfaces (*i.e.* floors) in the sensitive and other (*e.g.* agricultural disinfectant) industries.
- Oxicoal (20000.0 mg/l) could be added to water contaminated with enteric viruses and pathogens to form a slurry. This procedure could be used to remove enteric pathogens from water prior to the production of potable water.

Further work

- The production cost of oxicoal and oxifulvic acid needs to be determined to enable the cost-effectiveness of the various application of oxicoal and oxifulvic acid to be determined
- The application of synergistic formulations of oxifulvic acid as disinfectants warrants further investigation.

- The application of oxifulvic acid to control acid mine drainage needs to be investigated to determine the practical feasibility of this application.
- The use of oxicoal for the removal of enteric viruses from contaminated water needs to be investigated to determine the optimum conditions, contact time and range of enteric viruses removed.

Table listing key findings with regards to each area of application investigated

Area of application	Conclusion
Bactericidal	Oxifulvic acid was the most bactericidal coal-derived product evaluated since 150.0 mg/l was required for bactericidal activity against seventeen bacterial rest organisms.
Fungicidal	A concentration of oxifulvic acid > 4000.0 mg/l was required for fungicidal activity against twelve fungal isolates.
Algicidal	*Chlorella, Chlorococcum* and *Calothrix* species isolated from water-cooling systems were inhibited by 400.0 mg/l oxifulvic acid.
Control of acid mine drainage	Oxifulvic acid inhibited iron oxidation by iron-oxidizing bacteria at 100.0 mg/l.
Disinfectant	The combination of oxifulvic acid (25.0 mg/l) and SDS (250 mg/l) was the most synergistic combination evaluated.
Water purification	The 100% removal of coliphage from raw sewage with in 90 and 180 min by oxicoal at 20000.0 mg/l and 30000.0 mg/l, respectively, indicated that this product has a potential application for the removal of enteric viruses from polluted water