Fingerprinting of commercially available water treatment bactericides in South Africa

VS Brözel* and TE Cloete
Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa

Abstract

Eighteen dominant isolates from water-cooling systems were exposed to 50 mg ℓ^{-1} of commercially available bactericides, and the kill percentage was determined after 6 h. Application costs of all bactericides giving an average kill percentage of over 90%, were compared. Low cost bactericides were re-evaluated at cost-equivalent concentrations. Dichlorophen, sulphone, a thiocarbamate and biphenol performed best, killing the full spectrum of isolates cost-effectively. Certain expensive products performed rather poorly. e.g. isothiazoline and MBT. This study highlights the selective action of many bactericides and the inherent resistance of bacteria to a number of different bactericides. This implies the importance of matching bactericides to the dominant bacteria in systems.

Introduction

Bacterial colonisation of surfaces in an aqueous environment is a basic strategem for survival in nature as nutrients are more available at the solid - liquid interface (Hoppe, 1984; Lawrence, *et al.*, 1987). The resulting aggregates form microcolonies which develop into biofilms (McCoy *et al.*, 1981). These biofilms promote corrosion of metals by creating potential differences across surfaces and by harbouring sulphate-reducing bacteria (Iverson, 1987). They also increase fluid frictional resistance (McCoy *et al.*, 1981) and decrease the rate of heat energy transfer (Characklis and Cooksey, 1983). The above phenomena are termed collectively as microbially induced corrosion (MIC). As the costs attributable to microbially induced corrosion are high, ca. R400 million in 1988 to the South African Industry (Von Holy and Cloete, 1988), effective control of bacterial numbers in industrial aqueous environments is essential.

A range of bactericidal substances, commonly termed biocides or microbicides, are available, all of which are claimed by their agents to kill bacteria in aqueous sytems quantitatively. However, different bacteria react differently to bactericides, either due to differing cell wall properties (Paulus, 1987), or to other mechanisms of resistance, either inherent or inducible (Heinzel, 1988). It follows that evaluation of bactericide efficacy simply by determining the percentage kill against a standard laboratory pure culture could lead to misleading results (Allsop and Seal, 1986; Guthrie *et al.*, 1987). Bactericides should be evaluated against the organisms which they are expected to kill, i.e. the dominant ones in the system to be treated. The composition of microbial populations in systems varies with the type of water used (Cloete *et al.*, 1989b). A bacterial population structure of South African water-cooling systems was determined by Cloete *et al.* (1989a). Eighteen dominant isolates from this study were used to determine the bactericidal fingerprints of 32 commercially available non-oxidising water treatment bactericides.

Materials and methods

Bactericides and test organisms

Bactericides were obtained from the major South African suppliers and were coded for confidentiality. All were drawn from fresh stock. These are listed below according to their active constituents. No concentrations of active constituents were supplied by the suppliers.

Test isolates were taken from the culture collection of the Environmental Biotechnology Program at the Department of Microbiology and Plant Pathology, University of Pretoria, and were the dominant bacteria which had been isolated in a previous study (Cloete *et al.*, 1989a). These are listed in Table 2.

Bactericide evaluation procedure

Bacterial cultures were grown on Standard I nutrient agar (Std1) for 24 h at 30°C. Suspensions were prepared by washing cultures off with quarter strength Ringers solution using a sterile Drigalski spatula. Suspensions were diluted to ca. 3 x 10^8 cfu.mℓ^{-1} using the McFarland scale (McFarland, 1970). Initial counts of suspensions were determined by spreading 0,1 mℓ quantities of a serial dilution series in duplicate onto StdI agar plates. Concentrations at which bactericides should be applied were only specified by certain suppliers, and even then a broad specification was given. Therefore bactericides were initially added to the culture suspensions to a final concentration of 50 mg.ℓ^{-1} of product as supplied. Suspensions with bactericide were incubated at 25°C for 6 h, when viable counts were determined as above. Water-cooling systems have

TABLE 1
BACTERICIDES EVALUATED

Six isothiazoline samples
One dichlorophen sample
One sulphone sample
Two thiocarbamate samples
Four methylene bis-thiocyanate samples (MBT)
Two quaternary ammonium compound (QAC) samples
Two QAC-tin complex samples
One glutaraldehyde sample
Two isothiazoline-QAC samples
Three organosulphur samples
One organophosphorus sample
One broad spectrum sample (generic composition not specified)
One thiocyanomethylthiobenzothiazole (TCMTB) sample
Two phosphonium chloride samples
One biphenol sample

*To whom all correspondence should be addressed.
Received 13 February 1990; accepted in revised form 23 July 1990.

TABLE 2
TEST ORGANISMS USED FOR FINGERPRINTING BACTERICIDES

1	*Chromobacterium violaceum*
2	*Pseudomonas fluorescens*
3	*P. mendocina*
4	*P. vesicularis*
5	*P. putida*
6	*P. pickettii*
7	*Moraxella urethralis*
8	*P. alcaligenes*
9	*P. stutzeri*
10	*Alcaligenes faecalis*
11	*A. denitrificans* var.*xylosoxidans*
12	*Acinetobacter calcoaceticus*
13	C1 gram-positive isolate
14	C6 gram-positive isolate
15	C8 gram-positive isolate
16	C5 gram-positive isolate
17	J9 gram-positive isolate
18	K4 gram-positive isolate

various temperature zones, but as Berg *et al.* (1982) had shown *Escherichia coli* to be more resistant to chlorine dioxide at 15°C than at 37°C, it was therefore decided that exposure to bactericide should be at 20°C. Plates were incubated for 48h at 30°C.

The kill percentage was calculated for each individual culture-bactericide combination using the following formula.

$$\% \text{ Kill} = 100 - \frac{\text{Survivor count}}{\text{Initial count}} \times 100$$

Results were tabulated as bar charts grouped together for each bactericide. Average kill percentage and standard deviation were calculated for each set.

Cost-effectivity comparison calculations

All the bactericides exhibiting an average kill percentage greater than 90% were considered to be effective. All bactericides exhibiting a lower than 90% average kill percentage, were screened for their cost-effectivity. Each generic group was first considered separately. The cost-effective application concentration was calculated by comparing the relevant bactericide with the most effective related product as follows. The price of the best generic compound was divided by that of the cheaper one. The factor obtained was multiplied by 50 to obtain the cost-effective application concentration in $mg.\ell^{-1}$.

Cost-effective application concentration

$$= \frac{\text{Price of best product}}{\text{Price of test product}} \times 50\ mg.\ell^{-1}$$

Cost-effective application concentration meant applying as much bactericide as it would cost to apply 50 $mg.\ell^{-1}$ of the one which had been most effective initially. Where the new cost-equivalent application concentration was considerably higher than 50 $mg.\ell^{-1}$, fingerprints were again determined at the new application concentrations. Bar charts were again drawn and average kill percentage and standard deviation were calculated for each set. Finally the most effective bactericides were listed and their cost-effectivity index (CEI) calculated as follows:

$$CEI = \frac{\text{Cost x concentration}(mg.\ell^{-1})}{\% \text{ Kill}}$$

Results

Comparative analysis of bactericides

The data obtained are shown in Figs. 1 to 4. Average values and standard deviations for each bactericide are given in Table 3. Efficacy of generic bactericides varied considerably. In some cases lower price was parallel to lower efficacy, but in certain cases the opposite was found, e.g. bactericides E and AA. A high standard deviation was found for most bactericides, showing their selective nature. The quaternary ammonium compounds OACs, for example, killed most isolates well, but did not kill *Pseudomonas vesicularis* at all, with growth occurring in three of the QACs. The gram-positive isolate C6 also grew in three of the four QACs. The methylene bis-thiocyanate MBTs killed certain isolates well, but three of them did not kill *Moraxella urethralis* and three did not kill *P. alcaligenes.*

Re-evaluation of bactericides at cost-effective concentrations

Within generic groups, bactericides yielding an average kill percentage higher than 90% were taken to be cost-effective. Cost-effective application concentrations were calculated based on these prices. The data obtained were tabulated as bar chart fingerprints as depicted in Fig. 5. Table 4 shows the application concentrations, average percentage kill and standard deviations as well as application costs. Because some QACs foamed at concentrations higher than 50 $mg.\ell^{-1}$, they were not re-evaluated. The isothiazolins and MBTs (except MBT AA) were also discarded due to their low average percentage kill, high standard deviation and high cost. At the higher concentration both sulphone C and thiocarbamate D showed a smaller standard deviation (3,80 and 8,17), exhibiting a broad spectrum activity against the test strains. QAC M and organosulphur W showed a low average kill percentage and larger standard deviation (42,30 and 38,26), proving them not suitable for bactericidal application in water-cooling systems. Bactericides are listed according to their cost-effectivity in Table 5.

Discussion

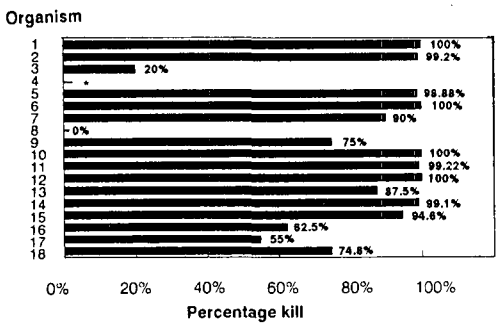
Method of evaluation

Evaluation of products for field application should always occur under conditions simulating the actual ones as close as possible. For large systems, *in situ* screening of a wide range of bactericides is not possible. However, the most important factors such as pH, temperature and nutrient concentration should be included in the experimental procedure when conducted in the laboratory. LeChevalier *et al.* (1988) showed that microbes attached to surfaces or entrapped in particles are shielded from antimicrobial agents. They showed that much higher concentrations are required to kill attached bacteria. Eigener (1988) reviewed a range of test procedures, where contact time and suspension method play

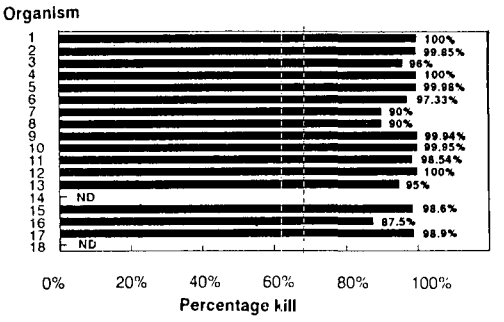
TABLE 3
COMPARATIVE ANALYSIS OF BACTERICIDES USED AT 50 mg.ℓ^{-1}

Biocide code	Generic group	% Kill after six hours* X	S	Cost per kg
B	Dichlorophen	96,97	4,18	R14,58
I	Phosphonium chloride/QAC	85,77	25,92	NS
L	Biphenol	92,98	16,51	R17,29
A	Isothiazolin	75,32	34,65	R19,60
U	Isothiazolin	68,40	44,18	R7,62
Q	Isothiazolin	61,35	39,74	R11,31
J	Isothiazolin	49,08	42,63	R11,65
G	Isothiazolin	38,10	41,03	NS
GG	Isothiazolin	35,75	39,84	R6,46
BB	TCMTB	51,57	40,01	R15,66
H	Broad spectrum	42,95	40,76	R12,00
N	Gluteraldehyde	53,66	47,54	R13,81
K	Organophosphorus	88,36	26,14	R14,70
V	Isothiazolin - QAC	51,31	48,14	R6,50
FF	Isothiazolin - QAC	47,28	45,72	R12,16
M	QAC	64,14	42,30	R6,70
DD	QAC	64,07	42,10	R7,26
F	QAC	62,03	45,37	NS
P	QAC	52,97	46,66	R4,56
C	Sulphone	74,60	36,85	R7,76
S	Thiocarbamate	90,35	25,50	R10,91
D	Thiocarbamate	42,28	46,28	R3,13
W	Organosulphur	80,21	38,26	R5,71
CC	Organosulphur	54,06	33,08	R3,01
EE	Organosulphur	26,89	19,00	R3,86
AA	MBT	75,89	31,72	R8,16
R	MBT	75,08	36,44	R13,57
E	MBT	73,79	41,26	R12,23
X	MBT	58,59	45,55	R6,50
T	QAC-Tin	81,57	31,61	R5,90
O	QAC-Tin	81,28	33,12	R8,96

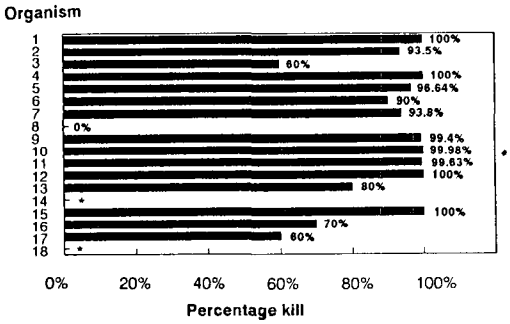
QAC = Quaternary ammonium compound
TCMTB = Thiocyanomethylthiobenzothiazole
NS = Not specified
X = Average kill percentage
S = Standard deviation
* = Data compiled from Figs. 1 to 4



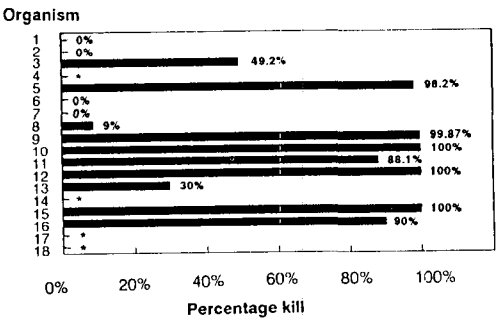
Isothiazolin: Product code A



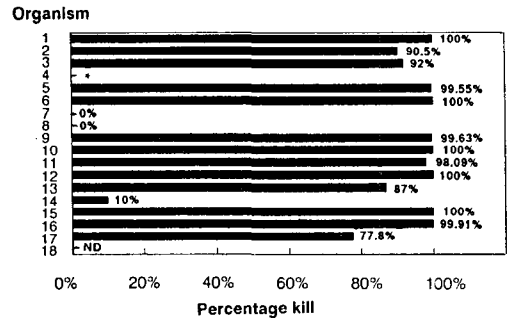
Dichlorophen: Product code B



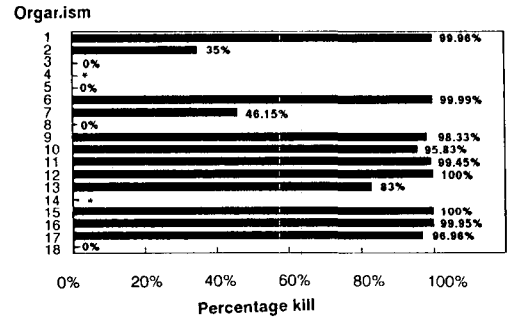
Sulphone: Product code C



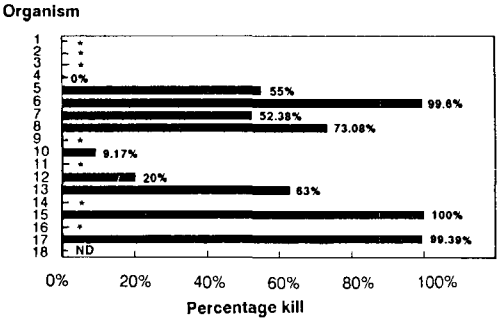
Thiocarbamate: Product code D



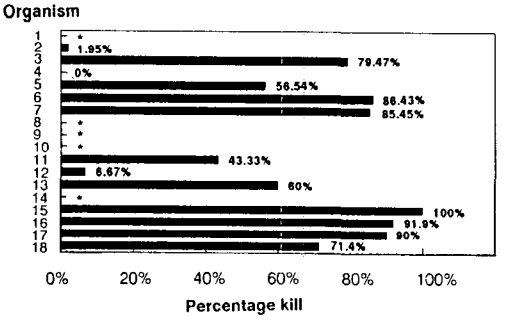
MBT: Product code E



QAC: Product code F

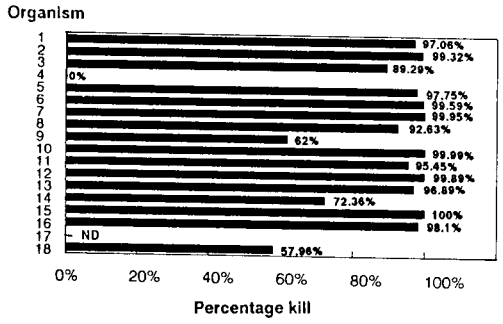


Isothiazolin: Product code G

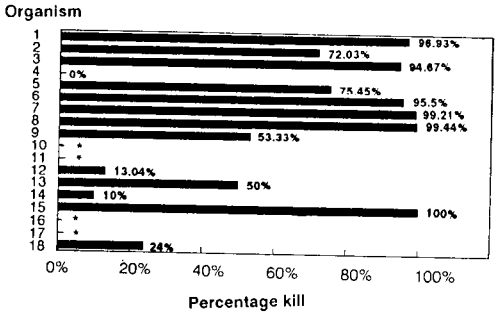


Broad spectrum: Product code H

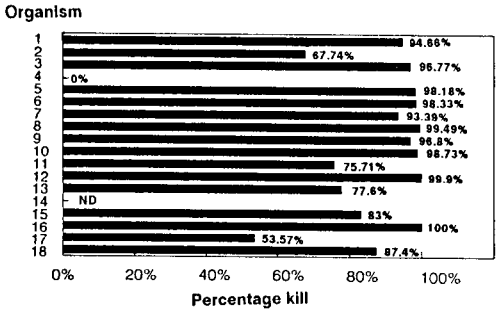
Figure 1
Percentage kill of 18 test strains after 6 h exposure to 50 mg.ℓ^{-1} of bactericides as listed in Table 1 (= growth; ND = not done).*



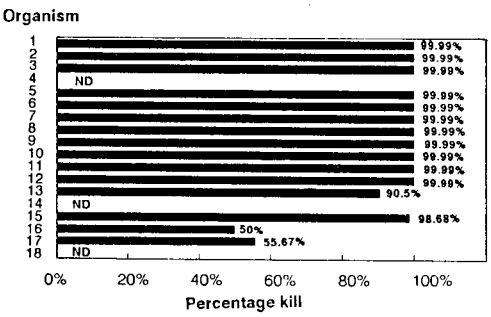
Phosphonium chloride/QAC: Product code I



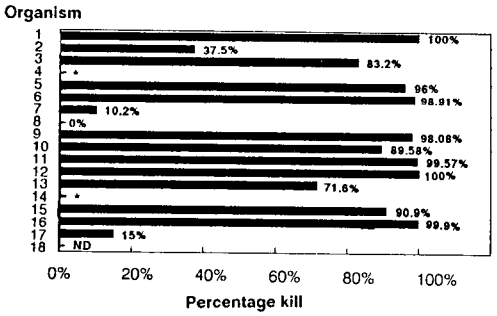
Isothiazolin: Product code J



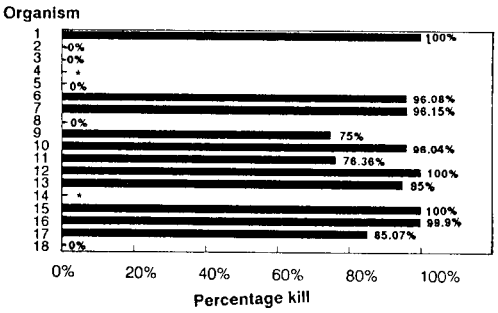
Organophosphorous: Product code K



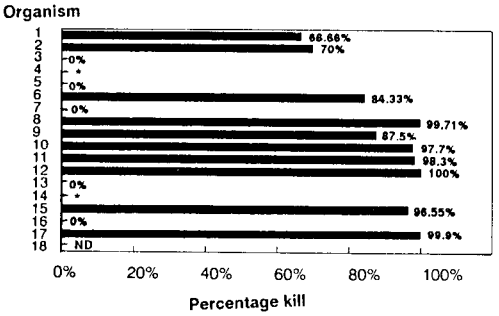
Biphenol: Product code L



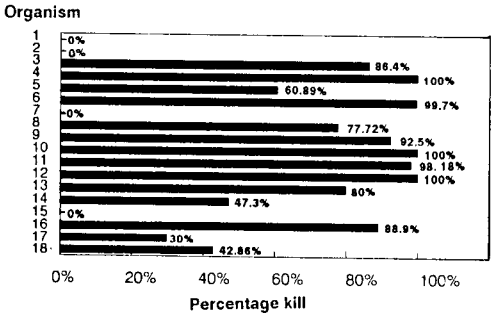
QAC: Product code M



Gluteraldehyde: Product code N

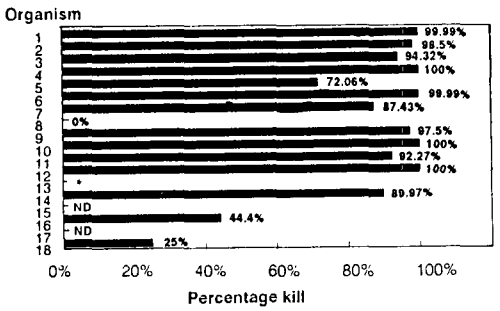


QAC: Product code P

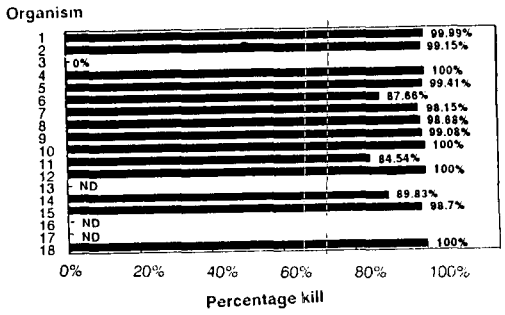


Isothiazolin: Product code Q

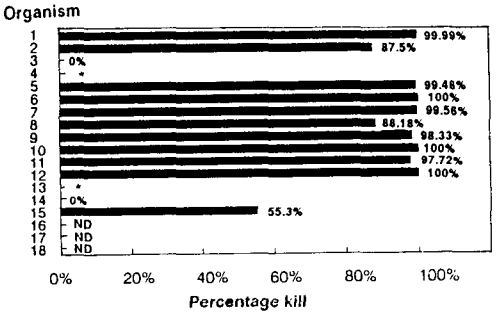
Figure 2
Percentage kill of 18 test strains after 6 h exposure to 50 mg.ℓ^{-1} of bactericides as listed in Table 1 (= growth; ND = not done).*



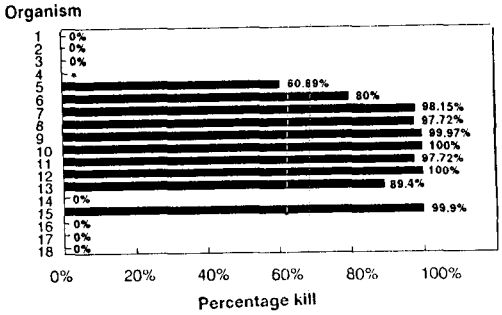
MBT: Product code R



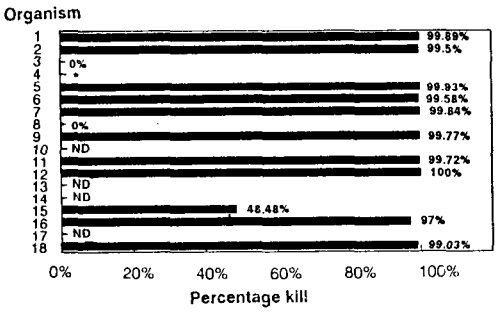
Thiocarbamate: Product code S



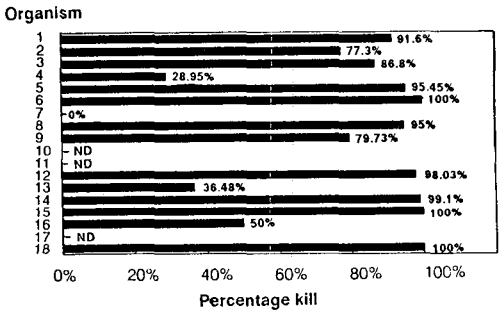
Isothiazolin: Product code U



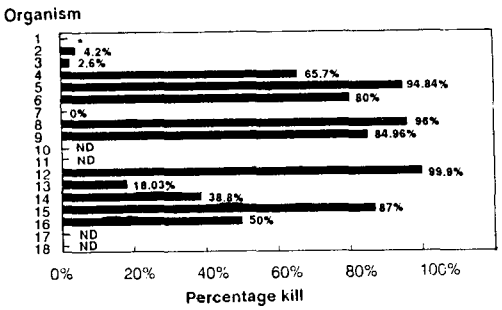
Isothiazolin-QAC: Product code V



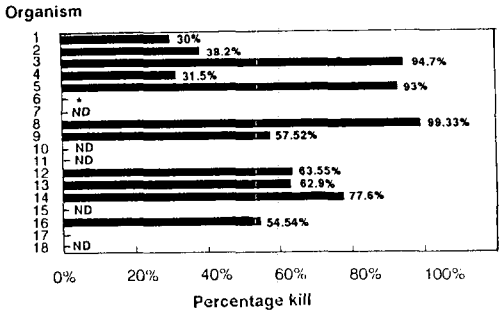
Organosulphur: Product code W



MBT: Product code AA

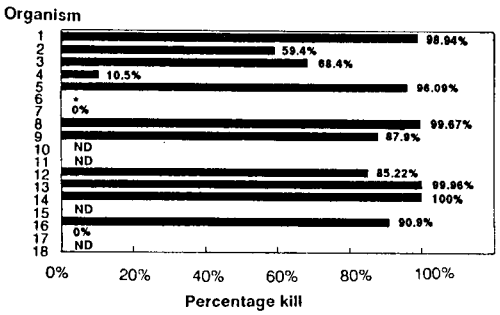


TCMBT: Product code BB

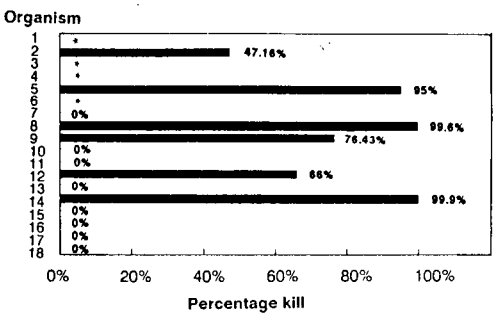


Organosulphur: Product code CC

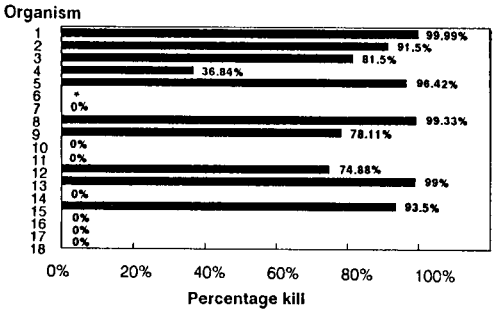
Figure 3
Percentage kill of 18 test strains after 6 h exposure to 50 mg.ℓ^{-1} of bactericides as listed in Table 1 (= growth; ND = not done).*



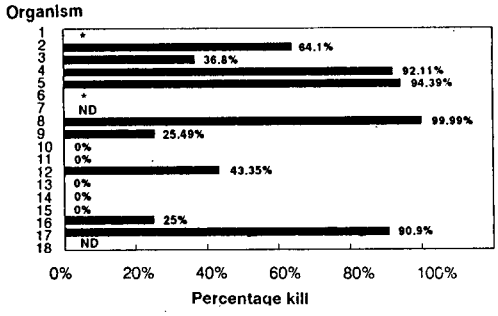
QAC: Product code DD



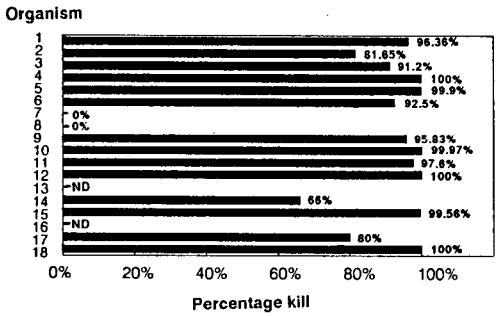
Organosulphur: Product code EE



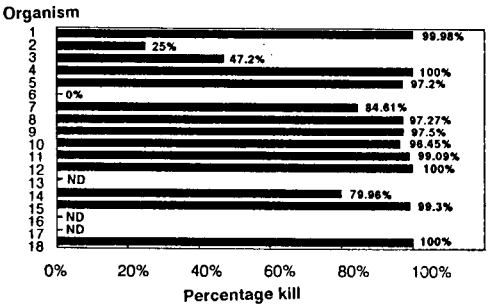
Isothiazolin-QAC: Product code FF



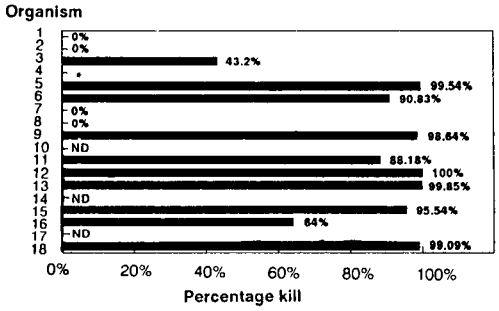
Isothiazolin: Product code GG



QAC-Tin: Product code O

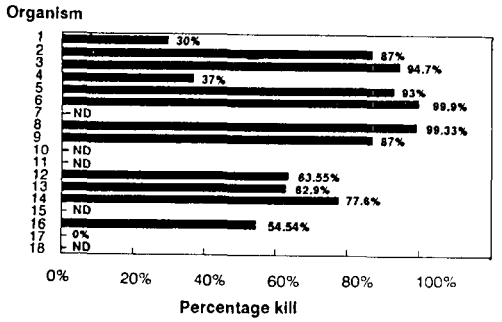


QAC-Tin: Product code T

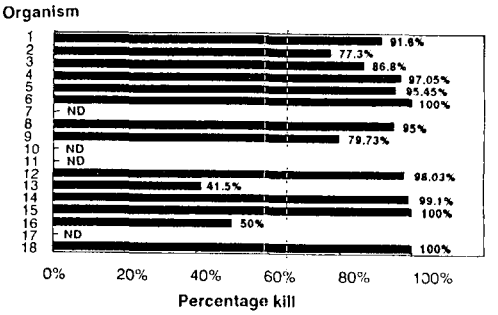


MBT: Product code X

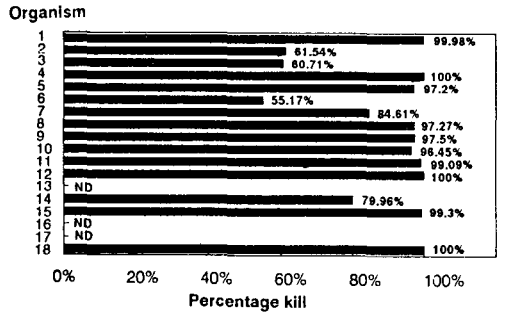
Figure 4
Percentage kill of 18 test strains after 6 h exposure to 50 mg.ℓ$^{-1}$ of bactericides as listed in Table 1 (= growth; ND = not done).*



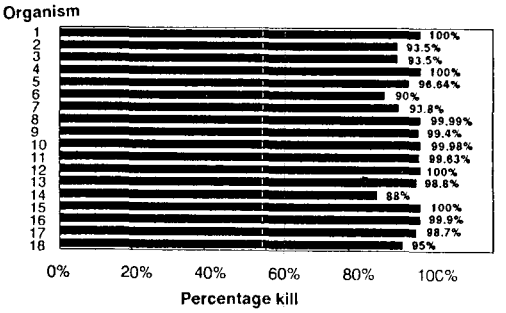
Organosulphur: Product code CC



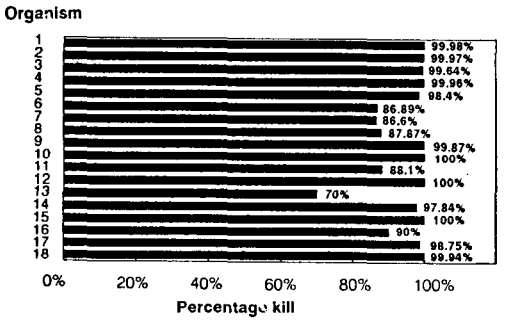
MBT: Product code AA



QAC-Tin: Product code T



Sulphone: Product code C



Thiocarbamate: Product code D

Figure 5
Percentage kill of 18 test strains after 6 h exposure to cost-equivalent concentrations of various bactericides
CC : 95 mg.ℓ⁻¹
AA : 83 mg.ℓ⁻¹
D : 174 mg.ℓ⁻¹
T : 70 mg.ℓ⁻¹
C : 100 mg.ℓ⁻¹

an important role. For the evaluation of bactericides against biofilm bacteria, tests should, strictly speaking, be performed on attached bacteria. However, we abandoned this approach due to the inherent problem of enumerating viable attached bacteria. Attached bacteria cannot be dispersed effectively enough to enumerate them by either plate or direct count (Caldwell, 1990). Whereas some methods yield aggregates of cells, others are too drastic, resulting in the death of many of the bacteria (Yoon and Rosson, 1990). Enumeration methods based on metabolic rate (e.g. ATP bioluminescence), pose restrictions due to the varying cell volumes of cells in natural surroundings (Karl, 1986).

Therefore tests were conducted on bacterial suspensions to screen bactericides. The aim was, after all, to determine the ability of bactericides to kill all, or at least most of the dominant bacteria in systems, and to obtain their fingerprints against these isolates. The bactericides used are all water soluble, and would therefore be able to penetrate the biofilm matrix. Although the concentration used (50 mg.ℓ⁻¹) would probably not suffice to kill attached bacteria (LeChevalier *et al.*, 1988), the fingerprints could be extrapolated for attached bacteria. Only the application concentration would have to be determined by multiplying the concentration from the fingerprint by a factor.

All the test isolates were taken from planktonic samples, and these could be different from the attached microflora. However, biofilms are in a continuous state of flux (Characklis and Cooksey, 1983; Delaquis *et al.*, 1989). Surface bacteria are continually attaching and detaching. In a large system in a state of equilibrium, the ratio among bacterial species in the upper layer of the biofilm should therefore be similar to that of planktonic cells. *P. fluorescens*, the most frequently occurring isolate in South African systems (Cloete *et al.*, 1989a) is a dominant member of biofilm populations on surfaces in water systems (Lawrence *et al.*, 1987). Even if the ratio were different, the fingerprints are still indicative of bactericide efficacy as an agent effective against 18 bacterial species would probably be effective against a 19th one as well.

TABLE 4
RE-EVALUATION OF PRODUCTS AT COST-EFFECTIVE APPLICATION CONCENTRATIONS

Code	Generic name	mg.ℓ^{-1}	% Kill after six hours*		Cost/kg	Use cost**
			X	S		
S	Tiocarbamate	50,0	90,35	25,20	R10,91	R10,91
D	Thiocarbamate	174,0	94,65	8,17	R3,13	R10,89
M	QAC	50,0	64,14	42,30	R6,70	R6,70
T	QAC-Tin	67,7	88,58	16,37	R5,90	R8,26
W	Organosulphur	50,0	80,21	38,26	R5,71	R5,71
CC	Organosulphur	95,0	74,90	22,59	R3,01	R5,71
C	Sulphone	100,0	97,00	3,80	R7,76	R15,52

* = Data compiled from Fig. 5.
** = Determined using the kg price and new application concentration.

TABLE 5
BACTERICIDES LISTED IN ORDER OF THEIR COST-EFFECTIVITY INDEX

Code	Generic name	mg.ℓ^{-1}	% Kill		Cost/kg	CEI*
			X	S		
W	Organosulphur	50	80,21	38,26	R5,71	R3,55
CC	Organosulphur	95	74,90	22,59	R3,01	R3,81
D	Thiocarbamate	174	94,65	8,17	R3,13	R5,79
S	Thiocarbamate	50	90,35	25,50	R10,91	R6,03
B	Dichlorophen	50	96,97	4,18	R14,58	R7,51
C	Sulphone	100	97,00	3,80	R7,76	R8,00
L	Biphenol	50	92,98	16,51	R17,29	R9,29

$$*CEI = \frac{\text{cost x mg.}\ell^{-1}}{\text{\% Kill}}$$

Bactericide fingerprints

As expected, most bactericides were selective in their action. The standard deviation can be taken as a criterion of selectivity; the higher the standard deviation, the more selectively the bactericide killed the test isolates. The test organisms used were all dominant in South African water-cooling systems (Cloete *et al*, 1989a). Most isolates exhibited resistance to at least one bactericide at 50 mg.ℓ^{-1}. *P. fluorescens,* which made up 35,5% of the population as reported by Cloete *et al.* (1989a), was not killed by thiocarbamate, isothiazoline, gluteraldehyde, isothiazoline-QAC and MBT at the application concentration used in this evaluation, whereas QAC-tin, organosulphur, TCMTB, QAC, and the broad spectrum compound showed a low kill percentage. *P.vesicularis* was resistant to 18 of the 31 bactericides tested. Although it made up only 2,2% of the population cited, death of its competitors could result in take-over of the population, a situation where re-application of the bactericide would have little effect in decreasing bacterial counts. Only dichlorophen and biphenol gave a standard deviation of less than 20 and only dichlorophen showed a high kill percentage against all 18 isolates at 50 mg.ℓ^{-1}. At the cost-equivalent application concentration sulphone and thiocarbamate D also killed all 18 isolates to a degree of 95% and over. This indicates the importance of considering the application cost of a bactericide when comparing its efficacy to that of another. Sulphone and thiocarbamate would have been rejected on the ground of selective killing at 50 mg.ℓ^{-1}. However, thiocarbamate proved much more effective when applied at cost equivalent concentration.

Some isolates multiplied in the presence of certain bactericides, and the count after six hours was higher than the initial inoculum. In these cases the bactericide was totally ineffective and allowed cells to divide. Such bactericides would have little or no effect in systems, and should not be used. Certain isothiazolins, the broad spectrum compound, organosulphur and thiocarbamate D at 50 mg.ℓ^{-1} all permitted growth of certain isolates.

The results represented here are based on tests performed with pure cultures of bacteria under laboratory conditions. The results cannot be applied directly to full-scale industrial systems where

populations are mixed and conditions are more variable. However, the results do provide a screening system. If a specific bactericide does not function efficiently under controlled conditions, it can hardly be expected to be efficient in full-scale systems.

We therefore concluded that only dichlorophen (50 mg.ℓ^{-1}), sulphone (100 mg.ℓ^{-1}), thiocarbamate D (174 mg.ℓ^{-1}) and biphenol (50 mg.ℓ^{-1}) were effective bactericides and are feasible for decreasing bacterial loads in water-cooling systems, as they were the only ones which killed all 18 isolates.

Acknowledgements

We thank I Boshoff, H Eybers and N Mienie for technical assistance, Anglo American Research Laboratories who donated bactericide samples and the Division of Energy Technology of the CSIR for financial assistance.

References

ALLSOP, D and SEAL, KJ (1986) *Introduction to biodeterioration.* Edward Arnold, London.

BERG, JD, MATIN, A and ROBERTS, PV (1982) Effect of antecedent growth conditions on sensitivity of *Escherichia coli* to chlorine dioxide. *Appl. Environ. Microbiol.* **44** 814-819.

CALDWELL, DE (1990) Personal communication. Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan S7N Owo, Canada.

CHARACKLIS, WG and COOKSEY, KE (1983) Biofilms and microbial fouling. *Adv. Appl. Microbiol.* **29** 93-138.

CLOETE, TE, PRESSLY, J and BRÖZEL, VS (1989a) A bacterial population structure study of water cooling systems in South Africa. *Water SA* **15**(1) 37-42.

CLOETE, TE, SMITH, F and STEYN, PL (1989b) The use of planktonic bacerial populations in open and closed recirculating water cooling systems for the evaluation of biocides. *Int. Biodeterioration* **25** 115-122.

DELAQUIS, PJ, CALDWELL, DE, LAWRENCE, JR and McCURDY, AR (1989) Detachment of *Pseudomonas fluorescens* from biofilms on glass surfaces in response to nutrient stress. *Microb. Ecol.* **18** 199-210.

EIGENER, U (1988) Disinfectant testing and its relevance in practical application. In: KR Payne (ed.) *Industrial biocides.* Chichester. John Wiley and Sons. 52-67.

HEINZEL, M (1988) The phenomena of resistance to disinfectants and preservatives. In: KR Payne (ed.) *Industrial Biocides.* Chichester. John Wiley and Sons. 52-67.

HOPPE, HG (1984) Attachment of bacteria: Advantage or disadvantage for survival in the aquatic environment. In: KC Marshall (ed.) *Microbial Adhesion and Aggregation.* Berlin Springer Verlag. 283-301.

IVERSON, WP (1987) Microbial corrosion of metals. *Adv. Appl. Microbiol.* **32** 1-36.

KARL, DM (1986) Determination of *in situ* microbial biomass, viability, metabolism, and growth. In: JS Pointdexter and ER Leadbetter (eds.) *Bacteria in Nature. Volume 2.* New York. Plenum Press. 49-84.

LAWRENCE, JR, DELAQUIS, PJ, KORBER, DR and CALDWELL, DE (1987) Behaviour of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microb. Ecol.* **14** 1-14.

LECHEVALIER, MW, CAWTHON, CD and LEE, RG (1988) Inactivation of biofilm bacteria. *Appl. Environ. Microbiol.* **54** 2492-2499.

McCOY, WF, BRYERS, JD, ROBBINS, J and COSTERTON, JW (1981) Observations of fouling biofilm formation. *Can. J. Microbiol.* **27** 910-917.

McFARLAND, J (1970) The nephelometer: An instrument for estimating the number of bacteria in suspensions used for calculating the opsonix index for vaccines. *J. Amer. Med. Assoc.* **49** 1176.

PAULUS, W (1987) Development of microbiocides for the protection of materials. In: DR Houghton, RN Smith and HOW Eggins (eds.) *Biodeterioration.* London. Elsevier Applied Science. 1-19.

VON HOLY, A and CLOETE, TE (1988) Practical aspects of monitoring biofilms and microbiologically induced corrosion in industrial water systems. *S. Afr. J. Science.* **84** 17-19.

YOON, WB and ROSSON, RA (1990) Improved method of enumeration of attached bacteria for study of fluctuation in the abundance of attached and free-living bacteria in response to diel variation in seawater turbidity. *Appl. Environ. Microbiol* **56** 595-600.