The effect of bactericide treatment on planktonic bacterial communities in water cooling systems+

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Abstract

Bactericides were applied to experimental open recirculating cooling-water systems at concentrations found to be effective under laboratory pure-culture conditions. Total aerobic plate counts and bacterial population structures were determined over a period of 48 h. In all cases the total aerobic count increased one day after bactericide addition, and decreased rapidly after ca. 36 to 40 h. Population shifts occurred during the course of all four treatments. In all cases different species became dominant concurring with fluctuations in the planktonic plate count, indicating stress reaction of the biofilm. The species diversity decreased after treatment with dichlorophen, thiocarbamate and methylene-bis-thiocyanate, and increased upon treatment with humic acid. Species susceptible to bactericides in pure culture were found to be the dominant planktonic survivors. An example is *Pseudomonas stutzeri* which was the dominant survivor after treatment with thiocarbamate and with dichlorophen.

Introduction

Bacteria in aqueous surroundings attach preferably to surfaces (Hoppe, 1984), where they produce copious amounts of adhesive exopolymers (Characklis and Cooksey, 1983). This adhesion and subsequent metabolism leads to formation of biofilms (McCoy et al., 1981). Such bacterial biofilms promote fouling and corrosion of metal and other surfaces (Costerton, 1981; Ford and Mitchell, 1990; Hamilton, 1985; Iverson, 1987). Many industrial water systems are, therefore, treated with bactericides to reduce the bacterial load and degree of corrosion. Because attached bacteria are more resistant to bactericides than are free-floating (planktonic) ones (Heinzel, 1988; LeChevalier et al., 1988), the design of effective treatment programmes poses certain difficulties.

Attached bacteria cannot effectively be dispersed without killing most (Costerton, 1981; Costerton et al., 1986). Therefore the enumeration of bacteria in biofilms does not yield representative results. As a result, quantitative evaluation of bactericides has been performed mostly on planktonic samples (Eigener, 1988). As cells in biofilms and the planktonic communities are in continuous exchange (Costerton et al., 1986), the planktonic community does reflect on the sessile one. Death of cells in the planktonic phase would influence the equilibrium and shifts would occur in both the planktonic and the sessile populations. Investigation of the planktonic phase would, therefore, yield insights into the sessile one, and into the system as a whole.

Bactericides attack targets of cell function (Wainwright, 1988), placing the bacterium under stress. It is well recognised that communities under stress have a lower species diversity and select for fitter species (Atlas, 1984). As bactericides are selective in their action (Heinzel, 1988), application of any one could result in selection for resistant bacteria. Where a bactericide is the stress factor, fitter species would be those resistant to or more tolerant of the specific bactericide. As diversity is inversely proportional to productivity, it would influence the degree of corrosivity of the biofilm. Although information on the selective activity (bactericide fingerprints) of a range of bactericides has been published (Brözel and Cloete, 1991a), the result of *in situ* application is unknown. Few studies have been reported regarding the reactions of aqueous bacterial populations to various bactericide treatments (Cloete et al., 1989b), and on the resulting species diversity in water-cooling systems.

We chose a range of bactericides from the reported series, based on their ability to kill a range of bacteria found dominant in South African cooling-water systems (Cloete et al., 1989a). A novel bactericidal product derived from oxidised coal and currently under evaluation in this laboratory was also used. The bactericides were applied to small open recirculating water-cooling units at concentrations found cost-effective under laboratory pure-culture conditions, in order to study their efficacies *in situ*.

Materials and methods

In situ application of bactericides

Experimental units

Three identical open recirculating water-cooling units with a volume of 100 ℓ each, and operating at ca. 14 °C, were used in this study. The water was originally taken from the municipal water supply, but no make-up water was added during the 48-h study period. A well developed biofilm was visible in each system. None of the systems had previously been treated with bactericide, and had been operating over a period exceeding three months. This study was performed during the third quarter of 1989.

Bactericides evaluated

A range of bactericides was chosen from the series evaluated previously (Brözel and Cloete, 1991a). In that study the selective

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TABLE 1
BACTERICIDES USED TO APPLY STRESS IN VARIOUS SYSTEMS

Bactericide	Concentration (mg·ℓ⁻¹)	S*
Dichlorophen	50	4,18
Thiocarbamate	174	8,17
Methylene-bis-thiocyanate	83	27,29
Humic acid	1 000	ND

* = Standard deviation of bactericide fingerprint as reported
ND = Not determined

ability of bactericides to kill bacteria found dominant in South African water cooling systems was measured by standard deviation (S). The lower S was, the wider was the range of kill of the bactericide. Bactericides were added at their cost-effective concentrations as listed in Table 1. Samples were taken every 2 h till 6 h after beginning. After 6 h the relevant bactericide was added at the appropriate concentration. After the first test of 48 h, make-up water was added from the municipal water supply to replace that lost, and the systems were allowed to recover for one month, after which the other bactericides were applied.

Bacteriological analysis

Total aerobic count

The aerobic planktonic load was determined on samples collected at two-hourly intervals over a period of 48 h. Four samples were taken, one every 2 h, before bactericide was added. All samples were collected in sterile glass tubes from the sump of each system, stoppered with a sterile aluminium cap, and analysed within 2 h after sampling. The aerobic plate count was determined by plating 0,1 mℓ aliquots of serial dilutions aseptically and in duplicate (Terplan and Zaadhof, 1982) onto Standard 1 Nutrient Agar (Biolab)(StdI) and incubating at 25°C for 48 h. Colonies visible to the naked eye were counted on plates having between 30 and 300 colonies, and the average of two plates was recorded.

The average growth rate constants were calculated using the generally accepted expression for growth rate (Stanier et al., 1986).

$$k = \frac{(\log Z - \log Z_o)}{(t - t_o)} \times 2{,}303$$

Average generation times were calculated as follows (Stanier et al., 1986):

$$g = \frac{\ln 2}{k}$$

Average generation time values are given in hours. These are not real generation times, but the theoretical value required to yield the increase in cell numbers in the planktonic phase taken that no cell death occurred.

Population structure study

Dominant organisms were obtained from plates carrying between 30 and 300 colonies. The Harrison's disk (Harrigan and McCance, 1976) was used to obtain representative colonies at random. These were isolated and purified by suspending in quarter strength Ringers solution (Merck), vortexing and plating out on Std 1 agar. This procedure was repeated twice more, after which colonies were regarded as pure. Incubation was always at 25 °C for 120 h as some isolates contained slower growing contaminants at first.

Isolates were cultured for 18 h and were then subjected to Gram staining, oxidase, catalase and O/F tests (medium of Hugh and Leifson containing glucose) (Hugh and Leifson, 1953). Gram-negative oxidative isolates were identified using the API 20 NE kit (API System, S.A.,-La Balme Les Grotes - 38390, Montalieu, Vercieu, France), and fermentative isolates were identified with the API 20 E kit. Gram-positive isolates were tested for spore-forming ability by pasteurising for 10 min at 80 °C and plating out on Std 1 agar. Spore-forming isolates were identified using the key of Norris, Berkeley, Logan and O'Donnel (Norris et al., 1981). Other gram-positive isolates were only identified to the genus level by the keys given in Bergey's Manual of Systematic Bacteriology, vol. II (Sneath et al., 1986).

Species diversity was calculated using the Shannon-Weaver diversity index (Atlas and Bartha, 1987b).

$$H = \frac{C}{N}(N \log_{10} N - \Sigma n_i \log_{10} n_i)$$

where:

C = 2,3
N = number of individuals
n_i = number of individuals in *i*th species.

Results

Bactericide 1: Dichlorophen (Fig. 1)

The planktonic count increased 16 h after the addition of dichlorophen (50 mg.ℓ⁻¹) and then fluctuated, but remaining higher than the initial count. The theoretical growth rate between 24 and 26 h was 0,35 h^{-1} and the doubling time was 119 min. Between 28 and 30 h it was 107 min. The *Bacillus* sp. and *Vibrio metschnikovii* populations were decreased below detectable levels by the dichlorophen (Table 2). *Pseudomonas cepacia* increased in numbers at the start of the logarithmic "growth phase", but then disappeared. A range of Gram-negative bacteria and a *Micrococcus* sp. were detected at 34 h. This indicates a shift in the bacterial community upon bactericide treatment. Only *P. stutzeri* kept a constant presence of between 25 and 50% throughout the study period. The species diversity index decreased from 1,492 to around 1,2, despite the higher numbers.

Bactericide 2: Thiocarbamate (Fig. 2)

The TAPC fluctuated around the initial value till 20 h after addition of thiocarbamate (174 mg·ℓ⁻¹). It then rose to around 10 times the initial value. The theoretical growth rate for the period 26 to 34 h is 0,44 h^{-1} or a division time of 95 min. *Pseudomonas stutzeri* increased in numbers above the rest of the community,

TABLE 2
BACTERIAL POPULATION STRUCTURE DURING 48-h TREATMENT WITH DICHLOROPHEN (% REPRESENTATION)

Species	0h	30h	32h	34h	46h
Bacillus megaterium	12,5	0,0	0,0	0,0	0,0
B. cereus	12,5	0,0	0,0	0,0	0,0
Micrococcus sp.	0,0	0,0	0,0	12,5	0,0
Vibrio metschnikovii	12,5	0,0	0,0	0,0	0,0
Pseudomonas stutzeri	25,0	50,0	37,5	50,0	43,0
P. cepacia	37,5	12,5	0,0	0,0	0,0
P. mendocina	0,0	12,5	0,0	12,5	14,0
P. vesicularis	0,0	0,0	0,0	0,0	29,0
P. pickettii	0,0	0,0	12,5	0,0	0,0
Flavobacterium sp.	0,0	0,0	0,0	0,0	14,0
CDC gr VD	0,0	25,0	0,0	0,0	0,0
CDC gr IV C-2	0,0	0,0	12,5	25,0	0,0
Gram-negative rod*	0,0	0,0	37,5	0,0	0,.0
Total number of species	**5**	**4**	**4**	**4**	**4**
Shannon-Weaver index	**1,492**	**1,212**	**1,254**	**1,212**	**1,276**

* Unidentified Gram-negative oxidative motile rod

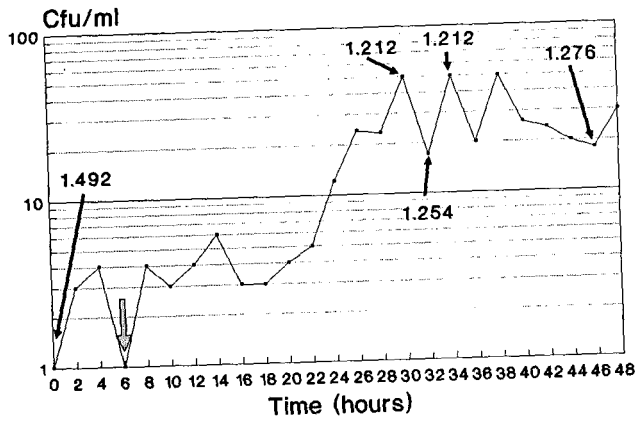

Figure 1
Planktonic aerobic count over 48 h after adding 50 mg·ℓ⁻¹ dichlorophen. The large arrow indicates the time of bactericide addition. The Shannon-Weaver species diversity indexes are indicated on the graph.

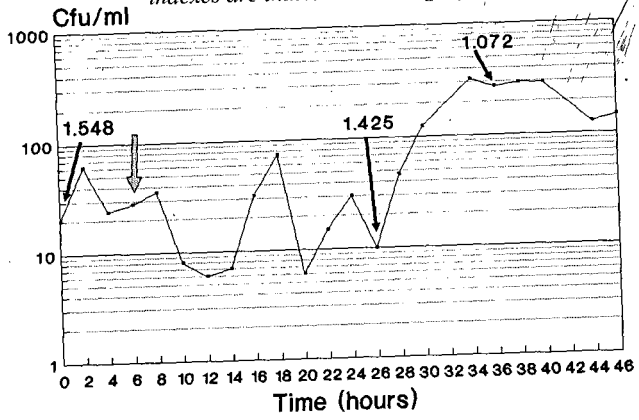

Figure 2
Planktonic aerobic count over 48 h after adding 174 mg·ℓ⁻¹ thiocarbamate. The large arrow indicates the time of bactericide addition. The Shannon-Weaver species diversity indexes are indicated on the graph.

TABLE 3
BACTERIAL POPULATION STRUCTURE DURING 48-h TREATMENT WITH THIOCARBAMATE (% REPRESENTATION)

Species	0h	26h	38h
Pseudomonas stutzeri	29,0	44,0	62,5
P. putrefaciens	14,0	22,0	0,0
P. cepacia	0,0	0,0	12,5
Bacillus cereus	0,0	0,0	12,5
Xanthomonas maltophilia	14,0	0,0	0,0
Aeromonas sp.	14,0	11,0	0,0
Micrococcus sp.	0,0	11,0	0,0
Gram-negative rod*	29,0	11,0	12,5
Total number of species	**5**	**5**	**4**
Shannon-Weaver index	**1,548**	**1,425**	**1,072**

*Unidentified Gram-negative oxidative motile rod.

becoming the dominant population after 38 h. *Bacillus cereus* and *P. cepacia* became detectable after 38 h, whereas *P. putrefaciens*, the *Aeromonas* and *Micrococcus* sp. dropped to below detectable levels (Table 3). Again the treatment resulted in a community shift and decrease in the diversity index.

Bactericide 3: Methylene-bis-thiocyanate (Fig. 3)

Addition of methylene-bis-thiocyanate (83 mg·ℓ⁻¹) caused a slight initial disturbance of the planktonic population, with an apparent growth stimulation (0,92 h^{-1}) or a division time of 45 min). Between 22 and 26 h the theoretical division time was 76 min. The planktonic community decreased in numbers to the initial level towards the end of the study. The diversity index decreased, however. In this treatment *Pseudomonas stutzeri* decreased to below detectable levels (Table 4). *P. cepacia* was the only gram-negative bacterium which appeared in the final planktonic population. It also appeared after thiocarbamate treatment (Table 3). Overall *Bacillus* species dominated.

TABLE 4
BACTERIAL POPULATION STRUCTURE DURING 48-h TREATMENT WITH METHYLENE-BIS-THIOCYANATE (% REPRESENTATION)

Species	0h	26h	38h
Bacillus megaterium	33,0	17,0	29,0
B. cereus	11,0	50,0	57,0
B. coagulans	11,0	33,0	0,0
Flavobacterium sp.	11,0	0,0	0,0
Pseudomonas stutzeri	22,0	0,0	0,0
P. cepacia	0,0	0,0	14,0
Aeromonas hydrophila	11,0	0,0	0,0
Total number of species	**6**	**3**	**3**
Shannon-Weaver Index	**1,675**	**1,010**	**0,955**

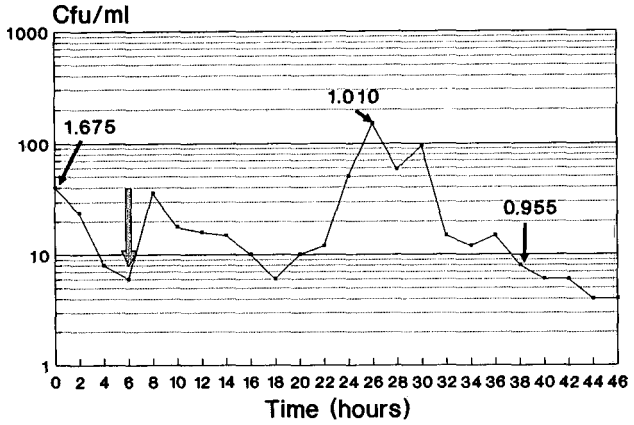

Figure 3
Planktonic aerobic count over 48 h after adding 83 mg·ℓ⁻¹ methylene-bis-thiocyanate. The large arrow indicates the time of bactericide addition. The Shannon-Weaver species diversity indexes are indicated on the graph.

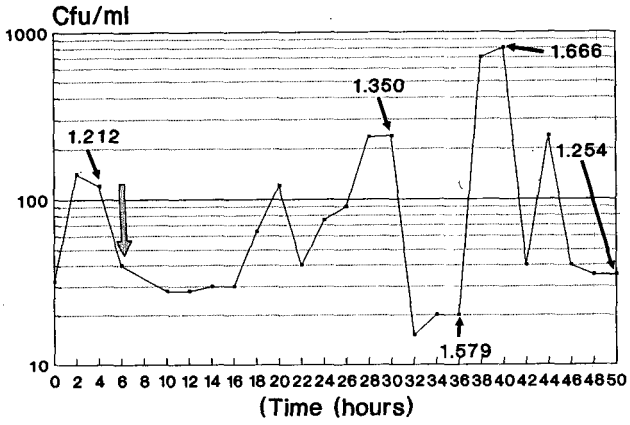

Figure 4
Planktonic aerobic count over 48 h after adding 1,00 mℓ·ℓ⁻¹ humic acid solution. The large arrow indicates the time of bactericide addition. The Shannon-Weaver species diversity indexes are indicated on the graph.

Bactericide 4: Humic acid (Fig. 4)

The TAPC decreased somewhat after addition of humic acid (4 h), went through two small growth and decline cycles, and increased 32 h after addition of humic acid at a theoretical division time of 23 min. However, it decreased rapidly 2 h later to the initial value (Fig. 4). Again the bacterial community underwent dynamic change during exposure to the bactericide. The species diversity index increased due to treatment, and decreased to the initial value at the end of the study. *P. stutzeri* was present until the end of the study, when it decreased in numbers to below the detectable level.

Discussion

Planktonic bacterial numbers

Although each bactericide resulted in a different reaction of the planktonic bacterial population, certain trends did appear during each stress application:

- The total planktonic count fluctuated $\log_{10}$l to $\log_{10}$1,7 upon treatment with bactericide.
- In two of the treatments the final planktonic count exceeded the initial count before bactericide application (dichlorophen and thiocarbamate).

The rapid increases in cell numbers cannot be ascribed solely to growth. The maximum recorded division time for *P. putida* is 45 min at 30 °C and for *B. subtilis* at 40 °C it was 26 min (Stanier et al., 1986). In the case of MBT and humic acid the theoretical doubling time at a stage was under 45 min, not taking cell death into account. In these cases the larger part of the population must have originated from elsewhere. As entry from outside is unlikely, they must have originated from the biofilm. These phenomena can, in our oppinion, not be unequivocally explained. We will, however, put forward a range of possible hypotheses.

The most apparent explanation for such a rapid increase in cell numbers is release of cells from the biofilm. The bactericides applied would exert a degradative force on the polysaccharides making up the biofilm, either by oxidative or dispersive properties. Bacteria would lose their support on the surface. As the bactericide would probably be quantitatively depleted by now, survival of released cells would depend solely on competition and predation. The rapid decrease in cell numbers could be explained by starvation of bacteria on the depletion of available nutrients. However, release of nutrients from dead cells would support others to multiply again. This theory does not adequately explain the rapid increase and decrease in numbers.

A second apparent explanation would be the more rapid death of bacterial predators such as phagotrophic flagellates and sarcodinads (Anderson, 1988; Gonzalez et al., 1990). As only unprotected bacteria would be engulfed, attached bacteria would survive and planktonic populations would be kept at a minimum until such time as the predators are removed. Such predator-prey interactions have on occasion been found to form stable communities in nature (Atlas and Bartha, 1987a). After a while the predators would recover and the planktonic population would decrease again. As the increase and decrease in bacterial numbers took place within a short period, death of predators cannot be seen as an important factor as protozoan growth rates are much slower than those of bacteria.

Cells could have been triggered to leave the biofilm due to the

stress exerted on attached bacteria. Attached bacteria are more resistant to bactericides than are planktonic ones (LeChevalier et al., 1988), so detachment seems illogical. Bacteria have been shown to detach from biofilms in response to nutrient stress (Delaquis et al., 1989). Could bacteria detach from biofilms in response to other forms of stress as well? As many free-floating bacteria actually occur in clumps, the increase in counts could result from declumping triggered by stress on free-floating microcolonies. The released cells would be more susceptible to bactericide (LeChevalier et al., 1988) and would subsequently die. This would explain the rapid increase and decrease in cell numbers.

A fourth possible explanation is the selective death of susceptible bacteria, leaving behind a culture of bacteria previously suppressed by amensal interactions (Atlas and Bartha, 1987a). As the concentration of suppressive agents decreased below its threshhold value, these bacteria would multiply rapidly by utilising the available nutrients. Due to the rather high growth rate, the nutrient level would drop below a threshhold value, resulting in halting of planktonic growth. This would then lead either to the starvation of cells, which would either die or lose their colony-forming ability on agar (Roszak and Colwell, 1987), or to reattachment.

Bacterial population structure

Again each bactericide gave a different result, but certain trends were observed:

- Planktonic species diversity fluctuated in all cases, decreasing over time in the case of dichlorophen, thiocarbamate and MBT and increasing in the case of humic acid.
- There was always a shift in the detectable planktonic bacterial population.
- In most cases bacteria effectively killed in pure culture by the relevant bactericide (Brözel and Cloete, 1991a), were the dominant planktonic survivors.

In habitats under stress, microbial diversity is normally low (Atlas, 1984) whereas stable communities are assocciated with a high degree of diversity. As the number of species dominant at any given time did not change markedly (Tables 2 to 5), one could prematurely conclude that the bacterial population was not under any stress. However, there was a shift in each community, in numbers, in the dominant species and in the Shannon-Weaver index of diversity. Therefore the bactericides must have had some disturbing effect. Stable biologically controlled communities such as mature biofilms have a high degree of interaction and stability, and a low growth rate. They are composed largely of K-strategists (Atlas and Bartha, 1987b), organisms which depend on physiological adaptation to their surroundings for survival (Andrews and Harris, 1986). If such an interactive community is disturbed, even if only one major constituent of the community is killed, the other K-strategists lose their competitive overhand and r-strategists take over. These are fast-growing bacteria which rapidly deplete the available nutrient (Andrews and Harris, 1986) and which would only dominate long-term in niches receiving a continuous nutrient supply. In the cooling water the nutrient supply would quickly be depleted, resulting in reversion to a K-strategist community. The bacterial communities actually underwent a considerable amount of stress which is reflected not so much in a change in numbers, but rather in the decrease in diversity and in the population shifts during treatment. The system treated with humic acid showed an increase in diversity after treatment, which questions its efficacy as a water-treatment bactericide.

As bacterial growth rates in nutrient-limited surroundings are much lower than the optimal laboratory rates (Koch, 1988; Roszak and Colwell, 1987), the rapid planktonic increase of some species could not originate solely in cell division. At least some must have been introduced into the system, and the most likely source would be the biofilm. Above (**Planktonic bacterial numbers**) we argued that bacteria could detach from surfaces due to stress other than nutrient stress. Could certain species found dominant after bactericide addition have selectively detached ? This would explain the rapid increase in numbers of only certain species. However, the available data are insufficient to elucidate this, and further work is vital to shed light on this question.

Such selective detachment would result in a biofilm of lower diversity. Because community diversity is inversely proportional to productivity (Atlas and Bartha, 1987b), the surviving biofilm

TABLE 5
BACTERIAL POPULATION STRUCTURE DURING 48-h TREATMENT WITH HUMIC ACID (% REPRESENTATION)

Species	4h	30h	36h	40h	50h
Pseudomonas stutzeri	25,0	29,0	44,0	12,5	0,0
P. pickettii	12,5	14,0	11,0	0,0	0,0
P. aeruginosa	0,0	0,0	0,0	37,5	37,5
B. cereus	12,5	29,0	11,0	0,0	12,5
B. megaterium	0,0	0,0	0,0	0,0	12,5
B. coagulans	0,0	0,0	11,0	0,0	0,0
Cytophaga sp.	0,0	29,0	0,0	0,0	0,0
Flavobacterium sp.	0,0	0,0	11,0	0,0	0,0
Vibrio sp.	0,0	0,0	0,0	12,5	0,0
Micrococcus sp.	0,0	0,0	0,0	12,5	0,0
Gram-neg.rod (agarolytic)	50,0	0,0	0,0	12,5	37,5
Gram-positive rod	0,0	0,0	0,0	12,5	0,0
Stalked bacterium	0,0	0,0	11,0	0,0	0,0
Total number of species	**4**	**4**	**6**	**6**	**4**
Shannon-Weaver index	**1,212**	**1,350**	**1,579**	**1,666**	**1,254**

would show a higher average metabolic rate, resulting in more corrosive excretion products such as sulphides and organic acids (Iverson, 1987). If the above inferment is taken to be true, we can deduce that the application concentrations for bactericides determined in planktonic pure culture (Brözel and Cloete, 1991a) are definitely too low. Further we believe that sublethal dosage of bactericide results in reactivation of the biofilm due to selective detachment. Therefore the rate of microbially induced corrosion would increase despite a decrease in planktonic numbers.

In many cases the dominant planktonic survivor after 48h was a species most effectively killed by the relevant bactericide (Brözel and Cloete, 1991a). An example is dichlorophen which killed 99,94% of *Pseudomonas stutzeri* at 50 mg·ℓ$^{-1}$ in pure culture and yet left this species the dominant isolate after 48h (43%). Also thiocarbamate killed 99,87% of *P. stutzeri* at 174 mg·ℓ$^{-1}$ in pure culture, and left it the dominant planktonic survivor (62,5%). We offer no unequivocal explanation for this phenomenon. It must in some way relate to complex population interaction mechanisms, or to the adaptation of certain populations to the bactericides. *P. stutzeri* and *B. cereus* have been shown to develop resistance to various water treatment bactericides (Brözel and Cloete, 1991b).

In conclusion the above results reflect some of the complex interactions taking place between bacterial populations in cooling waters. They show that planktonic laboratory data should not simply be extrapolated to field application design. Interactions between species and between planktonic and sessile communities are not simple, and have a marked influence on the bactericidal efficacy of bactericides.

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