Effect of storage time and temperature on the aerobic plate count and on the community structure of two water samples

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Abstract

The effect of storage at various temperatures on the bacterial community of a cooling-water sample and a tap-water sample was determined. Samples were stored at 4, 10, 20 and 30°C for 24, 48, 72, and 216 h and the aerobic plate count and bacterial community structure of each were determined using R2A and R3A agars. The culturable count (aerobic plate count on R2A/R3A agar) in both samples varied over time, even after 24 h storage at 4°C, showing that bacterial communities in water are dynamic, even at refrigerator temperatures. At 4°C the culturable count of cooling water initially decreased, followed by a tenfold increase. The tap-water count decreased at 4°C. At 10°C the pattern was similar. At 20 and 30°C there was a tenfold increase in the culturable count of the cooling water, even after 24 h. In the cooling-water sample, the dominant isolates throughout were *Pseudomonas stutzeri* and an unidentified Gram negative pink isolate. This isolate was not detected in previous studies where Std I nutrient agar was used. Possibly this isolate plays an important role in cooling-water ecology, but does not grow on the conventional agars. The other isolates appeared randomly on the agar plates. The tap-water sample showed great variation in dominance of species over time. No direct tendencies of rate of decrease or increase could be detected in any of the samples, either in the culturable count or in community structure. Therefore results of analysis after storage cannot be adapted by a pre-determined factor. They must be interpreted with extreme caution, as they do not of necessity reflect the bacterial composition of the sample as drawn, both in terms of total numbers and in terms of community structure. Only counts performed on fresh samples yield reliable results on the total culturable count, and only community structures performed immediately, reflect the state of the community in the system from which the sample was drawn.

Introduction

The enumeration of live bacteria from water samples is still performed mostly by the agar plate count technique (Brözel, 1990). As this technique requires specialised laboratory equipment, samples usually have to be transported to laboratories at locations distant from the sampling site. Lower temperatures are usually taken to imply lower enzymatic reaction rates (Laidler and Meiser, 1982; Stanier et al., 1986) and therefore to decrease the rate of bacterial cell division and death. Stanier et al. (1986) state: "From the effect of temperature on the rate of a chemical reaction one would predict that all bacteria would continue to grow (although at progressively lower rates) as the temperature is reduced, until the system freezes. However, most bacteria stop growing at a temperature well above the freezing point of water." Therefore samples are shipped cold when bacterial analysis has to be performed away from the sampling site (Harrigan and McCance, 1976). This practice has often led to the assumption that samples retain their bacterial composition when refrigerated. The authors have often received samples for analysis after these had been kept in a refrigerated state for a day or more. As some intensive studies are based on results obtained from samples drawn at far-away sites (Brözel, 1990; Brözel and Cloete, 1991), a change in the initial composition of a sample used, could produce results of reduced value.

Various factors influence the result obtained when performing a plate count. These include the ability of cells to form colonies on the nutrient agar used; cells are either in the so-called culturable state where they do form colonies (Roszak and Colwell, 1987) whereas many cells are unable to divide even on a nutrient agar optimal for the species, the viable but non-culturable state (Roszak et al., 1984). A further factor is the treatment of cells during analysis. The brief higher temperature to which cells are exposed during the pour plate method has repeatedly been shown to give lower recovery of cells (Klein and Wu, 1974). Lastly the nutrient composition of the agar used plays a decisive role in colony development by viable cells (Brözel, 1990) as well as in the species recovered (Gustafsson and Mårdén, 1989; Sørheim et al., 1989).

We studied the reactions of two water samples to storage at various temperatures and over various times to determine the effect on the culturable count and on the bacterial community structure of the sample.

Materials and methods

R2A agar was used for the tap-water sample and R3A was used for the cooling-water sample as these yield the highest plate count (Brözel, 1990; Reasoner and Geldreich, 1985). R3A was also found to yield the highest species diversity for cooling-water samples as measured by the Shannon-Weaver index (Brözel, unpublished results).

Samples

Two water samples were evaluated, one from an open water-cooling system treated weekly with 40 $\mu\ell \cdot \ell^{-1}$ sodium dimethyldithiocarbamate bactericide (a commercial solution of 500 $g \cdot \ell^{-1}$ supplied by DASA Services), and one from a drinking-water tap in the laboratory. The tap was left to run for 5 min before the sample was taken in order to ensure that the sample was representative of water in the main system. Five samples of each were taken by drawing 10 mℓ aliquots into sterile glass tubes and covering these with sterile aluminium caps. These were taken directly to the laboratory and 4 of each were then incubated at 4, 10, 20, and 30°C.

Aerobic plate count

The number of culturable aerobes was determined on the fresh samples within 10 min of drawing the sample. This was repeated

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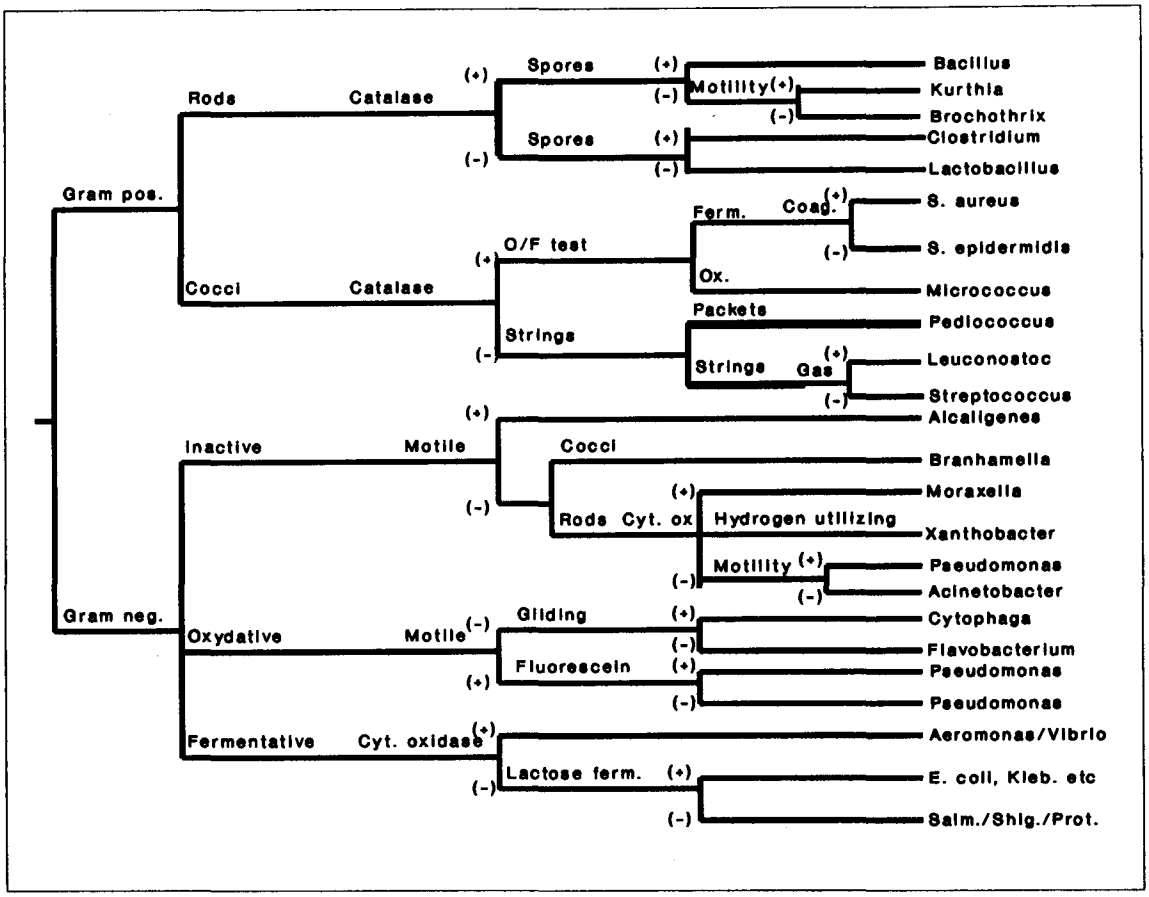

Figure 1
Flowchart used for the preliminary identification of bacterial isolates (as modified from Fischer et al., 1986)

after 24, 48, 72 and 216 h on samples stored at each of the four temperatures (i.e. 4, 10, 20, and 30°C). The count was performed by preparing a serial dilution series in sterile tap water and 0,1 mℓ aliquots of these were spread aseptically and in triplicate onto R2A agar (Reasoner and Geldreich, 1985) for the tap-water sample, and onto R3A agar (Reasoner and Geldreich, 1985) for the cooling-water sample using a Drigalski spatula, and incubated at 30°C for 120 h (Brözel, 1990; Morais and Da Costa, 1990).

Community structure of samples

The dominant bacterial species in each sample were determined by picking ca. 10 colonies off one of the 3 plates of highest dilution yielding a count over 30 but less than 300. Colonies were picked at random using the Harrison's disk method (Harrigan and McCance, 1976). These were suspended and vortexed in quarter strength Ringer's solution (Merck (Pty) Ltd.). A drop of the suspension was streaked out onto R3A agar using a platinum wire loop, and incubated at 25°C for 120 h as slow-growing isolates were masked by faster growing isolates in some cases. This procedure was repeated a further 2 times. Gram stains were performed to check for purity, after which colonies were regarded as pure. Cultures were kept on R3A agar slants.

Isolates were grown for 18 h and were then subjected to Gram staining, and to oxidase, catalase and O/F tests (medium of Hugh and Leifson containing glucose) (Hugh and Leifson, 1953). All isolates showing non-fermentative alkali-producing metabolism, were retested on O/F medium containing only 10% of the prescribed quantity of peptone (0,2 $g \cdot \ell^{-1}$) as acid production from glucose by some Gram negative oxidative rods is masked by alkali production from metabolism of amino acids in the peptone (Snell and Lapage, 1971; Ward et al., 1986). Motility of Gram negative isolates was determined by streaking onto motility agar (R3A agar containing only 0,4% agar) (Krieg and Gerhard, 1981). Some bacteria are immotile in agar but do possess flagella (Ward et al., 1986). Transmission electron microscopy was therefore performed on negative stains of 4 h old liquid cultures of all immotile isolates to show absence of flagella. Negative staining was by the method of Horne (1965). Preliminary identification was by a key modified from that of Fischer et al. (1986), as shown in Fig. 1.

Gram negative oxidative isolates were provisionally identified using the API 20 NE kit (API System, S.A.,-La Balme Les Grotes -38390, Montalieu, Vercieu, France), but were allocated to species by keys in Bergey's Manual of Systematic Bacteriology, Vol. I (Krieg and Holt, 1984). 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 onto R3A agar. Spore-forming isolates were assigned to the genus *Bacillus*. Other Gram positive isolates were only identified to the genus level by the keys given in Bergey's Manual of Systemic Bacteriology, Vol. II (Sneath et al., 1986).

Diversity

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

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

where:

C = 2,3
N = number of individuals
n_i = number of individuals in ith species.

Results

Aerobic plate count

General trends

All samples underwent a continuous change in culturable count (Figs. 2 and 3). Although the 2 samples gave different results, certain common trends were observed. Firstly, storage at the lower 2 temperatures (4 and 10°C) resulted in an initial decrease in the count, followed by an increase after 72 h. Storage at 30°C initially stimulated an increase in numbers, followed by a decrease after 48 h (Sample 1) or 72 h (Sample 2). At 20°C storage, the count increased, decreased and increased again. In Sample 1 the count increased, decreased after 48 h and increased to too numerous to count. In Sample 2 the count remained constant over 24 h, then increasing and again increasing after 72 h. None of the storage temperatures gave the same count initially and after 24 h. The cooling-water sample (Sample 1) gave a similar count at 10°C and less so at 4°C. In contrast the tap-water sample (Sample 2) gave the same count after storage at 30°C for 24 h.

Cooling water

The cooling-water sample culturable counts varied between 6,80 x 10^4 and 1,93 x 10^6 cfu·mℓ^{-1}, a factor of 28 times (Fig. 2). These two extremes were both achieved at 10°C. The highest count was after 216 h at 10°C and was 10,16 times the original count. However, after 24 h the counts also varied considerably, i.e. between 1,43 x 10^5 cfu·mℓ^{-1} or 75% of the original count at 4°C and 1,23 x 10^6 cfu·mℓ^{-1} at 20°C or a 6,47-fold increase.

Tap water

The tap-water sample showed more pronounced changes in culturable count than the cooling-water sample (Fig. 3). After 24 h the sample count remained constant when stored at 30°C. At 4°C it decreased to 8,7 x 10^2 cfu·mℓ^{-1} or 30% of the initial value. At 4 and 10°C the count did not vary much over the storage period and remained within $\log_{10}$ 0,82 below or $\log_{10}$ 0,23 above the initial value. Storage at 20 and 30°C however resulted in marked increases in the count.

After 48 h at 20°C it was $\log_{10}$ 2,09 greater or 122 times as much as the initial count. After 72 h at 30°C it was $\log_{10}$ 3,54 greater or 3 448 times as much. This is a large increase in colony-forming cell numbers.

Community structure

As opposed to the aerobic culturable counts, no general trends

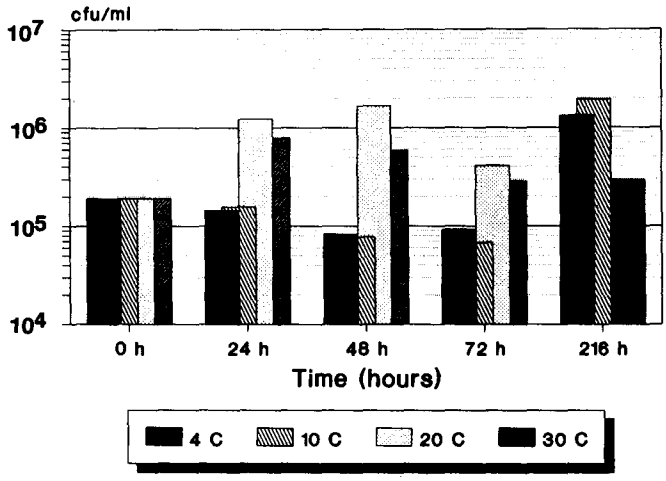

Figure 2
Total aerobic count of cooling-water sample stored at 4, 10, 20 and 30°C for 24, 48, 72 and 216 h (determined on R3A nutrient agar incubated at 30°C for 120 h). After 216 h the sample stored at 20°C was too numerous to count (TNTC)

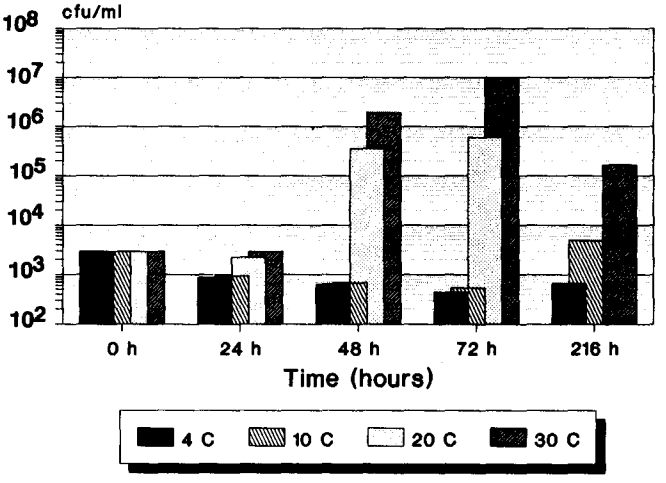

Figure 3
Total aerobic count of tap water sample stored at 4, 10, 20 and 30°C for 24, 48, 72 and 216 h (determined on R2A nutrient agar incubated at 30°C for 120 h). After 216 h the sample stored at 20°C was too numerous to count (TNTC)

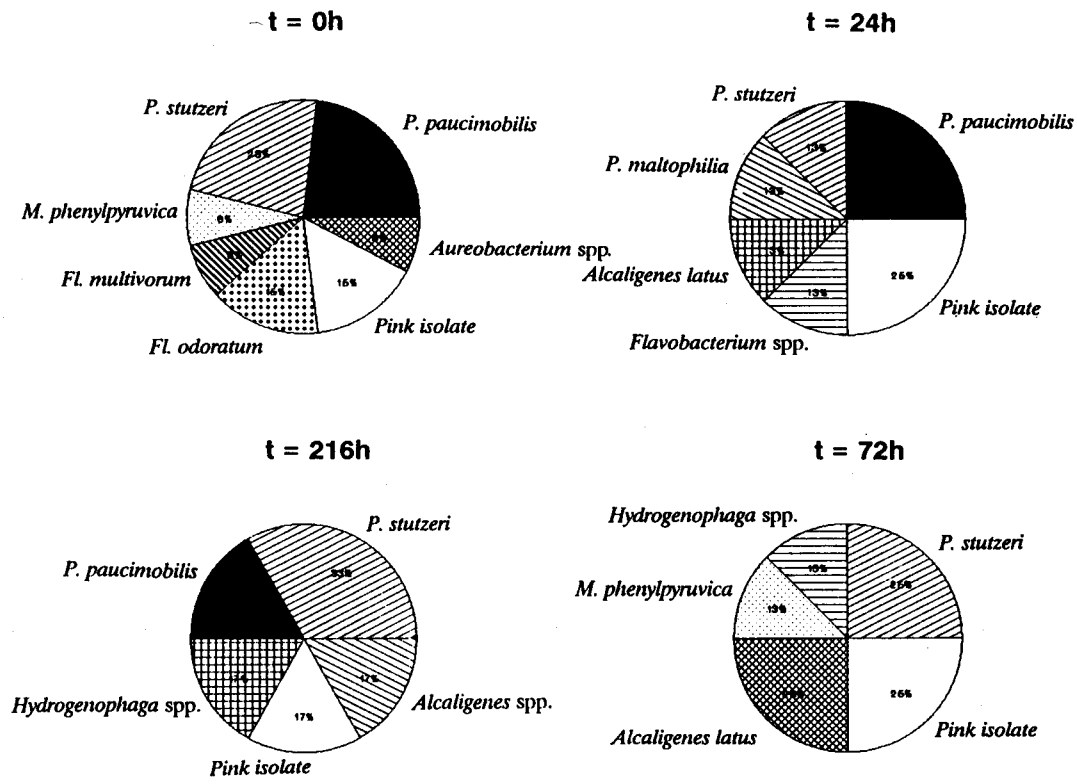

Figure 4
Community structure of cooling-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 4°C

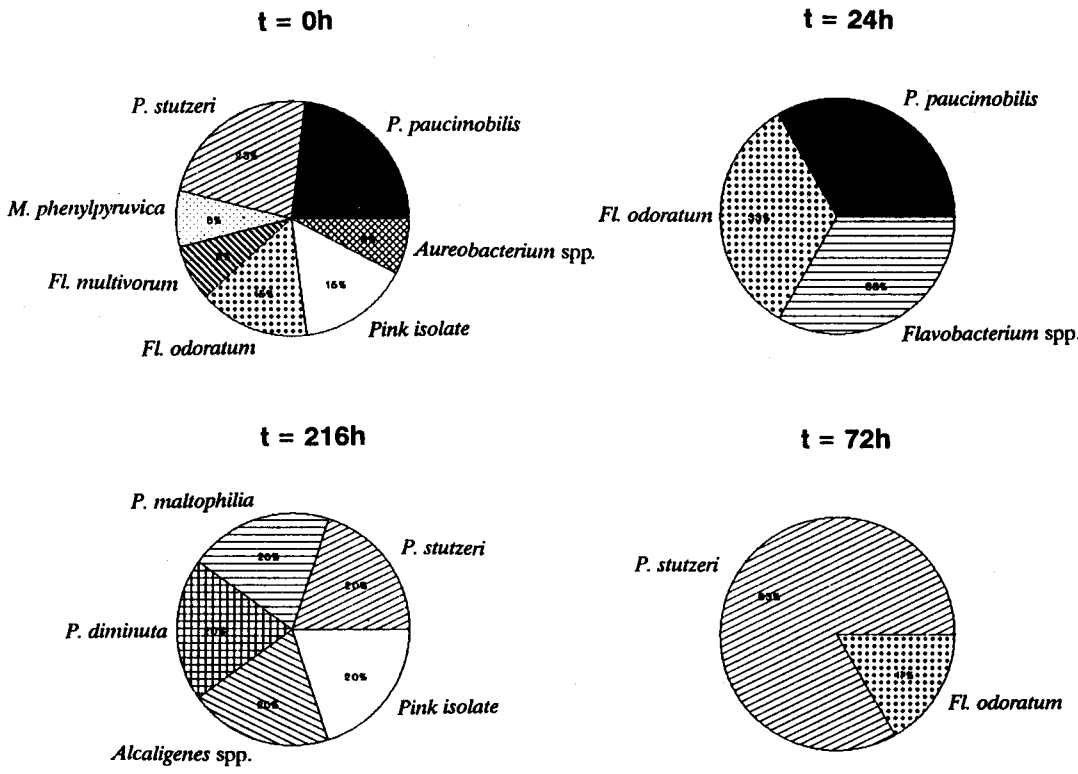

Figure 5
Community structure of cooling-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 10°C

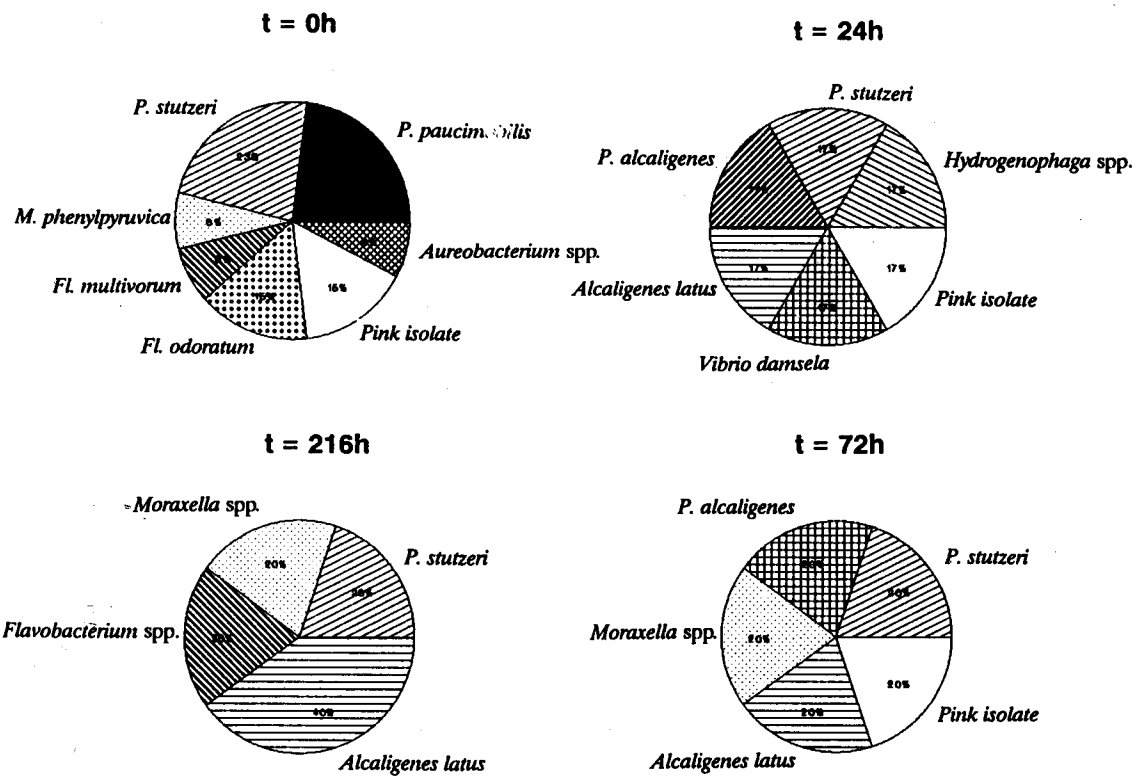

Figure 6
Community structure of cooling-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 20°C

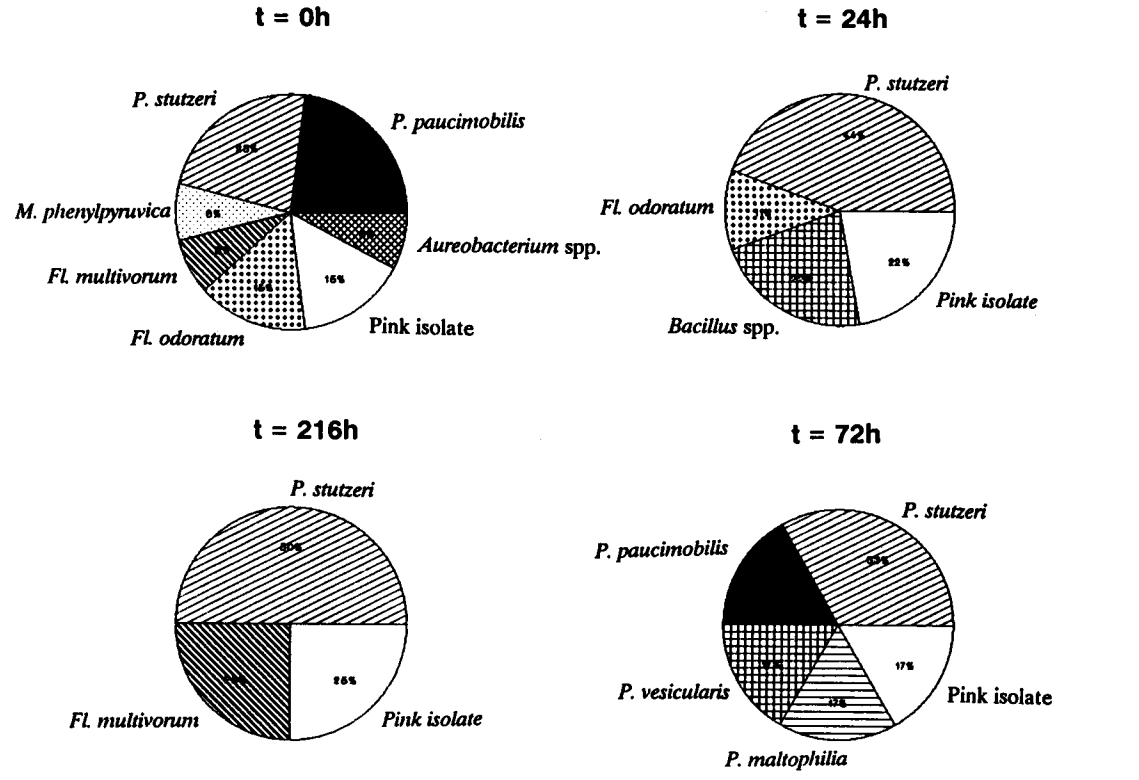

Figure 7
Community structure of cooling-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 30°C

TABLE 1
COMMUNITY STRUCTURE OF COOLING WATER STORED AT 4, 10, 20 AND 30°C FOR 24, 72 AND 216 h. PROPORTIONS OF SPECIES ARE GIVEN AS PERCENTAGE OF THE TOTAL COMMUNITY

Species		4°C			10°C			20°C			30°C		
	0h	24h	72h	216h	24h	72h	216h	24h	72h	216h	24h	72h	216h
P. paucimobilis	23	25		17	33							17	
P. stutzeri	23	13	25	32		83	20	17	20	20	44	33	50
P. maltophilia		13					20					17	
P. diminuta							20						
P. alcaligenes								17	20				
P. vesicularis												17	
Fl. odoratum	15				33	17					11		
Fl. multivorum	8												25
Flavobacterium sp.		13			33					20			
M. phenylpyruvica	8		12.5										
Moraxella sp.									20	20			
Pink isolate	15	25	25	17			20	17	20		22	17	25
Aureobacterium sp.	8												
Alc. latus		13	25					17	20	40			
Alcaligenes sp.				17			20						
Hydrogenophaga sp.			12.5	17				17					
Vibrio damsela								17					
Bacillus sp.											22		
Shannon-Weaver Index	**1.843**	**1.731**	**1.558**	**1.559**	**1.097**	**0.450**	**1.608**	**1.790**	**1.608**	**1.331**	**1.272**	**1.559**	**1.039**

were apparent in the species compositions of samples stored at various temperatures (Figs. 4 to 11). Certain species not detected at first appeared dominant after time, whereas others lost their dominant position. This does not of necessity imply death of members of a species which lost dominance, as the total count increased in certain cases and other species could have multiplied and thus masked the presence of those originally predominant.

Cooling water

The 2 isolates present under most of the conditions were *Pseudomonas stutzeri* and a Gram negative pink rod-shaped isolate which could not be identified to date. These unidentified organisms were all immotile, catalase positive, cytochrome oxidase positive and did not produce acid or alkali from glucose. They were mainly coccobacilli, but a few thin long rods were present, and in older cultures rods of medium length were also seen. Although there were signs of a rod - coccus cycle, this was not as clear as in members of the genus *Arthrobacter*. The results are tabulated in Table 1 and are depicted in Figs. 4 to 7. Certain species occurred only once, e.g. *Pseudomonas diminuta* and *Vibrio damsela*. However, most species occurred sporadically at various storage temperatures and after various storage times. *P. maltophilia* occurred after 24 h at 4°C, after 216 h at 10°C and after 72 h at 30°C. *Alcaligenes latus* occurred only at 4 and 20°C, but continuously at the latter temperature.

Tap water

The tap-water sample gave a variety of species after the various storage conditions (Table 2 and Figs. 8 to 11). *Alcaligenes latus* was the only species which retained dominance for 24 h at all temperatures whereas all others decreased or decreased and increased again in time. *Moraxella phenylpyruvica* retained a dominant position for 24 h at 10 and at 20°C, whereas it regained its dominance after 216 h at 4°C. *P. diminuta* at 20°C lost its dominant position after 24 h, but regained it again before 216 h. *M. lacunata* kept its relative dominance at 10°C, but lost it at 20°C. However, the total culturable count did not change much at 10°C, whereas it increased 205-fold after 72 h at 20°C. Therefore *M. lacunata* probably maintained a constant population, but its presence was masked by other species which multiplied at the higher temperatures.

Species diversity in samples

Figure 12 shows the course of numerical species diversity against time in the two samples stored at various temperatures. Species

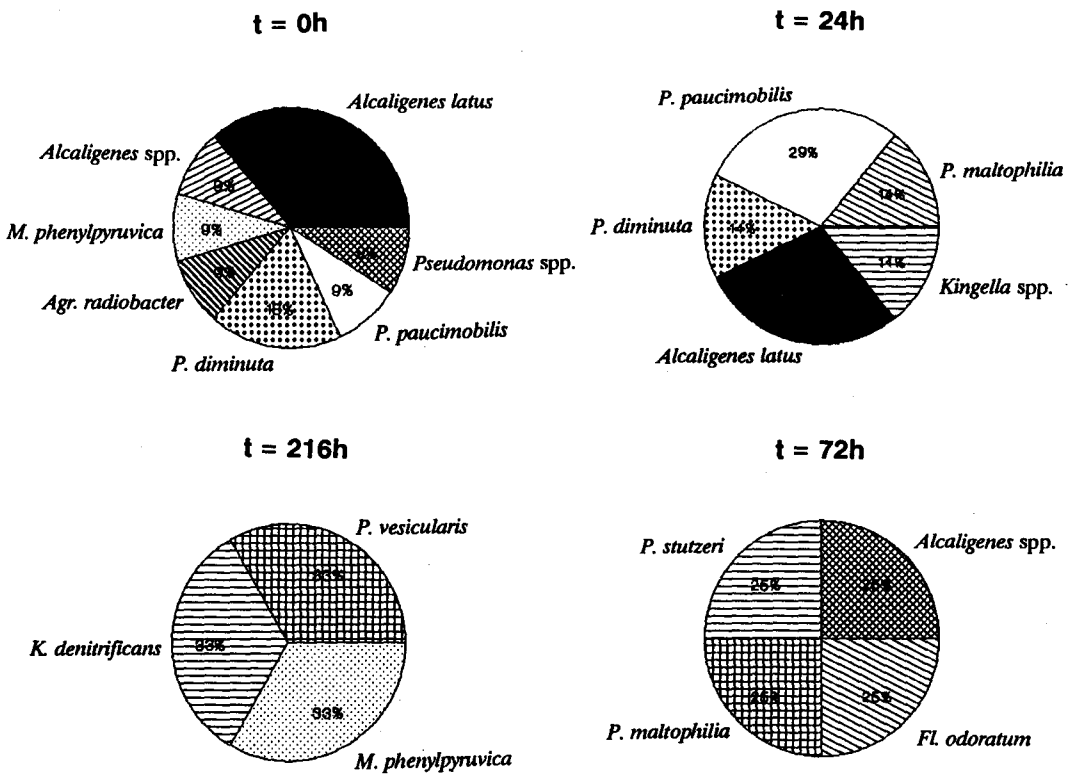

Figure 8
Community structure of tap-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 4°C

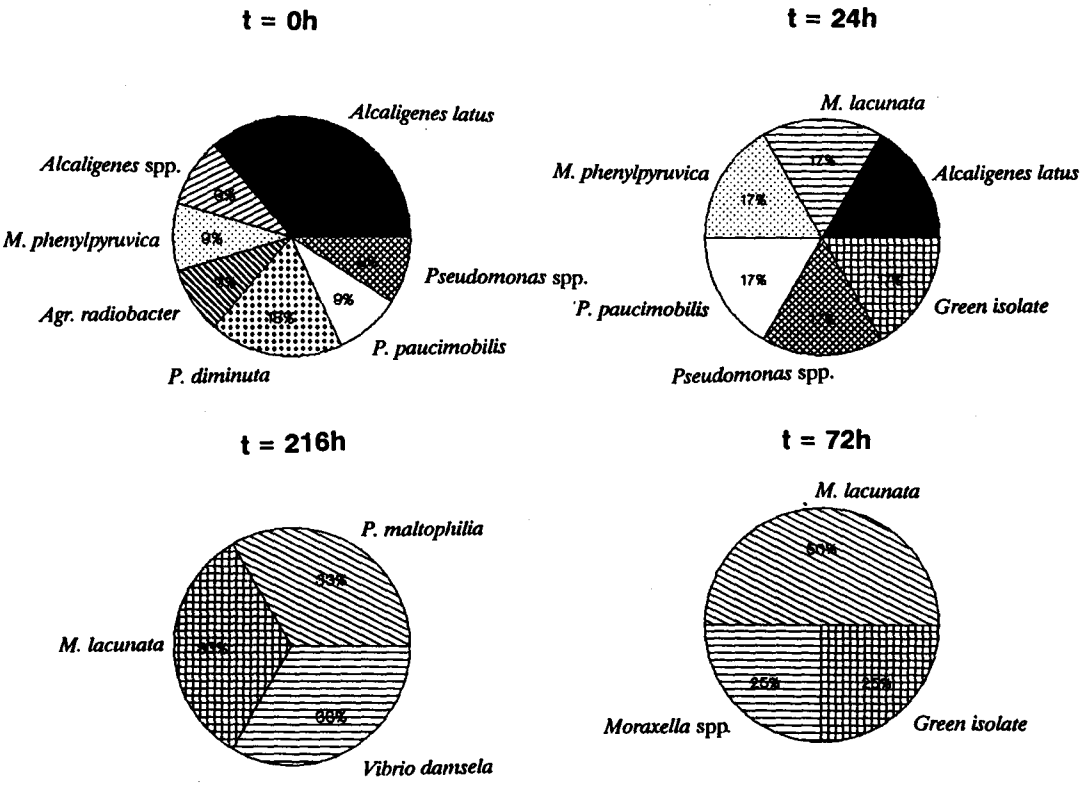

Figure 9
Community structure of tap-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 10°C

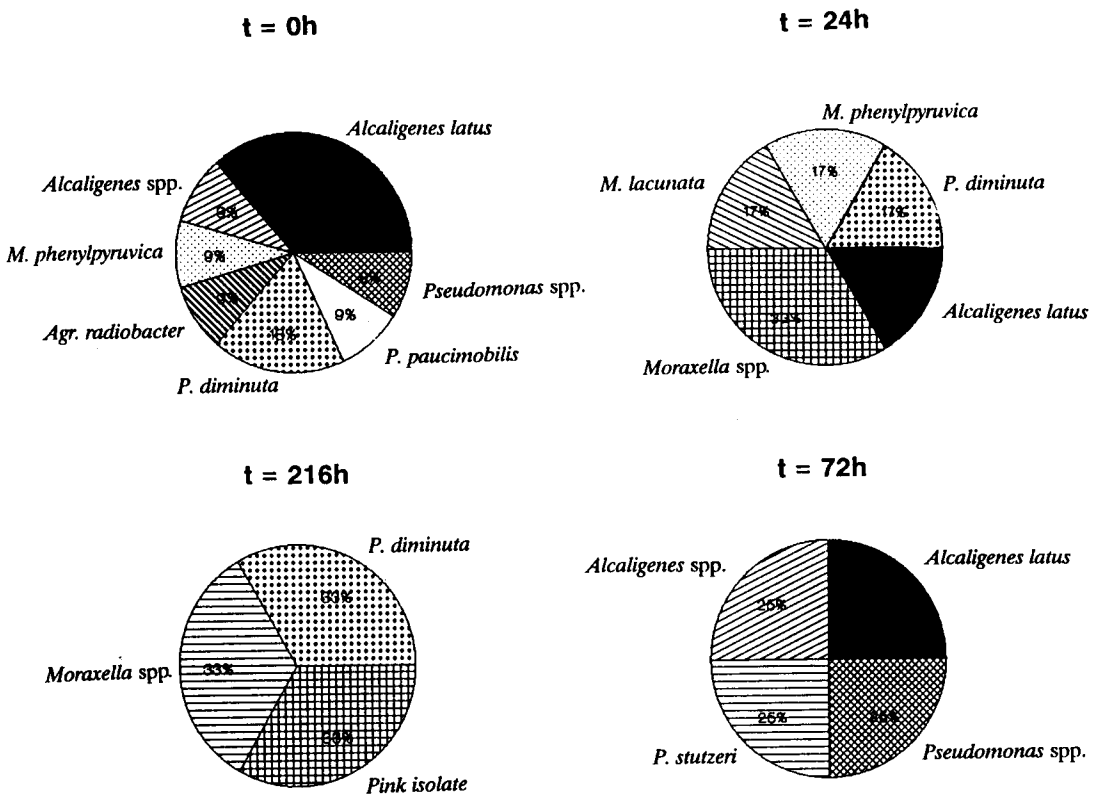

Figure 10
Community structure of tap-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 20°C

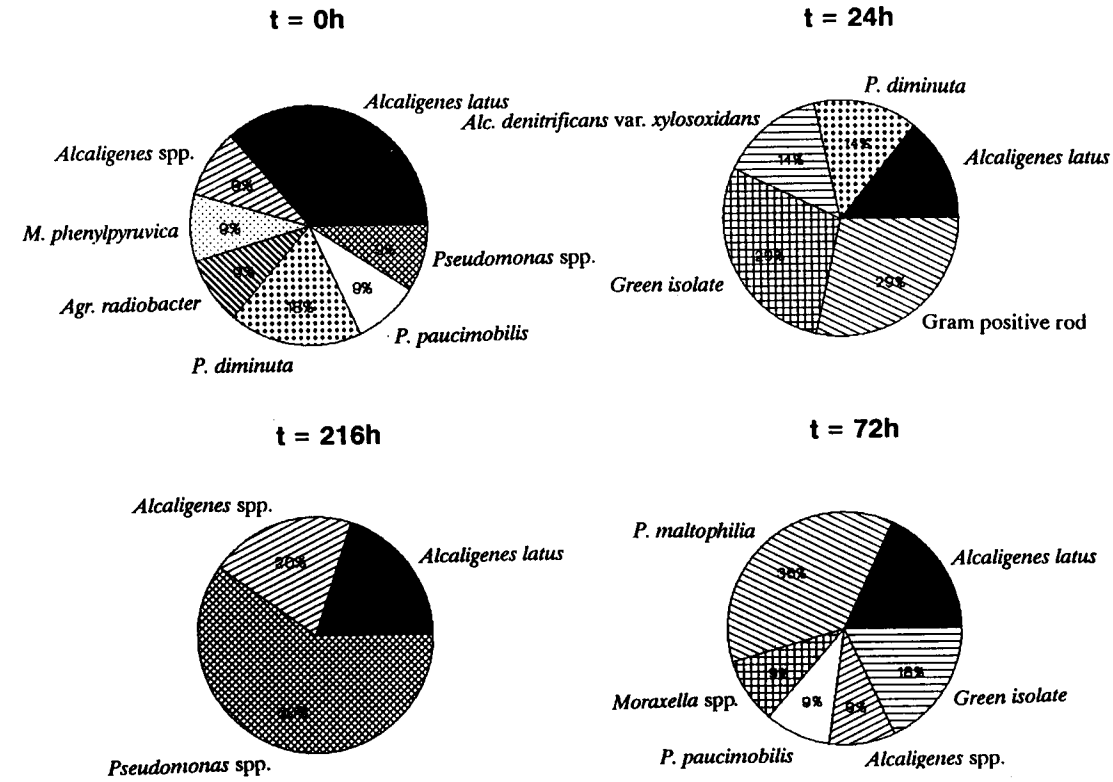

Figure 11
Community structure of tap-water sample as drawn (0 h) and after 24, 72 and 216 h storage at 30°C

TABLE 2
COMMUNITY STRUCTURE OF TAP WATER STORED AT 4, 10, 20 AND 30°C FOR 24, 72 AND 216 h. PROPORTIONS OF SPECIES ARE GIVEN AS PERCENTAGE OF THE TOTAL COMMUNITY

Species		4°C			10°C			20°C			30°C		
	0h	24h	72h	216h	24h	72h	216h	24h	72h	216h	24h	72h	216h
P. paucimobilis	9	29			17							9	
P. diminuta	18	14						17		33	14		
P. maltophilia		14	25				33					37	
P. vesicularis				33									
P. stutzeri			25						25				
Pseudomonas sp.	9				17				25				60
Alc. latus	37	29			17			17	25		14	18	20
Alc.denitr. subsp. *xyl.*											14		
Alcaligenes sp.	9		25						25			9	20
Agr. radiobacter	9												
M. phenylpyruvica	9			33	17			17					
M. lacunata					17	50	33	17					
Moraxella sp.						25		33		33		9	
Kingella sp.		29											
K. denitrificans				33									
Fl. odoratum			25										
Vibrio damsela							33						
Pink isolate										33			
Green isolate					17	25					29	18	
Gram positive rod											29		
Shannon-Weaver Index	**1.766**	**1.558**	**1.385**	**1.097**	**1.790**	**1.039**	**1.097**	**1.559**	**1.385**	**1.097**	**1.548**	**1.640**	**0.949**

diversity in samples stored at both 4°C and 20°C followed the same pattern. This was true for both the tap-water and the cooling-water sample. In these cases diversity also decreased steadily. Samples stored at 10°C and at 30°C followed differing routes. The net effect here was also a decrease in species diversity over time.

Discussion

Culturable count

Bacterial populations are dynamic by nature, with continuous cell division and death of cells taking place (Atlas and Bartha, 1987a). They can, therefore, be expected to occur in a constant state of flux, in terms of numbers, in terms of physiological state of the cells and in terms of their species composition and interaction. The results obtained prove that bacterial communities in water are dynamic. They react to external conditions such as temperature. As the various storage temperatures promoted either net decrease or net increase in the culturable count over time, total cell numbers and their physiological state changed over time during storage. Whereas 4 and 10°C induced a net decrease followed by an increase after 72 h, 20 and 30°C brought about an initial increase followed by either a decrease (30°C) or a further increase (20°C).

Drinking water is a low nutrient environment (Poindexter, 1981), and the little nutrient available would be depleted by cellular respiration over time. Various authors have shown that bacteria attach to surfaces as a response to low nutrient conditions (Kjelleberg et al., 1982; Zobell, 1943). The samples studied were stored in pre-sterilised glass tubes and aliquots were drawn after vortexing. The decrease in the culturable count could be partially ascribed to attachment of cells to the glass surface due to nutrient depletion in the water, the so-called bottle effect which can be decreased by reducing the surface to volume ratio of the container (Ferguson et al., 1984). The bottle effect takes about 4 h before numbers are decreased significantly, and the count at t_o would not have been affected. Where the nutrient concentration decreased even further, cells could have detached in search of possible nutrient sources. Delaquis et al. (1989) showed that *Pseudomonas fluorescens* detaches from surfaces due to low nutrient stress.

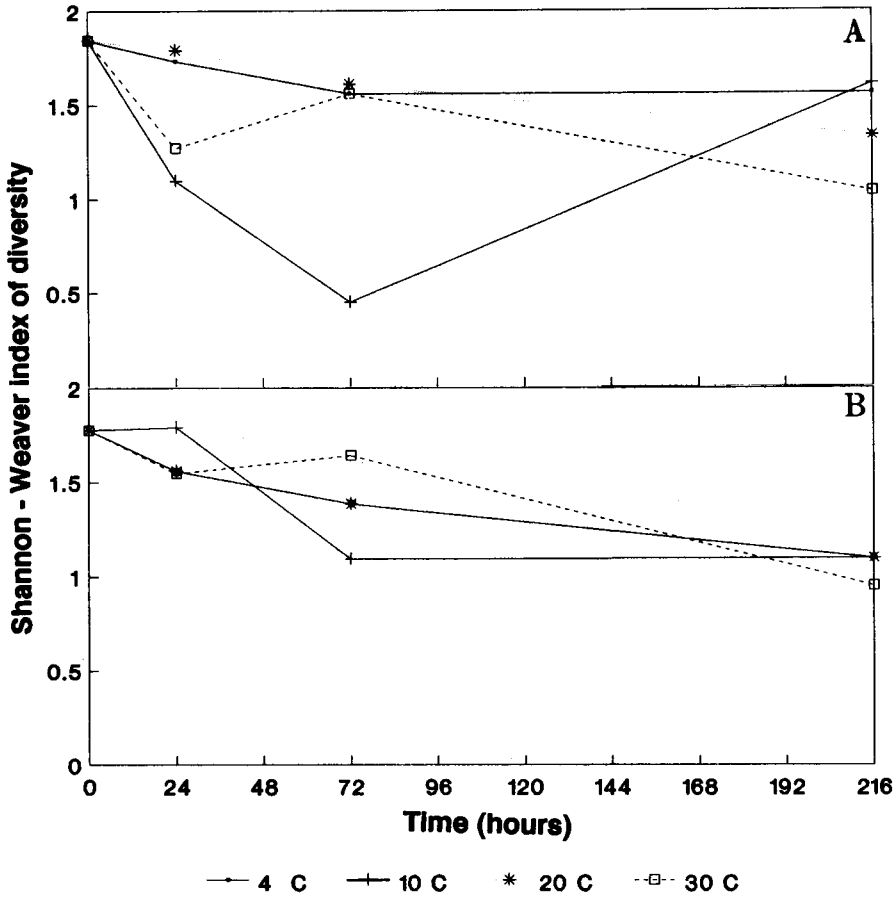

Figure 12
Shannon-Weaver index of diversity of samples of cooling-water (a) and of tap water (b) stored at 4, 10, 20 and 30°C over a period of 216 h

The tap-water sample showed a much larger increase in the culturable count after 24 h storage at 20 and 30°C (3 448-fold) than did the cooling-water sample (10,16-fold). This increase could not be ascribed to detachment, as the initial count was lower than subsequent ones. The sample would not have contained sufficient nutrients for such an increase in cell numbers. However, sub-lethally injured cells which survived chlorination, could have recovered over time and regained the culturable state (LeChevalier et al., 1982; Roszak et al., 1984). This would explain the increase over time at the higher storage temperatures (20 and 30°C), where residual chlorine would have dissipated more rapidly from the sample. Morais and Da Costa (1990) reported an increase in the heterotrophic plate count of bottled mineral water from $10 \cdot m\ell^{-1}$ to $10^5 \cdot m\ell^{-1}$ after 7 d. Carson et al. (1973) reported the growth of *Pseudomonas cepacia* in distilled water in hospitals, counts increasing from 10^2 to $10^7 \cdot m\ell^{-1}$ at 25°C. Therefore the high bacterial growth rate in tap water does not reflect poor sanitation or purification. It does, however, point to the inadequacy of the traditional culture media used to enumerate bacteria in drinking water (Gibbs and Hayes, 1988).

Community structure

No direct pattern of community development was detected in either of the samples, both samples showing a variety of resultant populations after storage. In both samples only one or two species attained any form of dominant position, while all others appeared randomly. In the cooling-water sample it was *P. stutzeri* and the pink Gram negative isolate which maintained a dominant position. Brözel (1990) found *P. stutzeri* to be the most dominant species in cooling water after sub-lethal bactericide treatments. This organism appears to be very important in cooling water, as it seems omnipresent due to extreme fitness and its ability to adapt. The pink isolate has not been isolated from cooling water in this laboratory before, but previous isolations were always performed on Std I nutrient agar (Biolab). Reasoner et al. (1989) reported the isolation of pink Gram negative rods from drinking water using R2A agar, although these were not identified. R3A and R2A agars were used in this study. The pink isolate is probably present in many cooling-water systems, but has to date eluded detection as it does not grow on Std I nutrient agar. This isolate warrants further investigation as to its role in cooling water and biofouling. All storage conditions resulted in community shifts, however, with the greatest change occurring at 10°C storage temperature.

The only isolate holding any dominant position in the tap-water sample, was *Alcaligenes latus*. However, it only held its dominant position at 30°C. At the lower temperatures various *Pseudomonas* and *Moraxella* species were dominant, whereas at the higher temperatures it was the *Alcaligenes* species. This reflects the high degree of species diversity in tap water. As mentioned above, many dominant species may have eluded detection as they were sub-lethally injured. Conversely, the results could show random selection, where many more species were present in more or less equal numbers but only some were chosen. However, clear shifts occurred upon storage at all 4 temperatures, even up to 24 h.

Numerical species diversity

Although the numerical species diversity followed similar routes at both 4°C and 20°C, the pattern of dominant species differed notably (Figs. 4, 6, 8 and 10). Species common to both water samples at 24 and at 72 h were *P. stutzeri, Alc. latus* and the pink isolate. There was a lower correlation between the tap-water samples at 4°C and at 20°C.

The similar routes of numerical species diversity at 4°C and at 20°C in the 2 samples point to 2 physiological groups present in water. Diversity at 10°C and at 30°C changed markedly in both samples over time. This indicates that the bacterial community was under some stress as decrease in diversity is indicative of stress (Atlas, 1984). The decrease at 10°C in the cooling-water sample indicates stress on the community. The tap-water sample reacted to a lesser degree, but the sample stored at 10°C also underwent the greatest changes in diversity. Because 4°C storage appeared to support species diversity, the community was under less stress, similar to the situation at 20°C. Therefore it can be said that 4°C and 20°C both exert a similar load of stress. Conversely the bacterial community can be said to have 2 temperature optima. The higher numbers at 20°C than at 4°C do not contradict this, as growth rate is temperature dependant (Stanier et al., 1986), i.e. stress is not directly related to growth rate.

A question worth asking is whether bacterial communities in cooling water are stressed at temperatures between the operating temperature and 4°C. If this would be so, bactericide treatment at such intermediate temperatures should be investigated. Bacterial communities in cooling-water experience stress in the presence of bactericides, so application of such stress under already stressed conditions should result in a higher percentage kill.

Conclusion

The results do show that a culturable count performed any period after drawing of the sample does give different results to those obtained upon immediate analysis. The count obtained after sample storage is sometimes lower and sometimes higher than the initial count, depending on the storage time, temperature and initial state of the cells. Therefore results of analysis after storage cannot be adapted by a known factor. They must be interpreted with extreme caution, as they do not of necessity reflect the bacterial composition of the sample as drawn, both in terms of total numbers and in terms of community structure. Only counts performed on fresh samples give reliable results on the total culturable count, and only community structures determined immediately, reflect the state of the community in the system from which the sample was drawn.

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