Viability of Heterotrophic Bacteria in Freshwater

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The viabilities of planktonic bacteria from nine freshwaters and one raw sewage were compared. Viabilities estimated from microcolony formation in slide culture (18.4–48.7%) were higher than a conventional plate count method (1.8–14.9%). Microcolony viability was estimated in four ways; the one recommended was the ratio of the microcolony count and total count of microcolonies and single cells after 24 or 72 h incubation. Bacterial viability strongly correlated with the percentage of bacteria $<0.2\ \mu\text{m}^3$ in size and the concentration of soluble carbohydrate in the water.

INTRODUCTION

Plate counts are generally accepted as inadequate for counting all the naturally occurring aquatic bacteria which are viable (Buck, 1979). Many alternative methods have been suggested which fall into two groups. The biochemical methods assess the proportion of active bacteria by counting those which take up radioactive organic materials (Hoppe, 1976; Meyer-Reil, 1978) or which respire (Zimmermann *et al.*, 1978). In the second group are the growth methods which rely on microscopic enumeration during the initial stages of growth in the laboratory. These include techniques which count microcolonies on membrane filters (Jannasch & Jones, 1959; Kunicka-Goldfinger & Stronkowska, 1977), microcolonies on agar (Fry & Zia, 1982) and division-inhibited cells in liquid medium (Kogure *et al.*, 1979). All these methods could be used to assess viability. However, no studies have yet attempted to find the factors which either cause, or are related to, the differences between viability observed in aquatic habitats. This is our aim in the research reported here. For this we use a growth method because there can be no doubt that growing cells are alive, whereas some biochemical function could remain in dead or moribund cells. Also a slide culture technique was selected because it offered substantial advantages over other methods (Fry & Zia, 1982).

METHODS

Sampling. Water and sewage samples (1 l) were obtained between June and September 1980. Four samples were taken from each site with approximately one month between each sampling occasion. Sewage samples were obtained from a Cardiff pumping station (Ordnance Survey grid reference, ST 169737) immediately after the sewage had been comminuted. Surface water samples were taken from nine freshwater habitats in South Wales. Three were from flowing waters, polluted mainly with sewage (National Water Council, 1981); these were the R. Rhymney at Draethen (ST 223877), the Afon Llwyd at Llantarnam (ST 308937) and the Taff feeder (ST 179770), a water course connecting the R. Taff at Blackweir to Cardiff docks. The other waters were unpolluted and included two mesotrophic water supply reservoirs (Scott, 1975); these were Ynysyfro Old Pond (ST 891285) and Pantyreos Reservoir (ST 258916). Two recreational waters were sampled, namely Roath Park Lake (ST 186794) in Cardiff and a disused section of the Monmouthshire–Brecon Canal at Llantarnam (ST 294922). The remaining two waters were a natural spring, which arose in a stone trough and flowed into a small stream called Nant Draethen (ST 204864), and one of the main drainage channels (reens) on the Monmouthshire levels (ST 268815; Scotter *et al.*,

Abbreviations: BOD, Biochemical oxygen demand; CPS, casein/peptone/starch medium; PCA, principal component analysis.

1977). Only the reën, the Monmouthshire-Brecon Canal and Ynysfro reservoir supported substantial stands of aquatic macrophytes; the other waters were almost devoid of such plants. After transport to the laboratory, samples were stored at 4 °C, shaken for 5 min by hand and used within 4 h of collection.

Sample concentration. Where necessary 10 ml samples were concentrated by centrifugation in glass centrifuge tubes with tapered ends at 2000 *g* for 20 min at room temperature (20–22 °C). After centrifugation, 9.7–9.9 ml of the supernatant was removed, the remainder was vortex mixed to resuspend the bacteria and pooled with other concentrated samples.

Medium. Casein/peptone/starch (CPS) agar was used for both slide culture and plate counts. It was prepared as described by Fry & Humphrey (1978), but for slide culture was filtered through glass fibre filter paper (Whatman GF/C).

Viable counts by slide culture (microcolonies). Agar coated slides were prepared by allowing 0.5 ml of molten (65 °C) CPS agar to solidify on three-quarters of one side of thin (0.8–1 mm), glass slides sterilized by flaming with 95% ethanol. Immediately after solidification, 10 µl of concentrated water sample was spread on 1 cm² of the agar and allowed to dry for 10 min at room temperature. The agar was then covered with a coverslip and incubated at 10 °C for 24 and 72 h. Numbers of microcolonies, which consisted of two or more bacteria, and single cells were counted before or after incubation with a Zeiss Universal microscope using either a 63X planapochromat (numerical aperture, NA = 1.4) or a 100X neofluor (NA = 1.3) oil immersion objective with phase contrast microscopy. Full details of the slide culture methodology are discussed elsewhere (Fry & Zia, 1982).

Viable counts on agar plates (macrocolonies). Samples were serially diluted in CPS medium without agar before plate counts were performed by the spotting method of Bousfield *et al.* (1973). Five samples (10 µl) of each dilution were put on to the surface of agar plates, allowed to dry and incubated at 18 °C for 8 d. After incubation, the number of macrocolonies in drops which contained between 2 and 20 colonies were counted.

Epifluorescence microscopy. Total counts of bacteria were obtained by an acridine orange epifluorescence method (Jones, 1979). A final concentration of acridine orange of 5 mg l⁻¹ was used to stain the bacteria for 3 min and filtration was through 0.2 µm pore size black Sartorius membrane filters. A Zeiss Universal microscope with two BG-12 exciter filters and a barrier filter 50 using a 63X (NA = 1.4) or a 100X (NA = 1.25) oil immersion lens was used to count 100–400 red or green fluorescing bacterially shaped objects. Bacteria on three replicate membrane filters were counted in each sample.

Calculations of viability. Estimates of microcolony viability (V , V_0 , V_e , V_i) and macrocolony viability (V_v) were calculated as follows: $V = m_i/t_0$; $V_0 = (m_i - m_0)/t_0$; $V_e = (m_i - m_0)/t_e$; $V_i = m_i/t_e$; $V_v = c/t_e$ where m_0 = the number of microcolonies before incubation, m_i = the number of microcolonies after incubation, t_0 = the total count of single cells and microcolonies before incubation, t_e = the total count of bacteria by epifluorescence microscopy and c = the plate count. To estimate V_e , V_i and V_v , all parameters in the equations had to be calculated as a number per unit volume. This was simply done for t_e and c , but for m_i and m_0 the estimates had to be corrected for losses of bacteria during centrifugation. This was determined from total counts by epifluorescence microscopy of the original and concentrated water samples. Viabilities were converted to percentages.

Cell size estimation. Bacteria were concentrated in 0.5% (v/v) formaldehyde (Powell & Errington, 1963) leaving 0.1 ml in each glass centrifuge tube. Although formalin might cause shrinkage of bacteria, its use is essential to prevent the enlargement of small cells which occurs on agar (Torrella & Morita, 1981). The concentrate (10 µl) was spread over approximately 0.5 cm² of the surface of an agar coated slide, allowed to dry, covered with a coverslip and examined under phase contrast microscopy, as described for slide culture. Black and white photographs were taken using an Olympus OM-2 camera. Developed negatives (36 mm × 24 mm) were viewed with a slide projector and the length and diameter of all the bacteria which were within a constantly placed central section (12 mm × 8 mm) of the negative were measured. Measurements were made of the first 100–150 bacteria that were observed. Absolute sizes were calculated from the proportions of a similarly projected negative of a slide micrometer. The cell volume of the fixed cells was calculated assuming them to be parallel sided cylinders with hemispherical ends using the formula:

$$\text{cell volume} = [d^2(\pi/4)] (1 - d) + \pi d^3/6$$

where d was the cell diameter and l was the length. This photographic method correlated well ($r = 0.88$; $P < 0.001$) with the classical method of size measurement using an ocular micrometer (Salonen, 1977).

Biochemical oxygen demand (BOD). The method of Taras *et al.* (1971) was used with the reagents of Golterman & Clymo (1969) and a back titration procedure (Talling, 1973) to assess the oxygen concentration. BOD measurements were made using aerated membrane-filtered water as diluent.

Estimation of soluble carbohydrate and protein. The dissolved materials in 1 l of 0.2 µm filtered water and sewage were concentrated by rotary evaporation at 50 °C under reduced pressure to approximately 10 ml. Soluble carbohydrates in the concentrated samples were measured by the phenol/sulphuric acid method (Gerchakov & Hatcher, 1972) using glucose as the calibration standard. The Lowry method was used to estimate the soluble protein in concentrated samples using bovine serum albumin as standard.

Statistical analysis. Means were compared by analysis of variance and differences between means were assessed with the least significant range procedure (Sokal & Rohlf, 1969). Percentages were transformed by $\arcsin \sqrt{x}/100$ and other variables by $\log_{10} x$. These transformations ensured normality of distribution and homogeneity of variances; the $F_{\max}$ and Bartlett's test were used to test for the latter condition. Confidence intervals and coefficients of variation were calculated on untransformed and transformed data, respectively. These tests and principal component analysis (PCA) were performed with the Statistical Package for the Social Sciences (Nie *et al.*, 1975; Hull & Nie, 1979). PCA was performed with subprogram FACTOR using PA1. Multiple regression was performed with Minitab (Ryan *et al.*, 1976) using transformed data unless otherwise stated. All significant differences between means and the significance of correlation coefficients are quoted for $P < 0.05$ unless otherwise stated. The squared correlation coefficient (r^2) and the squared multiple correlation coefficient (R^2) were used to describe the percentage variability explained by regression.

RESULTS

Comparison of methods for estimating viability

Viabilities of bacteria from ten sites show (Table 1) that estimates based solely on the microcolony method (V_0) were highest. Estimates which used microcolony and epifluorescence counts (V_e) were lower because the total counts of bacteria obtained by the microcolony method (t_0) were less ($55.7 \pm 1.6\%$; Table 2) than those obtained by epifluorescence (t_e). The lowest results were for the macrocolony viability (V_v), which were significantly lower than the other estimates at all but one site. Microcolony counts after 72 h incubation were not possible in almost all samples from the Taff feeder, R. Rhymney, Afon Llwyd and Nant Draethen spring. Hence, at these sites the optimum incubation time was 24 h. All other sites had optima after 72 h incubation. The three polluted waters and Nant Draethen spring showed the highest viabilities

Table 1. *Percentage viabilities obtained by slide culture and colony counts at ten sites*

Slide cultures were incubated at 10 °C for 24 h or 72 h and colony counts were made by a drop procedure incubated at 18 °C for 8 d; both used CPS medium. The values represent means of four samples taken at monthly intervals unless otherwise stated.

	Percentage microcolonies at 0 h	Percentage viability calculated by:			
		Slide culture (V_0) after incubation for		Ratio of microcolony and epifluorescence count (V_e) incubated for optimum period	Ratio of plate count and epifluorescence count (V_v)
		24 h	72 h		
Taff feeder	5.6	43.1*	NC	25.9‡	14.1§
R. Rhymney	2.6	36.6*	NC	20.2‡	9.1§
Afon Llwyd	4.9	38.7*	NC	21.4‡	12.5§
Sewage	11.8	20.8	NC	12.1	9.2
Roath Park Lake	0	21.9	25.9	13.9	2.3§
Monmouthshire-Brecon Canal	0	21.2	29.2	16.2	3.3§
Reen	0	19.1	21.7	12.4	7.1§
Ynysyfro res.	0	18.9	22.7	12.7	1.8§
Pantyreos res.	0	14.0	18.4	10.3	2.2§
Nant Draethen spring	0	38.4*	31.25†	21.3‡	14.9§
Average coefficient of variation (%)	—	9.5	11.5	11.2	25.6

NC, Counts not possible due to merging of microcolonies.

* Percentage viabilities at marked sites were significantly higher than those at other sites except that R. Rhymney was not significantly different from Monmouthshire-Brecon Canal.

† Counts only possible on one of the four sampling occasions.

‡ Percentage viabilities at marked sites were significantly higher than those at other sites except that R. Rhymney and Nant Draethen spring were not significantly different from the Monmouthshire-Brecon Canal.

§ V_v significantly lower than V_0 and V_e .

|| Percentage viabilities at marked sites were significantly lower than those at other sites.

Fig. 1. Principal component analysis solutions of the first two components for (a) all the variables used in this study and (b) the viability estimates only, using data from all sites. The abbreviations are defined in Methods, except the following: carb, soluble carbohydrate; prot, soluble protein; vol, mean bacterial volume; <0.2, bacteria <0.2 μm^3 in size.

with all three methods. The polluted waters and sewage were the only sites having microcolonies present before incubation (m_0). V was calculated by adding V_0 and m_0 , and V_i was a combination of V_e and m_0 . Thus V and V_i were highest in the polluted waters, Nant Draethen spring and sewage. In these waters V ranged from 32.6% to 48.7% and V_i ranged from 19.0% to 28.4%.

Despite these differences the viabilities were very highly correlated with each other; this correlation was stronger for the microcolony viabilities ($r^2 = 81\text{--}98\%$) than for the macrocolony viability ($r^2 = 50\text{--}66\%$). PCA clearly showed these relationships (Fig. 1) as the viabilities were tightly grouped whether other variables were present (Fig. 1a) or absent (Fig. 1b). In the latter solution the first two components explained 96% of the data's variability and the viabilities were in three closely related groups. The macrocolony viability (V_v) was separated from the microcolony viabilities, which were tightly grouped with V_0 close to V_e and V_i close to V . As the microcolony viabilities were so closely related, only one should be selected for further study. V_e and V_i were difficult to estimate because microcolony and epifluorescence total counts had to be related and this needed extra counts to allow for losses of bacteria during centrifugation. V_0 needed an estimate of the number of microcolonies present at zero time. Consequently, in the remaining results V will be the microcolony viability used, because it was simplest to estimate, and will be compared with the more traditional macrocolony viability (V_v).

Comparison of viabilities and other variables

Population estimates and sizes of bacteria, together with values of BOD, soluble protein and soluble carbohydrate at all sites are given in Table 2. The viable counts were lower than both of the total counts, the microcolony counts ($m_i - m_0$) being highest. There were significant differences between the sites for all variables. Sewage was significantly different from all other sites having the highest bacterial populations and values for the chemical variables. Sewage also contained the largest cells and had the lowest proportion of small cells. Nant Draethen spring water was also significantly different from the other sites with the lowest values for bacterial populations and chemical variables; however, its bacteria did not have the smallest mean volume, but they had the highest proportion of small cells. The values of all variables at all other sites fell between these two extremes, but showed no clear pattern, although most values for the Taff feeder, Roath Park Lake, the Monmouthshire-Brecon Canal and the reen site were higher than the rest.

The differences in the cell sizes can be seen in the typical frequency histograms of cell volumes (Fig. 2). They showed that the unpolluted sites such as Pantyreos and Ynysyfro reservoirs had

Table 2. Mean values of various bacterial and chemical variables at ten sites

The techniques used are described in Methods and values were obtained from four samples taken at monthly intervals. Symbols used for bacterial counts are defined in Methods.

Site	$10^{-5} \times \text{No. bacteria ml}^{-1}$, obtained by:					BOD ($\text{mg O}_2 \text{ l}^{-1}$)	Soluble protein (mg l^{-1})	Soluble carbo- hydrate (mg l^{-1})	Mean bacterial volume (μm^3)	Percentage bacteria < $0.2 \mu\text{m}^3$
	Total count by epifluor- escence (t_e)	Total count by slide culture at 0 h (t_0)	Plate count (c)	Microcolony counts after the optimum incubation period ($m_t - m_0$)						
Taff feeder	50.2 ^a	29.2 ^a	7.10 ^a	13.0	7.25	1.70	0.91	0.77	12.1	12.1
R. Rhymney	14.3 ^b	7.70 ^b	1.28	2.77	6.12	0.84	0.84	0.70	9.1	9.1
Afon Llwyd	7.62 ^b	4.30 ^b	0.88	1.59	4.66	0.36	0.62	0.62	19.1	19.1
Sewage	757*	442*	70.9*	92.4*	112.0*	4.98*	12.2*	1.00	4.9†	4.9†
Roath Park Lake	61.9 ^a	32.7 ^a	1.10	8.77	8.22	1.27	1.60	0.48	42.7	42.7
Monmouthshire- Brecon Canal	39.7 ^a	22.9 ^a	1.11	5.83	4.03	0.58	1.87	0.32	45.5	45.5
Reen	33.2 ^a	18.9 ^a	2.27	3.95	6.97	2.12	2.50	0.55	39.4	39.4
Ynysfiro res.	12.0 ^b	6.73 ^b	0.21†	1.49	6.48	1.12	1.12	0.39	37.7	37.7
Pantvres res.	11.2 ^b	5.85 ^b	0.24†	1.06	2.30	0.39	0.79	0.35	47.4	47.4
Nant Draethen spring	0.94†	0.51†	0.14†	0.21†	2.41	NM	0.21†	0.51	60.1	60.1
Average coefficient of variation (%)	12.2	14.6	7.6	31.4	19.8	4.7	4.0	12.6	44.5	44.5

NM, Protein content could not be measured as an interfering colour reaction was present in all samples.

a, b, Values in groups which were significantly different from values in other groups; unmarked values have complex patterns of significant differences not easily represented here.

* Values at marked sites were significantly higher than those at other sites.

† Values at marked sites were significantly lower than those at other sites.

Fig. 2. Frequency histograms of bacterial volumes from (a) Pantyreos reservoir, (b) Ynysfyo reservoir, (c) sewage and (d) Taff feeder water samples.

frequency distributions skewed very heavily to the left whilst those of the polluted sites were less skewed. This meant that unpolluted sites had bacteria with smaller mean volumes and most cells in the $0\text{--}0.2\ \mu\text{m}^3$ class, whilst polluted sites had higher mean volumes and few small cells. The smallest bacteria observed at the different sites varied between 0.02 and $0.08\ \mu\text{m}^3$ and the largest between 1.51 and $6.07\ \mu\text{m}^3$.

The types of organisms and microcolonies observed by slide culture during this work were similar to those reported previously (Fry & Zia, 1982). In the samples from Roath Park Lake about 5% of the bacteria were prosthecate forms resembling *Planctomyces* spp. Also, in samples from Pantyreos and Ynysfyo reservoir 5–7% of the bacteria were loosely aggregated clumps of pleomorphic rod-shaped bacteria. Neither of these distinct morphological types multiplied during incubation.

Further statistical analysis was required to determine the relationship that existed between the viabilities and other variables. The value of many variables in sewage was very high (Table 2), hence the analyses were performed both with and without the sewage results. This was necessary to check that these high values were not forcing linear relationships upon the data.

Correlation analysis (Table 3) between viability and the other variables gave similar results whether the sewage samples were included or omitted. The highest coefficients were with the cell size estimates, but these relationships only accounted for between 29 and 46% of the data's variability. The only other consistently significant coefficients were with the plate counts and soluble carbohydrate, which accounted for less variability (16–25%). There were few other significant correlations. The size variables were poorly correlated with population estimates and chemical variables; these correlations were rarely significant and never accounted for more than about 15% of the data's variability. The population estimates and chemical variables were all significantly correlated with each other, but no obvious pattern in the coefficients was evident.

PCA objectively confirmed these observations, revealed other relationships and provided similar solutions whether sewage was included (Fig. 1) or omitted. In the solution with all the data (Fig. 1a) the first two components accounted for 85% of the variability and the other components did not add to the explanation. This solution shows all the population and chemical

Table 3. *Correlation coefficients between percentage viabilities and other variables using data from all sites except sewage*

Viability estimate	Total count of bacteria by slide culture (t_0)	Total count of bacteria by epifluorescence (t_e)	Plate Count (c)	Micro-colony count ($m_t - m_0$)	BOD	Soluble protein	Soluble carbohydrate	Mean bacterial volume	Percentage bacteria < 0.2 μm^3
Microcolony viability (V)	-0.162	-0.170	0.399**	0.098	0.144	-0.130	-0.466**	0.616***	-0.677***
Macrocolony viability (V_c)	-0.369*	-0.388**	0.403**	-0.172	0.102	-0.009	-0.495**	0.537***	-0.542***

*, **, *** - Significant at $P \leq 0.05$, 0.01 or 0.001, respectively.

Table 4. *Multiple regression analysis of percentage viabilities with epifluorescence total counts, two bacterial size estimates and three chemical variables using data from all sites*

Dependent variable	Regression coefficients for the following selected independent variables:*					Constant term	Squared multiple correlation coefficient, R^2 (%)
	Percentage bacteria < 0.2 μm^3	Soluble carbohydrate	Mean bacterial volume	Epifluorescence total count	BOD		
Microcolony viability (V)	-0.194(41)	-13.2(12)	8.32(5)	5.22(8)	-	36.3	66
Macrocolony viability (V_c)	-0.124(31)	-9.14(6)	4.99(6)	-	6.98(7)	14.7	50

-, variable not selected.

* variables were selected by backward elimination until the minimum t -ratio was 1.0 (Chatterjee & Price, 1977).

variables to be closely grouped and loading very heavily (average = 0.925) on component one with the viability estimators loading equally highly (average = 0.93) on component two. The size variables also loaded heavily on component two, which were thus closely related to the viabilities. All other variables were close to zero on component two, but the plate count and soluble carbohydrate were furthest from zero, a reflection of the significant correlations between these variables and the viabilities (Table 3).

PCA of the independent variables used for the multiple regression (Table 4) showed that the data was multicollinear, because several eigenvalues were very small (Chatterjee & Price, 1977). Despite this condition the multiple regression solution was reliable. Similar solutions were obtained with transformed or untransformed data using stepwise or backward elimination procedures, and the regression coefficients changed little from step to step during variable selection. In both solutions four variables were selected, but two of these variables accounted for most of the variability explained by the equations (72 & 80%). Hence both viabilities were influenced predominantly by the percentage of bacteria smaller than $0.2 \mu\text{m}^3$ and the soluble carbohydrate.

DISCUSSION

The main method of estimating viability used here, with microcolony counts and slide culture, proved satisfactory and straightforward. The method always gave viability estimates much larger than the macrocolony technique (V_c). This agrees with other research which has shown that microcolony counts on membrane filters (Jannasch & Jones, 1959; Straskrbova, 1973; Kunicka-Goldfinger & Stronkowska, 1977) and division inhibition techniques (Kogure *et al.*, 1979) are superior to the traditional plate count. Slide culture is likely to be better than these other techniques as it is easier to use than membrane filtration and should be more realistic than the inhibition method as no inhibitors are used. Our slide culture is also likely to be superior to that of Torrella & Morita (1981) as we use a thinner layer of agar, which allows a better phase contrast image and avoids excessive concentration of the inoculum which leads to overgrowth of small microcolonies by large ones. As in previous work (Fry & Zia, 1982) the current experiments found the optimum incubation time varied between sites, so a single incubation time still cannot be recommended.

The inclusion of the microcolonies present at zero time in the calculation of viability is problematical. Their exclusion will prevent overestimates of viability. However, their inclusion makes viability estimates by slide culture much easier as an initial count does not have to be made. The only waters which had microcolonies at 0 h were from polluted sites, containing large bacteria which are more likely to be viable (Tempest *et al.*, 1967; Hoppe, 1976). Also, counts of cell pairs in aquatic bacterial populations have been used to estimate growth rates (Brock, 1971; Hagstrom *et al.*, 1979; Newell & Christian, 1981). Thus, microcolonies present before incubation are likely to be viable and so our omission of the zero time microcolony count when estimating viability as V seems reasonable. In further work V could be estimated from the ratio of the microcolony count and the total count of microcolonies and single cells after incubation as this would avoid any observation before incubation. This is possible because on CPS microcolonies or single cells are not lost during incubation (Fry & Zia, 1982). Consequently, we recommend this for routinely estimating microcolony viability.

The close association between the population densities and chemical variables noted here agree with the popular belief that the size of bacterial populations depends on the fertility, and hence the carbon content, of their aquatic habitat (Wetzel, 1975). In particular, the relationships observed with BOD correspond very well with those between BOD, direct counts, colony counts and microcolony counts found for a similar range of waters by Straskrbova (1968). Consequently, the results presented here probably have general relevance to freshwater habitats. In particular, the strong relationship between the viabilities and both the percentage of small cells and the soluble carbohydrate, rather than the population estimates or other chemical variables, is probably generally applicable. It cannot be said from the evidence presented whether this relationship is causal. The soluble carbohydrate is probably related to the

concentration of available carbon in the water and hence causally related to viability. The proportion of small cells might be related to growth rate, as it is in pure cultures (Tempest *et al.*, 1967), which in turn could cause changes in viability.

Marine and estuarine bacteria are reported to be very small, with average volumes ranging from about 0.05 to 0.19 μm^3 (Hoppe, 1976; Bowden, 1977; Wilson & Stevenson, 1980). Freshwater bacteria are also small, averaging 0.015–0.24 μm^3 (Salonen, 1977; Krambeck *et al.*, 1981). The mean volumes reported here are considerably larger (0.32–1.0 μm^3). This discrepancy could in part result from differences in preparation procedures before size estimation or from our use of fairly nutrient-rich sites. It is possible that the smallest bacteria were lost during centrifugation as Torrella & Morita (1981) believe. However, we have observed bacteria as small as 0.02 μm^3 in our samples, so such losses cannot completely explain our large average sizes.

The strong link between cell size and viability, which we have demonstrated, concurs with suggestions from other workers that small aquatic bacteria are dormant (Stevenson, 1978) and are unculturable (Hoppe, 1976). Consequently, it might be only the large bacteria in our samples that formed microcolonies. This theory seems tenable from a comparison of the viabilities (Table 1) and the percentage of small cells (Table 2). However, it is probably an oversimplification as microcolonies of both small and large cells are observed with slide culture (Torrella & Morita, 1981; Fry & Zia, 1982). An alternative theory is that the natural aquatic bacteria have evolved to be resistant to starvation by becoming very small with a minimum maintenance requirement and turnover of cell components. They might coincidentally become very difficult to culture by, for example, becoming particularly susceptible to substrate-accelerated death (Postgate, 1967) or similar phenomena. Many reports support this theory. Examples are the discovery of small, starvation-resistant mutants of *Klebsiella aerogenes* (Harrison & Lawrence, 1963), the lysine-accelerated death observed in a marine isolate of *Alteromonas esperjiana*, which was also starvation resistant (Chan, 1977), and the ability of many small, marine bacteria to form colonies of large cells on rich media (Torrella & Morita, 1981).

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