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Viability and Isolation of Marine Bacteria by Dilution Culture: Theory, Procedures, and Initial Results

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Dilution culture, a method for growing the typical small bacteria from natural aquatic assemblages, has been developed. Each of 11 experimental trials of the technique was successful. Populations are measured, diluted to a small and known number of cells, inoculated into unamended sterilized seawater, and examined three times for the presence of 10^4 or more cells per ml over a 9-week interval. Mean viability for assemblage members is obtained from the frequency of growth, and many of the cultures produced are pure. Statistical formulations for determining viability and the frequency of pure culture production are derived. Formulations for associated errors are derived as well. Computer simulations of experiments agreed with computed values within the expected error, which verified the formulations. These led to strategies for optimizing viability determinations and pure culture production. Viabilities were usually between 2 and 60% and decreased with >5 mg of amino acids per liter as carbon. In view of difficulties in growing marine oligobacteria, these high values are noteworthy. Significant differences in population characteristics during growth, observed by high-resolution flow cytometry, suggested substantial population diversity. Growth of total populations as well as of cytometry-resolved subpopulations sometimes were truncated at levels of near 10^4 cells per ml, showing that viable cells could escape detection. Viability is therefore defined as the ability to grow to that population; true viabilities could be even higher. Doubling times, based on whole populations as well as individual subpopulations, were in the 1-day to 1-week range. Data were examined for changes in viability with dilution suggesting cell-cell interactions, but none could be confirmed. The frequency of pure culture production can be adjusted by inoculum size if the viability is known. These apparently pure cultures produced retained the size and apparent DNA-content characteristic of the bulk of the organisms in the parent seawater. Three cultures are now available, two of which have been carried for 3 years. The method is thus seen as a useful step for improving our understanding of typical aquatic organisms.

While marine bacteria often dominate planktonic biomass (16), culturability seldom exceeds 0.01% (12, 15, 22) and knowledge of their nutrition, genetics, and biochemistry is mostly restricted to large and easily culturable, but rare, forms. Most others, called oligobacteria (6), are apparently able to sequester organic compounds from very dilute ambient levels present at sufficient rates for growth. Inhibition by added nutrients (12, 32), cell size (9) that is below convenient instrumental detection, and resistance to growth on agar plates combine to divert attention from these numerous organisms to rare copeotrophic varieties. Yet probes of metabolic activity (11, 12) indicate activity among many of these small organisms. Isotope incorporation by filter-fractionated populations shows that activity is concentrated in the smallest cells (8), results recently corroborated by flow cytometer sorting of cells (unpublished data, this laboratory). Bacterivores which help regulate and recycle bacteria (14, 24) are easily excluded. However, bacteriophages, another group of organisms affecting bacterioplankton ecology (4, 29, 35), can be confused with ultramicrobacteria during microscopic observation (5, 26, 31) and may further complicate cultivation. Whether oligobacteria are unique (16, 30) or are inactive dwarfs (21, 25, 34) is debated, although extensive production of nonreproducing bodies seems unlikely.

Bacterial abundances in 0.2-μm-filtered seawater will return to original levels without nutrient supplementation (7, 23, 27), and metabolic activity toward radiolabeled substrates in sterilized seawater can be returned by inoculation and incubation of only a few individuals from the original population (2). Cultures produced by dilution of the original population to near extinction of the ability to grow are called dilution cultures. These appear to have advantages for studying oligobacteria because culture selection then favors the most abundant, rather than the most nutrient-tolerant, organisms. Theory is well developed for obtaining the most probable number of organisms in the original population from the frequency of growth in samples diluted to extinction (17). However, formulae for evaluating the viability of individual organisms from the frequency of culture development together with the number of cells inoculated were formerly unavailable. Since these cultures can give information about the environmental responses of typical aquatic organisms, we extended the theory associated with this technique. The purpose of this communication is to report development of the dilution culture technique with attention to statistical theory, viability, nutritional requirements, population dynamics, allelopathic interactions, and pure culture generation.

MATERIALS AND METHODS

Seawater samples. Samples were collected in acid-washed, thoroughly rinsed Niskin bottles or dipped from the surface with fired (550°C) glassware. Locations were near the center

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of Resurrection Bay off the Gulf of Alaska for deep samples and the Seward Marine Station pier for shallow samples from this fjord (5 by 20 km) and at the oyster grounds between England and Holland, 250 km north of Texel in the North Sea.

Dilution cultures. The growth medium was prepared by collecting seawater from the site to be examined, filtering it through fired Gelman A/E glass fiber filters, autoclaving it, and refiltering it except as noted. Screw-topped culture tubes (60 ml) were cleaned, closed with caps lined with acid-washed Teflon to reduce accumulation of organic compounds from the vapor phase, autoclaved, and aseptically filled with 50 ml of the preparation. A combination of amino acids such as casein hydrolysate was added to some. Portions of the seawater sample were preserved with 0.5% formaldehyde and stored cold for immediate analysis by epifluorescence microscopy (10, 19, 28) and for more-precise later evaluation by flow cytometry. The sample was then diluted with the unamended growth medium and dispensed into dilution tubes on site. Statistically, the calculated inoculum size ranged from a total of 0.1 to 10^3 organisms per tube with 10 to 30 tubes at each dilution. The tubes were maintained at 10°C, which was within 3°C of sample temperatures, and, in the dark, transported to a laboratory and incubated with gentle swirling on a rotary shaker and attention to minimizing gas-phase organic carbon in the surrounding air. Examination for developing populations was normally done three times between 3 and 8 weeks after inoculation. When evaluated by microscopy, tubes were scored positive after location of 100 cells. For a negative, detritus was located on the filter to confirm focus but not more than 1 cell per 20 fields was observed. Flow cytometry required populations of >0.05-μm^3-volume organisms at $>10^4$ cells per ml to be considered positive.

Flow cytometry. Procedures were as previously described (8, 30) and modified (9). DNA is reported as apparent DNA per cell because even Triton X-permeabilized cells of some bacterial species give less 4′,6-diamidino-2-phenylindole (DAPI) fluorescence than a single *Escherichia coli* chromosome and are not always consistent (unpublished data, this laboratory), so that DNA values read from standard curves based on pure DNA could underestimate the true value. Bacterial subpopulations were enumerated from isolated clusters in bivariate histograms and characterized by electronic scanning of gated distributions of forward scatter (cell size) and blue fluorescence (DNA).

Computations. Statistical computations utilized a VAX 8800 computer with GLIM software (33). A description of the simulation techniques and the FORTRAN source code for the Monte Carlo simulations are available on request.

RESULTS

Preliminary observations. When the bacteria of seawater were diluted to very small numbers with sterilized water from the same source, populations consistently developed in some of 40 to 120 trials in each of 11 experiments. Typical results are shown in Table 1. They suggest that many of the cells were viable, that the ability to generate populations of $>10^4$ cells per ml persisted down into the oceanic saline water underlying the fresher glacial-silt-laden water where many of the organisms should have had an open-ocean origin (18), and that viability on a per-cell basis decreased with inoculum size. Also, cultures sometimes appeared in microscopic observation to fragment, and ruptured cells and particles resembling the lipid-containing phage PM2, of the family *Corticoviridae*, of marine *Pseudomonas* organisms (13) were observed in electron micrographs.

TABLE 1. Typical results from dilution culture experiments[a]

Date, depth, and condition	Days until last observation	Avg no. of cells per tube (X)[b]	No. of replicates (n)	No. of tubes showing growth (z)
July 1989, 0 m, calm water	44	1	10	0
		5	9	0
		10	5	4
		100	5	3
September 1989, 30 m, light wind	45	5	10	0
		10	10	2
		100	9	4
October 1989, 10 m, full storm	66	5	10	10
		10	10	10
		100	10	10

[a] Samples collected from Resurrection Bay; see Materials and Methods.
[b] Excluding small fluorescent bodies which were taken to be viruses.

Statistical theory. To interpret bacterial growth in dilution cultures in terms of parent population characteristics, we derived a number of formulae. One describes the viability of individual organisms from the frequency of population appearance. Another establishes confidence limits as an aid to experimental design and to test for the possibility of allelopathic or synergistic influences among cultures. A third examines the likelihood that the cultures generated will be pure and thus suitable for examination of the species obtained.

Viability. For a given set of cells in an incubation vessel (tube), viability V can be defined as the ratio of the number of viable cells, as evaluated from the development of an observable culture, to the total number of cells originally present. The average number X of cells prior to incubation can be estimated from X_0, the cell population in the original sample and the dilution factor d. Tubes within a set are replicates of each other in that each contains an equal volume of the same inoculum and that each of the tubes is prepared, inoculated, incubated, and observed in the same way. Under these conditions, V can be assumed to be identical in all tubes. It can be estimated from dilution level d, the number z of observed positives among n replicates, and the average number of cells X prior to incubation. Complete nomenclature is given in Table 2.

Formulations. In a set of n tubes, tube i ($i = 1,. . .n$) can be inoculated with X_i cells, among which x_i may be viable. If $x_i > 0$, so that at least one viable cell remains, then a culture can develop during incubation. If the organism divides a sufficient number of times to produce enough growth for observation by the techniques chosen, then the tube is scored as a positive. Otherwise, $x_i = 0$ and the observation is negative.

The value X_i is not explicitly known and may vary from tube to tube. However, its expected value is given by dX_0. Because x_i is not observable, we estimate V from stochastic models. To do this, V is redefined as the probability that a randomly picked cell from a freshly inoculated tube is viable. This probability is assumed to be the same for all the tubes. Then, in a cell population X_i, x_i is binomially distributed with parameters X_i and V:

TABLE 2. Nomenclature

Symbol		Definition
Theoretical	Observable	
CV		Coefficient of variation (SD/estimate)
	D	Scaled deviance
	d	Dilution coefficient (vol of water sample/diluted vol)
E		Expectation operator
	k	No. of sets of replicates
M		Moment-generating function
	n	No. of replicate tubes within a set of inocula prepared with the same no. of cells
	p	Portion of tubes with observed growth, $p = z/n$
	P	P value, or observed level of significance, is the probability of observing a hypothetical D larger than the value observed. Large values of P (e.g., $P > 0.1$) suggest a good fit.
π		Probability of observing growth in a tube
π_1		Probability of observing growth in a tube starting from a single cell, i.e., a pure culture
Pr		Probability
r		Hypothesized no. of viable cells; dummy variable
u		Expected no. of tubes of pure culture from a set of n tubes; $u = n\pi_1$
	$\hat{u}$	Estimated no. of tubes of pure cultures
u_s		No. of tubes of pure culture observed by simulation
V		Viability, i.e., probability that a cell selected at random is viable
	$\hat{V}$	Estimated viability
$\hat{V}'$		Estimated viability based on dilution-independent hypothesis
V_0		Expected viability
	X_0	Original population
	X	Avg no. of cells per tube ($X = dX_0$) before incubation
X_i		Actual no. of cells in tube i before incubation
x_i		No. of viable cells inoculated into tube i
	z	No. of tubes with observed populations, or positives

$$\Pr(x_i = r \mid X_i) = \left(\frac{X_i}{r}\right) V^r (1 - V)^{X_i - r},\ r = 1, \ldots, X_i \tag{1}$$

Equation 1 with $r = 0$ gives the probability of observing an absence of growth (obtaining a negative), when tube i initially contains X_i cells:

$$\Pr(x_i = 0 \mid X_i) = (1 - V)^{X_i} \tag{2}$$

While this probability cannot be calculated directly because X_i is unknown, the average probability of obtaining a negative can be calculated as a weighted sum of the quantities in equation 2, where the weights are the probabilities that each of the values X_i can take. This average probability $\Pr(x_i = 0)$, sometimes called the unconditional probability, is given by the expected value of the right side of equation 2:

$$\Pr(x_i = 0) = E[(1 - V)^{X_i}] \tag{3}$$

If π is the probability of obtaining a positive and t is defined as $\ln(1 - V)$, then equation 3 becomes

$$1 - \pi = E[\exp(tX_i)] \tag{4}$$

The right side of equation 4 is the moment-generating function $M(t)$ of X_i and can be calculated from the distribution of X_i (20).

Assuming that X_i is Poisson distributed with mean X:

$$\Pr(X_i = k) = \frac{X^k e^{-X}}{k!},\ k = 0, 1, \ldots \tag{5}$$

where each cell has the same chance of being anywhere in the undiluted inoculum (taxis-oriented motility is negligible and the nutrient distribution is homogeneous), the Poisson hypothesis appears to be appropriate (20). The moment-generating function of X_i is

$$M(t) = E[\exp(tX_i)] = \exp\{X[\exp(t) - 1]\} \tag{6}$$

Combining equations 4 and 6 and the value of t gives

$$1 - \pi = \exp(-VX) \tag{7}$$

When π is estimated by the proportion of observed positives $p = z/n$, equation 7 suggests that V can be estimated by

$$\hat{V} = -\frac{\ln(1 - p)}{X} \tag{8}$$

For example, the sample inoculum taken in July 1989 (Table 1) and incubated in $n = 5$ replicate tubes with an average $X = 10$ initial cells per tube showed $z = 4$ tubes with growth. Application of equation 8 with $p = 4/5$ gives a viability of 16%.

More extensive data are plotted in Fig. 1. Viability was generally higher, between 2 and 100%. The December 1989 data, like those of Table 1, suggested a decrease in viability with inoculum size. Because of the difficulty in locating small populations by epifluorescence microscopy, sensitivity of the experiments to systematic error, and the fact that inoculated cultures sometimes fail to grow in microbiological experiments for unknown reasons, the experiment was performed also in August 1989 and March 1990 as shown. Results again seemed to indicate allelopathic or inhibitory interactions due to the negative slope and gave a viability of 2.7 to 13.8%, but statistical tests were required to examine this question. None of 10 tubes at each higher dilution, 100, 1,000, and 10,000 cells per tube, failed to grow. These data are not plotted because equation 8 is invalid for $p = 1$. This reflects a limitation of statistical theory; because p is an

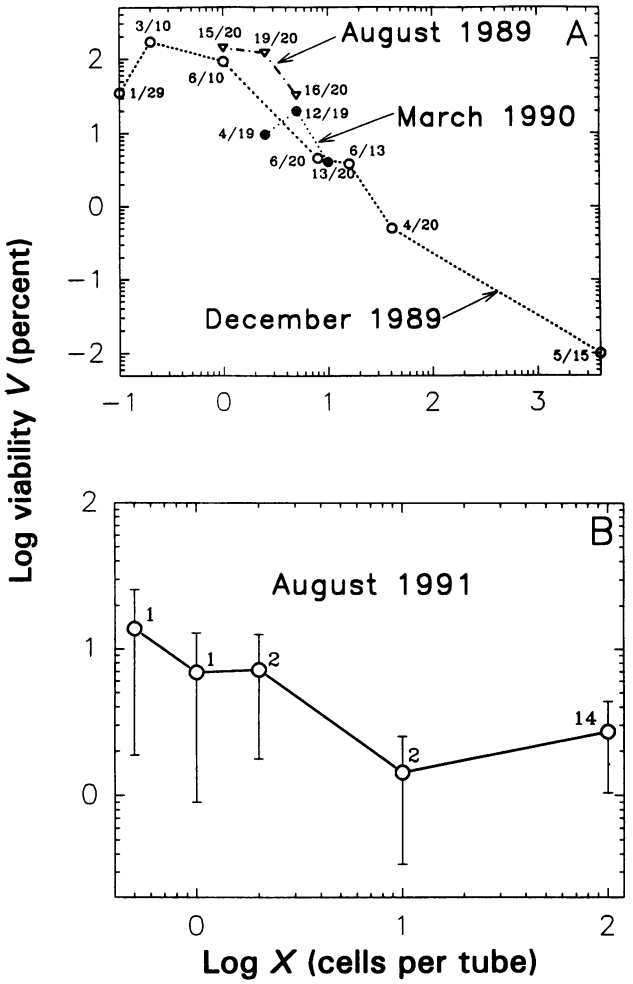

FIG. 1. (A) Viability with respect to inoculum size of dilution cultures collected from 10-m samples from Resurrection Bay, Alaska. Values were computed from equation 8. Dilution medium was filtered through 0.2-μm Gelman minicapsule filters, the second filtration was eliminated, and individual results, z/n, were evaluated by epifluorescence microscopy. (B) For this experiment only, the number of positives z is shown since the number of trials $n = 15$ in each case. Populations were located by flow cytometry. Error bars shown are values for which positive and negative legs were computed separately by using equation 9.

imprecise estimate of π, the possibility of all n cultures being positive implies non-null viability, requiring that the experimenter increase the dilution coefficient until some negative observations occur.

Error in viability. Quantitative measures of error are useful both for evaluating systematic trends in the data such as those shown in Fig. 1 and for choosing a midpoint for target dilutions to maximize precision. The estimate $\hat{V}$ was derived from a stochastic model and hence carries an error. The asymptotic standard error of $\hat{V}$, designated ASE($\hat{V}$), is a means to estimate the maximum magnitude of that error when the number n of tubes is large. Small values of ASE($\hat{V}$) suggest reliable estimates; indeed, V is within $\hat{V} \pm 1.96$ ASE($\hat{V}$) with 95% confidence. The following formulation was derived by the delta method (1):

$$\mathrm{ASE}(\hat{V}) = \frac{1}{X}\sqrt{\frac{p}{n(1-p)}} \tag{9}$$

If viability is expected to be near V_0, error associated with $\hat{V}$ can be minimized by choosing X as follows. Combining equations 7 and 9 gives

$$[\mathrm{ASE}(\hat{V})]^2 \approx \frac{1-\exp(-V_0X)}{nX^2\exp(-V_0X)} \tag{10}$$

The appropriate X now minimizes the right side of equation 10. Numerical methods yield $V_0X \approx 1.594$ and lead to a best choice for X:

$$\text{Optimal } X = \frac{1.6}{V_0} \tag{11}$$

Thus, if the expected viability is $V_0 = 10\%$, a dilution experiment for determining viability should contain a set of tubes each inoculated with 16 cells.

An alternative measure of the relative precision of $\hat{V}$ is the coefficient of variation CV($\hat{V}$) = ASE($\hat{V}$)/$\hat{V}$. Advantages are that it is dimensionless and, although small values of ASE($\hat{V}$) and CV($\hat{V}$) both suggest reliable estimates of V, CV($\hat{V}$) is preferred when V is close to zero. The value of the CV($\hat{V}$) can be shown from equations 8 and 9 to be never smaller than $1.24/\sqrt{n}$ and therefore can be decreased only by increasing n.

Table 3 demonstrates use of these formulations to analyze dilution culture data and gives estimated viabilities $\hat{V}$ along with their ASEs and CVs. It also shows the estimated number of tubes of pure cultures $\hat{u}$, with their own ASEs and CVs (see below). The CV of 0.36 for the August 1991 data (row 2) suggests that the viability estimate of 0.03 is statistically reliable because a CV for 15 tubes should not be smaller than $1.24/\sqrt{15} = 0.32$. Similarly, the viability estimates in rows 3 and 4 are reliable since a CV for 20 tubes cannot be smaller than 0.27. The ASE should not exceed half the viability value estimated for statistical reliability. Since row 1 shows a CV of 1.00, the value $\hat{V} = 0.14$ is unreliable. Row 5 shows a viability estimate exceeding 100%; since the CV is small, perhaps X was underestimated.

Pure culture production. A dilution culture will be genetically pure if inoculated with exactly one viable cell. The

TABLE 3. Some viability and pure culture calculations with experimental data

Expt	X	z	n	p	$\hat{V}$[a]	CV($\hat{V}$)	$\hat{u}$[a]	CV($\hat{u}$)
August 1991	0.5	1	15	0.07	0.14 (0.14)	1.00	0.97 (0.90)	0.93
August 1991	100	14	15	0.93	0.03 (0.01)	0.36	2.71 (1.65)	0.61
North Sea	6	11	20	0.55	0.13 (0.04)	0.31	7.19 (0.45)	0.06
August 1990	5	18	20	0.90	0.46 (0.13)	0.29	4.61 (1.75)	0.38
August 1990	1	15	20	0.75	1.39 (0.39)	0.28	6.93 (0.75)	0.19

[a] Numbers in parentheses are the ASEs.

probability $\pi_1 = \Pr(x_i = 1)$ of obtaining such a culture can be obtained from equation 1:

$$\Pr(x_i = 1 \mid X_i) = X_i V(1 - V)^{X_i - 1} \quad (12)$$

An argument similar to that giving equation 4 yields

$$\pi_1 = E[X_i V(1 - V)^{X_i - 1}] \quad (13)$$

giving

$$\pi_1 = \frac{V}{1 - V} E[X_i \exp(tX_i)] \quad (14)$$

Differentiation of equation 6 gives

$$\frac{d}{dt} M(t) = E[X_i \exp(tX_i)] = Xe^t \exp[X(e^t - 1)]$$

$$= X(1 - V) \exp(-VX) \quad (15)$$

Now combining equations 14 and 15:

$$\pi_1 = VX \exp(-VX) \quad (16)$$

which can be written as

$$\pi_1 = -(1 - \pi) \ln(1 - \pi) \quad (17)$$

An estimate p_1 of π_1 can be deduced from equation 17:

$$p_1 = -(1 - p) \ln(1 - p) \quad (18)$$

The expected number of pure culture tubes among the n replicates is $u = n\pi_1$. It is estimated from

$$\hat{u} = np_1 = -n(1 - p) \ln(1 - p) \quad (19)$$

Thus, u can be estimated simply from the number of tubes with growth among n trials.

It can be shown (1) that the ASE of $\hat{u}$ is

$$\mathrm{ASE}(\hat{u}) = |\ln(1 - p) + 1| \sqrt{np(1 - p)} \quad (20)$$

Simulations reported below suggest that equation 20 is valid for $n \geq 20$.

Computer simulations. These derivations were based on several assumptions: (i) the number of cells X_i in the replicate tubes is Poisson distributed; (ii) the mean number of cells X in each tube is known with good precision, and (iii) the number of replicate tubes n is large. Questions include whether the number of trials in the experiments (n = 10 to 30) was large enough for good viability estimates and whether the formulations developed to interpret the results are accurate.

Computer simulations were used to examine assumptions and verify the equations derived. To simulate typical results, values $n = 20$, $V = 0.2$, and $X = 9$ were chosen. According to equation 11, the choice $X = 9$ is near optimal. Integers $X_1, \ldots, X_n$ are then generated according to the Poisson law with the mean $X = 9$ to simulate the number of cells in each tube. For tube i, the integer x_i is generated according to the binomial law with parameters X_i and 0.2 to simulate the number of viable cells. Results are shown in Table 4.

The first two tubes contain no cells while the third contains 10 cells, 2 of which are viable. The fifth tube contains 7 cells, one of which is viable. Absent synergistic stimulation of the nonviable cells, the culture is pure but would appear to the experimenter only as another positive without further tests. There are $z = 15$ positives. The only data available to the experimenter are $n = 20$, $X = 9$, and $z = 15$. The observed ratio of positives is $p = z/n = 0.75$. The viability estimate is $\hat{V} = 0.154$ by equation 8, with an ASE of 0.043 by equation 9. The endpoints of a 95% confidence interval are given by $0.154 \pm 1.96 \times 0.043$, yielding [0.070, 0.238]. This interval spans the true viability of 0.2, and so the confidence intervals formulated are supported.

TABLE 4. Computer simulation of a dilution experiment

Tube no.	X_i	x_i
1	0	0
2	0	0
3	10	2
4	5	3
5	7	1
6	10	3
7	11	3
8	9	2
9	7	0
10	9	1
11	8	1
12	12	2
13	6	1
14	12	2
15	7	0
16	13	1
17	12	2
18	8	3
19	6	0
12	12	4

The expected number of pure cultures is $u = n\pi_1 = 5.95$, while the estimate from the simulation is, from equation 19, $\hat{u} = np_1 = 6.93$ with an ASE of 0.75 according to equation 20. The corresponding 95% interval estimate is [5.47, 8.40] tubes. The number of pure cultures in simulation, $u_s = 5$, varies significantly from the predicted value of 5.95 or its estimate of 6.93 and does not fall within the confidence limits. This discrepancy can be attributed to the small value of n; when it was increased to $n = 100$, the simulation results were $u = 29.75$, $\hat{u} = 32.77$, $u_s = 30$, and $28.30 \leq u \leq 37.25$ at 95% confidence. Thus, the formulations are corroborated by simulation, but they may be poor predictors for small sample sizes.

To examine the matter of sample size, experiments were simulated with various values of V, X, and n (Table 5). While rows 2 to 8 give good estimates of viability and the number of pure cultures, rows 9 to 12 underestimate the viability and overestimate the number of pure cultures. Results of rows 4 to 6 suggest that the best estimates of viability and the number of pure cultures are obtained when the true viability is near 0.10 and the sample size is $n = 30$ or larger. Results with $n = 20$ were acceptable although errors in viability were large. Results at a viability of 0.05 and $X = 1$ were misleading.

When the inoculum size is large, giving many positives and a value of p near 1, as in rows 10 to 12, the estimate of viability is poor. For example, row 11 shows that a quantity 10 cells per tube at a viability of 20% gives an estimate of only 15%.

Pooling data and cell-cell interactions. Statistical accuracy might be attained by combining data from several levels of dilution. Conversely, if allelopathic or other interactions transpire among the organisms present, the data from different dilutions should not be combined. However, these interactions can be identified.

TABLE 5. Some viability and pure culture calculations with simulated data

V	π	X	n	p	$\hat{V}$	ASE($\hat{V}$)	$\hat{u}$	ASE($\hat{u}$)	u_s
0.05	0.05	1	10	0.00	0.00		0.00		0
			20	0.05	0.05	0.05	0.97	0.92	1
			30	0.07	0.07	0.05	1.93	1.27	2
			100	0.03	0.03	0.02	2.95	1.65	3
0.10	0.63	10	10	0.60	0.09	0.05	3.67	0.13	4
			20	0.70	0.12	0.03	7.22	0.42	9
			30	0.67	0.11	0.03	10.99	0.25	11
			100	0.61	0.09	0.01	36.72	0.28	31
0.20	0.87	10	10	0.60	0.09	0.05	3.67	0.01	3
			20	0.75	0.14	0.04	6.93	0.75	5
			30	0.77	0.15	0.03	10.19	1.05	7
			100	0.81	0.17	0.02	31.35	2.59	23

Assuming that, for k sets of replicate experiments at different dilution levels $d_1,\ldots,d_k$ with experiment j having n_j replicate tubes, V does not depend on the level of dilution, the probability of growth in the jth experiment can be obtained from equation 7:

$$1 - \pi_j = \exp(-VX_j), j = 1, \ldots, k \quad (21)$$

which can be written as

$$\ln[-\ln(1 - \pi_j)] = \ln(X_j) + \ln(V), j = 1, \ldots, k \quad (22)$$

The parameter $\ln(V)$ satisfying equation 22 can be estimated by the maximum likelihood principle from experimental data (accomplished by use of the Newton-Raphson method of successive approximations together with GLIM software). This software provides a quantity D called scaled deviance, which can be used to test the hypothesis of constant viability. Values of D smaller than a value D_0 obtained from a chi-square table suggest that the hypothesis of constant viability should be accepted. We may also calculate a value P, which is the probability that a scaled deviance calculated from hypothetical data that one might encounter is larger than D which is calculated from actual data. In the present case, values of P larger than 0.05 indicate support for a hypothesis of constant viability at a 5% level of significance so that invoking explanations such as cell-cell interactions or systematic error is unnecessary. Smaller values indicate a trend in the data. P values are convenient since no further calculations or table consultations are necessary. Moreover, they provide a measure of the strength of evidence from the data either supporting or contradicting the hypothesis; the larger the P value, the stronger the evidence favoring constant viability with dilution.

Number of pure cultures. If an objective of dilution culture is to obtain populations which are pure and culture purity is not easily evaluated, it may be useful to maximize the ratio of pure cultures to the number of positives π_1/π. Equations 7 and 16 give

$$\frac{\pi_1}{\pi} = \frac{VX}{\exp(VX) - 1} \quad (23)$$

For example, inoculating $n = 1{,}000$ tubes with $X = 0.1$ cells of viability $V = 0.2$ will statistically yield $n\pi = 19.8$ cultures (equation 7), $u = 19.6$ of which are pure. This (19.8-19.6)/19.6 = 1% mixed-culture content would be large if synergistic influences increase viability and small if all bacteria from a given location are not genetically unique. A more conservative estimate of pure culture produced can be made by assuming a viability of 1 which allows for the presence of cells in a tube which shows growth that arise from those which may not attain detectable levels.

For economy of tubes, one may wish to maximize the number of pure cultures among a total of n tubes, i.e., to maximize π_1. This can be done if viability is known to be near a value V_0 by choosing $X = 1/V_0$. In fact, it can be established that π_1 of equation 17 attains its maximum value when $V_0X \approx 1$. Moreover, the value of the maximum is $e^{-1} \approx 0.368$; hence, the proportion of pure cultures to total tubes in a set can never exceed 0.368.

Effects of nutrient addition. Dilution culture medium usually contains only the native organics of seawater (3) as a carbon and energy source to avoid inhibition of the organisms by nutrient concentrations too high for the organisms to tolerate. Amino acid supplements were included in three 90-tube dilution experiments conducted in March, June, and August of 1990 using seawater from Resurrection Bay (Table 6). No change in viability was observed in response to the 0.01 to 1 mg/liter (as carbon) added; however, the large variance among the experiments raised questions about validity. Final populations did increase with supplementation; concentrations of 0.01, 0.1, and 1.0 mg/liter gave populations of 0.38 ± 0.14, 1.71 ± 0.78, and 4.12 ± 2.46 million cells per ml, respectively. When seawater from the North Sea was used, amino acid additions of 5 mg or more per liter inhibited growth. These data were internally consistent, as suggested by the small CVs (Table 3). Viabilities of 0.3% were obtained in other experiments (data not shown) with milligram-per-liter amino acid additions, which helps account for usual population estimates by plate count.

Population dynamics in dilution culture. To probe the population dynamics of dilution cultures in greater detail, we followed the development of each of 15 replicates of eight dilutions from the August 1991 Resurrection Bay sample (Fig. 1B) by high-resolution flow cytometry. Epifluorescence microscope counts of single and attached organisms gave a bacterial population of (1.7 ± 0.3) × 10^6 organisms per ml in each of five independent subsamples. Bacterial assemblages larger than two per group were not included in this count. Some bacteria were present in larger clumps as well. These usually contained fewer than five bacteria and were only (2.6 ± 1.2)% of the total. Another group of visible particles was red-fluorescing phytoplankton. They numbered a similar (0.05 ± 0.01) × 10^6 organisms per ml. The population of single bacteria and doublets was used to compute the likely

TABLE 6. Effect of amino acid additions on viability[a]

Location, depth, and date	Amino acids (mg of C/liter)	No. of cells (X)	No. of replicate tubes (n)	No. of positive tubes (z)	Viability $\hat{V}$
Resurrection Bay, 10 m, March 1990	0.00	2.5	8	2	0.12 (0.08)
	0.01	2.5	10	0	
	0.10	2.5	10	0	
	0.00	5.0	10	0	
	0.01	5.0	10	0	
	0.10	5.0	10	8	0.32 (0.13)
	0.00	10.0	8	2	0.03 (0.02)
	0.01	10.0	10	3	0.04 (0.02)
	0.10	10.0	10	4	0.05 (0.03)
Resurrection Bay, 10 m, June 1990	0.0	2.5	10	10	
	0.1	2.5	10	9	0.92 (0.38)
	1.0	2.5	10	10	
	0.0	5.0	10	10	
	0.1	5.0	10	4	0.10 (0.05)
	1.0	5.0	10	8	0.32 (0.13)
	0.0	10.0	10	8	0.16 (0.06)
	0.1	10.0	10	10	
	1.0	10.0	10	10	
Resurrection Bay, 10 m, August 1990	0.0	2.5	10	2	0.09 (0.06)
	0.1	2.5	10	5	0.28 (0.13)
	1.0	2.5	10	5	0.28 (0.13)
	0.0	5.0	10	7	0.24 (0.10)
	0.1	5.0	10	9	0.46 (0.19)
	1.0	5.0	10	10	
	0.0	10.0	10	6	0.09 (0.04)
	0.1	10.0	10	5	0.07 (0.03)
	1.0	10.0	10	8	0.16 (0.06)
North Sea, 10 m, February 1991	0	0.94	18	8	0.63 (0.22)
	0	1.88	20	11	0.42 (0.13)
	5	0.94	20	0	
	5	1.88	20	0	
	50	0.94	20	0	
	50	1.88	20	0	
	500	0.94	20	0	
	500	1.88	20	0	

[a] The ASEs are in parentheses. Note that $z = 0$ implies $\hat{V} = 0$ and that equation 9 is invalid when $n = z$.

level of inoculation of the dilution cultures. Subsequent analysis by flow cytometry gave the population as $(2.0 \pm 0.1) \times 10^6$, $n = 3$. Instrument settings excluded clumps and phytoplankton and agreed with the epifluorescence count given above, which indicated that the inoculum size was as close to that intended as the dilution process would allow. Complete growth curves from dilution tubes, normally scored only as positive or negative, are shown in Fig. 2. Controls gave a signal in only one of two subsamples (shown in panel A) from one of the 15 trials inoculated with sterile seawater, and that was only marginally above the detection limit of 0.1×10^5 cells per ml by flow cytometry. These data show that inoculation is required for population development; that difficulties from chemical, biological, or electronic contamination were negligible; and that scoring of false positives was unlikely. To decide whether mixing was adequate or whether organisms migrated and adhered to tube walls, escaping detection, we scraped down the sides of the tubes and reexamined the populations. This was done 11 months after inoculation. The seven cultures which were originally scored negative remained so. The four positives retained populations near the original values. However, these were difficult to evaluate by flow cytometry, and examination by microscopy showed that sheets of precipitate had formed. In other large-dilution cultures which were scored positive, homogeneous populations of cells appeared attached to the precipitate, amounting to populations of about 10^6 per ml. Growth curves for 21 of the 120 additional tubes are shown in panels B to H of Fig. 2. Growth rates computed (equation 8) from the number of viable cells inoculated together with early measurable populations were sometimes more than 1 day^{-1} as shown. Subsequently, at

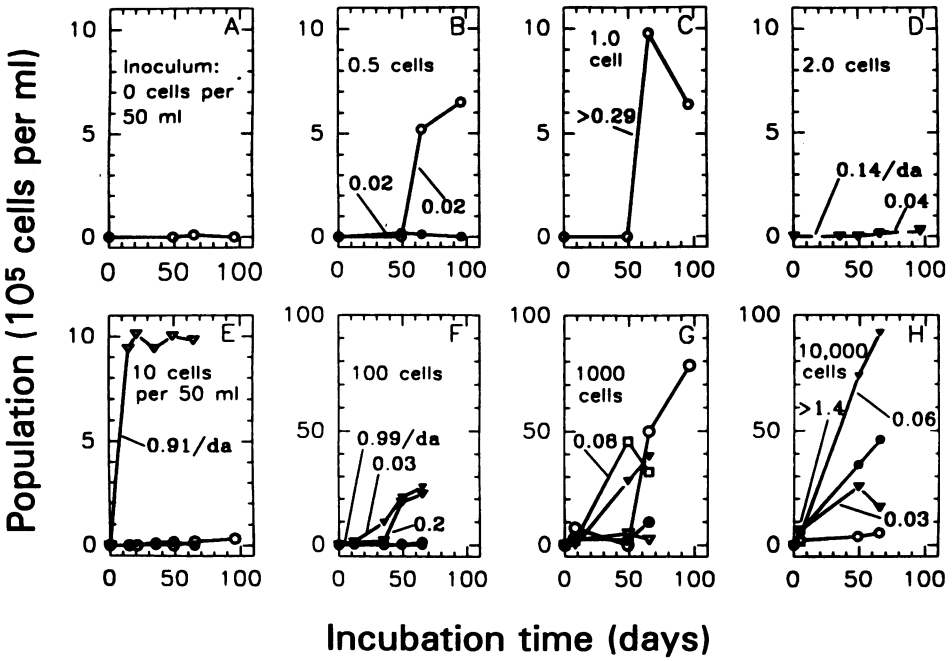

FIG. 2. Growth curves of bacterial populations in selected dilution culture tubes. The parent seawater was from Resurrection Bay, August 1991. The numbers of cells inoculated into the 50-ml incubations are shown. Growth rates of representative segments are indicated in units per day. For initial segments, the population was taken as VX or 1, whichever was larger, where V = 2.9%.

higher population values (10^4 to 10^7/ml), rates were usually 0.05 day^{-1} or lower. Rates obtained are likely to have been underestimated, considering the likely sigmoidal shape of the growth-curve segment used. These rates were generally higher with larger inocula for which the probability of selecting organisms capable of growing rapidly in the unamended seawater was increased. Curves were also more consistent at higher inoculum levels as shown in panel H, probably for the same reason, and total populations increased with inoculum size as well. Thus, rates at small inoculum size, which favor use of cultures of the most abundant organisms, were lowest. Doubling times of a month or more were often observed, and cultures sometimes took as long as 2 months to attain populations of 10^5/ml.

Developing populations exhibited substantial diversity. Discrete subpopulations, as compared with the parental seawater, could be resolved by flow cytometry based on cell size and apparent DNA content (Fig. 3). Both the characteristics of these bivariate histograms and the development of the subpopulations over time indicated this diversity. Thus, replots showing development of the populations within specific subpopulations were quite diverse as well, with some rising over time and others subsiding (Fig. 4). Like total population, some subpopulations did not appear for nearly 2 months. Again, development was more consistent at larger inoculum sizes as the probability of selecting organisms suited for extended growth in the media provided improved. As with the indications of population crashes noted above, declines in dilution culture population sometimes occurred (for example, Fig. 2C and 4D). These dynamics suggest the importance of recording multiple observations during culture development. Production of genetically pure cultures is favored at large dilutions because most bacteria appear microscopically to be separate and unattached to others and selection of cultures developing from single cells is favored. Successful production of pure cultures is suggested by bivariate histograms having two peaks lying at 45°. This reflects a binary distribution of size and DNA consistent with a growing culture with dividing cells, some having twice the size and DNA content of the others. Figure 3B shows such a culture in which the larger of the pair is indicated by contour-lobe direction. Retention of these characteristics is shown in Fig. 4A. Figure 3C shows a histogram from a growing isolate in which dividing doublets are distinct. This oligobacterium, designated RM 1, was obtained by dilution culture (2.5 cells per tube), repurified (1.0 cell per tube), and then subcultured in unamended sterilized seawater 13 times over nearly 2 years. DNA sequences used in phylogenetic studies confirmed the flow-cytometric evidence and statistical probability of culture purity.

The small size (0.002 to 0.1 μm^3) and apparent DNA content (1 to 8 fg/cell) characteristic of oligobacteria (Fig. 3A) were retained in both the dilution cultures from this experiment (Fig. 3B and D to F) and one carried from a similar experiment in 1989 (Fig. 3C). These characteristics are typical of organisms from near but slightly above the mean of the natural population (0.008 μm^3 and 2 fg of apparent DNA) rather than like those obtained by enrichment culture, which are often above scale even with the 3-decade span used.

Results of this dilution culture experiment were analyzed according to equations 8 and 9 and as shown in Fig. 1B and Table 7. The large P values obtained indicate that viability was independent of dilution. Column $\hat{V}$ shows dilution-dependent estimates of the viability V (equation 8) for comparison purposes.

The expected number of cultures that are pure is given in column $\hat{u}$. Because we underestimated viability (as well as the extent of cell-cell interactions), the number of pure cultures obtained was relatively small. For the 1991 data and assuming dilution-independent viability, the point estimate for viability $\hat{V}' = 0.03$ and the 95% interval estimate is 0.02

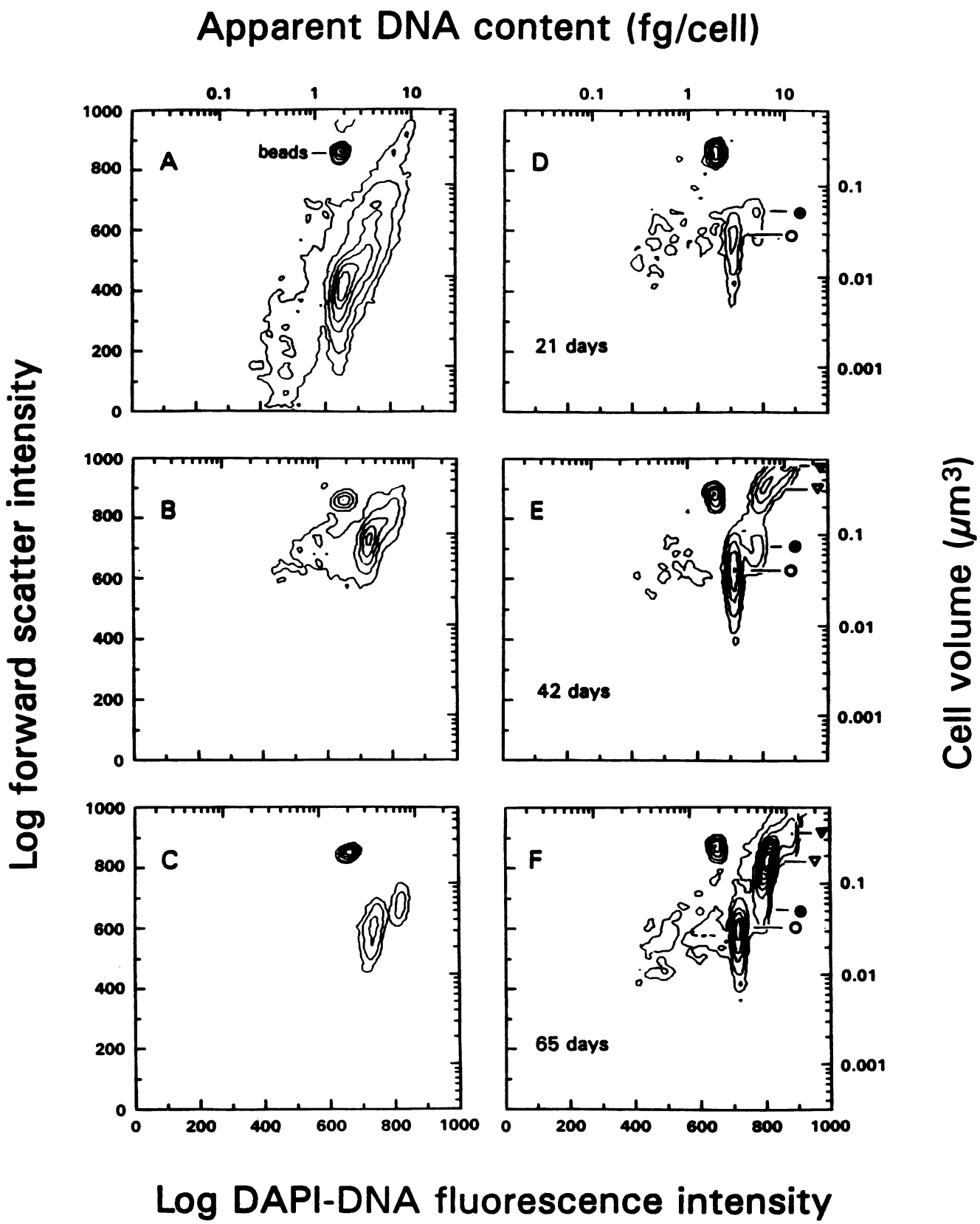

FIG. 3. Bivariate apparent DNA per cell versus cell volume histograms from dilution cultures. (A) Parent seawater. (B) Dilution culture inoculated with 10 cells from Fig. 2E (▽). (C) Two-year-old isolate RM 1. (D to F) Time course of population development of a dilution culture inoculated with $VX = 2.9$ cells from Fig. 2F (▽). Lengths of incubation are shown, and symbols identify subpopulations tracked in Fig. 4D.

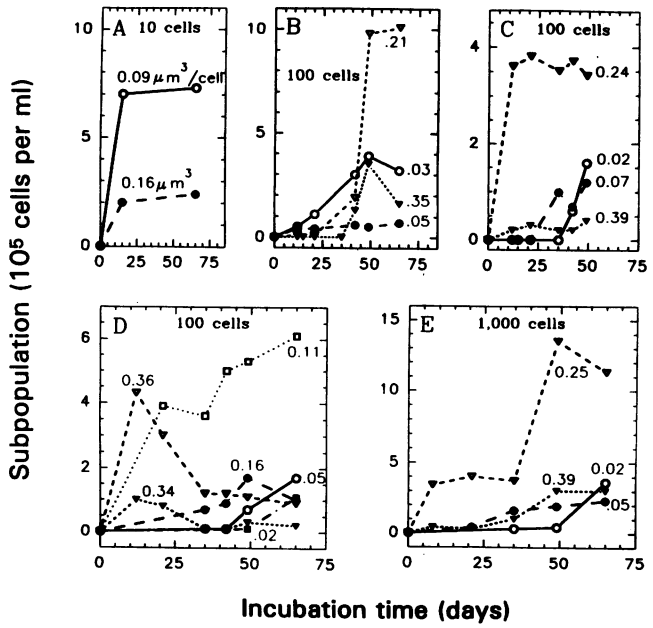

FIG. 4. Time course of individual subpopulation development. Inoculating population X was 10 to 1,000 cells into 50 ml. The subpopulations are identified by mean cell volume in cubic micrometers. The data source was histograms as shown above before contour smoothing: Fig. 3B for panel A, Fig. 3D to F for panel B, and others not shown for panels C to E.

$\leq V \leq 0.05$. The scaled deviance of $D = 4.67$ at 4 df gives $P = 0.32$ where P is calculated as the probability that a chi-square variable with 4 df exceeds 4.67. This P value, much larger than 0.05, suggests that a dilution-independent viability estimate is valid. (These data and conclusions were supported by a 120-tube experiment performed in August 1992.) Notice that rows 8, 14, and 15 are not used in the calculations because the software would not accept entries with $z = 0$ (all negatives) or $z = n$ (all positives) for mathematical reasons.

For the December 1989 data, the group estimate of viability gave $P = 0.00$; hence, the hypothesis of dilution-independent viability is untenable, i.e., absent systematic error, there is a trend in viability with dilution.

To further explore the 1991 data in terms of the trend suggested by Fig. 1B, we employed simulation. Resulting data, which are dilution independent by design, gave $\hat{V}' = 0.08$ and $0.06 \leq V \leq 0.11$ with $P = 0.39$, while the true viability is $V = 0.10$. The estimated total of pure culture tubes at all dilution levels is 18.18 tubes (sum of the last five entries of column $\hat{u}$), which compares favorably with the simulation result of 19 tubes and supports equation 13. While line-by-line estimates of viability vary, this analysis allows a conclusion of constant viability, and the apparent trend is attributed to chance.

DISCUSSION

The dilution culture technique reported has been attempted 11 times and was always successful. It is possible that most marine bacteria, defined as seawater particles between viruses and algae in size and containing femtogram quantities of DNA, are viable. Some of our results suggest that to be the case, although early data in which apparent viability exceeded 100% were probably due to imprecise total count data. Skill and hence confidence in viability estimates improved with time and gave values in the 2- to 60% range. This represents a major improvement in ability to propagate typical marine bacteria. Generation of populations with bias toward the most abundant, rather than the most culturable, organisms is noteworthy. That a large and statistically quantifiable number of cultures will be pure should allow a new focus on typical oligobacteria, environmentally dominant and important organisms which have escaped modern investigation. Production of such cultures rests on the assumption that most marine bacteria propagate independently rather than in associated groups. Microscopic evidence consistently supports this concept, particularly so in oligotrophic systems. Moreover, many cultures produced appear to be pure by flow cytometry. Because typical marine bacteria grow poorly on solid media, the method provides an attractive method of pure culture production.

The concept of viability acquires new dimensions. It is apparent that viability is an operational term which means ability of a single cell to attain a population discernible by the observer. One reason for low estimates of viability by traditional techniques is that most marine bacteria reach stationary phase before attaining visible turbidity. Epifluorescence microscopy and flow cytometry improve sensitivity about 3 orders of magnitude, but even then, populations sometimes are truncated near their sensitivity limit of 10^4 organisms per ml (Fig. 2), and growth rates clearly decrease in this region. Nutrient addition seems to stimulate higher populations for some, but the larger portion is inhibited by such additions (Table 6), and so refinement of this procedure is in order. Operationally, a viable organism is one that will, in the absence of cohorts, divide 13 or more times in unamended seawater. Sterile seawater is capable of supporting 10^6- to 10^7-cell-per-ml populations of many marine bacteria but not necessarily 10^4-cell-per-ml populations of all.

TABLE 7. The dilution-independent hypothesis; statistical evaluation of cell-cell interaction

Expt	X	z	n	$\hat{V}$	$\hat{u}$
Resurrection Bay, December 1989[a]	0.1	1	29	0.35	0.98
	0.2	3	10	1.78	2.50
	1.0	6	10	0.92	3.67
	8.0	6	20	0.04	4.99
	16.0	6	13	0.10	4.33
	41.0	4	20	0.01	3.57
	4,000.0	5	15	0.00	4.06
Resurrection Bay, August 1991[b]	0	0	15		
	0.5	1	15	0.14	0.97
	1	1	15	0.07	0.97
	2	2	15	0.07	1.86
	10	2	15	0.01	1.86
	100	14	15	0.03	2.71
	1,000	15	15		
	10,000	15	15		
Simulated data[c]	1	2	15	0.14	1.86
	2	1	15	0.04	0.97
	5	7	15	0.13	5.03
	10	10	15	0.11	5.49
	20	12	15	0.08	4.83

[a] $D = 73.20$, df = 5, $P = 0.00$.
[b] $D = 4.67$, df = 4, $P = 0.32$, $\hat{V}' = 0.03$, $0.02 \leq V \leq 0.05$.
[c] True viability: $V = 0.10$; $D = 5.23$; df = 5; $P = 0.39$; $\hat{V}' = 0.08$, $0.06 \leq V \leq 0.11$.

Oligobacterial nutrition now becomes a tractable investigation. With techniques to grow many or most organisms present and statistical tests for evaluating success, one can quantify subtle environmental effects. For example, the addition of small quantities of nutrients changes apparent viability, and allelopathic effects may elaborate a similar response. While our early results suggested such interaction, these could also have been due to an occasional recording of a small population as a negative or obtaining a false positive from fluorescence background. With the small populations sometimes generated, false negatives are possible with either evaluation technique used. In any case, later results in which populations were evaluated by flow cytometry were consistent with the hypothesis of no allelopathic interaction.

Optimal procedures for dilution culture-based examination of oligobacteria include use of unamended sterilized seawater as medium and recording developing populations at least three times over a 9-week interval. Evaluation of growth can be done by epifluorescence microscopy; however, less sensitive techniques are unacceptable and more sensitive techniques may be useful. Evaluation is much improved by flow cytometry because its ability to characterize a small population improves confidence in results so that both false positives and false negatives are less likely.

Theory now gives an analysis of viability with precision which exceeds our ability to detect it. The advantage is that it provides a theoretical framework for improved experimental investigations.

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