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Response of Marine Bacterioplankton to Differential Filtration and Confinement

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The bacterioplankton community of confined seawater at 25°C changed significantly within 16 h of collection. Confinement increased CFU, total cell number (by epifluorescence microscopy), and average cell volume of bacterioplankton and increased the turnover rate of amino acids in seawater sampled at Frying Pan Shoals, N.C. The bacterioplankton community was characterized by two components: differential doubling times during confinement shifted dominance from bacteria which were nonculturable to bacteria which were culturable on a complex nutrient medium. Culturable cells (especially those of the genera *Pseudomonas*, *Alcaligenes*, and *Acinetobacter*) increased from 0.08% of the total cell number in the seawater immediately after collection to 13% at 16 h and 41% at 32 h of confinement. Differential filtration before confinement indicated that particles passing through a 3.0-μm-, but retained by a 0.2-μm-, pore-size Nuclepore filter may be a major source of primary amines to the confined population. The 3.0-μm filtration increased growth rate and ultimate numbers of culturable cells through the removal of bacterial predators or the release of primary amines from cells damaged during filtration or both.

Many of the advances in our understanding of the dynamics of marine planktonic communities have resulted from the use of bottles to confine samples of water. Confinement terminates exchange of cells, nutrients, and metabolites with surrounding water and facilitates experimental manipulation, but results can be biased by what has long been referred to as the bottle effect.

The purpose of this study was to determine the nature and to quantify the extent of the response to confinement of natural communities of neritic marine bacterioplankton. We monitored changes in metabolic activity, total cell numbers, and the abundance of culturable species. We also determined the impact of partial removal from the water of particles ≥3.0 μm and ≥0.2 μm. The data demonstrated that large progressive changes occur within 16 and 32 h of confinement. The changes we observed are consistent with microbiological theory and must be considered in the design and interpretation of bottle experiments.

Early researchers in aquatic microbiology recognized the influence of the bottle effect on the number of culturable bacteria. Whipple (31) noted that when freshwater was stored in glass bottles, the number of culturable bacteria increased from 4-fold to >500-fold in 24 h. Stored seawater exhibited similar results; increases of more than three orders of magnitude were noted in some experiments (35, 37). Increases occurred in the light and in the dark at incubation temperatures ranging from 0 to 30°C and were most rapid in the dark and at higher temperatures (25). Potter (20) reported that the observed increases were inversely proportional to the size of the inoculum population. The apparent growth rate of culturable cells also increased more rapidly when the initial populations was reduced by filtration (28).

Many researchers have been critical of extrapolating data from bottles to the natural environment. In 1936, ZoBell and Anderson (36) questioned the use of controlled "test tube experiments" to predict bacterial dynamics in the ocean. Gunkel (7) noted that confinement of natural water interfered with quantitative analyses of the relationship between bacterial dynamics and organic substrate concentrations and stated, "it is still not possible to design experiments which would allow strict comparisons between results obtained in the laboratory and in the sea" (p. 1535).

Marine plankton ecologists generally have chosen either to minimize or ignore rather than attempt to measure the bias of the bottle effect. Large volumes (>10^3 liters) of confined water minimize the bottle effect by reducing the surface-to-volume ratio (1, 16, 17). Short-term bottle experiments of less than a few hours' duration minimize bias because the brief period of incubation precludes a significant bottle effect. For a number of reasons, however, plankton ecologists often have chosen to conduct bottle experiments with volumes of less than several liters and for incubation periods of more than several hours. Large-volume experiments generally are not feasible on oceanographic cruises. Longer-term incubations can reduce bias due to diurnal effects and circadian periodicities (24) and can overcome analytical detection limitations (32), but can also result in shifts in the relative abundance of species (1, 27). Metabolic rates of bacteria have been assumed to be constant over incubation periods of up to 24 h for rate computations (23) with the assumptions that nutrient partitioning, metabolic rates, and species shifts in the bottle are negligible. These assumptions, if violated, could result in estimates of metabolic rates that are not relevant to the natural environment.

MATERIALS AND METHODS

Surface water was collected from the upcurrent side of the Coast Guard light tower at Frying Pan Shoals, N.C. The light tower is ca. 150-km seaward of Cape Fear, N.C. (water depth, 10 m). We used a plastic bucket and a 20-liter

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polypropylene carboy to collect the water. The apparatus and bottles were cleaned and sequentially rinsed with 0.1% nitric acid, distilled water, and seawater before their use. Three bottle experiments were initiated at dawn on day 1 and at noon and midnight on the next day. Three 4-liter autoclaved polypropylene bottles were used in each experiment. Each bottle received a total of 3 liters of water. Water was filtered either through 3.0-μm-pore-size Nuclepore filters or through both Gelman A-E glass fiber and 0.2-μm-pore-size Nuclepore filters at a vacuum of ≤10 cm of mercury or was not filtered. Experimental treatments were: (i) 100% unfiltered water, (ii) 90% 0.2-μm-filtered water plus 10% unfiltered water, and (iii) 90% 3.0-μm-filtered water plus 10% unfiltered water. Bottles were incubated in a water bath directly under the tower and never received direct sunlight. Water and air temperatures were 25 ± 2°C during water sampling and incubation. Subsamples were taken (sterile pipetting or decanting) at 0, 16 to 18, and 30 to 34 h after filtration. Additional samples were collected during the first 16 h for direct microscopic counts of bacteria.

The acridine orange direct count technique (8) was used to determine the total number of bacterial cells and to categorize the cells according to size and shape. Triplicate subsamples were preserved in formaldehyde at a final concentration of 1.9% (wt/wt) and held at 0 to 5°C for 1 week before counting upon our return to shore. One filter was prepared and counted with 1 to 10 ml of water from each subsample. A total of 10 or 20 fields of view were counted, depending on the number of cells per field, so that ≈200 cells were counted for each subsample. The proportion of cells in five size and shape categories was determined, and mean volumes for the categories were used to estimate the average and total cell volumes of the bacteria.

The turnover rate of amino acids was measured with a mixture of tritium-labeled amino acids (NET 250; New England Nuclear Corp.) which approximates the amino acid composition of algal protein hydrolysate. Turnover rate was measured in 30-ml subsamples with three experimental and one control preparation for each experiment, treatment, and time combination. The final added concentration of amino acid mixture was 1 nmol · liter^{-1}. The turnover rate of the substrate is equal to the fraction of the added label that is taken up divided by the time of incubation (0.5 to 1.0 h). We also measured the concentration of dissolved primary amines (DPA) which includes dissolved free amino acids. DPA was measured with a Turner fluorometer and the reagent fluorescamine (18).

Standard spread-plate procedures were used to enumerate culturable bacteria (3). Each incubation bottle was mixed by shaking, and serial decimal dilutions were made in sterile salt solution (12). Volumes (0.1 ml) were then surface inoculated onto plates containing 15 ml of Marine Agar 2216 (Difco Laboratories) adjusted to 2% agar with Bacto-Agar (Difco) and spread uniformly. Each dilution was plated in triplicate. The plates were incubated at 25°C for 10 to 14 days, and the number of CFU was counted differentially by the morphology of the colonies. For calculations, each colony was assumed to originate from a single cell; few clumps of cells or colonies of cells on particles were detected by direct microscopy. Inocula were taken from the spread plates, and isolates were maintained on Marine Agar 2216 slants and in Marine Broth 2216 (Difco). Cultures (18 to 48 h old) of each isolate were Gram stained and were examined for motility with the hanging-drop slide method. Gram-negative rods were identified as to genus with the API 20E strip and the API profile recognition system (Analytab Products, Inc., Plainview, N.Y.). Because this procedure requires cell growth at 37°C, the ability of the isolates to grow at 37°C was confirmed with Marine Agar 2216 slants in an incubator. Statistical analyses were conducted on an IBM 360 computer with the SAS software package (2).

RESULTS

Time. The time of confinement, but not treatment or experiment, had a consistent effect on the bacterial assemblages. Multiple analysis of the variance of total cell volume, amino acid turnover rate, and CFU at each of the three observation times in the three experiments indicated no consistent experiment or treatment effect over time (multiple analysis of variance, $P < 0.05$ level). Averaged over treatment and experiment, CFU, turnover rate of amino acids, total number of cells, and average cell volume increased during confinement (Table 1). Large increases occurred by 16 to 18 h, particularly in CFU and amino acid turnover rate. The ratio of CFU to total cell numbers (± standard error, $n = 9$) increased from 0.08 ± 0.02% at time zero to 12.6 ± 6.2% at 16 to 18 h to 40.7 ± 11.5% at 30 to 34 h. The frequency distribution of cell sizes in the direct microscopic counts also changed dramatically during confinement (Table 2). Initial water samples were dominated by cells less than 0.6 μm in the major dimension. Subsequent to confinement the relative frequency of the smallest cells decreased from 80 to 50%, whereas that of all of the larger cell categories increased. Rods 1.2 to 1.8 μm in the major dimension had the largest relative increase. Rods greater than 1.8 μm in length increased but comprised only 1.6% of the cell numbers at the end of the incubations.

Both non-colony-forming cells and small cells increased in absolute abundance during confinement but did so much more slowly than CFU. During the initial 16 h of confinement, apparent doubling times were: CFU, 2.1 h; nonculturable cells (i.e., total cell number minus CFU), 53 h; and cells <0.6 μm, 75 h. From 16 to 32 h, however, nonculturable cells decreased, whereas CFU and cells <0.6 μm continued to increase but at a reduced rate.

The species composition of culturable bacteria changed during confinement. Nineteen colony types on the spread plates were counted and isolated. Eighteen of the isolates were gram-negative rods, 14 of which were identified as to genus (Table 3). Ten of the isolates were of the genus *Pseudomonas*. One isolate was an unidentified yeast. *Pseudomonas* sp. strain 1 (84%) and *Acinetobacter* sp. (12%) accounted for most of the total CFU in the initial water samples. The former species always was the most abundant type, but its contribution to the total CFU decreased to 53 and 55% in the 16- and 32-h observations, respectively. The latter species also became relatively less abundant during confinement and accounted for <1% of the total CFU observed after 16 and 32 h. Those species whose relative abundance increased during confinement were *Alcaligenes*

TABLE 1. Means over three treatments and three experiments ($n = 9$) of abundance, activity (amino acid turnover rate per day), and cell volume of confined bacteria

Time (h)	Dependent variable			
	CFU ($10^5 \cdot ml^{-1}$)	Amino acid turnover rate (d^{-1})	Total cell no. ($10^5 \cdot ml^{-1}$)	Avg cell vol (μm^3)
0	0.0043	0.038	5.7	0.053
16–18	0.91	2.9	7.9	0.072
30–34	5.4	6.7	12	0.10

TABLE 2. Mean and standard error of mean averaged over three experiments and three treatments (n = 9) of percentage of cells in size and shape categories at three observation times and average cell volume for each category

Category	% Cells (mean ± SE) at:			Vol ($\mu m^3 \cdot cell^{-1}$)
	0 h	16–18 h	30–34 h	
Rods (μm)				
<0.6	80.0 ± 2.7	70.9 ± 8.9	50.6 ± 5.9	0.033[a]
0.6–1.2	16.9 ± 2.9	20.9 ± 7.1	31.3 ± 3.7	0.11[b]
1.2–1.8	0.4 ± 0.2	4.8 ± 1.9	13.7 ± 2.8	0.25[b]
1.8–3.0	0.3 ± 0.2	1.3 ± 0.6	1.6 ± 0.7	0.57[b]
Cocci (0.6–1.2 μm)	1.6 ± 0.4	2.0 ± 0.5	3.4 ± 0.9	0.32[b]

[a] Volume calculated assuming volume equivalent to spherical cell 0.4 μm in diameter.

[b] Geometric mean of the calculated volumes of the smallest and largest cells in each category. Volume of rods was calculated assuming columnar cells. Cell width was estimated with a linear regression (width = 0.101 length + 0.323; r = 0.923; n = 5) of SEM data (34).

sp., *Pseudomonas* sp. strain 2, *Flavobacterium* sp., and *Vibrio* sp. All of these species increased in absolute abundance during 0 to 16 h and then continued to increase at a reduced rate through 32 h. Apparent doubling times for the culturable species during the first 16 h of confinement ranged from 1.1 h for *Alcaligenes* sp. to 3.4 h for *Acinetobacter* sp., whereas that for *Pseudomonas* sp. strain 1 was 2.2 h. Shifts in the relative abundance of the culturable bacteria, therefore, may have resulted from differential doubling times, all of which were measured in terms of a few hours or less. At all times, two species, *Pseudomonas* sp. strain 1 plus either *Acinetobacter* sp. or *Alcaligenes* sp., comprised 90% or more of the total CFU. The unidentified yeast isolate was not detected in the water incubated for 32 h.

Treatment. Although treatment effects were not consistent through time, significant treatment effects and patterns occurred in the data at time zero and for the dependent variable DPA at all observation times. Total number of cells, turnover rate of amino acids, and CFU were affected by experimental treatment at time zero (Table 4). The treatment effects for the total number of cells and CFU were statistically significant (analysis of variance, $P < 0.05$ level). The 0.2-μm-pore-size filtration treatment significantly reduced the total number of cells, CFU, and amino acid turnover rates by approximately an order of magnitude (t test, $P < 0.05$ level). The 3.0-μm filtration did not affect cell abundance, CFU, or amino acid turnover rate at time zero but did significantly (t test, $P < 0.05$) increase DPA (Table 5). At 16 to 18 h, the DPA concentrations were highest, and there were no significant differences among treatments. At 30 to 34 h, the concentration of DPA decreased in all treatments, and the concentration in the 0.2-μm filtration-treated water was significantly (t test, $P < 0.05$) lower than the concentrations in the unfiltered and 3.0-μm filtration-treated water.

TABLE 3. Differential plate count abundance and species of isolates obtained from incubation experiments

Relative abundance (%) at:		Species
0 h	32 h	
84.4	54.7	*Pseudomonas* sp. strain 1
10.8	0.8	*Acinetobacter* sp.
0.8	ND[a]	Unidentified yeast
0.6	2.7	*Pseudomonas* sp. strain 2
0.4	37.2	*Alcaligenes* sp.
ND	1.5	*Flavobacterium* sp.
ND	1.4	*Vibrio* sp.
3.0[b]	1.4	Eight pseudomonads and four unidentified bacteria

[a] ND, Not detected.

[b] These 12 isolates never individually accounted for more than 1% of the total CFU in any sample.

TABLE 4. Means and standard error (n = 3) for dependent variables as a function of treatment at time zero[a]

Filtration treatment[b]	Dependent variable					
	Total cells ($10^5 \cdot ml^{-1}$)		Amino acid turnover rate (d^{-1})[b]		CFU (colonies · ml^{-1})	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Unfiltered	7.34	1.13	0.070	0.023	578	60
0.2 μm[c]	1.04	0.08	0.003	0.002	62	5
3.0 μm	8.86	0.91	0.043	0.036	638	12
F test	Significant[c]		Not significant[c]		Significant[c]	

[a] Analysis of variance of F test for treatment effect, $P < 0.05$.

[b] See text.

[c] The approximate order of magnitude reduction in the 0.2-μm filtration compared with the unfiltered treatment was significant (t test, $P < 0.05$) for all three dependent variables.

Experiment. Within each of the three experiments, large increases with time were observed in CFU, the incorporation of amino acids, and the total cell number, but treatments that were significant at time zero were not significant at 16 and 32 h (analysis of variance, $P > 0.05$ level), owing to variability among experiments. In general, the most rapid increases and highest levels of CFU occurred in the filtered water (Fig. 1). CFU in the 3.0-μm filtration treatment and the unfiltered control increased from less than 700 CFU · ml^{-1} to greater than 10^5 CFU · ml^{-1}. In all three experiments, however, CFU in the 3.0-μm filtration treatment increased more rapidly and achieved higher densities than in the unfiltered control. The 0.2-μm filtration treatment started at less than 100 CFU · ml^{-1} and increased two orders of magnitude in experiment A, over three orders of magnitude in experiment B, and over four orders of magnitude in experiment C. In two of the experiments (B and C), the 0.2-μm filtration treatment achieved CFU levels observed in the 3.0-μm filtration treatment, and the final CFU exceeded the initial total cell number (Fig. 1a and b), suggesting substantial growth of culturable cells during confinement. The amino acid turnover rate closely paralleled the observed CFU in all three experiments (Fig. 1). Initially, in two of the three experiments, the turnover rate was lower and then increased more rapidly in the 3.0-μm filtration treatment than in the unfiltered control. The rate of increase

TABLE 5. Concentration of DPA ± standard error (n = 3) in different treatments through time and averaged over the three experiments

Time (h)	Concn (μmol · $liter^{-1}$)		
	Control	0.2-μm filtration	3.0-μm filtration
0	0.90 ± 0.21	0.98 ± 0.30	1.58 ± 0.12
16–18	3.04 ± 0.48	2.67 ± 0.07	2.42 ± 0.38
30–34	1.92 ± 0.09	0.93 ± 0.15	2.10 ± 0.50

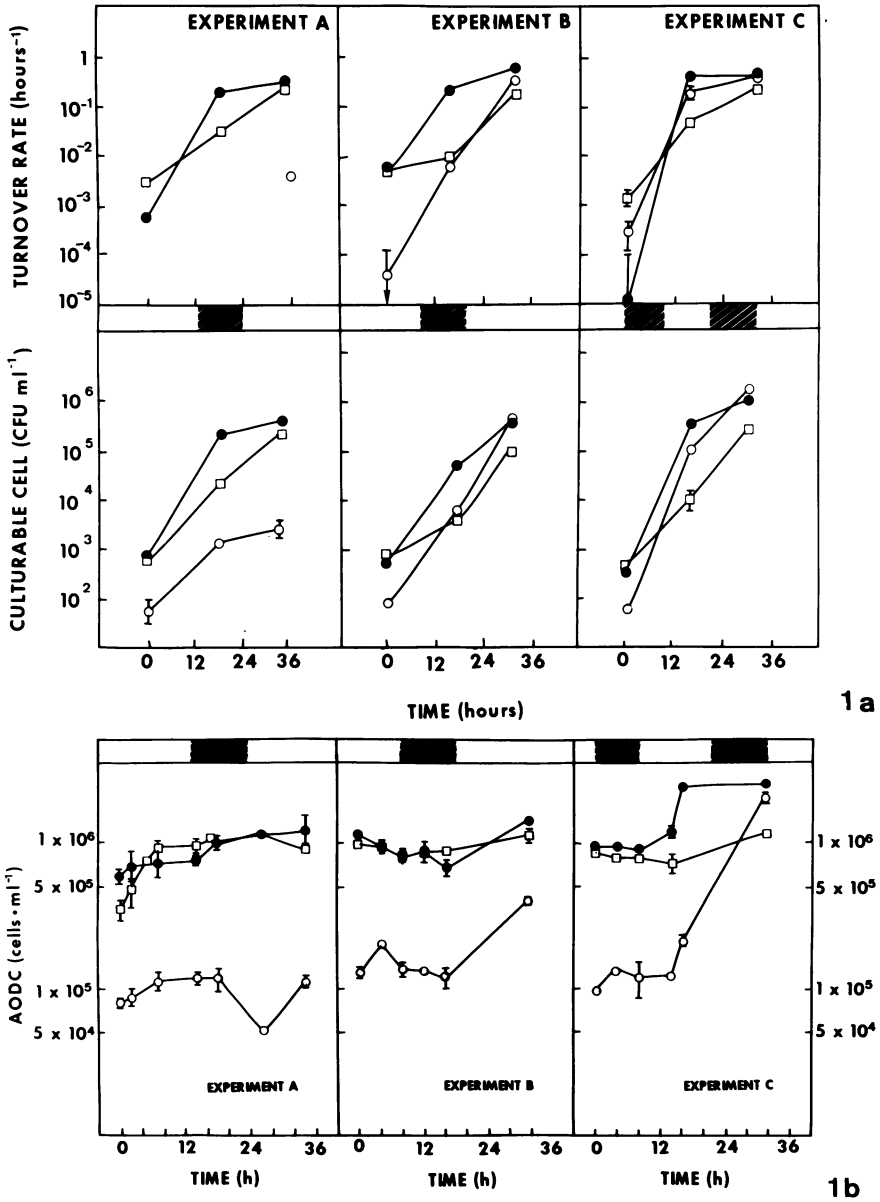

FIG. 1. Effect of duration of confinement and treatment in three experiments on amino acid turnover rate and CFU (mean ± standard error) (a) or acridine orange direct count (AODC) (mean ± standard error) (b). Symbols: □, unfiltered; ○, 0.2-μm filtered; ●, 3.0-μm filtered. The shaded areas indicate periods of darkness. Error bars smaller than the symbol used to denote the mean are omitted.

slowed from 16 to 32 h, but rates remained higher in the 3.0-μm filtration treatment than in the control. The amino acid turnover rate for the 0.2-μm filtration treatment generally was lowest at time zero and then either remained relatively low (experiment A) or increased rapidly to exceed that rate in the control (experiments B and C).

Total cell numbers generally increased through time (Table 1), but the increases occurred, for the most part, during daylight hours (Fig. 1b). The largest increases in total cell number were observed in the 0.2-μm filtration treatment from the 16- to 32-h observations in experiments B and C and in the 3.0-μm filtration treatment from the 0- to 16-h observation in experiment C. These increases in total cell numbers also corresponded to the largest increases in CFU (Fig. 1). In the initial 12 h of confinement, total cell numbers consistently increased only in experiment A; that experiment was initiated approximately at sunrise.

DISCUSSION

The data confirmed that nonculturable bacteria were abundant in the seawater and comprised >99.9% of the natural bacterioplankton community in the seawater tested. During confinement, however, the bacteria which were culturable on Marine Agar 2216 increased to 41% of the total cells present at 30 to 34 h. The two bacterial types defined here as culturable and nonculturable on Marine Agar 2216 are equivalent to the two types of bacteria distinguished by Torrella and Morita (26): (i) those which increase in cell size and rate of growth upon nutrient enrichment and quickly

colonize nutrient (e.g., Lib-X, 2216, etc.) agar as a result of rapid growth rate; and (ii) those which are smaller, do not increase in cell size or rate of growth upon nutrient enrichment, and form ultramicrocolonies not visible to the naked eye. Thus, the dramatic shift in dominance from nonculturable to culturable bacteria occurred as a result of differential net growth rates of the bacteria when confined. Nonculturable bacteria increased with an apparent net doubling time of 53 h, whereas culturable bacteria increased with an apparent net doubling time of 2.2 h.

Of the increase in total bacterial cell numbers subsequent to confinement, 41% at 16 to 18 h and 86% at 30 to 34 h was due to bacteria which grew rapidly at nutrient (DPA) concentrations only somewhat higher than those that originally occurred in the water. The dilute organic matter present in seawater, however, invariably supports the extensive growth of bacteria which are culturable on nutrient-rich media subsequent to confinement (28, 29, 36). Organic matter in seawater is in a state of dynamic equilibrium between formation and decomposition, and this equilibrium is disrupted as a result of confinement (11, 28).

It is unlikely that the rapid doubling rates of culturable bacteria observed in the confined seawater are representative of normal rates in the sea since culturable bacteria may not grow at the low in situ nutrient levels characteristic of seawater (11, 15). ZoBell (35) hypothesized that rapid growth of the culturable bacteria during confinement occurs in microhabitats near the container wall which are enriched by adsorption of nutrients from the adjacent water. The observation that the average cell volume increased during confinement rather than decreased, as starved cell volumes may do (19), also supports the enrichment hypothesis. Some of the increase in average cell size can be attributed to the enlargement of culturable cells upon nutrient enrichment (19). The observation that the smallest cells also were increasing in number indicates, however, that a large part of the increase in volume per cell is due to the shift in dominance to culturable cells that generally are larger than the nonculturable cells. Growth of culturable bacteria in unpolluted seawater probably occurs in enriched microhabitats, including surface microlayers (22) and water adjacent to solid surfaces and particles (30). Although in oligotrophic areas incorporation of organic matter is mainly due to nonculturable bacteria (9, 10), in eutrophic areas culturable bacteria play a more important role (21). This is due to the increased nutrient concentrations and the higher abundance of particles in eutrophic, relative to oligotrophic, waters. Culturable cells account for 0.1 to 3% of the total cell numbers in unpolluted estuarine water (E. N. Buckley III, Ph.D. thesis, University of North Carolina, Chapel Hill, 1980; Ferguson and Palumbo, unpublished data). That culturable cells increased most rapidly and achieved highest numbers in the 3.0-μm filtration treatment may be due to two factors: removal of bacterial predators larger than 3.0 μm from the filtered water and increased DPA, presumably due to cells damaged during filtration (this would increase the nutrients available to the confined bacterial community).

When compared with the change in dominance from nonculturable to culturable bacteria, changes in the species composition of culturable bacteria during confinement appear to be relatively minor. All of the culturable bacteria detected initially in the water also were present at 16 to 18 h and 30 to 34 h. *Vibrio* sp. and *Flavobacterium* sp. were the only bacteria not observed initially which were subsequently detected in the bottles. Most of the isolates were pseudomonads and were physiologically quite similar. Even those culturable species which decreased in relative abundance were dividing very rapidly compared with the nonculturable bacteria. Thus, changes in the dominance of physiological types and species composition resulted from differential net growth rates under the condition of confinement and may reflect differential intrinsic maxima as well as differences in growth response to conditions in confined seawater. Low intrinsic growth maxima of the nonculturable bacteria may explain the shift in dominance to culturable bacteria in confined seawater and enriched habitats.

DPA was increased ca. 76% by the filtration of sample water through a 3.0-μm-pore-size Nuclepore filter, which may explain why the most rapid growth and highest ultimate concentrations of CFU occurred in the 3.0-μm filtration treatment. The most likely sources of this DPA were cells damaged during filtration. Averaged over treatment and experiments, DPA ± standard error increased from 1.15 ± 0.13 μmol · liter^{-1} at time zero to 2.71 ± 0.22 μmol · liter^{-1} at 16 h, even though the bacteria were growing rapidly at this time and were consuming amino acids. Dissolved free amino acids were being incorporated at a rate of ca. 0.16% · h^{-1} at time zero, 6.0% · h^{-1} at 16 to 18 h, and 28% · h^{-1} at 30 to 34 h. Although, as discussed above, dissolved free amino acids do not comprise the entire DPA pool, it is not surprising that DPA decreased to 1.65 ± 0.24 μmol · liter^{-1} at 30 to 34 h.

Why did DPA increase and then decrease during confinement? The potential sources for DPA in the confined water are dissolved and colloidal macromolecules, particles containing combined primary amines, and living cells containing combined and free primary amines. Combined primary amines are subject to hydrolysis by exoenzymes excreted by bacteria (5). Phytoplankton release de novo primary amines while living (32) and subsequent to death or predation (14). The increase in DPA in the 3.0-μm filtration treatment relative to the control suggests that amines were released from phytoplankton which may have been damaged during filtration (Table 5). Water that had been filtered through a 0.2-μm-pore-size filter did not indicate DPA enrichment relative to the unfiltered control at time zero. In all incubations including controls, DPA increased from 0 to 16 to 18 h. Thus, one effect of confinement is an increase in DPA. Subsequently, DPA decreased, particularly in the 0.2-μm filtration treatment. A major source of primary amines to the confined population of bacteria, therefore, appears to be particles which are retained by a 0.2-μm-pore-size filter but which pass through a 3.0-μm-pore-size Nuclepore filter. The data also confirmed that the dilute dissolved and fine particulate organic matter in oligotrophic seawater can support extensive growth of bacteria under suitable conditions. These conditions include confinement in a bottle.

We recommend caution in the interpretation of measured increases in bacterial abundance by direct cell counts of confined communities. Such counts do not differentiate between the nonculturable bacteria which are dominant in unpolluted natural water and the culturable bacteria which appear to be stimulated by confinement. In recent reports of bacterial productivity, the number of CFU either was not monitored (6, 13) or was not measured within 16 h (33). Marine Agar 2216 generally gives higher CFU than other enriched agar media (4), and our results are not directly comparable with the earlier reports (33). Two independent techniques have been used to estimate in situ productivity of bacteria: short-term (≤3-h) incorporation of tritiated thymidine and longer-term (≤24-h) increases in total cell number (6, 13). In our samples, 41% of the growth occurring within 16 h was attributable to the bottle effect. If we had not

determined CFU, we would have overestimated the net growth of the bacteria by 70%. By following both CFU and total cell numbers, we estimated that the bottle effect (stimulation of culturable cells) increased total cell numbers by 10% in our samples after an incubation time of ca. 15 h. This time period may not be constant for different marine waters, in situ conditions, and sample pretreatments. With a starting ratio of 3.0% culturable cells to total cells (10) and a doubling time of 2.2 h when confined, culturable cell production could increase initial total cell numbers by 10% within 5 h. It also is possible that confinement or exposure of the water to contamination associated with sample collection or handling may have direct stimulatory or inhibitory effect on the nonculturable bacteria. Such effects are likely to increase with the time of exposure.

The containment effect on culturable bacteria results in doubling times which can affect estimates of total cell production within as few as 5 h of the sample collection. We agree with Hoppe (10) that total cell counts or spread-plate counts of bacteria in seawater alone do not provide a complete picture of the role of bacteria in the aquatic ecosystem. It is essential to combine these procedures with measures of rates of heterotrophic processes. Unfortunately, most approaches, including radiotracer techniques, are based on measured change over time in confined samples of water. During incubation periods in excess of 5 to 15 h at 25°C, significant changes occur in metabolic rates and species composition of confined communities of marine bacterioplankton. The containment effect on nonculturable bacterioplankton remains to be described. We also have not tested, owing to lack of appropriate controls, whether rates measured over incubation times of less than 5 h reflect those rates occurring in the environment. Demonstration of the relevance of short-term incubations of confined populations to natural water populations requires measurements of rates both in unconfined bodies of water and in confined samples to establish comparability. Measurements in the confined samples also should be made in a time series of minutes to hours to establish the duration of linear rates.

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