Growth of bacteria in seawater filtered through 0.2 µm Nuclepore membranes: implications for dilution experiments

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ABSTRACT: Dilution experiments were conducted in the Caribbean and Sargasso Seas in an attempt to measure bacterial growth under reduced grazing pressure. In this type of experiment, a small volume of the intact plankton sample is diluted into the same seawater from which all organisms have presumably been removed by filtration. We noted that diluent prepared by vacuum filtration (<100 mm Hg pressure) of natural seawater through 0.2 µm Nuclepore membranes contained bacteria which could be retained by the same membranes upon refiltration. Upon incubation, these bacteria rapidly accumulated ^{3}H-amino acids and grew at high rates (0.18 h^{-1}). The literature supports the evidence of ultramicrobacteria (those which might be expected to pass through 0.2 µm membranes): these may have passed into the diluent but were subsequently retained on 0.2 µm membranes possibly because they increased in cell size, formed ultramicrocolonies, or were aspherical in shape so that the orientation in relation to the membrane pore determined whether the cell was filtered or retained. Although the apparent specific growth rate of bacteria was in inverse relation to the degree of sample dilution, interpretable as a consequence of grazing on bacteria, the growth of bacteria in the diluent made it problematic to consider the dilution experiments in the conventional manner. It is likely that the 0.22 µm cellulose ester membranes used by many others who have performed such experiments would be much more effective in retaining ultramicrobacteria.

INTRODUCTION

Many studies have shown that bacteria in natural waters are anabolically active (e.g. Karl 1981, Cuhel et al. 1983). However, when samples of such waters are contained in small vessels and monitored over a few tens of hours, there is frequently little increase in the number of microscopically-detectable bacteria (Kirchman et al. 1982, Ferguson et al. 1984, Ducklow & Hill 1985a, b). This failure to observe net growth has been attributed to the removal of cells by bacteriovores (Wright & Coffin 1984a, b). One line of evidence that leads to this suggestion derives from so-called dilution experiments (Kirchman et al. 1982, Landry & Hassett 1982, Landry et al. 1984, Ducklow & Hill 1985a, b, Lovell & Konopka 1985). In this type of experiment, a small volume of the intact plankton sample is diluted into the same seawater from which all organisms have presumably been removed by filtration. The number of bacteria in these diluted samples often increases substantially over several hours and their apparent specific growth rate is in inverse proportion to the ratio of unfiltered to filtered water (Landry et al. 1984). These findings have been suggested as indicative of a decreasing rate of encounter between grazers and prey as dilution increases (Kirchman et al. 1982, Landry & Hassett 1982, Landry et al. 1984).

That bacteria are susceptible to grazing is supported by evidence of accelerated bacterial growth in plankton samples subjected to other sorts of manipulation before incubation. These include the removal of grazers by filtration (Fuhrman & Azam 1980, Wright & Coffin 1984a, b, Andersen & Fenchel 1985) and the suppression of grazing activity by selective metabolic inhibition (Newell et al. 1983, Fuhrman & McManus 1984).

Recently, there have been suggestions that some bacteria grazers are of the same order of size as their prey (<1 µm) (Fuhrman & McManus 1984, Wright & Coffin 1984b, Cynar et al. 1985) so that there would be no effective way of selectively eliminating all grazers by filtration. Also, the specificity of commonly used

inhibitors has been questioned (Li & Dickie 1985, Sanders et al. 1985). Nevertheless, as Waksman & Carey (1935) hypothesized half a century ago, it appears that at steady state, bacterial crop size reflects a balance between the supply rate of nutrients required for bacterial growth and the grazing or mortality rate at which bacteria are removed (Billen et al. 1980, Wright 1984, Wright & Coffin 1984a).

This paper describes dilution experiments conducted during cruises in the Caribbean and Sargasso Seas. Although dilution experiments circumvent the aforementioned problems of effective grazer removal and inhibition, we report here a complication of a different sort when Nuclepore filters are used. We noted that upon incubation, there developed in the diluent (seawater that had passed through a 0.2 μm Nuclepore polycarbonate filter) bacterial populations and heterotrophic activities that were retainable on 0.2 μm (and even 1 μm) filters. This observation, if generally valid, would greatly confound the interpretation of dilution experiments.

MATERIALS AND METHODS

Experiments were performed on board CSS 'Hudson' during Cruise 83-002 to the Sargasso Sea in April, 1983 and Cruise 84-049 to the Caribbean Sea in December, 1984. Water was sampled from 80 m at 14°40′ N, 64°53′ W (Exp. 1, Dec 4, 1984), from 60 m at 14°38′ N, 65°0′ W (Exp. 2, Dec 6, 1984), and from 10 m at 35°20′ N, 62°30′ W (Exp. 3, Apr 19, 1983) using a pump sampler system (Herman et al. 1984) as in previous work (Li 1984, Li & Dickie 1984, 1985).

All containers used for filtration and incubation of samples were acid-washed, rinsed with deionized water and with the sample itself before use. Diluent was prepared by filtering seawater through 0.2 μm Nuclepore polycarbonate filters (47 mm diameter) at a vacuum pressure of less than 100 mm Hg. This pressure differential (or a value slightly larger) has been the upper limit used by many others performing this type of experiment (Fuhrman & Azam 1980, Ferguson et al. 1984, Kirchman et al. 1984, Wright & Coffin 1984a, Ducklow & Hill 1985a, Sieracki et al. 1985). Each experiment consisted of a dilution series comprising the undiluted plankton sample, the diluent, and mixtures in various proportions of the plankton and diluent. For convenience, a mixture of x% plankton with (100 − x)% diluent will be referred to as an 'x% diluted sample'. All samples were placed into dark polycarbonate bottles. Experiments were initiated by adding ^{3}H-labelled-L-amino acid mixture (23 Ci mmol^{-1}, NET 250, New England Nuclear Corp.) to the contents of each bottle to a final activity of 0.1 μCi

ml^{-1}. Bottles were placed in incubators cooled with surface seawater. The assimilation of radioactivity by bacteria retained on 0.2 μm and 1 μm Nuclepore polycarbonate filters was monitored in time course fashion. The details of the filtration and radioassay procedures were as before (Li 1984, Li & Dickie 1984, 1985). In the experiments conducted in the Caribbean Sea, subsamples were removed (over time) from the incubation bottles, preserved with filtered (Millex-GS, 0.22 μm, Millipore Corp.) formalin (2 % final concentration), and enumerated for DAPI-stained cells (Porter & Feig 1980, Robarts & Sephton 1981) retained on 0.2 μm Nuclepore filters. Although the reagents were filtered before use, there were always a few DAPI-stained bodies observed in the blank preparations. These blank values were subtracted from the cell counts of the samples.

RESULTS

Bacterial density

Diluent was prepared by passing seawater through 0.2 μm Nuclepore filters. In Exp. 1, a cell count of formalin-preserved diluent refiltered onto 0.2 μm filters showed that the density of bacteria was reduced to an undetectable level from a value of 4.6×10^5 cells ml^{-1} in the undiluted sample (Fig. 1). In Exp. 2, bacterial density was reduced to 0.7×10^5 cells ml^{-1} from a value of 5.5×10^5 cells ml^{-1} in the undiluted sample (Fig. 2). In both experiments, there were significant increases in the number of 0.2 μm-retainable bacteria in the diluent after 4 h of incubation (Fig. 1 &
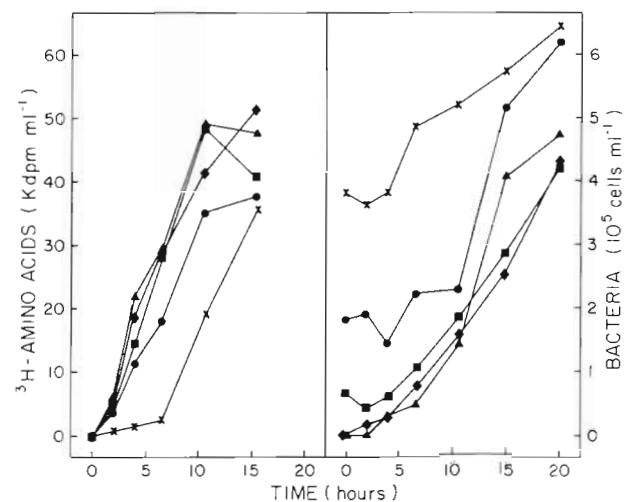
Fig. 1 Experiment 1 Left panel: accumulation of ^{3}H-amino acids in particles retained on 0.2 μm membranes. Right panel: development of bacterial populations retained on 0.2 μm membranes. x undiluted plankton (100 % sample); ● 50 % diluted sample; ■ 10 % diluted sample; ◆ 1 % diluted sample; ▲ diluent (0 % sample)
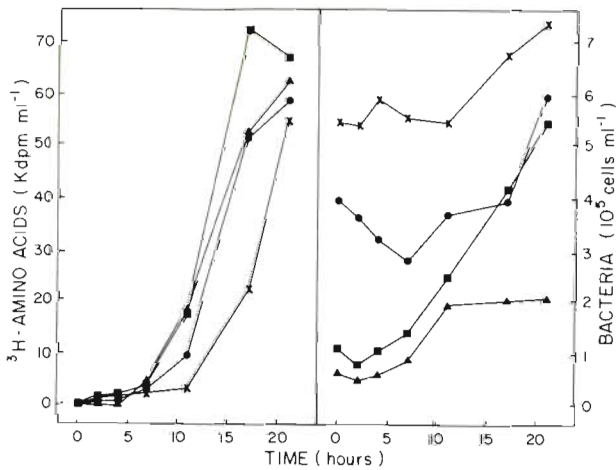
Fig. 2. Experiment 2. Left panel: accumulation of ³H-amino acids in particles retained on 0.2 µm membranes. Right panel: development of bacterial populations retained on 0.2 µm membranes. x undiluted plankton (100 % sample); ● 50 % diluted sample; ■ 10 % diluted sample; ▲ diluent (0 % sample)

2). The apparent specific growth rate of such bacteria after the lag period was 0.18 h^{-1} (apparent doubling time = 3.9 h) in both experiments (Fig. 3). In Exp. 1, this rate was sustained till the end of the incubation (20 h, Fig. 1); but in Exp. 2, there was no apparent further increase in cell density after 11 h (Fig. 2).

In both Exp. 1 & 2, there were measurable increases in bacterial density at all dilutions following a variable lag period (Fig. 1 & 2). The apparent specific growth rate (following the lag) was inversely related to the degree of dilution (Fig. 3). The apparent specific growth rate of bacteria in undiluted water was 0.03 h^{-1}
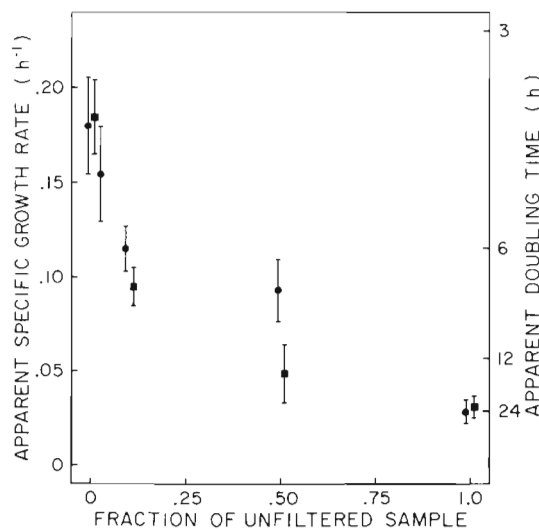

Fig. 3. Dependence of apparent specific growth rate, calculated from increase in cell density over time, on the fraction of the sample that was the original (unfiltered) plankton. ● Experiment 1; ■ Experiment 2. Standard errors indicated

(apparent doubling time = 23 h) in both experiments (Fig. 3).

A control experiment was performed in which diluent was incubated with formalin (2 %), chloramphenicol (100 µM), or cycloheximide (100 µM). The seawater sample originally contained $3.3 \pm 0.5 \times 10^5$ cells ml^{-1}; this value was reduced to $0.4 \pm 0.4 \times 10^5$ cells ml^{-1} in the freshly prepared diluent. After 20 h incubation, cell densities (units of 10^5 cells ml^{-1}) were 2.3 ± 0.3 in the uninhibited diluent, 0.03 ± 0.04 in the formalin-treated diluent, 0.2 ± 0.3 in the chloramphenicol-treated diluent, and 2.6 ± 0.6 in the cycloheximide-treated diluent.

Heterotrophic activity

The assimilation of ³H-amino acids by bacteria retained on 0.2 µm filters was evident at all dilutions, including the diluent. Formalin (2 %) and chloramphenicol (100 µm) were both effective at inhibiting such accumulation (100 % and 98 to 99 % inhibition, respectively).

Throughout Exp. 1, radioactivity in 0.2 µm-retainable bacteria in the diluent and the 1 and 10 % diluted samples were similar, but exceeded that in the 50 % diluted sample, which in turn exceeded that in the undiluted sample (Fig. 1).

In Exp. 2, radioactivity of 0.2 µm-retainable bacteria (Fig. 2) was partitioned (by post-incubation filtration) into 2 size classes using 1 µm Nuclepore filters (Fig. 4).
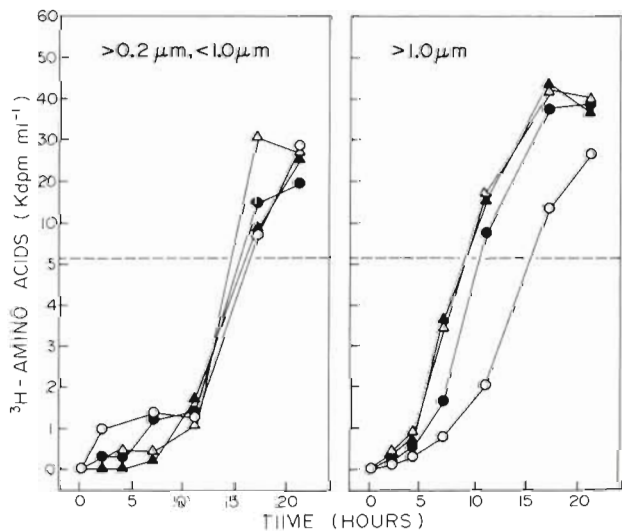

Fig. 4. Experiment 2. Left panel: Accumulation of ³H-amino acids in particles passing 1 µm membranes, but retained on 0.2 µm membranes; calculated as the difference of the values in Fig. 2 left panel and Fig. 4 right panel. Right panel: Accumulation of ³H-amino acids in particles retained on 1 µm membranes. ○ undiluted plankton (100 % sample); ● 50 % diluted sample; △ 10 % diluted sample; ▲ diluent (0 % sample)

Radioactivity in < 1 µm bacteria increased more slowly in the diluent and in the diluted samples than in the undiluted sample during the first 7 h. In fact, there was no detectable < 1 µm radioactivity in the diluent during the first 4 h (Fig. 4). From the 11th h onwards, < 1 µm radioactivity increased rapidly and to similar extents at all dilutions (Fig. 4). In contrast, at all stages of this experiment (#2), > 1 µm radioactivity in the diluent and the 10 % diluted sample exceeded that in the 50 % diluted sample, which in turn exceeded that in the undiluted sample (Fig. 4).

The design of Exp. 3 differed slightly from that of the first 2 experiments. Here, the diluent and the undiluted sample were separately incubated with ^{3}H-amino acids for 3 h before they were mixed in various proportions. Once again, radioactivity of 0.2 µm-retainable

bacteria was partitioned (by post-incubation filtration) into 2 size classes using 1 µm Nuclepore filters.

In this experiment, the time course of ^{3}H-amino acid assimilation differed greatly between the diluent and the undiluted plankton sample. In the diluent, < 1 µm radioactivity accumulated exponentially at a specific rate of 0.15 h^{-1} (Fig. 5F); > 1 µm radioactivity accumulated logistically: the specific rate during the early exponential phase was 0.29 h^{-1} (Fig. 6F). In contrast, in the undiluted plankton, the rate of accumulation decreased with time in both the < 1 and > 1 µm fractions, and the radioactivity in the former greatly exceeded that in the latter throughout the incubation (Fig. 5A & 6A). The course of events following mixture of the undiluted plankton and diluent was well-represented, in some cases, by the mean activities of the 2
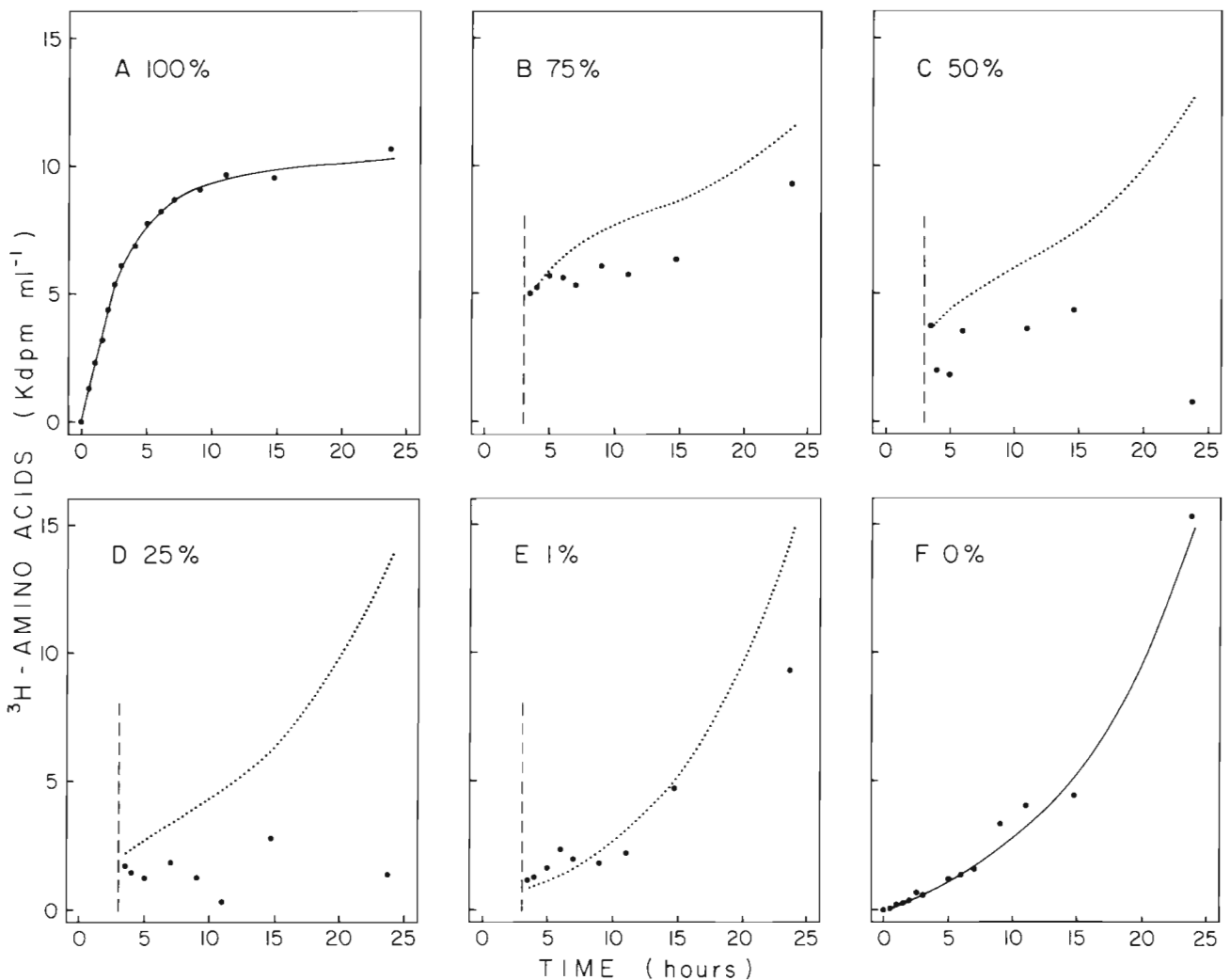

Fig. 5. Experiment 3. Accumulation of ^{3}H-amino acids in particles passing 1 µm membranes, but retained on 0.2 µm membranes. Dashed vertical line: time at which undiluted plankton and diluent were mixed to establish the various diluted samples. Solid curves (panels A,F): best fits ('by eye') to the data; dotted curves (panels B,C,D,E) constructed by calculating the average value of the activities in the undiluted plankton and diluent, weighted according to the proportion of the constituents in the diluted samples
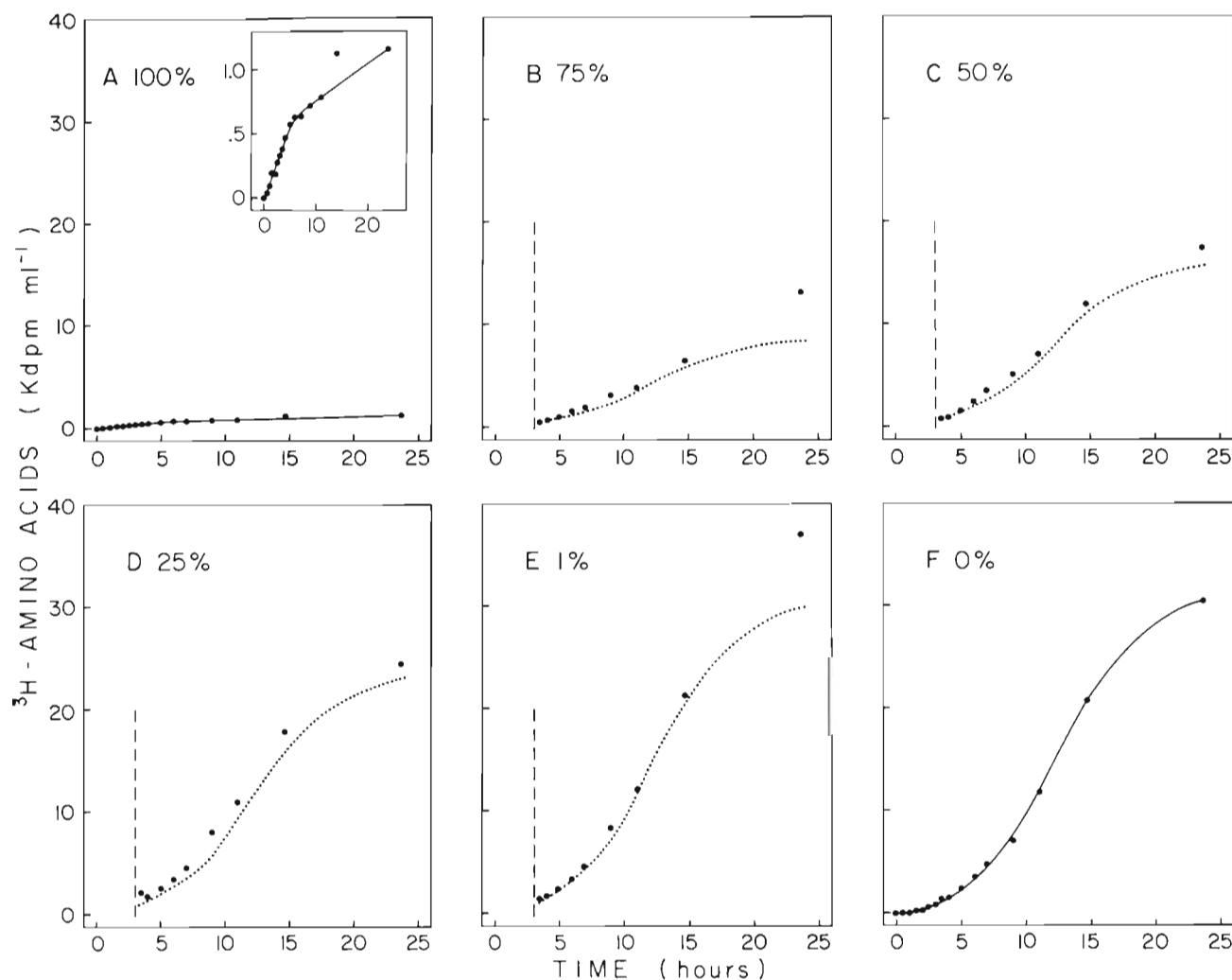
Fig. 6. Experiment 3. Accumulation of ³H-amino acids in particles retained on 1 µm membranes. Inset in panel A: data plotted on an expanded ordinate scale. See legend to Fig. 5 for explanation of dashed, solid and dotted lines

constituents, weighted according to the proportion of each constituent in the mixture. This was the case for the >1 µm fraction at all dilutions (Fig. 6B, C, D, E) and for the <1 µm fraction in the 1 % diluted sample (Fig. 5E). The increase in <1 µm radioactivity in the 75 % diluted sample was less than expected from weighted averaging (Fig. 5B); and there were no significant changes in the 50 % and 25 % diluted samples (Fig. 5C, D).

DISCUSSION

An implicit assumption in dilution experiments is that the diluent be free of bacteria. The results of our study indicate that this assumption cannot be taken for granted when diluent is prepared by vacuum filtration (<100 mm Hg pressure) of natural seawater through 0.2 µm Nuclepore filters. Although the number of bacteria and heterotrophic activity in freshly prepared

diluent may be low or even insignificantly different from background, this should not be taken as assurance that they remain so throughout the duration of an experiment.

Our results demonstrated that the increase in 0.2 µm-retainable DAPI-stained bodies and radioactivity in the diluent was a prokaryotic process (sensitive to formalin and chloramphenicol but insensitive to cycloheximide). The abiotic formation of particles from dissolved organic material (Jensen & Sondergaard 1982) would have been an unimportant process here.

The simplest explanation of the results is that during the preparation of diluent, some bacteria passed through the 0.2 µm filters. There is substantial evidence in the literature that this can happen. From 1 % (Hobbie et al. 1977) to 10 % (Zimmermann 1977) of the total number of bacteria have been found to pass through such filters. Sieburth (1984) observed 'minicells' approximately 0.1 µm in diameter. Fuhrman

(1981) presented a cumulative frequency distribution for bacteria in the California Bight from which may be estimated that about 5 % of the total number of bacteria were of equivalent spherical diameter less than 0.2 μm. Upon inspection of 2 Woods Hole seawater samples, Watson et al. (1977) found that small bacteria (<0.3 μm diameter) comprised 21 %, in one case, and 55 %, in the other, of all the bacteria present. Electron micrographs indicated that many of the small rods and vibrios which passed through 0.2 μm filters had actual diameters <0.2 μm, but their lengths reached 0.5 μm (Watson et al. 1977). A difference in the orientation of bacteria in relation to the membrane pore may explain why a refiltration of freshly prepared diluent might contain 0.2 μm-retainable bacteria. Additionally, there is usually overlapping of pores in Nuclepore filters as a consequence of the manufacturing process (Brock 1983) giving rise to pores whose effective size is much larger than the rest.

Bacteria filtered through 0.45 μm membranes (Tabor et al. 1981) and even 0.2 μm membranes (MacDonell & Hood 1982) have been recovered from the filtrate in a viable state. One of the reviewers (anonymous) of the present paper commented that he or she has also been able to culture freshwater and marine bacteria that passed through 0.2 μm filters. The term 'ultramicrobacteria' was suggested by Torrella & Morita (1981) to designate these very small cells. The literature on marine ultramicrobacteria has been summarized by Morita (1982). Recently, Smith et al. (1985) reported the results of one experiment conducted in eastern Canadian arctic waters wherein the community between 0.08 and 0.2 μm (established by serial differential filtration using Nuclepore filters) accounted for 34 % of ^{3}H-TTP and 5 % of ^{3}H-UTP taken up by the microbial plankton assemblage as a whole.

To account for retention of bacteria and heterotrophic activity on 0.2 μm filters in the diluent, we suggest that over the incubation period, the presumed ultramicrobacteria either increased their cell size, associated together to form a physical unit retainable on 0.2 μm filters, or both. It has been shown that when marine bacteria are presented with suitable nutrients at levels higher than previously available, some are capable of a rapid and substantial increase in cell size concurrent with an increase in the number of cells in the population (Torrella & Morita 1981, Morita 1982, Amy et al. 1983). Some ultramicrobacteria do not increase in cell size upon nutrient upshift; however, ultramicrocolonies may be formed (Torrella & Morita 1981) and these might be retained on 0.2 μm filters. Kirchman et al. (1982), Ammerman et al. (1984), Ferguson et al. (1984) and Sieracki et al. (1985) have all documented an increase in average cell volume of marine bacteria during incubations of plankton sam-

ples diluted with diluent prepared by vacuum filtration. It is very likely that the physical integrity of some cells is altered as a result of vacuum filtration, with a subsequent loss of cell contents (utilizable as nutrients by bacteria) to the filtrate (Ferguson et al. 1984, Goldman & Dennett 1985). The diluent would thus be a nutrient-enriched, grazer-free medium in which ultramicrobacteria could thrive.

The results of our 3 experiments differ in detail but not in the general aspects, which we now discuss. The apparent specific growth rate of bacteria was inversely related to the degree of dilution (Fig. 3), seemingly consistent with the grazing model of Landry & Hassett (1982). However there is an important difference. The ordinate intercept in the Landry & Hassett scheme refers to a hypothetical rate: that at which bacteria would grow in the absence of grazers. It is a measurement unachievable in practice because not only are there no grazers in a sample containing 0 % of the original undiluted plankton, there should also be no bacteria in that sample (i.e. the diluent). In our case, the ordinate intercept was an actual measurement pertaining to the growth of bacteria in the grazer-free diluent. Every sample that was diluted would also have had this growing population of diluent bacteria in addition to the larger bacteria. Only in the undiluted sample would the bacterial community have had been in its 'natural' (i.e. unfiltered) state, which may have been one in which ultramicrobacteria did not grow. If we had not monitored events in the diluent over time but assumed the validity of the Landry & Hassett scheme, we would have calculated a specific grazing rate of 0.10 ± 0.02 h^{-1} and a grazer-free specific growth rate of 0.13 ± 0.01 h^{-1} (i.e. slope and ordinate intercept respectively of the linear regression to the points in Fig. 3, omitting the data for the diluent). However, in light of the events that occurred in the diluent, these rates cannot be regarded as representative of those in situ.

It was apparent from the rapidity of ^{3}H-amino acids uptake in all 3 experiments that the diluent bacteria were highly active. The results of Exp. 3 were particularly interesting because the time course of uptake differed so greatly between the bacteria in the undiluted plankton (Fig. 5A & 6A) and in the diluent (Fig. 5F & 6F). The time course of radioisotope accumulation in the undiluted plankton (continual decrease in rate towards zero) was suggestive of a bacterial assemblage showing no net growth (Li 1982, 1984); in contrast, that in the diluent (continual acceleration in rate) was suggestive of an assemblage undergoing exponential net growth (Li 1982, 1984). This is a clear illustration of the potential for growth in marine bacteria once presumed controlling factors (e.g. grazing pressure, nutrient limitation) have been ameliorated. Furthermore, this

experiment suggested complex trophic interactions in the diluted samples: the time course of isotope accumulation in diluted samples could not always be predicted from the time courses in the undiluted plankton and diluent. In particular, it seemed that there was a process which curtailed the net accumulation of filterable radioactivity in 75, 50 and 25 % diluted samples in particles <1 μm (Fig. 5 B, C, D) but not in those >1 μm (Fig. 6 B, C, D). On the basis of these activity measurements alone, we are hesitant to designate this process as grazing because if very small grazers (< 1 μm) were present (Fuhrman & McManus 1984, Wright & Coffin 1984b, Cynar et al. 1985), they too would have become radiolabelled through their food. Our measurements of <1 μm radioactivity cannot distinguish between predator and prey. Furthermore, changes in the assimilation of radioactivity over time could be due to changes in the bacteria themselves: uptake rates, cell volumes and cell numbers (Kirchman et al. 1982). Nevertheless, the lower rates of cell number increase in less diluted samples (Fig. 3) were consistent with the notion that bacteria, including those developed from the diluent, were grazed.

We and others (e.g. Landry et al. 1984) used Nuclepore membranes to prepare our diluent. Nucleation-track membranes such as these act as sieves (Sheldon 1972, Brock 1983): that is to say, they tend to retain particles larger than their nominal pore size, allowing smaller particles to pass through. On the other hand, cellulose ester membranes (e.g. Millipore) are 'open-celled foam-like structures' whose 'pore diameters have no direct relationship to the size of the particles being filtered' (Brock 1983: p. 12). Thus, these filters tend to retain particles much smaller than their nominal pore size (Sheldon 1972, Brock 1983). It seems likely that when seawater is filtered through 0.22 μm cellulose ester membranes, few bacteria would be found in the filtrate.

In summary, our results serve to re-emphasize that 0.2 μm Nuclepore filters do not retain all marine bacteria (MacDonnell & Hood 1982). We note that Höfle (1984) used filtrate from 0.05 μm Nuclepore membranes as diluent in his experiments. It seems that for dilution experiments, diluent can best be prepared by using 0.22 μm cellulose ester membranes (e.g. Kirchman et al. 1982, 1984, Ammerman et al. 1984, Fuhrman & McManus 1984, Wright 1984, Ducklow & Hill 1985a).

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