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NOTE

Filter-type and sample handling affect determination of organic substrate uptake by bacterioplankton

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ABSTRACT: Natural assemblages of bacterioplankton (coastal seawater and seawater filtrates), treated with radiolabeled organic substrates, were used to test the effectiveness of various types of 0.2 µm pore size filters in capturing labeled cell materials. The highest retention of cell material was observed with filters made of either polyethersulfone (PES), mixed cellulose esters (MCE) or Nylon. PES filters retained up to 22% less than MCE or Nylon filters in some comparisons, but in most cases these filters gave similar, apparently high retention of cell materials. In contrast, retention of labeled cell material by track-etched polycarbonate (PC) or polyester (PE) filters was 10 to 80% less than for PES, MCE or Nylon filters, even at low filtration vacuum (5 cm Hg). Radiolabel lost from PC filters was recovered as dissolved compounds in the filtrates, and tests showed that cell breakage/leakage occurred, not during filtration per se, but rather at the end of filtration when filters ran dry. Higher filtration vacuum over the range 5 to 38 cm Hg caused greater losses with PC filters whereas retention of radiolabel by PES filters was nearly independent of vacuum. Losses on PC filters were greatest during early phases of incubations, when label in the cells was found mostly as untransformed parent substrate, and losses were greater for substrates known to function as intracellular osmolytes (e.g. glycine betaine and dimethylsulfoniopropionate) than for substrates (e.g. leucine and glucose) that were rapidly incorporated into macromolecules. Treatment of PES-filtered cells by a cold osmotic shock procedure resulted in nearly identical losses to those which occurred with non-shocked cells on PC filters. Likewise, addition of formalin (2% final concentration) to a seawater filtrate culture which had already taken up ^{14}C-glycine betaine caused rapid losses of ^{14}C from particulate materials, resulting in the same amount of loss as occurred when parallel live samples were filtered onto PC filters. The results suggest that a major fraction of the soluble components of bacterioplankton can be selectively lost during filtration through polycarbonate-type membranes, and by procedures which subject cells to osmotic shock and poisoning. Furthermore, our results indicate that PES, MCE and nylon filters function similarly well at retaining most cell materials.

KEY WORDS: Filtration · Artifact · Osmolyte · DOC · Filter comparison · Betaine

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Filtration is used widely in many aspects of microbial ecology research, therefore the performance of various filtration techniques needs to be thoroughly understood. One common application is the use of membrane filters to capture whole cells during uptake studies employing addition of radiolabeled substrates to aquatic samples (Wright & Hobbie 1966, Griffiths et al. 1974, Azam & Hodson 1981, Hodson et al. 1981, Coffin 1989, Griffith et al. 1990, Middelboe et al. 1995, Rich et al. 1996). Termination of incubations with radiolabeled substrates is often best achieved by filtering cells, rather than addition of poisons, because poisons such as formalin, $HgCl_2$ and strong acids can cause losses of label from the cells (Griffiths et al. 1974, Palumbo et al. 1983). Filtration itself can be problematic, however, if conditions are harsh (high vacuum shear, or desication on dry filters), as this can cause release of dissolved organic compounds, especially from eucaryotes like microzooplankton and phytoplankton (Fuhrman & Bell 1985, Goldman & Dennet 1985, Nagata & Kirchman 1990). While most 0.2 µm membrane filters do effectively remove the majority of bacterial cells from water, as evidenced by insignificant numbers of acridine orange- or DAPI-positive cells in the filtrates (Hobbie et al. 1977), few aquatic microbial ecology studies have addressed the efficiency of membrane filters for retention of *all* bacterial cell materials. Furthermore, a wide variety of membrane filters have been used by investigators carrying out microbial uptake studies, yet little information exists about the relative effectiveness of the different filters used.

We recently investigated uptake of the low molecular weight osmotic solutes, glycine betaine (GBT), and dimethylsulfoniopropionate (DMSP) by natural microbial communities (Kiene & Hoffmann Williams 1998, Kiene et al. 1998). We used radiolabeled substrates to measure uptake into filterable material (cells) and in preliminary tests we noted that 0.2 µm Supor filters

(polyethersulfone; PES) generally retained higher amounts of radioactivity when compared with other 0.2 µm membrane filters, including Nuclepore (polycarbonate; PC) and Anopore (aluminum oxide) types when a water sample was passed simultaneously through each filter type under identical vacuum conditions. This finding led us to investigate the performance of a wider variety of popular filter-types in uptake assays. In addition, we tested how factors such as type of radiolabeled substrate, time of incubation, and sample handling (filtration vacuum, osmotic shock and addition of poison [formalin]) affected retention of radiolabeled cell materials by selected filters.

Materials and methods. ***Filter types used:*** The filters used in this study were all rated at 0.2 µm pore size by the respective manufacturers. The types of filter materials tested included polyethersulfone (PES; Gelman, Supor-200); mixed cellulose esters (MCE, Poretics), track-etched polycarbonate (PC; Nuclepore or Poretics); track-etched polyester (PE; Poretics); Nylon (Poretics, Magna Nylon) and aluminum oxide (AO, Whatman, Anopore). Twenty-five mm diameter sized filters were used in all cases. General characteristics of the filter materials used are given in Table 1. Detailed information about the characteristics of the filter materials are widely available in catalogs of the respective manufacturers. Most of the comparisons in this study were between 0.2 µm Supor-PES and Nuclepore-PC filters, and most experiments utilized ^{14}C-GBT (see below) as the representative uptake substrate. We arbitrarily chose Supor-PES filters as a reference with which to compare other filters because we routinely used these filters and we had previously determined that they gave low abiotic adsorption blanks, and were amenable to extraction in organic solvents.

Water collection and culture generation: Coastal seawater samples used for microbial uptake experiments and filter comparisons were collected in the vicinity of Dauphin Island, Alabama, located in the Northern Gulf of Mexico (30° 15' N, 88° 05' W). Water was collected from the shore (pier or beach) into a polycarbonate carboy. In some experiments, freshly collected whole water was used, while in other experiments, filtrate cultures were used so that the microbial community consisted mainly of bacteria. The cultures were generated by filtering water through Whatman GF/F glass fiber filters (>0.7 µm nominal retention) using gravity only. Prior to the initiation of uptake experiments, the filtrate cultures were incubated in the dark for 24 to 48 h, during which time endogenous substrates became depleted and bacterial abundances typically doubled from 1–2 to 2–4 × 10^6 cells ml^{-1}. Microscopic examination showed that filtrate cultures contained mainly free living bacteria, with few microzooplankton or photoautotrophs observed at the time experiments were carried out.

Table 1. Filter-types used and their general characteristics

Filter membrane material and abbreviation	Pore-size (µm)	Filter manufacturer and trade name[a]	Characteristics[b]	Comments
Polyethersulfone (PES)	0.2	Gelman Supor-200 Poretics, Osmonics	Sponge-like matrix filter. Irregular surface. Rapid filtration	Mechanically strong, and stable in methanol: chloroform (12:5)
Polycarbonate, tracked-etched (PC)	0.2	Nuclepore, Costar Poretics, Osmonics	Well defined pore size. Slow filtration rate. Very flat surface	Little moisture retention
Polyester, track-etched (PE)		Poretics, Osmonics	Well defined pore size. Slow filtration rate. Very flat surface	Similar in most respects to track etched polycarbonate
Mixed esters of cellulose (MCE)	0.2	Poretics, Osmonics	Sponge-like matrix filter. Irregular surface. Rapid filtration	Mechanically weak, tears easily
Nylon		Poretics, Osmonics - Magna Nylon	Sponge-like matrix filter. Irregular surface. Rapid filtration	Mechanically strong, and stable in methanol: chloroform (12:5)
Aluminum oxide (AO)	0.2	Anopore, Whatman	Flat surface. Moderate filtration rate	Brittle

[a]These are the manufacturers of filters we used. Other sources of individual filter-types may be available
[b]Detailed information about filter membrane characteristics are available in the catalogs of respective manufacturers and retailers

Comparisons of filter-type, substrate-type and incubation time in live bacteria uptake assays: GF/F filtrate cultures were treated with either *methyl* ^{14}C-GBT, ^{35}S-DMSP, U-^{14}C-glucose, or U-^{14}C-leucine, with concentrations ranging from 5 to 16 nM (600 to 12700 dpm ml^{-1}), depending on the substrate and experiment. GBT and DMSP are examples of naturally-occurring, low molecular weight osmotic solutes, and both of these compounds appear to be taken up by the same transport system in marine microorganisms (Kiene et al. 1998). Leucine is an amino acid which is rapidly assimilated into proteins and is widely used in uptake assays by microbial ecologists (Kirchman et al. 1985, Simon & Azam 1989). Glucose was used because it is a major substrate for bacterioplankton in the sea (Rich et al. 1996), and available evidence suggests that marine bacteria take it up by a transport mechanism which differs from that used for most amino acids and osmolytes (DeLong & Yayanos 1987, Logan & Kirchman 1991).

After substrate addition to the filtrate cultures, 5 ml sub-samples were withdrawn by pipette at selected times (typically 10 min, 2 h and 24 h) and transferred to a 10-place Hoefer filtration manifold set up with several different filter types (in duplicate or triplicate). The samples were filtered using a vacuum which was typically 5 to 10 cm Hg. Vacuum was carefully monitored during all experiments and the effects of different vacuum levels were evaluated in 1 experiment (see below). After filters ran dry they were quickly rinsed with 3 × 1 ml of 0.2 µm filtered seawater (FSW) of the same salinity as the sample. After the final rinse, and with the vacuum still on, the filters were removed and placed into scintillation vials with 5 ml of Ecolume (ICN Biomedicals) for determination of radioactivity by liquid scintillation counting (Packard Tri-Carb model 2500 TR). In 1 experiment, 5 different filter types (PES, MCE, PC, PE and Nylon) were compared. In addition, a second set of Supor-PES filters was included and after rinsing with FSW, they were treated with 5 ml of ice-cold 5% trichloroacetic acid (TCA). The TCA was allowed to stand on the filters for 5 min prior to being drawn through the filters. Radioactivity remaining on filters after cold TCA treatment is generally considered to be material assimilated into cellular macromolecules (Kirchman et al. 1985).

Time course of ^{14}C-GBT and ^{35}S-DMSP uptake, retention, and assimilation: Information on the rates at which osmotic solute substrates were accumulated intracellularly and ultimately assimilated were obtained by following the amount of total uptake and the amount of untransformed substrate captured on the filters over time. Replicate samples (250 ml) from the same filtrate culture were treated with either ^{14}C-GBT (11.9 nM; 1500 dpm ml^{-1}), or ^{35}S-DMSP (9.5 nM; 410 dpm ml^{-1}). These samples were incubated in the dark in Teflon bottles at 27°C. At selected time points, replicate 10 ml sub-samples were taken by pipette for total uptake into particulates. Sub-samples were filtered onto Supor-PES filters, which were assayed for radioactivity in Ecolume. At the same time points, parallel PES filters (10 ml filtered) were taken for analysis of untransformed substrate by placing filters into 5 ml of an extraction fluid consisting of methanol, chloroform and water (MCW, 12:5:1). The extraction fluid contained 50 to 100 nmol of unlabeled GBT or DMSP to aid recovery of the respective isotopes. After >24 h, the extracted filters were removed from the vials and rinsed with methanol into the same extraction vial. The rinsed filters were placed in a clean scintillation vial, allowed to dry in a fume hood, then counted in Ecolume. The activity retained on these extracted filters represented non-extractable material, presumably macromolecules. The MCW extract was dried under N_2, reconstituted in pure water and injected into an HPLC. The peaks representing GBT or DMSP were collected and the radioactivity determined by liquid scintillation methods. Further details on these procedures are given elsewhere (Kiene & Hoffmann Williams 1998). Recovery of radioactivity as the untransformed parent compound (HPLC peak) plus the non-extractable material remaining on the PES filter averaged 80% (^{35}S-DMSP) and 90% (^{14}C-GBT) of the activity found on parallel non-extracted filters, indicating that these fractions comprised the majority of labeled material on the filters.

Effects of filtration vacuum: The effects of filtration vacuum on retention of ^{14}C by either Supor-PES or Nuclepore-PC filters was investigated using a 48 h old filtrate culture treated with 10 nM ^{14}C-GBT. Samples were taken approximately 3 h after addition of labeled GBT. Triplicate filters of each type were used at each vacuum level. Four vacuum levels (5, 13, 19 and 39 cm Hg) were tested within a 30 min period, with sampling proceeding from low to high vacuum.

Filter and filtrate label recovery: Ten ml samples of a ^{14}C-GBT-amended filtrate culture were filtered as described above onto 0.2 µm Supor-PES and Nuclepore-PC filters using the Hoefer filtration manifold. Triplicate samples for each filter type were taken and each was rinsed with exactly 2 × 0.5 ml of FSW. The filtrates (11 ml total) were captured in scintillation vials. Three 1 ml subsamples from each filtrate were taken for determination of total filtrate radioactivity. We tested whether any of the radioactivity passing the 0.2 µm filters might be cell fragments or macromolecules by passing the filtrates through Gelman Nanospin 4000 MWCO centrifugal ultrafilter units. Two ml of the 0.2 µm PES or PC filtrates were placed in the ultrafilter units and these were spun at 10000 × *g* for 15 min at 4°C. From each of the ultrafiltrates, duplicate

0.5 ml sub-samples were taken to determine the concentration of radioactivity (dpm ml^{-1}). The concentrations of ^{14}C activity in the <4000 MW ultrafiltrates were compared with that in the <0.2 µm Supor-PES or Nuclepore-PC filtrates.

A separate experiment tested whether losses from cells on Nuclepore-PC filters occurred during passage of the water sample through the filters or during the drying/rinsing steps at the end of filtration. Sub-samples (7 ml) of a ^{14}C-GBT-amended filtrate culture (1.2 h after substrate addition) were placed in the Hoefer filter towers over either 0.2 µm Supor-PES or Nuclepore-PC filters (duplicates of each). The vacuum (5 cm Hg) was turned on until approximately 5 ml of the sample had filtered through. At this point the vacuum was shut off and the partial filtrate (~5 ml) collected and subsampled for the concentration of radioactivity (dpm ml^{-1}). New filtrate receptacles were placed and the remaining 2 ml of sample filtered through, with filters being rinsed with 3 × 0.5 ml of FSW. The filters were counted and the secondary filtrates (~3.5 ml) were subsampled for the concentration of radioactivity (dpm ml^{-1}). Volumes of partial and secondary filtrates were recorded to the nearest 0.1 ml and total dpm in the respective filtrates were calculated from the concentration of radioactivity times the volume of the filtrate. Recovery of radioactivity in each of the 3 fractions (filter + partial filtrate + secondary filtrate and rinse) was >95% of the added amount. The range of duplicate determinations of each fraction was less than 10% of the mean of each.

Cold osmotic shock treatment: A cold osmotic shock procedure was used to test whether losses which occurred with Nuclepore filtration might be due to selective loss of periplasmic or outer membrane components of cells. Treatment of cells already filtered onto 0.2 µm Supor filters with a cold osmotic shock procedure was compared with filtration of replicate samples of cells onto 0.2 µm Nuclepore-PC filters or Supor-PES filters without the osmotic shock. The shock procedure, originally adapted from Neu & Heppel (1965), but optimized for a marine bacterial culture by Martinez & Azam (1993), is reported to specifically cause release of periplasmic and outer membrane components of bacterial cells, leaving the cell membrane intact. The procedure of Martinez & Azam (1993) was modified for use with the dilute bacterial suspensions (~2×10^6 cells ml^{-1}) used here. Replicate sub-samples of a filtrate culture which had been amended with ^{14}C-GBT or ^{14}C-glucose, and incubated for 1 to 2 h, were filtered (10 cm Hg vacuum) onto a series of PES and PC filters. After filtering to dryness and rinsing with isotonic FSW, 3 PES and 3 PC filters were taken and immediately placed in Ecolume for counting. With the vacuum turned off, a second set of PES (in triplicate) was covered with 5 ml of an upshock solution, consisting of 20% sucrose (w/v) solution prepared in 24 ppt 0.2 µm-filtered seawater (same water as used for the filtrate culture) and containing 10 mM Na_2-ethylenediamine tetra-acetic acid (EDTA). The upshock fluid was allowed to stand on the filters for 15 min, after which time the fluid was filtered through and the filters rinsed with 2 × 1 ml of upshock fluid. After shutting off the vacuum, the stainless steel filter towers of the Hoefer unit were replaced with new towers which had been sitting on ice (cold towers were dried to remove freshwater before placing on filters). Five ml of ice cold downshock fluid consisting of 0.2 µm filtered seawater (24 ppt) was added to each tower and allowed to sit for 10 min. The downshock fluid was filtered through, and the filters rinsed with 2 × 1 ml of ice cold downshock fluid before they were removed for counting in Ecolume. Tests showed that ^{14}C-actvity lost from the PES filters during osmotic shock procedures was recovered in the filtrates, with 26% of the loss occurring in the upshock phase and 74% of the loss occurring in the downshock phase.

Effects of formalin on ^{14}C retention by cells and different filters: A 36 h old filtrate culture (200 ml) was treated with approximately 11 nM ^{14}C-GBT. After 1.5 h the culture was split into two 100 ml aliquots and one of these was treated with 2 ml of borate buffered formalin (2% formalin). The other remained untreated. For the next 1.5 h, sub-samples (in duplicate) were alternately removed from the live and formalin-killed cultures and filtered onto both PES and PC filters.

Isotopes and other chemicals: *Methyl*-[^{14}C]glycine betaine (57 mCi $mmol^{-1}$) was synthesized from ^{14}C-choline (ICN Biomedicals) following the procedures outlined by King (1987). ^{35}S-DMSP (37 mCi $mmol^{-1}$) was synthesized by feeding ^{35}S L-methionine to a culture of the phytoplankter *Platymonas* (= *Tetraselmis*) *subcordiformis*. Details on this synthesis and on the purification of ^{35}S-DMSP are given in Kiene et al. (1998). Radiochemical purity of these isotopes was >99% based on HPLC and thin layer chromatography. U-^{14}C-Glucose (354 mCi $mmol^{-1}$) and ^{14}C-Leucine (320 mCi $mmol^{-1}$) were obtained from ICN Biomedicals.

Formalin (37% formaldehyde) was obtained from Fisher Scientific. All other organic chemicals were obtained from either Sigma or Aldrich and were ACS reagent grade, or better.

Results. Preliminary tests involving uptake of ^{14}C-GBT by microbial communities in seawater, showed that 0.2 µm Supor-PES filters gave 2- to 4-fold higher retention of radioactivity than Nuclepore-PC or Anopore-AO filters of comparable pore size. Tests with 0.2 µm filtered seawater showed that the higher dpm retained on Supor-PES filters was not the result of abiological absorption of label by these filters. Also, the

lower counts obtained with PC filters were not due to an effect of these filters on liquid scintillation counting of ^{14}C or ^{35}S. The retentive characteristics of PES filters in relation to other membrane filters, and the reasons for losses on PC-type filters were explored in greater detail.

Comparisons of filter type, substrate and incubation time: We conducted an experiment which compared 5 different filter types (PES, MCE, PC, PE and Nylon) in microbial isotope uptake assays, using 3 different substrates (^{14}C-glycine betaine, ^{14}C-leucine, and ^{14}C-glucose) and 3 different incubation times (10 min, 2 h and 24 h). Similar patterns with respect to filter and time were observed with each substrate (Fig. 1A,B,C), but the magnitude of responses differed among the substrates. For all 3 substrates, and at all times, the highest retention of radioactivity was observed with either PES, MCE or Nylon filters. These 3 filter types seemed to perform similarly, but in some cases MCE filters retained up to 22% more radioactivity than the reference Supor PES filters, and these differences were significant ($p < 0.05$; Tukey, pairwise multiple comparisons) with GBT and glucose at 10 min and 2 h sampling times. We reiterate that our choice of Supor-PES as the reference filter type was arbitrary, and serves only as a basis for comparison. After 24 h, incubation samples filtered onto PES, MCE or Nylon filters gave results within 10% of one another and the differences were statistically insignificant (Fig. 1A,B,C).

Retention of radioactivity by PC and PE filters was significantly lower than with the other filter types, especially at the 10 min and 2 h incubation times (Fig. 1A,B,C). For all 3 substrates, however, the amount of radioactivity collected on PC and PE filters (relative to that on Supor-PES) increased with time ($p < 0.05$; 2-way ANOVA). In almost every case, the results with PC and PE filters were very similar to those for PES filters that were treated with 5% TCA, suggesting that PC and PE filters retained most of the macromolecular fraction of the cells but lost the dissolved fraction. The most severe losses (>80%) on PC and PE filters were observed at 10 min and 2 h incubation times when GBT was the substrate (Fig. 1A). When leucine was the substrate (Fig. 1B) the loss was 70% at 10 min but only 25% at 2 h. Similarly, the losses when glucose was the substrate (Fig. 1C) were 46 and 22% at 10 min and 2 h respectively. After 24 h incubation, filtration of ^{14}C-leucine- and ^{14}C-glucose-treated samples onto PC and PE filters yielded radioactivity lower than, but within 20% of that found on PES, MCE and Nylon filters. This contrasted somewhat with the ^{14}C-GBT-treated samples, which showed large (~45%) losses with the PC and PE filters even after 24 h incubation.

In a separate experiment with more limited filter comparisons, a time-dependent pattern of relative re-

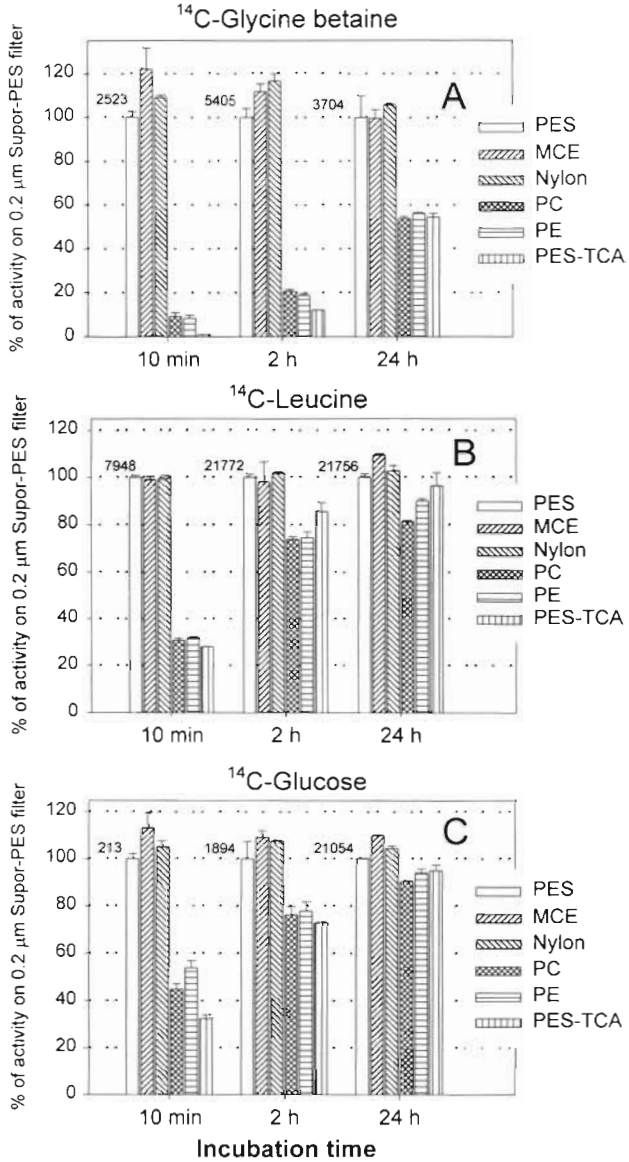

Fig. 1. The relative retention of radiolabeled bacterial cell materials by 5 different 0.2 µm pore-size filter types commonly used in microbial uptake studies. The filter types were: PES, polyethersulfone; MCE, Mixed cellulose esters; Nylon, PC, polycarbonate and PE, polyester. Shown are the results at 3 time points for water samples incubated with either (A) 11 nM ^{14}C-glycine betaine, (B) 10 nM ^{14}C-leucine or (C) 16 nM ^{14}C-glucose. Also shown for each time point and substrate are results for PES filters which were treated with 5% trichloroacetic acid for 5 min after filtration of cells. Other filters were rinsed only with isotonic filtered seawater. Data for each filter type and treatment are expressed as the percent of ^{14}C activity measured on seawater rinsed PES filters. Mean values of the dpm collected on PES filters are shown next to the white bars. Error bars indicate 1 standard deviation of 3 replicates. All samples were filtered with identical vacuum (5 cm Hg) using a multiple-place Hoefer filtration manifold. The filtrate culture used for this experiment was generated from a seawater sample (salinity = 28 ppt) collected from the west end beach on Dauphin Island, Alabama. The incubation was carried out in the dark at 25°C

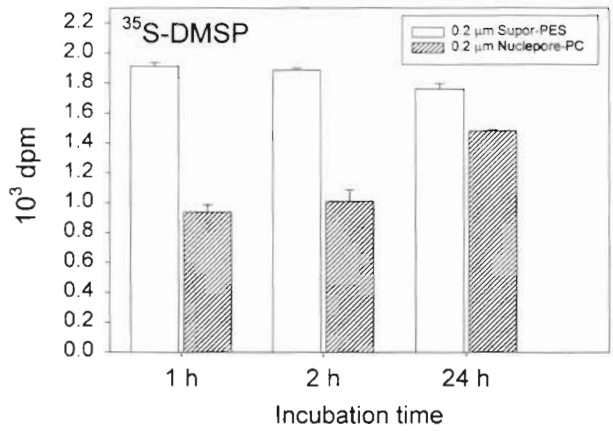

Fig. 2. The relative retention of ^{35}S-labeled cell materials by either 0.2 µm Supor-PES or 0.2 µm Nuclepore-PC filters. Shown are the results at 3 time points for a filtrate culture (GF/F filtered seawater) incubated with 5 nM ^{35}S-DMSP. Error bars indicate 1 standard deviation of 3 replicates. Vacuum was 10 cm Hg. The water sample (salinity = 18 ppt) was collected from the west end beach on Dauphin Island. Incubation was in the dark at the *in situ* temperature of 27°C

tention between 0.2 µm Supor-PES and Nuclepore-PC filters was also observed when 10 nM ^{35}S-DMSP was added as the labeled substrate to a 24 h old filtrate culture (Fig. 2). The PC filters yielded only 49, 53 and 84% of the ^{35}S activity on PES filters at 1, 2 and 24 h of incubation respectively.

Cellular partitioning of isotopes: A time course of uptake and retention of ^{14}C-GBT and ^{35}S-DMSP (using 0.2 µm Supor filters to capture cells) showed clearly that during the first few hours of incubation, when filtration losses on PC and PE filters were typically greatest (see Figs. 1 & 2), the majority (>70%) of the radioactivity retained on filters was found as the untransformed substrate (Fig. 3). With longer incubation times, cells apparently assimilated the acquired substrate and produced labeled cell materials which were non-extractable in MCW (Fig. 3). After 25 h incubation, when losses using PC and PE filters were typically less than at early time points, the untransformed substrate represented a much lower percentage (31 and 11% for ^{14}C-GBT and ^{35}S-DMSP respectively) of the total radioactivity captured on the filters. The non-extractable materials represented ~60% (GBT) and ~70% (DMSP) of the total radioactivity on filters after 25 h incubation.

Filter and filtrate label recovery: Using a ^{14}C-GBT-treated seawater filtrate culture, we found that radioactivity lost from Nuclepore-PC filters was recovered as excess activity in the filtrate (Table 2), with total recovery (filter + filtrate) being ≥95% for both PES and PC filters. Essentially 100% of the radioactivity in the 0.2 µm filtrates passed through a 4000

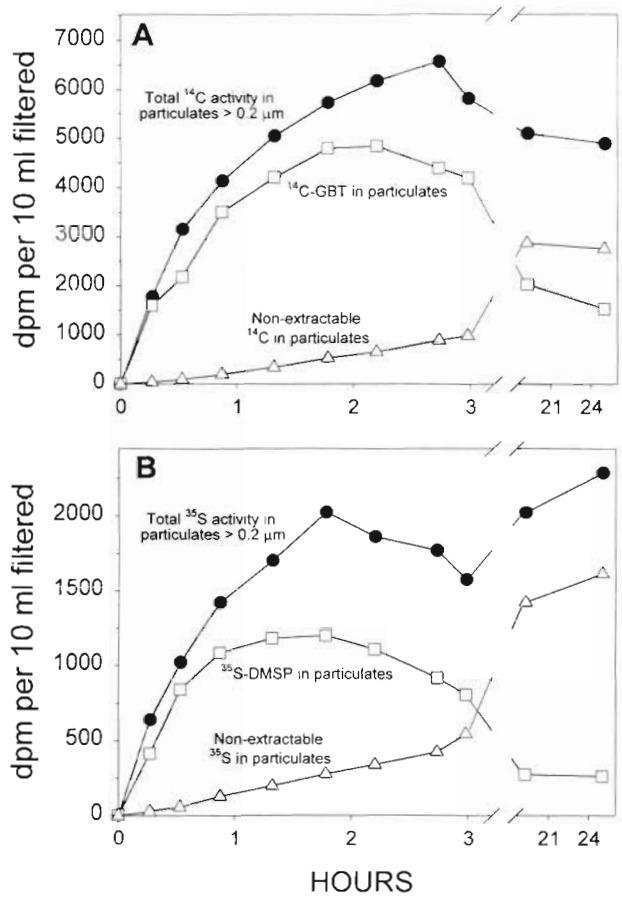

Fig. 3. Time courses of (●) total particulate radioactivity, (□) untransformed substrate in particulates, and (△) non-extractable radioactive materials in particulates in a seawater filtrate culture treated with either (A) ^{14}C-glycine betaine or (B) ^{35}S-DMSP. Sub-samples (10 ml) of the filtrate cultures were filtered onto 0.2 µm Supor-PES filters. Data represent single samples taken at each time. The untransformed substrate and the non-extractable materials represent sub-sets of the total radioactivity captured on filters. The added concentration of ^{14}C-GBT was 11 nM, while that of ^{35}S-DMSP was 9.5 nM. Water was collected from Mobile Bay and had a salinity of 17 ppt. Incubation was at 27°C

MWCO ultra-filter indicating that little of the labeled material, including the excess caused by PC filtration, was cell debris or macromolecules.

A subsequent experiment with ^{14}C-GBT as the substrate tested whether losses on PC filters occurred during the filtration or at the end of filtration in the rinse steps. Partial filtrates (5 ml out of 7 ml total) contained similar amounts of radioactivity for either PES or PC filters (Fig. 4). However, the final filtrate + rinses (3.5 ml) of the PC filters contained nearly 3 times the radioactivity of the final filtrate + rinses of the PES filters. As observed previously, the amount of dpm on the PC filters was much lower than for the PES filters, but the total recovery (filter + all filtrates) was similar for both filter types (Fig. 4).

Table 2. Recovery of ^{14}C after filtration of a ^{14}C-glycinebetaine-treated filtrate culture through 0.2 µm Supor-PES and Nuclepore-PC filters. SD: standard deviation

Pool	Supor-PES filters Mean[a]	SD	Nuclepore-PC filters Mean[a]	SD
Filter dpm	6673	84	1745	65
Filtrate dpm[a]	8319	624	12894	45
Filter + filtrate dpm	15014	575	14661	20
% of added isotope recovered	97.7	4	95	0
Ultrafiltrate of filtrate dpm[b]	8622	245	12985	286
Ultrafiltrate as % of filtrate dpm	104	6	101	2

[a]Values represent the mean of triplicate filters of each type
[b]Filtrate and ultrafiltrate dpm values represent the amount in the total filtrate estimated from the concentration of radioactivity in sub-samples of each fraction and the volume of each fraction

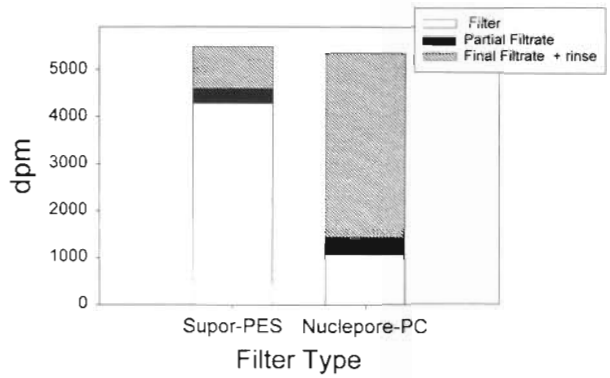

Fig. 4. Partitioning of ^{14}C after filtration of 7 ml sub-samples from a ^{14}C-glycine betaine-treated filtrate culture through either 0.2 µm Supor-PES or 0.2 µm Nuclepore-PC filters. Bars show the total radioactivity (dpm) on the filters, in the partial filtrates and in the final filtrate plus rinses. Approximately 5 ml of the 7 ml samples had passed through the filters when the partial filtrates were collected and counted. The 2 ml of sample remaining above the filters were subsequently drawn through and the filters rinsed with 3 × 0.5 ml of isotonic filtered seawater. The rinsed filters as well as the final filtrates plus rinses were collected and counted. Duplicates of each filter type were used, and the range of duplicate determinations of each fraction was less than 10% of the mean values of each

Effects of vacuum: The effect of vacuum on isotope retention by PES and PC filters was tested using a filtrate culture treated with ^{14}C-GBT for 2 h. Nuclepore-PC filters retained much less ^{14}C activity (26% of that on PES) at the lowest vacuum (5 cm Hg), and there was a 46% decrease in dpm on the filters from lowest to highest vacuum (Fig. 5). In contrast, the amount of ^{14}C retained by PES filters was largely independent of vacuum over the range tested (5 to 38 cm Hg) (Fig. 5). There was a slight increase of 6% in the dpm on PES filters from lowest to highest vacuum. Some of this increase may have been due to real changes in the amount of ^{14}C in the cells over the 30 min duration of this experiment.

Effects of cold osmotic shock: A cold osmotic shock treatment, which involved first an upshock and then a downshock, caused cells filtered onto Supor-PES filters to lose a substantial amount of radioactivity as compared with those not receiving the osmotic shock (Fig. 6). The amount of activity on PES filters after cells were osmotically shocked was very similar to the amount retained on PC filters when cells were not shocked, and this was true when either ^{14}C-GBT (Fig. 6A) or ^{14}C-glucose (Fig. 6B) were the substrates taken up. As observed previously, the losses upon filtration onto PC filters were much less for ^{14}C-glucose-treated samples than for ^{14}C-GBT-treated samples (Fig. 6). A subsequent experiment (data not shown) reveled that subjecting bacteria on PC filters to cold osmotic shock caused loss of an additional 50% of the radioactivity compared with unshocked cells on PC filters. In a separate test, it was found that even a modest osmotic downshock (no upshock involved) could result in significant losses of cellular radioactivity. When a high salinity (37 ppt), ^{14}C-GBT-treated filtrate culture was rinsed with either 24 ppt FSW or 37 ppt FSW, the amount of radioactivity on the 24 ppt-rinsed filters was 40% lower than that on the 37 ppt-rinsed filters.

Effects of formalin: The effects of formalin on the patterns of radioisotope retention by 0.2 µm Supor-PES

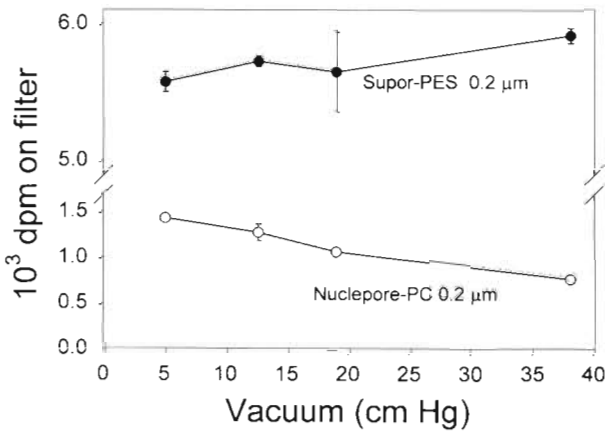

Fig. 5. Effects of filtration vacuum on retention of ^{14}C-activity by either 0.2 µm Supor-PES filters or 0.2 µm Nuclepore-PC filters. Five ml sub-samples (in triplicate) of a ^{14}C-glycine betaine-treated filtrate culture were passed through each filter type at each of the vacuums shown. Error bars represent 1 standard deviation of 3 replicates and are smaller than the symbol in some cases. Note the break in scale. The dpm on Supor filters increased 6% from the lowest to highest vacuum, whereas the dpm on Nuclepore filters decreased by 46% from the lowest to highest vacuum

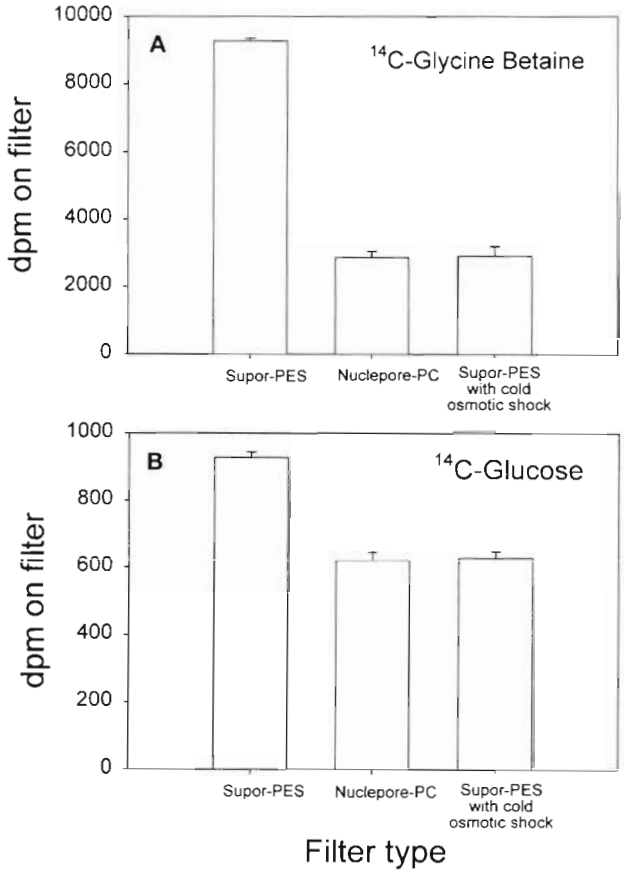

Fig. 6. The relative retention of ^{14}C-labeled cell materials by either 0.2 µm Supor-PES or 0.2 µm Nuclepore-PC filters taken with standard procedures (rinsing with isotonic filtered seawater) and that of 0.2 µm Supor-PES filters subjected to a cold osmotic shock procedure, as described in the text. Shown are data from filtrate cultures treated with (A) ^{14}C-glycine betaine and (B) ^{14}C-glucose, and sampled at 1 to 2 h after substrate addition. Error bars indicate one standard deviation of 3 replicates. Vacuum was 10 cm Hg. The water sample was collected from the east end pier on Dauphin Island and had a salinity of 24 ppt. Incubation was in the dark at the *in situ* temperature of 23°C

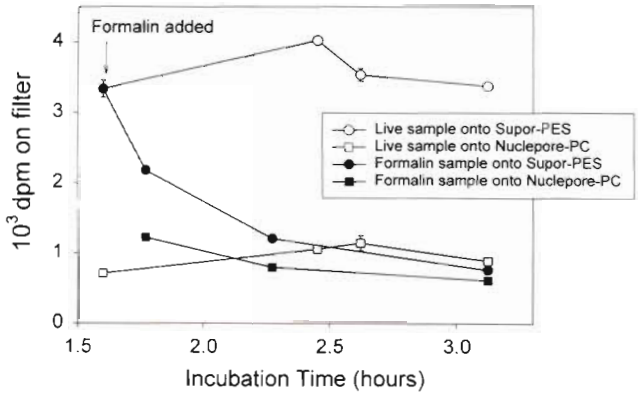

Fig. 7. The effects of adding 2% formalin (closed symbols) on the amount of radioactivity captured by either 0.2 µm Supor-PES (●, ○) or Nuclepore-PC (■, □) filters. Data from live samples (open symbols) are also shown. In this experiment, a filtrate culture was amended with 10 nM ^{14}C-glycine betaine 1.5 h prior to initiation of filter tests, so cells had accumulated ^{14}C-activity. Data points represent the mean of duplicate samples at each time, with bars indicating the range. Bars are smaller than the symbols in some cases

and Nuclepore-PC filters was tested by adding formalin (2% final concentration) to a filtrate culture which had already been allowed to take up ^{14}C-GBT for 1.5 h (Fig 7). Direct measurement confirmed that, at this time into the incubation, 82% of the label in cells was untransformed ^{14}C-GBT, while only 7% was non-extractable materials (see also results above). Parallel live samples (no formalin) filtered onto PES filters showed relatively constant amount of radioactivity at different time points over a 1.5 h period (Fig.7). In contrast, filterable radioactivity (onto PES filters) in formalin-treated samples decreased exponentially from 3400 dpm to 770 dpm 5 ml^{-1} in about 1.5 h. Sub-samples from the same formalin-treated bottle filtered onto PC filters showed similar amounts of radioactivity to live samples filtered onto PC filters, and both of these gave very much lower radioactivity than live samples filtered onto PES filters. Formalin-PES, formalin-PC and live-PC filter samples converged after 1.5 h at about 20% of the dpm obtained with live samples filtered onto PES filters (Fig. 7).

In other tests carried out with ^{14}C-GBT, there were no substantial differences between the performance of Nuclepore brand versus Poretics brand 0.2 µm PC filters; both gave poor retention of radioactivity compared with Supor-PES filters. It also made no difference whether a backing filter (a 0.2 µm Supor-PES) was used under the PC filters on the Hoefer filtration manifold. In addition, leaving the PC filter dry on the filter tower, after the sample had filtered through for ~1 to 2 s (the minimum time possible and the standard time used) gave results which agreed, within 10%, with filters left dry and the vacuum on, for up to 60 s (data not shown).

Discussion. Loss of organic matter during filtration of microorganisms is a serious concern because it results in underestimation of particulate material and overestimation of dissolved material. Conditions of filtration and the type of filter used are known to affect the release of dissolved organic matter (DOM) from natural aquatic microbial communities (Fuhrman & Bell 1985, Nagata & Kirchman 1990). DOM release is usually attributed to rupture of delicate phytoplankton and microzooplankton, but few studies have considered that bacteria might lose cell materials as well. The results of our study showed that major losses of labeled cell materials occurred when live assemblages of bac-

terioplankton were filtered onto 0.2 µm Nuclepore-PC or Poretics-PE filters, and that the magnitude of these losses depended on the incubation time and the type of substrate taken up (Figs. 1 & 2). The significant losses observed with these PC and PE filters (observed also with 0.2 µm Poretics-PC and Anopore-AO filters) probably resulted from the selective loss of dissolved cell components during the final stages of filtration, including the rinse steps (Fig. 4). That PC and PE filters produced results very similar to one another (Fig. 1) was not surprising because the specifications and characteristics of track-etched PC and PE membranes are similar in almost every respect (Poretics Products, Catalog 1997-98). Cells caught on the very flat, hydrophobic surfaces of PC and PE membranes may become desiccated and leaky when the filters become dry at the end of filtration. Leaked bacterial cell materials are probably lost from the filters during the rinse steps which are necessary to wash away non-cell associated radioactive substrates. Our findings are similar to those of Goldman & Dennett (1985), who concluded that certain delicate phytoplankton released DOM if glass fiber filters were allowed to dry during filtration.

In our study, which focused on bacterioplankton, filter membranes that captured cells in, or on, a sponge-like matrix (e.g. PES, MCE and Nylon types) generally had the highest retention of radiolabeled cell materials, and therefore did not appear to cause massive cell leakage. It should be noted, however, that PES filters sometimes retained less labeled cell material than MCE or Nylon filters (Fig 1A, C), therefore we cannot exclude the possibility that some cell leakage occurred with PES filters. We note that, with respect to uptake assays, no objective standard filter material exists, so we can only compare the performance of filter types with one another. Overall, retention of labeled cell materials by PES, MCE and Nylon filters differed by less than 22% in all cases and by less than 10% in most cases, therefore, depending on the needs of the study, each of these filter types may be suitable for uptake determinations. Matrix filters such as these probably hold sufficient water to prevent desiccation of trapped bacterial cells, thereby minimizing loss of cell materials during the filtration/rinsing procedures. Cell protection on PES filters was apparently quite effective because variations in filtration vacuum from 5 to 39 cm Hg had little effect on retention of labeled cell material by these filters (Fig. 5). This was in contrast to PC filters which retained less cell material at higher filtration vacuum (Fig. 5).

Mixed cellulose ester (MCE, cellulose acetate and cellulose nitrate) filters (e.g. Metricel and HA Millipore) are perhaps the most commonly used filters in uptake studies with natural assemblages of microorganisms (e.g. Wright & Hobbie 1966, Coffin 1989, Rich et al. 1996). However, 0.2 µm Nuclepore filters have also been used extensively (Azam & Hodson 1981, Palumbo et al. 1983, Ferguson & Sunda 1984, Griffith et al. 1990). Future studies employing filters for capture of live cells should consider potential differences in retention efficiency and the possible artifactual release of DOM from the bacterioplankton under conditions which subject cells to desiccation, osmotic shock or poisons (see also below).

An important conclusion which can be drawn from the present work is that harsh filtrations or other manipulations (osmotic shock and poisoning) caused substantial loss of dissolved components of bacterioplankton cells while cellular macromolecules remained in filterable form. The time dependence of filtration losses on PC and PE filters (greater relative losses at earlier time points) observed with either ^{14}C-GBT or ^{35}S-DMSP appeared to be related to the fraction of cellular radiolabel that was still in the untransformed state, and therefore dissolved in the cell. Losses with PC and PE filters were greatest (50 to 90 % loss relative to PES filters) during the first few hours of incubations (Figs. 1 & 2), when, in the case of ^{14}C-GBT and ^{35}S-DMSP, 70 to 90% of the radiolabel on the filters was present as the untransformed substrate (Fig. 3). During later phases of incubations (i.e. ~24 h), when untransformed substrate comprised a much smaller fraction (22 to 30%) of the label on the filters (Fig. 3) and when most of the radiolabeled material on filters was in a methanol-insoluble (macromolecule) pool, losses on PC filters were less severe (~20%). The fact that most of the material lost from cells upon filtration through PC filters was dissolved and low molecular weight (i.e. <4000 MW; Table 1), supports the hypothesis that the lost material was mostly untransformed parent compound (^{14}C-GBT in this case). If we assume that the newly acquired radiolabeled substrate acts as a proxy for other soluble cell components, then we can conclude that a major fraction of the dissolved, low molecular weight compounds associated with bacterial cells are lost upon filtration onto PC-type membranes and by procedures which subject cells to osmotic shock or poisoning. The osmotic solutes GBT and DMSP were particularly useful in revealing the loss of this dissolved pool from the cells because these compounds tend to accumulate intracellularly, perhaps to a greater degree than other substrates. We did not measure intracellular pooling of leucine or glucose, but these substrates appeared to be assimilated into TCA insoluble cellular macromolecules more rapidly than GBT or DMSP (Figs. 1 & 2). Simon & Azam (1989) have measured intracellular pools of leucine in marine bacteria, and observed that this pool turns over rapidly during bacterial growth as the leucine is assimilated into cellular proteins.

The exact location (periplasmic or cytosolic) of the easily-lost, dissolved cellular material is presently unknown. The results with cold osmotic shock treatment of cells which had taken up ^{14}C-GBT or ^{14}C-glucose (Fig. 6) suggest that periplasmic and outer membrane (rather than cytosolic) components of bacterial cells might be selectively lost upon filtration onto PC filters. However, this conclusion may not be valid, because of the way we carried out these tests. A key difference in our procedure compared with that of Martinez & Azam (1993) was that we filtered cells onto a membrane before subjecting them to the osmotic shocks while they centrifuged the cells into a pellet, which was resuspended in shock fluid, and then re-centrifuged. We suspect that the spheroplasts generated by the osmotic shock procedure may have been susceptible to breakage or leakage during vacuum filtration. While we cannot draw firm conclusions about the cellular location of material lost during osmotic shock, our results do suggest that the dissolved fraction of cells was lost. Cold osmotic shock of cells on PES filters produced nearly identical results as filtration of the same samples onto PC filters with no osmotic shock (Fig. 5), and this was true when either ^{14}C-GBT or ^{14}C-glucose were the substrates taken up. In addition, we found that simply rinsing PES filters with hypotonic FSW (24 ppt rinse vs 37 ppt natural salinity) caused substantial (40%) loss of radioactivity from PES filters (data not shown). Osmotic shock, therefore appears to be an important factor which can affect retention of cell materials on filters.

The results from the formalin addition experiment (Fig. 7) showed that addition of 2% buffered formalin to terminate uptake incubations can result in serious underestimation of total ^{14}C-GBT uptake. This was evident only when PES filters were used and not when PC filters were used, due to the fact that formalin caused release of ^{14}C label to the same extent as filtration onto PC filters (Fig. 7). Palumbo et al. (1983) concluded that addition of 0.6% formalin to terminate incubations did not affect uptake assays of tritiated amino acids. However, that study utilized 0.2 µm Nuclepore-PC filters, so all uptake may have been underestimated. The results presented here support earlier studies which found that formalin caused losses of labeled (Griffiths et al. 1974) and unlabeled (Wolfe 1996) substrates taken up by marine bacteria. Formalin does not appear to cause cell disruption because fixed bacterial cells are usually well defined when examined by epifluorescence microscopy on Nuclepore filters (Hobbie et al. 1977, Porter & Feig 1980). Formalin must therefore cause release of soluble compounds from the cells, which could include cytosolic and periplasmic components. The similar losses of radioactivity observed with filtration of live cells onto PC filters and of formalin treated samples onto PES filters suggests that these treatments cause the same pool of material to be lost from cells.

In conclusion, matrix-type filters including PES, MCE and Nylon appear to be the most suitable for capture of live cells with minimal release of dissolved cell components. Some differences in performance of these filters (lower retention on PES filters in some cases) makes it advisable that researchers compare selected filter-types and evaluate their suitability for the intended work. Most importantly, this study provided evidence that marine bacteria release a large fraction of their dissolved cellular material upon harsh filtration (e.g. onto PC-type filters), osmotic shock, or formaldehyde treatment. The phenomenon of DOM release may be a general response of stressed bacterial cells and of some concern to microbial ecologists studying material cycling by bacterioplankton.

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