Vol. 16: 251–264, 1999 | AQUATIC MICROBIAL ECOLOGY Aquat Microb Ecol | Published February 24

Effects of filtration on bacterial activity and picoplankton community structure as assessed by flow cytometry

Josep M. Gasol*, Xosé A. G. Morán

Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC, Passeig Joan de Borbó, s/n, E-08039 Barcelona, Spain

ABSTRACT: We used flow cytometry of autofluorescent and Syto13-stained marine bacteria and the uptake of tritiated leucine to assess the effects of different filter types on picoplankton abundance, community structure and bacterial activity in the filtrate. Coastal and oceanic samples from the NW and SW Mediterranean and the Atlantic coast of Galicia were size-fractionated using polycarbonate (PC), mixed cellulose esters (CE), aluminum oxide (IM) and glass fiber (GF) filters of 0.2 to 1.2 µm nominal pore size from different brands. Flow cytometry of Syto13-stained marine bacteria and autofluorescent photosynthetic prokaryotes was used to analyze picoplankton abundance, size structure and community composition before and after filtration. We combined this capability with the detection of the changes in cell-specific heterotrophic activity in the filtrates. We found that the CE filters retained picoplankton better than the PC filters. The PC filters did not discriminate prokaryotes according to size as much as the GF and the CE filters did. In our hands the IM filters were no better than the CE filters. Bacterial activity in the filtrates increased in the PC and in the CE filtrates and this stimulation of bacterial activity was more important in the less productive environments. We conclude that care must be taken when PC filters are used for generating bacteria-free water, and that the use of CE 0.22 µm filters is the best way of creating picoplankton-free water. However, the picoplankters that will go through the filters may encounter increased nutrient levels.

KEY WORDS: Filters · Filtration · Bacteria · Picoalgae · Flow cytometry · Bacterial activity

INTRODUCTION

Filtration is a method universally used in aquatic microbial ecology for a variety of purposes: the determination of particulate primary production (e.g. Anderson 1965); the retention of particulate organic matter or particles for bulk pigment or mass determinations, or for measuring light absorption (e.g. Yentsch 1957, Nagata 1986, Venrick et al. 1987, Stramski 1990); the concentration of bacteria for enumeration (Kepner & Pratt 1994) or for acid nucleic diversity studies (e.g. Lee & Fuhrman 1991); the generation of bacteria-free and predator-free water used, for example, for dilution-growth experiments (Kirchman et al. 1982, Wright & Coffin 1984, Li & Dickie 1985); fractionation for size-specific metabolism estimates (e.g. Azam & Hodson 1977, Palumbo et al. 1984, Griffith et al. 1990, Joint et al. 1993) or the separation of functional components of the microbial plankton (Larsson & Hagström, 1982) and the corresponding uncoupling of the microbial food web (e.g. Wikner & Hagström 1988, del Giorgio et al. 1996b). In general, the uses of filtration can be divided into 2 principal groups: those where the researcher is interested in the filtrate and those where the researcher is interested in the material collected in the filter. In the first case, the researcher wants the filtration to be as effective as possible, and also wants the filtrate not to be substrate enriched. This is more so if this filtrate will later be used e.g. for growing organisms in an environment as similar as possible to the unfiltered original. In the second case, the researcher

*E-mail: pepgasol@icm.csic.es

wants all the organisms of interest to be unselectively retained by the filter, or at least the researcher would like to know in what way filtration by a given filter type modifies community structure of what was collected in the filter.

For the purposes of separation and concentration, researchers can choose among a wide variety of filter types, made of both inorganic and organic compounds. Most frequently the filters used are either made of glass fiber (GF), polycarbonate (PC), inorganic aluminum polymers (IM) or mixed cellulose esters (CE). The GF/F filters and those of 0.2 µm pore size are commonly used to retain all the particles and are thus the filters of choice for retaining bacteria. The GF/C filters and those of sizes 0.6 up to 1.2 µm are used to discriminate pico- from nanoplankton, and are thus the filters of choice for separating bacteria from their predators and from algae.

However, it is well known that filtration is not always as effective as would be desired. Filtration at 0.2 µm may let bacteria through (Zimmerman 1977, Tabor et al. 1981, McDonnell & Hood 1982, Li & Dickie 1985; Stockner et al. 1990). It is also known that filtration at 0.6 or 0.8 µm may both let flagellates through (Fuhrman & McManus 1984, Cynar et al. 1985) and size-select the community composition of the bacterial community, and several studies have evidenced shifts in community taxonomic composition after filtration and enclosure of bacteria (Ferguson et al. 1984, Lee & Fuhrman 1991). Furthermore, some filters may retain dissolved organic matter (Maske & Garcia-Mendoza 1994), thus making the sample less rich in nutrients, while other filters may allow the breakage of some fragile cells, thus enriching the sample (Ferguson et al. 1984, Goldman & Dennett 1985, Fuhrman & Bell 1985). To what extent, then, can conclusions about the natural community be made by scaling the results obtained from the development of a filtrated population if this population was size-selected and enriched with organics?

In all the kinds of experiments and measurements cited above it is very desirable to have a good knowledge of the composition (in terms of number, size, and taxonomic identity) of the picoplankton community that crosses (or is retained by) the filter and also of the effects of filtration on the activity of the filtered organisms. In spite of the desirability of these controls, they are seldom performed in routine studies and experiments, or the results are not reported. This is in part caused by the time-consuming methods required to measure the stock, composition and activity of the bacterial community. Flow cytometry of natural bacteria, however, allows an extremely fast determination of bacterial abundance, relative size distribution and community composition (in terms of photo- vs chemotrophy) in sample volumes smaller than 1 ml and in less than 15 min (Olson et al. 1993, Li et al. 1995, del Giorgio et al. 1996a, Marie et al. 1996, Lebaron et al. 1998).

Here, we have tested the effects of filtration through some commonly used small pore-sized filters (0.2 to 1.2 µm) of the above-mentioned 4 types on picoplankton abundance, community structure and activity. Even though there is a large body of literature (reviewed in the paragraph above) offering data on the retentiveness of each type of filter for each kind of purpose, most of the studies reported comparisons between some types of filters only. Also, very few studies have compared the effects of filtration on community structure (in terms of size distribution), and even fewer studies have assayed the level of stimulation of bacteria produced by filtration. We thought it was interesting to systematically compare all the cited filters not only looking at total number of organisms, but also referring to some level of community structure and activity. The performance of the filters was assessed by flow cytometry of marine samples collected at 2 coastal NW Mediterranean localities, at 2 oceanic stations in the SW Mediterranean, and on a transect in the Atlantic Galician coast covering from estuarine to open-ocean waters.

MATERIAL AND METHODS

Water samples. Experiments were performed with surface water from the NW Mediterranean, offshore of Masnou and Blanes harbors (roughly 1.5 miles, 2.4 km, offshore; water-column depth approx. 20 m), from shelf-slope water of the Alborán Sea (SW Mediterranean) and from the Atlantic off the Galician coast along a gradient from the open sea to the Ría de Vigo estuary. In Masnou #1 and Blanes experiments, the water had been collected the day before, left in 2 l Nalgene bottles in a controlled-temperature chamber kept at the *in situ* temperature and sampled at intervals for 24 h. Irradiance was kept at approx. 250 µmol m^{-2} s^{-1} under a 12:12 h light-dark regime. For Masnou #2, #3 and the SW Mediterranean and Atlantic experiments, water was filtered within 1 h after collection. The measurements reported in Fig. 2 correspond to samples from the MESOMED experiment in mesocosms placed 1 mile (1.6 km) offshore of the Blanes coast. In these experiments, water was enriched with logarithmically increased nutrient additions every 2 d and for a total period of 3 wk. On Days 7 and 15 we filtered 500 ml samples from some of the treatments through PC 0.8 µm filters and counted and sized the bacteria that crossed the filters with the methods described below.

Filters used. The filters used in the experiments were of 4 different types: glass fiber (GF), mixed cellulose esters (acetate and nitrate; CE), polycarbonate (PC) and Al_2O_3 inorganic polymers (IM). GF filters used were Whatman GF/F (nominal pore size 0.7 µm) and Whatman GF/C (nominal pore size 1.2 µm). CE filters used were Millipore RAWP (nominal pore size 1.2 µm), Millipore AAWP (nominal pore size 0.8 µm) and Millipore GSWP (nominal pore size 0.22 µm). Polycarbonate filters used were Costar Nuclepore (pore size 0.8 µm) and Millipore GTTP (pore size 0.2 µm). IM filters used were Whatman Anodisc 0.2 µm filters. All filters were 25 mm in diameter. SEM pictures of some of these different types of filters can be seen in Jones et al. (1989) and in Stockner et al. (1990).

Filtration. When filtering large volumes through a filter, the probability of cell breakage and clogging ('overloading', Sheldon 1972) increases with the amount of water filtered (see also Lee et al. 1995). Because we wanted to detect the effects of filtration without the interference of leakage and clogging, we decided to use the smallest filters widely available (25 mm) and the smallest possible volumes. Thus, the volumes filtered were 15 ml in the Atlantic experiments and ranged from 5 to 20 ml in the Mediterranean experiments. These volumes are common for counting bacteria or measuring bacterial and algal production in coastal areas and are in the order of magnitude for extracting chlorophyll (e.g. Kemp et al. 1993, Kepner & Pratt 1994). These volumes were found not to release dissolved free amino acids in a study that compared different kinds of filters and different filtered volumes (Fuhrman & Bell 1985). A test for the possible effects of the volume filtered (in the scale studied) was performed in 2 of the Atlantic experiments, in which different volumes (5, 10 and 25 ml) were used. Filtration pressure was kept below 80 mm Hg in all experiments. Although these protocols have been recommended for avoiding cell breakage during filtration, our results suggest that they may not be enough (see 'Results').

Flow cytometric determination of bacterial and algal abundance. 1.2 ml samples were immediately fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final concentration) and stored frozen in liquid nitrogen. The samples for chemotrophic bacteria were later unfrozen, stained with Syto13 (Molecular Probes) at 2.5 µM and run through a flow cytometer. We used a B&D FACScalibur bench machine with a laser emitting at 488 nm. Samples were run at low speed (approx. 18 µl min^{-1}) and data were acquired in log mode until around 10000 events had been acquired. We added 10 µl per sample of a 10^6 ml^{-1} solution of yellow-green 0.92 µm Polysciences latex beads as an internal standard. Bacteria were detected by their signature in a plot of side scatter (SSC) versus FL1 (green fluorescence). In a plot of FL1 versus FL3 (red fluorescence) we could differentiate photosynthetic prokaryotes from non-photosynthetic prokaryotes. This method is based on that published by del Giorgio et al. (1996a) as adapted to seawater and the choice of stain among other possible blue nucleic acid dyes does not seem to be important for counting (Lebaron et al. 1998, author's unpubl. results). The average fluorescence of the bacterial population, as normalized to that of the beads, is a rough approximation of bacterial size (del Giorgio et al. unpubl.). These authors have shown that there is a relatively good relationship between relative FL1 and size (range analyzed: 0.028 to 0.072 $µm^3$) in which:

$$\text{Size } (\mu m^3) = 7.5 \times 10^{-3} + 0.11 \text{ Relative FL1}, \quad N = 20, R^2 = 0.66$$

Between 2 successive samples, we always let distilled water run through the cytometer for at least 1 min.

For the detection of photosynthetic prokaryotes, we ran the sample again without staining, and at high speed (approx. 44 µl min^{-1}). We added 10 µl per sample of a 10^5 ml^{-1} solution of yellow-green 0.92 µm Polysciences latex beads as an internal standard. *Synechococcus* were detected by their signature in a plot of orange fluorescence (FL2) versus red fluorescence (FL3). *Prochlorococcus* had a lower FL3 signal and no FL2 signal. Eukaryotic picoplankton had higher FL3 signals and no FL2 signals. Other algae had much higher FL3 signals. The settings were adapted for each sample as the populations and their fluorescence may change from sample to sample. Further details can be obtained from Olson et al. (1993).

The following populations and terms are used throughout the text: picoplankton includes chemotrophic ('heterotrophic') bacteria plus phototrophic bacteria (cyanobacteria and prochlorophytes) and picoeukaryotes. Flow cytometry of unstained samples allowed us to differentiate the phototrophic picoeukaryotes from the 2 groups of phototrophic bacteria. Flow cytometry of Syto13-stained samples let us enumerate all the chemotrophic bacteria. Prochlorophytes and *Synechococcus* were detected in both runs of the sample, but the numbers presented are those obtained with the unstained sample. The references to HDNA (high DNA) and LDNA (low DNA) bacteria (see below) refer always to HDNA and LDNA chemotrophic bacteria. We sometimes use the term picoalgae to refer to all the phototrophic picoplankters.

Bacterial activity. We estimated bacterial activity as the rate of radioactive leucine incorporation by bacteria using the method of Kirchman et al. (1985) as described in Kirchman (1993) but in Eppendorf vials as suggested by Smith & Azam (1992). We added 40 nM leucine to quadruplicate vials. This was the saturating

concentration as found in concentration-dependent incorporation experiments performed on the Blanes coast and on several occasions in Atlantic waters. The commercial leucine solution was brought to 1 µM and mixed with nonradioactive leucine at 10% hot:90% cold. We used 2 TCA-killed samples as controls. The Eppendorfs were incubated in the dark and at temperatures as close as possible to the original ones. Incubations lasted 60 to 150 min in accordance with the results of linearity experiments. After incubation, the samples were killed with 50% TCA and processed as described in the references cited above. The measurements of bacterial activity were done in subsamples of the unfiltered and the filtered volume and are expressed as activity in the filtrate as a % of the activity in the untouched sample.

RESULTS

Some characteristics of the samples used are shown in Table 1. Note the differences in chlorophyll and bacterial abundance in the 4 experiments that were performed on the NW Mediterranean coast. The increase in temperature from winter to fall is accompanied by an increase in bacterial abundance and a decrease in chlorophyll levels (see also Vaqué et al. 1997). The water samples from the Galician coast were also very different: bacterial abundance decreased from the Ría (estuary) to the open sea, as did *Synechococcus* and picoeukaryotes. Prochlorophytes, which were undetectable in the Ría, increased to up to 10% of the total bacterial numbers in the open sea sample. The 2 Alborán sea samples (SW Mediterranean) were quite similar except for chlorophyll concentration and bacterial abundance.

Flow cytometric signatures of bacteria and their size

The flow cytometric detection of Syto13-stained marine bacteria shows the presence of at least 2 distinct populations (Fig. 1) that have also been observed by other researchers working with different stains (Li et al. 1995, Jellett et al. 1996, Marie et al. 1997). We decided to call these 2 populations HDNA (high DNA bacteria) and LDNA (low DNA bacteria) because in marine samples Syto13 stains preferentially DNA (Guindulain et al. 1997). The HDNA population roughly corresponds to the Type 2 bacteria and the LDNA to the Type 1 bacteria described by Li et al. (1995). The %HDNA bacteria is, thus, equivalent to the 'active cell index' of Jellett et al. (1996).

We obtained indirect evidence that the intensity of fluorescence staining of the bacteria is related to cell size (Fig. 2): we followed nutrient additions in mesocosms and found that bacterial green fluorescence and bacterial abundance increased with the amount of nutrients added. The amount of bacteria that crossed a PC 0.8 µm filter decreased linearly with increased nutrient additions, with higher bacterial abundance, and with greater cell fluorescence (Fig. 2). The more nutrients added, the larger the bacteria; and the larger the bacteria, the less bacteria crossed the filter. Thus, the %HDNA value can be used as an index of community size structure of the bacterial population.

Filtration effects on abundance

Tables 2 & 3 present the results obtained at the different stations. In some areas we only assayed some of the filters.

Table 1. Stations sampled and the algal and bacterial concentrations in the water used for the experiments. All water was taken from 5 m. SW Mediterranean samples were taken during Cruise MATER1HESP. Atlantic waters were taken during Cruise INCOCEANO'97. BD: below detection (<1000 cells ml^{-1}). Chl: chlorophyll *a* at the beginning of the experiment and measured as retained in GF/F filters. Proc: prochlorophytes. Syn: *Synechococcus*. Pico: picoeukaryotes

	Date	Position	Temp. (°C)	Chl (µg l^{-1})	Bacteria (cells ml^{-1})	Proc (cells ml^{-1})	Syn (cells ml^{-1})	Pico (cells ml^{-1})
NW Mediterranean								
Masnou #1	14/1/97	41° 28' N, 2° 19' E	14	2.84	2.9×10^5	BD	BD	–
Blanes	13/3/97	41° 40' N, 2° 47' E	14	1.04	2.0×10^6	BD	2.9×10^4	–
Masnou #2	18/3/97	41° 28' N, 2° 19' E	15	–	2.6×10^6	BD	1.5×10^5	–
Masnou #3	29/9/97	41° 28' N, 2° 19' E	23	0.44	1.4×10^6	2.2×10^3	2.4×10^4	5.5×10^3
SW Mediterranean (Alborán Sea)								
Shelf slope 1	2/5/98	36° 23' N, 4° 14' W	16	0.34	1.7×10^6	BD	8.4×10^3	1.8×10^3
Shelf slope 2	4/5/98	36° 59' N, 4° 16' W	17	1.88	7.4×10^5	1.2×10^3	8.1×10^3	3.5×10^3
Atlantic								
Ría de Vigo estuary	2/5/97	42° 14' N, 8° 47' W	18	2.52	2.3×10^6	BD	5.2×10^4	2.1×10^4
Coastal	1/5/97	42° 9' N, 8° 55' W	17	1.07	1.7×10^6	1.9×10^3	4.9×10^4	1.1×10^4
Open sea	29/4/97	42° 7' N, 9° 26' W	16	0.38	5.8×10^5	6.6×10^4	4.3×10^4	5.0×10^3

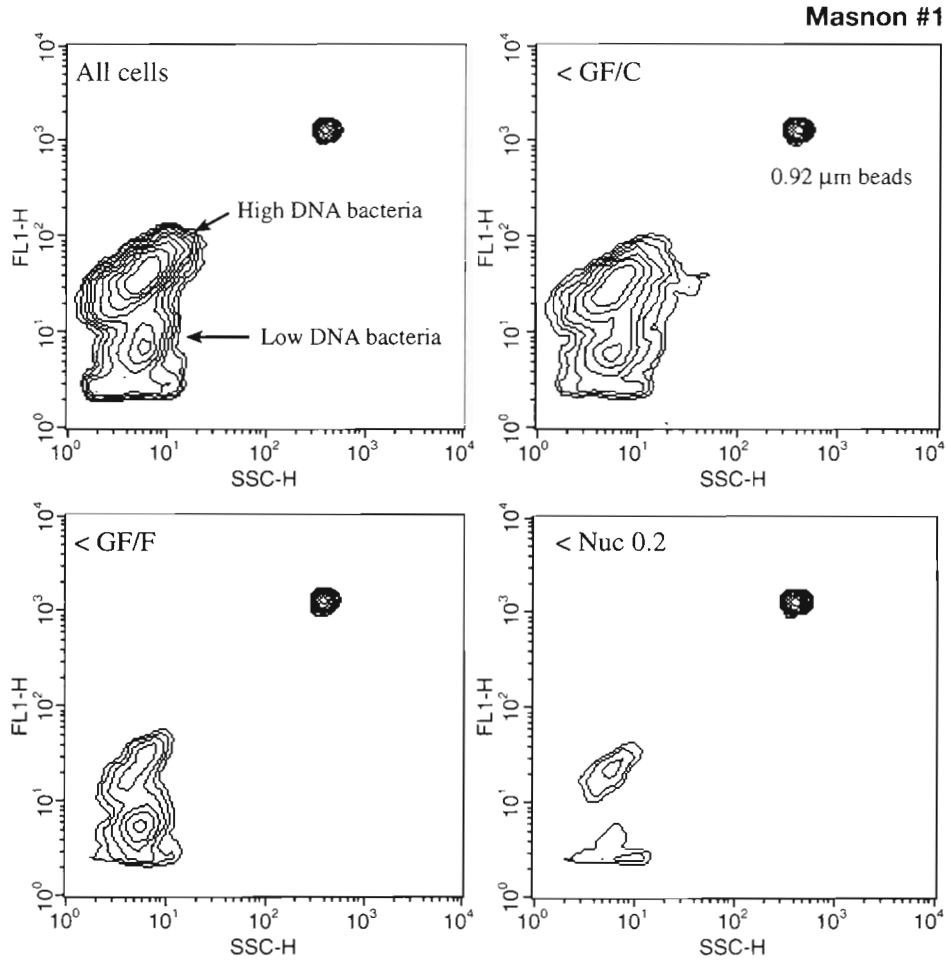

Fig. 1. Plot of side scatter (SSC) versus green fluorescence (FL1) for a Syto13-stained natural sample from the coast of Masnou (NW Mediterranean) before and after filtration through glass fiber GF/C, GF/F and polycarbonate 0.2 µm filters. Note the flow cytometric signature of the 0.92 µm yellow-green Polysciences beads, and the identification of the 2 bacterial populations, which we call High DNA and Low DNA

The glass fiber filters GF/C, which are sometimes used to retain chlorophyll and to separate picoplankton from nanoplankton, let through from 32 to 91% of the initial bacterial abundance, 60% on average (Table 2, Fig. 1). From 3 to 17% of the *Synechococcus* and 13 to 21% of the picoeukaryotes went through that filter, while at the oceanic station more than 30% of the prochlorophytes crossed it (Table 3). The GF/F filters, with nominal sizes of 0.7 µm, let through from 7 to 71% of the bacteria, on average 25% (Table 2). Almost no picoeukaryotes, less than 2% of the *Synechococcus* and 3% of the prochlorophytes passed the GF/F filters.

The PC 0.8 µm filter let through from 50 to 100% of the chemotrophic bacteria (average 81%). The filter retained a large amount of the Mediterranean *Synechococcus* but let through most of the Atlantic cyanobacteria. Most *Prochlorococcus* and picoeukaryotes crossed this filter. The PC 0.2 µm filter let from 1 to 23% (average 3%) of the bacteria through and <10% of all groups of algae in all stations.

The retention efficiency of the CE filters varied accordingly to their pore sizes: the 1.2 µm filter retained 74% of the bacteria from the estuarine sample and 27% of the oceanic bacteria (50% on average, Table 2). Between 62 and 79% of the bacteria were retained by the CE 0.8 µm filter (average 31% bacteria crossed the filter) and from 92 to 99% of the bacteria

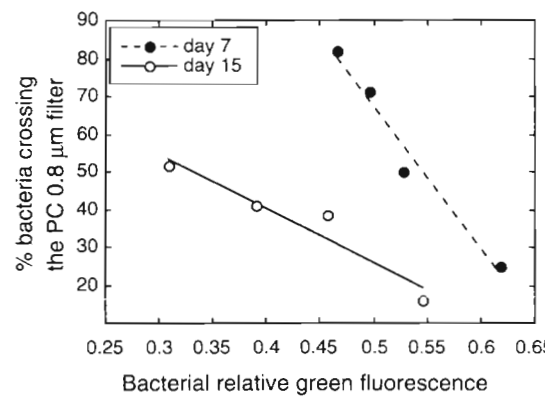

Fig. 2. Relationships between average bacterial green fluorescence and the % of the bacterial population crossing the polycarbonate 0.8 µm filter at 2 different times during a mesocosm experiment (MESOMED '97) in Blanes bay. The 4 points of each line correspond to increased nutrient additions (see text)

Table 2. Total chemotrophic bacterial concentrations in the filtrates of the different stations expressed as % of the unfiltered value plus SE of 4 replicates in all samples except in SW Mediterranean, Blanes and Masnou #3 (2 replicates) and Masnou #1 (5 replicates). GF: glass fiber, PC: polycarbonate, CE: mixed cellulose esters, IM: inorganic membranes. Community composition in terms of %HDNA of each filtrate. The average for HDNA is the average value after normalizing each filtrate composition to the unfiltered sample. –: not determined or below detection

Filter type	Estuary (2B2)	Atlantic Coastal (3B4)	Open sea (3B8)	Masnou #1	NW Mediterranean Blanes	Masnou #2	Masnou #3	SW Mediterranean Shelf slope 1	Shelf slope 2	Averages
All bacteria										
Unfiltered	100	100	100	100	100	100	100	100	100	–
<GF/C	55.7 ± 5.6	79.9 ± 2.8	91.1 ± 6.9	52.0 ± 3.8	44.5 ± 5.7	64.3 ± 10.7	32.4 ± 0.8	–	–	60 ± 8
<GF/F	30.1 ± 9.4	22.9 ± 0.8	71.0 ± 10.4	10.3 ± 1.4	6.5 ± 1.8	22.4 ± 4.6	9.5 ± 0.6	20.8 ± 2.6	31.9 ± 0.4	25 ± 6
<PC 0.8 µm	76.9 ± 7.8	106.7 ± 6.7	89.3 ± 8.4	–	–	–	50.4 ± 2.4	–	–	81 ± 12
<PC 0.2 µm	13.2 ± 3.4	2.2 ± 0.2	22.6 ± 5.4	2.7 ± 0.5	4.6 ± 1.6	6.0 ± 0.5	2.3 ± 1.2	1.1 ± 0.9	17.2 ± 15.2	8 ± 3
<CE 1.2 µm	26.4 ± 1.5	52.3 ± 3.7	73.3 ± 1.9	–	–	–	–	–	–	50 ± 14
<CE 0.8 µm	20.6	38.2 ± 1.7	35.0	–	–	–	–	–	–	31 ± 5
<CE 0.22 µm	3.5 ± 1.4	0.7 ± 0.1	7.5 ± 1.4	–	–	–	–	2.2 ± 0.1	2.9 ± 1.9	4 ± 1
<IM 0.2 µm	–	–	–	–	–	–	–	19.5 ± 1.9	17.1 ± 2.0	19 ± 2
% HDNA bacteria										
Unfiltered	65	71	43	81	81	62	68	55	63	–
<GF/C	61 ± 1	64 ± 1	30 ± 2	73 ± 2	67 ± 5	57 ± 1	65 ± 3	–	–	88 ± 4
<GF/F	36 ± 4	36 ± 6	23 ± 3	52 ± 2	27 ± 9	27 ± 3	57 ± 2	34 ± 2	38 ± 2	56 ± 5
<PC 0.8 µm	65 ± 3	68 ± 1	35 ± 1	–	–	–	66 ± 4	–	–	94 ± 4
<PC 0.2 µm	44 ± 12	40 ± 10	21 ± 5	83 ± 1	73 ± 4	49 ± 5	50 ± 11	65 ± 6	57 ± 2	81 ± 7
<CE 1.2 µm	61 ± 2	63 ± 1	32 ± 2	–	–	–	–	–	–	86 ± 6
<CE 0.8 µm	42	55 ± 1	31	–	–	–	–	–	–	71 ± 4
<CE 0.22 µm	33 ± 8	58 ± 8	22 ± 4	–	–	–	–	71 ± 4	62 ± 2	82 ± 15
<IM 0.2 µm	–	–	–	–	–	–	–	48 ± 11	37 ± 10	73 ± 14

were retained by the CE 0.22 µm filter. The different CE filters were quite efficient at retaining the algae (Table 3). The CE 0.22 µm filters gave the best results in terms of retention efficiency, followed by the PC 0.2 µm. The IM Anopore filters let through 19% of the bacterial population on average.

Table 2 also documents a tendency for more bacteria to be retained in the estuarine sample and less bacteria to be retained by filters in the oceanic samples. Thus, while almost all bacteria went through the GF/C filter at the most oceanic station, only 56% of the bacteria went through the filter in the estuarine sample of the Atlantic transect. The GF/F filter let through 71% of the bacterial population from the oceanic station and 30% in the estuarine sample. The CE 1.2 µm filter let through 26% of the estuarine bacteria, 52% of the coastal bacteria and 73% of the oceanic bacteria. However, this pattern did not hold for the PC filters nor for the smaller CE filters.

Community composition

In general, all types of filters retained the HDNA bacteria more effectively than the LDNA bacteria (Table 2). Thus, the effect of filtration on the %HDNA bacteria was a reduction of this value. The %HDNA was reduced on average by 12% after passage through the GF/C filter and by 44% after passage through the GF/F filter (Table 2). This reduction in the relative proportion of HDNA bacteria is consistent with the reduction in cyanobacterial numbers that we described in Table 3 and can also be seen in Fig. 1. The GF filters were more selective (decreased the %HDNA more) than the PC filters: the %HDNA was quite similar to that of the original sample after passage through the PC 0.8 µm filter (94%) and the PC 0.2 µm (81%) which was slightly selective. That this filter was much less size selective than the GF/F can be seen in Fig. 1: the flow cytometric signal of HDNA bacteria going through the PC 0.2 µm filter is clear. The CE and the IM filters were only slightly selective.

Table 3. Algal concentrations in the filtrates at 2 different stations on and off the Galician coast and at 3 of the Mediterranean coastal stations expressed as % of the unfiltered value plus SE of 4 replicates (where available), except for Blanes and Masnou #3 where values are averages of 2 replicates. PC: polycarbonate. CE: mixed cellulose esters. –: not determined or below detection

Filter type	Atlantic		NW Mediterranean		
	Estuary (2B2)	Open sea (3B8)	Blanes	Masnou #2	Masnou #3
Synechococcus					
Unfiltered	100	100	100	100	100
<GF/C	6.8 ± 1.9	16.9 ± 6.6	3.7 ± 0.5	14.2 ± 4.0	2.9 ± 1.5
<GF/F	0.2 ± 0.02	1.2 ± 0.6	2.0 ± 1.4	0	0.1 ± 0.1
<PC 0.8 µm	97.1 ± 13.9	80.4 ± 6.0	–	–	8.9 ± 0.3
<PC 0.2 µm	10.4 ± 3.6	10.2 ± 3.7	6.9 ± 3.3	4.1 ± 4.0	2.8 ± 2.3
<CE 1.2 µm	4.9 ± 1.7	14.5 ± 3.6	–	–	–
<CE 0.8 µm	0.3	1.5	–	–	–
<CE 0.22 µm	2.1 ± 1.0	2.2 ± 0.8	–	–	–
Prochlorococcus					
Unfiltered	–	100	–	–	–
<GF/C	–	33.6 ± 10.7	–	–	–
<GF/F	–	3.3 ± 1.7	–	–	–
<PC 0.8 µm	–	88.9 ± 6.1	–	–	–
<PC 0.2 µm	–	10.0 ± 2.7	–	–	–
<CE 1.2 µm	–	36.6 ± 3.6	–	–	–
<CE 0.8 µm	–	8.4	–	–	–
<CE 0.22 µm	–	2.0 ± 0.9	–	–	–
Picoeukaryotes					
Unfiltered	100	100	–	–	–
<GF/C	20.6 ± 3.5	13.1 ± 3.6	–	–	–
<GF/F	0.4 ± 0.03	0.3 ± 0.1	–	–	–
<PC 0.8 µm	108.3 ± 15.8	80.6 ± 8.6	–	–	–
<PC 0.2 µm	11.0 ± 4.1	9.9 ± 3.7	–	–	–
<CE 1.2 µm	13.1 ± 2.2	13.3 ± 2.4	–	–	–
<CE 0.8 µm	1.7	4.9	–	–	–
<CE 0.22 µm	1.8 ± 0.9	0.4 ± 0.2	–	–	–

Another effect of filtration on community composition concerns the relationship between picoalgae and bacteria. As an example, the ratio *Prochlorococcus*: chemotrophic bacteria was 11.3 in the oceanic Atlantic sample before filtration. After passage through PC 0.8 µm it still remained at 11.3, but after passage through GF/C it became 4.3 and after passage through GF/F it became 0.5. Similarly, passage through PC 0.2 µm and through CE filters decreased the value to ~4. *Prochlorococcus* were retained more than chemotrophic bacteria by all filters except the PC 0.8 µm.

Bacterial activity in the filtrates

In one of the experiments performed with Masnou water we measured bacterial activity in the filtrates several times during an *in situ* incubation. Bacterial abundance and activity increased in the unfiltered sample in the first 24 h of incubation (Fig. 3A) although activity increased before abundance did. It seemed clear that bacteria first incorporated leucine, the %HDNA bacteria increased, and then bacteria divided with a concomitant increase in bacterial abundance and a reduction in specific activity and %HDNA (Fig. 3A). The amount of bacteria let through by the GF/C filter decreased from 53% to 34% in the first 12 h while the amount of activity in the filtrate increased from 12% to 38%. Thus, activity was retained more efficiently than abundance and the increase in bacterial size that we presumed from the temporality of the changes in activity and abundance was reflected in a differential retention of activity and abundance by the filters. From 3 to 8% of the bacterial abundance crossed the GF/F filter. However, activity in the filtrate was as low as 0.5 to 3%. Because we know that, in bacteria, activity varies with cell size (Bird & Kalff 1993, Gasol et al. 1995), these results are consistent with the size selective filtration effect of this kind of filters discussed above. While 3% of the bacteria and 2% of the activity went through the PC 0.2 µm filter at the beginning of the incubation, these values increased in the first 12 h to 6% of the cells and 9% of the activity. At 24 h, only 1% of the cells and 0.5% of the activity passed the filter.

Changes in the specific activity of the bacteria in the filtrates can be interpreted as due to nutrient enrichment of the bacteria after having been filtered. At the

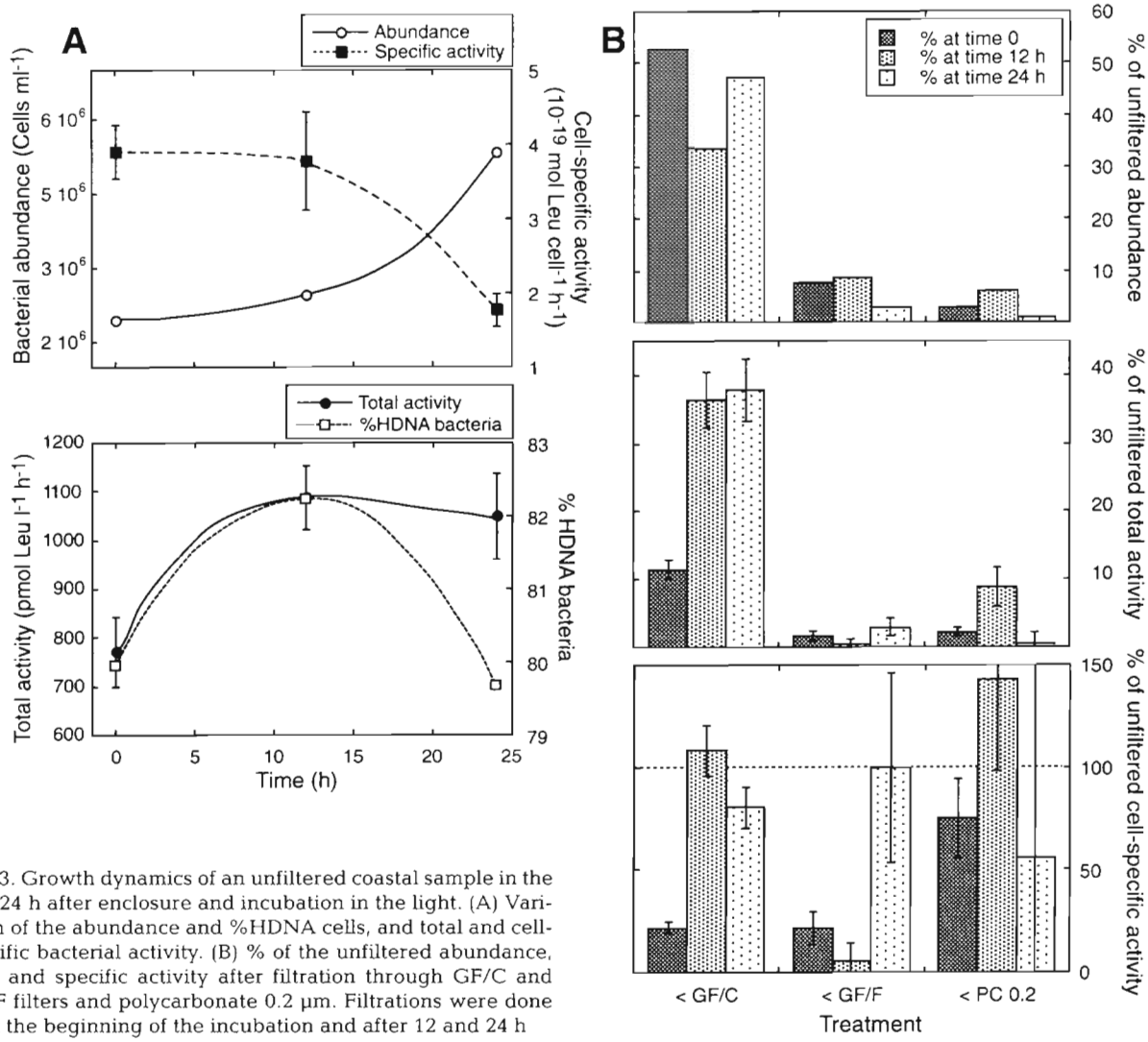

Fig. 3. Growth dynamics of an unfiltered coastal sample in the first 24 h after enclosure and incubation in the light. (A) Variation of the abundance and %HDNA cells, and total and cell-specific bacterial activity. (B) % of the unfiltered abundance, total and specific activity after filtration through GF/C and GF/F filters and polycarbonate 0.2 µm. Filtrations were done at the beginning of the incubation and after 12 and 24 h

start of the incubation, the bacteria in the filtrates had lower specific activities, more so in the GF filtrates than in the PC filtrates. However, at 12 h (Fig. 3B) those bacteria that crossed the filters increased their specific activities specially in the PC 0.2 µm filtrates, a phenomenon that we associate to nutrient enrichment caused by the filtration.

To further investigate the patterns shown in that experiment, we measured bacterial activities of the filtrates in 2 of the Atlantic samples and in several Mediterranean samples. As in the experiments reported in the previous section, the amount of bacteria crossing the filter was variable among stations (Table 4). Bacterial activity (leucine uptake rates) ranged from 8 to 190 pmol l^{-1} h^{-1}, with maximal values in the estuarine sample and minimal ones in the Alborán sea samples (Table 4). The cell-specific activity of the unfiltered estuarine sample was 40 times that of the unfiltered SW Mediterranean sample. At the different stations, however, the GF/C filtrate had ~20% of the unfiltered sample activity and the GF/F filtrate had ~2% of the unfiltered sample activity. Cell-specific activity values decreased in the different GF filtrates, an observation which is consistent with the decrease in the proportion of HDNA bacteria in these filtrates (Table 2) and in the assumed higher degree of activity of HDNA versus LDNA bacteria. Filtration through the GF filters seemed to act equally in the different samples, and seemed not to enrich the filtrates (as specific activity in the filtrates was always below 100%).

Bacterial activity in the PC filtrates was higher than expected. The 0.8 µm filter let through 145% of the activity of the unfiltered sample in the Atlantic oceanic water while 100% of the bacteria had gone through the filter (Table 4). In the coastal Mediterranean sam-

Table 4. Bacterial total and specific activities in the filtrates at 2 stations off the Galician coast (3B8 and 2B2) and at 3 Mediterranean stations. A volume of 15 ml was filtered by each filter type. PC: polycarbonate. CE: mixed cellulose esters. IM: inorganic membranes

	Abundance (% of initial cells in filtrate)	Total activity (pmol leu l^{-1} h^{-1}) Mean	SE	% change	Specific activity (10^{-19} mol leu $cell^{-1}$ h^{-1}) Mean	% change
Atlantic (open sea, 3B8)						
Unfiltered	100	24.14	0.66	100	0.42	100
<GF/C	76	4.97	1.97	21	0.11	26
<GF/F	31	0.42	1.50	2	0.02	5
<PC 0.8 µm	101	34.94	3.14	145	0.59	140
<PC 0.2 µm	9	6.94	0.29	29	1.32	314
<CE 1.2 µm	70	64.82	3.74	269	1.59	379
<CE 0.8 µm	35	24.21	5.90	100	1.19	283
<CE 0.22 µm	5	3.56	1.67	15	1.22	290
Atlantic (estuarine, 2B2)						
Unfiltered	100	190.13	9.41	100	0.82	100
<GF/C	69	48.04	3.20	25	0.30	37
<GF/F	13	4.34	1.05	2	0.15	18
<PC 0.8 µm	100	193.49	10.11	102	0.83	101
<PC 0.2 µm	7	7.60	1.97	4	0.47	57
<CE 1.2 µm	29	114.54	6.27	60	1.69	206
<CE 0.8 µm	21	20.31	3.40	11	0.43	52
<CE 0.22 µm	8	10.15	0.69	5	0.59	72
NW Mediterranean (coastal, Masnou #3)						
Unfiltered	100	83.62	11.00	100	0.33	100
<GF/C	32	16.56	3.18	20	0.20	61
<GF/F	10	0.92	0.51	1	0.04	12
<PC 0.8 µm	50	54.66	3.06	65	0.43	130
<PC 0.2 µm	2	2.87	0.70	3	0.51	155
<CE 1.2 µm	19	7.11	3.08	9	0.15	45
<CE 0.8 µm	16	4.30	2.35	5	0.11	33
<CE 0.22 µm	1	0.87	0.34	1	0.28	85
SW Mediterranean (shelf slope 1)						
Unfiltered	100	8.26	1.52	100	0.05	100
<GF/F	21	0.48	0.12	6	0.01	28
<PC 0.2 µm	2	0.80	0.05	10	0.22	460
<CE 0.22 µm	2	0.44	0.18	5	0.12	243
<IM 0.2 µm	20	0.57	0.02	7	0.02	36
SW Mediterranean (shelf slope 2)						
Unfiltered	100	10.65	0.68	100	0.14	100
<GF/F	32	2.48	0.91	23	0.11	73
<PC 0.2 µm	17	2.33	1.75	22	0.29	199
<CE 0.22 µm	3	0.57	0.13	5	0.51	357
<IM 0.2 µm	17	1.55	0.19	15	0.13	87

ple, 65% of the activity crossed the filter for 50% of the bacteria. Thus, cell-specific activity was higher in the bacteria that passed the filters than in those that had not been filtered in the coastal Mediterranean sample and in the oceanic sample (up to 130 and 140% of the unfiltered). The activity of the cells that passed the PC 0.2 µm filter was high in the oceanic samples (30% for 9% of the cells). Filtration through polycarbonate filters produced a stimulation of bacterial activity in the filtrates that was especially relevant in the oceanic stations: cell-specific production increasing up to 4.5-fold from the untouched sample to the <PC 0.2 µm one (Table 4).

The stimulation of bacterial activity after filtration was more apparent in some of the CE filtrates. The stimulation was higher in the open ocean sample (arriving at cell-specific activities >3 times those of the untouched sample), and less apparent in the other samples (Table 4). As these filters selected against HDNA bacteria (Table 2), the variations of cell-specific activity in the CE 0.22 µm filtrates of the other 2 samples (72 to 85% of the unfiltered specific activity, Table 4) also indicate some nutrient enrichment of the filtrate. Filtration through the IM Anopore filters did not stimulate bacterial production. From 17 to 20% of the cells crossed the filter, and 7 to 15% of the activity was measured in the filtrate.

DISCUSSION

We have presented data that confirm previous reports of the incomplete effect of filtration on phototrophic and chemotrophic bacterial abundance. We further describe that the effects of filtration produce not only a reduction in abundance, but also changes in community structure and, perhaps more importantly, stimulation of cell-specific bacterial activity in some kinds of samples.

Filtration effects on abundance

It is well known that the nominal sizes of the filters used in ecological studies cannot be taken as an absolute standard (Brock 1983). Microorganisms, specially flagellates (Cynar et al. 1985), are quite flexible and can squeeze through pores of sizes smaller than their diameter. Furthermore, the track-etched polycarbonate membranes can have holes several times wider than the stated pore size (Stockner et al. 1990).

Our results are comparable to those found by other studies: our GF/C filters let through a large fraction of the bacteria, from 32 to 90%. Nagata (1986) found in Lake Biwa that 28% of the bacteria crossed that kind of filter. This indicates that at some sites a large fraction of the bacterial community can be retained by these filters and the bacterial community crossing a GF/C filter may be quite different from the original. The GF/F filter retained bacteria with varying efficiencies: from 7 to 71% of the bacteria crossed the filters. These values compare to the 35 to 47% of bacteria crossing the filters reported by Lee & Fuhrman (1987) and Lee et al. (1995). It is interesting to note the variation in filtration efficiency detected on the Atlantic transect: the GF/C filters retained 44% of the bacteria in the estuary, 20% on the coast, and 10% in the open ocean (Table 2). Similarly, the GF/F filters retained ~74% of the bacteria in the estuary and on the coast, but only 29% at the open ocean station. We think that the differences among stations are due to their different trophic condition (Table 1) which probably translates into different average bacterial size and a different degree of attachment of bacteria to particles. The studies by Lee & Fuhrman (1987) and Lee et al. (1995) were done in coastal Atlantic and Antarctic stations, and their values compare well with what we found at our coastal stations. The GF filters performed much better in the retention of phototrophic bacteria: the GF/C filter let through 3 to 17% of the *Synechococcus* and 34% of the *Prochlorococcus*, but the GF/F filter let through 0 to 2% of the *Synechococcus* and 3% of the *Prochlorococcus*. These values are between those found by Li (1990; 8% of *Synechococcus* crossing the GF/F filter) and those found by Chavez et al. (1995; <1% of phototrophic bacteria crossing the GF/F), but tend to side with the latter authors in the recognition that the GF/F filters are an adequate method of collecting all chlorophyll-containing cells for bulk measurements. Our results, however, show that the GF filters are not adequate for collection of all heterotrophic bacteria, specially at oligotrophic oceanic stations.

Polycarbonate 0.8 µm filters let through most of the bacteria (50 to 100%) and of the picoalgae (>80%). Polycarbonate 0.2 µm filters were relatively effective at removing bacteria: from 2 to 26 % of the bacteria were found in the filtrates. Again, the largest amount of bacteria crossing the filter was found in the most oceanic station. Many authors have found varying numbers of bacteria after PC 0.2 µm filtration (Hobbie et al. 1977, Zimmermann 1977) with extremes at 0 % (Wikner & Hagström 1988) and up to 13% (Li & Dickie 1985). The presence of large holes in those kinds of filters (Stockner et al. 1990) and the way in which they are produced (Brock 1983) suggest that different batches and brands may have different retention efficiencies. As Li (1990) has pointed out, however, the presumption that water filtered through PC 0.2 µm filters is void of bacteria will never be warranted. We also encountered that from 3 to 10% of the cyanobacteria and of the prochlorophytes crossed the PC 0.2 µm filter. These values are slightly larger than those cited by Li (1990) and Chavez et al. (1995), which were ~1%, but compare well with some of the results cited by Li (1986) for experiments performed with Bedford Basin water and with different cyanobacterial strains. Jones et al. (1989) proposed the use of inorganic polymer Anopore filters as substitutes for polycarbonate track-etched filters because they detected 21 to 33% higher counts in Anopore than in Nuclepore filters. However, our results did not replicate theirs. In our hands the IM Anopore filters let through more than 15% of the bacteria and were less efficient than the CE 0.22 and the PC 0.2 µm filters (Table 2).

One of the suggestions of Li & Dickie (1985) was that because the CE membranes 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)', they should retain particles much smaller than their nominal pore size and, after filtration through CE 0.22 µm membranes, few bacteria should be found. We are not aware of any test of this suggestion other than the data presented here and the amount of total Coulter particles that Stockner et al. (1990) saw after filtration through 0.22 µm CE filters (7%, this may have included things other than bacteria).

The 1.2 µm CE filters let through on average 50% of the heterotrophic bacteria, 37% of the prochlorophytes

and 5 to 15% of the cyanobacteria. The CE 0.22 filter retained most of the heterotrophic bacteria (8% of the most oceanic sample crossed the filter) and of the phototrophic bacteria (<2%). Li (1986, 1990) checked the efficiency of CE filters and found very good retention efficiencies (<0.1% crossing the filter) for most of the samples and strains analyzed, although one specific cyanobacterial strain seemed to cross the CE filters easily too. Even though these CE 0.22 µm filters were not completely effective at retaining all bacteria, they were always better than the PC 0.2 µm and the IM 0.2 µm filters tested (Tables 2 & 3).

We chose to test the performance of the different filters with a very small volume because we were concerned not only with the amount of cells crossing the filters but also with the nutrient environment these cells would find. In the range of volumes analyzed, we did not detect any consistent trends with changes in the volume filtered (range 5 to 25 ml on a 25 mm filter). We are, thus, confident that we minimized clogging of the filters and excess vacuum pressures. The volumes and pressures used were based on the recommendations of Fuhrman & Bell (1985), who detected an increase in DFAA in the filtrates when higher volumes or pressures were used. It is possible that the effects that we have presented would have been less important if larger volumes had been filtered, at least in the GF and CE filters, but we did not investigate these effects further.

Filtration effects on community structure

Community structure in bacterioplankton communities can be analyzed at different levels: metabolic (chemo- vs phototrophy, prochlorophytes and *Synechococcus* vs other bacteria; Campbell et al. 1994); size distributions (Gasol et al. 1997); and group-specific genomic probes (Amann et al. 1995) or RNA or DNA genetic analysis (e.g. Pedrós-Alió 1993). The type of community structure analysis performed with flow cytometry, as presented here, combines the metabolic and the size approaches, and allows one to discriminate groups of bacteria with different main metabolisms and with different levels of nucleic acids that can be correlated to size.

Figs. 1 & 2 suggest that the population that we call HDNA bacteria is comprised of cells that are larger than those comprising the population that we call LDNA bacteria. The negative relationship between the relative fluorescence of the bacteria and the % of bacteria crossing a PC 0.8 µm filter (Fig. 2) supports the good relationship obtained by del Giorgio et al. (unpubl.) between cell size, as measured with image-analysis of DAPI preparations, and relative green fluorescence, which is the basis of separation between HDNA and LDNA. Whether the amount of green fluorescence is strictly proportional to cell size, and whether it is constant from flow cytometer to flow cytometer are matters of discussion, but even if they are not constant these values can be related to cell size in a relative way, thus allowing for the fast comparison of samples treated in the same way. The %HDNA can thus be used as a simple and fast index of community size structure.

Filtration through GF/C, and specially through GF/F filters, reduced selectively the amount of HDNA bacteria, forcing the %HDNA value to decrease. Fig. 1 shows an example where the filtrate through the PC 0.2 µm filter has quite a similar structure (in terms of %HDNA) to the original unfiltered sample. Lee et al. (1995) described similar changes in bacterial size spectra produced by filtration through GF/F filters. Furthermore, and for samples that harbor varying numbers of phototrophic and chemotrophic bacteria, filtration may alter the biomass distribution between both groups. For example, filtration of the oceanic Atlantic sample through GF/F let 71% of the chemotrophic bacteria but only 3% of the *Prochlorococcus* through, while filtration through CE 1.2 µm let 73% of the chemotrophic and 37% of the phototrophic bacteria through. Community composition, thus, was greatly affected by filtration through these types of filters.

The different effects of the different filters are probably related to their morphologies: the pore membrane PC filters act as sieves but have a certain number of wide holes that may let through a small representation of the initial community with little, or no, size discrimination (at least for bacteria). These filters may change their effectiveness depending on cell shape and rigidity, volume filtered and filtration pressure (Brock 1983). The GF and the CE filters act more like true filters that can trap particles both on the surface but also within the polymer and the chance of the presence of larger 'holes' is much lower. These filters may always retain particles proportionally to their size, and may even retain particles that are smaller than the factory-reported filter size.

Filtration effects on bacterial activity

The amount of heterotrophic activity that crossed ~1 µm filters was one part of the evidence that led researchers to admit that the ocean was harboring a very active submicron microbial community (Williams 1970, Azam & Holm-Hansen 1973). However, and to the best of our knowledge, it has never been shown how bacterial activity changes after filtration. As we have shown above, the CE and the GF filters tend to size-select the bacterial community and relatively

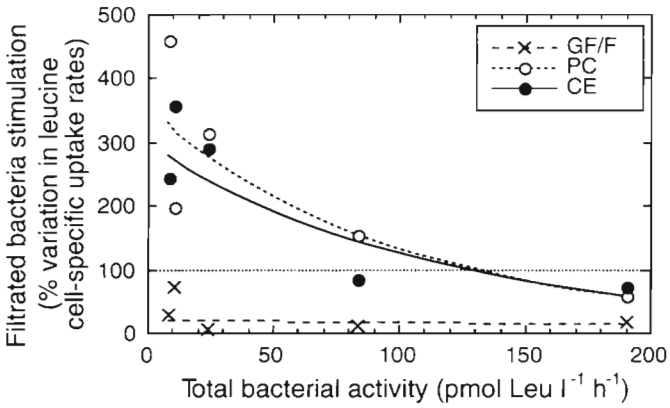

Fig. 4. Variation of the response of bacteria to filtration depending on the origin of the sample and the type of filter used. Different samples were ordered according to system productivity. Leucine uptake rates were taken as a measure of system productivity. The response was evaluated as the increase in cell-specific uptake rates produced by filtration through CE 0.22 µm (●), PC 0.2 µm (○) or GF/F (×) filters

'enrich' the filtrate with LDNA (smaller) bacteria. Because it has been shown that activity is largely related to size (Bird & Kalff 1993, Gasol et al. 1995; but see Posch et al. 1997 for an exception) we would tend to expect lower specific bacterial activities in the filtrates than in the unfiltered sample. However, Ferguson et al. (1984) showed increased levels of amines in filtrates, and Fuhrman & Bell (1985) described increased levels of dissolved free amino acids (DFAA), a source of N and C for bacteria, after filtration through GF, CE and PC filters if volumes filtered or pressures used were large. For a long time, some researchers have also warned that the ^{14}C method for measuring extracellular carbon release by phytoplankton could confound cell breakage with true release (Arthur & Rigler 1967, Goldman & Dennett 1985). This evidence would suggest that filtration is often associated with cell breakage, and that bacteria that cross the filters should find a relatively enriched environment.

Cell-specific bacterial activity decreased in the filtrates from GF/C (26 to 61%) and GF/F (5 to 73%, Table 4) filters. These results are consistent with the selection against HDNA bacteria that these filters produce (see Fig. 1) and the assumed higher activity of this type of bacteria. They also indicate that the bacteria crossing the filters did not seem to find increased nutrient levels. Bacterial activity after filtration through PC 0.2 µm was higher than the unfiltered values in 4 out of 5 stations. Even filtration through PC 0.8 µm increased specific activity in 2 of the samples. Both PC filters seemed, thus, to increase the nutrient levels for the bacteria. The CE filters did increase bacterial activity in the filtrates of the estuarine and specially the oceanic stations. In these oligotrophic (Table 1) stations, specific activity in the filtrates was up to 3.8 times higher than in the unfiltered replicate, in spite of the low volumes and low pressures used for filtration.

Bacteria from oligotrophic stations seemed to be much more sensitive to the effects of filtration than those of estuarine or coastal stations: when we plotted the % increase in cell-specific leucine uptake rates produced by different kinds of filters we realized that at the oligotrophic stations this increase was much higher than at the more productive stations (Fig. 4) and it only held for the PC and the CE filters, not for the GF filters. Among others, Kirchman (1990) has provided evidence of carbon limitation of bacteria in the subarctic Pacific, and Rivkin & Anderson (1997) of inorganic nutrient limitation in the tropical Atlantic. We cannot, however, discard the possibility that the differences in the response of coastal versus oceanic bacterioplankton communities to filtration presented in Fig. 4 and Table 4 were due, not to different degrees of nutrient limitation but to different compositions of the nano- and microplankton communities (Table 1) with different susceptibilities to breakage during filtration. Different amounts or types of POC and particulate nutrients could also explain the difference. Another factor that cannot be discarded from our results is that the increase in bacterial production in the filtrates through 1.2 and 0.8 µm filters could be generated by the trophic uncoupling between bacteria and their predators and not by increased nutrient levels after cell breakage, as Hopkinson et al. (1989) have suggested.

Because flow cytometric analysis of picoplankton community structure is a fast and simple way of checking for the effects of filtration, we recommend the systematic use of this or another method of analyzing community structure. The results of these controls, as well as the filter types, volumes filtered, and pressures applied, should be reported in the methods sections of the studies.

Acknowledgements. We thank S. Canut for providing the usual guidance, the people in the IIM and on board 'Cornide de Saavedra' for help, ambiance, and octopuses; Ramon Massana for the leucine measurements in the Alborán Sea and to Marta Estrada and some reviewers for helpful comments. X.A.G.M. was a recipient of a FPI fellowship. The work reported here was supported by grants MAS3-CT95-0016 to C. Marrasé, CYCYT AMB 94-1019 and 94-0853 to M. Estrada, DGICYT PB91-075, MAR95-1901-C03-03 and MAST3-CT97-0154 to C. Pedrós-Alió, and MAS3-CT96-0045 to C. M. Duarte.

LITERATURE CITED

Amann RJ, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Anderson GC (1965) Fractionation of phytoplankton communities off the Washington and Oregon coasts. Limnol Oceanogr 10:477–480

Arthur CR, Rigler FH (1967) A possible source of error in the ^{14}C method of measuring primary productivity. Limnol Oceanogr 12:121–124

Azam F, Hodson RE (1977) Size distribution and activity of marine microheterotrophs. Limnol Oceanogr 22:492–501

Azam F, Holm-Hansen O (1973) Use of tritiated substrates in the study of heterotrophy in seawater. Mar Biol 23: 191–196

Bird DF, Kalff J (1993) Protozoan grazing and size-activity structure in limnetic bacterial communities. Can J Fish Aquat Sci 50:370–380

Brock TD (1983) Membrane filtration: user's guide and reference manual. Science Tech., Madison, WI

Campbell L, Nolla HA, Vaulot D (1994) The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol Oceanogr 39:954–961

Chavez FP, Buck KR, Bidigare RR, Karl DM, Hebel D, Latasa M, Campbell L, Newton J (1995). On the chlorophyll a retention properties of glass-fiber GF/F filters. Limnol Oceanogr 40:428–433

Cynar FJ, Estep KW, Sieburth JMcN (1985) The detection and characterization of bacteria-sized protists in 'protist-free' filtrates and their potential impact on experimental marine ecology. Microb Ecol 11:281–288

del Giorgio PA, Bird DF, Prairie YT, Planas D (1996a) The flow cytometric determination of lake bacterioplankton abundance using the nucleic acid stain SYTO-13. Limnol Oceanogr 41:783–789

del Giorgio PA, Gasol JM, Vaqué D, Mura P, Agustí S, Duarte CM (1996b) Bacterioplankton community structure: protists control net production and the proportion of active bacteria in a coastal marine community. Limnol Oceanogr 41:1169–1179

Ferguson RL, Buckley EN, Palumbo AV (1984) Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microbiol 47:49–55

Fuhrman JA, Bell TM (1985) Biological considerations in the measurement of dissolved free amino acids in seawater and implications for chemical and microbiological studies. Mar Ecol Prog Ser 25:13–21

Fuhrman JA, McManus GB (1984) Do bacteria-sized marine eukaryotes consume significant bacterial production? Science 224:1257–1260

Gasol JM, del Giorgio PA, Massana R, Duarte CM (1995) Active versus inactive bacteria: size-dependence in a coastal marine plankton community. Mar Ecol Prog Ser 128:91–97

Gasol JM, Massana R, Pedrós-Alió C (1997) Bacterial size structure as a method to analyze communities. In: Martins MT, Sato MIZ, Tiedje JM, Hagler LCN, Döbereiner J, Sanchez PS (eds) Progress in microbial ecology. Sociedade Brasileira de Microbiologia/ICOME, São Paulo, p 155–160

Goldman JC, Dennett MR (1985) Susceptibility of some marine phytoplankton species to cell breakage during filtration and post-filtration rinsing. J Exp Mar Biol Ecol 86: 47–58

Griffith PC, Douglas DJ, Wainright SC (1990) Metabolic activity of size-fractionated microbial plankton in estuarine, nearshore, and continental shelf waters of Georgia. Mar Ecol Prog Ser 59:263–270

Guindulain T, Comas J, Vives-Rego J (1997) Use of nucleic acid dyes Syto-13, ToTo-1, and YoYo-1 in the study of *Escherichia coli* and marine prokaryotic populations by flow cytometry. Appl Environ Microbiol 63:4608–4611

Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225–1228

Hopkinson CS, Sherr B, Wiebe WJ (1989) Size fractionated metabolism of coastal microbial plankton. Mar Ecol Prog Ser 51:155–166

Jellett JF, Li WKW, Dickie PM, Boraie A, Kepkay PE (1996) Metabolic activity of bacterioplankton communities assessed by flow cytometry and single carbon substrate utilization. Mar Ecol Prog Ser 136:213–225

Joint I, Pomroy A, Savidge G, Boyd P (1993) Size-fractionated primary productivity in the northeast Atlantic in May-July 1989. Deep-Sea Res 40:423–440

Jones SE, Ditner SA, Freeman C, Whitaker CJ, Lock MA (1989) Comparison of a new organic membrane filter (Anopore) with a track-etched polycarbonate membrane filter (Nucleopore) for direct counting of bacteria. Appl Environ Microbiol 55:529–530

Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) (1993). Handbook of methods in aquatic microbial ecology. Lewis Pub, Boca Raton, Florida

Kepner RL Jr, Pratt JR (1994) Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microb Rev 58:603–615

Kirchman DL (1990) Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. Mar Ecol Prog Ser 62:47–54

Kirchman DL (1993) Leucine incorporation as a measure of biomass production by heterotrophic bacteria. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis Pub, Boca Raton, p 509–512

Kirchman DL, Ducklow H, Mitchell R (1982) Estimates of bacterial growth from changes in uptake rates and biomass. Appl Environ Microbiol 44:1296–1307

Kirchman DL, K'nees E, Hodson R (1985) Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl Environ Microbiol 49:599–607

Larsson U, Hagström Å (1982) Fractionated phytoplankton production, exudate release and bacterial production in a Baltic eutrophication gradient. Mar Biol 67:57–70

Lebaron P, Parthuisot N, Catala P (1998) Comparison of blue acid dyes for flow cytometric enumeration of bacteria in aquatic systems. Appl Environ Microbiol 64:1725–1730

Lee S, Fuhrman JA (1987) Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl Environ Microbiol 53:1298–1303

Lee SH, Fuhrman JA (1991) Species composition shift of confined bacterioplankton studied at the level of community DNA. Mar Ecol Prog Ser 79:195–201

Lee SH, Kang YC, Fuhrman JA (1995) Imperfect retention of natural bacterioplankton cells by glass fiber filters. Mar Ecol Prog Ser 119:285–290

Li WKW (1986) Experimental approaches to field measurements: methods and interpretation. In: Platt T, Li WKW (eds) Photosynthetic picoplankton. Can Bull Fish Aquat Sci 214:251–286

Li WKW (1990) Particles in 'particle-free' seawater: growth of ultraphytoplankton and implications for dilution experiments. Can J Fish Aquat Sciences 47:1258–1268

Li WKW, Dickie PM (1985) Growth of bacteria in seawater filtered through 0.2 µm Nucleopore membranes: implications for dilution experiments. Mar Ecol Prog Ser 26: 245–252

Li WKW, Jellett JF, Dickie PM (1995) DNA distributions in planktonic bacteria stained with TOTO or TO-PRO. Limnol Oceanogr 40:1485–1495

Marie D, Vaulot D, Partensky F (1996) Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and Pico-

Green for flow cytometric analysis of marine prokaryotes. Appl Environ Microbiol 62:1649–1655
Marie D, Partensky F, Jacquet S, Vaulot D (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. Appl Environ Microbiol 63:186–193
Maske H, Garcia-Mendoza E (1994) Adsorption of dissolved organic matter to the inorganic filter substrate and its implications for ^{14}C uptake measurements. Appl Environ Microbiol 60:3887–3889
McDonnell MT, Hood MA (1982) Isolation and characterization of ultramicrobacteria from a Gulf Coast estuary. Appl Environ Microbiol 43:566–571
Nagata T (1986) Carbon and nitrogen content of natural planktonic bacteria. Appl Environ Microbiol 52:28–32
Olson RJ, Zettler ER, DuRand MD (1993) Phytoplankton analysis using flow cytometry. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis Pub, Boca Raton, p 175–186
Palumbo AV, Ferguson RL, Rublee PA (1984) Size of suspended bacterial cells and association of heterotrophic activity with size fractions of particles in estuarine and coastal waters. Appl Environ Microbiol 48:157–164
Pedrós-Alió C (1993) Diversity of bacterioplankton. Trends Ecol Evol 8:86–90
Posch T, Pernthaler J, Alfreider A, Psenner R (1997) Cell-specific respiratory activity of aquatic bacteria studied with the tetrazolium reduction method, cyto-clear slides, and image analysis. Appl Environ Microbiol 63:863–873
Rivkin R, Anderson MR (1997) Inorganic nutrient limitation of oceanic bacterioplankton. Limnol Oceanogr 42:730–740
Sheldon RW (1972) Size separation of marine seston by membrane and glass-fiber filtres. Limnol Oceanogr 17:494–498
Smith DC, Azam F (1992) A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^{3}H-leucine. Mar Microb Food Webs 6:107–114

Editorial responsibility: William Li, Dartmouth, Nova Scotia, Canada

Stockner JG, Klut ME, Cochlan WP (1990) Leaky filters: a warning to aquatic ecologists. Can J Fish Aquat Sci 47: 16–23
Stramski D (1990) Artifacts in measuring absorption spectra of phytoplankton collected in a filter. Limnol Oceanogr 35: 1804–1809
Tabor PS, Ohwada K, Colwell RR (1981) Filterable marine bacteria found in the deep sea: distribution, taxonomy, and response to starvation. Microb Ecol 7:67–83
Vaqué D, Blough HA, Duarte CM (1997) Dynamics of ciliate abundance, biomass and community composition in an oligotrophic coastal environment (NW Mediterranean). Aquat Microb Ecol 12:71–83
Venrick EL, Cummings SL, Kemper CA (1987) Picoplankton and the resulting bias in chlorophyll retained by traditional glass-fiber filters. Deep-Sea Res 34:1951–1956
Wikner J, Hagström Å (1988) Evidence for a tightly coupled nanoplanktonic predator-prey link regulating the bacterivores in the marine environment. Mar Ecol Prog Ser 50: 137–145
Williams PJleB (1970) Heterotrophic utilization of dissolved organic compounds in the sea. I. Size distribution of population and relationship between respiration and incorporation of growth substrates. J Mar Biol Assoc UK 50: 859–870
Wright RT, Coffin RB (1984) Measuring microzooplankton grazing on planktonic marine bacteria by its impact on bacterial production. Microb Ecol 10:137–149
Yentsch CS (1957) A non-extractive method for the quantitative extraction of chlorophyll in algal cultures. Nature 179: 1302–1304
Zimmermann R (1977) Estimation of bacterial number and biomass by epifluorescence microscopy and scanning electron microscopy. In: Rheinheimer G (ed) Microbial ecology of a brackish water environment. Springer-Verlag, Berlin, p 103–120

Submitted: January 5, 1998; Accepted: July 22, 1998
Proofs received from author(s): December 28, 1998