Vol. 11: 65–77, 1996 | AQUATIC MICROBIAL ECOLOGY Aquat Microb Ecol | Published August 29

Cycling of marine dissolved organic matter. I. An experimental system

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ABSTRACT: The degradation of dissolved organic carbon (DOC), together with bacterial growth and mortality, was studied in a multi-stage flow-through system. Particle-free sterile seawater was used as growth substrate and fed into vessels containing bacteria and viruses with or without flagellates. The residence time spanned from 30 to 1800 h. The rate of net organic carbon consumption decreased 3 orders of magnitude with increasing residence time (from 0.45 to 0.001 µM C h^{-1}). Interestingly, we found a significantly higher DOC consumption in the presence of flagellates (1.3 to 10 times higher). Viruses were abundant in all culture vessels (1.7 to 6.5 × 10^{10} l^{-1}), indicating active virus production within the system. The number of nucleoid-containing bacteria decreased with increasing residence time from 68% of the total count at the shortest residence time to 30% at the longest residence time. Also, with bacteria only, the total number of bacteria decreased at the longest residence time. We argue that with a long residence time the internal cycling of organic matter increased and that the fraction of viable bacteria decreased substantially resulting in a large fraction of 'ghosts'

KEY WORDS: DOM · Degradation · Bacteria · Viability · Remineralization · Virus

INTRODUCTION

Degradation of organic matter in the microbial food web bears a resemblance to the processing of food in a digestive system. It is a mixture of physical and enzymatic processes resulting in products that can be absorbed by osmotrophic organisms. This complex transformation has been the concern of several studies aimed at explaining the distribution of organic matter in the sea (Ducklow 1983, Taylor & Joint 1990, Baretta-Bekker et al. 1994). Judging from seawater culture experiments, only a small fraction of the dissolved organic matter (DOM) in seawater is biologically utilizable (Ammerman et al. 1984, Coffin et al. 1993, Middelboe & Søndergaard 1993, Zweifel et al. 1993). The composition of the utilizable and non-degradable fractions however shows little difference; both fractions contain small and large molecules (Sundh 1992, Keil & Kirchman 1993, Amon & Benner 1994), and carbohydrates and amino acids are important components in both fractions. This paradox has been elegantly solved by the work of Keil & Kirchman (1993), who demonstrated that amino acids and sugars added to seawater can condense to non-degradable DOM. Although this rate is fairly slow, the resulting flow of carbon can support the build-up of a dissolved organic carbon (DOC) pool with a turnover time similar to the deep ocean's mean residence time. It is therefore understandable that at any given time the majority of the reduced organic matter is found as dissolved material <0.2 µm although it originates from particles >0.2 µm (i.e. primary producers).

Azam et al. (1993) have outlined a likely scenario for organic matter degradation. Organized biological structures undergo a gradual transfer to macromolecules through physical action by predators or cell lysis caused by senescence and viruses. Following this structural 'withering', enzymatic attacks on carbohydrates, proteins, lipids and nucleic acids are initiated. The rate of cleavage depends on the complexity displayed by the substrate towards enzymatic

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attack and differs depending on the molecular environment.

Most studies conducted on bacterial degradation of DOC have been concerned with the transfer efficiency of organic matter to higher trophic levels mediated by bacteria assimilating DOC. However, since bacterial mortality is a continuous process in the food web, a constant recycling of DOC is likely. For the carbon cycle this means that cell lysis, release of DOC and subsequent uptake by bacteria will increase the bacterial production but reduce transfer of carbon to higher trophic levels due to bacteria respiring (Bratbak et al. 1992, Fuhrman 1992). This may be even more pronounced in deep waters, where lack of new production forces the organisms to recycle the existing material.

In order to establish a conceptual model of the complex system responsible for DOC degradation in the sea we designed an experimental system to study degradation during a wide range of dilution rates. We argue that steady-state values of rates and state variables to be used in modelling can be obtained by such a system that ideally replicates the increasing turnover times found in a depth gradient. In this paper we report the results of an experiment with a flow-through system harbouring mixed natural bacteria, viruses and protozoan predators in vessels with residence times ranging over 2 orders of magnitude.

MATERIALS AND METHODS

Medium and experimental design. Water for the experiment (200 l) was collected 21 September, 1991, from 4 m depth at a coastal sampling station in the northern Skagerrak (58° 56' N, 11° 45' E). The water temperature was 15°C and the salinity 28‰ on the sampling occasion. A 40 l pressure vessel was filled on deck using a diaphragm pump with an on line prefilter (3 µm Millipore). The water was subsequently filtered through a 0.2 µm filter (Millipore) at 200 mm Hg and transferred to acid rinsed polycarbonate bottles (20 l). The glass dispensers and tubes (silicone) used in the experimental set up were fitted to the bottles and they were autoclaved twice and stored in the dark at 5°C. Water for inoculum was sampled from the same station as the medium and kept at 10°C until the start of the experiment.

The multi-stage flow-through system was constructed as shown in Fig. 1, including 3 stages (designated 0, 1, 2), with different mean residence time. The volume of water was 0.7 l in Stage 0, 2 l in Stage 1 and 10 l in Stage 2. The incoming water was fed drop-wise through a sealed glass tube to prevent back growth (Hagström et al. 1984). Ultra-pure synthetic air (AGA) was passed through a 0.2 µm acrodisc (Gelman) and used to gently force the inflow to the cultures to prevent water column building in the glass tube. The pressure and gas flow were maintained to allow small bubbles to be continuously produced (~1 s^{-1}). The bubbling also served as a 'stirrer' for the culture. Predator-free inoculum was prepared by gravity filtration, 3 times through 0.6 µm polycarbonate filters (MSI) using Nalgene polycarbonate filtration units. Transfer between filtration units was done through the sidearm, without removing the filters, to prevent protozoan transfer. The inoculum was added to the first vessel (B_0). The medium flowed into the first stage (B_0) and the outflow from B_0 was branched into 2 lines. One branch (B_1, B_2) was continued with bacteria only. The second branch (BF_1, BF_2) was inoculated with flagellates, introduced by the addition of 10 ml of 10 µm filtered water to the BF_1 vessel. The bottle was then left standing for 3 d to allow initiation of flagellate growth before the flow was turned on. The experimental system was kept in the dark in a temperature controlled unit at 15°C. Samples from Stages 0 and 2 could either be drawn from a sampling port or collected from an overflow of water from the vessels. In Stage 1, all outflow was transferred to Stage 2 and samples could only be taken from the sampling port. All filters and equipment used in preparing medium and inoculum were soaked in 1 M HCl and thereafter extensively rinsed in ultra-pure water (Millipore Milli-Q) before use.

Virus, bacteria, and flagellate enumeration. Samples for bacterial and flagellate enumeration were preserved with 0.2 µm filtered formaldehyde (4% v/v). A total of 5 to 10 ml of the samples was used for counting bacteria either by 4',6-diamidino-2-phenylindole (DAPI) or acridine orange staining and 10 to 20 ml was used for counting flagellates by proflavin staining (Hobbie et al. 1977, Porter & Feig 1980, Haas 1982). Cells were counted in an epifluorescence microscope

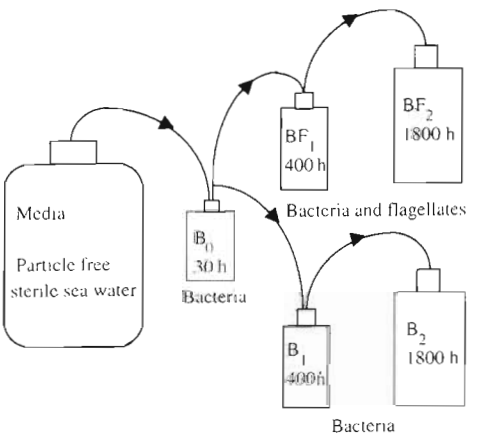

Fig. 1. Schematic view of the experimental flow-through system. For details see 'Materials and methods'

(Zeiss universal). At least 20 fields and 200 objects were counted for bacteria and 40 fields for flagellates. Numbers of nucleoid-containing cells (NucC) were determined essentially as described by Zweifel & Hagström (1995). In the original protocol cells were preserved with sodium azide. From this experiment, samples preserved with formaldehyde were the only ones available. This complicated the procedure since formaldehyde itself forms surfaces that adsorb DAPI. To solve this problem the samples fixed with formaldehyde were filtered, extensively rinsed with particle-free sterile seawater (200 ml) and then stained in the filtration funnels according to the original protocol. The accuracy of this procedure was tested by comparing NucC in samples preserved both with sodium azide and formaldehyde and treated with the respective protocol (data not shown). No significant difference was found although the original protocol is superior in reducing background staining. Bacterial strains were isolated on marine agar plates (DIFCO).

Samples for enumeration of viruses were fixed with glutaraldehyde (2% v/v). The samples were centrifuged and collected on grids as described by Cochlan et al. (1993). The grids were examined in a Zeiss EM-109 transmission electron microscope (TEM) with a magnification of ×50000. A minimum of 100 view fields were counted from at least 5 different grid openings.

Nutrient measurements. Inorganic as well as total nitrogen and phosphorus concentrations were determined using an autoanalyzer (Technicon TRAACS 800) and standard seawater methods (Grasshoff et al. 1983). Baseline and calibration solutions contained sodium chloride at concentrations close to that of the samples in order to minimize refractive index artifacts in the TRAACS flow cell. Using 50 mm flow cells, the detection limits, defined as twice the noise levels, were 0.3 µg l^{-1} for orthophosphate, 0.7 µg l^{-1} for nitrite and nitrate, and 1.0 µg l^{-1} for ammonia. The procedure for the determination of total nitrogen and phosphorus included oxidation of samples with potassium peroxodisulphate in an autoclave at 120°C for 30 min (Grasshof et al. 1983). Dissolved organic nitrogen (DON) was calculated from measurements of total nitrogen in filtered samples (GF/F Whatman filters, precombusted at 600°C for 4 h) minus the sum of inorganic nitrogen. Dissolved organic phosphorus (DOP) was calculated accordingly. Particulate organic nitrogen (PON) and particulate organic phosphorus (POP) were calculated by subtracting total N and P in filtered samples from unfiltered samples.

Dissolved organic carbon (DOC). DOC samples were filtered through 0.2 µm filters (Gelman Supor) using disposable syringes (Terumo) connected to filter holders (Millipore, Swinnex 25) or by use of polycarbonate filtration units (Nalgene). 7.5 ml of filtered water was transferred to polypropylene test tubes (Falcon, 15 ml) and immediately acidified with 100 µl 1.2 M HCl and kept at 4°C until analysis. All materials in contact with the samples, including the filters and filter units, were carefully acid rinsed with 1 M HCl and washed with ultra-pure water (Millipore Milli-Q) before use. DOC was measured with a high temperature carbon analyzer (Shimadzu TOC 5000) using a 4-point calibration curve with potassium biphtalate as standard. Standard solutions were run at each analysis to check for instrumental shifts. Blanks were tested before each analysis by injection of ultra-pure water (Millipore Milli-Q). The total blank (Milli-Q water + system blank) was in all cases less than 10 µM C. Samples were not corrected for this blank. Triplicate injections showed standard deviations of 0 to 2%. In most cases at least 2 individually filtered samples were analysed and showed standard deviations of 0 to 1% from the mean value of triplicate injections.

Analysis of dissolved DNA. Samples (500 ml) were collected from each vessel and filtered through 0.2 µm filters (Gelman Supor). The DNA was precipitated with 2 volumes of 99.5% ethanol. The samples were further treated according to Deflaun et al. (1986). All samples were corrected for losses by the addition of the plasmid pUC18 as an internal standard. DNA recovery was 50 to 90%. The DNA was hydrolyzed and the nucleoside content was determined by high-performance liquid chromatography (HPLC) analysis as described elsewhere (Pomerantz & McCloskey 1990).

Analysis of dissolved lipids. Samples were collected from the outflow of the vessels and filtered through 0.2 µm filters (Gelman Supor). The samples were stored at 4°C and pooled until a sufficient amount of sample was collected. Lipids were extracted twice from 2 to 4 l of filtered sample with 100 ml cyclohexane (Merck, analytical grade). The cyclohexane phases were pooled and evaporated at <40°C. The remainder was collected and dried under N_2 atmosphere, and the sample was dissolved in hexane-tetrahydrofuran (99/1 vol%). The samples were run with HPLC analysis using column LichroCART 125-4 diol and a mobile phase with hexane, tetrahydrofuran, isopropanol and water. The detector was an evaporative light-scattering detector (ELSD, Sedex modell 45) with a Hewlett Packard 3395 integrator. Commercial lipid standards (Larodan fine chemicals AB) were used to evaluate the amount of lipids in the samples. The total lipid content was calculated by summing the amount of hydrocarbons, triglycerides and stereoles. Lipid content by weight was converted to carbon mass by multiplying by a factor of 0.8. Phosphorus content was calculated by assuming a phospholipid content of 2 to 3% of total lipids in biological material.

Particulate organic matter (POM). Samples (200 to 500 ml) were filtered through precombusted (30 min, 200°C) 0.2 µm silver filters (Poretics) at maximum 200 mm Hg. Blanks were prepared by filtering the particle-free medium onto precombusted filters. The total blank (filter + non-specific adhesion of <0.2 µm organic material) made up 3 to 10% of the total sample (filter + non-specific adhesion of <0.2 µm organic material + sample) and were subtracted. The filters were air dried and thereafter stored frozen prior to analysis of carbon and nitrogen with a CHN analyser (Carlo Erba).

Biovolumes and carbon biomass. Bacterial biovolumes were estimated using an automated image analyzis system (Optilab). The flagellate volumes (50 μm^3) were estimated using an ocular micrometer. Bacterial biovolumes were converted to carbon equivalents using a conversion factor of 180 fg C μm^3 (Simon & Azam 1989). Bacterial cells lacking DNA (ghosts) were assumed to have 30% less carbon than intact cells due to loss of internal soluble pools. Flagellate biovolumes were converted to carbon equivalents using a conversion factor of 220 fg C μm^3 (Børsheim & Bratbak 1987).

Bacterial production. Bacterial production was measured using the [3H]thymidine incorporation method (Fuhrman & Azam 1982). Samples (5 ml) were incubated for 1 h with 10 nM [3H]thymidine (final concentration) under *in situ* conditions and processed according to the method description. Thymidine incorporation was converted to cell numbers using a range of $0.2 \times 10^{18} - 2.0 \times 10^{18}$ produced cells per mole incorporated thymidine.

Limiting nutrient assay. Samples (160 ml) were drawn from the culture vessels and filtered through 0.2 µm filters (Gelman Supor). An inoculum was prepared by mixing water from all 5 culture vessels, and the samples were subsequently filtered 3 times through 0.6 µm filters (MSI) to remove predators. The inoculum was added to the filtered water to give an initial number of ~ 5×10^7 cells l^{-1}. The sample from each vessel was then divided into 8 subsamples and enriched with different combinations of C, N, P, leaving an untreated control. Nutrients were added to give an enrichment of 0.6 µM PO_4-P (Na_2HPO_4) and 2 µM NH_4-N (NH_4Cl). Carbon was added as a mixed sucrose + glucose solution to give an increase in carbon of 40 µM C. All solutions used were cross-checked for inorganic nutrient and organic carbon contamination. Yield of bacteria was counted at entry to stationary phase.

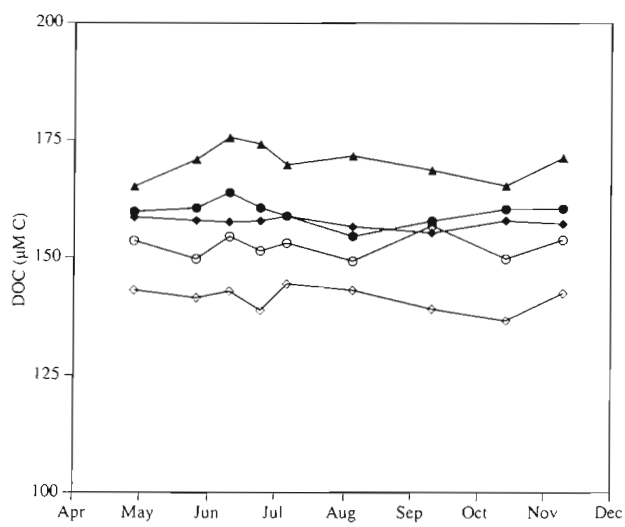

Fig 2. DOC concentrations during the experimental period. (▲) B_0; (●) B_1; (◆) B_2; (O) BF_1; (◇) BF_2

RESULTS

Steady state in the flow-through system

A flow-through system was constructed to reproduce degradation of organic matter in an experiment with increasing turnover time (Fig. 1). The experiment was run for 11 mo and samples were drawn at regular intervals. Table 1 gives a summary of the mean levels and standard deviation of biota, mineral nutrients, and organic carbon during the experimental period. Data from B_0 was collected from 1 wk after the start of the experiment, B_1/BF_1 data were added to the database after 1 mo and B_2/BF_2 data after 4 mo. A regression analysis of the compiled mean values failed to demonstrate significant trends with time ($p > 0.05$) of the measured parameters in any of the 5 different culture-vessels (except for nitrate in the B_2 vessel, see 'Inorganic nutrients' below). In the B_0 vessel bacterial numbers reached 1.10×10^9 l^{-1}, with minor variations around the mean (SD ± 0.14×10^9), which is consistent with *in situ* numbers in surface water in the Skagerrak (Rosenberg et al. 1990). Bacterial and flagellate numbers in the other vessels also displayed the same stable conditions (Table 1). The overall stability of the system can be illustrated by the constant levels of DOC maintained during the experiment (Fig. 2).

Bacteria and flagellates

Different levels of bacterial numbers were reached in accordance with the different conditions in the vessels. The bacterial numbers were slightly higher in B_1 compared with the inflow from B_0. In B_2, with the longest residence time, bacterial counts were lower compared with the inflow from B_1, suggesting cell lysis (Table 1). Due to grazing, bacterial numbers decreased 3-fold in the parallel vessels with flagellates (bodo-

Table 1 Mean values of chemical and biological parameters measured during the time course of the experiment. B_0, B_1 and B_2 defined in 'Medium and experimental design' Standard deviation and (n) given below values. –: no data

	Medium →	B_0 →	B_1 →	B_2	BF_1 →	BF_2
Residence time (h):	Medium	B_0 33	B_1 369	B_2 1809	BF_1 369	BF_2 1809
µM C						
DOC	185 6 (5)	170 4 (10)	160 3 (10)	159 3 (10)	155 2 (10)	143 3 (10)
POC	–	10.8 2 (4)	11.0 2 (2)	10.0 2 (4)	9.9 2 (2)	6.9 1 (4)
Total C	185	181	171	169	165	150
µM N						
NH_4-N	1.30 0.11 (9)	0.39 0.07 (8)	0.48 0.12 (7)	0.54 0.05 (7)	1.00 0.38 (11)	1.02 0.24 (9)
NO_3-N	0.15 0.04 (9)	0.22 0.05 (8)	0.14 0.05 (7)	0.29 0.21 (7)	0.18 0.13 (7)	0.14 0.06 (9)
DON	9.10 0.74 (10)	8.64 0.67 (4)	8.11 0.70 (4)	9.43 0.17 (4)	8.40 0.96 (7)	8.58 1.19 (5)
PON	–	1.21 0.29 (4)	1.23 0.09 (4)	0.64 0.06 (4)	0.77 0.22 (6)	0.56 0.21 (5)
Total N	10.5	10.5	10.0	10.9	10.4	10.3
nM P						
PO_4-P	76 17 (9)	31 8 (16)	28 10 (17)	22 6 (15)	36 10 (17)	28 5 (15)
DOP	117 26 (9)	104 8 (6)	95 11 (6)	97 11 (4)	119 6 (6)	103 13 (4)
POP	–	56 7 (6)	60 6 (6)	38 9 (4)	25 5 (6)	16 2 (4)
Total P	193	191	183	157	180	147
Biota						
Bacteria (10^9 l^{-1})	–	1.10 0.14 (24)	1.45 0.23 (25)	1.10 0.11 (21)	0.58 0.14 (24)	0.41 0.11 (21)
Flagellates (10^5 l^{-1})	–	–	–	–	5.2 1.0 (15)	3.8 0.8 (15)
Bact. vol. (µm³)	–	0.27 0.008 (352)	0.25 0.007 (363)	0.25 0.009 (232)	0.24 0.009 (225)	0.20 0.009 (207)
Total biomass (µM C)	–	4.5	5.1	3.8	2.0	1.4

and ochromonas shape) although the numbers of flagellates were modest.

Bacterial biovolumes decreased significantly with increasing residence time both in the presence and absence of flagellates (Table 1). Compared with B_0, the average individual volume decreased 8% in B_1 and B_2 with no significant difference between the latter two. In the presence of flagellates the average bacterial biovolume decreased by 13% in BF_1 and 25% in BF_2 compared with B_0 and a significant difference was also found between BF_1 and BF_2. The bacterial biovolumes obtained were relatively high compared with bacterial biomass in natural waters but comparable to data from similar experimental systems (Hagström et al. 1984, Andersson et al. 1986). Judged from phenotypic expression of colony-forming bacteria, the experimental system harboured at least 14 different bacterial species that were maintained in all culture vessels (data not shown).

Effect of residence time on DOC degradation

DOC degradation was significantly higher in the B_0 vessel than in any other vessel and the net carbon degradation rate amounted to 0.45 µM C h^{-1} (Table 2). In the consecutive vessels the degradation rates decreased 1 order of magnitude. In the last predator-free vessel (B_2) the DOC degradation was very low but there was also a decrease in particulate organic carbon (POC). Hence, the summed degradation of DOC and POC is also shown in Table 2.

Table 2. Effect of decreasing dilution rate and presence of flagellates on net rates of organic carbon degradation

Dilution rate (h^{-1})	Organic carbon degradation ($\mu M\ C\ h^{-1}$)			
	Bacteria only		Bacteria and flagellates	
	DOC	DOC+POC	DOC	DOC+POC
0.03	0.45	0.45	–	–
0.003	0.03	0.03	0.04	0.04
0.0006	0.0006	0.001	0.006	0.008

Table 3. CNP composition of dissolved lipids and DNA (nucleic acids) in the experimental system. nd: not determined

	Medium	B_0	B_1	B_2	BF_1	BF_2
Lipids						
C in total lipids (μM C)	1.8	1.7	nd	11	nd	3.9
P in phospholipids (nM P)	0.7	0.6	nd	4	nd	1.5
C in phospholipids (nM C)	31	28	nd	181	nd	67
Nucleic acids						
Carbon (nM C)	12	6	0[a]	11	15	19
Phosphorus (nM P)	1.2	0.6	0[a]	1.1	1.5	1.9
Nitrogen (nM N)	2.0	1.0	0[a]	1.8	2.5	3.1

[a]Not detectable

The degradation of DOC was significantly higher in the presence of flagellates both in BF_1 and BF_2 compared with B_1 and B2 (Fig. 3). The total amount of degraded DOC made up 16% of the total DOC pool with bacteria only and 23% in the presence of flagellates.

Dissolved lipids were measured at one occasion at the end of the experiment in the medium, B_0, B_2 and BF_2 vessel (Table 3). The concentrations of dissolved lipids were relatively similar in the medium and B_0 vessel but increased in vessel B_2 and BF_2 (Table 3). Dissolved DNA was degraded in B_0 and B_1 while the concentration increased in the B_2 vessel. In BF_1 and BF_2 the concentration of DNA increased compared with the incoming water.

Inorganic nutrients

The phosphate and ammonium concentration tended to decrease in the bacterial line although the only 2 consecutive vessels with significant difference were the medium and B_0 (Table 4). In BF_1 and BF_2 the concentrations of both phosphate and ammonium were higher than in the corresponding vessels lacking flagellates (Fig. 4). Nitrate concentrations were highly variable, and there were no significant differences in the mean values between bottles except between the medium and B_0, where nitrate increased significantly. In B_2, however, nitrate tended to increase with time, indicating a possible nitrification process.

The N:P ratio of inorganic nutrients in the medium was 19 (Table 1) indicating a surplus of nitrogen compared with a balanced bacterial growth substrate (N:P = 10). The inorganic N:P ratio increased with residence time to 37 in B_2, indicating an increasing P-limitation in the system. In the presence of flagellates the ratio was even higher (42 in BF_2), but this was due to increased ammonium concentration and not to further reduction in phosphate concentration.

The concentration of total nitrogen was constant in all 5 bottles whereas there was a significant decrease in total phosphorus concentration between B_1 and B_2

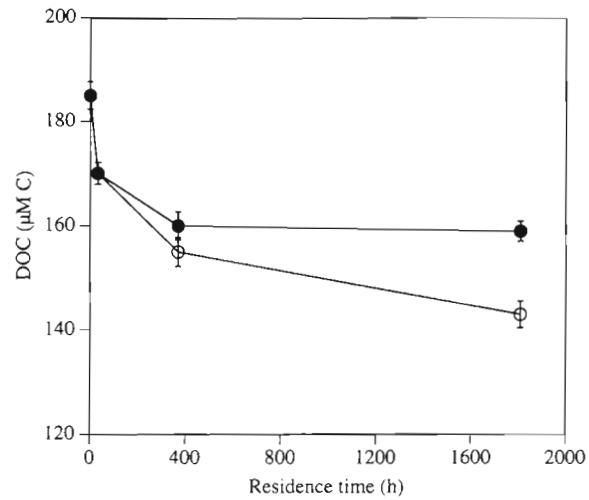

Fig. 3. Effect of increasing residence time and flagellate activity on DOC degradation. (●) Bacteria; (○) bacteria and flagellates

Table 4. Compilation of statistics for ammonium and phosphate. Mean levels measured over the experimental period in the different vessels compared using independent *t*-test. M: medium; n: no significant difference at 95% confidence level

		Ammonium				
Phosphate	Medium	B_0	B_1	B_2	BF_1	BF_2
Medium		$B_0<M$	$B_1<M$	$B_2<M$	n	n
B_0	$B_0<M$		n	$B_2>B_0$	$BF_1>B_0$	$BF_2>B_0$
B_1	$B_1<M$	n		n	$BF_1>B_1$	$BF_2>B_1$
B_2	$B_2<M$	$B_2<B_0$	n		$BF_1>B_2$	$BF_2>B_2$
BF_1	$BF_1<M$	n	$B_1<BF_1$	$B_2<BF_1$		n
BF_2	$BF_2<M$	n	n	$B_2<BF_2$	$BF_2<BF_1$	

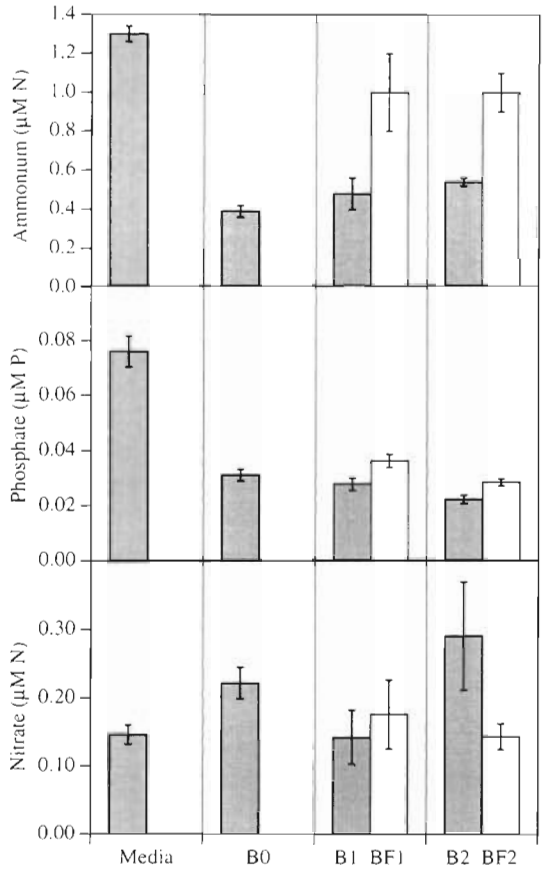

Fig. 4. Inorganic nutrients concentration in the medium and 5 culture vessels

and between BF_1 and BF_2. This decrease was immediate and did not proceed with time. Neither wall growth nor abiotic adhesion to the walls could explain this loss since the 'lost' phosphorus could not be found when the bottles were acid rinsed at the end of the experiment. We have currently no explanation for the loss in total phosphorus but suggest that the phosphorus with the longest residence time was modified into a structure that was not hydrolyzed to soluble reactive phosphorus by the analytical procedure.

Limiting nutrient

Direct evidence of limiting nutrient status in the bottles was determined by nutrient additions to subsamples from the 5 vessels. Yield of bacteria in water from the outflow of the culture vessels was recorded in separate experiments using additions of phosphate, ammonium and a glucose/sucrose mixture. In B_0 bacterial numbers increased in the control and the addition of nutrients had minor effects on growth except when combined CNP was added (Table 5). This indicates that at the prevailing dilution rate (0.03 h^{-1}) the medium were not exhausted and that utilizable substrate still was available in the outflow from B_0. In B_1 there was also a slight increase in bacterial numbers in the control and additions had little or no effect except for the combined CNP addition. In subsamples from B_2 there was no detectable growth in the control bottle, showing that the substrate was completely exhausted and could not support further growth. The subcultures responded slightly to P additions and considerably to combined CP and CNP. Thus, the order of limiting nutrients in the bacterial line in the flow-through system was P < C < N and nitrogen limitation was never met. The same order of limiting nutrients was found in the flagellate line and nitrogen limitation was not met there either.

Bacterial viability, production and virus production

Bacterial production measured by uptake of tritiated thymidine (TTI) decreased with increasing residence time (Table 6). In B_1 and B_2 TTI production was significantly higher than the production estimated from increase in actual bacterial numbers. The yield of bacteria in unenriched subsamples (see 'Limiting nutrient' above) supported the TTI estimate of bacterial production. The yield of bacteria in subsamples from B_0 showed that the residual substrate (outflow from B_0) could maintain an additional growth of 1.2×10^9 bacteria l^{-1} (Table 6). In B_1 the residual growth was 0.3×10^9 bacteria l^{-1}. This indicates that substrate equivalent to 0.9×10^9 bacteria l^{-1} was used in B_1 and, thus, if added to the recorded increase of bacteria in the first vessel, B_0, the potential number of bacteria in B_1 would be 2.0×10^9 bacteria l^{-1}. Compared to the actual counts of bacteria in B_1 (1.5×10^9 bacteria l^{-1}), it follows that 0.5×10^9 bacteria l^{-1} must have disintegrated. In B_2 the substrate was completely exhausted of nutrients and no further growth was possible on 0.2 µm filtered sub-

Table 5. Limiting nutrient experiment on subsamples from the 5 vessels. Numbers are given in percent growth compared with numbers of bacteria in the original vessels calculated as: (Yield in subsample/Bacterial numbers in flow-through vessel) × 100. Zero percent indicates no further growth

Bottle	Control	C	N	CN	P	NP	CP	CNP
B_0	110	100	105	90	130	140	170	460
B_1	20	10	20	30	30	20	30	60
B_2	0	0	0	0	5	5	50	90
BF_1	50	40	40	40	90	100	200	440
BF_2	40	40	40	40	50	50	80	115

Table 6. Bacterial production according to actual and potential increase in bacterial numbers compared to bacterial production measured as uptake of tritiated thymidine. nd: not determined; not possible to quantify due to flagellate predation

		B_0	B_1	B_2	BF_1	BF_2
		A	*B*	*C*	*D*	*E*
Yield of bacteria in subsamples ($\times 10^9$ l^{-1})	*1*	1.2	0.3	0	0.3	0.2
Actual number of bacteria ($\times 10^9$ l^{-1})	*2*	1.1	1.5	1.1	0.6	0.4
Potential number of bacteria ($\times 10^9$ l^{-1})	*3*	1.1[a]	2.0 *[A3+(A1–B1)]*	2.3 *[B3+(B1–C1)]*	nd	nd
Disintegrated bacteria ($\times 10^9$ l^{-1})	*4*	–	0.5 *(B3–B2)*	0.7 *(B2–C2+B1)*	nd	nd
Bact. prod. based on actual number ($\times 10^6$ cells h^{-1} l^{-1})		33	0.9	0	nd	nd
Bact. prod. based on potential number ($\times 10^6$ cells h^{-1} l^{-1})		33	5.1	1.3	nd	nd
Bact. prod. based on TTI[b] ($\times 10^6$ cells h^{-1} l^{-1})		8–80	4–40	0.8–8	1–10	0.7–7

[a]Minimum estimate. Lysis in first vessel not accounted for
[b]Conversion factor 0.2×10^{18} to 2.0×10^{18} produced cells per mole incorporated thymidine

samples. Thus, substrate equivalent to 0.3×10^9 bacteria l^{-1} (residual from B_1) was used in B_2 for bacterial growth. Compared with the incoming water from B_1, the number of bacteria in B_2 decreased by 0.4×10^9 bacteria l^{-1}. Thus, in B_2 0.7×10^9 bacteria l^{-1} were disintegrated per each residence time. As seen in Table 6, the production rates estimated from TTI were in between the range of production estimated from the yield data. We therefore concluded that bacterial growth did occur at long residence times as well, although little or no increase in total bacterial count could be detected.

The potential viability of the bacterial community was assessed by counting the number of NucC. This number represents a maximum number of actively growing bacteria since moribund phage-infected bacteria are included. The fraction of NucC was at most 68% of total count in B_0 and decreased to 20% with increasing residence time (Table 7, Fig. 5). Accordingly, a high number of bacterial ghosts were included in the total counts of bacteria. The number of NucC decreased more rapidly in the presence of flagellates, possibly due to selective grazing on live bacteria.

A substantial number of viruses was found in all stages of the flow-through system (Table 7). The number of viruses increased with increasing residence in the bacterial line. In the presence of flagellates the number of viruses increased in BF_1 compared with the inflow but decreased in BF_2. In B_0 the number of viruses and ghosts was 1.7×10^{10} l^{-1} and 3.5×10^8 l^{-1} respectively, resulting in an appar-

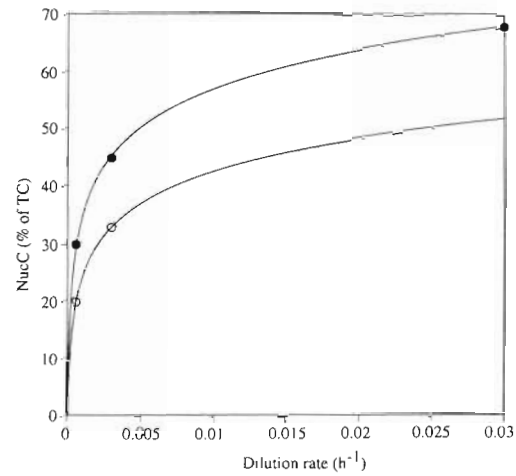

Fig. 5. Nucleoid-containing bacteria (NucC) versus dilution rate. TC: total count of bacteria. Line represents exponential curve fits. (●) Bacteria; (○) bacteria and flagellates

Table 7. Effect of changing dilution rate and flagellate grazing on abundance of virus, nucleoid-containing bacteria (NucC) and ghosts. TC: total count of bacteria. Standard deviation and (n) given below values

Vessel	Dilution rate (h^{-1})	Bacteria (10^9 l^{-1})	Virus (10^{10} l^{-1})	NucC (10^9 l^{-1})	Ghosts (10^9 l^{-1})	Fraction NucC (% of TC)
Bacteria only (B_0)	0.03	1.10 0.14 (24)	1.7 0.6 (2)	0.75 0.05 (2)	0.35	68
Bacteria only (B_1)	0.003	1.45 0.23 (25)	2.1 0.01 (2)	0.65 0.06 (2)	0.80	45
Bacteria only (B_2)	0.0006	1.10 0.11 (21)	6.5 1.3 (2)	0.33 0.04 (2)	0.77	30
Bacteria +flag. (BF_1)	0.003	0.52 0.14 (24)	4.8 0.2 (2)	0.17 0.03 (2)	0.25	33
Bacteria +flag. (BF_2)	0.0006	0.41 0.11 (21)	3.8 0.9 (2)	0.08 0.02 (2)	0.33	20

ent burst size of 50 viruses per lysed cell. This is a crude estimate since part of the lysed cells may not be microscopically identifiable as ghosts and part of the ghosts may have been generated through autolysis.

Particulate organic matter

Total POC (retention on 0.2 µm Ag-filter) was high throughout the system and could not be solely attributed to bacterial and flagellate carbon. Using the estimate of carbon biomass (Table 1), the contribution of bacterial and flagellate carbon to the total particulate carbon pool was only 42% in B_0, 50% in B_1, 46% in B_2, 21% in BF_1 and 18% in BF_2. Unspecific binding of initially present DOC did not 'contaminate' the filters since filtered medium water was used as a blank for the POC measurements. The nitrogen and phosphorus contents in the biota were calculated from the PON and POP estimates derived from nutrient analysis (Table 1). C:P ratios were ~80 to 85 and relatively constant in all culture vessels. The C:N ratios were 4 in B_0 and B_1 and increased to 6 in vessel B_2. In the vessels including flagellates the C:N ratio of the biota was ~2. This value appears to be low for the organisms present but indicates a trend of increasing nitrogen availability in the presence of flagellates. The C:N ratio of filterable POM in all vessels was 10 to 15, indicating a surplus of carbon compared with the biota. Thus, carbon-rich material with characteristics allowing retention on 0.2 µm filters must have been produced within the system. A figure of POC distribution, showing the contribution of NucC and ghosts, was compiled (Fig. 6). We have assumed that ghosts have 30% less carbon than intact bacteria due to loss of internal soluble pools. Detrital carbon ('invisible POC') made up the largest pool of POC in all culture vessels.

Utilizable pools of essential nutrients

The consumption of inorganic versus organic nitrogen and phosphorus was calculated in B_0. An increase of 0.056 µM P-POP was matched by a decrease of 0.012 µM P-DOP and 0.045 µM P-DIP (dissolved inorganic phosporus). Thus, 21% of the bacterial P-demand was met from the DOP pool while 80% was obtained from the DIP pool. The corresponding figures for nitrogen utilization were an increase in 1.23 µM N-PON and a decrease of 0.36 µM N-DON and 0.89 µM N-DIN corresponding to a 30% contribution from the DON pool and 72% from the DIN pool.

In addition, the total pool of utilizable substrate was estimated using the yield of bacteria in the limiting nutrient experiments. Utilizable phosphorus was 0.11 µM P, calculated from the yield of bacteria in the CN-enriched cultures using the bacterial P-content obtained in this study. Accordingly, the pool of utilizable nitrogen was 2.33 µM N, calculated from the yield in CP-enriched cultures. The utilizable pool of carbon was 20 µM C in the bacterial line and 35 µM C in the flagellate line (difference between the medium and Stage 2). These values imply pools of utilizable substrates with C:N:P ratios of 180:22:1 in the bacterial line and 320:22:1 in the flagellate line.

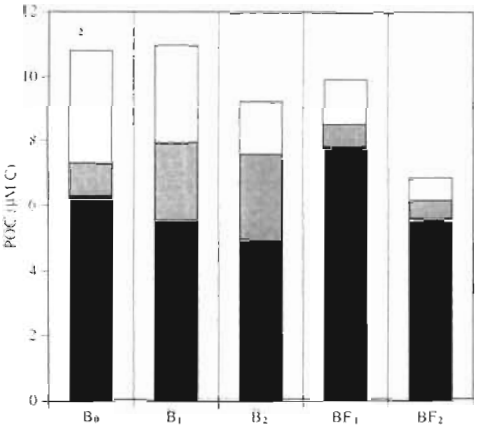

Fig. 6. POC distribution in the 5 cultures vessels. Dark colour represents dead organic material not visible in the microscope. Grey colour represents dead bacteria still visible in the microscope. Light colour represents live bacteria

DISCUSSION

In the experimental system, the inflow from the medium may be considered as an autotrophic compartment renewing the organic substrate in B_0 approximately every day. The steady-state values in the B_0 vessel indicated that the system mimicked the turnover of organic matter and the bacterial community at rates similar to those found in surface waters (Sieburth et al. 1977, Fuhrman & Azam 1980). In Stages 1 and 2, the organisms were supplied at a slower rate with a substrate that was partly exhausted. These stages were set to mimic the growth conditions of heterotrophic organisms below the autotrophic layer of the sea. While the levels and rates obtained are true for this particular system only, we believe the trends to be true indicators of general processes.

Several of the obtained results agree with earlier studies, especially results concerning processes induced by flagellate predation. Size-selective grazing (Andersson et al. 1986), excretion of DNA (Turk et al. 1992), and release of inorganic nutrients (Johannes 1965, Andersson et al. 1985, Goldman et al. 1985) by flagel-

lates have been extensively discussed elsewhere. We have thus focused the discussion on results concerning organic carbon cycling and bacterial proliferation.

DOC degradation

A surprising finding was the increased utilization of DOC in the presence of flagellates. This could be attributed to either enhanced bacterial degradation or flagellate assimilation of DOC. Since flagellates mediate remineralization, increasing both the availability of inorganic nutrients and organic substrates, this could obviously enhance further bacterial degradation of DOC. The nutrient addition experiments, however, showed that additions of inorganic nutrients could not stimulate bacterial growth (Table 5). Thus, with bacteria only, the remaining DOC was apparently non-degradable and the bacteria were unable to sequester more carbon. It has been suggested that co-metabolism could enhance bacterial degradation of refractory DOC, i.e. that additional substrates could support bacteria with energy to degrade refractory material (Brock et al. 1994). The possibility that the flagellates released such substrate and thus enabled the bacteria to break down additional DOC cannot be excluded based on our data. However, in a recent mesocosm experiment in which glucose was added to enhance degradation of non-degradable DOC (algal produced, stable isotope labelled), no further degradation of DOC could be demonstrated (Fry et al. 1996). Thus we feel justified in looking for alternative explanations.

Direct consumption of DOM by flagellates is known to occur but not in rates considered to be important for the transport of carbon in the microbial food web (Fenchel 1987). Flagellates are also able to ingest 'colloidal' DOM (Sherr 1988, Tranvik et al. 1993) as well as virus particles (González & Suttle 1993). Under conditions similar to those in BF_1 and BF_2, flagellates have been shown to ingest virus-like particles with an ingestion rate of 0.054 particles $cell^{-1}$ min^{-1} (González & Suttle 1993). Assuming a viral carbon content of 7×10^{-18} mol C (Bratbak et al. 1992), the loss of dissolved carbon due to flagellate grazing on viruses in our system would be about 9 pM C h^{-1}. If DOM uptake is associated with vacuole formation (pinocytosis), the maximum volume ingested by a flagellate is about 100% of the cell volume per hour (Fenchel 1987). Using the biovolumes and numbers of flagellates derived in this study, about 20 nl water will be passed through the flagellate community per hour. With a DOC pool of 150 µM C, a maximum of 3 pM DOC h^{-1} is passed through the food vacuoles of the flagellates. Since the increased consumption of DOC in the presence of flagellates was 5 to 10 nM C h^{-1}, virus ingestion or pinocytosis of DOC by flagellates could not explain the enhanced DOC degradation.

Relatively high assimilation rates can be accounted for, however, if the surface of the flagellates can serve as a collector of DOM. The encounter rates depend much on the structure of the DOM (Shimeta 1993). Stringy structures diffuse slowly but are efficiently encountered due to the high swimming velocities of flagellates. Smaller particles and molecules may be deflected along stream-lines as the flagellates swim past and are missed and not intercepted directly. However, encounter probability can nevertheless be high due to rapid diffusion towards the flagellate as it approaches, a process which depends on the diffusion rate of the matter to be encountered. It is illustrative to evaluate encounter rates for the 2 extreme situations of non-deflective interception and encounter by diffusion. Assuming a flagellate radius (r) of 2 µm and a swimming velocity (v) of 100 µm s^{-1}, a flagellate clears $\pi r^2 v = 4.5$ nl h^{-1} by simply moving through the water. Assuming a diffusion coefficient (D) of 10^{-6} cm^2 s^{-1} (molecular weight on the order of 1000 Da), it clears $4\pi rD = 9$ nl h^{-1} by diffusion alone irrespective of swimming speed. At 4×10^5 flagellates l^{-1} and 150 µM C, both potential encounter rates are high (on the order of 500 nM C h^{-1}) in relation to the observed reduction in DOC in the presence of flagellates (on the order of 5 to 10 nM C h^{-1})

If this scenario can be considered as a possible DOM pathway, a secondary question is then why flagellates are able to degrade DOM that was not accessible for the bacteria. Non-degradable DOM consists of molecules that most likely have been formed through abiotic processes (Azam et al. 1993, Keil & Kirchman 1994). If these molecules cannot be recognized and cleaved by bacterial enzymes, they should not be accessible to flagellate enzymes either. However, it can be speculated that due to low pH and hydrolyzis processes in the food vacuoles DOM may be chemically 'pre-processed' into degradable products by the flagellate and either taken up directly by the flagellates or released and taken up by bacteria. While this question cannot be fully resolved based on the present data, we conclude that the presence of flagellates induced enhanced DOM degradation.

Particulate organic matter

The high concentration of POC found in the system indicated that detrital carbon (not seen with fluorescent dyes in the microscope) was produced. The detrital POC produced in the experiment made up maximally 3% of the total DOC pool. Bacterial lysis may have caused production of 'invisible' particles, or bac-

teria may have produced material whose size or adsorptive tendencies ('stickiness') made them filterable. Biotic production of POC is likely related to the release of exopolymer material by bacteria and formation of TEP particles (transparent exopolymers) as observed in seawater (Alldredge et al. 1993, Heissenberger & Herndl 1994). In the presence of flagellates the detrital POC increased additionally, and this may be attributed to egestion of small particles from the flagellate food vacuoles (Koike et al. 1990). An artefact that may have caused abiotic particle formation was the air stream which created bubbles in the culture vessels (see 'Material and methods'). Bubbling of seawater is known to create particles, both small and large in size (Johnson et al. 1986). Whether the detrital organic carbon was produced by biological or abiotic activity cannot be determined from the present data. However, the additional formation of detritus in the presence of flagellates suggest biological activity.

Wall growth and medium stability

When seawater is contained, the walls of the container are never free of growing organisms. To evaluate the importance of possible wall growth in the experimental system 2 different approaches were used. Midway through the experiment the culture vessels were changed by aseptic transfer of the cultures to new acid washed bottles (Ammerman et al. 1984). The old empty bottles were rinsed and shaken several times with particle-free sterile seawater with 0.1% Triton X-100 and the numbers of released bacteria were counted. Secondly, at the end of the experiment the accumulation of N and P on the walls was determined by rinsing the empty bottles twice with 50 ml 1 M HCl and subsequently measuring the total N and P. The bacterial counts midway through the experiment showed that wall-growing bacteria made up a maximum of 18% of total bacteria in B_0, decreasing to 4% in B_2. This amounts to <1‰ of the total production of bacteria in the vessels up to the time when the bottles were changed (after 5.5 mo). The total N and P that was recovered from the walls at the end of the experiment made up 1 to 5% of total N and P in the bulk. If back-calculated to bacterial equivalents, a maximum of 17% of total bacteria would be present on the wall of the smallest bottle (B_0) and 3% in B_2. The difference in amount of colonization between the bottles can in part be explained by the decreasing area:volume ratio (from 6 to 2) in bottles with increasing residence time. Together with the fact that none of the biological or chemical variables (except nitrate) showed any significant trend with time, we concluded that initial colonization of the walls rapidly reached equilibrium with the bacterial community of the bulk phase, and we have not made any corrections for wall growth. Another concern was aging of the medium seawater. Transformation of fresh material to non-degradable DOC has been shown to occur as the result of unspecific chemical reactions, such as glycosylation and racemic transformation. The factors controlling these processes are poorly known, but facilitated under light conditions. Being initially rapid and slowing with time, the rates appear to be high enough to significantly influence the DOC dynamics in the surface water (Keil & Kirchman 1994). In our system we did not find evidence of transformation of fresh to non-degradable DOC during the 11 mo that the system was operated. If the medium were subjected to continuous 'humification', a decrease in bacterial abundance during the time course of the experiment would have been observed. Hence, we believe that transformation of utilizable to non-degradable DOC may have been prevented due to the cold and dark storage of the experimental water or that possible reactions occurred before the experiment was started (i.e. between sampling of the medium and start of experiment).

Bacterial growth at long residence time

Although the experimental system resembles a multi-stage chemostat, it is important to recognize that in contrast to a true chemostat, this system was controlled by several mortality factors. The only loss term in a true chemostat is the outflow, while in this experimental system flagellate predation, virus lysis and autolysis must also be accounted for. In a similar flow-through system Tempest et al. (1967) showed that the viability of *Aeromonas aerogenes* decreased to 35% at a dilution rate of 0.004 h^{-1}. This in turn gave the fraction of living bacteria a specific growth rate of 0.01 h^{-1}, i.e. substantially higher than the dilution rate (Tempest et al. 1967). Our results indicate several internal loops supporting rapid growth of a small but active bacterial community. In Fig. 7 a conceptual model is depicted to illustrate the carbon flows. Bacterial biomass production is only attributed to uninfected bacteria (or bacteria with lysogenic phages incorporated into the chromosome). Lysis of bacteria results in release of DOM-creating options for 'cryptic' growth in a closed system. POM are produced either as bacterial envelopes still visible in the microscope or as partly disintegrated membranes not visible in the microscope. Via enzymatic degradation (dashed lines on Fig. 7), the POM is finally returned to the DOM pool and subsequently reutilized by the bacteria. This internal circulation of organic carbon and the presence of flagellates makes it difficult to quantify production/

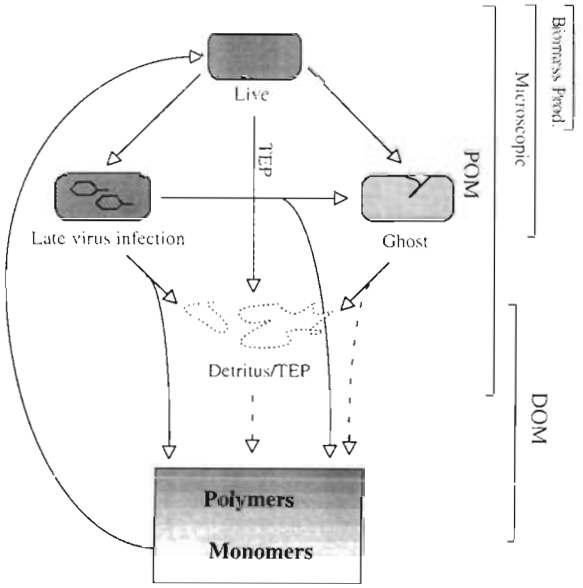

Fig. 7. Conceptual model of bacterial organic carbon turnover

degradation rates from the obtained steady-state values. In B_1 the increase in total numbers of bacteria was low compared with the inflow and in B_2 bacterial counts decreased. This could be interpreted as bacteria barely surviving on maintenance energy or dying (Table 6). However, at the same time uptake of TTI and estimates of growth from the yield experiments indicated a substantial production of cells in all 5 vessels. To evaluate the importance of internal circulation of material a dynamic model based on common concepts of microbial processing of organic carbon is presented in an accompanying paper (Blackburn et al. 1996 in this issue).

Acknowledgements. Skilful technical assistance was provided by Åsa Rudehäll, Susanne Berggren, Carl Stangenberg, Johan Nordbäck and Kerstin Jacobsson. Johan Wikner is acknowledged for kindly providing the data on viral numbers. This work was supported by grants from the Swedish National Science Council and Commission of the European Communities, MAST II grant MAS 2-CT93-0063 'Medipelagos'

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Responsible Subject Editor: P. M. Glibert, Cambridge, Maryland, USA

Manuscript first received: July 12, 1995
Revised version accepted: April 8, 1996