Loss rate of an oligotrophic bacterial assemblage as measured by $^3$H-thymidine and $^{32}$PO$_4$; good agreement and near-balance with production

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ABSTRACT Growth and loss of planktonic bacteria are thought to be roughly in balance, but are rarely measured together. The loss rate for a bacterial assemblage in surface waters of Villefranche Bay (NW Mediterranean Sea) was estimated using 2 independent techniques. The disappearance rate of $^3$H from cold-TCA-insoluble material following a labeling of the natural assemblage with $^3$H-thymidine gave a turnover of 2.2% h$^{-1}$, while the disappearance of $^{32}$P from the bacterial size fraction (0.2 to 1 $\mu$m) following an initial uptake period and a subsequent cold chase with orthophosphate gave a bacterial turnover rate of 2.5% h$^{-1}$. The similarity of the 2 estimates suggests that the same loss processes were measured and that processes independent of bacterial population turnover, such as rapid uptake and release of labels, were of minor importance. The mortality estimates were close to thymidine-based production estimates of 2 to 2.3% h$^{-1}$. Viral abundance (ca $2 \times 10^6$ ml$^{-1}$) was about 3 to 4 times that of bacteria, and relatively constant. Attempts to measure bacterial mortality due to viral infection were complicated by filtration artifacts. Passage of the thymidine-labelled assemblage through a 0.6 $\mu$m filter in order to separate bacteria and viruses from larger bacterivorous organisms removed 60% of the bacterial label. Label loss rates were undetectable in the filtered assemblage over 96 h incubations, suggesting that viruses were minor loss agents of this (minority) size fraction of bacteria. In the experiments with $^{32}$P, most of the label was transferred directly from the bacterial size fraction to dissolved compounds, with relatively minor amounts (10 to 20%) transferred to larger size fractions.

KEY WORDS: Bacterial decay - Phosphate metabolism - Viruses

INTRODUCTION

Population changes reflect the net difference between growth and loss processes. For bacteria, methods aimed at measuring growth include estimating DNA (Fuhrman & Azam 1980, 1982) or protein synthesis (Kirchman et al. 1985, Simon & Azam 1989), or counting the frequency of dividing cells (Hagström et al. 1979). These methods, in principle, attempt to measure the total bacterial growth process. In contrast, most methods for estimating loss rates are specific for individual loss processes such as predation (e.g. McManus & Fuhrman 1988) or viral lysis (Proctor & Fuhrman 1990, Steward et al. 1992). An exception to this is the approach introduced by Servais et al. (1985, 1989), who labelled a natural bacterial population using $^3$H-thymidine and subsequently followed the rate of disappearance of label from the cold-TCA-insoluble fraction. This method presumably measures the total turnover rate of bacterial DNA.

In many systems, phosphate uptake has been found to be dominated by bacteria (e.g. Faust & Correll 1976, Harrison et al. 1977, Lebo 1990), particularly when orthophosphate turnover time is short (Thingstad et al. 1993). When short turnover time and bacterial dominance of the uptake coincide, a rapid and 'hot' labeling of the bacteria is possible. Adding a subsequent cold chase of unlabelled orthophosphate to stop reassimilation of remineralized phosphate allowed Thingstad et al. (1993) to estimate a turnover rate for bacterial P.
After 3 h of incubation, a cold chase of KH$_2$PO$_4$ was TCA at 98°C for 1 h, then chilled and filtered as above. 10$^3$ dpm ml$^{-1}$. The radioactive concentration of ca 280% of the hot extraction, nucleic acids were hydrolyzed in 5 ml bottles. Carrier-free 32P$_0$ (Amersham) was added to a cold TCA extractions (Hollibaugh et al. 1980); for the experiments. Two 1 1 subsamples were incubated in acid-washed 1 l translucent polypropylene bottles. Thymidine incorporation basically followed Fuhrman et al. 1989, and regeneration of these nutrients is probably affected significantly by the processes and mechanisms of bacterial loss. It is well known that protists graze bacteria and regenerate nutrients (McManus & Fuhrman 1988). The recent recognition of viruses as active parts of microbial food webs (reviewed by Fuhrman & Suttle 1993, Bratbak et al. 1994) raises the interesting question of how these P-rich agents are involved in such systems. As part of a project to investigate the roles of microorganisms in the P cycling of the Mediterranean, we investigated the turnover of bacterial biomass in this system, with an eye to understanding the contributions of bacteria and viruses to P cycling. Of particular interest were (1) a comparison of P- and thymidine-based loss estimates, (2) comparison of loss and production estimates, and (3) the possible roles of viruses in bacterial turnover.

MATERIALS AND METHODS

Sampling and incubation. Water was collected with a 5 l Niskin bottle from 5 m depth at Point 'B' at the mouth of Villefranche Bay (43° 41' 10" N, 7° 19' 00" E) and pooled in a 20 l carboy. This was brought to the Station Zoologique for distribution into individual bottles for experiments. All incubations were done in a running seawater incubator with a neutral screen removing 70% of natural light.

Size distribution of chlorophyll and particulate P. Samples (500 ml) for chlorophyll and particulate P were filtered through 47 mm polycarbonate filters of 5, 1 and 0.2 μm pore size mounted serially in a tower arrangement with a suction of ≤2 × 10$^4$ Pa applied below the 0.2 μm filter at the bottom. Chlorophyll was extracted overnight in 90% acetone and measured fluorometrically (Strickland & Parsons 1972). Particulate P was measured as soluble reactive phosphorus after wet oxidation in acid persulphate (Koroleff 1976) using standard autoanalyzer techniques.

32P$_4$ experiments. Two 1 l subsamples were incubated in acid-washed 1 l translucent polypropylene bottles. Carrier-free 32P$_4$ (Amersham) was added to a radioactive concentration of ca 280 × 10$^3$ dpm ml$^{-1}$. After 3 h of incubation, a cold chase of KH$_2$PO$_4$ was added (0.1 mM final concentration) to one of the bottles. At various intervals during the total incubation period of 24 h, 5 ml aliquots were filtered in parallel through 25 mm polycarbonate filters of 5, 1 and 0.2 μm pore size, supported on Whatman GF/F filters soaked with 10 mM KH$_2$PO$_4$. Suction was regulated by a needle valve that was initially open and was closed successively as the aliquots passed through the filters of larger pore-size, but was not allowed to exceed 10$^4$ Pa (0.1 bar) before the water had passed through all the filters. The suction was then increased to ca 4 × 10$^4$ Pa, and the polycarbonate filters transferred without washing to polyethylene scintillation vials. Three ml of the 0.2 μm filtrate was transferred to a separate vial and distilled water was added to 10 ml final volume for counting of Cerenkov radiation from 32P using a Packard Tri-Carb scintillation spectrometer. Values were checked for unequal quenching based on a quench curve made with Irgalan Black as a color quencher and are reported as % of a triplicate measurement of radioactivity added to each bottle.

Thymidine. Methods for estimating thymidine-based loss were modified from Servais et al. 1985. A 1 l sample was incubated with 0.25 nM tritiated thymidine (Dupont New England Nuclear) in a 1 l polypropylene bottle. At intervals of several hours, duplicate 20 ml subsamples were taken, filtered onto 0.45 μm pore-size Millipore mixed-ester filters and extracted with 5% cold trichloroacetic acid (TCA), then rinsed 7 times with TCA (twice with filter towers removed). The filters were placed into scintillation vials and the nucleic acids were hydrolyzed at 95°C in 0.2 ml of 0.5 N HCl, then cooled and counted with Ready-Safe scintillation fluid (Beckman). After about 24 h of incubation, when radioactivity in the bacteria was already declining, half of the water was filtered gently through a 47 mm diameter, 0.6 μm pore-size Nuclepore polycarbonate filter, and this was subsampled throughout the rest of the experiment and filtered as above.

Thymidine incorporation basically followed Fuhrman & Azam (1982): two 40 ml water samples and a 1% formalin-killed control were incubated with 5 nM tritiated thymidine (Du Pont) New England Nuclear) in a 1 l polypropylene bottle. The organisms on the filter were extracted in cold 5% TCA for 2 min, then rinsed and counted as described above. Radioactivity was converted into a production estimate assuming 2 × 10$^{10}$ cells produced per mole incorporated (Fuhrman & Azam 1982).

An estimate of the portion of label in nucleic acid and 'protein' fractions was made by comparing hot and cold TCA extractions (Hollibaugh et al. 1980), for the hot extraction, nucleic acids were hydrolyzed in 5% TCA at 98°C for 1 h, then chilled and filtered as above.
Bacteria were enumerated by acridine orange direct counts (Hobbie et al. 1977). Viruses were counted after ultracentrifugation (200,000 × g, 1.5 h) onto C-stabilized formvar-coated Cu grids, staining them for 20 s with 1% uranyl acetate, blotting excess stain, and viewing on a Jeol 100 CX electron microscope at 27,000× magnification (modified from Børshøj et al. 1990).

RESULTS

Size distributions of chlorophyll and particulate P. Total particulate P in samples from 6 and 7 July was 41 and 53 nmol P l⁻¹ respectively. Most of the particulate P (avg. = 48 %) was in the 0.2–1 μm fraction with the remainder distributed approximately equally between the 1–5 and >5 μm fractions (Fig. 1). Total chlorophyll was 0.52 and 0.23 mg m⁻³ on 5 and 6 July, respectively, with the 1–5 μm size fraction containing an average of 63 % of the chlorophyll (Fig. 1). On average, 10 % of the chlorophyll passed the 1 μm filter and was collected on the 0.2 μm filter.

Counts of bacteria and viruses. Bacterial abundance averaged ca 5 × 10⁵ ml⁻¹ and viral abundance was nearly invariant at about 2 × 10⁶ ml⁻¹ over the study period (Table 1).

³²PO₄ labeling. After 1 h incubation about 4.5 % of the added label was found in the >5 μm size fraction, 18 % in the 1–5 μm fraction and 40 % in the 0.2–1 μm fraction (Fig. 2). A total of about 63 % of the label assimilated in 1 h corresponds to an approximate turnover time of \( T = \frac{-1}{\text{h} \ln(1 - 0.63)} = 1.0 \text{ h} \).

At the time of addition of the cold chase (8 July, 18:00 h), ca 50 % of the added label was found in the bacterial size fraction (Fig. 2). In the control, this remained relatively constant throughout the rest of the experiment. In the bottle with cold chase, the label disappeared from this fraction at a rate of 2.5 % h⁻¹ (estimated from a linear regression of log-transformed data for the period from 6 to 24 h after the start of the experiment). In both bottles, label in the 1–5 μm size fraction remained at about 20 %. In the period before addition of the cold chase, only a minor fraction of the label was incorporated in the size fraction >5 μm (6 % and 3 % in

<table>
<thead>
<tr>
<th>Date</th>
<th>Viruses (×10⁶ ml⁻¹)</th>
<th>Bacteria (×10⁵ ml⁻¹)</th>
<th>Bacterial production: TdR-based (×10⁶ cells l⁻¹ h⁻¹)</th>
<th>Bacterial loss: TdR-based (% h⁻¹)</th>
<th>Bacterial loss: ³²P-based (% h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Jul</td>
<td>1.8 ± 0.1</td>
<td>4.4</td>
<td>nd</td>
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<td>nd</td>
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<tr>
<td>6 Jul</td>
<td>2.0 ± 0.3</td>
<td>nd</td>
<td>8.8 ± 0.4</td>
<td>-2(^a)</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>7 Jul</td>
<td>1.7 ± 0.1</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
</tr>
<tr>
<td>8 Jul</td>
<td>2.0 ± 0.3</td>
<td>6.6 ± 0.1</td>
<td>15.0 ± 0.1</td>
<td>2.3</td>
<td>2.2 ± 0.1(^\ast)</td>
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\(^a\)Calculated with bacterial abundance from previous day.
control and cold chase, respectively); this appeared to increase slowly in both bottles during the experiment, reaching 10% and 7%, respectively, at the end of the experiment. The label released from the bacterial size fraction in the bottle with cold chase appeared in the dissolved fraction, reaching 39% of the total added label at the end of the experiment.

Thymidine-based production and loss. Bacterial production values obtained in cells ml⁻¹ h⁻¹ were divided by bacterial abundance to obtain values in % h⁻¹, suitable for comparison to the loss data (Table 1). The loss rate data fit reasonably well to the expected exponential decline over time (Fig. 3). However, in both thymidine experiments, the declines in the 0.6 µm filtrates were not significant (p > 0.05), although there appeared to be a small decline between the first 2 time points. The 0.6 µm filtration removed between 60 and 70% of the total label, suggesting that most of the active bacterial biomass was larger than this size. Overall, there was good agreement (variation of means <15%) between the production estimates and loss rate estimates even though they were independent of one another (Table 1).

The percentage of thymidine-derived radioactivity appearing in the 'protein' fraction (hot-TCA-precipitable) increased over the long incubations, starting near 37% and increasing to 58% after 4 d. The 0.6 µm prefilttered samples, in which no declines in cold-TCA-precipitable material were detectable (Fig. 3), showed a very similar trend, although based on only 2 time points (Fig. 4).

DISCUSSION

Community size structure

The distributions of chlorophyll a (chl a) (Fig. 1) suggest that most of the photosynthetic organisms were in...
The loss rates on 8 July found with the 2 methods (2.5 and 2.2% h\(^{-1}\)) would balance steady-state bacterial generation times of 1.2 and 1.3 d \([\ln(2)/\text{rate}]\) for the \(^{32}\text{P}\)- and \(^{3}\text{H}\)-thymidine-based methods respectively, and these were both close to the thymidine-based growth rate estimate of 2.3% h\(^{-1}\). Similarly, the production and loss rate estimates from 6 July (both with thymidine) were close to each other. Our values agree with those of Billen et al. (1990), who found that bacterial growth and decay rates based on thymidine techniques similar to those used here fluctuated around a mean value of 2% h\(^{-1}\). Based on the cold-chase approach, Thingstad et al. (1993) also estimated a decay rate of 2% h\(^{-1}\) in the brackish layer of a Norwegian fjord. A fairly uniform value for growth and decay rates was interpreted by Billen et al. (1990) as an indication of bottom-up rather than top-down control of bacterial growth.

The 0.6 \(\mu\)m filtration in the thymidine loss experiments was planned to remove protists and leave most of the bacteria and viruses behind so that we could examine virus-induced loss. Generally, filtration through a 0.6 \(\mu\)m pore-size filter does not remove most of the bacterioplankton. For example, Azam (1977) usually found >60% of the uptake of the protein (residue after hot TCA hydrolysis) fraction of labelled glucose to be in the <0.6 \(\mu\)m size fraction. However, we found that up to 70% of the label from 6 July thymidine experiment (see Fig. 3) upon duplicate determinations of both hot and cold TCA treatments, with an average coefficient of variation of 5.7%.

### Bacterial turnover

The temporal changes in the portion of \(^{3}\text{H}\)-thymidine in the "protein" (residue after hot TCA hydrolysis) fraction of cold-TCA-precipitable matter from samples taken during the 6 July thymidine experiment (see Fig. 3). Values are based upon duplicate determinations of both hot and cold TCA treatments, with an average coefficient of variation of 5.7%.

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Quite variable estimates of bacterial mortality due to viral attack can be obtained using literature estimates of viral burst size and viral decay rates, and assuming that viral production equals loss, an approach similar to the procedure used by Jiang & Paul (1994). Based on experiments with 3 phages in Texas, USA, coastal waters, Suttle & Chen (1992) calculated the effect of solar radiation on decay rates of marine viruses; their estimate of a probable decay rate for viruses at 5 m depth in clear water is equivalent to 10% h\(^{-1}\) averaged over 24 h. Based on a review of the literature, Borsheim (1993) estimated an average marine viral burst size of...
about 200. However, Fuhrman & Noble (1995) recently estimated decay rates at 7 to 9% h\(^{-1}\) and burst sizes of about 20 in coastal Californian waters. In Villefranche Bay at 5 m depth, viral concentration varied little, around a value of 2 \(\times 10^6\) ml\(^{-1}\) (Table 1), if we use the range of decay and burst size parameters from above, and assume that viral production balances decay, net viral production represents a bacterial loss rate of 2 to 0.14% h\(^{-1}\). This type of calculation thus suggests that virus-induced mortality could range from 7 to 100% of total bacterial mortality as obtained using \(^3\)H-thymidine and \(^32\)P-PO\(_4\). Combining even the low estimate with the very low label loss (cold-TCA-precipitable) observed among cells passing through a 0.6 \(\mu\)m filter seems to suggest that the larger bacterial cells may be the ones most susceptible to viral attack. However it should be noted that viral dynamics are perhaps very different in the NW Mediterranean (in terms of burst sizes or decay rates) and/or that viral mortality may not be measurable using declines in cold-TCA-precipitable material in these waters. In an investigation including Mediterranean samples, Servais et al. (1989) used 2 \(\mu\)m filtration and estimated from this that 20 to 90% of the bacterial mortality rate was due to >2 \(\mu\)m protozoans, i.e. 10 to 80% due to viral loss if all loss in the <2 \(\mu\)m fraction is attributed to viruses.

Both of the 2 measured loss rate estimates contain potential errors. The loss rate based upon loss of labelled P would primarily be expected to overestimate the carbon loss rate if there is a preferential leakage of P over the membrane. Such leakage of P is known to occur in some bacterial species (Rosenberg et al. 1977) and P leakage has been claimed to be an important process in freshwater algae (Lean & Nalewajko 1976). The method based on \(^3\)H-thymidine would, on the other hand, be expected to underestimate bacterial loss rates if label was transferred from bacteria into cold-TCA-precipitable material in viruses or protistan predators, or into free but TCA-precipitable matter. The former pathway would not be unexpected, since label associated with bacterial DNA has been shown to be released in the form of TCA-insoluble viral particles (Servais et al. 1989). The agreement in estimates produced by the 2 methods, one of which is likely to give an overestimate, the other an underestimate, strengthens our confidence that both methods gave a valid estimate of bacterial decay rate in the incubated samples. Both kinds of loss estimates are based on relatively long incubation periods (24 and 40 h respectively). This may in principle affect loss rates due to predation, since protozoan populations may be suspected to decline due to confinement in incubation bottles for such long periods. As long as the mechanisms controlling viral lysis in natural populations are unknown (Fuhrman & Suttle 1993), the effect of confinement in bottles on this loss process is difficult to evaluate. The coincidence between production estimates obtained from relatively short incubations (1 h) and the decay rate estimates suggests, however, that the relatively long incubation times required to obtain the decay estimates may not be too disturbing.

Transfer of P between fractions

The pattern of change in distribution of \(^{32}\)P in the bottle with cold chase (Fig. 2) suggests a simple flow pattern dominated by a flux of P from orthophosphate into bacteria and then a release into dissolved forms, either directly as orthophosphate or indirectly as dissolved organic forms that are more slowly hydrolyzed to orthophosphate. Mechanisms (not mutually exclusive) leading to such a cycle could be (1) leakage or excretion of P-containing compounds through the bacterial membrane, (2) bacterial loss dominated by viral lysis releasing the P contained in bacterial biomass as dissolved forms, or (3) a protozoan grazer population with very low efficiency of P assimilation.

The similarity of turnover rates for \(^3\)H-thymidine in cold-TCA-precipitable material and of \(^{32}\)P in the bacterial size fraction makes the first mechanism relatively improbable, although theoretically an excretion of DNA carrying both the thymidine-bound \(^3\)H and the \(^{32}\)P out of the bacteria could occur. The second mechanism is difficult to reconcile with our data, which show little loss from 0.6 \(\mu\)m prefiltered samples. Mechanism 3 would require that a low, but not unrealistic, value be assumed for the assimilation efficiency of P by bacterial predators in the >5 \(\mu\)m size fraction.

If the numbers from the cold chase experiments are used directly, the >5 \(\mu\)m fraction gained about 0.2% of total label \(h^{-1}\), which is about 10% of the label lost from the bacterial size fraction. Label in the intermediate size fraction (1–5 \(\mu\)m) remained relatively constant. One model explaining this pattern would be bacterial predators >5 \(\mu\)m with an assimilation efficiency of 10% for phosphorus.

In general terms, the relationship between C and P assimilation efficiencies can be derived as follows: the amount of phosphorus excreted \((\Delta P_{\text{ex}})\) per amount of carbon consumed \((\Delta C_{\text{cons}})\) can be derived from the mass balance requirement that the difference between P taken up and P incorporated in biomass is excreted:

\[
\frac{\Delta P_{\text{ex}}}{\Delta C_{\text{cons}}} = \left(\frac{P}{C}\right) - (1 - r)\left(\frac{P}{C}\right)
\]

Here, \(r\) is the fraction of carbon in the bacterial food lost through respiration, and \((P/C)_b\) and \((P/C)_p\) are the P:C ratios in bacterial and protist biomass. If \(f\) is the fraction of P consumed that is excreted:
we obtain the relationship $(1 - f)(P/C)_b = (1 - r)(P/C)_{prot}$. Bacteria are known to have a high P:C ratio (Norland et al., 1995). Assuming the bacterial P:C ratio, $(P/C)_b$, to be twice that in protists, $(P/C)_{prot}$, a protistan carbon respiration coefficient of 0.8 is calculated from this relationship. This number may underestimate the in situ assimilation efficiency, since the addition of cold orthophosphate would be expected to lead to rapid P uptake into the P-limited osmotrophic microorganisms (Thingstad et al. 1993) thereby increasing the $(P/C)_b$ value. Assuming a doubling in $(P/C)_b$ gives an estimated C respiration coefficient of 0.6. In independent measurements from the same site, the increase of particulate P in the bacterial size fraction following such an addition of orthophosphate has been found to be on the order of 40 to 60% (Thingstad unpubl.).

The relatively crude technique of size fractionation does not allow separation of the trophic groups within the chlorophyll-containing size fraction, 1–5 μm. The apparent slow turnover of P within this size fraction may be real, but may also be an artifact caused by balancing of gain from predation on bacteria and loss from predation by organisms in the size class >5 μm. A partial transfer of P via organisms in the 1–5 μm size class would partly explain the low estimates given above of efficiency in P transfer from the 0.2–1 μm size class to the >5 μm size class.

Chemical measurement of bioavailable orthophosphate (s) at low concentrations is inherently difficult. This problem also affects the direct computation of the orthophosphate uptake rate (v) from isotope uptake data since this involves the formula $v = s/T$ where $T$ is the orthophosphate turnover time obtained from isotope uptake. However, knowledge of the decay rate allows an independent estimate of $v$, and then an estimate of s from $s = vT$, as follows: combining a decay rate of 2% h$^{-1}$ with a bacterial P content of 23 nM (particulate P in the 0.2–1 μm fraction) gives a loss rate of approximately 0.5 nM h$^{-1}$. Assuming this to be in equilibrium with a P uptake dominated by orthophosphate, this is also an estimate of the bacterial orthophosphate consumption rate. Using the initial (at 1 h incubation; Fig. 2) distribution of about 20% of the added label in phytoplankton (>1 μm) and about 40% in bacteria (0.2–1 μm fraction), total orthophosphate consumption can be estimated at $v = 0.75$ nM h$^{-1}$. A total of 60% of the label incorporated in 1 h corresponds to an orthophosphate turnover time of $T = -0.6$ h$^{-1}$ = 1.1 h, corresponding further to a bioavailable orthophosphate concentration of $s = vT = 0.8$ nM. This is similar to the estimate obtained for the brackish layer of a Norwegian fjord (Thingstad et al. 1993). If there are other sources for P uptake the estimate of bioavailable orthophosphate will be even lower.

The model suggested from these measurements is a P cycle which at times may be dominated by bacterial uptake. The subsequent release of P through processes representing the decay of bacterial cell components and recycling appears to occur without major transport upwards in the food chain. In this picture, P must be linked to other elements of biogeochemical importance through the stoichiometry in decaying bacterial biomass. Such a structure of the P cycle must therefore also have important consequences for the microbial cycling of these elements.

Acknowledgements. This work was supported by the Commission of the European Communities, MAST grant MAS2-CT93-0063 'Medipelagos'.

**LITERATURE CITED**


Manuscript first received: August 7, 1995
Revised version accepted: November 7, 1995

Responsible Subject Editor: C. Bratbak, Bergen, Norway