Journal of Microbiological Methods 3 (1985) 321–330
Elsevier

JMM 00108

A convenient method for measuring rates of phospholipid synthesis in seawater and sediments: its relevance to the determination of bacterial productivity and the disturbance artifacts introduced by measurements

D.J.W. Moriarty[a], D.C. White[b] and T.J. Wassenberg[a]

[a] *CSIRO Marine Laboratories, P.O. Box 120, Cleveland, Queensland 4163 (Australia) and* [b] *Department of Biological Sciences, Florida State University, Tallahassee, FL 32306 (USA)*

(Received 28 November 1984) (Revised version received 21 February 1985) (Accepted 22 February 1985)

Summary

A rapid and convenient method for estimating microbial activity based on radioactive isotope incorporation into lipids is presented. The methods, utilizing disposable equipment, quantitatively measure bacterial and microeukaryotic activity separately. Bacterial activity was measured from the rate of incorporation of [^{32}P]phosphate into phospholipids during the first 10 to 20 min of an incubation when rates were linear. This rate of phospholipid synthesis was unaffected by cycloheximide. When isotope dilution methods were used to define specific activity, the productivity of bacterial suspensions, measured with the phospholipid method, was equivalent to productivity of suspensions measured by rates of [^{3}H]thymidine incorporation into DNA. The rate of [^{35}S]sulfate incorporation into sulfolipids was inhibited by cycloheximide. This provided an estimate of microeukaryotic activity. As DNA synthesis is not affected by disturbance as rapidly as phospholipid synthesis, the differences in bacterial production rates given by these two methods can be utilized to estimate disturbance artifacts induced by adding labelled precursors to sediments.

Key words: *Bacterial activity - Bacterial productivity - Disturbance artifact - Phospholipid synthesis - Sediment - Sulfolipid synthesis*

Introduction

Bacteria in aquatic sediments are essential components of food chains, particularly in areas such as seagrass beds where few animals feed directly on the primary producers. Although many methods are available for estimating bacterial biomass, activity or productivity [1] difficulties remain in applying or interpreting them. Further developments

or techniques are needed, especially in the measurement of production. A very useful method at present is based on the incorporation of tritiated thymidine into DNA [2].

The measurement with [^{32}P]phosphate of relative rates of bacterial lipid synthesis on particulate detritus in water has been proposed as a way to study bacterial activities [3]. Phospholipids are the major lipids in bacteria and their concentration is well correlated with biomass [4]. There is also a correlation between the rate of phospholipid synthesis and growth rate of bacteria [5]. In order to quantify the absolute rates of phospholipid synthesis, and thus estimate growth rates, we show in this paper how the specific activity of [^{32}P]phosphate at the site of synthesis may be measured using an isotope-dilution procedure [6–8]. Although all organisms synthesize phospholipids, short incubation times favour bacterial activity as shown here in experiments with cycloheximide, an inhibitor of growth in eukaryotes but not in prokaryotes [9, 10]. As a control for the effect of cycloheximide, a primarily eukaryotic activity [11], viz., ^{35}S incorporation into lipids, was also measured. We give a comparison of the bacterial productivities estimated from the rates of phospholipid synthesis and DNA synthesis. The combination of phospholipid biosynthesis and DNA synthesis can give a quantitative estimate of the disturbance artifact that occurs when labelled precursors are added to highly stratified sediments.

Materials and Methods

Materials

$H_3{}^{32}PO_4$ and $H_2{}^{35}SO_4$ (carrier free) were supplied by Amersham, U.K. and New England Nuclear, Boston, MA, U.S.A. [5-*methyl*-^{3}H]thymidine (40 Ci/mmol) was supplied by Amersham Inc., U.K. Cycloheximide was supplied by Sigma Chemical Company, St. Louis, MO, U.S.A. Radioactive incorporations with $^{32}PO_4$ and $^{35}SO_4$ were made in 50 ml disposable polypropylene syringes with eccentric luer tips, to each of which a 10 cm length of polyvinyl tubing was attached.

Lipid extraction

Glass fibre filters or sediments were transferred to disposable syringes from which plungers had been removed and with tubing fastened to the eccentric luer tip. The plungers were inserted and 4 ml chloroform and 8 ml methanol were drawn up through the plastic tube into the syringe. The syringe was shaken and then left standing with the vinyl tubing clamped to the syringe with a rubber band. After at least 2 h in ice, a mixture of 4 ml chloroform and 4 ml water, containing a suspension of $Ca(OH)_2$ (40 mg) to precipitate unincorporated phosphate or sulfate, was drawn into the syringe through the tubing. The syringe was shaken well and left to stand overnight at an angle of 50° with the luer tip uppermost so that precipitates settled away from the opening. The lower (chloroform) phase was expelled through the tubing into pre-weighed scintillation vials and the volume was calculated from the weight. Pigments were destroyed by bleaching in sunlight or strong photographic flood lights. The chloroform was then removed by evaporation. Fifteen ml of scintillation fluid (Aquasol; New England Nuclear) were added and the radioactivity was determined in a scintillation spectrometer. Quenching was corrected in ^{35}S samples with an external standard procedure.

Addition of isotopes to sediments

(a) Slurry method

Sediments were collected in 25 mm diameter corers and the upper 10 mm of each were transferred to a beaker, mixed gently with an equal volume of water and one ml spooned into syringes (from which plungers were removed) containing 20 μCi $^{32}PO_4$ or $^{35}SO_4$ and, in some experiments, cycloheximide (100 μg in 10 μl) in 0.5 ml filtered seawater. The plungers were replaced. The syringes had been fitted previously with a tube attached to the luer tip, which formed a valve when fastened to the side of the syringe with a rubber band. After incubation at ambient seawater temperature for various time intervals, the reaction was stopped by drawing in 4 ml of chloroform and 8 ml methanol through the tube as described above. In one experiment, the slurry was mixed for 15 min before the incubation with [^{32}P]phosphate.

(b) Mild slurry method

Sediments were collected in 8 mm diameter corers and the upper 10 mm were immediately transferred to syringes containing 20 μCi of [^{32}P]phosphate in 0.1 ml of filtered seawater. The plungers were then inserted and tubing, fixed previously to the luer tip, was clamped to the side with a rubber band. After swirling the sediment briefly to mix it, the syringes were incubated at ambient seawater temperature for 15 min. For isotope dilution experiments, syringes were prepared with 0, 20 or 50 nmol of phosphate in addition to the radioactive phosphate.

(c) Injection method

Cores of sediment were collected in 8 mm diameter corers (3 ml disposable syringes with base cut off). A total of 150 μl of isotope (20 μCi $^{32}PO_4$) and, in some cores, an extra 50 nmol phosphate, were injected through the core and the cut end was then closed with a rubber stopper. Cores were incubated at ambient seawater temperature for 15 min. Each core was then expelled into the 50 ml syringe and lipids were immediately extracted.

Measurement of DNA synthesis

Procedures for determining rates of DNA synthesis in sediment were similar to those described by Moriarty and Pollard [12, 13] with the following modifications. Cores were collected in 8 mm diameter corers, and, for some experiments, the top 10 mm were transferred to polypropylene centrifuge tubes containing 0.5 ml filtered seawater and 50 μCi [^{3}H]thymidine. The sediments were mixed briefly by swirling, and then incubated at ambient seawater temperature for 15 min. Reactions were stopped by adding 10 ml of 80% (v/v) ethanol and the tubes were stored on ice. For other experiments, the tritiated thymidine was injected into the cores, which were incubated for 15 min and then expelled into 10 ml of 80% (v/v) ethanol. In the laboratory, the ethanol was removed after centrifuging, and the sediments were extracted in 0.3 M NaOH as described elsewhere [12, 13]. The extracted DNA was dialyzed and radioactivity was measured in portions of the extracts. Experiments were carried out to show that recoveries of DNA with this method were usually complete, and that results were the same as corrected results ob-

tained with the earlier procedure [12, 13]. Thus, in the short time interval of the incubations, DNA was the only macromolecule that was detectably labelled.

Suspensions of microbes

Seawater (1 l) and about 50 leaves of *Halodule wrightii* were placed in a bottle and shaken for a minute. Leaves and large particles were removed by filtering the water through a screen of 150 μm mesh.

Bacterial growth rates were measured with the thymidine method on 20 ml subsamples as described elsewhere [12] with the following modifications. Reactions were stopped by adding 1 ml of 36% (v/v) formaldehyde. Samples were chilled on crushed ice, then sufficient 100% (v/v) trichloroacetic acid (TCA) was added to give a final concentration of 5%. After standing on ice for 15 min, the samples were filtered through 25 mm Whatman GF/F filters and washed 5 times with 3% (v/v) TCA.

Rates of phospholipid synthesis were measured as follows: Disposable syringes (30 ml) with eccentric luer tips were prepared containing a triplicate series of 0, 10, 20 and 100 nmol of phosphate and 20 μCi of [^{32}P]phosphate in a final volume of 100 μl. Plungers were inserted into the syringes. Incubations were started by drawing up 20 ml of water into the syringes and capping the luer tips. Blanks, which contained 100 μmol phosphate to dilute out the uptake of radioactive phosphate, were immediately filtered through GF/F filters in a holder attached to the syringe. Other incubations were stopped after 15 min by filtering. Filters were immediately transferred to a second set of disposable syringes that were fitted with plastic tubing on the luer tips. Plungers were inserted and the lipid was extracted by drawing a mixture of chloroform (4 ml) and methanol (8 ml) into each syringe. The syringes were sealed by clamping the tubing to the side of the syringe with a rubber band. Glass-fibre filters were necessary, because cellulose acetate filters degraded in the chloroform, causing high and variable blank values.

Calculations

Specific activity of [^{32}P]phosphate at the site of lipid synthesis was calculated from the results of an isotope-dilution analysis [6–8, 12, 13]. The concentration or amount of phosphate added was plotted against the reciprocal of radioactivity in phospholipid. The negative intercept is a measure of the relative pool size in the cell at the site of synthesis.

Bacterial production was calculated from rates of thymidine incorporation into DNA as described by Moriarty and Pollard [13], using conversion factors of 2.5×10^{-11} mg C per cell and 2×10^{18} cells produced per mol thymidine incorporated. Production was calculated from rates of phospholipid synthesis using the conversion factor of 50 μmol P per g dry weight of bacteria [5], and a carbon content of 50%. Thus, μmol P incorporated $\times 10$ = mg C bacterial biomass produced.

Results

Phospholipid synthesis

Phosphate was incorporated into lipid at a linear rate for about 20 min in sediments

from a seagrass bed and a coral reef. Cycloheximide had no effect during this period, but did inhibit phospholipid synthesis after 20 min (Fig. 1a, c).

Sulfolipid synthesis

Sulfate was incorporated into sulfur lipids at a linear rate, and this was markedly inhibited after 5 min by cycloheximide (Fig. 1b, d). There was no incorporation of ^{35}S into lipid in anaerobic sediment in the seagrass beds.

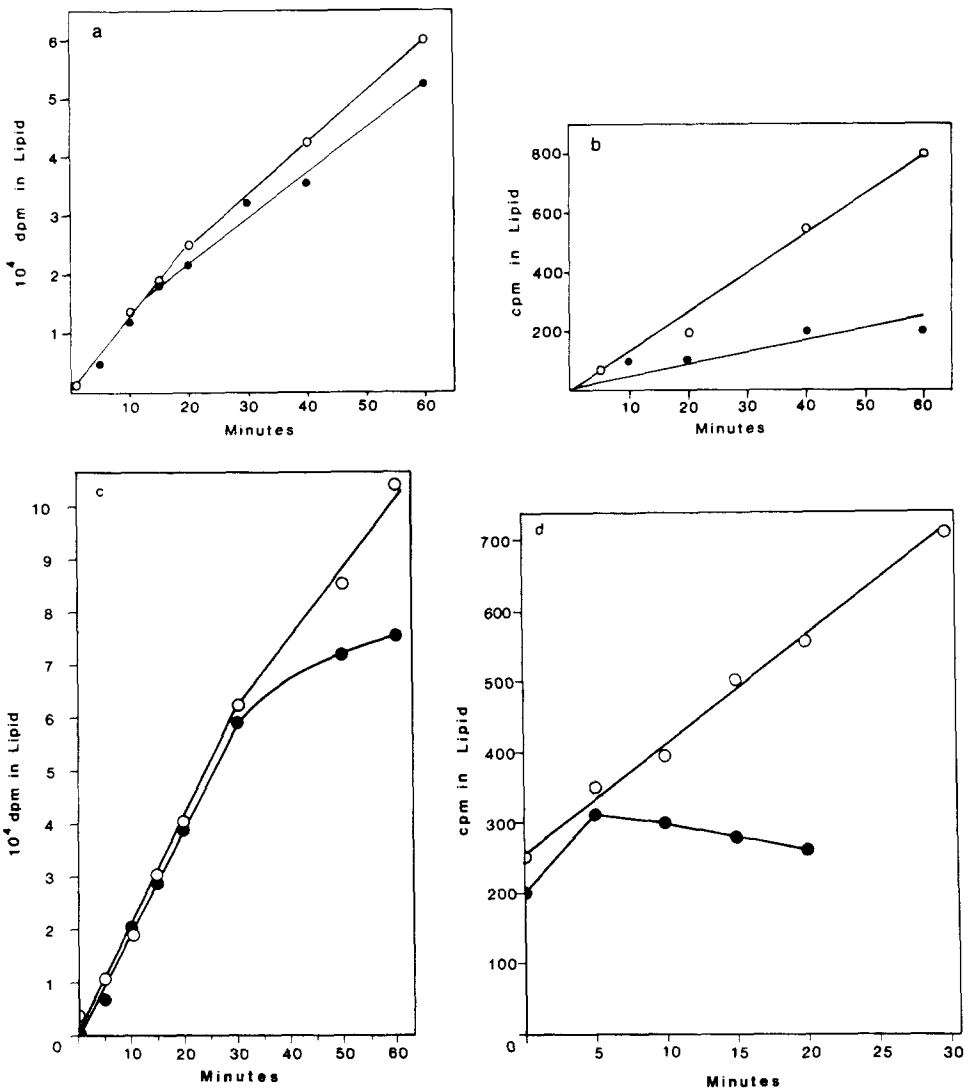

Fig. 1. Effect of cycloheximide on lipid synthesis. Rate of ^{32}P incorporation into phospholipids (a), and ^{35}S in sulfolipids (b) are shown in seagrass bed sediments from Moreton Bay, Queensland (153°20'W, 27°30'S) and rates of ^{32}P incorporation into lipids (c) and ^{35}S into lipids (d) in a coral reef sediment from Lizard Island, Queensland (145°20'W, 14°30'S) are shown. Control, O; cycloheximide, ●.

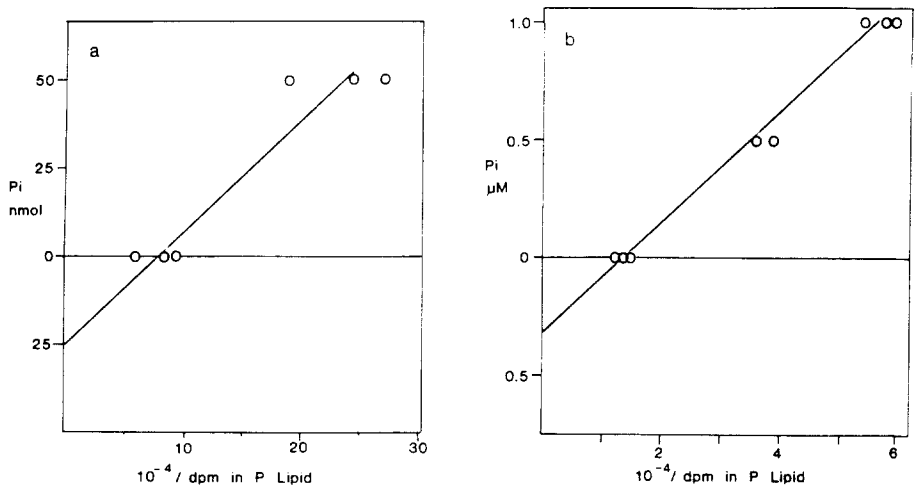

Fig. 2. Isotope-dilution plots for incorporation of [^{32}P]phosphate into lipid. a, each point was the result from an individual core of sediment injected with isotope; b, an experiment with water containing detritus from seagrass leaves.

Productivity

The specific activity of phosphate at the rate-limiting step for incorporation into phospholipid was determined with isotope dilution experiments (Fig. 2). More variation was observed when individual cores were injected or used for mild slurries (Fig. 2a), than when mixed slurries of sediment were used. The specific activity is the ratio of ^{32}P (dpm or μCi) added to each sample divided by the value of the negative intercept on the y-axis, in terms of amounts of P added. In the water column, which contained suspended detritus from seagrass leaves, rates of bacterial production estimated with the thymidine method agreed well with rates estimated from the phospholipid method (Table 1). Compared to sediment, these water samples were homogeneous and aerated.

TABLE 1

COMPARISON OF RATES OF BACTERIAL PRODUCTION MEASURED USING RATES OF PHOSPHOLIPID SYNTHESIS AND DNA SYNTHESIS

Measurements were made in seawater, which contained detritus from seagrass leaves.

	Method	
	Phospholipid	DNA
Incorporation (nmol l^{-1} h^{-1})	0.47 ± 0.03(5)[a]	0.14 ± 0.02(6)
Production (μg C l^{-1} h^{-1})	4.7 ± 0.3 (5)[b]	5.8 ± 0.8 (6)[b]

[a] Data given as $\bar{x}$ ± standard deviation (n).

[b] Analysis of variance: not significantly different.

TABLE 2

COMPARISON OF BACTERIAL PRODUCTION RATES ESTIMATED FROM RATES OF PHOSPHOLIPID AND DNA SYNTHESIS IN SEDIMENT SLURRIES AND CORES

Sediments were from a seagrass bed in August; water temperature 28 °C.

	Bacterial production (mg C m^{-2} h^{-1})	
	Phospholipid method	Thymidine method
Mixed slurry (n=8)	12.0 ± 0.3(8)[a]	9.4 ± 0.8(8)[c]
Injected cores (n=5)	9.1 ± 1.5(5)[b]	8.9 ± 1.6(5)[b]
Well-mixed slurry (n=6)	50.0 ± 2.0(6)	not determined

Data are presented as $\bar{x}$ ± standard deviation (n).
Analysis of variance: a > b ($P < 0.05$); a > c ($P = 0.05$).
Values labelled b, c not significantly different.

Bacterial productivities in surface sediment, when measured with the phospholipid and thymidine methods by injection into cores agreed well (Table 2).

Disturbance artifact

There was no significant effect on bacterial growth rates measured with the thymidine method when a slurry was made, whereas when the phospholipid method was used there was a significant difference ($P<0.05$). In this experiment, the surface sediment was collected from cores, gently mixed and then dispensed within a period of about 10 min. In another experiment, when the surface sediment was well mixed and aerated for 15 min, the rates of phospholipid synthesis increased by about 5 times (Table 2).

Discussion

Bacterial activity

As all organisms contain phospholipids, the measurement of the rate of phospholipid synthesis in a mixed community such as sediment is a useful measure of microbial activity [3]. During incubation periods of up to 2 h with [^{32}P]phosphate, it was predominantly bacterial activity that was measured [11]. Because the eukaryote inhibitor, cycloheximide, had no effect on the rate of ^{32}P incorporation into lipid for 20 min, we conclude that phospholipids were being synthesized mainly in bacteria (Fig. 1a, c). A control experiment to show that the cycloheximide was active, and not adsorbed to sediment, was the effect of cycloheximide on ^{35}S incorporation into lipid. The incorporation of [^{35}S]sulfate slowed or stopped within 5 min in the presence of cycloheximide. Results were similar in both coral reef sediments and siliceous sand (with some silt and clay) (Fig. 1b, d).

Further evidence that most of the phospholipid synthesis was due to bacteria is given by the good agreement with growth rates measured by the thymidine technique in the water samples (Table 1). The thymidine method specifically measures the growth of heterotrophic bacteria [2].

In order to use the rates of phospholipid synthesis as measures of growth or production, it is necessary to show that turnover does not account for a significant portion of the [^{32}P]phosphate incorporation. In monocultures of *Haemophilus*, 1.7–3.9 (1–3 h) generations were required for the ^{32}P of the most labile phospholipid, phosphatidyl glycerol (PG), to turnover in pulse-chase experiments [4]. In a microbial biofilm formed on estuarine detritus, the phosphate of PG, which is the most metabolically active of all the phospholipids, lost 50% of its ^{32}P in 2 h [14]. In this system, muramic acid showed a biphasic turnover with half times of 3.2 and 78.5 h. In sediments phospholipids lost half their ^{32}P in 2 days when incubated aerobically and in 12 days when incubated anaerobically [15]. Consequently in the short incubations used here, the ^{32}P incorporation represented phospholipid biosynthesis.

Sulfolipid synthesis

Rates of sulfolipid synthesis are correlated with growth and activity of microeukaryotes. This has been shown in biofilms of estuarine microbiota that were manipulated with antibiotics and nutrients to stimulate either prokaryotic or microeukaryotic growth [11]. Sulfolipid synthesis from $^{35}SO_4^{2-}$ was significantly slowed within 5 min in the presence of cycloheximide in both coral reef and siliceous sand sediments (Fig. 1b, d). Thus, sulfolipid synthesis in these sediments is a convenient marker for microeukaryotic activity. This has been shown for fungi in biofilms incubated in darkness [11] or in diatoms when incubated in the light [16].

Productivity

To utilize either thymidine or phospholipid synthesis as a measure of bacterial productivity, the specific activity of the precursor at the rate-limiting step in synthesis must be determined. The isotope-dilution procedure of Forsdyke provides a simple method to determine the specific activity at the site of synthesis [6, 7]. The problems of $^{32}PO_4$ binding in the sediment results in decreases in the isotope available for phospholipid synthesis, but it does not change the specific activity. Using the specific activities of $^{32}PO_4$ from Fig. 2, and a value of 50 μmol phospholipid/g dry weight of bacteria [5], the rate of phospholipid synthesis gives values for microbial productivity close to those determined from the rate of [^{3}H]thymidine incorporation into DNA. As tritiated thymidine incorporation into DNA in short-term experiments is an exclusively bacterial process [2], this is additional evidence that short-term phospholipid synthesis is a measure of bacterial activity. Because all microorganisms form phospholipids, the comparison of rates of DNA synthesis and phospholipid synthesis could provide insight into activity of those bacteria that do not incorporate [^{3}H]thymidine into DNA. Sulfate-reducing bacterial monocultures do not incorporate [^{3}H]thymidine into DNA (Moriarty, unpublished experiments). Consequently a measure of these anaerobic processes may be possible with a combination of phospholipid and DNA synthesis.

Disturbance artifact

Findlay et al. [17] have shown that the method of adding labelled precursors to highly stratified microbial environments such as sediments can have marked effects on rates of synthesis of macromolecules.

The data in Table 1 show that it is possible to detect a disturbance artifact by a combination of [^{3}H]thymidine incorporation into DNA and $^{32}PO_4$ incorporation into phospholipid.

The injection method of adding isotope showed significantly less phospholipid synthesis than the slurry method. When the slurry procedure was preceded by vigorous mixing for 10–15 min, the rate of phospholipid synthesis increased 5-fold in these sediments. The initial rate of [^{3}H]thymidine incorporation into DNA was not immediately affected by the method of addition of labelled precursor, because there is a lag in changes to rates of DNA synthesis in a 'shift up' experiment [18].

The technique described in this paper represents a rapid and convenient method of measuring short-term phospholipid or sulfolipid synthesis. It can give insight into bacterial and microeukaryotic activity and, when combined with [^{3}H]thymidine assays of DNA synthesis, can give a measure of disturbance artifacts. It also has potential for measuring anaerobic bacterial productivity in sediments.

Acknowledgements

We thank the Director and staff of the Lizard Island Research Station for their assistance. The following grants to D.C. White are acknowledged: OCE-80-19757 from Biological Oceanography, National Science Foundation; NAG-2-149 from the Advanced Life Support System Office, National Aeronautics and Space Administration; and Contracts N0014-82-C-0404 and N0014-83-K0056 from the Department of the Navy, Office of Naval Research; and CR-80-9994 of the Robert S. Kerr Environmental Research Laboratory of the Environmental Protection Agency.

References

1 van Es, F.B. and Meyer-Reil, L.-A. (1983) Biomass and metabolic activity of heterotrophic marine bacteria. Adv. Microb. Ecol. 6, 111–170.

2 Moriarty, D.J.W. (1985) Measurement of bacterial growth rates in aquatic systems using rates of nucleic acid synthesis. Adv. Microb. Ecol. 9, in press.

3 White, D.C., Bobbie, R.J., Morrison, S.J., Oosterhof, D.K., Taylor, C.W. and Meeter, D.A. (1977) Determination of microbial activity of estuarine detritus by relative rates of lipid biosynthesis. Limnol. Oceanogr. 22, 1089–1098.

4 White, D.C. and Tucker, A.N. (1969) Phospholipid metabolism during changes in the proportions of membrane-bound respiratory pigments in *Haemophilus parainfluenzae*. J. Bacteriol. 97, 199–209.

5 White, D.C., Bobbie, R.J., Herron, J.S., King, J.D. and Morrison, S.J. (1979) Biochemical measurements of microbial mass and activity from environmental samples. In: Native Aquatic Bacteria: Enumeration, Activity, and Ecology, ASTM STP 695, (Costerton, J.W. and Colwell, R.R., eds.) pp. 69–81, American Society for Testing and Materials.

6 Forsdyke, D.R. (1968) Studies of the incorporation of [5-^{3}H]uridine during activation and transformation of lymphocytes induced by phytohaemagglutinin. Biochem. J. 107, 197–205.

7 Forsdyke, D.R. (1971) Application of the isotope-dilution principle to the analysis of factors affecting the incorporation of [^{3}H]uridine and [^{3}H]cytidine into cultured lymphocytes. Biochem. J. 125, 721–732.

8 Sjostrom, D.A. and Forsdyke, D.R. (1974) Isotope-dilution analysis of rate-limiting steps and pools affecting the incorporation of thymidine and deoxycytidine into cultured thymus cells. Biochem. J. 138, 253–262.

9 Cooney, W.J. and Bradley, S.G. (1961) Action of cycloheximide on animal cells. In: Antimicrobial agents and chemotherapy (Finland, M. and Savage, G. M. eds.) pp. 237–244. American Society of Microbiology, Michigan.

10 Venkatesan, N. (1977) Mechanism of inhibition of DNA synthesis by cycloheximide in Balb 3T3 cells. Biochim. Biophys. Acta 478, 437–453.

11 White, D.C., Bobbie, R.J., Nickels, J.S., Fazio, S.D. and Davis, W.M. (1980) Non-selective biochemical methods for the determination of fungal mass and community structure in estuarine detrital microflora. Bot. Mar. 23, 239–250.

12 Pollard, P.C. and Moriarty, D.J.W. (1984) Validity of the tritiated thymidine method for estimating bacterial growth rates: the measurement of isotope dilution during DNA synthesis. Appl. Environ. Microbiol. 48, 1076–1083.

13 Moriarty, D.J.W. and Pollard, P.C. (1982) Diel variation of bacterial productivity in seagrass (*Zostera capricorni*) beds measured by rate of thymidine incorporation into DNA. Mar. Biol. 72, 165–173.

14 King, J.D., White, D.C. and Taylor, C.W. (1977) Use of lipid composition and metabolism to examine structure and activity of estuarine detrital microflora. Appl. Environ. Microbiol. 33, 1177–1183.

15 White, D.C., Davis, W.M., Nickels, J.S., King, J.D. and Bobbie, R.J. (1979) Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40, 51–62.

16 Bobbie, R.J., Nickels, J.S., Smith, G.A., Fazio, S.D., Findlay, R.J., Davis, W.M. and White, D.C. (1981) Effect of light on the biomass and community structure of the estuarine detrital microbiota. Appl. Environ. Microbiol. 423, 150–158.

17 Findlay, R.H., Pollard, P.C., Moriarty, D.J.W. and White, D.C. (1985) Quantitative determination of microbial activity and community nutritional status in estuarine sediments: Evidence for a disturbance artifact. Can. J. Microbiol. (in press).

18 Schaecter, M., Maaloe, O. and Kjeldgaard, N. O. (1958) Dependency on medium and temperature on the cell size and chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. 19, 592–606.