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A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^{3}H-leucine [1]

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Abstract

A simplified method is presented for using ^{3}H-leucine (^{3}H-leu) incorporation to measure bacterial protein synthesis rates in seawater. This method uses small volumes (< 2.0 ml) of seawater and centrifugation instead of filtration. Using high specific activity ^{3}H-leu (~ 5 TBq mmol^{-1}), this method is sensitive enough for measurements in open ocean and mesopelagic samples. The centrifugation method provides substantial savings in cost per sample as well as a reduction in the amount of radioactive waste generated. This method is preferable to the filtration method especially for large scale oceanographic programs involving extensive measurements of bacterial carbon production. This centrifugation method should also allow the investigation of small scale patchiness of bacterial carbon production.

Key words: Bacteria, Production, Method.

Résumé

Une méthode simple et économique utilisant la ^{3}H-leucine pour mesurer les taux de synthèse des protéines bactériennes dans le milieu marin

Ce travail présente une méthode simplifiée de mesure des taux de synthèse des protéines bactériennes dans le milieu marin, utilisant l'incorporation de la ^{3}H-leucine. La méthode utilise des petits volumes (< 2,0 ml) d'eau de mer et une centrifugation à la place de la filtration. Se basant sur la forte activité spécifique de la ^{3}H leucine (~ 5 TBq mmol^{-1}), elle est suffisamment sensible pour traiter des échantillons de milieu océanique ou mésopélagique. La centrifugation est économique car elle permet de réduire les pertes de matériel marqué.

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Elle est à utiliser de préférence à la filtration dans les programmes océanographiques à grande échelle, impliquant un grand nombre de mesures de la production de carbone bactérien. L'emploi de la centrifugation devrait également permettre l'étude de cette production dans des petits essaims ("microscale-patchiness").

Introduction

Bacterial carbon production has become a key parameter in quantifying carbon flows through aquatic food webs. Its measurement over a wide range of aquatic environments has led to the conclusion that approximately 40 % of the primary production is required to support the bacterial carbon demand (Cole *et al.*, 1988). Bacterial protein synthesis, as measured by the incorporation of ^{3}H-leu (Kirchman *et al.*, 1985; Simon and Azam, 1989), provides the most direct estimate of bacterial carbon production. As such, it has become the method of choice over other means of measuring bacterial carbon production.

Biogeochemical studies which emphasize carbon cycling on an ocean-basin scale require extensive spatial and temporal sampling. Such studies would benefit from a simple, economical method of measuring bacterial carbon production given the large number of samples processed. In addition, due to high extracellular isotope dilution in eutrophic water, Riemann and Azam (1992) recommend measuring ^{3}H-leu incorporation at several ^{3}H-leu concentrations to calculate the V_{max} of bacterial protein synthesis. This increases the number of incubations per sample.

Here we propose a simple, economical method of using ^{3}H-leu to measure bacterial protein synthesis. This method employs centrifugation rather than filtration to separate the ^{3}H-leu incorporated into protein from the unincorporated label. In addition to being more economical, this method substantially reduces the amount of radioactive waste generated as compared to the filtration method. We find this method preferable to the filtration method for both shipboard and laboratory studies.

Materials and Methods

Sampling

Seawater was collected in sterile 1 liter polycarbonate flasks from the Scripps Institution of Oceanography pier (32° 53' N, 117° 15' W) and maintained at *in situ* temperature (18 °C). Incubations were started within 30 min of collection.

Centrifugation method

A flow diagram of the method is presented in Figure 1. Prior to collecting the sample water, 5.1 µl of L-[4,5-^{3}H] leucine (Amersham, TRK.636; specific activity

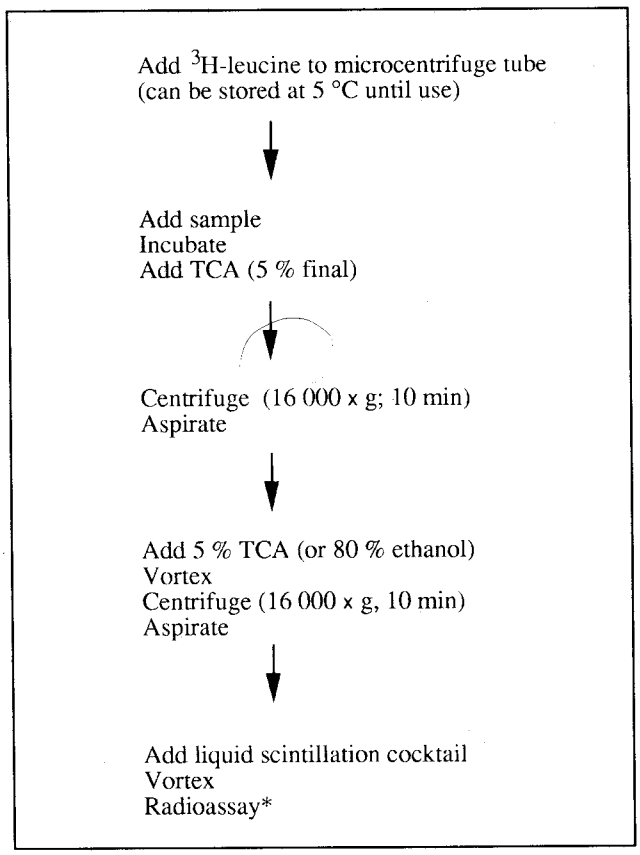

Fig. 1. A flow diagram of the centrifugation method.

* Place the microcentrifuge tube directly into a liquid scintillation vial and radioassay. The scintillation vial can be reused.

= 5 TBq mmol^{-1}), was added into sterile 2.0 ml capacity screw cap microcentrifuge tubes (Fisher, 05-664-34) with o-rings (Fisher, 05-664-57) to yield a final concentration of 20 nM upon the addition of 1.7 ml of seawater. To achieve a final concentration of 5 %, 89 µl of 100 % (w/v) trichloroacetic acid (TCA) were added to those tubes which served as blanks. All tubes were stored at 5 °C and brought to incubation temperature just prior to the start of the incubations.

The incubations were started by the addition of 1.7 ml of seawater to each of the tubes. Samples were run with eight replicates and four blanks. Incubations were terminated by the addition of TCA (5 % final concentration) after one hour. The samples stood at 18 °C for 30 min prior to centrifugation.

The tubes were centrifuged (Eppendorf, 5415C) for ten minutes at 16 000 x g and aspirated. The samples were washed by the addition of 1.5 ml of 5 % TCA and vortex mixing. Samples were centrifuged again (10 min, 16 000 x g) and aspirated. Liquid

scintillation cocktail (0.5 ml; Ecoscint, National Diagnostics) was added directly to the microcentrifuge tube and vortexed. The microcentrifuge tubes were then placed into scintillation vials and radioassayed in a liquid scintillation counter (Beckman LS 6000TA). After radioassaying, the microcentrifuge tubes are removed from the scintillation vials which are then reused.

In three experiments, samples were extracted with 80 % ethanol after precipitating with 5 % TCA or precipitating and washing with 5 % TCA. These samples were compared to samples treated with TCA only to determine whether a significant portion of the ^{3}H-leu apparently incorporated is ethanol-extractable as has been reported for filtration studies (Wicks and Robarts, 1988; Hollibaugh and Wong, 1992).

In the initial development of this method, bovine serum albumin (BSA) was added as a co-precipitant (0.3-300 μg ml^{-1} final concentration) to the samples after the incubation was terminated with TCA. Sodium deoxycholate (125 μg ml^{-1} final concentration) was also tried as it has been reported to increase the efficiency of precipitating small amounts of protein with TCA (Bensadoun and Weinstein, 1976).

Microcentrifuge tubes with hinged snap-caps (National Scientific, RN2000-GMT and Intermountain, C-3261-1) as well as screw cap tubes (Fisher, 05-664-34) with o-rings (Fisher, 05-664-57) and without o-rings (Fisher, 05-664-43) were tested for this assay.

Filtration method

Prior to collecting the sample water, 16.5 μl of L-[4,5-^{3}H] leucine was added into sterile 13.5 ml capacity culture tubes (Falcon, 2051). This yielded a final ^{3}H-leu concentration of 20 nM upon the addition of 5.0 ml of seawater. To achieve a final concentration of 5 %, 263 μl of 100 % TCA were added to those tubes which served as blanks. All tubes were stored at 5 °C until just prior to the start of the incubations. Samples were run with eight replicates and four blanks. Incubations were terminated by the addition of TCA (5 % final concentration) after incubating one hour. The samples stood at 18 °C for 30 min prior to filtration.

The samples were filtered onto 0.45 μm filters (Millipore, HAWP) in a filtration manifold (Millipore, 1225). The filters were rinsed twice with ~1 ml of 5 % TCA and then edge-rinsed. The filters were placed into scintillation vials and dissolved in 1 ml of ethyl acetate. The samples were radioassayed after the addition of 7 ml of liquid scintillation cocktail.

In order to evaluated the effects of incubating in 2.0 ml microcentrifuge tubes compared to that of the larger tubes we incubated 5.0 ml samples in 13.5 ml tubes as in the filtration method but processed 1.7 ml aliquots in microcentrifuge tubes as above.

The samples were extracted with TCA at room temperature (18 °C) rather than with hot TCA prior to centrifugation or filtration (Chin-Leo and Kirchman, 1988; Wicks and Robarts, 1988). The data were normalized to volume, corrected for the blanks and compared using a one factor ANOVA.

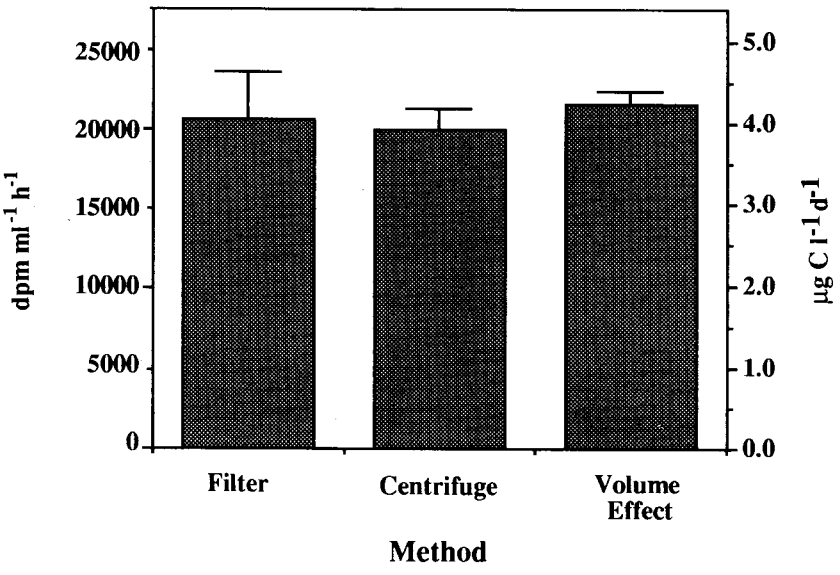

Fig. 2. Comparison of the centrifugation and filtration methods and the effect of incubation in small tubes. Disintegrations ml^{-1} h^{-1} are converted to µg C l^{-1} d^{-1} using the conversion factor of Simon and Azam (1989). The bars represent the mean of 8 replicates ± one standard deviation.

Results and Discussion

There were no significant differences among the centrifugation method, the filtration method and the test of the sample volume (Fig. 2; ANOVA, $p = 0.26$), indicating that the centrifugation method is as reliable as the filtration method for measuring bacterial protein synthesis rates. One advantage is that the centrifugation blanks were much lower than the filtration blanks (t-test; $p = 0.005$). The centrifugation blanks averaged 0.90 % ± 0.06 (mean ± SD) of the total counts whereas the filter blanks averaged 6.49 % ± 0.76 (mean ± SD). In our experience, the use of high specific activity ^{3}H-leu coupled with the low blanks makes the microcentrifuge method sensitive enough to use in open ocean and mesopelagic samples (results to be published elsewhere).

Samples washed with 80 % ethanol after TCA precipitation retained 98.7 % ± 2.8 (mean ± SD; $n = 9$) of the label incorporated in samples treated with TCA only. Therefore, label incorporated into protein is not lost with an ethanol wash. Previous studies, using the filtration method, have reported that 1-46 % of the ^{3}H is removed from the filter with an 80 % ethanol rinse (Wicks and Robarts, 1988; Hollibaugh and

Wong, 1992). This ethanol-extractable ^3H pool is not incorporated into protein and is presumably lipid associated (Wicks and Robarts, 1988). The absence of an ethanol-extractable ^3H pool in our samples may indicate that these substances were not produced during the incubations or that our ethanol extraction was inefficient. Alternatively, the ethanol-extractable ^3H pool retained by filtration may not sediment under the centrifugation conditions employed here.

No significant differences were found with the addition of BSA as a co-precipitant ($p > 0.05$; $n = 8$). Although the addition of BSA allows visualization of the precipitated pellet, which is useful during the washing step, it also results in higher blanks possibly due to unincorporated ^3H-leu retained in the void volume of the pellet. There was also no significant difference when sodium deoxycholate was used in precipitating protein in our samples ($p = 0.18$; $n = 4$). We therefore eliminated these steps.

We also found no significant difference ($p > 0.05$; $n = 8$) when comparing bacterial protein synthesis rates in the different types of microcentrifuge tubes tested. The microcentrifuge tubs with screw-caps and o-rings are preferred for better containment of the radioactivity during centrifugation. Snap cap microcentrifuge tubes are easier to use, although the hinge on the lid must be cut off before the tube will fit into a scintillation vial. This sometimes results in leakage of sample from the microcentrifuge tube which prevents the reuse of the scintillation vial as well as increases the possibility of radioactive contamination. We recommend the use of the screw cap tubes with o-rings.

We have successfully used this method on several cruises. The microcentrifuge does not require a gimbal, unlike large centrifuges, and has performed well in the seas we have encountered (calm to moderately rough). We find it convenient to dispense ^3H-leu into sterile microcentrifuge tubes just prior to a cruise and store them at 5 °C. Because microcentrifuge tubes are small and pack tightly into racks, enough tubes can be prepared for most cruises. A constant temperature during the incubation is maintained by floating the microcentrifuge tubes in a water bath using plastic rafts. The size of the microcentrifuge tubes allows more samples to be incubated in a given water bath than the larger tubes commonly used in the filtration method. Alternatively, commercially available water baths specifically designed for microcentrifuge tubes can be used. These have the advantages of being water tight and requiring less bench space, both of which are useful for shipboard use.

The cost per measurement by the centrifugation method is < 25 % of samples done with the traditional filtration method. Although the actual cost per sample will vary, the ratio of the costs should remain fairly constant. The cost could be a significant factor particularly for large scale programs which require extensive sampling. The main savings are due to the elimination of the filter, a reduction in the amount of ^3H-leu required and the reuse of the scintillation vials.

Another important advantage of this method is a reduction in the amount of both liquid and solid radioactive waste generated. Since the same tube is used from

incubation to radioassay and because liquid scintillation vials are reused, the generation of solid radioactive waste is greatly reduced. Liquid radioactive waste is also reduced due to smaller incubation volumes, smaller volumes of 5 % TCA needed for rinsing the samples and the elimination of the filtration manifold rinse. This is especially useful on cruises where the use of radioactivity is restricted to isolation vans. Not only is the radioactive waste reduced but it is also more contained. After aspirating the samples the small volume of liquid waste generated is simply poured from the trap into a radioactive waste container.

There is also a savings of time when processing a large number of samples using the centrifugation method as compared with the filtration method. More samples can be processed at one time (18 place microcentrifuge rotor versus 12 places in the filtration manifold) and another set of samples can be spun while aspirating the previous set, whereas the filters must be carried through the rinsing stages prior to reloading the manifold with new filters. There are microcentrifuges available which handle more tubes per rotor (*e.g.* Hermle Z23M which can handle 44 tubes at one time) which would greatly increase the efficiency of sample processing.

We are currently exploring the possibility of storing samples which have been terminated with TCA for later processing. This would be especially useful for cruise samples. Microcentrifuge tubes with ^{3}H-leu can be sent out on a cruise, incubated with sample water, terminated with TCA and stored. These samples can be processed later in the laboratory. Our initial attempt to store samples was done with BSA added a co-precipitant and resulted in increasing blanks with prolonged storage (4-75 days). No significant difference ($p = 0.45$; $n = 8$) was detected in samples stored 1-2 days as compared to those samples processed immediately.

It is possible that the centrifugation method presented here could be modified for use in measuring bacterial production by incorporation of ^{3}H-thymidine (Fuhrman and Azam, 1980, 1982). This would require the use of a refrigerated microcentrifuge and washing the samples with ice-cold 5 % TCA. The use of DNA as a co-precipitant may be necessary.

In addition to the advantages of the centrifugation method presented here it may also be useful in studying micro-scale patchiness of bacterial carbon production. Recently Duarte and Vaqué (1992) reported on the existence of patchiness of bacteria abundance at the centimeter scale. The sample volume for the production assay presented here can be reduced further and smaller microcentrifuge tubes (1.7 or 0.5 ml capacity) can be used. The ^{3}H-leu can be diluted in order to have convenient volumes to pipet and then dried down in the microcentrifuge tubes prior to the addition of small volumes of sample seawater. We chose 2.0 ml capacity microcentrifuge tubes in order to maximize the amount of sample water per incubation which is important in waters with low bacterial productivity. It is interesting to note that in our samples we did not see any patchiness in the bacterial carbon production at the 1.7 ml scale (as evidenced by the small (± 6.8 %) standard deviation of the 8 replicates).

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Note added in proof:

We have determined that thymidine incorporation rates can be measured by the microcentrifugation method as well. The only modification is that samples are precipitated on ice and maintained at 5 °C during centrifugation.