NOTE
BRAZILIAN JOURNAL OF OCEANOGRAPHY, 52(3/4): 237-241, 2004

ON THE DETERMINATION OF A CONVERSION FACTOR FROM LABELLED THYMIDINE INCORPORATION BY BACTERIA TO CELL PRODUCTION IN A SUB-TROPICAL ESTUARY: PRELIMINARY RESULTS

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Bacterial carbon production has become a key parameter in quantifying carbon flows through aquatic food webs (Smith & Azam, 1992). Measurement of ^{3}H-Thymidine (^{3}H-TdR) incorporation into bacterial DNA is a useful method to determine bacterial cell production in seawater, freshwater and sediment (Fuhrman & Azam, 1980; Riemann *et al*., 1982; Moriarty & Pollard, 1981). Fuhrman & Azam (1980) related the need of an adequate conversion factor to estimate of bacterial cell production from ^{3}H-TdR incorporation into DNA, and since then several authors have discussed methodological aspects.

On the basis of theoretical considerations, a factor ranging between 0.2 to 1.3 x 10^{18} cells per mol of incorporated ^{3}H-TdR has been proposed (Fuhrman & Azam, 1980). Empirical conversion factors have also been determined by simultaneous measurements of rates of ^{3}H-TdR incorporation and cell production computed from direct counts for different marine environments around the world. The large range of variation for this factor may be found among these studies due to both high conversion factors derived from (^{3}H-TdR) isotope dilution (Moriarty, 1984) and to the characteristics of the different environment and climatic condition under consideration, and it was demonstrated that the coupling between population growth and ^{3}H-TdR is not uniform (Riemann *et al.,* 1987). Up to this moment, no conversion factor was proposed in the literature on tropical or sub-tropical estuaries, as well. In this sense, the present work represents a first effort to determine a conversion factor for a sub-tropical estuary.

On May 2004, estuarine water (salinity 22; temperature 24ºC) from the inner portion of the Cananéia Estuarine System, at Cananéia Sea (25º00'S, 47º54'W), Brazil, was collected to estimate an empirical conversion factor for labeled thymidine incorporation to cells produced. Cell production rate was empirically estimated on dilution culture experiment by correlation of changes in cell abundance and ^{3}H-TdR uptake rates (Kirchman *et al*., 1982). The experiment used bacterial culture media prepared by filtering an aliquot of estuarine water sample onto 1µm NUCLEPORE polycarbonate filters. Another aliquot was filtered through a GELMAN pleated capsule with 0.2 µm Versapor membrane. The sample filtered in 1µm was then diluted in a ratio of 1:10 with the 0.2 µm filtered water. A total bacterial culture volume of 2000 ml was incubated for 40h in the dark, at room temperature, which varied from 22.5 to 26.2ºC. The culture was sub-sampled at 5h intervals for determination of cell abundance and bacterial secondary production experiments (^{3}H-TdR incorporation rate). For cell abundance determination three aliquots of 2-3 ml were stained with DAPI (1 µg ml^{-1} final concentration) for 10 minutes and filtered onto black 0.2 µm PORETICS polycarbonate membrane filters. The filters were laid on microscope slides between layers of immersion oil, and cells were counted under a ZEISS JENALUMAR epifluorescence microscope under 1000x magnification using a UV filter combination (Porter & Feig, 1980). Secondary production followed the method proposed by Smith & Azam (1992) but using ^{3}H-TdR instead of ^{3}H-Leucine. Ten sterile 1.5 ml capacity micro-centrifuge tubes received 20 µl ^{3}H-TdR (Thymidine-(Methyl-^{3}H) SIGMA FW 242.2; 0.9 mCi ml^{-1} 64 Ci mmol^{-1}) to yield a final concentration of 20 nM upon the addition of 1.23 ml of culture media. Two of these tubes received immediately 50 µl of formaldehyde p.a. 37% for representing the blank and the other eight were incubated for an hour in the dark, at room temperature. Incubation was stopped by the addition of 50 µl of formaldehyde p.a. 37%. Samples were kept at 4ºC until laboratory processing. Samples received 7µl of ice-cold TCA 100% (final concentration 5%) and after 30 min they were centrifuged (EPPENDORF 5804R) at 4°C for 15 min

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at 12000 rpm and the liquid was aspirated. The samples were washed by the addition of 1.25 ml of ice-cold TCA 5%, vortex mixed, centrifuged and aspirated. This process was repeated three times and then 1.5 ml of scintillation cocktail (Bray, 1960) was added and vortexed. The micro-centrifuge tubes were placed into scintillation vials and radioassayed in a liquid scintillation spectrometer (PACKARD Tri-Carb1600). DPM counts were converted to mols of incorporated ^{3}H-TdR l^{-1} h^{-1} according to the equation proposed by Bell (1993):

$$v = \left(\frac{[dpm_{sample} - dpm_{blank}] * (4.5 * 10^{-13})}{SA * t * V} \right) * 10^{-3} \quad \text{(mol } ^3\text{H-TdR } l^{-1} h^{-1}\text{)} \quad (1)$$

where 4.5 x 10^{-13} is the number of curies per dpm; SA is the specific activity of the ^{3}H-TdR added to the sample in curies per mmol; t is the incubation time in h; V is the incubation volume in l; and 10^{-3} is the correction from mmol to mol.

The conversion factor (C) from ^{3}H-TdR incorporation to the number of cells produced at the same time interval was computed by the derivative method described by Kirchman *et al*. (1982) and modified by Bell *et al.* (1983):

$$C = \frac{\mu N(t)}{v(t)} \quad \text{cells (mol } ^3\text{H-TdR)}^{-1} \quad (2)$$

where N(t) and ν(t) are, respectively, the bacterial abundance and the incorporation rate at any time, and μ is the slope of lnN(t) versus time, i.e. the growth rate (h^{-1}).

The conversion factor was also computed by the integrative method described by Riemann *et al.* (1987) which considers the number of bacterial cells produced at a selected time interval of the experiment (the final abundance minus the initial abundance at the time interval) divided by the total amount of ^{3}H-TdR incorporated during the same interval:

$$C = \frac{N(t_f) - N(t_i)}{v_{\Delta t}} \quad \text{cells (mol } ^3\text{H-TdR)}^{-1} \quad (3)$$

Another way to calculate the conversion factor was presented by Ducklow & Hill (1985):

$$C = \frac{\delta * No}{e^b} \approx \frac{\mu * No}{To} \quad \text{cells (mol } ^3\text{H-TdR)}^{-1} \quad (4)$$

where N_o and T_o are, respectively, the bacterial abundance and the incorporation rate at time = 0, μ is the slope of linear regression of ln(N) vs. time, and δ and b are respectively the slope and *y*-intercept of linear regression of ln(T) vs. time.

As bacterial grazing was minimized by filtration and dilution with 0.2 μm-filtered water, bacterial abundance increased exponentially as indicated on Figure 1A. After 25h of incubation, the growth rate decreased, and the last 10 hours represent a stationary phase. A maximum cell number of $1.1x10^9$ cells l^{-1} was recorded at the end of the experiment (Table 1). The maximum growth rate was 0.0421 h^{-1}, a value comparable with those obtained by Kirchman & Hoch (1988) in the Delaware Estuary (Table 2). The incorporation rate of ^{3}H-TdR both per h^{-1} and per cell, showed an increase after 15 h, corresponding to the end of the exponential phase of the growth curve (Figs 1A and 1C). Some researchers observed an uncoupling between TdR incorporation and cell number increase, especially due to a lag phase experienced by bacteria after the beginning of the experiment with filtered or diluted water (Rieman *et al.,* 1987).

The conversion factors obtained both by derivative (equation 2) and integrative (equation 3) methods computed for the first 5h interval were higher than the other ones showed (Fig. 1B and Table 1). Kirchman & Hoch (1988) demonstrated that an initial isotope dilution of the added ^{3}H-TdR occurs due to high extra-cellular concentrations of amino-acids, and TdR as well, released as a consequence of the initial filtration. According to these authors, this fact could explain the high initial conversion factors obtained and represents no ecological relevance. So, in general, the initial conversion factors (< 5 h) are not included in average value computation. Thymidine concentration used in the present work was high enough to avoid isotope dilution, as well as to hamper the thymidine degradation due to the action of thymidine phosphorilase (Moriarty, 1984). We used this TdR concentration based in previous experiment to settle the appropriate concentration to be used (Gianesella *et al.,* unpublished data). The average conversion factor obtained was $0.94x10^{18}$ and $1.80x10^{18}$ cells mol^{-1} ^{3}H-TdR, respectively by the derivative (eq. 2) and integrative (eq. 3) methods, when all conversion factors are considered (Table 2). Without the initial high value, mean conversion factors were $0.50x10^{18}$ and $0.52x10^{18}$ cells mol^{-1} ^{3}H-TdR, respectively. These values are in the theoretical range proposed by Fuhrman & Azam (1980) and are similar to the theoretical value also proposed by Bell (1993). In spite of being lower than other values presented in Table 2, like the range obtained by Kirchman & Hoch (1988) for the Delaware Estuary. Conversion factor calculated by Ducklow & Hill (1985) varied between 5.32 and $5.64x10^{18}$ cells mol^{-1} ^{3}H-TdR, higher to those presented by these authors. However, as this method is

based on the initial incorporation rate, it can be biased by the artifacts discussed by Kirchman & Hoch (1988), resulting in an overestimated conversion factor. These results show the difficulty of this task since there is a high variation depending on the considered algorithm, and there is little information about which one was used by each author to compare our results.

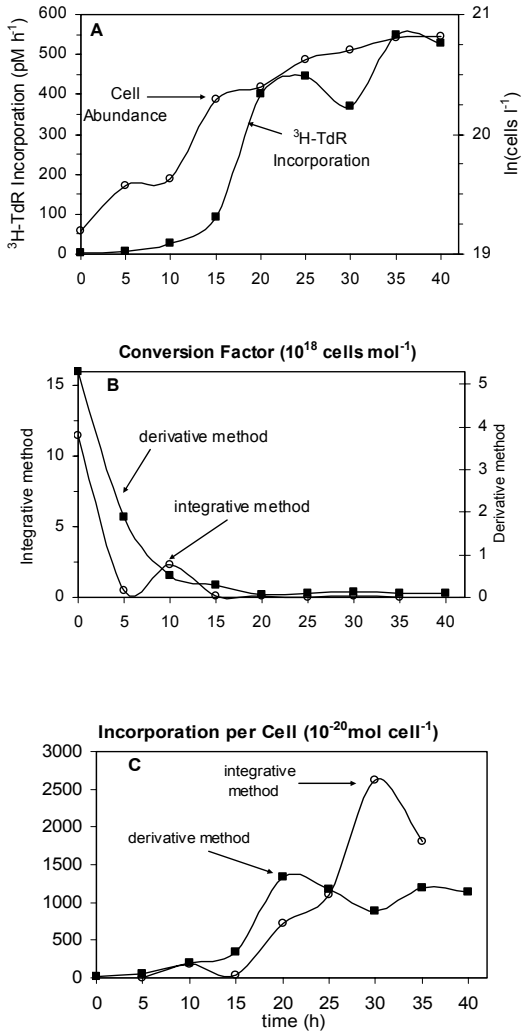

Fig. 1. Bacterial growth experiment in May 2004 to estimate conversion factor for the thymidine (^{3}H-TdR) method. (A) Bacterial abundance (cells l^{-1}) and Thy incorporation rate pM (^{3}H-TdR) h^{-1}; (B) Conversion factors (10^{18} cells (mol ^{3}H-TdR)$^{-1}$) calculated by the derivative (eq. 2) and integrative (eq. 3) methods and (C) ^{3}H-TdR Incorporation rates of per cell (10^{-20} mol ^{3}H-TdR cell^{-1}).

It should be noted that even though the present paper considered a single trial from a single location, it is a necessary step to establish an appropriate set ofconversion factors to a sub-tropical estuary, providing a basis to compare the obtained factors with those obtained from temperate environments. TdR metabolism could also result in tritium incorporation in macromolecules indistinguishable from DNA by the simple extraction and hydrolysis procedures typically used to separate incorporated from unincorporated TdR (Hollibaugh, 1988). A number of authors have reported that ^{3}H-TdR labels other macromolecules as well as DNA, and it can be a significant source of error in bacterioplankton productivity estimates in certain environments (*see in* Hollibaugh, 1988). But, in spite of this, and based on: 1- the theoretical assumption that ^{3}H-TdR is incorporated exclusively into cellular DNA (Fuhrman & Azam, 1980); 2- that in most environments, ^{3}H-TdR appears to be incorporated specifically, or almost specifically, into DNA by microbial assemblages (*see in* Hollibaugh, 1988); and 3- that our conversion factors, both calculated by integrative and derivative methods, were similar to those found in different estuarine systems and inside the range of theoretical ones, the present data constitute a reference for future studies on bacterial production in Brazilian estuaries.

The great variety of environmental conditions in tropical estuaries both due to hydrographic gradients and to temporal variation (tidal stages, annual cycle, etc) can hinder the establishment of a unique conversion factor. Another source of uncertainty for the measurement of bacterial production is the carbon content per bacterial cell. Therefore, a complete study to provide both a set of conversion factors and an estimation of carbon content per bacterial cell over an annual cycle, comparing different estuaries or along salinity gradients and tidal stages (e.g.) is, actually, necessary to an improved determination of bacterial productivity.

Acknowledgments

We thank Dr. Rubens Mendes Lopes of the Instituto Oceanográfico USP (IOUSP) for his valuable help during field work and the crew of "B/Pq. Albacora" for their help during sampling. We also thank Dra. June Ferraz Dias (IOUSP), for allowing us to use the micro-centrifuge.

Table 1. Mean cell abundance (N), standard deviation of abundance and incorporation rates of ^{3}H-TdR along the incubation experiment.

Time (h)	0	5	10	15	20	25	30	35	40
N(x 10^{8} cells l^{-1})	2.16	3.15	3.34	6.52	7.17	9.00	9.85	10.88	11.01
std.dev. (x 10^{8} cells l^{-1})	0.88	0.89	0.84	0.85	0.89	0.93	0.78	0.75	0.84
v(x 10^{12} mole ^{3}H-TdR l^{-1} h^{-1})	1.72	7.00	27.81	92.18	402.75	445.37	369.53	550.26	528.64

Table 2. Growth rate and mean conversion factors calculated for this experiment and by others authors.

Author	Study area	Conversion factors (10^{18} cells mol^{-1} TdR incorporated)		Growth rate
			Method used	(h^{-1})
present work	Cananéia Estuary,	0.50[a]; 0.94[b]	derivative	0.0421
	Brazil	0.52[a]; 1.80[b]	integrative	
Fuhrman & Azam 1980	theoretical	0.2 - 1.3	not definided	
Ducklow & Hill, 1985	Gulf Stream	4.0	Ducklow & Hill	
Alldredge *et al.,*1986	Gulf Stream	1.4	not definided	
Riemann *et al*., 1987	Danish fjord	1.1	integrative	
Kirchman & Hoch, 1988	Delaware Estuary	0.68[a]-1.53[a]	derivative	0.017 - 0.06
		0.70[a] -1.59[a]	integrative	
Findlay *et al*., 1991	Hudson River Estuary	1.0	not definided	
Zohary & Robarts 1992		1.0	not definided	
Bell, 1993	theoretical	0.5	not definided	
Hoch & Kirchman, 1993	Delaware Estuary	1.1	not definided	
Goosen *et al*.,1995	Holland estuaries	2.0	not definided	

[a] calculated without considering < 5h factor conversion

[b] calculated considering < 5h factor conversion

Rest of factor conversion has not information about algorithm used.

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(Manuscript received 16 June 2004: revised 15 October 2004; accepted 21 October 2004)