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Validity of the empirical conversion factors for assessing bacterial production from ^3H thymidine incorporation rates

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Keywords : Freshwater bacteria, thymidine incorporation rates, conversion factors.

Empirical factors for converting ^3H thymidine incorporation into bacterial production were determined from samples taken in a mesotrophic lake. Twenty five diluted water cultures were conducted at « *in situ* » temperature (4-22° C) from April 1987 to February 1989. The conversion factors varied through the year ; the average conversion factor was 6.29×10^9 cells (2-10.7) per nanomole of thymidine incorporated into cold TCA precipitate and 7.18×10^9 cells (3.47-10.7) $nmol^{-1}$, when corrected for the increase in cell biomass which occured during the incubations.

The validity of these factors, higher than the theoretical conversion factors values, is discussed considering the diluted water culture conditions and the data analysis methods.

Validité des facteurs utilisés pour convertir l'incorporation de thymidine tritiée en production bactérienne.

Mots clés : Eau douce, production bactérienne, thymidine, facteurs de conversion.

Les facteurs de conversion permettant d'estimer la production bactérienne à partir de l'incorporation de ^3H thymidine ont été déterminés de façon empirique à partir d'échantillons d'eau prélevés dans un lac mésotrophe. Vingt-cinq cultures en eau diluée ont été faites à température « *in situ* » entre avril 1987 et février 1989. Les facteurs de conversion varient au cours de l'année ; ils sont en moyenne de $6,29 \times 10^9$ cellules (2-10,7) par nanomole de thymidine incorporée dans la fraction TCA insoluble et de $7,18 \times 10^9$ cellules (3,47-10,7) $nmol^{-1}$, si l'on tient compte de l'accroissement de taille des cellules.

La validité de ces facteurs, supérieurs aux facteurs de conversion théoriques, est discutée en fonction des conditions de culture et du mode d'analyse des données.

1. Introduction

^3H thymidine incorporation (Fuhrman & Azam, 1980, 1982) is currently the most widely used method for estimating bacterial production in aquatic ecosystems. The amount of thymidine incorporated is converted into bacterial production using conversion factors which are either derived from biochemical considerations or, more commonly, based on simultaneous measures of growth and assimilation rates of bacterial populations in diluted or grazer-free samples. They are expressed as number of cells produced per nanomole of thymidine incorporated into the purified DNA or into the insoluble fraction of the cold-TCA extract and are converted to carbon biomass using the mean bacterial cell volume. These values are then applied to the thymidine incorporation rates measured during short-term *in situ* incubations. In marine environments, Riemann et al. (1987) found the conversion factor to be quite uniform, close to 1×10^9 cells $nmol^{-1}$ of thymidine incorporated into cold-TCA precipitate. In freshwater environments, the conversion factor estimates are much greater and variable from 1 to 60×10^9 $nmol^{-1}$ (see Bell 1988). The coupling of laboratory experiments and field measurements and the accuracy and validity of the conversion factors have recently been questioned (Pollard & Moriarty 1984, Bell 1988, Bjornsen & Riemann 1988, Cho & Azam 1988, Moriarty 1988). New measurements are

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required to better understand the degree of variation of these factors. We report in this paper 25 conversion factors obtained for the bacterioplankton of a mesotrophic lake over a one and half year period by simultaneously measuring ^3H thymidine incorporation and growth in bacterial cell numbers (and biomass) from natural bacterioplankton cultures. We examine the problems related to experimental conditions, in particular those resulting from the long incubation periods, and the increase in cell size which is often observed during such experiments.

2. Materials and Methods

Sample collection. From April 1987 to September 1988, 24 integrated water samples were taken from surface water layers (0-9 m.) and deep water layers (15-25 m.) of the mesotrophic reservoir, Lake Pareloup (France). In July 1988, five experiments were conducted using five different samples to test the variability of the results. One experiment was conducted in February 1989.

Experimental conditions. Conversion factors were empirically determined from simultaneous measurements of ^3H thymidine incorporation rates and increase in bacterial cell numbers rates in diluted water samples. The lake water was diluted 7-10 times with the original medium filtered through 0.2 μm pore-size nucleopore filter. The samples (about 1 liter) were incubated in the dark, at *in situ* temperature except for one in June, for which the temperature was raised from 8 to 16° C.

The incubation bottle was only stirred manually at sampling times. For two experiments in July and August the samples were gently swirled during all the incubation period.

Subsamples from the incubation bottle were taken initially and at time intervals (2 hours during the exponential growth phase) for cells counts, biomass estimates and ^3H thymidine incorporation rates.

Thymidine incorporation. Thymidine incorporation was measured according to Fuhrman & Azam (1982). Duplicate or triplicate 8 ml samples and duplicate formalin-killed blanks (2 % final conc.) were incubated with 20 nM methyl ^{-3}H thymidine (40-60 Ci mmol^{-1}CEA France). Incubation times varied from 20 to 40 mn depending on the temperature and bacterial activity. The incubations were stopped by adding formalin (2 % final conc.). After extraction in 5 % ice-cold TCA (final conc.) for about half an hour, the samples were filtered onto 25 mm, 0.2 μm pore-sized Nucleopore polycarbonate filters and rinsed 3 times with 3 ml of ice-cold 5 % TCA. The dry filters were put into scintillation vials, 5 ml of scintillation cocktail was added and radioactivity was counted in a Beckman liquid scintillation counter. To be sure that the concentration of 20 nM. ^3H thymidine used for the experiments was sufficient to eliminate isotopic dilution, some incubations were performed by adding 25 and 35 nM at the beginning and at the end of the incubation period in April, July, August and September.

Determination of ^3H thymidine incorporation into DNA and proteins. In July and September 1988 and February 1989 macromolecular fractionation was performered by acid and base hydrolysis (Fuhrman & Azam 1982, Riemann 1984) at the beginning and at the end of the growth cycle, for estimating the percentage of incorporated radioactivity in DNA and proteins.

Bacterial numbers and biomass. Cell counts were made using the acridine orange procedure (Honnie et al., 1987) from fixed samples (formalin 2 % final concentration). Cell size was measured from photographs of acridine orange-stained cells. Cell volume was converted to carbon biomass using the factor 1.21 10^{-7} μg carbon μm^{-3} (Watson et al., 1977). Counts were done in duplicate or triplicate : at least 1 000 cells were counted and 150 cells were measured for each subsample. The mean difference between bacteria counts was 6 %.

Diversity of the bacteria. In February, June and July, bacterial strains were isolated via spread plates incubated at the « *in situ* » temperature, during the lag and stationnary phases. Two different media were used : autoclaved lake water prefiltered through 0.2 μm pore-size filter and C.P.S. medium (Jones 1970). The isolated strains were characterized from about 30 morphological, physiological and nutritional tests (A.P.I. system for characterization of heterotrophic bacteria).

Data analysis. The data (^3H thymidine incorporation, cell numbers and biomass) were plotted vs. time. The curves show lag, exponential and stationnary phases. Only the exponential phase is considered and linear regressions of Ln (thymidine incorporation rate), Ln (cell number) and Ln (biomass) vs. time were calculated. The conversion factors were estimated from the period ranging from the first subsample taken during the exponential growth phase, to the time at which the incorporation rate reached the value of the incorporation rate as it was measured in the lake on this date (Fig. 1). They were calculated by dividing the difference in cell number at the beginning and at the end of the selected time period by the integrated ^3H thymidine incorporation.

3. Results

Thymidine incorporation and growth exhibited the typical phases of bacterial cultures : lag, exponential growth and stationnary phases when the carrying capacity of the medium was reached.

The lag periods preceding bacterial growth ranged from 12 h (September) to over 80 h (February).

The rates of increase in cell numbers (δ) ranged from 0.008 to 0.225 h^{-1} (δ = 0,0927 ± 0,07) and were always significantly lower than the ^3H thymidine incorporation rates (μ), ranging from 0,022 to 0.42 h^{-1} (μ = 0,191 ± 0,124) (Table I).

			t	T°C	n	μ	V_o	r	δ	N_o	r	γ	B_o	r
1987														
April	1	s	40	8	6	0.034	0.82	0.97	0.015	5.8	0.85	0.029	4.82	0.98
August	2*	s	10	19.5	4	0.340	3.11	0.99	0.191	1.89	0.98			
	3	s	14	19.5	4	0.328	2.50	0.98	0.225	2.05	0.97			
	4	d	28	12.5	4	0.110	1.00	0.98	0.035	3.02	0.92	0.058	3.00	0.94
November	5	s	29	7	5	0.049	1.17	0.98	0.017	5.6	0.94	0.034	0.86	0.96
December	6	s	38	6	5	0.031	0.35	0.98	0.008	4.7	0.95	0.020	3.82	0.92
1988														
January	7	s	36	7	4	0.041	0.37	0.99	0.009	3.6	0.97	0.023	1.82	0.96
	8	d	36	7	4	0.040	0.50	0.97	0.010	3.0	0.96	0.018	1.70	0.95
February	9	s	84	7	5	0.071	1.32	0.99	0.026	8.7	0.93	0.032	5.50	0.93
March	10	s	73	7	5	0.090	2.70	0.98	0.027	7.1	0.96	0.079	2.76	0.98
	11	s	38	16	4	0.170	1.60	0.99	0.033	7.6	0.83	0.074	3.92	0.97
June	12	d	44	10	5	0.124	0.71	0.94	0.039	3.36	0.92	0.061	1.84	0.95
	13	s	30	16	4	0.167	4.32	0.97	0.122	3.29	0.96			
July	14	s	16	17	4	0.380	3.00	0.97	0.140	3.5	0.93	0.250	3.50	0.94
	15*	s	10	17	5	0.320	7.04	0.99	0.177	4.5	0.94	0.230	5.27	0.96
	16	s	16	17	5	0.310	4.40	0.99	0.160	2.8	0.97	0.290	2.55	0.92
	17	s	16	17	3	0.300	5.00	0.98	0.120	5.02	0.89			
	18	s	16	17	4	0.420	6.00	0.97	0.180	5.48	0.92			
	19	d	26	13	5	0.160	1.65	0.99	0.069	2.8	0.98	0.120	2.97	0.98
	20	d	26	13	3	0.250	1.05	0.96	0.050	2.52	0.94	0.094	2.30	0.97
September	21	s	18	20	5	0.317	3.36	0.99	0.198	3.1	0.96			
	22	s	20	20	5	0.298	3.00	0.98	0.152	3.45	0.97	0.188	2.80	0.93
	23	d	53	15	5	0.205	0.63	0.99	0.070	1.16	0.93	0.127	1.05	0.72
	24	d	53	15	4	0.210	0.60	0.98	0.074	1.24	0.99	0.120	1.00	0.97
1989														
February	25	s	65	5	4	0.022	3.50	0.98	0.019	16	0.97			

Table I. Regression results from dilution growth experiments

— slope of ln-transformered ^3H thymidine incorporation rate (μ), cell abundance (δ) and cell biomass (γ) curves (h^{-1})

— intercept of ln- transformered ^3H thymidine incorporation (Vo, picomoles $l^{-1}h^{-1}$), cell abundance (No, 10^8 cells l^{-1}) and biomass (Bo, μg C l^{-1} curves).

— r = correlation coefficient.

— t = duration of the period from the dilution time to the first point time of the exponential growth phase.

— s = sample from surface water, d = sample from deep water.

— n = number of measurements.

— * = sample swirled during all the incubation period.

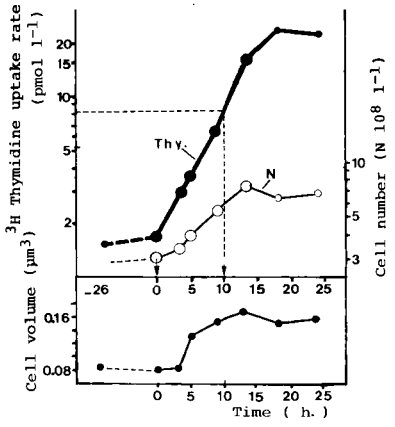

Fig. 1. An example of the time-course changes of the ^{3}H thymidine incorporation rate (Thy), bacterial numbers (N) and bacterial mean cell volume (experiment 19d). The big circles indicate the time points used for calculating the regression equations. The arrows indicate the selected period for estimating the conversion factor. At the end of this period ^{3}H thymidine uptake is equal to the incorporation rate measured in the natural environment.

Table II. Empirical conversion factors derived from the diluted water experiments.
— C_1N and C_2N conversion factors (10^9 cells nmol^{-1}) calculated by using respectively the ^{3}H thymidine incorporation rates μ and μ' (see text).
— C_1B, C_2B, CB conversion factors expressed in carbon units (μg C nmol^{-1}):
$C_1B = C_1N \times$ the mean cell carbon content during the experiment.
$C_2B = C_2N \times$ the mean carbon content of a cell at the start point of the experiment.
CB = Increase of carbon biomass divided by ^{3}H thymidine incorporation.

		C_1N	C_1B	CB	C_2N	C_2B
1987						
April	1	4.6	50.0	85.0	8.5	51.0
August	2	9.0	108.0			
	3	9.3	100.0			
	4	5.5	82.5	111.0	6.8	81.6
November	5	3.8	28.5	56.2	5.6	30.2
December	6	3.8	37.0	101.0	5.5	40.0
1988						
January	7	2.8	28.0	48.3	4.9	28.0
	8	2.0	24.0	27.5	3.5	18.6
February	9	8.8	58.0	62.4	9.8	58.0
March	10	4.3	27.0	59.8	7.4	32.0
	11	4.8	34.5	78.0	7.2	38.8
June	12	5.7	40.0	65.5	7.5	44.7
	13	8.1	58.7			
July	14	7.2	94.5	185.0	8.2	78.7
	15	8.2	113.0	145.0	9.5	114.0
	16	5.2	77.0	129.0	9.2	77.0
	17	6.2	59.5			
	18	9.3	100.0			
	19	7.0	109.0	172.0	9.3	89.0
	20	4.1	49.5	92.7	5.5	53.0
September	21	10.7	103.0		12.3	
	22	8.8	105.0	124.0	10.3	99.0
	23	4.2	66.0	99.0	7.0	66.8
	24	4.9	65.0	94.0	6.0	63.3
1989						
February	25	9.0	70.0			

For eighteen experiments, individual bacterial size increased during the first part of the exponential growth phase, then remained rather constant (Fig. 1). The biomass growth rates (γ) which incorporate increases in both cell number and individual cell biomass varied from 0,018 to 0,29 h^{-1} ($\bar{\gamma}$ = 0.116 ± 0,08). The number of bacteria produced per nanomole of thymidine incorporated into the insoluble cold TCA fraction (C_1N in Table II) varied from 2 to 10.7 × 10^9 ($\overline{C_1N}$ = 6.29 ± 2.35 × 10^9). By multiplying the values by the mean carbon content of the cells during the incubation, the factors (C_1B, Table II) ranged from 24 to 113 μg C per nanomole of thymidine incorporated ($\overline{C_1B}$ = 68.3 ± 28.7). When growth in cell numbers was slow but cell volume increased, these conversion factors were much lower than the conversion factors calculated directly by dividing the biomass increase during the selected time period by the integrated ^{3}H thymidine incorporation ($\overline{CB}$ = 93.3 ± 36.9 μg C nmol^{-1}, ranging from 27.5 to 185). This difference, noted by Kuparinen (1988), arises from the fact that population biomass production is a combination of the new production of large cells and the biovolume increase of the cells initially present in the diluted water sample.

Influence of culture conditions on bacterial population dynamics.

Swirling the culture decreased the lag period without modifying the bacterial growth and ^{3}H thymidine incorporation dynamics (experiments 2s and 15s in July and August). In March, the increase in temperature from 7 to 16° C decreased the lag period from 73 to 38 h ; it increased much more the

thymidine uptake rate than the growth rate in cell number. However, as the specific incorporation rate was surprisingly low at the beginning of the exponential phase, this did not yield a diminution of the conversion factor.

The increasing amounts of ^3H thymidine added to verify if the thymidine uptake rate was saturated at the concentration of 20 nM. (the concentration used for the routine experiments) did not change the incorporation rates except for the water surface sample of September in which the incorporation rates were slightly higher at the concentration of 35 nM.

The variation coefficient (S/X × 100) of the 5 conversion factors estimated in July was about 20 %. So we can consider the estimates to be accurate enough and reproducible.

4. Discussion

Assuming an exponential growth, Kirchman et al. (1982) derived conversion factors (C) from the thymidine incorporation rate (μ) (or the growth rate in number, δ) from the initial values of the number of bacteria (No) and the ^3H thymidine incorporation, (To) : $C = \mu NoTo^{-1}$. However, this model assumes a close coupling between the increase in bacterial numbers and substrate incorporation i. e. $\mu = \delta$. In our experiments, the uptake rate μ, was always higher than bacterial growth rate δ. This discrepancy has been reported and analysed in several works (Ducklow & Hill 1985 a, b, Kirchman et al. 1982, Scavia et al. 1986, Nagata 1987). When the added amount of ^3H thymidine is sufficient to eliminate isotopic dilution, the discoupling between the two processes may result from the presence of inactive cells or (and) the changes of physiological state of the bacterial assemblage, including the possible changes in bacterial composition.

The increase in size, often noted in such experiments (Kirchman et al. 1982, Lovell & Konopka 1985, Nagata 1987, Riemann et al. 1987), may be the expression of the metabolism change. Indeed, the diagrams of the bacterial size frequency (Fig. 2) showed that the increase in cell size did not only result from a preferential growth of large cells in the absence of bacterivores, but also from a uniform growth in size of the cells in the culture. Moreover the fractionation of macromolecules carried out during two experiments in July and September 1988

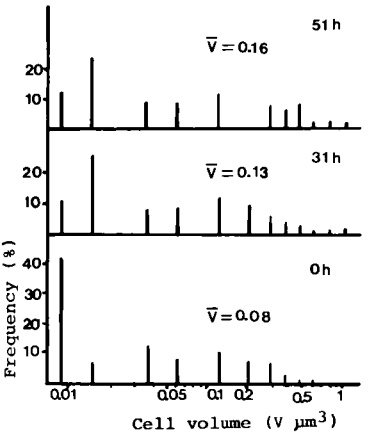

Fig. 2. Volume-frequency distribution of the cells at 3 different times of a bacterial culture (experiment 19 d) : - 0 h. lag phase, - 31 h. exponential growth phase - 51 h. stationnary phase.

showed that the proportion of radioactive marker in the proteins increased during the incubation with a respective increase in cell size (Table III). On the other hand, in February 1988, the cell size remained constant and the partition of ^3H thymidine incorporated did not change during the experiment. This increase in size may result from a stimulation of cell metabolism due to the dilution of samples which provided an increase in nutrients available per cell (Chrost et al. 1988). In these conditions, how does one apply the experimental results to the natural environment where the size of the bacteria remained small ? A rough estimate of the conversion factors can be made by not taking into account the proportion of ^3H thymidine which entered the biomass corresponding to the increase in cell size. This proportion can be determined by the difference of the growth rates ($\gamma - \delta$) and the thymidine incorporation rate (μ') directly linked to the increase in bacterial numbers. Hence, μ' was calculated from the relation $\mu' = \mu - (\gamma - \delta)$. μ' usually remained greater than δ, probably due to the presence of

Table III. (^{3}H) in DNA, RNA and proteins as a percentage of total (^{3}H) in TCA insoluble material at the beginning (lag period, l.p.) and at the end (stationnary period, s.p.) of 3 diluted-water experiments (values = mean % ± standard deviation of the 5 samples treated at each date).

	DNA	RNA	Proteins	Cell size (μm^3)
July				
l.p.	72.6 ± 8	10 ± 5.2	17.4 ± 4.1	0.07
s.p.	54 ± 8.2	16.4 ± 2.8	29 ± 6.7	0.18
September				
l.p.	77.4 ± 5.7	6.8 ± 1.9	15.8 ± 4.8	0.08
s.p.	58.2 ± 7.1	14.8 ± 4.5	27 ± 5.7	0.27
February				
l.p.	72.4 ± 4.6	10.2 ± 5.4	17 ± 6	0.045
s.p.	74 ± 8	10 ± 4	15 ± 8	0.045

inactive bacteria. This rate is independant of the individual cell volume increase and the conversion factors are calculated by dividing the cell number increase during the growth phase by the integrated ^{3}H thymidine incorporation calculated with μ'. As calculated, the conversion factors (C_2N, Table II), vary from 3.47 to 10.7 × 10^9 cells nM^{-1} thymidine incorporated (C_2N = 7.8 ± 1.77). They are much greater and variable than the theoretical conversion factors < 2 × 10^9 (see Bell 1988) ; on the other hand, they are in the range of the results of Nagata (1987), Scavia & Laird (1987) and Smits & Riemann (1988) for the fast growing bacteria in freshwater environments. The number of cells formed per nanomole of thymidine incorporated is then multiplied by the mean cellular carbon concentration at the start of incubation and the average conversion factor expressed in carbon units was 66.5 μg C $nmol^{-1}$ (ranging from 18.6 to 114).

For Bell (1988), the high conversion factors resulted mainly from an underestimate of the isotopic dilution. Our experiments, performed with increasing concentrations of ^{3}H thymidine at various times during the study, suggested that the 20 nM concentration was generally sufficient to saturate the thymidine uptake. This is consistent with several works showing that a 10-15 nM added concentration of ^{3}H thymidine was sufficient to inhibit the completely *de novo* synthesis (Riemann & Sondergaard 1986, Smits & Riemann 1988). We nevertheless think the high conversion factors from the September experiments may result from ^{3}H thymidine unsaturated samples.

The high values may also result from the selection of active strains. The bacterial assemblages, as characterizied from the strains isolated from spread plates, showed changes in their properties and overall a strong decrease in their diversity during the incubations. Although only a low proportion of the bacteria develop on plate cultures, these results emphasized the changes which may occur during the experiments, particulary when the lag phase is long (Fuhrman & Azam 1982, Lovell & Konopka 1985). Likewise, the asynchronous evolution of the numbers and the mean individual cell volume of the bacteria in experiment 11s (Fig. 3) suggested the development of a little diversified population after the lag phase. Note, however, that these observations are in partial disagreement with the rather uniform increase in cell size which occurred during the incubations.

^{3}H thymidine incorporation may be uptake limited if the tritiated thymidine was transported into the cell slower than TTP was incorporated into DNA, or if thymidine phosphorylase activity is so great that thymidine is degraded as fast as it is taken up

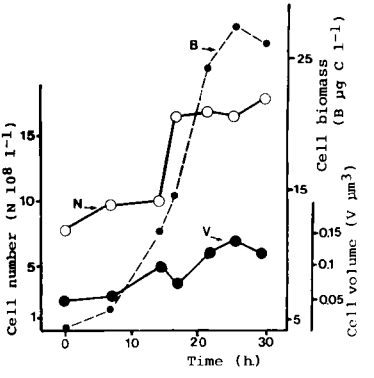

Fig. 3. Time-courses of mean cell volume, cell numbers and total cell biomass for experiment 11s (Note the asynchronous evolution of the mean individual cell volume and the cell numbers).

(Moriarty 1988). This, which yields high conversion factors, would mainly occur for the fast growing bacteria (Smits & Riemann 1988), which is the case for the majority of our experiments, in which the doubling time was lower than thirty hours. However, it is not clear if the limitation of uptake completely explains the high conversion factors assessed from the radioactivity incorporated into the cold- TCA precipitate, i. e. into all the macromolecules. On the other hand, it may explain the strong changes of the conversion factors through the growth cycle (Kirchman & Hoch 1988, Smits & Riemann 1988, our results) : during the lag and stationary phase, as the growth is low, thymidine incorporation rates were not uptake limited, contrary to what happened during the exponential phase.

These diverse results and considerations underline the problems of the interpretation and ecological use of the results from « *in vitro* » experiments. In particular, when there is a discrepency between the curves of the incorporation rates and cell numbers the choice of the period for calculating the conversion factors may greatly influence the results (Fig. 4). Even if the whole growth period is considered, the results depend on the frequency of the measurements for determining exactly the end of the lag period and overall the beginning of the stationnary phase ; indeed, thymidine incorporation continues after the growth in number ceased.

As noted by Smits & Riemann 1988, the production of fast growing bacteria is probably underestimated when the thymidine incorporation rates measured in natural conditions are converted into bacterial production by using the theoretical conversion factors. This may occur when the bacterial populations strongly increase (for example, during the spring algal bloom) and justify the estimating of the conversion factors several times during an annual cycle. Indeed, the seasonal variations of the conversion factors (also observed in Lake Michigan by Scavia & Laird 1987), probably represented the succession of various different dominant forms. On the other hand, the application of these high conversion factors in natural environments where the bacterioplankton is constituted by many subpopulations growing with different growth rates is doubtful ; indeed they probably resulted only from the growth of the most active part of the natural assemblage « *in vitro* ».

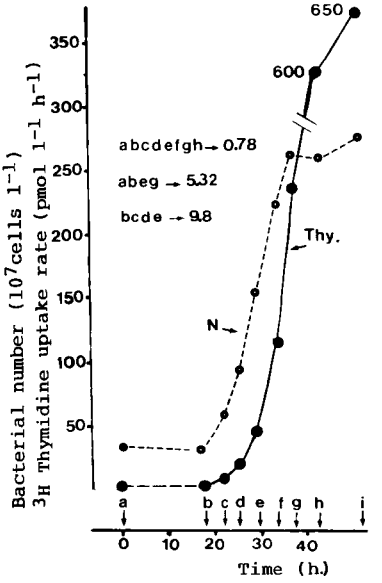

Fig. 4. Conversion factors estimates from changes in ^3H thymidine uptake and in bacterial numbers. Different values may be obtained according to the selected time periods. (Ex. from the measurements b, c, d, e, the conversion factor = 9.8×10^9 cells nmol^{-1}).

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