

## **Bacterioplankton Production Determined by DNA Synthesis, Protein Synthesis, and Frequency of Dividing Cells in Tuamotu Atoll Lagoons and Surrounding Ocean**

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**Abstract.** This study compares three independent methods used for estimating bacterioplankton production in waters from the lagoon (mesotrophic) and the surrounding ocean (oligotrophic) of two atolls from the Tuamotu archipelago (French Polynesia).

Thymidine and leucine incorporation were calibrated in dilution cultures and gave consistent results when the first was calibrated against cell multiplication and the second against protein synthesis. This study demonstrates that determining conversion factors strongly depends on the selected calculation method (modified derivative, integrative, and cumulative). These different estimates are reconciled when the very low proportion of active cells is accounted for.

Frequency of dividing-divided cells (FDDC) calibrated using the same dilution cultures led to unrealistically high estimates of bacterial production. However, highly significant correlations between FDDC and either thymidine- or leucine-specific incorporation per cell were found in lagoon waters in situ. These correlations became more positive when oceanic data were added. This suggests that the FDDC method is also potentially valid to determine bacterioplankton growth rates after cross calibration with thymidine or leucine methods. If recommended precautions are observed, the three methods tested in the present study would give reliable production estimates.

### **Introduction**

Planktonic bacteria play an important role in most aquatic ecosystems. The development of epifluorescent microscopy and tracer approaches has led to a better understanding of their contribution to the cycling of energy and matter in various pelagic ecosystems. However, little is known about their importance in atoll lagoons [50],

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as most of the studies dealing with coral reef environments were carried out in waters overlying coral reefs [10, 28, 30, 31].

Among the methods proposed to determine bacterial production in aquatic ecosystems, three are widely employed. Frequency of dividing cells (FDC) [18, 33], [*methyl-<sup>3</sup>H*]thymidine incorporation (TdR) [14, 15], and [*4,5-<sup>3</sup>H*]leucine incorporation (Leu) [24] have been used as indexes of bacterial growth rates, DNA synthesis, and protein synthesis, respectively. These methods provide bacterial production estimates only if adequate conversion factors (CF) are known. Due to the strong variations reported, thymidine [40], leucine [7, 12, 24, 25, 43], and FDC [1, 9, 17, 19, 28, 32, 33] conversion factors must be determined when investigating an unknown ecosystem.

The objective of this study was to test the adequacy of these three techniques for the purpose of estimating the contribution of heterotrophic bacteria to carbon fluxes in Tuamotu atoll lagoons (French Polynesia). These three methods were thus used together in situ and calibrated in dilution cultures. A recent work [12] emphasized the differences of TdR and Leu CF values obtained using different calculation methods. Therefore, special attention was devoted to the calculation of CF for isotope incorporations. The CF calculation was first implemented for Tikehau lagoon waters. The three methods were then compared for determining bacterioplankton production in Tikehau and Takapoto lagoons and oceanic waters.

## Materials and Methods

### *Sites of Study, Sampling*

Water samples were collected during five field trips in the lagoon of Tikehau (148°15'W, 14°55'S, Tuamotu archipelago, French Polynesia) 9–15 November, 1991, 15–21 March, 1992 (start and end of the rainy season, respectively), 8–22 November, 1992, 16 May–3 June, 1993, and 31 July–19 August, 1994 (dry season). Samples were collected daily (at 9:00 a.m. or at 4:00 p.m.), mostly from the Faufaa reference station at 0.5 m depth (total depth 20 m) representative of the main part of Tikehau lagoon (average depth 25 m, [3]). The Tikehau data set includes also two diel studies (17–18 November, 1992 and 24–25 May, 1993) and ten vertical profiles at different seasons. This lagoon has been described previously for phytoplankton and phytobenthos productivity [5], zooplankton [27], and particulate organic matter [3].

Samples were also collected from ten stations in the lagoon of Takapoto (145°20'W, 14°30'S, average depth 23 m [41]) during 16–25 January, 1994 at various depths and daylight hours.

Occasionally, water was sampled 0.5 m deep in oligotrophic ocean waters surrounding Tikehau (50–500 m from the reef rim, depth 50 m to >400 m, respectively, on 19 November 92, 22 May 93, and 24 May 93).

Single water samples collected using acid-washed 1.7-liter Niskin bottles were immediately transferred into acid-washed 1-liter polycarbonate bottles and stored in the dark at in situ temperature until treatment at the ORSTOM station within 1 hr after sampling. Samples were then processed at the laboratory, as described below.

### *Abundance, Biovolume, Biomass*

Single 5-ml subsamples were filtered onto 0.2 µm Nuclepore DMF, Gonesse, France membranes. Bacteria were stained with DAPI [38] and counted under epifluorescence (magnification ×1000) immediately after sampling, or on microscopic slides that had been frozen at –20°C. Enumeration

of cyanobacteria was made on the same slides by switching from the ultraviolet filter set required for DAPI (Leitz Ploemopak A, Leica, Rueil-Malmaison, France with BP340-380 excitation filter, RKP 400 dichroic mirror, LP 430 barrier filter) to the blue filter set (Leitz Ploemopak I2/3, with BP 450-490 excitation filter, RKP 510 dichroic mirror, LP 515 barrier filter). Cyanobacteria were then easily distinguished by their bright orange fluorescence. More than 400 cells in at least 20 fields were counted. A total of 50 duplicate subsamples were enumerated and values of each set of duplicate samples differed on average by 9.9% of the mean. Mean cell volumes were estimated by delimiting bacterial contours on photographic slides projected on a sheet at a final magnification of 10,000. Length and width of individual cells were determined with a digitizing tablet. Bacterial volumes were computed by assuming bacteria to be cylindrical with a hemisphere at both ends. At least 2 photographic slides were used per sample for a total of at least 150 cells. Mean cell volume estimation differed on average by less than 20% (range 4–36%) on the 2 slides of the same sample. While we know that there are considerable uncertainties about the carbon volume ratio for planktonic bacteria, we used carbon contents proposed by Simon and Azam [44], as their comprehensive study includes carbon, protein, and DNA contents on a wide range of cell volumes.

#### *Frequency of Dividing-Divided Cells (FDDC)*

Dividing-divided cells (DDC) were counted according to Davis and Sieburth [9], including daughter cells with a clear separation if the space between them was less than a cell radius. In this case the two cells were counted as one "dividing-divided cell." To enable comparison with other works, we estimated the frequency of dividing cells (FDC) according to Hagström et al. [18] (excluding daughter cells) on selected samples. In our samples  $FDC = 0.82 \times FDDC$  ( $SE = 0.02$ ,  $n = 19$ ) on average. More than 30 dividing cells on at least 20 fields were enumerated. A total of 50 duplicate subsamples were enumerated and values of each set of duplicate samples differed on average by 15% of the mean.

#### *TdR Incorporation*

TdR incorporation was performed routinely by amending duplicate 10-ml or 30-ml subsamples (for lagoon water and oceanic water, respectively) with 10 nM [*methyl*-<sup>3</sup>H]thymidine (final concentration, Amersham France, Les Vlis, France, 1.74 TBq mmol<sup>-1</sup>). After 30 min incubation with the label at 30°C ( $\pm 1^\circ\text{C}$  in situ temperature), the duplicates were chilled in a 2°C water bath for 10 min. Samples were then filtered onto 0.2- $\mu\text{m}$  Nuclepore polycarbonate membranes and rinsed with 5 ml of 0.2- $\mu\text{m}$  filtered lagoon water. The vacuum was disconnected and filters received 15 ml ice-cold 5% TCA. After 15 min, vacuum was reapplied and the membranes were rinsed 3 times with 5 ml of ice-cold 5% TCA. Membranes were stored at  $-20^\circ\text{C}$  in scintillation vials before radioactivity determination (within 2 weeks). DNA was hydrolyzed by 0.5 ml of 0.5N HCl at 100°C during 30 min in the vials [14, 47]. Radioactivity was determined after allowing samples to stay in scintillation cocktail overnight to minimize chemiluminescence. Quench correction was made with external standards. Incorporation was calculated after subtracting a zero time blank. Incorporation was linear for more than 90 min while incorporation rate always saturated at less than 5 nM TdR (checked on every field trip, data not shown).

Labeled DNA was extracted enzymatically from selected in situ samples following a modification [47] of Wicks and Robert's [48] procedure. Label recovered in DNA averaged 73.6% with minor fluctuations ( $SE = 1.7\%$ ,  $n = 65$ ). Thus, on the remaining samples, incorporation into DNA was calculated as incorporation into cold TCA-precipitable material multiplied by 0.736. Of the 26% not removed by DNase activity, ethanol soluble compounds represented 8.7% of TCA precipitable radioactivity on average ( $SE = 1.8$ ,  $n = 8$ ). Isotope dilution assays [37] performed at different seasons showed that labeled TdR constituted 98.7% on average of TdR incorporated according to this procedure ( $SE = 0.7\%$ ,  $n = 18$ ).

#### *Leucine Incorporation*

Leucine incorporation into TCA-precipitable material was assayed using an isotope dilution procedure [37] on all samples during March and November 1992 Tikehau experiments and on selected samples

in Takapoto, (January 94). Four 10-ml replicates received 4 nM L-[4,5-<sup>3</sup>H]leucine each (6.11 TBq mmol<sup>-1</sup>) and 0, 20, 40, and 60 nM unlabeled leucine (final concentrations). Incubation, filtration, and precipitation of labeled macromolecules were performed as described for TdR. In addition, cold TCA precipitate recovered on the membranes was rinsed 2 times with 5 ml of ice-cold 80% ethanol [49]. A zero-time blank was subtracted. Maximum incorporation rates ( $V_{max}$ ) were computed from the regression of leucine concentration (sum of labeled and unlabeled added) versus the reciprocal of incorporation rates [37]. The rate was determined using 24 nM Leu and represented  $89.3 \pm 2.3\%$  ( $n = 51$ ) and  $86.9 \pm 4.4\%$  ( $n = 5$ ) of  $V_{max}$  on average ( $\pm$ SE) in Tikehau and Takapoto, respectively. Leu incorporation into TCA-precipitable material was thus assayed routinely on 10-ml duplicates amended with 24 nM leucine and corrected with these values to obtain  $V_{max}$ .

### *Specificity of Bacterioplankton for TdR and Leu Incorporation*

In atoll lagoons like Tikehau and Takapoto, large numbers of active cyanobacteria of the genus *Synechococcus* are observed [6]. The specificity of incorporation of both labels for bacteria was verified by comparing the pattern of label incorporations and abundance of both bacteria and cyanobacteria in different size classes using Nuclepore membranes of 0.2-, 0.6-, and 0.8- $\mu$ m porosity.

### *Experimental Calibrations*

Dilution cultures were obtained by inoculating 10–30% of 0.6- or 1- $\mu$ m filtered lagoon water into 0.2- $\mu$ m filtered lagoon water in order to remove potential limitation of bacterial growth by available nutrients and grazing by eukaryotes greater than 0.6 or 1  $\mu$ m [14]. The absence of flagellates was verified during microscopic enumeration of bacteria. Bacteria were allowed to grow in the dark at 30°C with gentle stirring (100 rpm). Periodically (1.5–2 h) water samples were removed and bacterial abundance, biovolume, and FDDC were determined as described above. Exponential growth was assessed by the significance of the regression of  $\ln$  (cells liter<sup>-1</sup>) versus time, from the beginning of the culture until a plateau was reached. Exponential rates of increase for other bacterial parameters were determined by the slopes of the regressions during that period. TdR was added at a concentration of 20 nM in order to prevent isotope dilution and/or unsaturation of incorporation into fast-growing populations in these cultures. Comparison of rates with 20 nM and 40 nM concentrations were made occasionally and showed no significant differences. Recovery of the label into the DNA and total cold-TCA fractions were estimated systematically in dilution experiments. Labeled DNA constituted a constant percentage of labeled total cold-TCA fraction during exponential growth (mean 88%, SD 12%,  $n = 32$ ), and this percentage was significantly higher than in situ ( $P < 0.001$ , Student's *t*-test). Leucine incorporation was determined only in the 5 last cultures (C to G, see below), using the isotope dilution approach as described above. In order to relate leucine incorporation directly to protein synthesis, bacterial proteins ( $P$  in fg) were computed from average volumes ( $V$  in  $\mu$ m<sup>3</sup>) by the power function  $P = 105 V^{0.59}$  [44].

### *Statistical Analysis*

Correlation and regression analyses were performed according to Snedecor and Cochran [46].

## **Results**

### *In Situ Values*

Average parameters recorded in Tikehau, Takapoto, and oceanic waters surrounding the atoll are summarized in Table 1. With  $1.8 \times 10^9$  cells l<sup>-1</sup> in Tikehau lagoon, average bacterial abundance was rather typical of coastal waters and was significantly

**Table 1.** Average parameters recorded during this study<sup>a</sup>

		CELL	FDDC	TdR	LEU	TdR/ Cell	LEU/ Cell	LEU/ TdR
Tikehau lagoon	Mean	1.84	8.7	12.2	157	7.3	94	13.1
( <i>n</i> = 77)	SD	0.58	2.7	2.8	38	2.8	40	2.8
Takapoto lagoon	Mean	0.64	12.1	6.9	105	11.1	167	15.5
( <i>n</i> = 40)	SD	0.10	2.4	1.2	18	2.4	38	3.4
Both lagoons	Mean	1.43	9.9	10.4	139	8.6	119	13.9
( <i>n</i> = 117)	SD	0.74	3.0	3.4	41	3.2	52	3.2
Oceanic water	Mean	0.50	5.1	1.9	36	3.6	71	23.4
( <i>n</i> = 13)	SD	0.10	0.8	1.4	22	2.0	35	10.5
All data	Mean	1.34	9.4	9.5	129	8.1	114	14.9
( <i>n</i> = 130)	SD	0.76	3.2	4.2	51	3.4	53	5.2

<sup>a</sup>CELL, Bacterial abundance ( $10^9 \text{ l}^{-1}$ ); FDDC, frequency of dividing-divided cells (% of total); TdR, thymidine incorporation into DNA; LEU, leucine incorporation into cold TCA-precipitate (both in  $\text{pmol l}^{-1}\text{h}^{-1}$ ); TdR/Cell and Leu/Cell, specific thymidine and leucine incorporation rates per cell (both in  $10^{-21} \text{ mol cell}^{-1}\text{h}^{-1}$ ). Lagoon waters represent data pooled from Tikehau and Takapoto lagoons

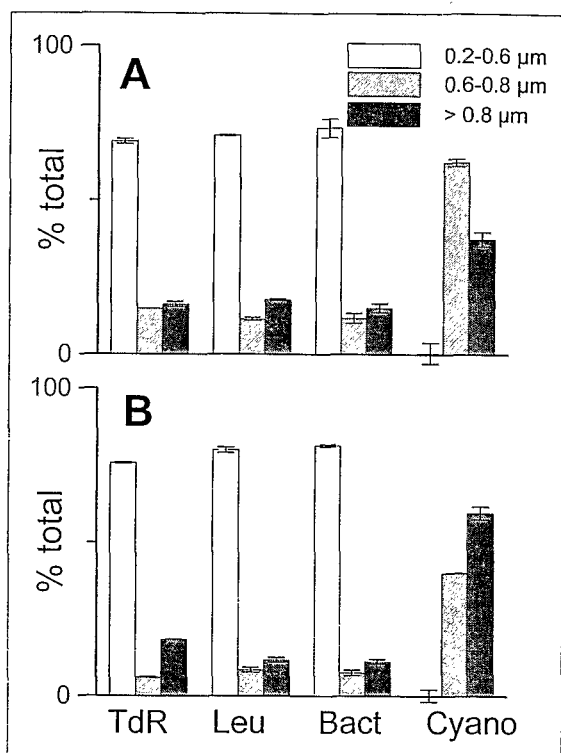
greater than in Takapoto lagoon. Values inside the lagoons studied were both significantly greater than in oceanic waters ( $P < 0.001$ , Mann-Whitney test). Average cell volumes were small and stable in these lagoons, ranging from  $0.03$  to  $0.06 \mu\text{m}^3 \text{ cell}^{-1}$  (average =  $0.050$ , SD =  $0.008$ ,  $n = 25$ ). Based on Simon and Azam's [44] relationships, lagoon bacteria would contain  $15.2 \text{ fg C}$  and  $17.7 \text{ fg proteins}$  per cell on average. FDDC averaged  $8.7\%$ ,  $12.1\%$ , and  $5.1\%$ , and were significantly different in Tikehau lagoon, Takapoto lagoon, and oceanic waters, respectively ( $P < 0.001$ , Mann-Whitney test). TdR and Leu incorporation varied little inside each lagoon and were also significantly different in the three sites ( $P < 0.001$ ).

#### *Specificity of Bacterioplankton for TdR and Leu Incorporation*

Results of two size fractionation experiments (Fig. 1) showed that the patterns of the incorporated labels into the three size classes were very similar to that of bacterial abundance. The very different shape for abundance of cyanobacteria suggested that these organisms were very unlikely to incorporate significant amounts of Leu and TdR in our experimental conditions.

#### *Growth in Dilution Cultures*

Average growth rates based on abundance were always significant ( $P < 0.05$ ) from inoculation of the culture to the beginning of the stationary phase and ranged from  $0.03$  to  $0.27 \text{ h}^{-1}$  (Table 2). Bacteria increased in size from  $0.05 \mu\text{m}^3$  ( $17.7 \text{ fg proteins}$ ) at the beginning of the cultures (average cell volume in situ) to  $0.18 \mu\text{m}^3$  ( $38 \text{ fg proteins}$ ) at most. Thus, proteins increased at a higher rate than abundance. TdR and Leu incorporation rates also increased exponentially and with higher rates than biomass. Exponential rates of increase were the same for TdR and Leu incorporation (Table 2). Both were generally faster than the exponential increase in bacterial protein. The lowest exponential rates of increase were recorded for cell numbers (Table 2).



**Fig. 1.** Fractionation of  $^3\text{H}$ -TdR label,  $^3\text{H}$ -Leu label, abundance of bacteria, and abundance of cyanobacteria in three size classes in Tikehau (A 19 May 93, B 26 May 93). Error bars represent replicate determinations.

**Table 2.** Exponential growth rates ( $\text{h}^{-1}$ ;  $\pm\text{SE}$ ) for bacterial parameters determined in the dilution cultures<sup>a</sup>

Exp.	Date	N	CELL	PROT	TDR	LEU
A	16 Nov 91	3	$0.271 \pm 0.011$	ND <sup>b</sup>	$0.239 \pm 0.058$	ND
B	17 Nov 91	4	$0.090 \pm 0.020$	ND	$0.089 \pm 0.020$	ND
C	19 Mar 92	6	$0.169 \pm 0.025$	$0.200 \pm 0.017$	$0.256 \pm 0.037$	$0.271 \pm 0.012$
D	15 Nov 92 a	5	$0.104 \pm 0.025$	$0.210 \pm 0.022$	$0.375 \pm 0.062$	$0.428 \pm 0.029$
E	15 Nov 92 b	7	$0.063 \pm 0.012$	$0.178 \pm 0.011$	$0.243 \pm 0.023$	$0.259 \pm 0.029$
F	27 May 93 a	10	$0.033 \pm 0.009$	$0.092 \pm 0.017$	$0.382 \pm 0.037$	$0.387 \pm 0.039$
G	27 May 93 b	9	$0.030 \pm 0.006$	$0.061 \pm 0.012$	$0.327 \pm 0.037$	$0.336 \pm 0.042$

<sup>a</sup>N, Number of points; CELL, PROT, TDR, LEU, slopes of ln-transformed bacterial abundance, proteins, thymidine incorporation rate into DNA and leucine incorporation rate into cold TCA-precipitate, respectively, versus time during exponential growth of abundance

<sup>b</sup>ND, not determined

## Discussion

### Calculation of Isotope Conversion Factors

Conversion factors may be calculated by three different methods: the modified derivative, integrative, and cumulative methods, which are summarized and dis-

**Table 3.** Thymidine/cell and Leucine/protein conversion factors determined from the dilution cultures

Exp.	Date	10 <sup>18</sup> cells per mol TdR				g proteins per mol Leu			
		Modified derivative	Integrative	Cumulative	r <sup>a</sup>	Modified derivative	Integrative	Cumulative	r <sup>a</sup>
A	16 Nov 91	0.698	0.913	0.989	1.000				
B	17 Nov 91	1.066	1.071	0.710	1.000				
C	19 Mar 92	1.956	0.625	0.533	0.971	3597	1377	1276	0.957
D	15 Nov 92 a	4.553	0.685	0.378	0.998	7810	1188	990	0.998
E	15 Nov 92 b	4.008	0.812	0.792	0.956	4969	2245	2194	0.979
F	27 May 93 a	202.7	1.062	1.234	0.998	296813	2854	2309	0.982
G	27 May 93 b	56.64	1.091	0.954	0.994	59543	1369	1248	0.994
	Mean	38.8	0.894	0.798		74546	1807	1603	
	SD	75.0	0.192	0.290		126448	716	604	

<sup>a</sup>r, Pearson correlation coefficient of the linear relation between integrated incorporation of the label and cumulated biomass index for TdR and Leu, respectively

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cussed by Ducklow et al. [12]. Thymidine conversion factors (TCF) and leucine conversion factors (LCF) were determined from the data by means of the modified derivative and cumulative methods (Table 3). As noted by Bjornsen and Kuparinen [2], the integrative method uses only initial and final values of biomass and activity in the cultures. In order to use the whole data set for the integrative method, initial and final values were estimated from the regression equations (Table 3).

#### *Experimental Calibrations of TdR Incorporation*

Ducklow et al. [12] reported that CF values calculated with the three methods are equivalent when the growth rates for biomass and label incorporation are similar. This is observed in the two first cultures (A and B, Table 3) characterized by similar growth rates for abundance and TdR incorporation (see Table 2). In the last five cultures (C–G, Table 2), while the integrative and cumulative methods still give comparable results, the modified derivative method yields substantially higher estimates. This difference is explained by the much faster increase of label incorporation relative to the biomass index. This tends to decrease the CF with increasing time intervals [12]. Both integrative and cumulative methods, putting maximum weight on the high values at the end of the incubation, lead to lower CF in this case. On the other hand, the integrative and cumulative approaches have the advantage of limiting the influence of transient phenomena such as temporary uncoupling between macromolecule synthesis and cell multiplication.

This apparent uncoupling has been reported frequently [11, 23, 36, 42] and has not been explained satisfactorily [12]. In our experiments, as in others [12], the possible increase in TdR catabolism in the cultures is not supported by the high and constant proportion of TdR incorporated into DNA ( $88 \pm 12\%$ , see Materials and Methods). Other explanations could be a significant proportion of nongrowing cells at the beginning of the cultures, or a progressive shift of the community from a significant proportion of cells unable to take up TdR at the beginning of the culture to a predominance of TdR-active cells during exponential growth [36].

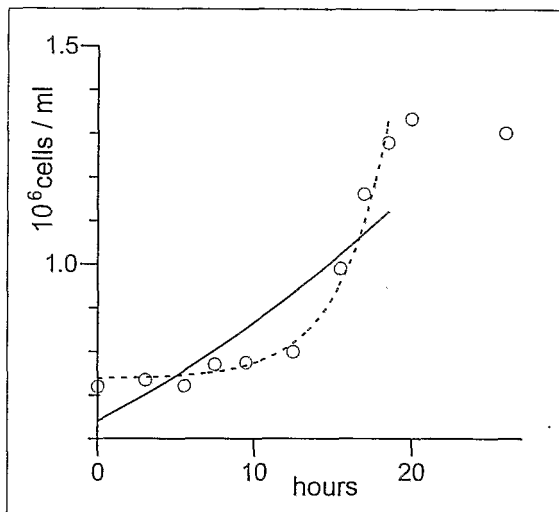
**Table 4.** Calculation of thymidine conversion factors (TCF) using the modified derivative method considering active and inactive cells

Exp.	Date	N = N(0) e <sup>μt</sup>		N = Na(0) e <sup>μat</sup> + Ni			TCF (10 <sup>18</sup> cells mol <sup>-1</sup> )	
		μ cells (h <sup>-1</sup> )	r <sup>a</sup>	active cells μa <sup>b</sup> (h <sup>-1</sup> )	active cells Na(0) (10 <sup>6</sup> l <sup>-1</sup> )	inactive cells Ni (10 <sup>9</sup> l <sup>-1</sup> )		r <sup>c</sup>
C	19 Mar 92	0.169	0.960	0.256	11.4	0.202	0.955	0.555
D	15 Nov 92 a	0.104	0.922	0.375	4.6	0.205	0.996	0.519
E	15 Nov 92 b	0.063	0.917	0.243	11.7	0.250	0.962	0.843
F	27 May 93 a	0.033	0.786	0.382	0.2	0.260	0.970	1.565
G	27 May 93 b	0.030	0.892	0.327	1.4	0.738	0.987	1.329
						Mean		0.962
						SD		0.467

<sup>a</sup>Correlation coefficient for the linear regression

<sup>b</sup>Assuming μa = slope of Log-transformed TdR vs. time

<sup>c</sup>Correlation coefficient for the nonlinear regression



**Fig. 2.** Increase of bacterial abundance in a dilution culture (27 May 93 B). *Solid line*  $N(t) = N(0)e^{\mu t}$ , *dashed line*  $N(t) = Na(0)e^{\mu a t} + Ni$ . See text for details.

Without excluding the second possibility, a low proportion of actively growing bacteria at the beginning of the cultures would be in agreement with the lag observed before exponential increase of bacterial numbers that was not observed for TdR incorporation. The modified derivative method assumes that all cells are growing exponentially, and could be biased if nongrowing cells are a significant proportion of the population. In order to test this hypothesis, the expression (1):  $N(t) = N(0) e^{\mu t}$  (implying that all cells are multiplying) may be replaced by the expression (2):  $N(t) = Na(0) e^{\mu a t} + Ni$  where Na represents "active cells" and Ni "inactive cells." Na(0) and Ni are determined by nonlinear regression, assuming that the active cells grow with a growth rate ( $\mu a$ ) identical to the slope of log-transformed TdR incorporation. Results presented in Table 4 (see also Fig. 2) show



that the correlation coefficient for expression (2) is equivalent to that for expression (1) for culture C, and greater in the last four cultures (D–G). Moreover, when the modified derivative method is applied to the active bacterial numbers, the average conversion factor ( $0.96 \pm 0.47$ , Table 4) is very close to those obtained by the two “model-free” approaches, i.e., integrative and cumulative methods ( $0.89 \pm 0.19$  and  $0.80 \pm 0.29$ , Table 3). Therefore, a very low proportion of active bacteria (0.1–5.4%, Table 4) at the beginning of the cultures is a consistent explanation for the lag phase and the apparent uncoupling between cell multiplication and TdR incorporation. This very small fraction of actively growing cells at the beginning of the cultures implies a low proportion of actively growing bacteria in the lagoon. However, filtration through 0.6- and 1- $\mu\text{m}$  membranes may also lower the proportion of active cells in the culture by removing larger ones such as dividing cells.

Even though all three methods yield comparable results after correcting the derivative methods for the proportion of active cells, we use the cumulative conversion factor, as its adequacy may be judged from the linear regression fit [2] (see  $r$  in Table 3).

#### *Experimental Calibrations of Leu Incorporation*

Leu incorporation is an estimation of protein synthesis. Therefore it must be related to biomass rather than to abundance of bacteria because we found that bacterial size increased during culture. This is confirmed by a Leu/TdR incorporation ratio always greater in the cultured cells ( $>20$ ) than in situ (13.1). The Leu conversion factor (using cumulative method) averages  $1603 (\pm 604)$  grams of proteins synthesized per mole of Leu incorporated (Table 3). With an average protein content of 17.7 fg for in situ cells (see above), LCF would thus be equivalent to  $1603 / 17.7 \times 10^{-15} = 0.091 \times 10^{18}$  cells  $\text{mol}^{-1}$  of leucine incorporated. This conversion factor is of course greater than the LCF derived directly from cell numbers ( $0.031 \pm 0.012 \times 10^{18}$  cells  $\text{mol}^{-1}$  using the cumulative method, not shown) as bacterial cells are larger in the cultures than in the lagoon.

#### *Experimental Calibrations of FDDC*

Published conversion factors of  $\mu$  versus FDDC or FDC [1, 9, 17, 19, 28, 32, 33] are of little help in our ecosystems as temperature dependency [18, 19] would prevent us from using these values in tropical waters. Moreover, with one exception [28], all of them are derived from experiments in natural conditions far from that prevailing in Tikehau or Takapoto lagoons or even in artificial conditions (bacterial isolates, enrichments, etc.). When applied to our values in Tikehau lagoon, these factors give a wide range of possible growth rates ( $-2.6$  to  $7.7 \text{ day}^{-1}$ ).

Estimated on the five last dilution cultures, FDDC is related to the growth rate of bacterial abundance calculated for the interval following FDDC determination according to Newell and Christian [33]. A linear relationship is found in only two out of five experiments (Table 5a). These fitted equations give very different results, limiting the interpretability of the data. However, using all the data in the same regression gives a significant correlation ( $P < 0.001$ ). Using  $\ln \mu$  as some authors

**Table 5a.** Regressions of growth rate for abundance ( $\mu$ ) versus frequency of dividing-divided cells (FDDC) in dilution cultures<sup>a</sup>

Exp.	Date	Slope	$\pm$ SE	Intercept	$\pm$ SE	<i>r</i>	<i>P</i>	<i>n</i>
C	19 Mar 92	0.0205	0.0066	-0.15	0.10	0.812	*	7
D	15 Nov 92 a	0.0008	0.0092	0.05	0.17	0.037	ns	8
E	15 Nov 92 b	0.0087	0.0014	-0.12	0.04	0.965	**	5
F	27 May 93 a	0.0150	0.0073	-0.08	0.06	0.610	ns <sup>b</sup>	9
G	27 May 93 b	0.0065	0.0057	-0.01	0.04	0.397	ns	9
All data		0.0061	0.0014	-0.01	0.02	0.596	***	38

**Table 5b.** Regressions of bacterial production (BP) versus dividing-divided cells (DDC) in dilution cultures<sup>a</sup>

Exp.	Date	Slope	$\pm$ SE	Intercept	$\pm$ SE	<i>r</i>	<i>P</i>	<i>n</i>
C	19 Mar 92	0.523	0.348	0.056	0.044	0.558	ns	7
D	15 Nov 92 a	0.544	0.634	-0.007	0.042	0.331	ns	8
E	15 Nov 92 b	0.954	0.135	-0.032	0.012	0.971	***	5
F	27 May 93 a	1.315	0.344	-0.020	0.011	0.822	**	9
G	27 May 93 b	0.639	0.343	-0.009	0.025	0.576	ns	9
All data		0.813	0.130	-0.009	0.010	0.722	***	38

<sup>a</sup>Units are:  $\mu$ , ( $\text{h}^{-1}$ ); FDDC, (%); BP, ( $10^9 \text{ cell l}^{-1} \text{ h}^{-1}$ ); DDC, ( $10^9 \text{ cell l}^{-1}$ )

<sup>b</sup>ns, Not significant ( $P > 0.05$ )

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

have [1, 19, 32, 33] does not improve individual regressions and leads to nonsignificant correlation with all data (not shown).

A stronger correlation is obtained when the five data sets are pooled and bacterial production (BP) during the following period is plotted directly against the number of dividing-divided cells (DDC, Table 5b). This correlation leads to a relationship very similar to the preceding one.

#### *Comparison Between TdR, Leu, and FDDC Estimates of Bacterioplankton Production*

The leucine conversion factor (LCF) is  $0.091 \times 10^{18} \text{ cells mol}^{-1}$  and is thus 9 times lower than the thymidine conversion factor ( $\text{TCF} = 0.80 \times 10^{18} \text{ cells mol}^{-1}$ ). The Leu/TdR ratio averages 13.1 in Tikehau lagoon (Table 1). Therefore, in situ production estimated from Leu incorporation is on average 47% greater than production estimated from TdR incorporation (Table 6). Considering all the potential sources of errors, a 1.5-fold difference may be considered a good agreement between these two independent methods. In fact, average TCF and average LCF-based TCF (LCF in cells per mole multiplied by the average Leu/TdR incorporation ratio in Tikehau waters) do not differ significantly ( $P > 0.05$ , Mann-Whitney test, Table 7). Both TCF and LCF values are not far from the minimum theoretical values, assuming no isotope dilution ( $0.51 \times 10^{18} \text{ cells mol}^{-1}$  TdR with 2.5 fg DNA  $\text{cell}^{-1}$  and a GC% of 50, and  $1797 \pm 468 \text{ g protein mol}^{-1}$  Leu with  $7.3 \pm 1.9\%$  Leu into

**Table 6.** Average bacterioplankton production and growth rates estimates in Tikehau lagoon water column from TDR, LEU, FDDC, and DDC calibrated in dilution experiments. Average depth is 25 m and bacterial parameters are homogeneous vertically

Derived from (average value)	TDR <sup>a</sup> (12.2 pmol l <sup>-1</sup> h <sup>-1</sup> )	LEU <sup>b</sup> (157 pmol l <sup>-1</sup> h <sup>-1</sup> )	FDDC <sup>c</sup> (8.7%)	DDC <sup>d</sup> (0.15 × 10 <sup>9</sup> cell l <sup>-1</sup> )
Growth rate <sup>f</sup> (day <sup>-1</sup> )	0.13	0.19	1.08	1.47
Production (10 <sup>6</sup> cells l <sup>-1</sup> h <sup>-1</sup> )	10	14	83	113
Production <sup>e</sup> (g C m <sup>-2</sup> day <sup>-1</sup> )	0.089	0.131	0.763	1.037

<sup>a</sup>With TCF = 0.798 × 10<sup>18</sup> cells mol<sup>-1</sup><sup>b</sup>With LCF = 0.091 × 10<sup>18</sup> cells mol<sup>-1</sup><sup>c</sup>With μ(h<sup>-1</sup>) = 0.0061 FDDC - 0.008<sup>d</sup>With BP (10<sup>9</sup> cells l<sup>-1</sup>h<sup>-1</sup>) = 0.813 DDC - 0.009<sup>e</sup>Using 15.3 fgC per 0.05 μm<sup>3</sup> cell (Simon and Azam [44])<sup>f</sup>Daily production = 24 times hourly production**Table 7.** Comparison between TCF and LCF determined using the cumulative method

Date	TCF (10 <sup>18</sup> cells mol <sup>-1</sup> )	LCF (g proteins mol <sup>-1</sup> )	LCF <sup>a</sup> (10 <sup>18</sup> cells mol <sup>-1</sup> )	LCF-based TCF <sup>b</sup> (10 <sup>18</sup> cells mol <sup>-1</sup> )
16 Nov 91	0.989	—	—	—
17 Nov 91	0.710	—	—	—
19 Mar 92	0.533	1276	0.0721	0.932
15 Nov 92 a	0.378	990	0.0559	0.723
15 Nov 92 b	0.792	2194	0.1240	1.602
27 May 93 a	1.234	2309	0.1305	1.686
27 May 93 b	0.954	1248	0.0705	0.911
Mean	0.798	1603	0.0906	1.171
SD	0.290	604	0.0341	0.441

<sup>a</sup>Obtained by dividing LCF in Fg proteins mol<sup>-1</sup> by 17.7 Fg protein per 0.05 μm<sup>3</sup> cell (Simon and Azam [44])<sup>b</sup>Obtained by multiplying LCF in 10<sup>18</sup> cells mol<sup>-1</sup> by the average Leu/TdR ratio of 13.1 for Tikehau bacterioplankton

proteins, both according to Simon and Azam [44]). This agreement with theoretical values is, however, not a strong argument for their validity as it was shown that even using the classical isotope dilution procedure, DNA synthesis may be greatly underestimated by TdR incorporation [21].

Average in situ growth rates calculated using FDDC and DDC calibrations from dilution cultures would be 1.08 and 1.47 day<sup>-1</sup> respectively (Table 6). These growth rates are 6–12 times greater than those derived from tracers. Although the correlation between FDC and TdR-specific incorporation rate is reported to be fairly good [32, 34, 39], bacterial production estimate with the FDC method is often much greater than production estimated by TdR incorporation (2–21 times greater in water samples [16, 20, 34, 39] and 20–114 times greater in sediments [13]).

There are three possible reasons for these discrepancies: (1) a bias due to

synchronization of growth in situ, (2) underestimation by the tracer methods, or (3) inappropriate FDDC calibration leading to an overestimation.

Synchronization of growth could lead to discrepancies as label incorporations and FDDC would not necessarily peak at the same time. However, our samples were collected at different times of the day and this should remove any trend or systematic bias between tracer and FDDC methods. Moreover, two diurnal cycles in Tikehau lagoon revealed very moderate variations of FDDC and specific incorporation rates per cell with no diurnal pattern (CV: 14.9%, 19.6%, 12.3% for FDDC, TdR, and Leu respectively, data not shown). Synchronization of growth is clearly not responsible for the discrepancies observed in our ecosystem.

Bacterial production could be underestimated by both tracer approaches. Fuhrman et al. [16] suggested that difference between FDC and TdR estimates might be due to grazing during TdR incubations, lowering net incorporation of label into DNA. But this is likely to be insignificant as our incubation times are very short (0.5 h) with regard to turn-over time of the whole bacterial population (16 h, using the shortest estimation given by our DDC/BP relationship, Table 6). Several other limitations have been reported for the isotope methods, including uncertainty in the rate-limiting step of the incorporation processes, the proportion of bacteria able to incorporate the label, and the extent of isotope dilution. But without dismissing possible artifacts, we believe that underestimation of bacterioplankton production by nearly the same factor when using the TdR and Leu methods is very improbable.

Another possible reason might be the inappropriate application of FDDC/ $\mu$  relationships obtained from cultures with a probably high percent of active cells to in situ samples with a likely lower proportion of active bacteria [33]. But a low proportion of active cells in situ would lead to an underestimation of FDDC and thus an underestimation of bacterial production. And this is not the case. Another possible explanation is the increase of the time from the constriction of the bacterial cell to its division as  $\mu$  decreases [33]. The FDDC method would thus be more sensitive to community changes in batch cultures than tracer approaches and this would preclude the use of FDDC calibrations from fast-growing dilution cultures.

Moreover, with a total primary production (pelagic + benthic) of about  $0.6 \text{ g C m}^{-2}\text{day}^{-1}$  in Tikehau lagoon [5], FDDC estimates of bacterioplankton production would lead to unrealistic values compared to primary production in this ecosystem receiving minor terrestrial inputs (Table 6). Hence, improper calibration of the FDDC method in dilution cultures is the most likely explanation for these discrepancies. We thus analyzed the correlations between FDDC and growth rates determined with TdR and Leu in order to determine if a cross calibration of FDDC values with tracers incorporation would make sense.

#### *In Situ Correlations Between Leucine and Thymidine Incorporations*

Thymidine and leucine incorporation show a strong covariation over the whole range of observations (Fig. 3). This has been reported in several studies [7, 8, 22, 25, 26, 29, 43, 45] and suggests that both provide reliable potential estimates of bacterial production. Leu/TdR ratios are rather constant (Table 1) in Tikehau, Takapoto and oceanic waters but differ significantly from each other ( $P < 0.001$ , Mann-Whitney test). These rather constant ratios indicate near steady-state condi-

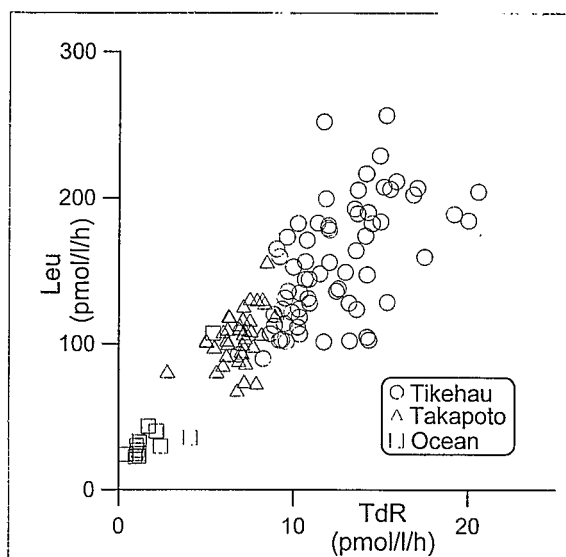


Fig. 3. Scatter plot of  $^3\text{H}$ -leucine and  $^3\text{H}$ -TdR incorporation in Tikehau lagoon, Takapoto lagoon, and ocean water samples.

Table 8. Matrix of Pearson's correlation coefficients between  $\log(\text{specific TdR and Leu incorporation rates per cell})$  and  $\log(\text{FDDC})$

Tikehau lagoon ( $n = 77$ )			Takapoto lagoon ( $n = 40$ )		
	$\log(\text{Leu/Cell})$	$\log(\text{FDDC})$		$\log(\text{Leu/Cell})$	$\log(\text{FDDC})$
$\log(\text{TdR/Cell})$	0.809***	0.657***	$\log(\text{TdR/Cell})$	0.575***	0.201 <sup>a</sup>
$\log(\text{Leu/Cell})$		0.755***	$\log(\text{Leu/Cell})$		-0.007 <sup>a</sup>
Tikehau and Takapoto lagoons ( $n = 117$ )			Oceanic waters ( $n = 13$ )		
	$\log(\text{Leu/Cell})$	$\log(\text{FDDC})$		$\log(\text{Leu/Cell})$	$\log(\text{FDDC})$
$\log(\text{TdR/Cell})$	0.846***	0.699***	$\log(\text{TdR/Cell})$	0.680***	0.326 <sup>ab</sup>
$\log(\text{Leu/Cell})$		0.747***	$\log(\text{Leu/Cell})$		0.447 <sup>a</sup>
All data ( $n = 130$ )					
	$\log(\text{Leu/Cell})$	$\log(\text{FDDC})$			
$\log(\text{TdR/Cell})$	0.821***	0.752***			
$\log(\text{Leu/Cell})$		0.759***			

<sup>a</sup>Nonsignificant

<sup>b</sup>0.577\* without the aberrant point

<sup>c</sup>0.769\*\*\* without the aberrant point (see text and Fig. 4A)

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

tions for bacterioplankton assemblages. The observed Leu/TdR ratio greater in oceanic waters than in lagoon waters is consistent with results from a comparable ecosystem [50] and may correspond to an increase of protein turn-over rate relative to cell turn-over rate in more oligotrophic waters. However, incorporation rates per cell also show significant correlations for each of the three ecosystems sampled and for all data pooled together (Table 8). Correlations between FDDC and both tracer methods were thus determined.

**Table 9.** Linear relationships of log(specific incorporation rates per cell)<sup>a</sup> and log ( $\mu$ )<sup>b</sup> versus log(FDDC)<sup>c</sup>

	Slope	$\pm$ SE	Intercept	$\pm$ SE	<i>r</i>	<i>P</i>	CT <sup>d</sup>	<i>n</i>
<b>A. Thymidine</b>								
Tikehau lagoon	0.83	0.11	0.06	0.10	0.657	***	1.017	77
Takapoto lagoon	0.23	0.19	0.78	0.20	0.201	ns <sup>e</sup>	1.012	40
Both lagoons	0.88	0.08	0.04	0.08	0.699	***	1.017	117
Ocean	1.71	0.77	-0.67	0.54	0.577	*	1.034	12
All data	1.03	0.08	-0.11	0.07	0.769	***	1.020	129
<b>B. Leucine</b>								
Tikehau lagoon	0.93	0.09	1.09	0.09	0.755	***	1.012	77
Takapoto lagoon	-0.01	0.18	2.22	0.19	-0.007	ns	1.011	40
Both lagoons	1.04	0.09	1.02	0.08	0.747	***	1.018	117
Ocean	1.27	0.48	0.93	0.50	0.477	ns	1.029	13
All data	0.98	0.07	1.09	0.07	0.759	***	1.019	130
<b>C. Growth rate</b>								
Tikehau lagoon	0.89	0.09	-1.61	0.09	0.747	***	1.011	77
Takapoto lagoon	0.08	0.16	-0.64	0.17	0.087	ns	1.009	40
Both lagoons	0.98	0.08	-1.66	0.08	0.756	***	1.015	117
Ocean	1.53	0.63	-2.04	0.44	0.611	*	1.023	12
All data	0.98	0.07	-1.66	0.06	0.790	***	1.016	129

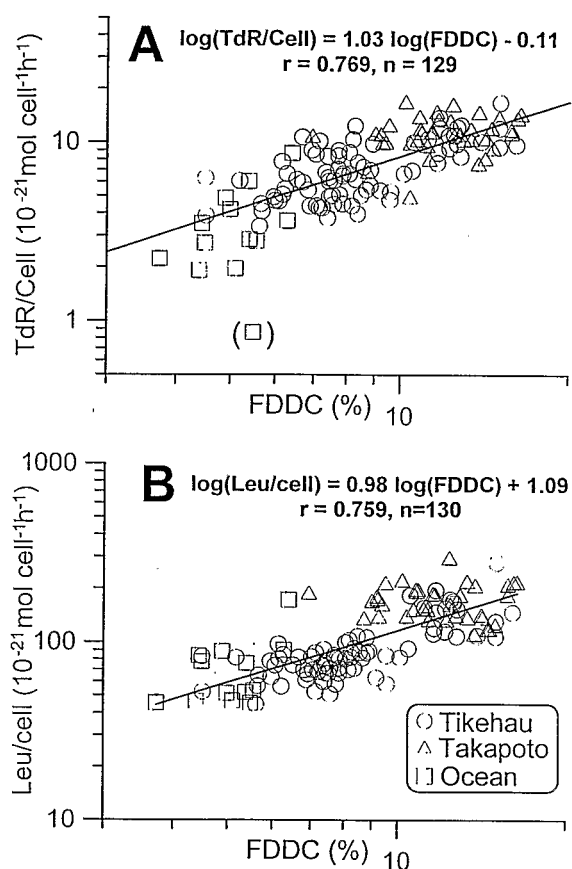
<sup>a</sup>Leu/cell and TdR/cell in  $10^{-21}$ mol h<sup>-1</sup>cell<sup>-1</sup><sup>b</sup> $\mu$  in day<sup>-1</sup>, calculated by averaging Leu and TdR based production (cell l<sup>-1</sup>h<sup>-1</sup>) divided by abundance of bacteria<sup>c</sup>FDDC in %<sup>d</sup>Multiplication by a correction term, CT =  $10^{\text{MSE}^2}$ , where MSE is the mean square of the error from the regression, is necessary to correct the bias created after back transformation from logarithmic to arithmetic scale [35]<sup>e</sup>ns, nonsignificant ( $P > 0.05$ )\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ 

### *In Situ Correlations Between Specific Incorporations per Cell and FDDC*

In order to estimate the correlations between tracers and FDDC approaches, data were log transformed to equalize the variance over the range of observations and to improve the normality of the data required by the statistical analysis. Least squares linear regressions were used. The correlation matrix (Table 8) shows that relationships of FDDC with TdR/Cell or Leu/Cell are significant in most of the cases. The absence of significant correlation in Takapoto lagoon might be explained in part by the very narrow range of TdR/Cell and Leu/Cell estimated during the single field trip. However, data from Takapoto reinforce the correlations when they are pooled with data from Tikehau. These relationships are not significant in oceanic waters alone, but once again, pooling oceanic data with lagoon data improves the correlations.

Hence, while the conversion of FDDC or DDC into growth rate or production values appears inadequate for as yet unexplained reasons, the proportion of FDDC is well correlated with cell-specific incorporation rates in situ. This would suggest that all three methods employed measure the same process in our ecosystem and that the discrepancies come from empirical calibration of, at least, DDC or FDDC.

We thus tried to quantify the relationships between growth rates obtained by tracer approaches and FDDC. When they are significant, the regression slopes of



**Fig. 4.** Plot of specific  $^3\text{H}$ -TdR incorporation per cell (**A**) and  $^3\text{H}$ -leucine incorporation per cell (**B**) versus FDDC. The point in brackets has been excluded from the regression.

$\log(\text{TdR}/\text{Cell})$  versus  $\log(\text{FDDC})$  are similar for the different water bodies (Table 9A, Fig. 4A). The same results are observed with  $\log(\text{Leu}/\text{Cell})$  versus  $\log(\text{FDDC})$  (Table 9B, Fig. 4B). When all the data are pooled together, these slopes are not significantly different from 1 indicating that direct relationships exist between FDDC and specific incorporation rates per cell.

Therefore, in order to calibrate FDDC to growth rate determined with both TdR and Leu estimates, we used the regression of growth rate values (averaged from Leu and TdR incorporations using their respective CF  $0.091$  and  $0.798 \times 10^{18}$  cells  $\text{mol}^{-1}$ ) versus FDDC obtained from in situ determinations to provide bacterial production estimates in this kind of ecosystem. These data generally lead to better relationships than those obtained by separate tracer approaches (Table 9C). Once again, pooling the oceanic data with lagoon data improves the correlations. Conversion factors for FDDC values in lagoon ecosystems would thus be:

$$\mu \text{ (day}^{-1}\text{)} = 10^{-1.66} \times \text{FDDC}^{0.98} \times 1.016 = 0.022 \times \text{FDDC}^{0.98}$$

Finally, after taking care of choosing the appropriate calculation method to determine the conversion factors for labels and by calibrating FDDC against tracers,

all three methods seem appropriate for estimating bacterioplankton growth rates in our study. We suggest the use of a cross calibration between FDDC and growth rates determined with tracer methods to obtain a realistic relationship rather than using a direct estimation of FDDC/ $\mu$  relationship in dilution cultures. The use of FDDC values to estimate bacterial production might be of particular interest whenever the use of radioisotopes or incubations of the samples are excluded. The strongest limitation of this method is its very tedious character when counted by eye. The improvements of automatic analysis such as image analysis [4] or flow cytometry might lead to a better use of this powerful and elegant technique.

In the two atoll lagoons studied, bacterioplankton production values are low compared to their biomass. This demonstrates generation times to be very long despite average temperature of 30°C. The ecological implications of biomass, production, and generation time values in these atoll lagoons will be treated in a separate publication.

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