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Measurements of Diel Rates of Bacterial Secondary Production in Aquatic Environments†

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Measurements of bacterial secondary production were carried out during 13 diel studies at one coastal marine station and in five lakes differing with respect to nutrient concentration and primary production. Bacterial secondary production was measured in situ every 3 to 5 h by [^{3}H]thymidine incorporation into DNA. In some of the diel studies, these results were compared with results obtained from dark $^{14}CO_2$ uptake and frequency of dividing cells. Only minor diel changes were observed. The rate of [^{3}H]thymidine incorporation into DNA and the frequency of dividing cells varied from 23 to 194% of the diel mean. The dark CO_2 uptake rate varied from 12 to 259% of the diel mean. An analysis of variance demonstrated that no specific time periods during 24 h showed significantly different production rates, supporting the idea that bacterial activities in natural assemblages are controlled by a variety of events. The best correction ($r^2 = 0.74$) was obtained between the [^{3}H]thymidine incorporation and frequency of dividing cells procedures from the lake water samples. The actual production rates calculated by [^{3}H]thymidine incorporation into DNA were appreciably lower than those obtained by the frequency of dividing cells and the dark CO_2 uptake techniques. Diel rates of bacterial production are discussed in relation to sampling frequency, statistical errors, and choice of method.

Particulate and dissolved organic matter in lakes is mainly decomposed by bacteria (11, 26). To estimate the rates of metabolism and amounts of organic matter metabolized by aquatic bacteria, it is useful to determine bacterial production as a basis for such estimates. Due to a lack of suitable techniques, such measurements have not been possible in the past. During the last few years, however, several new methods have been developed, particularly those based on [^{3}H]thymidine incorporation into DNA (2, 16), frequency of dividing cells (FDC) (6), and $^{35}SO_4$ incorporation into protein (1, 12). The dark uptake of inorganic carbon by bacteria was introduced by Romanenko (24) as a measure of bacterial production. The method was reexamined by Overbeck (20).

Much work is now in progress on methodological improvements and on relationships between bacterial production rates and physical, chemical, and biological events (9), which is adding to our knowledge of the ecological role of bacteria in aquatic environments.

Considering the observed short-term changes in some of the easily assimilated compounds of the dissolved carbon pool (12a), marked diel changes may be expected in the bacterial production rates, and in fact, appreciable diel changes in bacterial production in the water column over a seagrass bed have been reported (17). Such changes must be evaluated before any significant changes due to season can be recognized.

This study reports diel changes in bacterial secondary production estimated by means of (i) [^{3}H]thymidine incorporation into DNA, (ii) FDC, and (iii) dark CO_2 uptake. The objective was to evaluate diel changes in bacterial secondary production and to design a sampling program that would allow calculation of the diel production rates of bacteria from aquatic environments.

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MATERIALS AND METHODS

Water samples were taken from the central parts of five lakes in Denmark differing with respect to nutrient content and primary production. Lake Esrom and Frederiksborg Slotssø are situated on Zealand and have primary production rates of about 240 and 500 g of C m^{-2} $year^{-1}$. Lake Almind, Lake Mossø, and Lake Ørn are situated in the central part of Jutland; their primary production rates are about 60, 300, and 600 g of C m^{-2} $year^{-1}$, respectively (22; unpublished data).

The marine station, Rønbjerg harbor, is situated in the Limfjord in northern Jutland. The average depth is 8 m, and the salinity fluctuates around 27‰ at the sampling station near the marine laboratory. All experiments were carried out with water samples collected at sunrise and incubated in glass bottles just below the surface of the water. The subsampling method from enclosed water samples was used to ensure that diel changes were measured on the same population throughout. The water for the FDC and [^{3}H]thymidine incorporation measurements was contained in 5-liter bottles, whereas 1- and 0.5-liter bottles were used for the ^{14}C dark uptake experiments. Subsamples were taken every 3, 4, or 5 h.

Tritiated thymidine incorporation into DNA. The procedure of Fuhrman and Azam (2) was followed for the seawater samples, and a modification of this procedure was followed for the lake water samples (21). During 17 to 18 November 1980 and 6 to 7 April 1981 in Frederiksborg Slotssø, samples were assayed for radioactivity with material insoluble in cold trichloroacetic acid (TCA). An average of 26% of ^{3}H in cold TCA precipitate has been found in DNA (21). During the remaining 11 diel studies, DNA, RNA, and protein were assayed separately by the following procedure. Samples (5 to 10 ml) were extracted in (i) equal volumes of 10% ice-cold TCA for 5 to 15 min, (ii) 1 N NaOH (final concentration) at 60°C for 1 h and then chilled and acidified with TCA (1 g ml^{-1}, 1.4 ml per 5-ml water sample), or (iii)

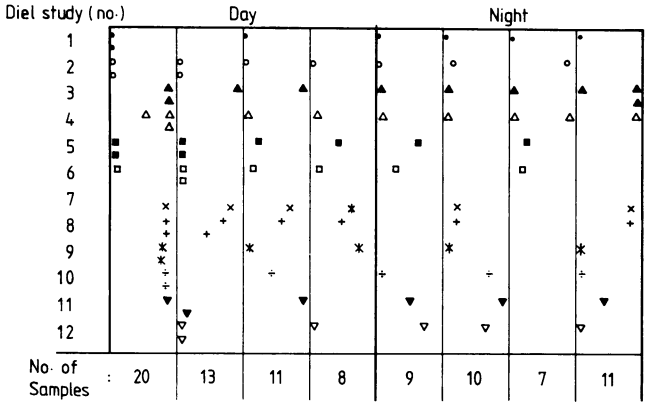

FIG. 1. Relative position of the sampling times during 12 diel studies in aquatic environments in relation to light and dark regimes indicated on a relative scale. Numbers 1 through 8 indicate freshwater diel studies and numbers 10 through 12 indicate diel studies from the marine station. Each symbol refers to individual diel studies.

equal volumes of 40% TCA at 95 to 100°C for 30 min and then chilled. Previous experiments with the macromolecular fractionations showed appreciable amounts of ^3H in the RNA and protein fractions (21). However, later experiments with DNase and RNase have suggested that the modifications in (ii) and (iii) should be used (unpublished data). In the freshwater samples, about 60 to 80% of ^3H incorporation was in the DNA fraction. The seawater samples showed diel mean values from 74 to 102% in DNA.

Incubation times varied from 20 to 60 min, depending on temperature and bacterial activity. All incubations were carried out in 100-ml clear Jena bottles, and 12.5 nM [^3H]thymidine was added, except on 17 to 18 November and 6 to 7 April in Frederiksborg Slotssø, when 24 nM [^3H]thymidine was added. Duplicate samples were assayed for all specific macromolecular fractions. Blanks (Formalin-fixed samples, 1% final concentration) were treated the same way. All filtrations were carried out with a ten-place Filter Holder (model 342-0013; Bio-Rad Laboratories). The steel funnels were cooled before filtration to 0°C. This ensured that samples remained below 3°C during filtration.

HCl (0.5 ml; 0.5 N) was added in the scintillation vials to hydrolyze DNA and improve counting, and there were found to be no measurable effects. Consequently, HCl treatment was omitted. Furthermore, the addition of a "carrier solution" (cold DNA) to improve retention efficiency of ^3H-labeled macromolecules was omitted. We found no changes in ^3H activity in DNA, RNA, or protein fractions from samples with no added cold DNA compared with results obtained from samples treated with 50 to 500 μg of DNA. When cold DNA was added at more than 100 μg per sample, the filtration speed decreased appreciably.

Enumeration of bacteria and FDC. Basically, the method of Hobbie et al. (8) was used with minor changes (23). The samples were filtered through 0.2-μm Uni-Pore filters (24 or 47 mm in diameter, prestained in Irgalan black). At least 300 cells were counted, and the cell size was obtained from measurements of at least 100 cells per filter. Volume was converted to biomass by multiplying by 1.21×10^{-13} to give grams of carbon per cubic micrometer (25).

The determination of FDC was carried out as described by Hagström et al. (6). Specific growth rate (μ) was plotted against temperature for various FDC (percent) values (data from Hagström et al. [6]). Lines were fitted by eye through points with the same FDC (percent) at temperatures of 5, 10, and 15°C and were extrapolated to 20°C (the highest measured temperature was 21.8°C). This procedure made allowance for the marked effect of temperature on the growth rate between 15 and 20°C. In contrast, Larsson and Hagström (14) calculated the specific growth rates from FDC (percent) at temperatures above 15°C as if they were 15°C. Bacterial secondary production (milligrams of carbon per cubic meter per hour) was calculated by multiplying the specific growth rate, μ ($hour^{-1}$), and the bacterial biomass (milligrams of carbon per cubic meter).

The statistical treatment included analysis of variance from unbalanced data with a comparison of data sets from stochastic independent results. The relative deviation of single results was calculated in relation to the diel mean value, and each single value was plotted on a relative day and night axis with eight equidistant time columns (Fig. 1). The aim of the analysis of variance was to test whether any of the eight selected time periods showed significantly different results with 95% confidence limits.

$^{14}CO_2$ dark uptake. The dark uptake of $^{14}CO_2$ was followed with time in darkened bottles, with [^{14}C]$NaHCO_3$ added at sunrise. One bottle (1 liter) contained natural water and another bottle (0.5 liter) was filled with water prefiltered through 1.0-μm pore-size Uni-Pore membranes. The prefiltered samples were used in an attempt to avoid interference from algal CO_2 fixation and zooplankton grazing. At time intervals, subsamples were taken from each bottle, and ^{14}C incorporation into particles was measured. The samples from the bottle with natural water were filtered through 1.0-μm Uni-Pore membranes to remove the algae. The microorganisms in these filtrates were recovered on a 0.2-μm Uni-Pore filter. The samples from the prefiltered water were filtered directly through 0.2-μm filters. The filters were washed with water from the sample location, and the activity was assayed by liquid scintillation counting.

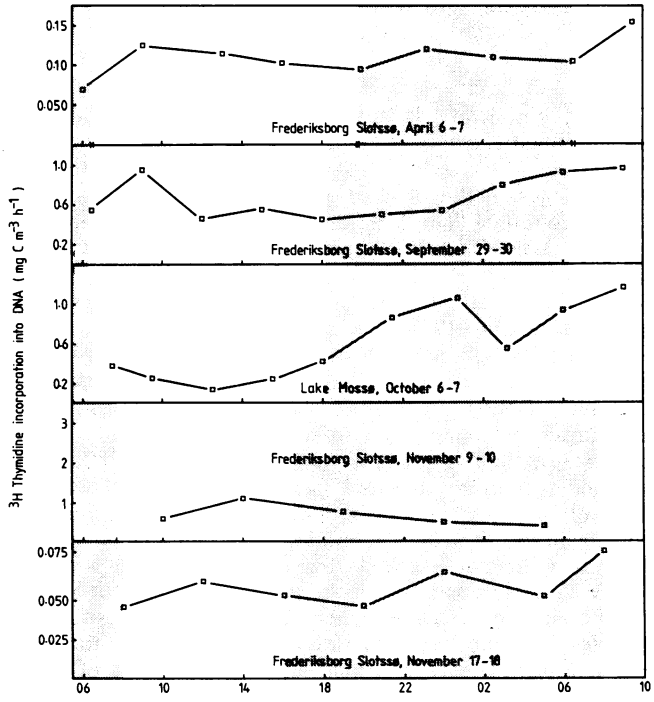

FIG. 2. Diel changes in bacterial production measured by [^3H]thymidine incorporation into DNA during five diel cycles.

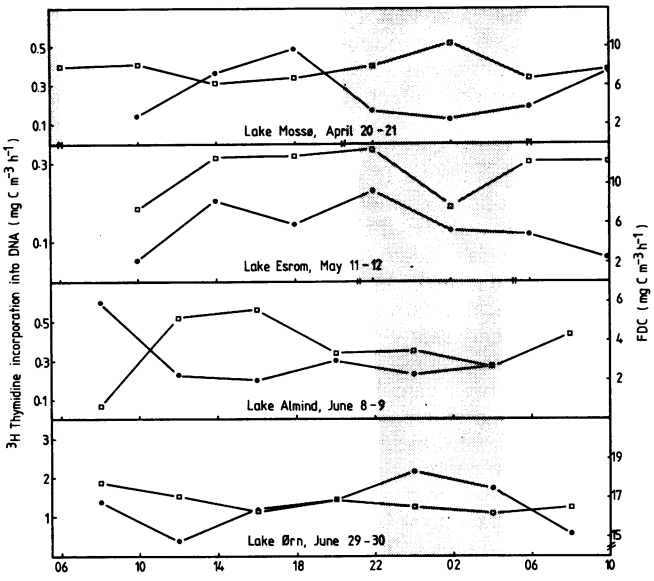

FIG. 3. Diel changes in bacterial production measured by [^{3}H]thymidine incorporation into DNA (□) and FDC (●).

The ^{14}C taken up by particles found on the 0.2-μm filters was defined as small free bacteria of <1.0 and >0.2 μm. To correct for the size distribution of microheterotrophic activity, the distribution of [^{3}H]glucose uptake in the two size fractions of >1.0 μm and <1.0 and >0.2 μm were measured. The results were used to correct for the ^{14}C uptake by small free bacteria relative to the total population.

The anaplerotic uptake of $^{14}CO_2$ by the bacteria was calculated to estimate the bacterial production, assuming that the anaplerotic fixation averaged 6% of the total carbon assimilation (12, 20). The measured ^{14}C fixation represents cumulative values; therefore, subtraction was used to calculate the production rates during the respective time periods.

RESULTS

Incorporation of [^{3}H]thymidine into DNA was followed during nine diel cycles in lakes (Fig. 2 and 3) and during four diel cycles in seawater (Fig. 4). There were no systematic diel changes in the production rate. Production estimates varied from 0.04 (The Limfjord, November) to 2.2 mg of C m^{-3} h^{-1} (Lake Ørn). In eight of the studies, a rise was found shortly after sunrise. In all cases, diel changes were minor. Maximum deviations from the calculated diel mean values varied from 23 to 194%.

Bacterial production rates in the lake water were higher when measured by FDC than by [^{3}H]thymidine incorporation into DNA (0.04 to 18.35 mg of C m^{-3} h^{-1}) (Fig. 3 and 5). During the four studies in seawater, similar rates were obtained by the two methods, and in a few cases [^{3}H]thymidine incorporation into DNA gave even higher values than those obtained by the FDC technique (Fig. 4). A correlation coefficient (r^2) of 0.74 was obtained from an analysis of the results of the two methods from the lake water samples (Table 1). A summary of all the data is presented in Fig. 5. Prefiltered samples gave higher values than the postfiltered samples for bacterial production rates measured by the dark ^{14}C methods. The correlation between the results of these two methods was poor (Table 1).

Diel estimates of the bacterial production were calculated based on integration over 24 h of the production rates per cubic meter and hour (Table 2). In lake water, the results obtained by [^{3}H]thymidine incorporation into DNA were appreciably lower than results obtained by FDC and the two dark CO_2 uptake measurements (Fig. 6). Results from the latter three methods were often close to each other; exceptions, however, were found with FDC in Lake Frederiksborg Slotssø, 9 to 10 November, and with prefiltered dark CO_2 from Limfjord, 15 to 16 September.

An analysis of variance was carried out on the basis of the matrix diagram in Fig. 1 to test whether there were any systematic diel cycles (Table 3). For all four methods, the calculated test values were smaller than the tabulated values (95% level). Thus, the hypothesis that any of the eight selected time periods gave significantly different results from the means was rejected.

On the basis of the total variance calculated by [^{3}H]thymidine incorporation into DNA (0.085; Table 3A), we calculated the standard error (SE) and 95% confidence limits (CL) for estimating diel bacterial production by means of two, three, or five samplings during 24 h (Table 4). If [^{3}H]thymidine were used, three samplings would allow diel bacterial production estimates to be made with ±42% (95% CL) of the mean value. In a similar way, only two samples during 24 h gave ±185% of the mean.

Considering FDC and dark CO_2 uptake, 95% CL were appreciably higher than those calculated from the [^{3}H]thymidine results.

DISCUSSION

Our measured production rates fell into two groups. [^{3}H]thymidine incorporation into DNA gave appreciably lower rates (7 to 21 times) than FDC and the dark CO_2 uptake measurements. Newell and Fallon (19) found production rates in seawater measured by FDC to be two to seven

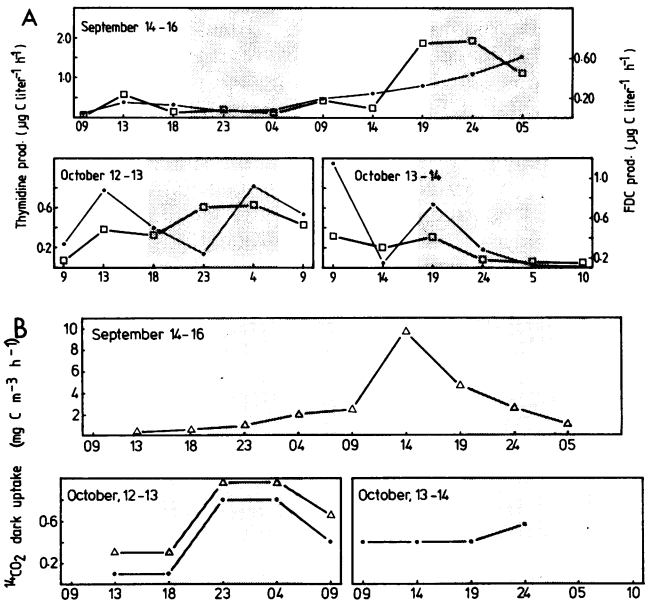

FIG. 4. Diel changes in bacterial secondary production measured by [^{3}H]thymidine incorporation into DNA (□) and FDC (●) (A) and dark $^{14}CO_2$ uptake (●) and dark CO_2 prefiltered (△) (B) from a coastal marine station. During the two diel periods in September, water samples were not changed in the bottles, but during the October experiments, fresh water was put into the bottles at 9 a.m. October 13.

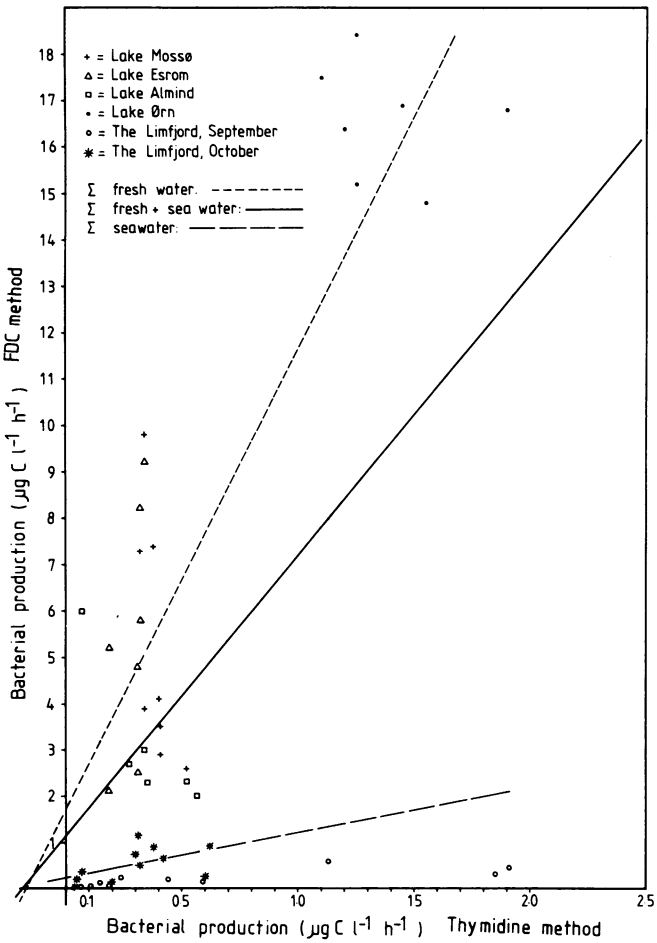

FIG. 5. Correlation between bacterial production estimated by [^{3}H]thymidine incorporation into DNA and FDC.

times higher than rates obtained by the [^{3}H]thymidine method. All these results suggest that the [^{3}H]thymidine incorporation technique in many pelagic environments gives lower production rates than those obtained from FDC and CO_2 uptake measurements. However, our seawater samples did not show low results by the [^{3}H]thymidine technique. In fact, some of the values were even higher than those obtained by the FDC technique (Fig. 4 and Table 2).

A question that needs to be answered is whether [^{3}H]thymidine incorporation into DNA underestimates the true production rate or whether dark CO_2 and FDC procedures overestimate, and sometimes even underestimate, bacterial production. Also, what effects do environment, season, and time of day have on the results?

Fuhrman and Azam (2) have listed uncertainties in the [^{3}H]thymidine technique and concluded that all would lead to underestimates of the production rates. Several of these assumptions were later evaluated (3). Serious drawbacks in the [^{3}H]thymidine technique included lack of knowledge about the rate-limiting step in the uptake and incorporation of [^{3}H]thymidine in natural populations of bacteria and lack of knowledge about the role of de novo synthesis of thymidine, which results in a reduction of the specific ^{3}H activity of the precursor to DNA.

Moriarty and Pollard (16, 17) have described an approach for determining the magnitude of exogenous and endogenous pools of thymidine and for correcting for dilution of isotope by de novo synthesis. They added increasing amounts of nonradioactive thymidine to samples and measured dilution of radioactivity in DNA. We have tested this approach in freshwater (21) and in marine samples (unpublished data). Sometimes, plots deviated from linearity (multiphasic plots) above certain levels of exogenous thymidine. Later we lowered the amount of nonradioactive thymidine and made the plots only from purified DNA (D. W. J. Moriarty, personal communication). The results indicate that when linear plots are obtained, most often only a small dilution of [^{3}H]thymidine incorporation into DNA is found (unpublished data).

The question is, what fraction of the total DNA synthesis rate do results obtained here by the Fuhrman and Azam (2) procedure represent? Does this fraction vary from time to time and from place to place? The correlation between FDC and [^{3}H]thymidine incorporation measured by the Fuhrman and Azam (2) technique was fairly good for the freshwater samples ($r^2 = 0.74$). Clearly, more data are needed. However, our present conclusion is that the [^{3}H]thymidine incorporation technique in many freshwater environments gives lower values compared with values obtained by the FDC and the two dark CO_2 procedures.

Similar values were observed between FDC and dark CO_2 procedures when calculated as integrated diel values. Diel changes did not match, and all correlations were poor. Neither of the two procedures is yet well understood. Major drawbacks of the FDC procedure were discussed recently by Newell and Christian (18). These include difficulties with a clear identification of dividing cells and limited information about the relationship between the specific growth rate and temperature, especially between 15 and 20°C and between 0 and 5°C (6, 20a). Furthermore, when the amount of carbon is calculated from the turnover rates of the biomass, an accurate determination of cell volumes is required, especially when changes in volume are large between sampling periods (5, 13).

The dark CO_2 procedure has been criticized (12, 20, 23). Recently, Li (15) concluded from chemostat experiments that CO_2 fixation was not constant with changing growth rate, but it is doubtful whether such conclusions can be made from experiments in which organisms are removed from the chemostat for incubation with $^{14}CO_2$. The dark CO_2 procedure is extremely sensitive to optimum size fractionation between algae and bacteria and to the choice of conversion

TABLE 1. Correlation coefficients (r^2) between [^{3}H]thymidine incorporation into DNA, FDC, dark CO_2 uptake, and dark CO_2 uptake from prefiltered samples

Procedure	Sample location[a]	r^2 (n) for the following procedures:		
		FDC	CO_2	CO_2 prefiltered
Thymidine	F	0.74 (28)	0.16 (26)	0.08 (15)
	F+S	0.31 (50)	0.20 (32)	0.06 (24)
FDC	F		0.29 (15)	0.42 (11)
	F+S		0.42 (21)	0.53 (20)
CO_2	F			0.38
	FS			0.48 (12)

[a] F, Freshwater samples; S, seawater samples.

TABLE 2. Estimates of diel bacterial secondary production rates measured by [^{3}H]thymidine incorporation into DNA, FDC, dark CO_2 uptake, and dark CO_2 uptake from prefiltered samples

Location	Date	Bacterial production (mg of C m^{-3} 24 h^{-1}) by the following methods:			
		Thymidine	FDC	Dark CO_2	Dark CO_2 prefiltered
Lake Almind	8–9 June	7.8	55.8	15.7	47
Lake Ørn	29–30 June	32.5	400.0	300.0	518
Lake Esrom	11–12 May	6.8	142.0	83.0	184
Lake Mossø	20–21 April	10.9	143.0	180.0	
	29–30 September	5.1		66.6	
Frederiksborg Slotssø	9–10 November	20.7	0[a]	36.0	50
	6–7 October	5.2		30.5	
	6–7 April	5.2			
	17–18 November	1.7			
Limfjord	14–15 September	6.1	2.6	17.0[b]	30.2
	15–16 September	23.9	7.0		94.0
	12–13 October	10.6	14.5	11.0	15.6
	13–14 October	3.8	8.5	11.2	12.1[b]

[a] Growth rate was 0 due to low temperature.
[b] Based on 24-h incubations.

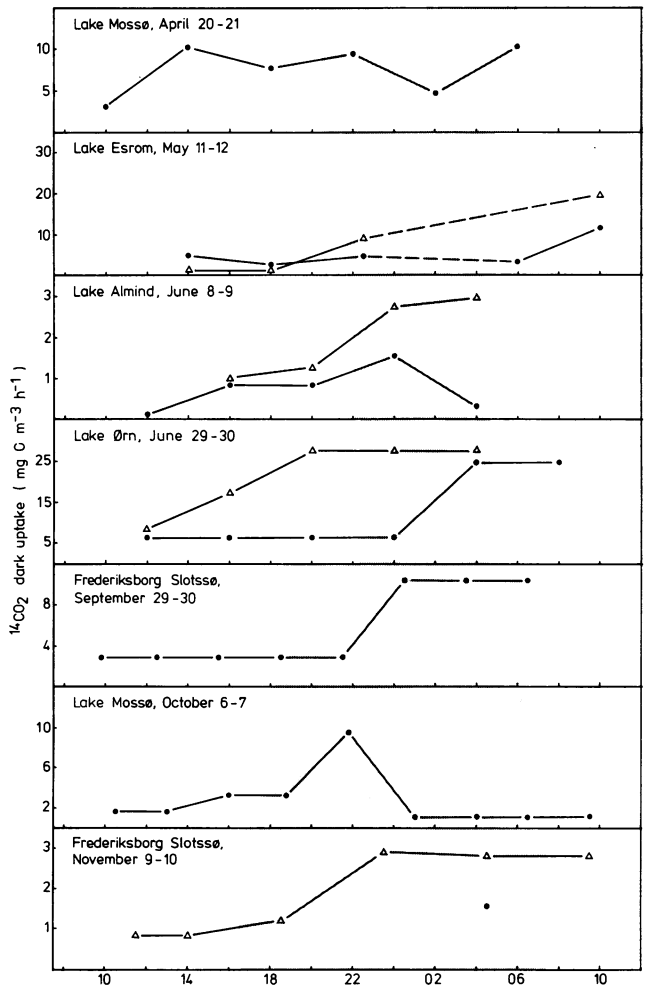

FIG. 6. Diel changes in bacterial secondary production measured during seven diel studies by dark CO_2 uptake (●) and dark CO_2 uptake with prefiltered samples (△).

factor between $^{14}CO_2$ uptake and uptake of total organic carbon.

In a comparison among the methods applied in this study, it must be emphasized that each method measures something different. The [^{3}H]thymidine method is a short-term measurement of the instantaneous rate of DNA replication, whereas FDC is an integrated measurement of events which took place before sampling. Furthermore, the anaplerotic dark fixation is coupled with the instantaneous metabolism in the citric acid cycle and does not necessarily reflect the production of new biomass. Despite the differences in results obtained by the four techniques, none of the methods gave results which revealed significant diel cycles. A more detailed analysis of these observations in relation to the coupling between new inputs of bacterial nutrients and

TABLE 3. Data matrix from the analysis of variance from the eight selected time periods (Fig. 1)[a]

Comparison	Sum of square	f	Variance	Test
A. Between sets	0.3629	7	0.052	0.56
Inside sets	7.4844	81	0.092	$f_B f_I$[b] = 2.13
Total	7.8473	88	0.085	
B. Between sets	1.986	7	0.284	0.93
Inside sets	11.286	37	0.305	$f_B f_I$ = 2.27
Total	13.272	44	0.302	
C. Between sets	2.821	7	0.403	0.73
Inside sets	23.657	43	0.550	$f_B f_I$ = 2.24
Total	26.478	50	0.530	
D. Between sets	1.162	7	0.166	0.41
Inside sets	10.140	25	0.406	$f_B F_I$ = 2.40
Total	11.302	32	0.353	

[a] The data matrix was determined by [^{3}H]thymidine incorporation into DNA (A), FDC (B), dark CO_2 (C), and dark CO_2 from prefiltered samples (D).
[b] $f_B f_I$ is the tabulated value.

TABLE 4. SE and 95% CL (percentage of the mean value) calculated on the basis of two, three, or five sampling periods during 24 h[a]

No. of samples	Thymidine		FDC		Dark CO_2		Dark CO_2 prefiltered	
	SE (%)	95% CL (%)	SE (%)	95% CL (%)	SE (%)	95% CL (%)	SE (%)	95% CL (%)
2	15	185	28	350	37	474	41	528
3	10	42	18	79	25	107	58	248
5	6	16	11	31	15	41	17	46

[a] The values were calculated by using the variance from Table 3 and the mean value of the results. The statistical errors are calculated by the four methods.

growth of the bacterial populations and grazing of the bacteria by micropredators is nevertheless not possible. In some of the diel studies, a morning rise in the bacterial production rate was seen. This may be ascribed to either phytoplankton release of low-molecular-weight photosynthesis products (10) or morning activities caused by the grazing of zooplankton (4, 7) or both. The sudden increase in the production rate observed from the Limfjord data on September 15 at 19 p.m. (Fig. 4A) is probably too high and may represent a bottle effect caused by the long storage period of water in our incubation bottles. Whether enclosure of the water samples in the experimental bottles changed the production rate in the rest of the diel studies is difficult to evaluate. In some of the diel studies, a continuous increase was found in the bacterial production rate throughout a 24-h period. Fuhrman and Azam (2) have demonstrated an increasd bacterial production rate after 20 to 30 h of containment of coastal marine waters. During most of our diel studies, the temperature was lower than during their experiments. Furthermore, we did not find any irregularities in the phytoplankton production and biomass, except during the Limfjord study (September 15), when primary production increased about three times compared with the results from September 14. The light conditions were identical on the two days (unpublished data). During the diel study with the highest temperatures (Lake Almind, 21.8°C), bacterial production rates were not significantly different in 1-, 0.5-, and 0.25-liter bottles compared with the production rates obtained from 5- and 10-liter bottles (unpublished data). Although this does not prove whether changes occurred in the 5-liter bottles compared with the bacterial production rates in the open water, we believe that such changes would increase rather than decrease true diel changes. The influence of allochthonic materials from sewage inlets and transport of labile organic materials from deeper parts of the water column were not measured. Nevertheless, the role of phyto- and zooplankton on diel rates of bacterial production rates are probably much more pronounced in open pelagic environments. As a consequence, the confidence limits from Table 4 represent overestimations rather than underestimations.

Based on the total variance obtained from the analysis of variance on each individual technique, and assuming that this variance could be applied to a new system, we calculated the expected error by using various sampling periods. The lowest error was obtained by [^{3}H]thymidine incorporation into DNA, and progressively higher errors were obtained by FDC, dark CO_2 uptake, and dark CO_2 uptake with prefiltered samples.

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