

The use of oxygen microprobes to measure bacterial respiration for determining bacterioplankton growth efficiency

Enora Briand^{1,2}, Olivier Pringault^{1*}, Séverine Jacquet¹, and Jean Pascal Torréton¹

¹UR 103 Camélia, Centre IRD de Nouméa BP A5, 98848 Nouméa Cedex, Nouvelle Calédonie

²UR 98 Flag, Centre IRD de Bel Air, BP 1386 CP 18524 Dakar, Sénégal

Abstract

Heterotrophic bacterial growth efficiency (BGE), the ratio between the carbon consumed and the bacterial biomass produced, is a key factor in understanding flows of organic matter in aquatic ecosystems. Methods generally used to estimate bacterial respiration require long incubations (24 to 36 h) to measure significant rates during which nonlinear patterns of oxygen decrease may bias BGE computation. These respiration estimates are generally compared to bacterial production rates determined from radiotracer incorporation from much shorter incubations. The aim of this study was to improve the determination of bacterial respiration to better estimate BGE. For this purpose, we employed oxygen microprobes in predator free (0.6 μm filtered) seawater samples and determined in parallel bacterial abundance. The use of oxygen microprobes allowed us to continuously monitor oxygen concentration during the incubation. Hence, the length of incubation can be adjusted as soon as a significant decrease of oxygen is observed. At the most productive sites, respiration was measurable from the beginning of the incubation and varied with time. In contrast, at the oligotrophic sites, respiration was often detectable only after a lag-phase of 5 to 10 h and remained constant thereafter. BGE was computed from the changes in bacterial abundance observed during the respiration measurements. This way, both processes were determined in similar incubation conditions. In comparison, the use of radiotracer derived bacterial production systematically resulted in an underestimation of BGE.

Heterotrophic bacteria play a predominant role in aquatic ecosystems. In terms of their numerical abundance, they are the most important biological component involved in the transformation and mineralization of organic matter in the biosphere (Cho and Azam 1988). It is well established that they process at least about one half of the total carbon flux passing through marine planktonic network (Fuhrman 1992). Heterotrophic bacteria contribute to the cycles of nutrients and carbon in two major ways: by the production of new bacterial biomass (secondary production) and by the remineralization of organic carbon and nutrients (del Giorgio and Cole 1998). Understanding this dual character of planktonic bacteria in aquatic ecosystems is a central paradigm of contemporary microbial ecology (Pomeroy et al. 1991; Ducklow and Carlson 1992).

Consequently, knowledge of BGE, the ratio between carbon consumed and bacterial biomass produced, is a prerequisite for the evaluation of carbon budgets in aquatic ecosystems.

Many authors clearly emphasize the fact that BGE varies in time (Lemée et al. 2002) and space (Griffith et al. 1990). In fact, BGE depends on a variety of factors such as the quantity and quality of organic matter and minerals. More precisely, BGE appears dependent upon substrate C/N ratios (Kroer 1993; Jorgensen et al. 1994) and substrate molecular weight (Tulonen et al. 1992; Amon and Benner 1996). BGE also seems positively correlated with inorganic nutrient availability (Tulonen et al. 1992; Kroer 1993; Zweifel et al. 1993), the concentration of the labile carbon pool (Barillier and Garnier 1993; Middelboe and Sondergaard 1993) and to bacterioplankton growth rates (Middelboe et al. 1992). BGE was reported to be negatively correlated to temperature (Bjornsen 1986, Daneri et al. 1994), but other results did not support this hypothesis (Barillier and Garnier 1993; Kroer 1993). Therefore, many processes are likely to govern BGE and these processes can themselves answer different factors of control (del Giorgio and Cole 1998). The very large variability of BGE in any one system suggests that at the present time, BGE values may not be extrapolated from literature data when a new ecosystem is investigated.

*Corresponding author. Telephone: +687 26 08 12; fax: +687 26 43 26; e-mail: olivier.pringault@noumea.ird.nc

Acknowledgments

This work was supported by the "Institut Français de Recherche pour le Développement" (IRD) and a grant from the French "Programme National Environnement Côtier" (PNEC). We express our gratitude to S. Tereua and A. Lapetite for their efficient help during sample collection. Emma Rochelle-Newall is thanked for her comments and suggestions.

BGE can be determined by two different ways. First estimations have been based on use of simple sources of organic carbon by radiotracers (Hobbie and Crawford 1969). In spite of the advantage brought by this approach (high sensitivity, short times of incubation, no uncoupling), the use of simple compounds, generally labile, cannot be representative of the diversity of substrates used by bacteria (del Giorgio and Cole 1998). As a consequence, this method presents the major drawback of overestimating BGE, which can reach values of 97% depending on substrate used and the measurement conditions (Bjornsen 1986). The other methods need uncoupling (e.g., bacteria must be physically separated from other planktonic components) in an aim to specifically determine activities of heterotrophic bacteria. This is usually attempted by filtration in the 2- to 0.6- μm range. These methods consist of measuring bacterial consumption of available organic matter in situ and comparing it to an estimator of bacterial production. Bacterial production is most often determined using radiotracer incorporation (^3H -thymidine or leucine) but can also be estimated from changes in bacterial abundances. To measure bacterial consumption of organic matter, different indicators have been proposed: dissolved organic carbon (DOC) consumption (Middelboe et al. 1992; Kroer 1993; Zweifel et al. 1993; Carlson and Ducklow 1996), O_2 consumption (Pomeroy et al. 1994; Pomeroy et al. 1995), or CO_2 production (Bjornsen 1986). However, these methods most often require long incubation times (24, 36, or 72 h) to measure significant variations of DOC, O_2 , or CO_2 . Therefore, bottle effect may induce changes of bacterial community composition (Massana et al. 2001; Gattuso et al. 2002) and/or exhaust trophic resources (Pradeep Ram et al. 2003). Consequently, the results may not be representative of the initial bacterial assemblage. It is therefore important to estimate the BGE with a method that allows continuous measurement of consumption of organic matter during an incubation time less than 24 h.

Oxygen may be continuously monitored using different techniques such as respirometers with oxygen macroprobes, which have been developed in the last few years (Langdon 1993; Taylor et al. 2003). Recently, a semicontinuous technique based on mass spectrometry determination of oxygen (Kana 1994) has been employed to measure bacterial respiration in estuarine waters (Bouvier and del Giorgio 2002; del Giorgio and Bouvier 2002). In this study, we present a method using oxygen microprobes to estimate bacterial respiration with the aim to determine bacterial growth efficiency. Oxygen microprobes have been in use for more than 20 years in benthic systems, however this technique has yet to be applied to planktonic systems. We first describe the advantages of this method, which allows a continuous monitoring of the oxygen concentration. Then, we have used this procedure to estimate BGE in contrasted trophic situations in the southwest lagoon of New Caledonia.

Materials and procedures

Study sites—Water samples were collected in the southwest of the New Caledonian lagoon (2,000 km^2), mostly in the

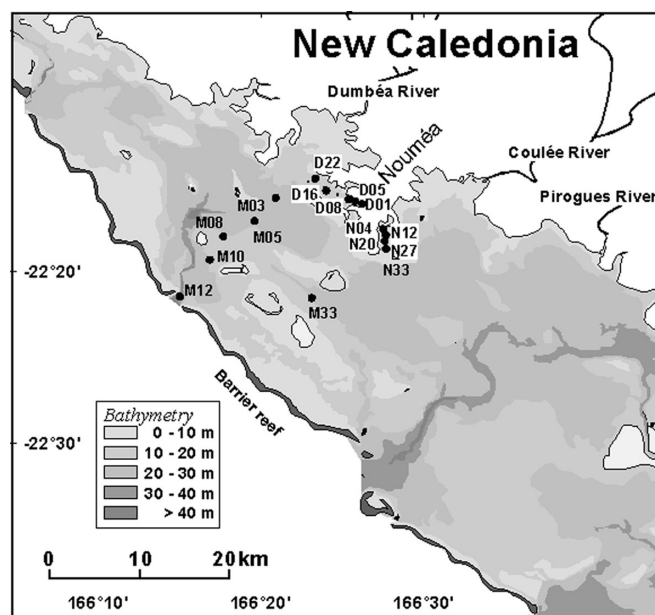


Fig. 1. SW Lagoon of New Caledonia and location of study sites.

vicinity of the city of Nouméa (125,000 inhabitants) and surrounding bays (Fig. 1). The average depth of the SW lagoon is 21 m, and the main channel is 25 m deep on average, whereas bay stations are somewhat shallower (Table 1). A total of 16 stations were sampled along 3 transects with the objective to obtain a large trophic range.

The stations of “open lagoon” transect (M03, M05, M08, M10, M12; Fig. 1) are rapidly renewed by oceanic waters entering through the southern part (Douillet 1998). Far from the terrigenous and anthropogenic influences, these stations are generally the most oligotrophic of this area. The “Sainte Marie” transect (N04, N12, N20, N27, N33, M33) is located in the eutrophic Sainte Marie Bay. This bay receives untreated wastewaters from the surrounding urban area. Finally, the “Grande Rade” stations (D01, D05, D08, D16, D22) are impacted by urban effluents and contaminants from the nickel industry. Both these latter sites present a decreasing degree of eutrophication between coast and opening of the bays.

The availability of oxygen-measuring equipment allowed the sampling of only one site at a time. For each sampling, water column variables (conductivity, temperature, in vivo chlorophyll fluorescence, and photosynthetically active radiations) were recorded with a Seabird SBE 19 CTD probe. Water samples were collected at 3 m depth using acid-washed 5-L Niskin bottles. Previous work has shown that this depth of sampling provides a representative sample of the whole water column (Jacquet et al. unpubl. data unref.). Samples were kept in Niskin bottles until return to the laboratory within 90 min.

Bacterial respiration—Upon return to the laboratory, 600 μL of lagoon water were immediately prescreened through a 125- μm mesh to eliminate the larger, less abundant organisms that

Table 1. Average characteristics of the stations sampled over the whole water column

Date	Station	Maximum depth (m)	Salinity (psu)	Temperature (°C)	Turbidity (ftu)	Chl (µg/L)
3 Mar 03	D01	6.5	33.291	29.13	5.83	1.37
6 Mar 03	D01	6.5	35.317	27.71	5.80	1.08
10 Mar 03	N04	11.5	35.275	28.19	1.52	0.99
12 Mar 03	M12	38.5	35.380	27.31	0.31	0.54
18 Mar 03	M03	25.2	35.134	26.50	1.12	1.33
19 Mar 03	M10	28.5	35.281	25.60	0.66	0.87
20 Mar 03	M08	15.5	35.216	26.27	0.56	0.66
21 Mar 03	M05	33.0	35.245	26.11	0.78	0.44
24 Mar 03	N04	11.5	35.036	28.22	4.03	4.47
25 Mar 03	N12	14.5	35.160	27.34	2.52	1.23
26 Mar 03	N20	12.5	35.127	27.66	0.84	1.16
27 Mar 03	N27	15.0	35.167	27.65	1.46	2.23
28 Mar 03	N33	17.6	35.233	27.27	1.12	0.71
31 Mar 03	D01	6.5	35.325	27.25	2.17	1.46
1 Apr 03	D05	12.3	35.302	27.15	1.8	0.80
2 Apr 03	D08	13.0	35.271	26.96	1.27	0.89
3 Apr 03	D16	15.8	35.301	26.72	1.11	0.61
4 Apr 03	D22	17.0	35.263	26.73	0.89	0.43
8 Apr 03	N33	17.6	35.250	26.41	1.15	1.21
10 Apr 03	N12	14.5	34.842	26.43	3.20	2.64
15 Apr 03	N12	14.5	34.948	25.72	1.23	0.81
22 Apr 03	M33	19.0	35.452	25.33	0.17	0.55
22 Apr 03	N12	14.5	35.168	25.61	1.79	0.68
6 May 03	N12	14.5	35.080	24.84	2.04	1.00

D01, N04, and N12 were sampled twice and processed independently.

were likely to increase the variability of the oxygen consumption in the total community. The filtered water was then distributed in two sets of water samples: an unfiltered water sample that served to assess total community respiration and a water sample filtered onto a 0.6-µm pore size Nuclepore membrane. Filtration was done under low differential pressure (<100 mm Hg) to avoid disruption of fragile cells. Samples were then poured in 250 mL glass vials hermetically closed by pierced silicon stoppers into which were inserted the microprobes and were incubated at ± 1°C in situ temperature in the dark. Gentle homogenization of the water was achieved by use of a magnetic stirrer. Incubation vessels, microprobes, stoppers, and stirrers were acid-washed (hydrochloric acid, 10% final volume) before each experiment.

Respiration was continuously measured by oxygen microprobes. The microprobes (Unisense, Denmark) are designed with an exterior guard cathode (Revsbech 1989), which results in extremely low oxygen consumption by the electrodes themselves (4.7 to 47 × 10⁻⁷ mmol O₂ h⁻¹). Probes have a response time shorter than 1 s and a precision of 0.1 µM. Oxygen concentration was collected by a computer every 10 s during 14 h (Sainte Marie and Grande Rade samples) or 24 h (open lagoon samples). Bacterial respiration was estimated

from oxygen consumption in the 0.6-µm filtered water sample. Bacterial respiration was computed from the slope of O₂ versus time measured during a 5-h period, which we assumed to be sufficiently short to minimize bacterial production and abundance changes. The starting point was chosen from observation of significant oxygen decrease (0.5 µM). Oxygen consumption was converted to carbon respired (µgC L⁻¹ h⁻¹) assuming a respiratory quotient of 1.

Chlorophyll a—Chlorophyll *a* concentration from unfiltered samples was determined after filtration onto GF/F filters immediately stored at -20°C until analysis. Chlorophyll *a* concentration was later estimated fluorometrically according to Yentsch and Menzel method (1963). Pigments concentrations were computed from Lorenzen (1966), modified by Jeffrey and Humphrey (1975).

Bacterial abundance—Bacterial abundance was estimated from samples immediately preserved with borax-buffered formalin (2% final concentration), filtered onto 0.2 µm black polycarbonate membrane, stained with DAPI (4'-diamidino-2-phenylindole), and stored at -20°C until enumeration epifluorescence microscopy. More than 400 bacteria were counted in at least 20 fields in duplicates to obtain a coefficient of variation of 10% (Kirchman et al. 1982). Bacterial

abundance was determined at the beginning and at the end of each respiration incubation.

Incorporation of ^3H -thymidine—Bacterial DNA synthesis, a proxy of the bacterial production was measured by the incorporation of ^3H -thymidine into cold trichloroacetic acid (TCA) precipitable material. Duplicate or triplicate 5 to 10 mL water samples were incubated in the dark with ^3H -[methyl]thymidine (final concentration 15 nM, 1.8 TBq mmol⁻¹, Amersham) for 1 h at in situ temperature ($\pm 1^\circ\text{C}$). Previous experiments have shown that saturation is always obtained at this concentration. The activity was stopped by the addition of buffered formalin (2% final concentration). Labeled material was collected by filtration under low pressure (<100 mm Hg) through a 0.2- μm polycarbonate membrane and allowed to precipitate 15 min at 4°C with ice-cold TCA (5% w/v). Membranes were rinsed 3 times with 5 mL cold 5% TCA. DNA was then hydrolyzed by heating at 100°C for 30 min with 0.5 N HCl. After addition of 4 mL of scintillation cocktail, radioactivity was measured with a Packard TriCarb scintillation counter after quench correction. Bacterial DNA synthesis was determined at the beginning and at the end of each respiration incubation. Rates of ^3H -thymidine incorporation were converted to cell production using a conversion factor of 2.91×10^{18} cells mol⁻¹ thymidine (Jacquet unpubl. data unref.).

BGE determination—Generally, BGE is computed from bacterial production estimated from thymidine or leucine incorporation determined from short incubation time (i.e., ~ 1 h) and from bacterial respiration measured over 24 h incubation (using Winkler titration of O₂). In this study, BGE was computed from:

$$\text{BGE}(\%) = \frac{\text{BBP}_{\text{net}}}{(\text{BBP}_{\text{net}} + \text{BR})} \times 100 \quad (1)$$

with BBP_{net} being net bacterial biomass production, i.e., the difference of bacterial biomass between the beginning and the end of the incubation time considered, and BR, bacterial respiration, converted in carbon units. Conversion of bacterial abundance (10⁶ cell mL⁻¹) to carbon biomass ($\mu\text{g C L}^{-1}$) was done using a conversion factor of 20 fgC cell⁻¹ (Lee and Fuhrman 1987), although considerable uncertainties remain on this factor (Fukuda et al. 1998).

Assessment

Methodological approach—Oxygen consumption of the probes. In order to insure that oxygen consumption by the microprobes was negligible compared to bacterial consumption, we monitored oxygen concentration in 0.6- μm and 0.2- μm filtered subsamples of the same water in parallel incubations during 24 h (Fig. 2). Oxygen concentration in 0.2- μm filtered water was constant (215 μM O₂, Fig. 2) from 0 to 15 h of incubation. The decrease observed after 15 h of incubation was caused by growth of residual small bacteria, which passed through the filter, and/or contamination of the sample because the apparatus could not be autoclaved. This residual

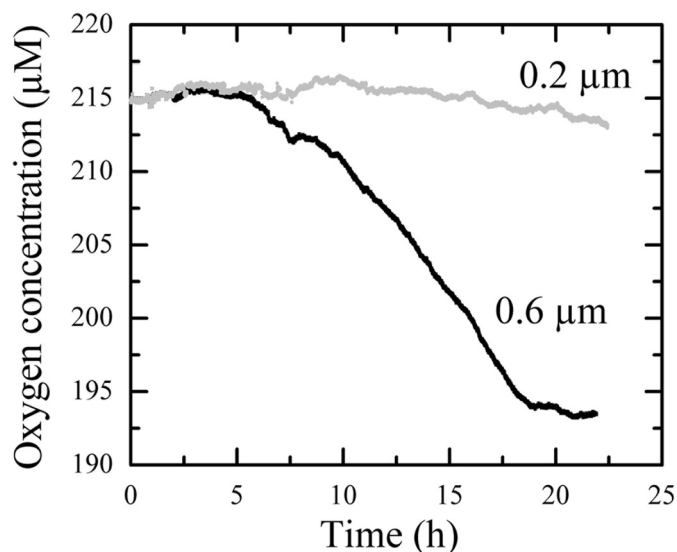


Fig. 2. Oxygen concentration during dark incubation in 0.6- and 0.2- μm filtered subsamples of station N12 (6 May 03).

contamination is negligible for samples filtered on 0.6 μm (Fig. 2) containing most of the bacteria (average $90\% \pm 4\%$, $n = 31$, Torr ton et al. unpubl. data unref.). The absence of oxygen consumption by the microprobes is a considerable advantage, because most of the oxygen-measuring equipment used in planktonic waters involve oxygen macroprobes (Griffith 1988; Langdon 1993), which exhibit an internal oxygen consumption, leading to an overestimation of the oxygen consumption unless corrected.

Reproducibility. Oxygen consumption estimates using the Winkler method are usually performed in replicates. In our study, the availability of the equipment to measure oxygen concentration prevented us from estimating O₂ consumption systematically in replicates. However, on several occasions, we checked reproducibility in duplicate water samples. Fig. 3 shows that the time course of oxygen concentration is very similar in two samples of the same station.

Precision of the oxygen microprobe. Table 2 presents different techniques used to measure oxygen concentration in planktonic systems. The Winkler technique is the most commonly used, and many works have been performed to increase the precision of this chemical determination of oxygen concentration. The precision of oxygen microprobe (0.05%) is equivalent to highly precise Winkler techniques described by Roland et al. (1999) and by Sherr and Sherr (2003).

Trend of respiration in <0.6- μm filtered samples—A major drawback of discrete O₂ or CO₂ measurements to estimate respiration is that they require rather long incubation times to observe significant differences with initial oxygen concentration. Oxygen consumption is then assumed to be linear, and this is usually not easy to verify on short time scales using Winkler titration. In a previous study, Pomeroy et al. (1994)

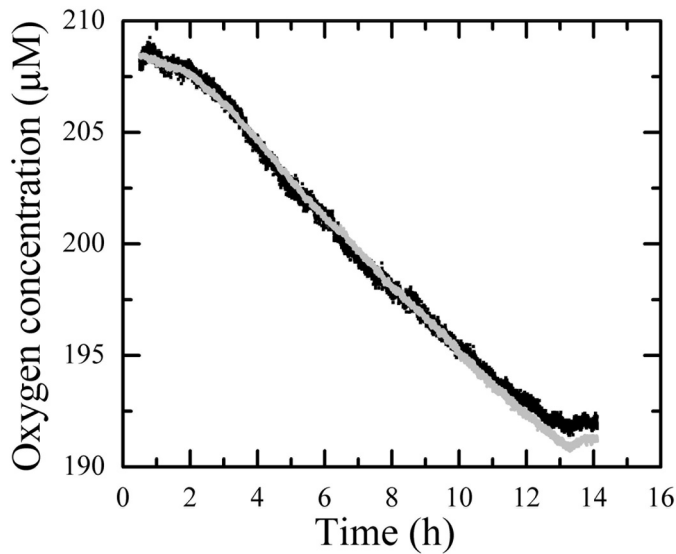


Fig. 3. Oxygen concentration during dark incubation in duplicate 0.6-µm filtered subsamples of station N12 (10 Apr 03).

observed different trends of oxygen concentration time course during 24 h incubations with discrete oxygen measurements every 5 h. One third of their data did not show a linear decrease of oxygen concentration as a function of time. Therefore, they suggest a continuous monitoring of oxygen concentration for a better estimation of the bacterial respiration.

The use of oxygen microprobes allowed us to follow continuously the oxygen concentration in the respiration bottles. Oxygen concentration data versus time were then fitted to an exponential decay equation, and the time course of respiration were then calculated from the first derivative of the fitted function. In parallel, ³H-thymidine incorporation and bacterial abundance were determined every 5 h during the 24 h dark incubation on replicate 0.6-µm filtered samples. Fig. 4 presents the time course of respiration, bacterial abundance, and bacterial production in two contrasted trophic situations (M10, oligotrophic site, and D01, eutrophic site, see Fig. 1).

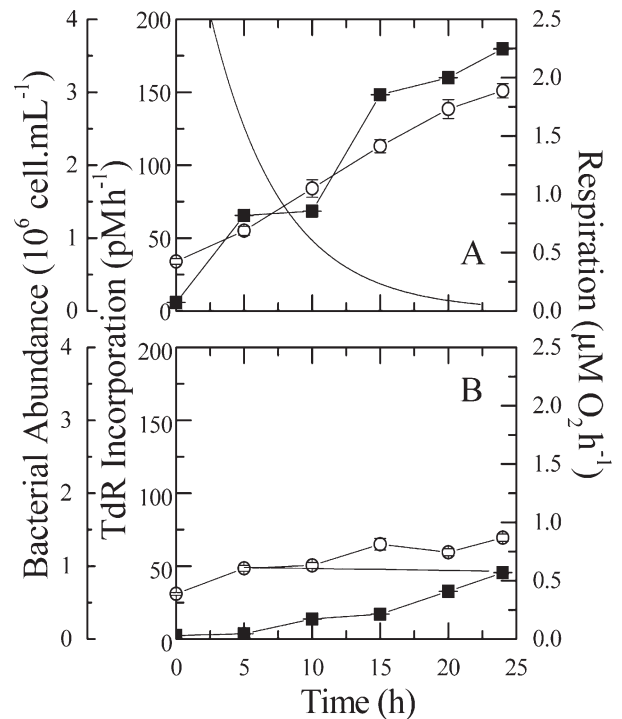


Fig. 4. Bacterial abundance (○), TdR incorporation (■), and respiration (—) of 0.6 µm filtrates from a eutrophic, D01 (A), and oligotrophic site, M10 (B). The bars represent standard deviation.

A significant respiration appeared only after 2 to 3 h in the eutrophic site sample (Fig. 4A), whereas it appeared only after 8 to 10 h in the oligotrophic site sample (Fig. 4B).

In D01 (Fig. 4A), TdR incorporation rate increased considerably, from 6 to 180 pM h⁻¹ in 24 h of incubation. Bacterial abundance at the end of incubation reached up to 5 times initial value. Respiration was maximal after 2 h of incubation (2.5 µM O₂ h⁻¹), and then decreased until becoming practically null after 15 h of incubation. The increase in TdR and bacterial abundance suggest that bacteria were likely not resource limited. The observed discrepancies between production and con-

Table 2. Different methods to measure oxygen concentration in planktonic environments

Methodology	Precision (%)	Precision* (µM)	Measurements	Reference
Winkler	0.12	0.28	Discrete	Sherr and Sherr (2003)
Winkler	0.03	0.07	Discrete	Smith and Prairie (2004)
Winkler	0.1 to 0.8	0.23 to 1.85	Discrete	Roland et al. (1999)
Mass spectrometry	<0.5	<1.15	Discrete	Kana et al. (1994)
Oxygen macroprobe	0.3	0.8	Continuous	Langdon (1993)
Oxygen macroprobe	0.65-1.52	1.5-3.5	Continuous	Coffin et al. (1993)
Respirometer	0.3	0.8	Continuous	Griffith (1988)
Respirometer	1	2.3	Continuous	Taylor et al. (2003)
Oxygen microprobe	0.05	0.1	Continuous	This study

*Precision in µM corresponds to the precision calculated for an oxygen concentration of 230.9 µM (oxygen solubility at 20°C and 35‰)

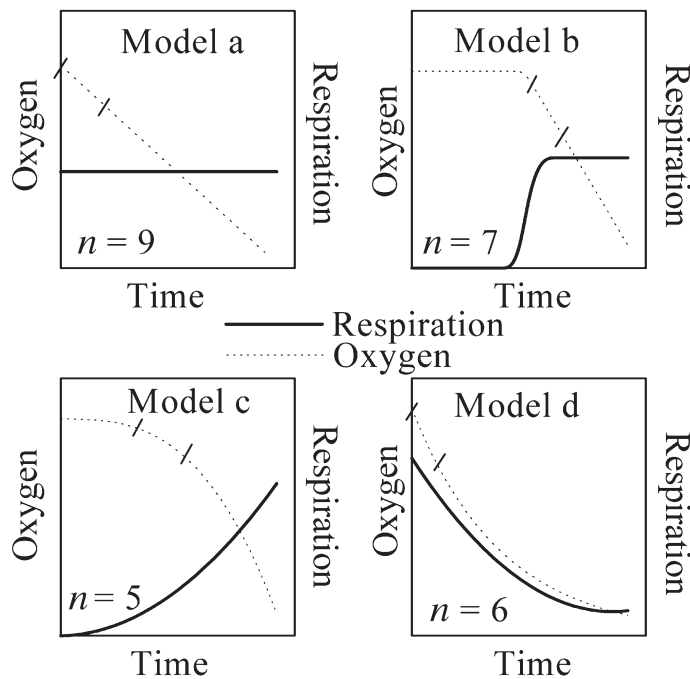


Fig. 5. Representation of the different trends of the time course of the oxygen concentration and the predicted respiration versus time. *n* represents the number of corresponding cases observed during our study. The two lines indicate the interval (5 h) used to calculate the bacterial respiration. For models b and c, the starting point was set from a minimum decrease of 0.5 $\mu\text{M O}_2$ (see text).

sumption indicate a temporal uncoupling between both processes as suggested by del Giorgio and Cole (1998).

The sample from the oligotrophic site (Fig. 4B) displayed an increase in thymidine incorporation after a lag phase of a few

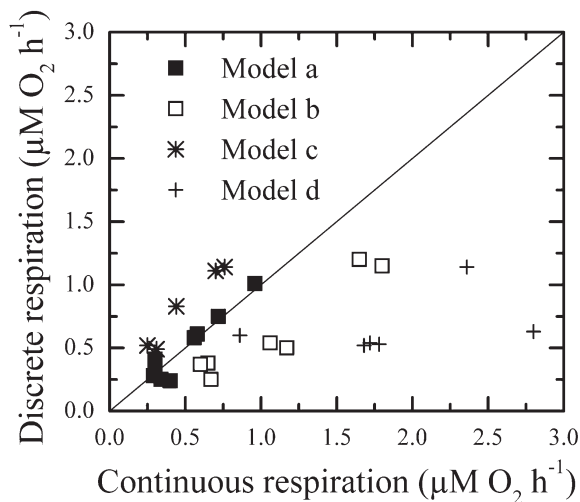


Fig. 6. Comparison of the respiration estimated from discrete and continuous O_2 measurements. For the discrete approach, respiration was calculated from the difference between the oxygen concentration at the beginning and at the end of the incubation (14 h or 24 h).

hours. Similarly, significant respiration was only measurable after a lag phase of 6 h and the rate ($0.6 \mu\text{M O}_2 \text{ h}^{-1}$) remained almost constant during the next 15 h, whereas thymidine incorporation rate kept increasing until reaching 45 pM TdR h^{-1} at the end of incubation. Bacterial abundance increased 2.5-fold for the first 15 h and reached a plateau near $1.4 \times 10^6 \text{ cell mL}^{-1}$.

These results allowed the optimization of incubation times to measure oxygen consumption by heterotrophic bacteria in oligotrophic and eutrophic sites. Additionally, parallel trends in ^3H -thymidine incorporation and bacterial abundance documented the limits of this method. In fact, interpretation of the results should be made with caution, considering that what is measured is more representative of process occurring during incubation in bottles rather than initial in situ conditions.

Results from station M10 show that significant oxygen consumption was only observed when bacterial abundance and activity increased. This increase in activity can result from both release of predation by $>0.6 \mu\text{m}$ organisms, enrichment by disruption of fragile cells during filtration, bottle effect like adsorption and concentration of the organic matter on glass material, or by changes of bacterial communities during incubations (Schäfer et al. 2000; Massana et al. 2001; Fuchs et al. 2000; Gattuso et al. 2002). Biddanda et al. (1994) have already shown such increases of bacterial abundance and activity during incubation in waters from the northern Gulf of Mexico of various trophic status. In their study, at the end of 20 h incubations, bacterial production increased up to 12- and 8-fold in highly productive shelf waters and in less productive slope waters, respectively. Likewise, bacterial abundance in the eutrophic site increased 1.5-fold, whereas it remained constant in the oligotrophic site. Consequently, during these incubations the community evolves and activity measurements are not truly representative of initial conditions.

It is therefore highly preferable to incubate during the shortest period to stay the closest to initial conditions. Hence, we have chosen to measure the bacterial respiration with the slope calculated on a 5-h interval. This time was the best compromise between the minimum time to ensure a significant decrease of oxygen concentration and a minimized increase of bacterial rate of DNA synthesis (1.19 ± 0.21 fold) and abundance (1.42 ± 0.09 fold). The beginning of the interval was chosen as soon as we observed a significant decrease of oxygen.

Continuous O_2 measurements versus discrete O_2 measurements—We collected a total of 27 samples from 16 stations of contrasted trophic status (Table 1). Fig. 5 shows the different time courses of oxygen concentration observed during our study. Fig. 6 presents the comparison between the discrete method and the continuous method for the estimation of bacterial respiration.

Only 9 of 27 cases presented a constant O_2 consumption during incubation (model a, Fig. 5 and Fig. 6) and, hence, do not show important bias to compute bacterial respiration. In model a, discrete methods to estimate bacterial respiration

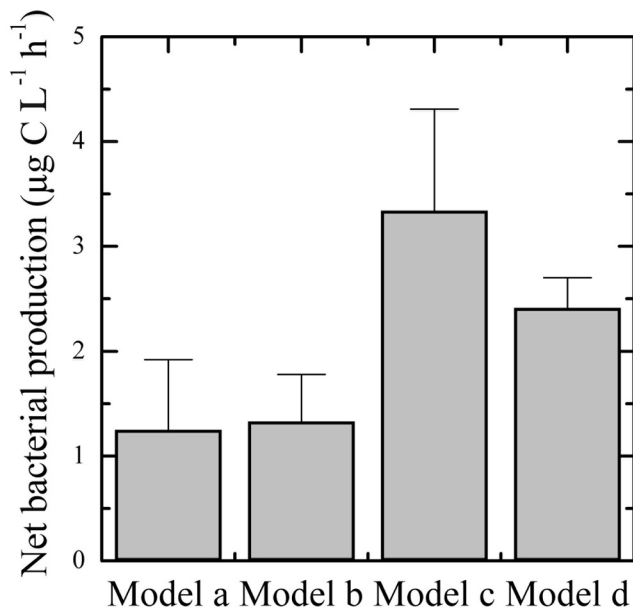


Fig. 7. Distribution of the average net bacterial biomass production as a function of the model of oxygen consumption observed during incubation. Error bars represent the standard deviation.

would give results similar to those estimated by continuous monitoring of oxygen concentration. This model characterized respiration of water samples displaying a low net bacterial production (Fig. 7).

Model b presents a 5- to 10-h lag phase before oxygen decrease. This model was, in general, characteristic of the oligotrophic samples (4 of 7) from the open lagoon transect (Fig. 1). In model b, respiration estimated by the discrete method would result in an underestimation of the oxygen consumption rates (Fig. 6). As observed for model a, model b is characteristic of water samples exhibiting a low net bacterial production (Fig. 7). In model c, consumption increases as a function of time due to increasing biomass and production. The discrete method would therefore overestimate BR compared to continuous oxygen recording during the 5-h time interval (Fig. 6). Samples respiring according to model c are characterized by the highest net bacterial production (Fig. 7). Finally, in model d, the slope is maximal at the beginning of the incubation and decreases thereafter. This trend is characteristic of rather eutrophic sites and may be explained by an exhaustion of the trophic resources and subsequent decrease of the heterotrophic bacterial activity. For model d, the discrete method for the estimation of the bacterial respiration would result in a lower rate than the value determined using continuous monitoring (Fig. 6).

These different models show the great variability of oxygen kinetics and therefore the difficulty in choosing how to compute the bacterial oxygen consumption. Similarly, in a previous study, Pomeroy et al. (1994) observed 4 different oxygen trends from discrete oxygen measurements performed during

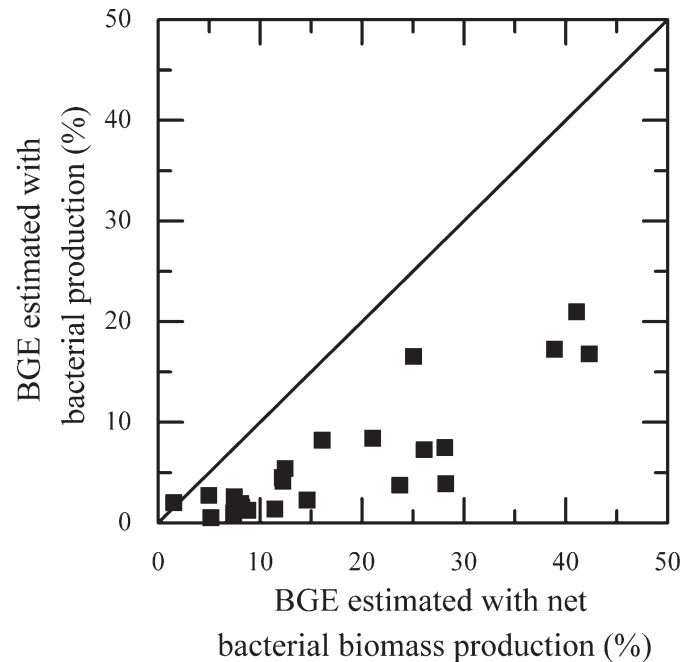


Fig. 8. Relationship between BGE estimated with the net bacterial biomass production (BGE_{BBPnet}) and the BGE estimated with the bacterial production determined by the ³H-thymidine incorporation (BGE_{BP}).

24 h incubation. Even if, in our study, the models a/b and c/d can be assigned to water of different trophic status as revealed by bacterial net production of biomass (Fig. 7), we cannot assign one model to any characteristic site. This result emphasizes the need to monitor continuously the oxygen consumption of planktonic microorganisms in order to assess respiration rates with minimum biases.

Estimation of BGE—BGE is most often determined from a proxy of bacterial production (measured with ³H-leucine or ³H-thymidine incorporation) computed with bacterial respiration. In our study, we estimated the net bacterial biomass production from the change of abundance observed during the incubation used for the bacterial respiration determination (see Materials and procedures). This way, both processes that govern BGE are determined under the same conditions. Fig. 8 presents the relationships between the BGE estimated with the net bacterial biomass production (BGE_{BBPnet}) and the BGE estimated with the bacterial production determined from ³H-thymidine incorporation (BGE_{BP}). Except for one station, BGE_{BBPnet} is always higher than BGE_{BP} . BGE_{BBPnet} and BGE_{BP} can be correlated using a linear regression ($R^2 = 0.75$, $n = 24$, $P < 0.001$) with the following equation:

$$BGE_{PB} = 0.4323 \times BGE_{BBPnet} - 1.4829 \quad (2)$$

This trend would suggest that when BGE is estimated from the bacterial production estimated from tracer incorporation, it would result in an underestimation of the bacterial growth

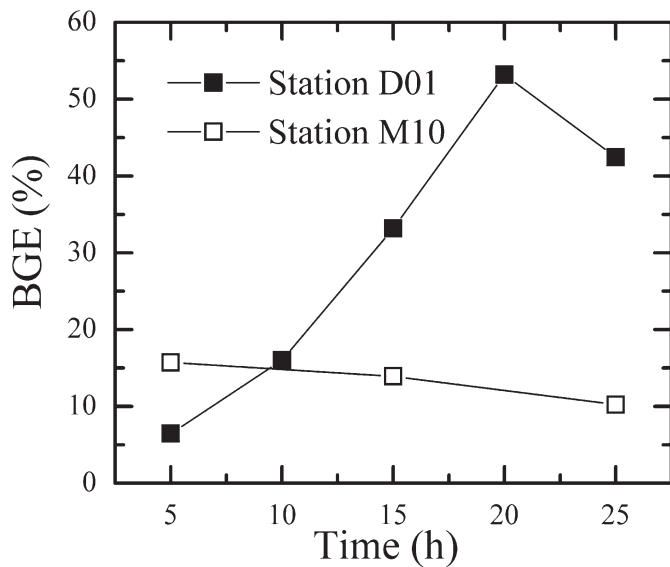


Fig. 9. Time course of BGE during a 24-h incubation in two contrasted trophic status. Data used to compute BGE are from Fig. 4. For station M10, the calculation of the net biomass production was performed under a 10-h interval to get a significant change in bacterial abundance.

efficiency. This underestimation might be due to the discrepancy in incubation time to determine both processes (1 h for TdR-derived bacterial production versus 12 to 24 h for the net bacterial biomass production).

The 24-h incubation used to estimate BGE could lead to strong changes of the biomass and bacterial production as shown in Fig. 4. These changes in bacterial biomass and production can strongly affect the determination of the BGE. Fig. 9 presents the time course of the BGE during 24-h incubation computed from the data presented in Fig. 4. At the eutrophic site (D01), BGE was equal to 7% at the beginning of incubation, and strongly increased with time to reach a maximum value of 53% after 20 h of incubation. This increase in BGE is due to the strong decrease in the respiration rate during the incubation, concomitant with a constant net biomass bacterial production (Fig. 4A). Coffin et al. (1993) have observed similar patterns for bacterial abundances and respiration during 24 h incubations leading to a regular increase in BGE. Similarly, Pomeroy et al. (1994) have observed a decrease in oxygen respiration (using multi-point Winkler titration) concomitant with a regular increase in bacterial production determined from leucine incorporation. These observed time courses of both processes result in a regular increase in BGE during incubation. High initial respiration rates concomitant with low bacterial production (resulting in a low BGE value) could indicate that both processes are not coupled as previously suggested by del Giorgio and Cole (1998).

In contrast, at the oligotrophic site M10, respiration and net bacterial biomass production were constant with time (Fig. 4B). As a consequence BGE did not exhibit significant variations

during incubation (Fig. 9). Possible variations of BGE during a 24-h incubation, such as those observed for the eutrophic site, emphasize the necessity to reduce as much as possible the time of incubation in order to minimize the bias in the BGE determination.

Discussion

The Winkler technique is the most commonly used technique to estimate bacterial respiration in planktonic systems. This technique offers high sensitivity (see Table 2), however it has the disadvantage of preventing the continuous monitoring of oxygen concentration with time. Respiration is most often computed from the difference between initial and final oxygen concentrations assuming a linear decrease of oxygen during incubation. Previous studies have already shown that oxygen decrease in long incubation is not always linear but can exhibit different patterns like exponential decay or exponential increase (Biddanda et al. 1994; Pomeroy et al. 1994). In addition, despite a high sensitivity, long incubation times are often required to detect significant respiration rates, especially in oligotrophic waters where incubations can reach up to 36 h. The main consequences of these long incubations are well documented; this includes changes in bacterial number and activity (see del Giorgio and Cole 1998, for a review) but also changes in the community composition (Massana et al. 2001; Gattuso et al. 2002).

The use of oxygen microprobes to measure bacterial respiration can solve one of the main problems encountered with discrete measurements: the monitoring of the oxygen decrease during dark incubations. Among the 27 measurements performed in this study, only 9 of them exhibited a linear decrease of oxygen concentration, the others show trends that were somehow related to the trophic status of the water. This monitoring offers two main advantages. First, by following the oxygen concentration versus time, it is possible to detect the onset of significant oxygen consumption. Oxygen microprobes do not consume oxygen due to the implementation of a guard cathode (Revsbech 1989) and exhibit a high precision around 0.1 μM of O_2 , a value that is similar to the one observed in high precision Winkler measurements (see Table 2). However this high sensitivity is counterbalanced by the background noise, which often occurs during oxygen measurements with a microprobe. As a consequence, this theoretical precision of 0.1 μM is in practice reduced to 0.5 μM of O_2 for oxygen measurements performed in planktonic waters.

The second advantage is that, as soon as a significant oxygen decrease is noticed, it is possible to considerably reduce the time of incubation and therefore stop the incubation when enough data points have been recorded. Thus, by minimizing bottle effects and the concomitant community changes, measurements are performed under conditions that remain the closest possible to the initial in situ conditions.

The precision of the oxygen microprobes is however not sufficient enough to measure bacterial respiration in oligo-

otrophic waters with a short incubation time. Monitoring of oxygen concentration in oligotrophic waters has demonstrated that oxygen decrease became measurable by the oxygen microprobe only after bacterial activity and biomass increased during the incubation (Fig. 4B). This clearly shows that measurements of respiration in such waters are still problematic since, at the present time, there is no technique with sensitivity sufficient enough to detect these very low in situ respiration rates. Gattuso et al. (2002) have proposed the applications of alternative techniques, which would offer higher oxygen sensitivity and therefore potentially considerably reduce the incubation time such as the use of membrane inlet ion trap mass spectrometry (Cowie and Lloyd 1999), to estimate rates of respiration.

Comments and recommendations

The determination of BGE requires the estimation of bacterial production. This is generally done by measuring the rate of protein or DNA synthesis, using radiolabeled leucine or thymidine, although the changes in bacterial abundance and size can also be used. Estimation of bacterial production from the incorporation of radiotracers can be performed with very short incubation times and is considered as a good proxy of the in situ rate. However, BGE is afterward computed from bacterial respiration estimated over incubation times longer than those used for the determination of bacterial production. BGE is therefore computed from the rate of two metabolic processes estimated in two different incubation conditions, which is likely to bias it (i.e., the production rate measured over a short-time interval may not correspond to the respiration rate over a much longer time scale). The estimation of net bacterial production from the changes of bacterial abundances during the incubations for respiration measurements can be an alternative solution. By using a non-destructive method to measure oxygen change, sub-samples can be obtained at the end of the incubation to determine the net bacterial biomass production. This way both processes would be estimated with the same time scale and the same conditions of incubation.

The possibility to reduce incubation length by continuous monitoring of oxygen changes during bacterial respiration measurements needs the determination of the net bacterial biomass production with a sufficient accuracy. To achieve the required sensitivity, determination of bacterial counts using epifluorescence microscopy will require the enumeration of a large number of bacteria and the use of several replicates, especially in oligotrophic waters. This will dramatically increase the workload associated with the measurements. Flow cytometry might be an alternative technique to determine net bacterial biomass production during respiration incubations. This technique offers the possibility to measure bacterial numbers with a higher sensitivity compared to epifluorescence microscopy (Troussellier et al. 1999; Lemarchand et al. 2001). In addition, flow cytometry can be used to estimate the bio-

volume of the cells, or even the protein content (Zubkov et al. 1999), at the beginning and at the end of the incubation and thus, allows a better calculation of the net bacterial production, since changes in the biovolume of bacterial cells have been often reported during incubation for BGE determination (Gattuso et al. 2002).

References

- Amon, R. M. W., and R. Benner. 1996. Bacterial utilization of different size classes of dissolved organic matter. *Limnol. Oceanogr.* 41:41-51.
- Barillier, A., and J. Garnier. 1993. Influence of temperature and substrate concentration on bacterial growth yield in Seine River water batch cultures. *Appl. Environ. Microbiol.* 59: 1678-1682.
- Biddanda, B., S. Opsahl, and R. Benner. 1994. Plankton respiration and carbon flux through bacterioplankton on the Louisiana shelf. *Limnol. Oceanogr.* 39:1259-1275.
- Bjornsen, P. K. 1986. Bacterioplankton growth yield in continuous seawater cultures. *Mar. Ecol. Prog. Ser.* 30:191-196.
- Bouvier, T. C., and P. A. del Giorgio. 2002. Compositional changes in free-living bacterial communities along a salinity gradient in two temperate estuaries. *Limnol. Oceanogr.* 47:453-470.
- Carlson, C. A., and H. W. Ducklow. 1996. Growth of bacterioplankton and consumption of dissolved organic carbon in the Sargasso Sea. *Aquat. Microb. Ecol.* 10:69-85.
- Cho, B. C., and F. Azam. 1988. Major role of bacteria in biogeochemical fluxes in the ocean's interior. *Nature* 332:441-443.
- Coffin, R., J. Connolly, and P. S. Harris. 1993. Availability of dissolved organic carbon to bacterioplankton examined by oxygen utilization. *Mar. Ecol. Prog. Ser.* 101:9-22.
- Cowie, G., and D. J. Lloyd. 1999. Membrane inlet ion trap mass spectrometry for the direct measurement of dissolved gas in ecological samples. *J. Microbiol. Meth.* 35:1-12.
- Daneri, G., B. Riemann, and P. J. L. Williams. 1994. In situ bacterial production and growth yield measured by thymidine, leucine and fractionated dark oxygen uptake. *J. Plankt. Res.* 16:105-113
- del Giorgio, P. A., and T. C. Bouvier. 2002. Linking the physiologic and phylogenetic successions in free-living bacterial communities along an estuarine salinity gradient. *Limnol. Oceanogr.* 47:471-486.
- and J. J. Cole. 1998. Bacterial growth efficiency in natural aquatic systems. *Annu. Rev. Ecol. Syst.* 29:503-541.
- Douillet, P. 1998. Tidal dynamics of the south-west lagoon of New-Caledonia: observations and 2D numerical modeling. *Oceanologica Acta* 21:69-79.
- Ducklow, H. W., and C. A. Carlson. 1992. Oceanic bacterial production. *Adv. Microb. Ecol.* 12:113-181.
- Fuchs, B. M., M. V. Zubkov, K. Sahm, P. H. Burkill, and R. Amann. 2000. Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow

- cytometric and molecular biological techniques. *Environ. Microbiol.* 2:191-201.
- Fuhrman, J. A. 1992. Bacterioplankton role in cycling of organic matter: the microbial food web, p. 361-383. In: P. G. Falkowski and A. D. Woodhead [eds.], *Primary productivity and biogeochemical cycles in the sea*. Plenum Press, New York.
- Fukuda, R., H. Ogawa, T. Nagata, I. Koike. 1998. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. *Appl. Environ. Microbiol.* 64:3352-3358.
- Gattuso, J. P., S. Peduzzi, M. D. Pizay, and M. Tonolla. 2002. Changes in freshwater bacterial community composition during measurements of microbial and community respiration. *J. Plankt. Res.* 24:1197-1206.
- Griffith, P. C. 1988. A high-precision respirometer for measuring small rates of change in the oxygen concentration of natural waters. *Limnol. Oceanogr.* 33:632-638.
- , D. J. Douglas, and S. C. Wainright. 1990. Metabolic activity of size-fractionated microbial plankton in estuarine, nearshore and continental shelf waters of Georgia. *Mar. Ecol. Prog. Ser.* 59:263-270.
- Hobbie, J. E., and C. C. Crawford. 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. *Limnol. Oceanogr.* 14:528-532.
- Jeffrey, S. W., and G. F. Humphrey. 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, *c*₁ and *c*₂ in algae, phytoplankton and higher plants. *Biochem. Physiol. Pflanz.* 167:191-194.
- Jorgensen, N. O. G., N. Kroer, and R. B. Coffin. 1994. Utilization of dissolved nitrogen by heterotrophic bacterioplankton: effect of substrate C/N ratio. *Appl. Environ. Microbiol.* 60:4124-4133.
- Kana, T., C. Darkangelo, M. D. Hunt, J. B. Oldham, G. E. Bennett, and J. C. Cornwell. 1994. Membrane inlet mass spectrometer for rapid high-precision determination of N₂, O₂, and Ar in environmental water samples. *Anal. Chem.* 66:4166-4170.
- Kirchman, D. L., J. Sigda, R. Kapuscinski, and R. Mitchell. 1982. Statistical analysis of the direct count method for enumerating bacteria. *Appl. Environ. Microbiol.* 44:376-382.
- Kroer, N. 1993. Bacterial growth efficiency on natural dissolved organic matter. *Limnol. Oceanogr.* 38:1282-1290.
- Langdon, C. 1993. Community respiration measurements using a pulsed O₂ electrode, p. 447-453. In: P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole [eds.], *Handbook of methods in aquatic microbial ecology*, Lewis Publisher.
- Lee, S., and J. A. Fuhrman. 1987. Relationship between biovolume and biomass of naturally derived marine bacterioplankton. *Appl. Environ. Microbiol.* 53:1298-1303.
- Lemarchand, K., N. Parthuisot, P. Catala, and P. Lebaron. 2001. Comparative assessment of epifluorescence microscopy, flow cytometry and solid-phase cytometry used in the enumeration of specific bacteria in water. *Aquat. Microb. Ecol.* 25:301-309.
- Lemée, R., E. Rochelle-Newall, F. Van Wambeke, M. D. Pizay, P. Rinaldi, and J. P. Gattuso. 2002. Seasonal variation of bacterial production, respiration and growth efficiency in the open NW Mediterranean Sea. *Aquat. Microb. Ecol.* 29:227-237.
- Lorenzen, C. J. 1966. A method for the continuous measurement of in vivo chlorophyll concentration. *Deep-Sea Res.* 13:223-227.
- Massana, R., C. Pedros-Alio, E. O. Casamayor, and J. M. Gasol. 2001. Changes in marine bacterioplankton phylogenetic composition during incubations designed to measure biogeochemically significant parameters. *Limnol. Oceanogr.* 46:1181-1188.
- Middelboe, M., and M. Sondergaard. 1993. Bacterioplankton growth yield: seasonal variations and coupling to substrate lability and β -glucosidase activity. *Appl. Environ. Microbiol.* 59:3916-3921.
- , B. Nielsen, and M. Sondergaard. 1992. Bacterial utilization of dissolved organic carbon (DOC) in coastal waters—determination of growth yield. *Arch. Hydrobiol. Ergebn. Limnol.* 37:51-61.
- Pomeroy, L. R., W. J. Wiebe, D. Deibel, R. J. Thompon, G. T. Rowe, and J. D. Pakulski. 1991. Bacterial responses to temperature and substrate concentration during the Newfoundland spring bloom. *Mar. Ecol. Prog. Ser.* 75:143-159.
- , J. E. Sheldon, and W. M. Sheldon. 1994. Changes in bacterial numbers and leucine assimilation during estimations of microbial respiratory rates in seawater by the precision Winkler method. *Appl. Environ. Microbiol.* 60:328-332.
- , J. E. Sheldon, W. M. Sheldon, and F. Peters. 1995. Limits to growth and respiration of bacterioplankton in the Gulf of Mexico. *Mar. Ecol. Prog. Ser.* 117:259-268.
- Pradeep Ram, A. S., S. Nair, D. Chandramohan. 2003. Bacterial growth efficiency in the tropical estuarine and coastal waters of Goa, southwest coast of India. *Microb. Ecol.* 45:88-96.
- Revsbech, N. P. 1989. An oxygen microsensor with a guard cathode. *Limnol. Oceanogr.* 34:472-476.
- Roland, F., N. F. Caraco, and J. J. Cole. 1999. Rapid and precise determination of dissolved oxygen by spectrophotometry: Evaluation of interference from color and turbidity. *Limnol. Oceanogr.* 44:1148-1154.
- Schäfer, H., P. Servais, and G. Muyzer. 2000. Successional changes in the genetic diversity of a marine bacterial assemblage during confinement. *Arch. Microbiol.* 173:138-145.
- Sherr, B. F., and E. B. Sherr. 2003. Community respiration/production and bacterial activity in the upper water column of the central Arctic Ocean. *Deep-Sea Res. Part I* 50:529-542.
- Smith, E. M., and Y. T. Prairie. 2004. Bacterial metabolism and growth efficiency in lakes: The importance of phosphorus availability. *Limnol. Oceanogr.* 49:137-147.
- Taylor, G. T., J. Way, and M. Scranton. 2003. Planktonic carbon cycling and transport in surface waters of the highly urbanized Hudson river estuary. *Limnol. Oceanogr.* 48:1779-1795.

- Troussellier, M., C. Courties, P. Lebaron, and P. Servais. 1999. Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. *FEMS Microbiol. Ecol.* 29:319-330.
- Tulonen, T, Salonen K, Arvola L. 1992. Effects of different molecular weight fractions of dissolved organic matter on the growth of bacteria, algae and protozoa from a highly humic lake. *Hydrobiologia* 229:239-252.
- Yentsch, C. S., and D. W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and pheophytin by fluorescence. *Deep-Sea Res.* 10:221-231.
- Zweifel, U. L., B. Norrman, and A. Hagström. 1993. Consumption of dissolved organic carbon by marine bacteria and demand for inorganic nutrients. *Mar. Ecol. Prog. Ser.* 101:23-32.
- Zubkov, M. V., B. M. Fuchs, H. Eilers, P. H. Burkill, and R. Amann. 1999. Determination of total protein content of bacterial cells by SYPRO staining and flow cytometry. *Appl. Environ. Microbiol.* 65:3251-3257.

Submitted 18 May 2004

Revised 29 October 2004

Accepted 15 November 2004