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HOW IMPORTANT IS VIRAL EFFECT ON THE MEASURE BACTERIAL GROWTH IN WESTERN SUBTROPICAL PACIFIC COASTAL WATER?

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Key words: dilution method, growth rates, virus.

ABSTRACT

Bacterial growth rates were determined in natural seawater samples using two different methodological approaches: incubation in dialysis bags, and a modification of the dilution approach. Bacterial growth rates estimated for the dialysis bag varied from 0.026 h^{-1} (April) to 0.208 h^{-1} (August). Bacterial growth rates in 0.2 μm and 30 kDa size-fractionated series ranged from 0.038 to 0.094 h^{-1} and 0.078 to 0.42 h^{-1}, respectively. Furthermore, in all of our samples, growth rates in the 30 kDa fractionated series (grazer and virus-free diluent) were higher than in the 0.2 μm series (grazer-free diluent) or in the dialysis bag incubations.

I. INTRODUCTION

Bacteria are important components of the aquatic food web both in terms of biomass and activity. The role of bacteria in organic matter processing and nutrient recycling is well established (Azam *et al*. [1]). Furthermore, bacteria play an important role in functioning of the microbial food web in relation to environmental conditions such as temperature (Felip *et al*. [6]; Tsai *et al*. [34]), resource availability (Felip *et al*. [6]; Pace and Cole [25]), predation pressure (Pace and Cole [25]; Tsai *et al*. [35]) and viral infection (Winter *et al*. [40]). They exhibit high level of short-term variations in activity because their metabolism can change rapidly in response to changes in environmental conditions, probably more rapidly than in other planktonic organisms (Straskrabova and Fuksa [30]).

Various methods have been used to determine the bacterial growth or production in aquatic ecosystems, but may result in different estimates of specific growth rates on the same sample (Christian *et al*. [3]). Methods based on the dilution method (Landry *et al*. [20]), size fractionation (Wright and Coffin [41]), incubation in dialysis bags (Herndl *et al*. [13]), 3H-thymidine and adenine incorporation into DNA (Fuhrman and Azam [8]), and 3H-leucine incorporation into protein (Kirchman [17]) have been developed. Each technique has advantages and disadvantages. Methodological shortcomings probably represent the greatest impediment to our understanding of microbial ecology and the factors influencing the growth and fate of microorganisms in natural environments. To our knowledge, there have been limited seasonal studies in which simultaneously estimate bacterial growth using various approaches (Jugnia *et al*. [15]).

The biotic factors (top-down control) that may control bacterial production in aquatic systems is predation by protists (Pace [24]) and it can be lost through viral infection and lysis (Wilhelm *et al*. [39]; Taira *et al*. [31]). It is now accepted that a significant fraction of the bacterial population is lost through viral infection and lysis (Fuhrman and Noble [10]; Bettarel *et al*. [2]). Previous estimates of bacterial growth rate and production might have been underestimated because almost all viruses could remain in the incubation treatments and the methods did not take into account viral infection. In the following study, bacterial growth rates were determined by means of size fractionation used the incubation method as dialysis bags. To date, no studies have investigated the effect of viral-mediation of bacterial growth in western subtropical Pacific coastal water. Moreover, experiments were set up based on the original protocol of the modified dilution method of Evans *et al*. [5] to determine the impact of viruses on bacterial growth rates. Our goal in this study was to test the hypothesis that viral-dependent mortality was an important loss process during determinations of bacterial production and greatly affected the calculated variations of bacterial growth rates at our study site.

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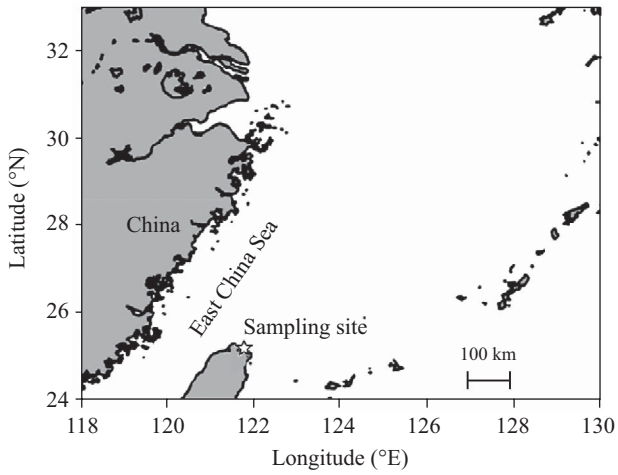

Fig. 1. Map of sampling station.

II. MATERIAL AND METHODS

1. Sampling

Samples for the bacterial growth rate study were collected two to four times a month from March to October 2011 (dialysis bags) and once time a month from April to December 2011 (modified dilution method) at an established coastal station (25°09.4'N, 121°46.3'E) along a rocky shore in northeastern Taiwan (Fig. 1). The environment of this site was previously described based on data gathered from 1999 to 2001 (Tsai *et al*. [33]). Surface water temperature was around 16°C in March and increased gradually to 29°C in July (Tsai *et al*. [33]). Annually, salinity ranges from 33.1 to 34.3, lower salinity within this range likely reflecting the influence of rainfall runoff. During this study, water temperature was measured immediately after the bucket-cast, and all samples were brought to the laboratory within 30 minutes.

2. Incubation Experiments

Using the size fractionation method (Wright and Coffin [41]), bacterial growth rates were measured using dialysis bags. A 2-μm pore polycarbonate filter was used to remove predators of bacteria; this fraction was designed to exclude picoplankton grazers (grazer-free). Each filtered water sample was then transferred into dialysis bags (Spectro/POR type, 25.5-mm inflated diameter, molecular weight cutoff 12-14 k MWCO) (Turley and Lochte [36]) of 250 ml in triplicate. Then dialysis bags were transported to the sampling site and incubated in situ at a depth of 0.5-1 m for 24 h.

We determine the impact of viruses on bacterial growth rates using a modified technique of parallel dilution experiments – a "standard" set that reduces grazers and a set that reduces both grazers and viruses (Evans *et al*. [5]). To prepare the "standard" diluent, the natural sample was passed through 10 μm mesh and then filtered through 47 mm Nuclepore filter (type PC, pore size of 0.2 μm). Filtered seawater sample (<10 μm) was then diluted with the 0.2 μm filtered seawater in a 4-point dilution series: 25, 50, 75, and 100% seawater (<10 μm). The mixtures were incubated for 24 h in triplicate in 50-mL polycarbonate bottles under natural light in a water bath set at the same temperature that seawater was at the time of sampling. The size fractionation used for grazers (<10 μm) was chosen based on previous studies at this site to eliminate ciliates but not nanoflagellates (Tsai *et al*. [35]). An additional dilution series, which used 30 kDa filtered seawater in place of 0.2 μm filtered water, modified both grazing and viral mortalities. The net growth rate of bacteria (k, h^{-1}) was calculated for each sample based on microscopic cell counts at the start and the end of the experiment (N_t and Nt_0), assuming exponential growth (Landry and Hassett [19]):

$$k = \ln (N_t / Nt_0)/(t\text{-}t_o)$$

Subsamples of 1-2 mL were filtered onto 0.2 μm black Nuclepore filters for bacteria. Samples were stained with DAPI at a final concentration of 1 μg mL^{-1} (Porter and Feig [26]). Bacteria were counted using an epifluorescence microscope (Nikon Optiphot-2) at 1000 × magnification and were identified by their blue fluorescence under UV illumination.

III. RESULTS AND DISCUSSION

Seasonal variations of bacterial growth rates estimated with for the dialysis bag varied from 0.026 h^{-1} (April) to 0.208 h^{-1} (August) (Table 1, Fig. 2). Theoretically, dialysis bag incubations are useful for determining bacterial growth rates (Scheffers *et al*. [27]). It can be demonstrated that use of a dialysis bag most closely simulates natural conditions, with very little sample processing (Turley and Lochte [36]) and incubation in dialysis bags enables a chemical connection between the encaged microbial population and the surrounding water (Ferrier-Pagès *et al*. [7]). In this study, we used dialysis bags to separate nanoflagellates from their prey (picoplankton) and allow nutrient diffusion. One shortcoming of this method is the necessity of filtration procedures to exclude bacterivores and the poorly understood effect of confinement on bacterial activity. In the present study, we used 2-μm pore polycarbonate filters through which almost the entire bacterial population passed. The 2-μm pore filter was very effective in removing grazers of bacteria because no nanoflagellate could be found in the filtrate drawn from dialysis bags after in situ incubation for 24 h, when at least 50 fields of view were examined under a microscope. Furthermore, development of bacteria on the walls of dialysis bags, the main source of error associated with the dialysis bags has been considered to be negligible for 24 h incubations (Velimirov and Walenta-Simon [37], Jugnia *et al*. [15]).

We used a modified dilution method to estimate bacterial growth rates for 7 different months (Table 1). The y-intercepts of the regression lines (bacterial growth rates) for 0.2 μm and 30 kDa fractionated series ranged from 0.038 to 0.094 h^{-1} and

Table 1. Temperature, bacterial abundance and bacterial growth rate in dialysis bags were observed and the y-intercepts of the linear regression analysis for 0.2 μm and 30 kDa dilution series.

Month	temperature (°C)	bacterial abundance (10^6 cells ml^{-1})	growth rate (0.2 μm) μ (h^{-1})	growth rate (30 kDa) μ (h^{-1})	growth rate (dialysis bag) μ (h^{-1})
Mar	19	0.62 ± 0.04			0.041
Apr	19.7-23.0	0.6 ± 0.08	0.043	0.078	0.026
May	21.1-27.0	0.63 ± 0.11	0.052	0.083	0.05-0.072
Jun	27-28	0.81 ± 0.12	–	–	0.057-0.11
Jul	27.5-29.5	0.79 ± 0.04	0.071	0.36	0.075-0.131
Aug	25-28	0.88 ± 0.06	–	–	0.064-0.21
Sep	27-28.1	0.64 ± 0.14	0.084	0.42	0.071-0.11
Oct	23.5-25.3	0.75 ± 0.07	0.093	0.27	0.016-0.054
Nov	21.0	0.77 ± 0.05	0.046	0.17	–
Dec	18.4	0.54 ± 0.04	0.038	0.15	–

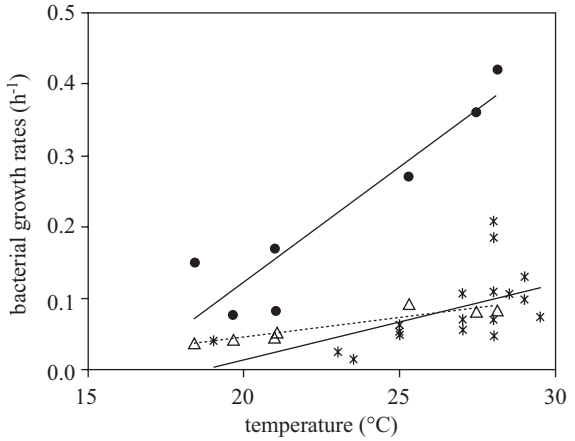

Fig. 2. Apparent bacterial growth rate plotted against the surface seawater temperature during the study period. Linear regression of bacterial growth rates of 30 kDa dilution series *vs.* temperature (●; y = 0.032x-0.52, r^2 = 0.86). Solid and broken line in lower part of figure: linear regression for bacterial growth rates in dialysis bag (∗; y = 0.11x-0.20, r^2 = 0.31) and in 0.2 μm dilution series (△; y = 0.006x-0.06, r^2 = 0.87), respectively.

0.078 to 0.42 h^{-1}, respectively (Table 1). It is obvious that our data, in all of our samples, growths in the 30 kDa fractionated series (grazer and virus-free) were higher than in the 0.2 μm (grazer-free) and incubation in the dialysis bags (ANOVA, $p < 0.05$) (Table 1). Although some limitations and drawbacks were either unavoidable for either method, growth in the polycarbonate bottles seems to be controlled by other factors, such as substrate supply or availability of nutrients (Keil and Kirchman [16]). In the present study, there was no significant difference in bacterial growth rates between in the polycarbonate bottles and incubation in the dialysis bags after 0.2 μm (grazer-free) filtered (*t*-test, $p > 0.05$). When polycarbonate bottle and dialysis bag incubations were performed at the similar temperature, bacterial growth rates varied non-significantly between treatments (Fig. 2). This implies that factors of substrate supply or availability of nutrients did not affect bacterial growth in both incubated treatments. However, the marked difference in the 30 kDa fractionated series (grazer and virus-free) may reflect the degree of bacterial mortality caused by viral lysis.

Most studies have assessed factors that control bacterial growth, such as temperature (Shiah and Ducklow [29]; Kirchman *et al*. [18]; Tibbles [32]; McManus *et al*. [21]; Tsai *et al*. [34]) and substrate availability (Fuhrman and Bell [9]; Gasol and Morán [11]). However, the impacts of environmental factors on bacterial growth with different culture treatments are still poorly understood. Significant positive correlations exist between bacterial growth and temperature (Hoch and Kirchman [14]; Shiah and Ducklow [29]; Kirchman *et al*. [18]; Tibbles [32]; McManus *et al*. [21]; Tsai *et al*. [34]). Our study is consistent with these previous studies indicating that temperature can be important in controlling seasonal variations of bacterial growth; positive relationships existed between the growth rates and temperature for bacteria incubated in dialysis bags and polycarbonate bottles using modified dilution method (Fig. 2). As mentioned above, however, the estimated the slope of growth rates with temperature was significantly different between the treatments of 30 kDa fractionated series (virus-free) and dialysis bags (30 kDa: growth rate (h−1) = 0.032 temp−0.52, $r^2 = 0.86$, $p < 0.05$; dialysis bag: growth rate (h−1) = 0.011 temp−0.20, $r^2 = 0.31$, $p < 0.05$) (Fig. 2). Our observations suggest that under conditions where lytic pressure is relatively high, the data obtained from the standard protocol underestimated growth rates because almost all viruses could remain in the incubation treatments. Previous estimates of biogeochemical fluxes and carbon budgets as well as nutrients, might be modified by the new dilution approach which takes into account viral infection.

The relative importance of the bottom-up (temperature, substrate availability and nutrients) and top-down control (grazing and viral lysis) on bacterial production demonstrates a high degree of both spatial and temporal variability. Viruses may exert a more subtle influence by generating a relatively labile lysate which, though a small component of dissolved organic carbon, may nonetheless be an important source of organic matter to other microorganisms (Gobler *et al*. [12]),

especially under oligotrophic conditions (Noble and Fuhrman [23]). In fact, a laboratory study has shown that viral lysate can solely support bacterial growth (Middelboe *et al*. [22]). It is important to note, we observed higher bacterial growth rates in the 30 kDa (virus-free) than in the 0.2 μm and incubation in the dialysis bags. All results revealed that viruses played an important part in the determination of bacterial growth rates, and suggest strong top-down (viral lysis) control of bacterial production in the current study.

It is worth noting that the ratio of bacterial production: primary production (BP:PP) can be considered as an indication of the trophic state of a system. Shelford *et al*. [28] showed that the removal of viruses reduced ammonium production and decreased phytoplankton growth, supporting evidence from other studies in which the removal of viruses decreased growth rates of *Synechococcus* (Weinbauer *et al*. [38]). However, in this case, growth rates of bacteria were higher in treatment of which the concentration of viruses was reduced. The result of this study highlights the complex interactions within bacteria, primary producers and viruses.

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