Is the Dilution Technique Underestimating the Picophytoplankton Growth Measurements?

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Abstract: In oceanic communities, picophytoplankton often dominates phytoplankton biomass and productivity. Diel variations in picophytoplankton abundance and growth have been well documented. In the current study, we used flow cytometry to assess the short-term variations (3 h) of the abundance of the most dominant picophytoplankton, *Synechococcus* spp. and picoeukaryotes, in the coastal regions of northeastern Taiwan. To explore the change in growth and mortality rate in the daytime and over 24 h incubation, we performed a two-point modified dilution experiment for measuring growth, viral lysis, and nanoflagellate grazing rate. In this study, the growth rates of picoeukaryotes were 0.21 and 0.06 h⁻¹, and those of *Synechococcus* spp. were 0.15 and 0.06 h⁻¹ for daytime and 24 h incubation, respectively, and the values were higher at significant levels in the daytime than those for 24 h incubation. These growth rate values of picoeukaryote and *Synechococcus* spp. after incubation for 24 h were approximately underestimated at 71% and 55%, respectively. This finding suggests that estimates based on 24 h sampling may not accurately reflect the true growth rate of these populations on ecologically relevant timescales.

Keywords: picophytoplankton; diel variations; growth rate; picoeukaryote; *Synechococcus* spp.

1. Introduction

Picophytoplanktons (0.2–2 µm), comprising picocyanobacteria and photosynthetic picoeukaryotes, are important components of aquatic ecosystems. In the oligotrophic waters, picophytoplankton contribute to approximately 60–80% of the total marine primary productivity [1], suggesting that picophytoplankton has crucial roles in primary productivity in oligotrophic regions. In natural aquatic environments, the primary factors that affect picophytoplankton are temperature, nutrient availability, and irradiance level. Among these, the daily light–dark alternation is undoubtedly a very important external stimulus. Indeed, picophytoplankton is highly synchronized to the light–dark alternation [2,3] and the timing of division varies among species, groups, and even strains [2]. The diel variations in picophytoplankton abundance, growth, and division have been well documented [2,4,5].

Picophytoplankton is the major group in the oligotrophic ocean; therefore, much interest has been shown in determining their growth. Among some incubation approaches currently available, the dilution technique has been used in a wide range of aquatic environments and is now being accepted as a standard method for estimating picophytoplankton growth and mortality rates [6–11]. However, in these studies, the dilution treatments were done in 24 h incubation, and these may have ignored the fact that the picophytoplankton are possibly the most affected by the day–night cycle in the natural irradiance; besides, their diel periodicity in cell division and photosynthesis in marine ecosystems are well documented [2,4]. Some previous studies have shown that *Synechococcus* spp. have distinct
diel changes in growth, with higher division rates at dusk \[2,3\]. In this situation, considering that the picophytoplankton grow at the same rate during 24 h incubation, calculating the growth rate may lead to a possible underestimation because 24 h incubation duration is longer over the growth phase of picophytoplankton, and these studies may not have been able to capture the maximal growth rate within a diel cycle. This implies that the estimates based on 24 h sampling may not accurately reflect the true growth rate of these picophytoplankton populations on ecologically relevant timescales. We hypothesize that the growth rate of picophytoplankton may be higher during the daytime with higher rates of division than that during their incubation for 24 h, as estimated using the dilution method.

In the current study, we used flow cytometry to assess the short-term variations (3 h) of the abundance of the most dominant picophytoplankton, *Synechococcus* spp. and picoeukaryotes, in the coastal regions of northeastern Taiwan. In addition, we also attempted to understand the difference in growth rates under different incubation periods (daytime and 24 h) of *Synechococcus* spp. and picoeukaryotes by using a modified dilution method \[9,12,13\]. Using these observations on picophytoplankton, we evaluated the changes in picophytoplankton growth rates over the course of the day.

### 2. Materials and Methods

#### 2.1. Water Sampling

Samples were collected in August 2020 (12 and 13) from the surface waters at an established station located in the coastal waters of the northeastern Taiwan coast (25°09.4′ N, 121°46.3′ E), and all samples reached the lab within 30 min of sampling. Samples of *Synechococcus* spp. and picoeukaryotes were collected at 3 h intervals for 24 h observation of the diel variations, and their abundance from natural, as well as experimental, samples was enumerated within one week after sampling using a flow cytometer. Picophytoplankton (*Synechococcus* spp. and picoeukaryotes) were detected and measured using flow cytometry (FCM) (BD FACSCalibur™; United States). Fluorescent beads (1 μm) (Molecular Probes) were added to each sample to a final concentration (10^5 beads mL^{-1}) \[14\]. Forward-angle light scatter (FSC), side-angle light scatter (SSC), and green (SYBR-I) fluorescence were recorded. *Synechococcus* spp. and picoeukaryotic cells were distinguished using pigment autofluorescence and FSC.

#### 2.2. Experimental Design for the Incubation

To explore the change in growth and mortality rate in the daytime and over 24 h incubation, we performed a two-point modified dilution experiment and measured picophytoplankton (*Synechococcus* spp., and picoeukaryotes) growth, viral lysis, and nanoflagellate grazing rates \[9,12,13\]. Samples for modified dilution experiments were collected in 2 L polyethylene containers and transported to a local field laboratory. Then, 1.0 L of seawater was screened through 200 μm Nitex mesh to remove larger mesozooplankton grazers (SW). First, a dilution was performed using 0.2 μm mesh (47 mm diameter polycarbonate filters; AMD Manufacturing)-filtered water operated at low pressure (<50 mm Hg), which represented grazer-free diluents. This filtered water was then processed by tangential flow filtration through a 30 kDa cartridge to create virus-free water. Then, fresh SW was collected as described above and combined with grazer- and virus-free diluents at a proportion of 25% SW.

All treatments were incubated for 9 h (10:00 am to 19:00 pm) and 24 h in triplicate in 100 mL polycarbonate bottles under natural light in a water bath to match the in situ temperatures at the time of sampling (29 °C). Subsamples were taken at the beginning (t₀) and after incubating for 9 h (t₉) or 24 h (t₂₄) to estimate picophytoplankton (*Synechococcus* spp. and picoeukaryotes) abundance. The net growth rate of picophytoplankton (k, d⁻¹) was calculated as k = (lnN_t − lnN₀)/t, where t is the incubation time, and N₀ and N_t are the picophytoplankton abundance at the end and the start of the experiments.

The net growth rate of picophytoplankton in SW treatment signifies the difference between the gross growth rate (µ) and total mortality rate (grazing rates, Mₕ; and viral
Thus, the net growth rate of picophytoplankton in SW treatment is expressed in terms of Equation (1) as:

$$k_{SW} = \mu - (M_g + M_v)$$  \hspace{1cm} (1)

where $k$ is the measured net growth rate of picophytoplankton (d$^{-1}$), $\mu$ is the instantaneous growth rate of picophytoplankton, $M_g$ is the mortality due to grazing, and $M_v$ is the mortality due to viral lysis.

In diluted water with 25% of 0.2 $\mu$m filtration treatments, picophytoplankton mortality grazing activity is reduced to 25%. Thus, the net growth rate is as shown in the equation below:

$$k_{0.2 \mu m \text{ diluted}} = (\mu - M_v) - 0.25 \times (M_g)$$  \hspace{1cm} (2)

Finally, in sample diluted with 25% of 30 kDa filtration-treated water, picophytoplankton mortality is caused by 25% of grazing and viral lysis ($M_g + M_v$) in this incubated experiment. Thus, the equation is expressed as:

$$k_{30 \text{ kDa diluted}} = \mu - 0.25 \times (M_g + M_v)$$  \hspace{1cm} (3)

Picoplankton grazing rate ($M_g$) was calculated using Equation (1) minus Equation (2):

$$M_g = \left(\frac{4}{3}\right) \times (k_{0.2 \mu m \text{ diluted}} - k_{SW})$$  \hspace{1cm} (4)

Furthermore, $M_v$ was also calculated using Equation (3) minus Equation (2):

$$M_v = \left(\frac{4}{3}\right) \times (k_{30 \text{ kDa diluted}} - k_{0.2 \mu m \text{ diluted}})$$  \hspace{1cm} (5)

We thereby obtained the values of $M_g$ and $M_v$, and the value of the gross growth rate ($\mu$) was calculated as $\mu = k_{SW} + (M_g + M_v)$.

The Kruskal–Wallis test was used to test for significant differences in net growth rates (k) of picoplankton under diluted treatments. The differences in means of two treatments were tested using Welch’s t-test. When the picoplankton net growth rate (k) is lower in the diluted than in the undiluted treatments, this indicates a violation of the central assumption of the dilution method [6]. Thus, in this case, losses were considered undetectable. The significance level for all tests was set at <0.05.

3. Results

Picophytoplankton populations, analyzed by flow cytometry, revealed mainly two types of cells in our study region: *Synechococcus* spp. and picoeukaryotes (Figure 1). We found that the total abundance of *Synechococcus* spp. and picoeukaryotes had a pattern with an increased abundance during the day, and reached a peak at nearly 22:00 pm, followed by a gradual decrease during the nighttime (Figure 1). During the study period, picoeukaryotes were less abundant than *Synechococcus* spp. (3.6 $\times$ 10$^3$ and 10.2 $\times$ 10$^3$ cells mL$^{-1}$, respectively), and diel changes of *Synechococcus* spp. abundance varied more than five-fold (2.1 $\times$ 10$^4$ and 11.6 $\times$ 10$^4$ cells mL$^{-1}$) (Figure 1A,B). Investigations into their short-term variability show that *Synechococcus* spp. divide about once every 24 h, and diel changes in the abundance of picophytoplankton populations are driven primarily by two processes: cell division, which increases cell abundance, and cell mortality, which decreases cell numbers. Furthermore, studies of small-scale variability in picophytoplankton abundance have found that temporary imbalances between growth and mortality rates throughout a day generate important daily variation in aquatic environments.
Results of modified dilution experiments are summarized in Table 1. In our modified dilution experiments, net growth rates of picoeukaryotes were estimated to be 0.19 and 0.10 h\(^{-1}\) in 30 kDa diluted and undiluted water, respectively, during daytime (Table 1). However, in the 24 h incubation treatment, net growth rates for picoeukaryotes averaged 0.05 and 0.01 h\(^{-1}\) in 30 kDa diluted and undiluted water, respectively (Table 1), with a higher value in the 30 kDa diluted sample than that in undiluted water for picoeukaryotes (Welch’s \(t\)-test, \(p < 0.05\)). However, the net growth rates of picoeukaryotes were not significant (Welch’s \(t\)-test, \(p > 0.05\)) in the 0.2 \(\mu\)m filtered and 30 kDa diluted water during the daytime and 24 h incubation, respectively (Table 1). The estimated growth rates of \textit{Synechococcus} spp. were higher during the daytime than those during 24 h incubation in all experimental treatments (Kruskal–Wallis test, \(p < 0.05\)) (Table 1).
Table 1. Net growth rates of picoeukaryotes and *Synechococcus* spp. in each treatment at daytime, nighttime, and 24 h incubation. Growth, grazing, and viral lysis rates of picoeukaryotes and *Synechococcus* spp. were calculated based on net growth rates in each treatment at daytime, nighttime, and 24 h incubation. * Significance of the difference between the value of daytime or nighttime and 24 h incubation. ± represents the SD estimated from triplicate measurements. nd: not detected.

|                          | Picoeukaryotes                     | *Synechococcus* spp.       |
|--------------------------|-----------------------------------|----------------------------|
|                          | 25% (0.2 µm)                      | 25% (30 kDa)               |
|                          | 100%                              | 100%                       |
| Net growth rate (h⁻¹)    |                                   |                            |
| (daytime)                | 0.18 ± 0.03                       | 0.19 ± 0.04                |
| (nighttime)              | −0.03 ± 0.01                      | −0.03 ± 0.02               |
| (24 h)                   | 0.03 ± 0.01                       | 0.04 ± 0.01                |

|                          | growth rate (h⁻¹) | grazing rate (h⁻¹) | viral lysis | growth rate (h⁻¹) | grazing rate (h⁻¹) | viral lysis |
|--------------------------|-------------------|--------------------|-------------|-------------------|--------------------|-------------|
|                          | (daytime)         | 0.21 ± 0.04 *      | 0.11 ± 0.03 * | nd                | 0.15 ± 0.06 *      | nd          |
|                          | (nighttime)       | −0.02 ± 0.01       | 0.05 ± 0.02  | nd                | 0.02 ± 0.02 *      | 0.09 ± 0.02 * |
|                          | (24 h)            | 0.06 ± 0.01        | 0.05 ± 0.01  | nd                | 0.06 ± 0.02        | 0.05 ± 0.01  |

4. Discussion

In this study, we addressed the potential importance of diel variations in growth activity. Based on our calculation, the growth rates of picoeukaryotes were 0.21 and 0.06 h⁻¹, and those of *Synechococcus* spp. were 0.15 and 0.06 h⁻¹ after daytime and 24 h incubations, respectively (Table 1). The values were higher at significant levels in the daytime than that after 24 h incubation (Welch’s t-test, p < 0.05). Interestingly, using dilution experiments, we could show that the growth rate of the picophytoplankton had clear temporal variations for different incubation periods examined. For picophytoplankton, these growth rate values of picoeukaryote and *Synechococcus* spp. after incubation for 24 h were approximately underestimated at 71% (1 − (0.06/0.21) × 100%) and 55% (1 − (0.05/0.11) × 100%), respectively (Table 1). Our results support the hypothesis that the growth rate of picophytoplankton is higher during the daytime with higher division rates than that during 24 h incubation. This could be a significant step forward, as accurately measuring the growth of the major producers in the aquatic ecosystem is important to understanding the functions and changes in the system over time.

The dilution technique has been used in a large variety of marine and freshwater systems and has now become the standard technique for measuring phytoplankton growth and grazing mortality rates [9,11,12,15–22]. However, it is noteworthy that in all these previous studies on dilution experiments, treatments were done for 24 h in incubators. Thus, the values obtained in these studies could lead to underestimation of the picophytoplankton growth rates based on our daytime measurement. This difference was probably due to longer incubation times over the growth phase. To our knowledge, the data presented in this study are novel and unique, with good agreement between replicates, and coherent trends among the distinct picophytoplankton groups, giving commonly accepted diel changes in picophytoplankton growth.

Our experiments also reveal other significant results. Grazing and viral lysis are the two main factors responsible for the mortality of *Synechococcus* spp. in aquatic environments [15,23–26]. Understanding the fate of this carbon, whether it undergoes processes such as grazing or viral lysis, is a necessary step toward understanding carbon fluxes in marine systems. In this study, we determined that the largest cause of *Synechococcus* spp. mortality during the study period was nanoflagellate grazing, which accounted for 0.09 h⁻¹ and 0.05 h⁻¹ of the grazing rates at the nighttime and 24 h incubation, respectively (Table 1). In this study, using relatively short incubation periods at the nighttime, we were able to assess diel changes in mortality and found higher diel mortality activity of *Synechococcus* spp. at night (Table 1). In comparison to the predators, we found that the impact of viruses on mortality was only minor at nighttime incubation and that they
exerted a small but noticeable effect on Synechococcus spp. (viral lysis: 0.01 h\(^{-1}\)) (Table 1). This observation may help explain the imbalance between the rates of growth and loss that resulted in a general diel change in Synechococcus spp. abundance. Tsai et al. [3] studied the correlation of the higher grazing rates with the shift from the larger size of dividing Synechococcus cells (daytime) to the smaller size of non-dividing cells at night and suggested that size-selective feeding is the basis of the diurnal differences in ingestion of Synechococcus spp. by nanoflagellates.

5. Conclusions

In conclusion, in this study conducted during summer, there was a pronounced increase in picophytoplankton abundance during the growth phase. Therefore, we translated gross growth rates (h\(^{-1}\)) by multiplying them by the duration of the growth period. However, these gross growth rates of picophytoplankton were higher than the growth rates measured by the dilution method of samples incubated for 24 h. These results are worth considering as the dilution approach provides direct estimates of picophytoplankton growth rates in specified growth phases.

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References

1. Mann, N.H. Phages of the marine cyanobacterial picophytoplankton. FEMS Microbiol. Rev. 2003, 27, 17–34. [CrossRef]
2. Vaulot, D.; Marie, D. Diel variability of photosynthetic picoplankton in the equatorial Pacific. J. Geophys. Res. Ocean. 1999, 104, 3297–3310. [CrossRef]
3. Tsai, A.-Y.; Chin, W.-M.; Chiang, K.-P. Diel patterns of grazing by pigmented nanoflagellates on Synechococcus spp. In the coastal ecosystem of subtropical western Pacific. Hydrobiologia 2009, 636, 249–256. [CrossRef]
4. Tsai, A.-Y.; Chiang, K.-P.; Chang, J.; Gong, G.-C. Seasonal diel variations of picoplankton and nanoplankton in a subtropical western Pacific coastal ecosystem. Limnol. Oceanogr. 2005, 50, 1221–1231. [CrossRef]
5. Lefort, T.; Gasol, J.M. Short-time scale coupling of picoplankton community structure and single-cell heterotrophic activity in winter in coastal NW Mediterranean Sea waters. J. Plankton Res. 2014, 36, 243–258. [CrossRef]
6. Landry, M.R.; Hassett, R.P. Estimating the grazing impact of marine microzooplankton. Mar. Biol. 1982, 67, 283–288. [CrossRef]
7. Reckermann, M.; Veldhuis, M.J.W. Trophic interactions between picophytoplankton and micro- and nanozooplankton in the western Arabian Sea during the NE monsoon 1993. Aquat. Microb. Ecol. 1997, 12, 263–273. [CrossRef]
8. Kuipers, B.R.; Witte, H.J. Prochlorophytes as secondary prey for heterotrophic nanoflagellates in the deep chlorophyll maximum layer of the (sub)tropical North Atlantic. Mar. Ecol. Prog. Ser. 2000, 204, 53–63. [CrossRef]
9. Worden, A.Z.; Binder, B.J. Application of dilution experiments for measuring growth and mortality rates among Prochlorococcus and Synechococcus populations in oligotrophic environments. Aquat. Microb. Ecol. 2003, 30, 159–174. [CrossRef]
10. Gutierrez-Rodriguez, A.; Selph, K.E.; Landry, M.R. Phytoplankton growth and microzooplankton grazing dynamics across vertical environmental gradients determined by transplant in situ dilution experiments. J. Plankton Res. 2016, 38, 271–289. [CrossRef] [PubMed]
11. Kanayama, T.; Kobari, T.; Suzuki, K.; Yoshie, N.; Honma, T.; Karu, F.; Kume, G. Impact of microzooplankton grazing on the phytoplankton community in the Kuroshio of the East China sea: A major trophic pathway of the Kuroshio ecosystem. Deep Sea Res. Part I Oceanogr. Res. Pap. 2020, 163, 103337. [CrossRef]
12. Taniguchi, D.A.A.; Landry, M.R.; Franks, P.J.S.; Selph, K.E. Size-specific growth and grazing rates for picophytoplankton in coastal and oceanic regions of the eastern Pacific. Mar. Ecol. Prog. Ser. 2014, 509, 87–101. [CrossRef]
13. Anderson, S.R.; Harvey, E.L. Seasonal variability and drivers of microzooplankton grazing and phytoplankton growth in a subtropical estuary. *Front. Mar. Sci.* 2019, 6, 174. [CrossRef]

14. Gasol, J.M.; del Giorgio, P.A. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.* 2000, 64, 197–224. [CrossRef]

15. Christaki, U.; Courties, C.; Karayanni, H.; Giannakourou, A.; Maravelias, C.; Kormas, K.A.; Lebaron, P. Dynamic characteristics of *Prochlorococcus* and *Synechococcus* consumption by bacterivorous nanoflagellates. *Microb. Ecol.* 2002, 43, 341–352. [CrossRef]

16. Ayukai, T. Possible limitation of the dilution technique for estimating growth and grazing mortality rates of picoplanktonic cyanobacteria in oligotrophic tropical waters. *J. Exp. Mar. Bio. Ecol.* 1996, 198, 101–111. [CrossRef]

17. Liu, H.; Suzuki, K.; Saino, T. Phytoplankton growth and microzooplankton grazing in the subarctic Pacific Ocean and the Bering Sea during summer 1999. *Deep Sea Res. Part I Oceanogr. Res. Pap.* 2002, 49, 363–375. [CrossRef]

18. Umant, S.F.; Tirelli, V.; Beran, A.; Guardiani, B. Relationships between microzooplankton and mesozooplankton: Competition versus predation on natural assemblages of the Gulf of Trieste (northern Adriatic Sea). *J. Plankton Res.* 2005, 27, 973–986. [CrossRef]

19. Agis, M.; Granda, A.; Dolan, J.R. A cautionary note: Examples of possible microbial community dynamics in dilution grazing experiments. *J. Exp. Mar. Bio. Ecol.* 2007, 341, 176–183. [CrossRef]

20. Paterson, H.L.; Knott, B.; Koslow, A.J.; Waite, A.M. The grazing impact of microzooplankton off south west Western Australia: As measured by the dilution technique. *J. Plankton Res.* 2008, 30, 379–392. [CrossRef]

21. Pasulka, A.L.; Samo, T.J.; Landry, M.R. Grazer and viral impacts on microbial growth and mortality in the southern California Current Ecosystem. *J. Plankton Res.* 2015, 37, 320–336. [CrossRef]

22. Sooria, P.M.; Menon, N.N.; Ranith, R.; Nair, M.; Anjusha, A.; Shivaprasad, A.; Joseph, K.A. Occurrence of enhanced herbivory in the microbial food web of a tropical estuary during southwest monsoon. *Estuar. Coast. Shelf Sci.* 2020, 246, 107017. [CrossRef]

23. Suttle, C.A.; Chan, A.M. Dynamics and distribution of cyanophages and their effect on marine *Synechococcus* spp. *Appl. Environ. Microbiol.* 1994, 60, 3167–3174. [CrossRef]

24. Dolan, J.R.; Šimek, K. Diel periodicity in *Synechococcus* populations and grazing by heterotrophic nanoflagellates: Analysis of food vacuole contents. *Limnol. Oceanogr.* 1999, 44, 1565–1570. [CrossRef]

25. Suttle, C.A. Cyanophages and their role in the ecology of cyanobacteria. In *The Ecology of Cyanobacteria: Their Diversity in Time and Space*; Whitton, B.A., Potts, M., Eds.; Springer: Dordrecht, The Netherlands, 2002; pp. 563–589, ISBN 978-0-306-46855-1.

26. Tsai, A.-Y.; Gong, G.-C.; Sanders, R.W.; Chiang, K.-P.; Huang, J.-K.; Chan, Y.-F. Viral lysis and nanoflagellate grazing as factors controlling diel variations of *Synechococcus* spp. summer abundance in coastal waters of Taiwan. *Aquat. Microb. Ecol.* 2012, 66, 159–167. [CrossRef]