植物プランクトンの濁度式継続培養装置の構造とPhaeodactylum tricornutum及びSkeletonema costatumの光合成能の日周期性についての実験

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Turbidometric Continuous Culture of Phytoplankton

Constructions of the apparatus and experiments on the daily periodicity in photosynthetic activity of *Phaeodactylum tricornutum* and *Skeletonema costatum*.

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Abstract

A continuous culture apparatus was designed and built. The population density in the growth chamber was constantly monitored by a turbidometric system and was maintained at a given concentration by automatically diluting the culture with fresh medium. The temperature was automatically controlled within a range of 0-40°C and the illumination was selected at any desired level in a range of 2,000-90,000 lux.

Observations on the daily variations of chlorophyll, phaeopigments, particulate organic carbon, photosynthetic activity and volume of medium consumed during culture experiments using this apparatus for *Skeletonema costatum* and *Phaeodactylum tricornutum* showed that the turbidity of culture increased only in the light period and decreased in the dark period, and that the daily variations of chlorophyll and carbon content were significant particularly in the *Phaeodactylum* culture.

Introduction

It has been a common experience that the physiological and photosynthetic behaviors of algal cells depend within rather wide limits upon the previous history of the conditions in laboratory culture or natural environments. In the field of marine biology, however, only a few attempts have been made to relate the conditions of culture or natural environments to the subsequent type of cell behavior. The ordinary method of "batch culture" is handicapped in any of such investigations by its inability to eliminate the internal variation of conditions that occurs with time within any one culture. These studies usually reveal effects of nutrient levels and of physical factors on rates of growth of a culture. Such studies, however, are necessarily complicated by the uptake of nutrients from the medium, by the production of metabolites, and by the crowding effect. Myers and Clark (1944) have pointed out that as a...
culture of *Chlorococcal* matures, there occur marked variations in the culture conditions such as H\(^+\) and NO\(_3\) ion concentrations of the medium, carbon dioxide supply, and the effective intensity of light. All of them are reasonably considered to be functions of the population. Observations so made seem to be applicable to conditions occurring in nature only during the late stages of blooms.

If a growing culture could be continuously diluted so as to be maintained at one point on its growth curve, then the effects of changing internal conditions might be eliminated. The continuous culture technique offers this possibility. This would be able to afford at once, 1) a permanent source of experimental material of high uniformity and 2) a means to stabilize internal variables so that relations of culture conditions to cell physiology and to photosynthetic behavior might be systematically explored.

Paasche (1967) performed experiments using the semi-continuous culture of synchronised phytoplankton. Maddux and Jones (1964) constructed a continuous culture apparatus which was equipped with a turbidity monitoring system for a growing phytoplankton culture. The most profitable point of this continuous culture apparatus is that a photosynthetically growing culture can be automatically maintained at a given constant concentration irrespective of varying rate of reproduction of the culture. In a chemostat system, the cultured phytoplankton density would be constantly diluted irrespective of its rate of growth. Although the semi-continuous culture system using synchronised cells is useful for physiological as well as ecological experiments, a combination of the light intensity and its illumination time is necessarily fixed in order to keep the culture well synchronised. Anyway, the selection of a culturing system depends upon the purpose of the experiment.

The apparatus described here has been developed along the scheme presented by Maddux and Jones (1964), and a few modification have been made. The apparatus was considered to be particularly suitable for the study of daily periodicity in various activities of phytoplankton in terms of photosynthetic potential, pigment and organic carbon contents. Results of a few experiments on daily periodicity of two common phytoplankton species, *Phaeodactylum tricornutum* and *Skeletonema costatum*, are described below.

**Continuous culture apparatus**

The continuous culture apparatus used in the present experiments was of a turbidometric type which was a modification of that employed by Maddux and Jones (1964). Although the apparatus was already described in the author's master thesis (Graduate School of Fisheries Sciences, Hokkaido University, 1969), it has not been published; therefore a detailed description of the apparatus is given below.

Any culture of photosynthetic organisms that was growing in a flask under a controlled light intensity and water temperature was continuously monitored by a system of turbidity meter and a temperature control system. When the cell concentration in the flask increased, the turbidity signal was fed, after amplification, to a solenoid coil which magnetically lifted up a valve permitting the passage of an amount of new medium solution into the growth chamber and decreased the turbidity of the original culture down to a pre-determined level. When the system of turbidity meter recovered the balance, the solenoid valve was closed automatically. Thus the culture could be maintained at a constant turbidity for an indefinite period of time.
as far as the organisms kept growing under the experimental conditions. The schematic diagram of the whole assembly is shown in Fig. 1. The actual construction is shown in Fig. 2.

The growth chamber (Fig. 3) was a 2.0 l hardglass flask which had 3 necks: a temperature well, an air and medium inlet, and an outlet. The chamber was seated on the centre of an octagonal water bath which was made of clean acryl-resin and surrounded by four "cool white" fluorescent circular tubes. Each tube was lit up separately, and the light intensity as measured at the centre of the chamber was regulated in a wide range from 2,000 lux to 90,000 lux by the use of a special transformer (Light-Regulator, Tokyo Shibaura Electric Co.) which
controlled the light intensity alone without causing any change in color temperature of the light. The voltage supply for the tubes was incorporated with a bell timer (BM-205, Taihei
Co.) to provide any required periodicity of illumination with a 5-minute step interval. The monitoring beam of the turbidity meter, described below, could pass through a pair of parallel opposite faces of the octagonal bath. The water of the bath was circulated through a Coolnics (Komatsu-Yamato Co.) and a monitor thermometer was put in the bath. The variation in temperature as observed in the thermometer well of the chamber did not exceed 0.05°C from the set point of the monitor-thermometer. The medium reservoir was a 5l dark glass bottle set high above the flask. The medium was siphoned out down through a solenoid-actuated glass valve directly into an intermediate air chamber which was connected with the growth chamber by a bent and narrow tubing that ended with a fritted glass head immersed deep under the free surface of the culture medium. The air chamber was constantly supplied through a side neck with a flow of bombed air that was strained through a set of filters composed of cotton wool and a Whatman GF/C filter, and pressed down the added medium into the growth chamber through the fritted glass head. Equivalent amounts of culture escaped through the outlet hole. When the solenoid valve was closed, the culture was aerated by the air flow. The culture was constantly stirred by a rotor driven by a magnetic stirrer placed under the chamber.

The light source of the monitor turbidometric system was a 6V, 5A tungsten filament lamp. The light flux from the lamp was split into two beams through an optical splitter that was composed of symmetrically set mirrors and condenser lenses. One beam was collimated and passed into the water bath and the culture flask through the central part of the latter. The light path within the chamber was 17.5 cm long and 1.0 cm diam. The beam, after passing through the chamber and the octagonal bath, was converged by a condenser lens onto a Cad-

Fig. 4. Transmittance of Kodak Wratten Filters No. 29 and No. 46, relative intensity of fluorescent light source, and relative sensitivity of the Cds cell.
Fig 5. Electric circuit of the turbidometric monitor system. Two KΩ variable resistor VR is controlled to set a desired density of the culture.

A calibration of the working condition of the whole assembly was carried out using an aqueous copper sulphide solution as a test solution (Fig. 6). A filter of 7.6% transmission at
Fig. 6. Calibration curves of the turbidometric monitor system. Saturated copper sulphide solution in the growth chamber was serially diluted with distilled water in the medium container at each set point of VR, with and without the filters of specified transmittance.

720 mμ in wavelength was placed in front of the No. 2 CdS. A concentrated copper sulphide solution was added to the chamber flask and a set point of the adjustable resistor was selected for the highest concentration (Dial reading 10.0). The solenoid valve was automatically lifted and the distilled water contained in the medium reservoir flowed down into the chamber. When the concentration in the chamber arrived at the pre-determined value (10.0), the valve was closed, and an aliquot of the copper sulphide solution was taken from the chamber. The absorbance of the sample at 720 mμ was measured using a Hitachi 139 Spectrophotometer. The same procedure was repeated at eleven set points of the resistor for each of the other two filters set in front of the No. 2 CdS. The optical transmittance of these filters was 15.7 and 51.3% at 720 mμ, respectively. The results are shown in Fig. 6. It was found that with the use of these filters any desired optical density of the solution within a range of 0.01-0.20/cm could be obtained in the growth chamber for an indefinite period of time, simply by setting an appropriately selected reading point of the variable resistor.

Methods

The clone of Skeletonema costatum used was isolated from the water sample taken in Hakodate
Table 1. Composition of the medium used.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$SiO$_3$ (3 g/100 ml)</td>
<td>2 ml</td>
<td>Trace Metal Mix Solution</td>
</tr>
<tr>
<td>NH$_4$Cl (5.3 g/100 ml)</td>
<td>2 ml</td>
<td>ZnSO$_4$ (4.4 g)</td>
</tr>
<tr>
<td>NaNO$_3$ (15 g/100 ml)</td>
<td>2 ml</td>
<td>CuSO$_4$ (1.96 g)</td>
</tr>
<tr>
<td>NaN$_3$O$_3$ (1 g/100 ml)</td>
<td>2 ml</td>
<td>MnCl$_2$ (36.0 g)</td>
</tr>
<tr>
<td>Trace Metal Mix</td>
<td>2 ml</td>
<td>Na$_2$MoO$_4$ (1.26 g)</td>
</tr>
<tr>
<td>Natural Sea Water</td>
<td>5 l</td>
<td>CoCl$_2$ (2.0 g)</td>
</tr>
<tr>
<td>Pure Water</td>
<td>100 l</td>
<td></td>
</tr>
</tbody>
</table>

Bay. A pure culture of *Phaeodactylum tricornutum* was kindly supplied by Mr. O. Umebayashi, Tokai Regional Fisheries Research Laboratory. This was a clone sub-cultured from the original strain isolated in the Plymouth Marine Biological Laboratory. These clones were not bacteria free. The inocula were preconditioned by growing them at 17-22°C for a few weeks in a flask under a fluorescent light of 1,000 lux. The medium solution used was of the same composition as that used for the subsequent continuous culture. The composition was rather simple as shown in Table 1; four inorganic salts and a trace metal mix were added to natural sea water taken from Hakodate Bay. It was boiled for about half an hour and filtered through a Whatman GF/C glass fibre filter before use.

A few ml of the pre-conditioned culture were transferred to the growth chamber of the continuous culture apparatus which was filled with the medium solution and pre-set with temperature and light conditions but with the turbidity system switched off. The light intensity at the centre of the chamber in the light period and the water temperature were kept constant throughout each experiment; for *Skeletonema* 12,000 lux, 19°C and for *Phaeodactylum*, 39,000 lux, 18°C. The former combination was close to the optimum condition for *Skeletonema* as obtained by Curl and McLeod (1961), but the latter was more or less an arbitrary selection. In a week or so after the incubation, a visual examination showed that the cell concentration in the chamber was likely to be sufficiently high, and the turbidity monitor system was switched on with an appropriate setting of balancing point.

The culture density was immediately regulated to a constant value pre-set by the dial if the original density was high enough. For a further few days, the pre-conditioning of the culture was continued to ensure a regular growth taking place in the chamber under the given conditions. When the culture attained a regular growth as observed by the daily consumption of fresh medium solution, a series of successive sampling from the culture was started and continued for 52-58 hours with an interval of 2 hours. At each sampling time, a total of 50 ml of culture was sampled; a first 20 ml sample was used for particulate organic carbon analysis, a second 10 ml sample for chlorophyll and phaeopigments determination and last triplicate 5 ml samples for photosynthetic activity determination. In a later half period of the *Skeletonema* experiment (sampling No. 18 and hereafter), the volumes of the first and second had to be reduced to 10 ml and 5 ml, respectively, because of a decline of the growth rate of the culture that occurred at this final stage of the experiment.

Each sample for chlorophyll and phaeopigments was filtered through a Whatman GF/C filter 25 μm in diam. which, in advance, was covered by a thin layer of magnesium carbonate. The precipitate was used to determine chlorophyll-α and phaeopigments contents by the fluorometric
method (Yentsch and Menzel, 1963). The apparatus used was a fluorometry attachment incorporated with the Hitachi-Perkin-Elmer UV-VIS 139 Spectrophotometer. Each sample for organic carbon analysis was filtered through a pre-ignitioned (450°C, 30 min) Whatman GF/C filter 25 m/m in diam., and the precipitate was washed by a 3% NaCl solution. The filter pad was stored in a deep freezer at -20°C until later analysis. Particulate organic carbon was determined with a Hitachi 026 CHN Analyzer after drying the sample. All carbon values obtained were corrected by a common value of 14.8 µgC/sheet inclusive of “adsorption” and “filter” blanks (Nishizawa, 1969). The photosynthetic activity was measured by the 14C technique; 0.5 ml of Na214CO3 solution was added to each of the three 5 ml samples contained in 2 light test tubes and one dark tube, and these were incubated at a temperature of 20°C for 15 minutes under 1,200 lux for Skeletonema and 4,000 lux for Phaeodactylum. At every sampling time, the decrease in volume of the medium in the reservoir was read to every 10 ml to derive the volume of medium consumed during each successive sampling interval.

Results

The experiment for Phaeodactylum tricornutum was carried out on July 21-23, 1969 and obtained variations in consumption of new medium as well as photosynthetic activity, pigments concentration, and organic carbon concentration during the 58 hours are shown in Fig. 7.

During the experiment, the medium consumption occurred only in light periods. In dark periods, a slight decrease in turbidity usually occurred. Total daily consumption of medium was in a narrow range of 1.0-1.3 l with the average of 1.1 l during the period. This showed that the population performed an active growth with a fairly constant rate during the two and half days, and the average rate of division was about 0.5 per day or once every two days. It was noticeable that the growth rate has two distinct peaks in each light period, one in the early light period and the other in the late light period. In the middle of each light period, the growth rate decreased markedly although the midday decrease in the first light period was somewhat out of phase.

Photosynthetic activity as observed under a constant illumination (4,000 lux) varied quite differently from the actual growth rate. Most striking was a consistent increase in the photosynthetic activity in the dark period, although the rate of increase was much different between the two dark periods. The same activity decreased with time in the light period although this variation was fully observed only in the second light period.

The midday decrease in growth was slightly reflected in the observed variation in the photosynthetic activity of the second light period, but no adequate data were available in the first and third light periods.

The variation in photosynthetic activity seemed to be accompanied by a similar trend of variation in chlorophyll concentration. In particular, the chlorophyll content nearly doubled in the first dark period. However, in the second dark period the increase was only 20%, and the decrease in the next light period was not much significant. No significant correlation was found between chlorophyll and phaeopigments concentrations.

The variation in organic carbon concentration was most regularly periodic with a notable day-time increase and a similarly notable decrease in night-time. The increase was very rapid in the early light period. Thus the variation in carbon was nearly inverse to that of chloro-
Fig. 7. Daily variation of chlorophyll, phaeopigments, organic carbon, photosynthetic activity and medium consumed observed on *Phaeodactylum tricornutum* population cultured in the turbidometric continuous culture apparatus.

The experiment for *Skeletonema costatum* was done on Dec. 24-26, 1970 and the results are shown in Fig. 8.

In this series of culture experiment, the medium consumption in the second day was about 50% of that in the first day, and the photosynthetic activity was also much reduced in the second day. This reduction in growth rate was accompanied by a sudden increase in phaeopigments which nearly doubled in a few hours in the later stage of the second light period. The variation also did not show any significant periodicity. However, carbon progressively increased...
Fig. 8. Daily variation of chlorophyll, phaeopigments, organic carbon photosynthetic activity and medium consumed observed on Skeletonema costatum population cultured in the turbidometric continuous culture apparatus.

and chlorophyll decreased all through the entire period of the experiment, although the extents of variations were less than 10%.

In spite of various different trends of variation in these parameters between the two experiments, there remained one common feature that the photosynthetic activity showed a more
or less marked increase in dark periods which in the case of Skeletonema was nearly 100% in the first dark period.

Discussion

It is essential that the present experiments were carried out under the condition of constant “turbidity” which means the attenuation rate per unit distance of incident light (720 µ) that passes through the culture medium. A constancy of turbidity does not necessarily mean a constancy in cell concentration. Thus, the obtained rate of medium consumption is simply the rate of turbidity increase which only under the assumption of uniform cell morphology throughout the experiment can be interpreted as the rate of cell division or growth. The assumption is, of course unrealistic because the growth and division of a cell is necessarily concomitant with various changes in cell morphology. The discussion that follows is based on the assumption that the “average” cell morphology is constant at least in the short experimental period of about 50 hours in which the population grew and performed cell division at a rate of less than 0.5 a day.

The two experiments shown above indicated that the increase in culture turbidity occurred only in light periods. The increase in culture turbidity, however, may be caused either by cell growth or cell division. If the assumption just made is essentially correct, both growth and division should have occurred in light periods only. This means that the cultures were not fully synchronised, because the volume of fresh medium consumed in every light period was less than one half the volume of the incubation chamber (2013 ml). In the dark periods, the culture turbidity gradually decreased; the decrease was more pronounced in the culture of Skeletonema and the maximum decrease at the end of a dark period was about 10-15%. This decrease was not recovered in this culture in the early half of the next light period. Thus the next increase was confined in the later half of the light period. In the culture of Phaeodactylum, the turbidity decrease in the dark period was less pronounced and was recovered in less than one hour under illumination. The total net consumption of new medium in the Skeletonema culture was 200 ml in the first day and 100 ml in the second day. This culture seemed to be either in a late logarithmic phase or physiologically unhealthy.

There are some literature which indicate occurrence of a “midday depression of photosynthesis” on the terrestrial crops (e.g. Yabuki, 1963). Various causes have been ascribed to this phenomenon. Yabuki interpreted it in terms of the daily change on absorptivity of incoming radiation in the plant community. However, in the present experiment on Phaeodactylum, no such a daily change in illumination could occur. This is a phenomenon that is difficult to explain and seems to warrant a further study.

In spite of these several differences in culture condition, there appeared a trend that was apparently common to the two experiments; the potential photosynthetic activity of both cultures markedly increased through the dark period and attained the maximum at the end of the dark period or at the beginning of the next light period. The actual volume consumption of new medium in the Phaeodactylum culture was nearly parallel to the day-time potential as obtained by incubating an aliquot of culture for 15 minutes under the light one order of magnitude lower in intensity. A deviation in the early hours of the light period would be due to the slight decrease in turbidity which occurred in the previous dark period. In the Skeletonema culture.
the deviation was large and this would again be ascribed to the marked turbidity decrease in the dark mentioned above. It is not precisely known what has caused the dark time decrease in turbidity. Most probably it was due to the respiration loss during the dark period, although there is another possibility that it was the effect of cell division which perhaps occurred in some well grown cells.

The results obtained are not in good agreement with those reported in literature either of culture experiments (Honjo and Hanaoka, 1969; Palmer et al., 1964; Jørgensen, 1966) or of field observations (Doty, 1957, 1967; McAllister, 1963). Most of these suggested, more or less unanimously, that the potential photosynthetic activity rises in day-time and falls in night-time. The steady increase in potential photosynthetic activity during night obtained here would probably be a direct result of the turbidometrical control of culture density.

As for the variations in chlorophyll and carbon contents per unit volume, the first experiment (*Phaeodactylum*) showed fairly regular patterns of daily periodicity; chlorophyll increased in the dark and decreased in the light, while organic carbon decreased in the dark and increased in the light. The two patterns that were inverse to each other, however, were not well recognized in the second experiment (*Skeletonema*). Average carbon and chlorophyll contents in light and dark periods for the two cultures are tabulated in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Chlorophyll (µg/l)</th>
<th>Carbon (µgC/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phaeodactylum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>122</td>
<td>5.71</td>
</tr>
<tr>
<td>Dark</td>
<td>164</td>
<td>4.87</td>
</tr>
<tr>
<td>Light</td>
<td>106</td>
<td>5.46</td>
</tr>
<tr>
<td>Dark</td>
<td>123</td>
<td>4.62</td>
</tr>
<tr>
<td>Light</td>
<td>115</td>
<td>5.56</td>
</tr>
<tr>
<td><em>Skeletonema</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>30</td>
<td>2.97</td>
</tr>
<tr>
<td>Dark</td>
<td>30</td>
<td>3.08</td>
</tr>
<tr>
<td>Light</td>
<td>28</td>
<td>3.22</td>
</tr>
<tr>
<td>Dark</td>
<td>29</td>
<td>3.44</td>
</tr>
</tbody>
</table>

Shimada (1958), and Yentsch and Ryther (1957) obtained in field observations that the chlorophyll concentration in sea water increased in a dark period and decreased in a light period. However, McAllister (1963) observed two peaks (11h and 23h) of chlorophyll concentration in a day at an oceanic station in the north-east Pacific. For culture of *Skeletonema*, Honjo and Hanaoka (1969) and Jørgensen (1966) obtained a daily periodic variation in chlorophyll which was, however, inverse to that obtained in the present experiment for *Phaeodactylum*. Paasche (1967) found that the chlorophyll concentration in a synchronous culture of *Coccolithus huxleyi* increased linearly with time without regard to light or dark period. In this culture, the population divided only in the dark period. Paasche (1968) performed a similar experiment on *Ditylum brightwellii* and found a day-time increase in chlorophyll content per volume which was, however, accompanied by a parallel increase in cell number. All of these natural or cultured populations were not, of course, turbidometrically controlled in population density and are not
directly comparable with the present results. However, the daily rhythms in chlorophyll and carbon in the first experiment were significant and the amplitudes of variation were 10–20% of the total carbon and 10–45% of the total chlorophyll, respectively. Further, the calculated ratio of change in carbon/change in chlorophyll in each period was in a range 10–49. This is close to the average carbon/chlorophyll ratio of the population, 40. The culture of Skeletonema did not show any recognizable periodicity except for a general increase in carbon and a decrease in chlorophyll. The ratio of increase in carbon/decrease in chlorophyll in the incubation period excluding the final dark period in which the culture was extremely senescent was 120, and this is again close to the average carbon/chlorophyll ratio of the mother population, 100.

I am indebted to Associate Professor S. Nishizawa for sincere advice and criticism throughout this study.

References