The Journal of Shimonoseki University of Fisheries 38 (1) 23—70 (1989)

Studies on Chemical and Biological Factors Influencing the Growth of *Uroglena americana*, a Red Tide Chrysophyceae in Lake Biwa*

Bon Kimura

Contents

水産大学校研究業績 第1245号, 1989年7月13日受理.
Contribution from Shimonoseki University of Fisheries, No. 1245. Received July 13, 1989.

* Submitted to Faculty of Agriculture, Kyoto University, Kyoto, in the requirements for the degree of Doctor of Agriculture, 1987.

1 Introduction

A heavy bloom of *Uroglena americana* (Chrysophyceae) occurred from the end of May through the beginning of June, 1977, in the Northern Lake Biwa, which had been believed to be an oligotrophic lake (Yoshimura, 1976). Every year since, the freshwater red tide of *U. americana* occurred from late spring to early summer (Ishida and Kadota, 1983). Although serious incidence of fish kills usually does not occur during the algal bloom of *U. americana*, the dense growth of this alga blocks the filter of treatment plants of public water supply systems. A very offensive odor such as cod liver oil is also present, deteriorating the purity of drinking water or causing economic loss to the tourist industry of Shiga prefecture. Moreover, the fact that algal bloom characteristic of eutrophic water occurred in Lake Biwa caused anxiety to the residents that the lake has been changing consistently and rapidly by the inflow of human, industrial and agricultural waste waters during recent years.

U. americana belongs to Chrysophyceae and is a free swimming and colonial species where numerous individual ellipsoidal cells form a spherical colony by excreting a gelatious matrix around the cells (Fig. 1). Each cell (Fig. 2) is provided with two flagella of unequal length, an eye spot, and two golden-brown chromatophore (chlorophyll *a* and *c*). The cells are about 6—10 μm in length and the colonies range from 300 to 400 μm in diameter. *U. americana* was originally reported by Calkins (1892). Since then several studies of its morphology and taxonomy have been conducted (Casper, 1972; Hibberd, 1976; Wujek, 1976). The presence of *U. americana* in Lake Biwa was originally described by Kondo (1933), but no one had found the bloom of *U. americana* until 1976 (Yoshida *et al.*, 1983).

It is a common experience that axenic cultivation of Chrysophyceae in chemically defined media is difficult, and, thus, there is a serious lack of detailed knowledge about nutrition and physiology in this group, except for genus *Ochromonas* (Pringsheim, 1952; Hutner *et al.*, 1953; Aaronson and Baker, 1959). *U. americana* is not exceptional. Although structure and taxonomy of this alga have been well described using natural algal samples (Casper, 1972; Hibberd, 1976; Wujek, 1976), there is virtually no *in vitro* nutritional and physiological investigation concerning this species. To understand the mechanism related to the development of the bloom of *U. americana*, it is essential to have a basic knowledge of the growth physiology of this species.

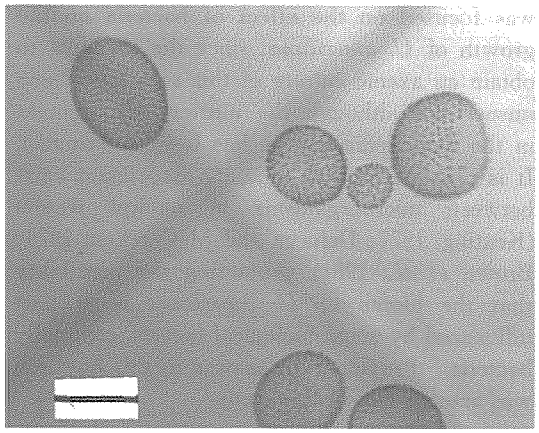

Fig. 1. Light micrograph of colonies of *U. americana*. The bar represents 200 μm.

Fig. 2. Vegetative cells of *U. americana*. The bar represents 2 μm.

The objective of this study was to elucidate chemical and biological factors influencing the growth of *U. americana* in order to understand the mechanisms of its growth.

To achieve the former purpose, the author attempted to use an algal assay method to clarify what nutrients are controlling the algal bloom in Lake Biwa. There is often a serious gap between the algal nutritional requirements obtained *in vitro* and the *in situ* chemical composition of nutrients in lake water (Skulberg, 1964; Chu, 1942). In this sense, the application of an algal assay will produce good results for the analysis of the mechanism of algal blooms.

To achieve the latter purpose, an attention was focused on the effect of bacteria on the growth of *U. americana*, since the attempts to obtain an axenic culture of this alga have been unsuccesful although the nutrient requirement in the monoxenic culture has been determined. It is possible that an intimate association exist between microorganisms in natural waters (Keating, 1977; Delucca and McCracken, 1976; Brown *et al.*, 1981), promoting the possibility that the bloom of *U. americana* is in part influenced by some biological factors, especially bacteria. Providing an answer to this question was another aim of this study.

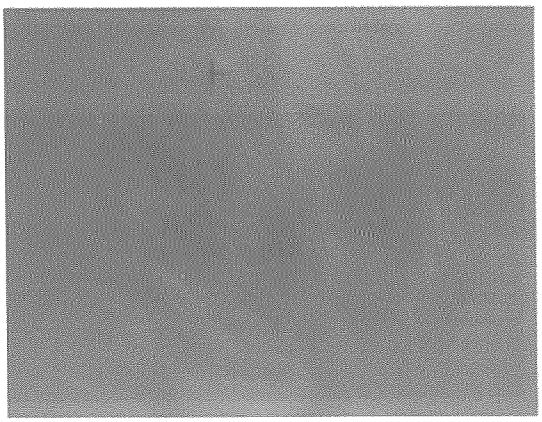

Fig. 3. The bloom of *U. americana* in Northern Lake Biwa in 1978 (Photograph courtesy of Shiga Prefecture).

2 Analysis of major nutrients influencing *U. americana* bloom by use of algal assay

2.1 Introduction

The common AAP (Algal Assay Procedure bottle test) is based on Liebigh's Law of the Minimum where the biomass produced during a given time is related to the available concentration of the limiting nutrient. It has received widespread acceptance for assessing the potential fertility of waters (Smayda, 1970; Skulberg, 1970; Nakamoto, 1977). In its simplest form, algal assay consists of the addition of a given algal species, so-called test alga, to a series of water samples which are then exposed to constant conditions of light and temperature over a given time. The growth of the test alga in the water samples indicates the relative potential fertility of the water. However, few attempts have been made to employ the algal assay method using natural phytoplankton isolates for the test organisms to determine the limiting nutrients that control *in situ* algal growth (Ryther and Guillard, 1959; Glooschenko and Curl, 1971; Frey and Small, 1980). This chapter describes an algal assay that used a monoxenic culture of *U. americana* isolated from the 1978-bloom to identify growth-limiting nutrients for bloom alga in Northern Lake Biwa.

Algal assay of this type is a point of controversy from a methodological viewpoint. Few researchers have examined the potential role of nutrients during bloom by algal assay using bloom algae (Colllier *et al.*, 1969; Hirayama *et al.*, 1972; Lund *et al.*, 1975; Hitchcock and Smayda, 1977). They differ in the methods used for treating the water samples. Hitchcock and Smayda (1977), Collier *et al.* (1969), and Hirayama *et al.* (1972) used water samples filtered through glass fiber filters and frozen before use. Lund *et al.* (1975) also filtered the water through glass fiber filters; however, without freezing. In both of these

samples, bacteria were not removed. It would be expected that treatments such as freezing or incomplete elimination of bacteria from water (e. g. GF/C filtration) would produce undesirable changes in the water samples before and during the test.

Therefore, in this study, algal assay was conducted on water samples aseptically filtered immediately after collection using a sterilized 0.22 μm membrane filter. *U. americana* (monoxenic culture) was then inoculated.

2.2 Materials and methods

2.2.1 Isolation and cultivation of *U. americana*

U. americana strain 78 was isolated in 1978 from Lake Biwa. The monoxenic culture of *U. americana*, which was accomanied by a bacterial strain NA-1 (*Vibrio-Aeromonas* sp.), was obtained by washing the algal colonies 10 to 15 times in petri-dishes containing 0.5 m*l* of sterilized Ur-1 medium using capillary pipettes. Stock cultures of *U. americana* were maintained in autoclaved paper-plugged (New Steri-Plug; W. Germany) 100 m*l* Erlenmeyer's flasks containing 40 m*l* of Ur-2 medium and transferred at intervals of every 2 weeks. The composition of Ur-2 medium is as follows (per liter): NH_4NO_3 5 mg, Na_2-glycerophosphate 2.0 mg, $MgSO_4 \cdot 7H_2O$ 10 mg, $CaCl_2 \cdot 2H_2O$ 10 mg, KCl 1mg, vitamin B_1 10 μg, vitamin B_{12} 0.1 μg, biotin 0.1 μg, Provasoli's PIV metal solution (Provasoli and Pintner, 1960) ($FeCl_3 \cdot 6H_2O$ 19. 6 mg, $MnCl_2 \cdot 4H_2O$ 3.6 mg, $ZnCl_2$ 1.05 mg, $CoCl_2 \cdot 6H_2O$ 0.4 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.25 mg, and Na_2-EDTA 100 mg in 100 m*l*) 1 m*l* ; pH 7.0.

2.2.2 Sampling station and water samples

Sampling stations in Northen Lake Biwa are shown in Fig. 4. Water samples were collected with a Van Dorn sampler (5 liters) from 3 m depth at station A from January 1980 through June 1981; from 3 m depth at station C and from 10 m depth at station A during April-December 1980; and from 3 m depth of station N4 during April-September 1980. The water samples collected were maintained in a sterilized 500 m*l* Pyrex glass bottle (precombusted for 1 h at 450 ℃) on ice. The bottles were brought back to the laboratory within 3 h and treated according to the following procedure.

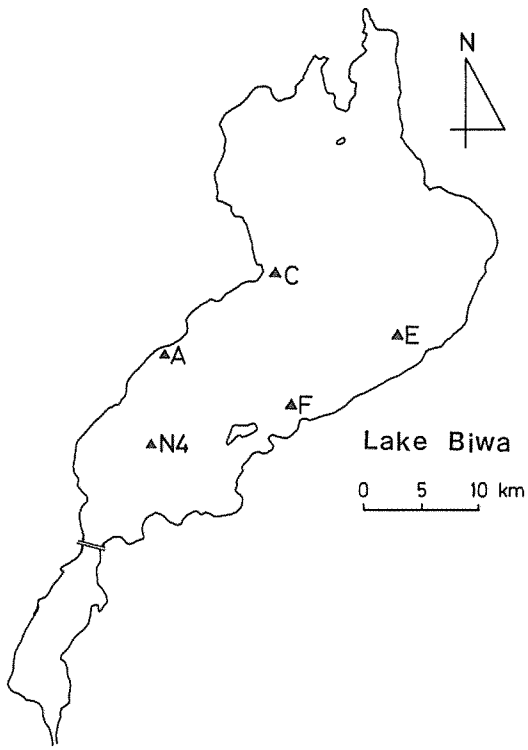

Fig. 4. Location of sampling stations of Lake Biwa.

2.2.3 Procedure of the algal bioassay

Procedure of algal bioassay was described in Fig. 5.

Process-1: The water samples were aseptically filtered through a sterilized 0.22 μm membrane filter (Millipore filter GS).

Process-2: Four m*l* aliquots of the filter-sterilized water samples were transferred to prerinsed and precombusted glass tubes (13 × 105 mm) sealed with polypropylene caps. Prior

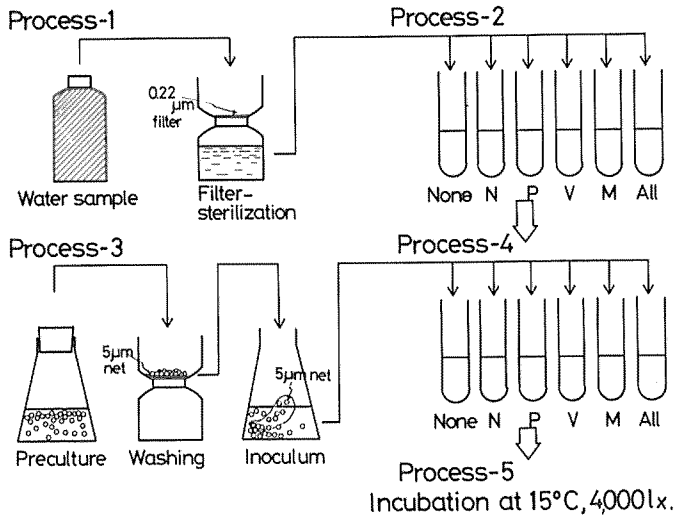

Fig. 5. Diagram of experimental procedure of the algal bioassay. None; no addition, N; NH_4NO_3 added, P: glycerophosphate added, V; vitamin B_1 + vitamin B_{12} + biotin added, M; PIV metal added, All; N + P + V + M added.

to the transfer the tubes were enriched using the following six procedures (at final concentration) and then autoclaved: None; no nutrient added, N; ammonium nitrate (1.75 mgN/*l*), P; Na_2-glycerophosphate (0.42 mg P/*l*), V; vitamin mixture (vitamin B_1 10, vitamin B_{12} 0.1 and biotin 0.1 μg/*l*), M; PIV metal mixture (EDTA-Na 1.0, Fe (as $FeCl_3$) 0.04, Mn (as $MnCl_2$) 0.01, Zn (as $ZnCl_2$) 0.005, Co (as $CoCl_2$) 0.001, Mo (as Ma_2MoO_4) 0.005 mg/*l*), All; all four components added.

Process-3: An exponentially growing culture of *U. americana* in Ur-2 medium was washed in a sterilized 5 μm Nylon net (Nytal, Switzerland) with unenriched filtered lake water to eliminate carry-over of the culture medium, and was resuspended in filter-sterilized lake water. The population density of the algal suspension was approximately 30 colonies per m*l*.

Process-4: Four m*l* of unenriched and enriched tubes (in triplicate) were inoculated with 0.2 m*l* of the algal suspension.

Process-5: The tubes were incubated at 15 ℃, and 4000 lx on a 14L: 10D photoperiod cycle.

Growth in the tubes was determined by measuring the concentration of chlorophyll *a* every two days with a modified Turner fluorophotometer (110 type) equipped with a photomultiplier RCA−R136 and a Corning 2−64 filter (765 nm). Growth in unenriched water (none) was estimated as an average of three of Turner readings in the stationary phase. Growth in the enriched cultures was expressed as a difference in Turner readings (in triplicates) between an unenriched culture and an enriched culture in the stationary phase.

It would be very convenient to do bioassay experiments if filtered lake water sterilized by

freezing or autoclaving could be stored for long periods without undesirable changes in quality. However, the bioassay values of a water sample frozen or autoclaved after 0.22 μm filtration were lower than than those of water samples treated with a 0.22 μm sterile filtration technique (Fig. 6). Therefore, in this study, the filter-sterilized waters were assayed on the day of collection as soon as possible.

The amount of chlorophyll *a* was determined by the Lorenzen method (Lorenzen, 1967).

2.3 Results

2.3.1 Bioassay for unenriched water samples

Mean growth yields of *U. americana* in an unenriched filtered sample of lake water collected from station A at 3 m depth are shown in Fig. 7. Algal growth exceeding a Turner reading of 20 (0.3 μg chl. *a*/m*l*), corresponnding to a value approximately twice as high as initial concentrations of *U. americana*, was regarded as effective growth.

In the period from January 1980 through June 1981, the bioassay in unenriched water gave effective growth for five experiments (March, early June, and November of 1980; and, January and early June of 1981), among which two were just after the bloom (early June). The *in situ* concentrations of DIN (dissolved inorganic nitrogen), DIP (dissolved inorganic phosphorus), filtered vitamin B_{12} and filtered iron for the two in early June were not significantly different from those of each component at other times; DIN 3 − 17 μg atoms/*l*, DIP 0.01 − 0.14 μg atoms/*l*, vitamin B_{12} 0.1 − 3.5 ng/*l* and iron 90 − 170 μg/*l* (data of B_{12} and iron were provided by A. Kurata, personal communication).

Results were also similar at stations E and F, where the inflow of a nutrient would be larger than that at stations A, C, and N4 (Fig. 8).

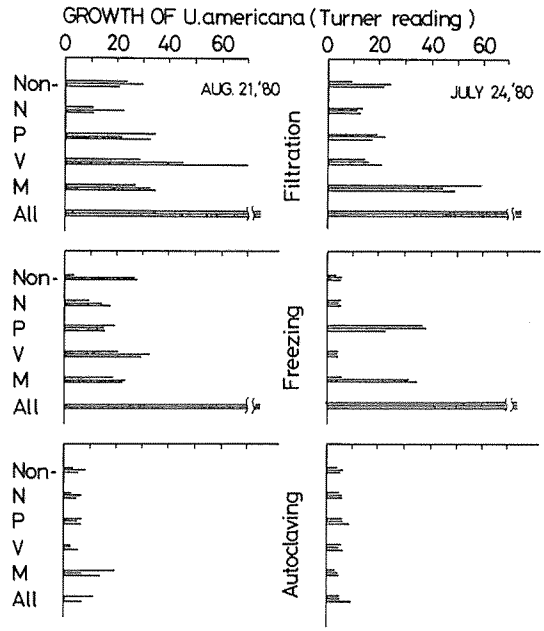

Fig. 6. Algal bioassay of water samples frozen or autoclaved after 0.22 μm filtration. Filtration; 0.22 μ m sterilized water samples, Freezing; water sample frozen after 0.22 μ m filtration, Autoclaving; water sample autoclaved after 0.22 μm filtration.

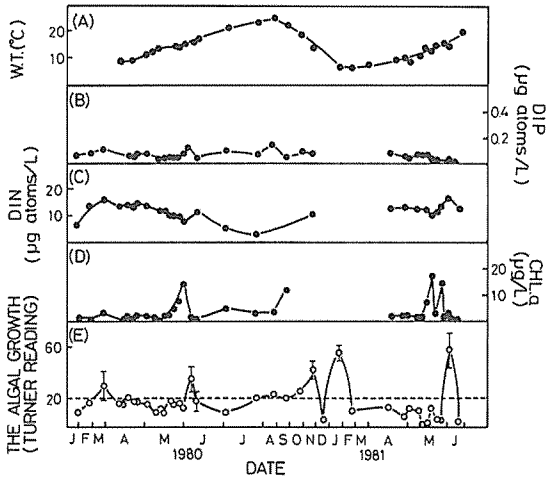

Fig. 7. Seasonal changes in water temparature (A), DIP (B), DIN (C), the amounts of chlorophyll *a* (D), and the algal bioassay values (E) for unenriched filtered lake waters collected from station A at a depth of 3 m in Lake Biwa, 1980—1981.

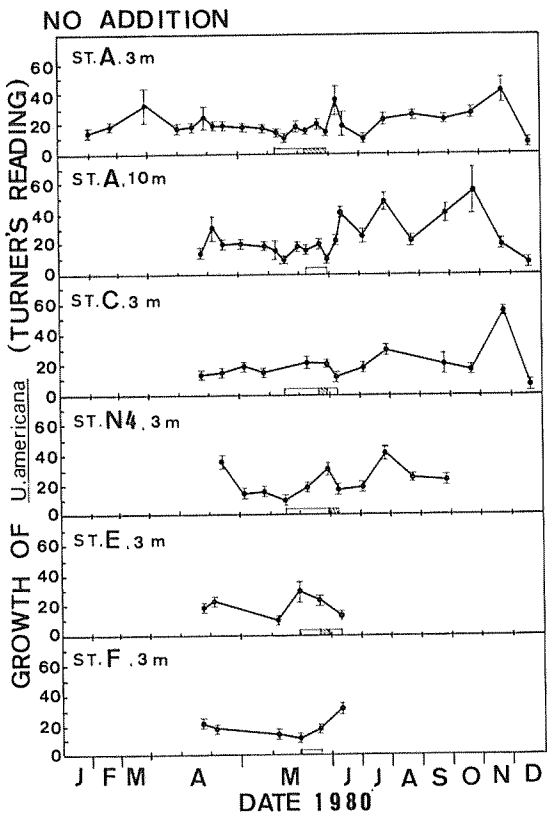

Fig. 8. Horizontal profiles of the algal bioassay values for unenriched filtered water samples at each station in Lake Biwa.

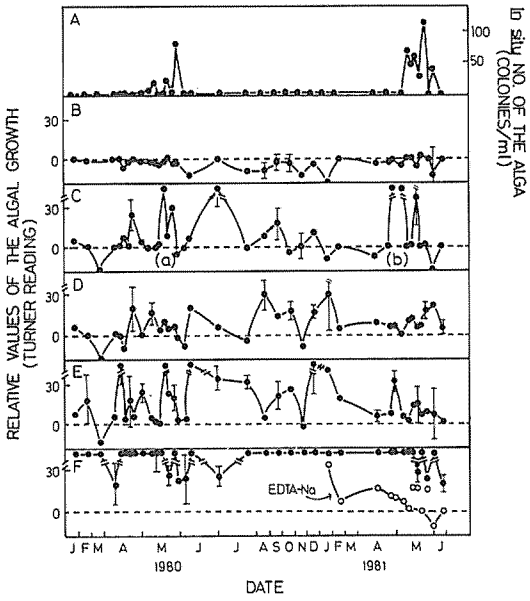

Fig. 9. Seasonal changes in the colony number of *U. americana* (A) and in the algal bioassay value with the addition of nitrogen (B), phosphorus (C), vitamins (D), metals (E), and all four components (F) in the filtered lake waters collected at station A at a depth of 3 m.

2.3.2 Effectiveness of the addition of each component to unenriched filtered lake water

Growth of *U. americana* population in each enriched medium was illustrated as a difference in a Turner reading between an unenriched culture and an enriched culture. Therefore, a positive result obtained in an enriched culture shows the effectiveness of addition of each component on *U. americana* growth. Results for enriched samples of lake water at station A at a depth of 3 m is shown in Fig. 9. With the addition of ammonium nitrate there was no significant stimulation of growth in the bioassay during 1980－1981 (Fig. 9－B). When Na_2－glycerophosphate was added, positive results were obtained during the second half of May 1980 (a) and early May 1981 (b), just before *U. americana* was abundant at station A (Fig. 9－C). Increased growth was also observed during in late April, early July, late September and late December of 1980. Effective growth of *U. americana* by phosphate addition was not observed during the spring of both years (January－early April) (Fig. 9－D).

Generally, the addition of the metal mixture improved growth more than that of the other component alone (Fig. 9－E). During the large bloom of *U. americana* in Lake Biwa during 1980 and 1981, however, positive results were weak, although the concentration of iron in the filtered lake water (100 to 150 $\mu g/l$) during this period was not higher than that in the other

period. The experiment carried out with addition of Na_2-EDTA alone gave a result similar to that with the metal mixture (Fig. 9—F).

When vitamin mixture was added, positive results were sporadically obtained. The improvement in growth did not correspond with a fluctuation in the concentration of vitamin B_{12} essential for growth of *U. americana*, which began to fall on March, reached low levels (1 to less than 0. 2 ng B_{12}/l) in March — June and was high from the end of July to December (Fig. 9—D).

The results obtained for bioassay of water samples from 3 m depth of station A with the addition of nitrogen, phosphorus, vitamins, or metals alone was similar to the results for water samples from 10 m depth of station A, and 3 m depth of stations N4, C, E, and F (Fig. 10—A, —B, —C, and —D).

Addition of all four components (complete enrichment) to unenriched filtered lake water at station A generally gave high growth, but weak positive results were observed on several occasions, especially in large blooms (late May 1980, and late May, early June 1981) (Fig. 9—

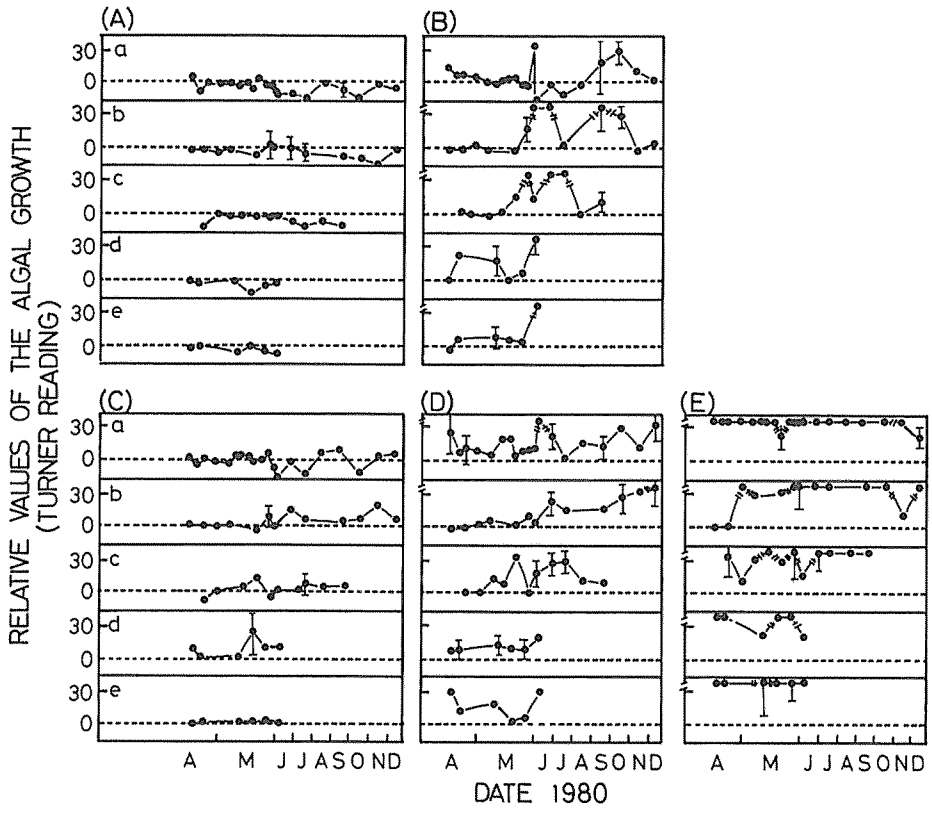

Fig. 10. Seasonal changes in the algal bioassay values with the addition of nitrogen (A), phosphorus (B), vitamins (C), metals (D), and all four components (E) to unenriched lake water at 10 m depth of station A (a), and 3 m depth of station C (b), N4 (c), E (d), and F (e).

F). In most cases strong positive results were obtained at the other stations with the exception of station C in middle April 1980. In this case the water of the Ado river which flows into Lake Biwa may have contained a growth inhibitor.

2.3.3 Bioassy with the addition of two or three components

As shown in Fig. 11 the addition of both components (phosphorus + vitamins, phosphorus + metals, and vitamins + metals) commonly gave better growth than either component alone. The growth was greater with the three components (phosphorous + vitamins + metals) rather than with two components during the period from October 1980 to June 1981, except on April 16, 1981.

2.4 Discussion

Laboratory results show that the concentrations of nutrients essential for minimum growth of *U. americana* can be defined as follows: 14－55 μg N/l as NH_4^+ or NO_3^-, 10 μg P/l as PO_4^-, 40 μg Fe/l as Fe－EDTA, and 10 ng B_{12}/l as cyanocobalamin. When comparing these values with the *in situ* concentration of dissolved inorganic phosphate, dissolved inorganic nitrogen (NO_3^- + NO_2^- + NH_4^+), filtered vitamin B_{12} and filtered iron in the lake water, it is obvious that the lake water

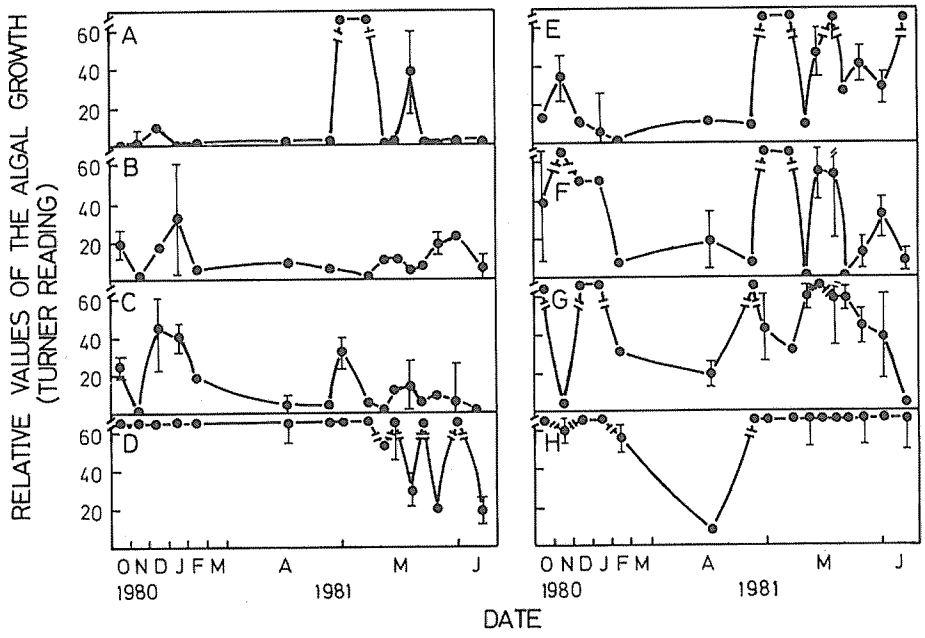

Fig. 11. Comparison of the algal bioassay values of the lake waters added with two or three components and those added with either component alone. Water samples were collected at 3 m depth of station A.
A); phosphorus, B); vitamins, C); metals, D); all four components, E); phosphorus + vitamins, F); phosphorus, + metals, G); vitamins + metals, and H); phosphorus + vitamins + metals.

contains a sufficient quantity of nitrogen to support minimum growth for bloom occurrence (final cell yield corresponds to 6 — 10 μg chl. *a* per liter of water). From algal bioassay results, however, it seems that the concentrations of phosphorus (less than 3 $\mu g/l$), vitamin B_{12} (less than 1 $\mu g/l$), and iron (less than 200 $\mu g/l$) contained in the lake water are not sufficient for the growth at least during late April-early June (bloom period). The amount of iron that is likely to be in the soluble state is negligible in the aerobic condition. This fact nearly corresponds with results obtained from the bioassay experiments that phosphorus, vitamin B_{12} and soluble iron are generally the major limiting factors in Northern Lake Biwa. More detailed observations show that phosphorus is the major limiting factor in the time period just before the large bloom of *U. americana* occurs (Fig. 9 — A and 9 — B). From the amount of intracellular phosphorus (2 μg per chlorophyll *a* μg of *U. americana*) determined from laboratory experiments, the concentration of phosphorus in the lake water necessary for the bloom was estimated roughly as 12 — 20 μg P/*l*. This concentration is about 10 times higher than dissolved inorganic phosphorus in the lake water. As this period is on stratification, it is possible that land drainage is major source of dissolved phosphorus. Of course the possibility of iron as the major limiting factor, in addition to the phosphate, must also be considered during this period. The concentration of filtered iron (undissolved iron) in the lake water during April — November 1980 ranged from 100 to 150 μg Fe per liter, and, from laboratory data, these values are sufficient for *U. americana* growth. Bioassay experiments show, however, that iron is one of the major limiting factors. This inconsistency can be explained if it is assumed that the large amount of iron is in an oxidized, undissolved state in the lake water. The fact that the addition of EDTA — Na in the algal assay generally gave similar results to that of EDTA — Fe (as PIV metal mixture) would support the idea that the concentration of iron utilizable for *U. americana* in the lake is negligible.

From the bioassay it appears that vitamin B_{12} was also a major limiting factor, at least during April — June and August — January, but not during February — middle April in 1980 and 1981, although the *in situ* concentration of vitamin B_{12} was less than 3.5 ng/*l* and was apparently insufficient for the minimum growth of *U. americana*. It is possible that the bacterial strain NA-1 contaminated in the *U. americana* culture used as an inoculum may produce vitamin B_{12}.

In general, phosphorus is a major limiting factor for algae in oligotrophic fresh water (Schindler, 1978; Richey, 1979). According to Yoshimura's definition (Yoshimura, 1976), Northern Lake Biwa is still considered in the category of oligotroph with respect to the *in situ* concentration of total phosphorus (less than 20 $\mu g/l$). Nevertheless, why did the bloom of *U. americana* suddenly occur in 1977? At present it is not possible to provide an accurate answer to the question. It is probable that phosphorus, soluble iron, and vitamin B_{12} were the major limiting chemical factors of algal growth even before 1977, when the algal bloom began in the lake. One speculation is that with a gradual increase of the load of phosphorus and organic substances from the catchement area the water quality of the lake reached a critical point and initiated the bloom of *U. americana* in 1977. On the other hand, another possibility is that some other factors may be involved in the mechanism initiating the bloom. In particular, the bacterial effect which may produce organic chelators, vitamin B_{12}, and other substances is of interest. This possibility is investigated in Chapter 3.

2.5 Summary

In an attempt to discover the major nutrients controlling *U. americana* bloom in Northern Lake Biwa, an algal bioassay was conducted using a monoxenic culture of *U. americana* isolated from the bloom. From the

bioassay with or without the addition of nitrogen, phosphorus, iron and/or vitamins to unenriched lake water, it was discovered that phosphorus and iron were the major limiting nutrients for the algal bloom, and that the lake water contained a sufficient quantity of nitrogen (Ishida *et al*., 1982).

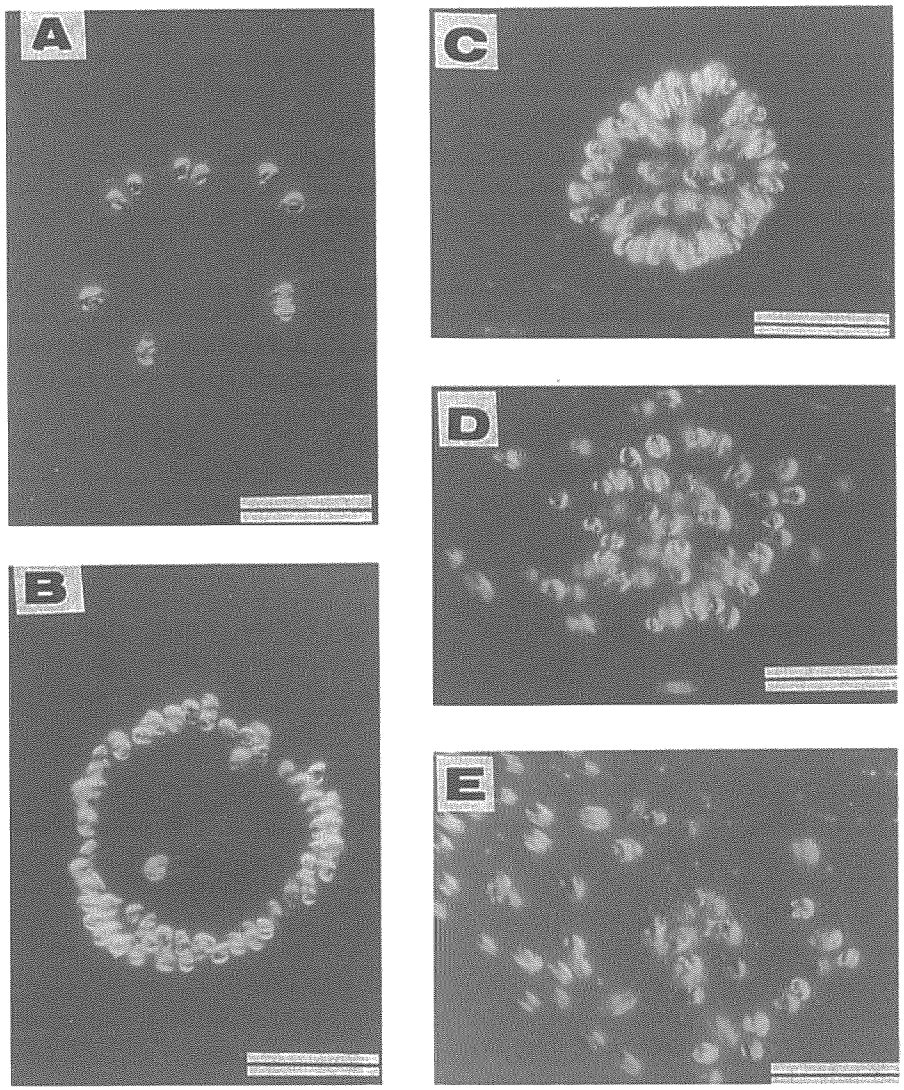

Fig. 12. Colonies of *U. americana* in mixed cultures with a bacterial strain NA-1. Bacterial attachment to the extracellular matrix surrouding the colonies is shown. A; an expanded 8-celled stage colony, B; an expanded 64-celled stage colony, C-E; the process of artifitial degradation of a packed 64-celled colony. Bacterial presence is increasingly evident in the inner portion of the colony. Scale bars represent 30 μm.

3 Effect of naturally collected bacteria on the growth of *U. americana*

3.1 Introduction

The culture of *U. americana* used in algal assay experiments described in Chapter 2 was not axenic, but monoxenic accompanied by a strain of bacteria NA-1 (Fig. 12). The alga, originally isolated from Lake Biwa during its bloom, was successfully grown in a bacterialized unialgal cultlure using a chemically defined medium Ur-1 (Table 1), but failed to grow under bacteria-free conditions.

This chapter describes the algal growth which is dependent on the presence of an associated bacteria in a laboratory culture. This chapter also describes the effect on algal growth of bacteria collected from Lake Biwa during the algal bloom in order to analyze some roles of bacteria for the development of the bloom.

3.2 Materials and methods

3.2.1 Culture of *U. americana*

The monoxenic culture of *U. americana* strain 78 used in this experiment was accompanied by an erythromycin-sensitive bacterium strain NA-1 (*Vibrio-Aeromonas*). Stock and experimental cultures of *U. americana* were grown in Ur-1 medium whose composition is the same as Ur-2 medium described in Chapter 2, except that glycerophosphate was replaced with 1.7 mg/*l* of NaH_2PO_4 (Table 1). NaH_2PO_4 gave the same growth of the alga as glycerophosphate. To prepare a temporaty axenic culture of *U. americana*, 1.5 μg/*l* of erythromycin was added to the culture.

3.2.2 Growth conditions and measurement of *U. americana*

For all growth experiments, 0.1 m*l* of the subcultures incubated for one week was inoculated into triplicate sterile Pyrex test-tubes plugged with P. P caps (Eiken Co.) containing 4 m*l* of Ur-1 medium. Conditions for incubation were as follows: 15 ℃ and 60000 lx cool white or daylight fluorescent light on a 14L: 10D photoperiod cycle. The growth of the cultures was monitored by measuring *in vivo* chlorophyll fluorescence of a 4 m*l* sample tube. Measurements were made with a Turner Model 110 Fluorometer, modified for chlorophyll analysis as described in Chapter 2.

Table 1. A compositon of defined medium (Ur-1 mesium) for the multiplication of monoxenic *U. americana*

$MgSO_4 \cdot 7H_2O$	10 mg
$CaCl_2 \cdot 2H_2O$	10 mg
KCl	1 mg
NH_4NO_3	5 mg
$NaH_2PO_4 \cdot 2H_2O$	2 mg
Fe-EDTA	0.5mg
PIV metal soln.	1 m*l*
Vitamin B_{12}	0.1 μg
Biotin	0.1 μg
Thiamin HCl	10 μg
Distilled water to	1000 m*l*
pH	7.5

3.2.3 Experimental procedure for bacterial effect on the growth of *U. americana*

(1) Preparation of a temporary axenic cultue of *U. americana.*

To prepare axenic *U. americana* culture, 1.5 μg/m*l* of filter-sterilized erythromycin was added to Ur-1 medium. A bacterial strain NA-1 associated with *U. americana* did not grow in the presence of more than 0.19 μg (per m*l*) of erythromycin (Table 2). After inoculating the alga, viability of a bacterial strain NA−1 was examined by use of $LT10^{-1}$ medium (Ishida *et al.*, 1980) containing 0.5 g of trypticase peptone (BBL), 0.05 g of yeast extract (Difco), and 1 liter of filtered aged lake water.

(2) Stability test of antibiotic in the algal culture.

Stability of the antibiotic (erythromycin) in the culture was assayed by the agar diffusion method. *Bacillus subtillus* (erythromycin-sensitive) as a test organism was spread on agar plates and metal cups containing samples were placed on the surface. After 24 h of incubation at 30 ℃, the clear zones on the plates were measured.

(3) A cullture system for bacterial effect on the growth of *U. americana*

To examine bacterial effects on the algal growth, 177 bacterial strains were isolated from Lake Biwa during April 16 to June 18, 1981, and among them 68 erythromycin-resistant strains were selected for an experiment employing a temporary axenic culture treated with erythromycin.

However, testing of erythromycin-sensitive bacteria is limited in this system. Therefore, a new monoxenic culture system was prepared in which the bacterial strain NA-1 associated with *U. americana* in the stock culture was replaced by a much slower growing bacterium 2B-11. To this culture system, each of 24 strains of bacteria isolated from Lake Biwa on Apr. 27; May 9, 13 and 25; and, Jun. 23 (1983) were inoculated, and the algal growth was assayed after days of incubation.

(4) The effect of sterilized and nonsterilized lake waters on the growth of *U. americana.*

Lake water samples were collected from Lake Biwa during the period from the initial phase (Apr. 27, 1983) to the final phase (Jun. 23, 1983) of the algal bloom. The samples were filtered through a combusted galss fiber filter immediately after sampling the water. A portion of the filtrate was sterilized by autoclaving at 121 ℃ for 15 min. For the growth response test, 0.1 m*l* of either sample was added to the test tubes containing 4 m*l* of Ur-1 medium. The cultlures were incubated for 14 days under light.

(5) *In situ* population of *U. americana* and bacteria

Population density of *U. americana in situ* was determined by counting the colony number in 0.5 m*l* of each water sample using a

Table 2. Growth response of bacteria to erythromycin

Bacterial strain	Growth response of bacteria*							
	Concentration of erythromycin (μg per m*l*)							
	12.0	6.0	3.0	1.5	0.75	0.38	0.19	0
NA-1	−	−	−	−	−	−	−	+
NbA-1	+	+	+	+	+	+	+	+

* +; growth, −; no growth

hemocytometer immediately after sampling the water.

The number of heterotrophic bacteria in the sample was counted after three weeks of incubation at 20 ℃ by the spread plate method with LT 10^{-1} medium.

3.3 Results

3.3.1 Growth response of *U. americana* to bacteria in the presence of erythromycin

Chemically defined medium Ur-1 (Table 1) supported good growth of the monoxenic culture of *U. americana* which was accompanied by an erythromycin-sensitive bacterial strain NA-1 (Fig. 13). However, after being made axenic by the addition of erythromycin, the alga failed to grow (Fig. 13). The growth in the erythromycin-added culture recovered when inoculated with an erythromycin-resistant bacterial strain NbA-1 (Fig. 13). The erythromycin-stability test employing the agar diffusion method demonstrated that erythromycin was not decomposed by the bacteria in this culture during the course of the experiments (Fig. 14). These results present evidence that the presence of bacteria is an essential factor for the growth of *U. americana*.

Sixty-six strains among 68 erythromycin-resistant bacteria isolatead from Lake Biwa in 1981 supported algal growth when examined in a similar system with that shown in Fig. 13. The extent of the algal growth varied, depending on the species of the associated bacteria. Figure 15 shows two typical examples. The growth of *U. americana* in a mixed culture with bacterial strain 3A-3 showed a distinct increase over 7—10 days, whereas the alga in a mixed culture with bacterial strain 2B-11 increased very slowly over a period of 40 days and subsequently reached only 40—60 % of the yield of the cultures containing bacterial strain 3A-3. During the course of experiments the number of bacterial strain 2B-11 remained remarkably low as compared with that of bacterial strain 3A-3 (Fig. 15). Subsequent transfer of these cultures yielded virtually identical results. The growth of *U. americana* accompanied by bacterium 2B-11 was enhanced by additionally inoculating a bacterium 3A-3 to the culture (Fig. 16). The growth of *U. americana* accompanied by bacterium 2B-11 was also greatly enhanced by adding a small amount of peptone to promote the bacterial growth (Fig. 17-B), or by making the initial number of bacteria higher (ca. 1×10^6 oer m*l*) (Fig. 17-C).

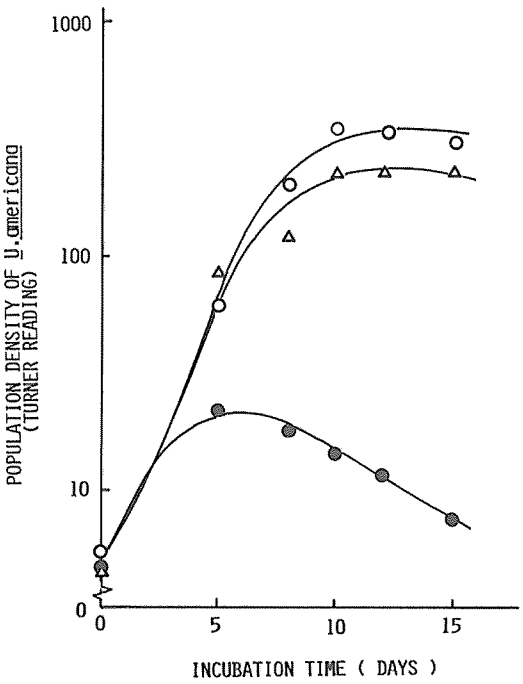

Fig. 13. Growth of *U. americana* in the presence and absence of associated bacteria. *U. americana* culture accompanied by an erythromycin-sensitive bacterium NA-1 (○) was added with 1.5 μg/m*l* of erythromycin (●) or erythromycin-resistant bacterium NbA-1 (△).

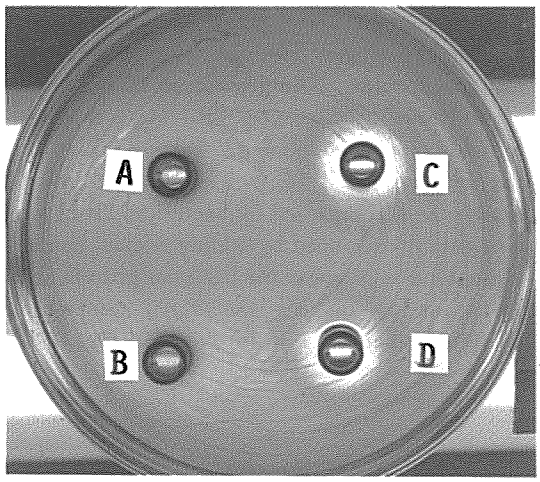

Fig. 14. Stability of erythromycin added to *U. americana* clulture after 12 days of incubation. *Bacillus subtillis* as a test organism was spread on an agar plate, and metal cups placed in the agar were filled with the algal cultures which had been incubated for 12 days with no addition (A), with erythromycin-resistant bacterium NbA-1 (B), with 1.5 μg/m*l* of erythromycin (C), and with 1.5 μg/m*l* erythromycin plus the bacterium NbA-1 (D). Note the same inhibition zone size in (D) as in (C).

3.3.2 The effect on algal growth of various bacterial strains isolated from Lake Biwa during the bloom (1983)

To examine the effects of various bacterial strains on algal growth, it is disadvantageous to employ the temporary axenic culture with erythromycin as an assay system, because erythromycin-sensitive bacteria can not be tested in this system. One hundred and twenty stains of bacteria isolated from Lake Biwa during the period from the initial phase to the final phase of the bloom of *U. americana* (1983) were, therefore, each inoculated to the algal culture accompanied by a slow growing bacterium 2B-11 without erythromycin. The algal growth was then assayed. Results are summarized in Table 3. All bacteria except one enhanced the growth of *U. americana*. In preliminary experiments, several bacteria inhibited algal growth. These inhibitory effects appeared mostly due to excessive growth of bacteria, but, making inoculum size lower, most of these inhibitory effects were eliminated.

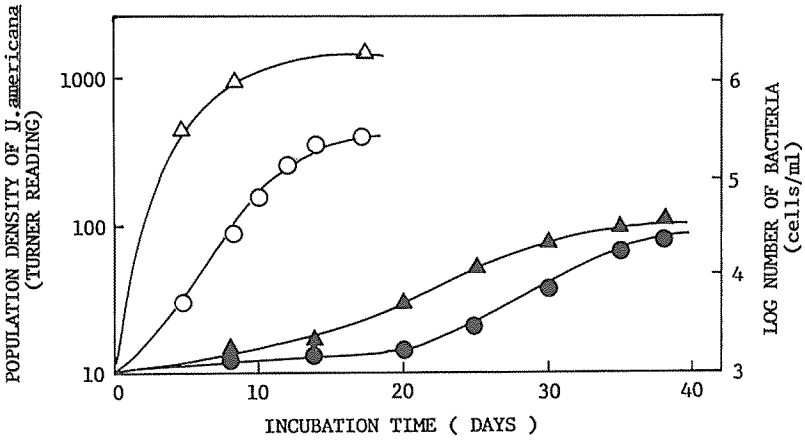

Fig. 15. Growth of *U. americana* and erythromycin-resistant bacteria in mixed cultures. Conditions for inoculum and incubation were the same as described in the legend of Fig. 1. Symbols: ○; *U. americana* with a rapid growing bacterium 3A-3, △; the bacterium 3A-3, with *U. americana*, ●; *U. americana* with a slow growing bacterium 2B-11, ▲; the bacterium 2B-11 with *U. americana*.

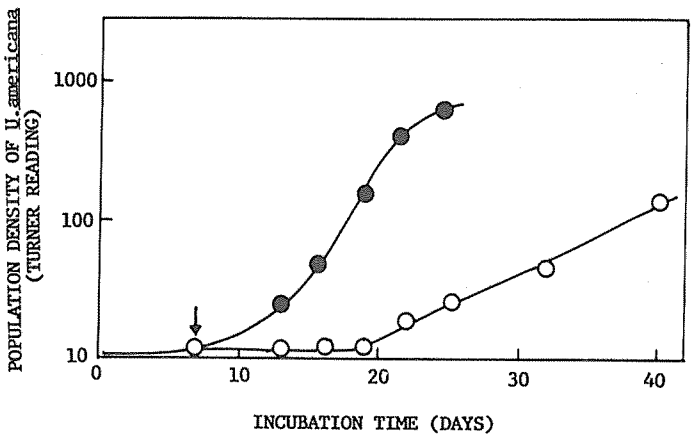

Fig. 16. Effect of the supplemental inoculation of bacterium 3A-3 on the growth of *U. americana* accompanied by bacterium 2B-11. The arrow indicates the addition of bacterium 3A-3.
Symbols: ○; *U. americana* with bacterium 2B-11, ●; *U. americana* with bacterium 2B-11 and 3A-3.

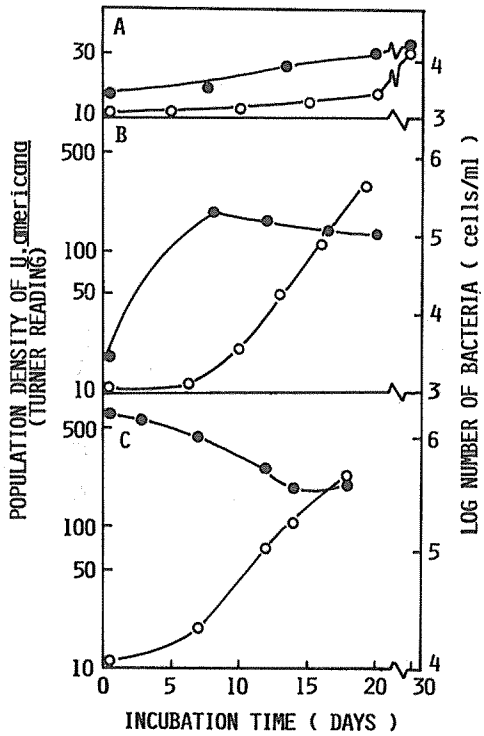

Fig. 17. Growth of *U. americana* (○) and bacterium 2B-11 (●) in the mixed culture system in Ur-1 medium.
(A); no addition, (B); with 50 mg/*l* trypticase peptone, (C); with additional supplement of a washed suspension of the bacterium (the initial number of the bacterium 1×10^6/m*l*).

3.3.3 *In situ* relationships between *U. americana* and bacteria

Figure 18 shows the population density of *U. americana* and heterotrophic bacteria during the algal bloom in Lake Biwa in 1983. The number of bacteria was less than 10^5 per m*l* before and during the algal bloom, and then increased after the bloom.

Figure 19 shows the results of the growth of *U. americana* accompanied by a bacterial

Table 3. Growth response of *U. americana* in addition of various living bacteria. Bacterial strains collected from Lake Biwa during Apr. 27 and Jun. 23, 1983 were inoculated to *U. americana* culture accompanied by a slow growing bacterium 2B-11

Date	No. of bacterial strains			
	Total	Growth promoted*	Growth nonpromoted	% promoted
Apr. 27	24	24	0	100
May 9	24	24	0	100
May 13	24	24	0	100
May 25	24	24	1	96
Jun. 23	24	24	0	100

* Growth of experimental cultures was compared with that of a control culture (no addition) after 14 of incubation. Positive growth effects were considered to be those which gave more than 200% growth of the control culture (taken as 100%).

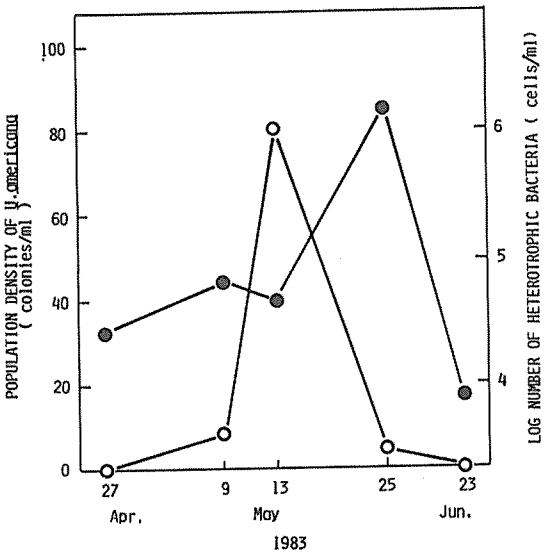

Fig. 18. Changes in the population of *U. americana* (○) and heterotrophic bacteria (●) during the algal bloom in Lake Biwa, 1983.

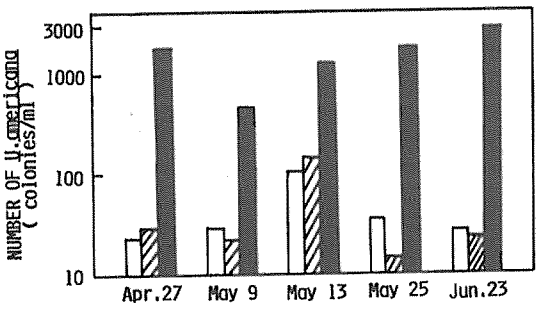

Fig. 19. Effect of natural and autoclaved lake water on the growth of *U. americana*. Lake water was collected from Lake Biwa on each date. 0.1 m*l* of natural (Solid bars) or autoclaved (hatched bars) lake water was added to 4 m*l* of Ur-1 medium (open bars). *U. americana* culture accompanied by a bacterium 2B-11 was inoculated and incubated for 14 days.

strains 2B-11 (a slow growing strain) with the addition of heat-sterilized and nonsterilized lake water to the Ur-1 medium. In all instances, the growth of *U. americana* was enhanced with the addition of nonsterilized lake water, but not when sterilized lake water was added. In some instances, these experiments were repeated with filter sterilization, but virtually identical results were obtained.

3.4 Discussion

The inability of *U. americana* to grow without the presence of bacteria in the culture at first seemed surprising. Although numerous workers have found that algae release many substances that could be potential nutrients for bacteria (Thomas, 1971; Bell and Mitchell, 1972; Bauld and Brock, 1974), there is little information that algal growth is dependent on the presence of bacterial associates in cultures. Lange (1970 & 1971) observed that under the condition of carbon dioxide limitation the addition of organic carbon stimulated the growth of blue green algae via carbon dioxide production from bacteria. Berland *et al.* (1970) observed that several species grew much better in bacterialized rather than axenic cultures and suggested that the release of vitamin substances by bacteria was a likely explanation of algal stimulation. Several other reports have been made for bacterial commitment for algal growth and physiology (Machilis, 1973; Haines and Guillard, 1974; Ukeles and Bishop, 1975; Paerl and Kellere, 1978; Provasoli and Pintner, 1980).

Whatever the mechanism involved, the fact that bacteria support the growth of *U. americana* in cultures suggests that there is a significant relationship between bacteria and *U. americana* in nature. An interesting question is: can bacteria be a limiting factor leading to the bloom ? In Chapter 2, it was suggested that phosphorus and iron were the primary limiting nutrients in Lake Biwa by using an algal assay technique. Results of the present study showed that ca. 10^5 per m*l* of bacteria were required to maintain exponential growth of *U. americana* in the laboratory (Fig. 17). Although there is difficulty in directly applying results from controlled laboratory experiments to natural phenomenon, the observation that the number of bacteria has never exceeded 10^5 per m*l* during the initial phase of the bloom and increased after the bloom in Lake Biwa (Fig. 18) suggests that algal growth in this period might be controlled by an interaction with bacteria in a way similar to the phenomenon in the mixed cultures used in this study. However, it is not possible to estimate the precise role of bacteria in initiating the bloom because of the ubiquitousness of bacteria in the environment to support algal growth (Fig. 19, Table 3). The problem would be better described by the study of the mechanism undergoing the algal-bacterial association. This is considered in Chapter 4.

3.5 Summary

It was shown that the presence of bacteria is essential for the growth of *U. americana*, a freshwater red tide Chrysophyceae, in a laboratory culture system. The effect on the algal growth of bacteria collected from Lake Biwa during the bloom was studied. All bacteria tested supported algal growth and the extent of algal growth was dependent on the strains of the associated bacteria. Algal growth was also promoted with the addition of nonsterilized natural lake waters. The possible role of bacteria in natural algal growth was discussed (Kimura, 1984; Kimura *et al.*, 1986).

4 Analysis of mechanism of the interaction between *U. americana* and bacteria

4.1 Introduction

In Chapter 3, it was shown that the presence of associated bacteria is essential for the growth of *U. americana* in a laboratory culture.

This chapter describes studies performed to analyze the relationships between *U. americana*

and bacteria in cultures and provides evidence of phagotrophic feeding of bacteria in *U. americana*.

4.2 Materials and methods

4.2.1 Cultures, growth conditions, and measurements

Cultures, growth conditions and measurement of the alga were those described in Chapter 3.

4.2.2 Preparation of culture filtrate of bacteria

A 24 h culture of a bacterium strain 3A-3 (an erythromycin-resistant strain collected from Lake Biwa) which reached approximately 2×10^8 cells/ml in LT 10^{-1} medium (see Chapter 3) was centrifuged. The supernatant obtained was filtered through a 0.22 μm Millipore filter and was aseptically stored at 4 ℃ in dark until needed. For algal growth experiments, a given amount of the bacterial filtrate was added to the tubes containing a temporary axenic culture of *U. americana*.

4.2.3 Dialysis culture experiments

The E-20 Ecologen (New Brunswick Sci. Co.) diffusion chamber used in this study has two 300 ml glass growth-chambers separated by a Millipore filter (0.1 μm pore size) and a GF/C glass fiber filter. After the entire assembly was autoclaved, 150 ml of sterilized Ur-1 medium was added to each chamber. To each chamber A the monoxenic culture of *U. americana* was inoculated. Each chamber B received either "no addition" (series 1), 1.5 μg/ml of erythromycin (series 2), 1.5 μg/ml of erythromycin plus 5 μg/ml of trypticase peptone plus an erythromycin-resistant bacterium 3A-3 (series 3), or 1.5 μg/ml erythromycin plus a bacterium 3A-3 plus *U. americana* (series 4). The chambers were incubated at 15 ℃ under fluorescent light (5000 lx, 14L : 10D photoperiod cycle). The growth of *U. americana* in chambaer A was determined during 17 daily periods.

4.2.4 Addition of heat-killed bacteria

Ten bacterial species were examined in this experiment, one (strain 3A-3) was native to the lake, and the others were *Escherichia coli* (ATCC 1105), *Micrococcus luteus* (ATCC 4698), *M. conglomeratus*, *Streptococcus lactis* (IFO 12007), *Staphylococcus aureus*, *Lactobacillus casei* (IFO 3425), *Bacillus subtilis* (ATCC 6051), *B. megaterium* (ATCC 1923), *B. megaterium* (QMB 1551). A 24 h culture of bacteria was harvested by centrifugation, washed twice with Ur-1 medium, suspended in Ur-1 medium, and autoclaved at 121 ℃ for 20 min. Various amounts of a suspension containing killed bacterial cells were added to the experimental tubes.

4.3 Results

4.3.1 Growth effect of a bacterial culture filtrate added to the algal culture

When a culture filtrate of bacterial strain 3A-3 was added to a temporary axenic culture of *U. americana*, no positive effect on the algal growth was noticed (Fig. 20). One possible explanation for this is that a substance supporting the algal growth may be either labile or produced in such small amount as to be undetectable in the culture filtrate.

4.3.2 Effect of dialyzable substance produced by bacteria

In order to answer the question mentioned above, dialysis culture experiments were conducted. An apparatus consisting of 2 chambers separated with a Millipore membrane filter (pore size 0.1 μm) was employed (Fig. 21). Algal growth in chamber A was detected only in series 1 (direct association with bacteria) as shown in Fig. 22. When associated bacterium NA-1 in chamber A was killed using erythromycin diffused from chamber B, it failed to grow even if an erythromycin-resistant bacterium 3A-3 was present in chamber B (series 3 and 4). These results show that a dialyzable substance produced by the bacteria was not effective for the growth of *U.*

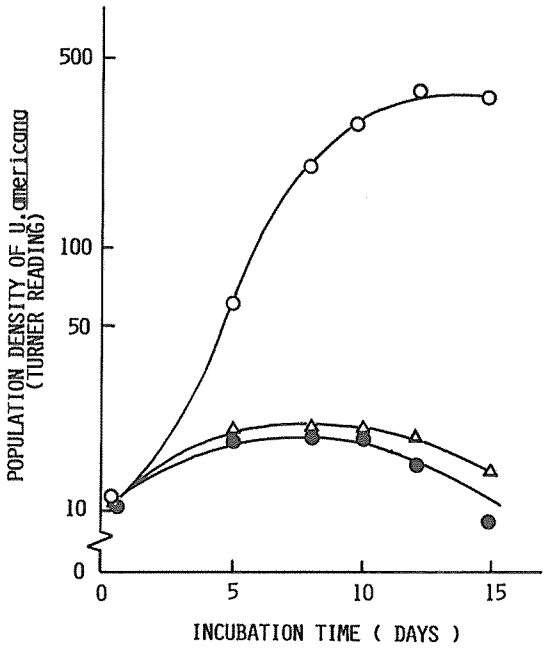

Fig. 20. Effect of a bacterial culture filtrate (strain 3A-3) on the growth of *U. americana*.
Symbols: ○: a monoxenic culture (with bacterium NA-1), ●: a temporary axenic culture, △; a temporary axenic culture added with the culture filtrate of a bacterium 3A-3.

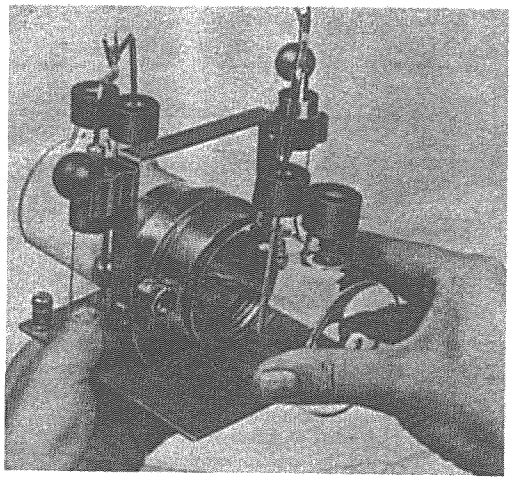

Fig. 21. An apparatus employed for the dialysis cultivation of *U. americana* and bacteria.

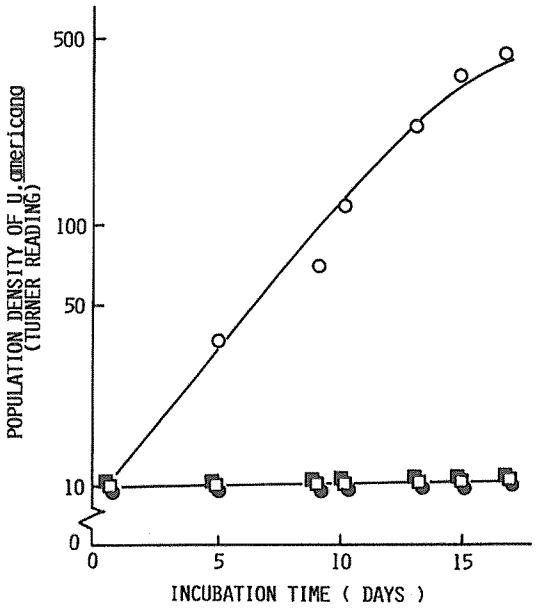

Fig. 22. Growth of *U. americana* in a dialysis culture. The apparatus used for the experiments is described in the text. In chamber A, *U. americana* accompanied by an erythromycin-sensitive bacterium NA-1 was inoculated. The algal growth in chamber A was determined with each chamber B receiving either no addition (○), erythromycin (●), erythromycin plus trypticase peptone plus an erythromycin-resistant bacterium 3A-3 (□), or erythromycin plus an erythromycin-resistant bacterium 3A-3 plus *U. americana* (■).

americana, and that cell-to-cell contact with bacteria was essential for algal growth.

4.3.3 Effect of heat-killed bacteria added to the algal culture

It was examined whether a living state of bacteria was necessary for algal growth. In place of a living bacterium, heat-killed cells (4 μg dry weight/ml) of a bacterium (strain 3A-3) were added to a temporary axenic culture of *U. americana*(Fig. 23). The effect of *U. americana* (Fig. 23). The effect of

supplemental addition of heat-killed cells was also determined. Two μg/ml of the killed cells was supplimentally added to a stationary phase culture (13 days of incubation) which had initially been supplied with 1 μg/ml of the killed cells, As shown in Fig. 24, growth of *U. americana* was recovered with the additional supplement of killed cells.

Heat-killed cells of six different bacteria, except two strains of *B. megaterium*, also supported algal growth (Figs. 25 and 26). The cell size of *B. megaterium* was 3-5 holds larger than the other bacteria and nearly half the length of *U. americana* cells.

4.4 Discussion

Algal cultures are frequently accompanied by several bacterial strains when algal cells have been isolated from natural waters (Blasco, 1965; Droop and Elson, 1966; Vance, 1966; Jolley and Jones, 1977). In the process of preparation of an axenic culture, the author's experience frequently indicated that *U. americana* can not grow when accompanying bacteria have been eliminated. Interactions between algae and bacteria have been reported in several laboratory culture systems (Vance, 1965; Delucca and McCracken, 1976;

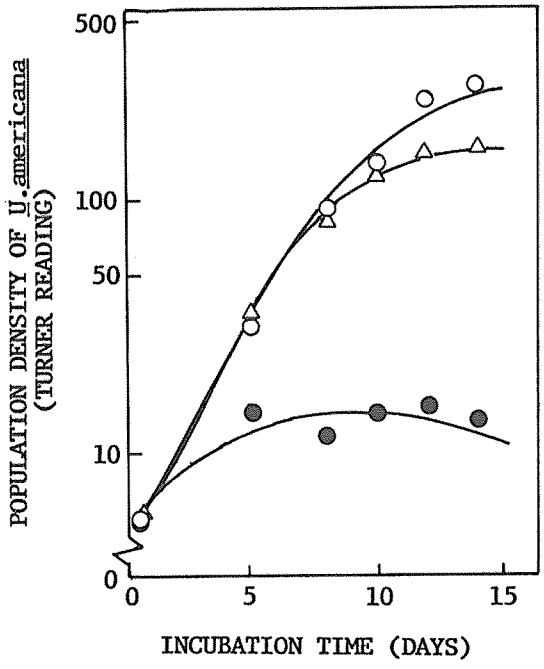

Fig. 23. Effect of heat-killed bacteria (strain 3A-3) on the growth of *U. americana* in a temporary axenic culture. Symbols: ○; a monoxenic culture (with bacterium NA-1), ●; a temporary axenic culture, △; a temporary axenic culture added with 4 μ g/ml (dry weight) of heat-killed bacteria.

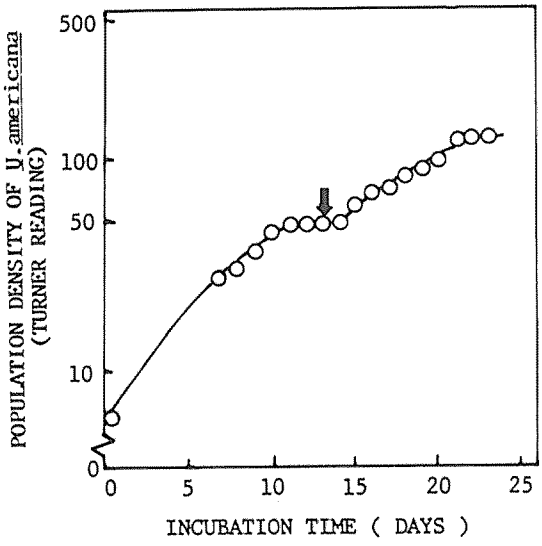

Fig. 24. Effect of supplemental addition of heat-killed bacteria (strain 3A-3) on the growth of *U. americana* in a temporary axenic culture. 2 μ g/ml of heat-killed cells was supplementally added to a stationary phase culture which has initially been supplied with 1 μg/ml of killed cells. Arrow indicates the time of supplementation.

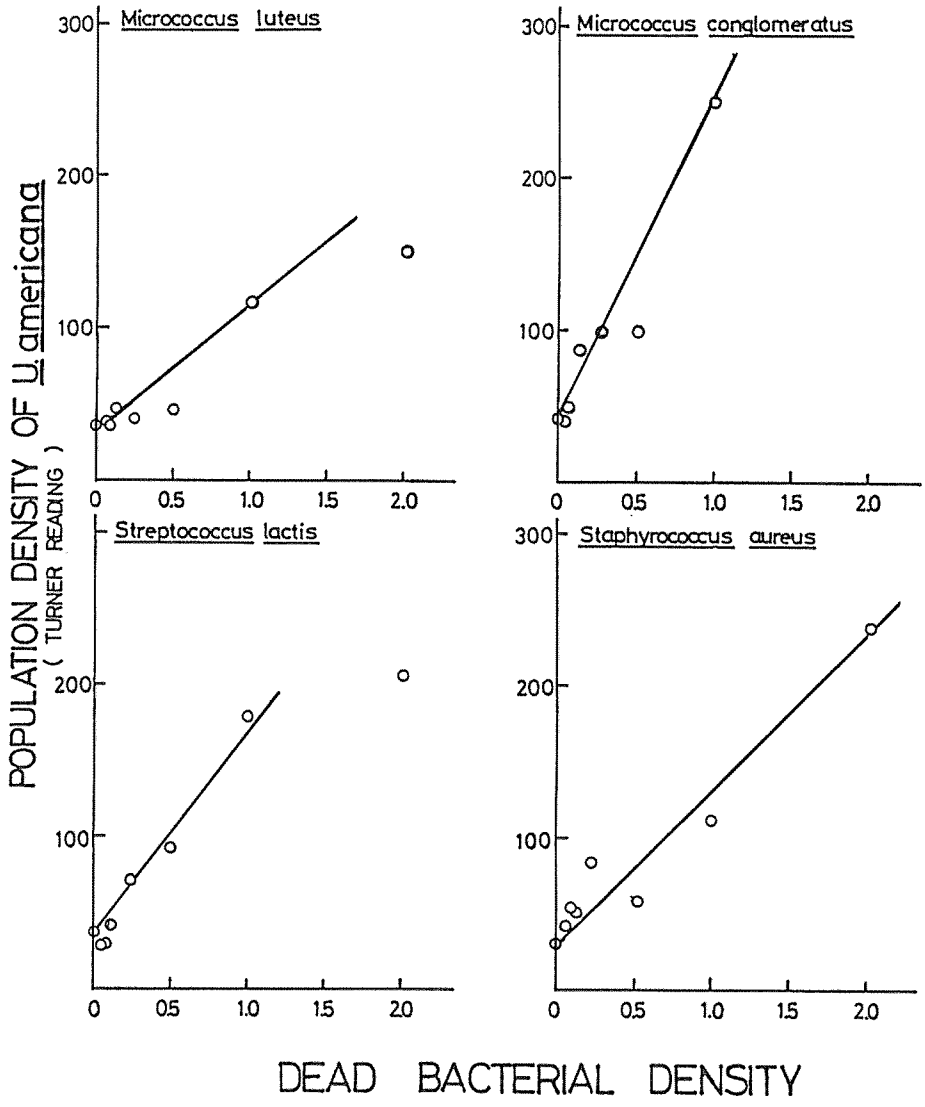

Fig. 25. Yield of *U. americana* as a function of initial density of dead bacteria. Temporary axenic cultures of *U. americana* were incubated with the addition of various densities of heat-killed bacterial cells.

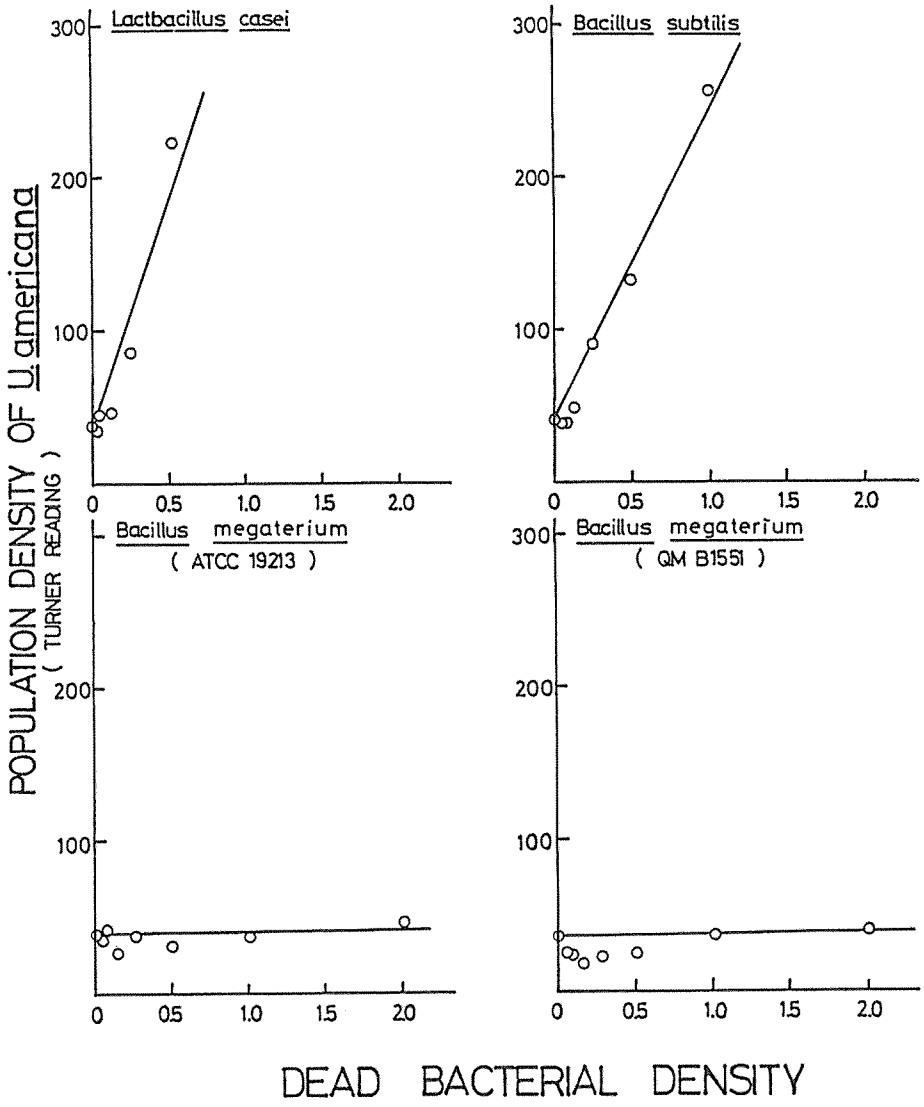

Fig. 26. Yield of *U. americana* as a function of initial density of dead bacteria. Temporary axenic cultures of *U. americana* were incubated with the addition of various densities of heat-killed bacterial cells.

Fredrickson, 1977; Mayfield and Inniss, 1978; Baker and Herson, 1978). Those bacteria utilized organic substances released by living or dead algal cells (Nalewajko *et al.*, 1976 & 1980; McFeters *et al.*, 1978; Bell, 1980). Algal growth, too, are not completely independent of bacteria. Observations by Provasoli (1958), Parker and Bold (1961), Nakamura (1963), Vance (1966), Berland *et al.* (1970), and Ukels and Bishop (1975) suggest that bacteria can enhance the growth of algae. In most of these studies, however, the mechanism of the interaction was not presented. Since the production of physiologically significant amounts of the vitamin B group, such as biotin and thiamine and lesser amounts of B_{12} and nicotinic acid, was demonstrated in many isolates of marine and freshwater bacteria in pure culture (Provasoli, 1974; Haines, 1974), it is the most likely explanation that bacteria provide these vitamins required by algae in the laboratory cultures.

However, the requirement of bacteria for the growth of *U. americana* described in this study could not be attributed to the vitamin production by the associated bacteria, since both the addition of the culture filtrate of bacteria to the algal culture and the dialysis cultivation of *U. americana* with bacteria terminated algal growth. Meanwhile, the observation that bacterial dead cells supported good growth of *U. americana* suggests that the algal growth factor must be closely associated with the cell components of bacteria. It is suggested that the incapability of two strains of *B. megaterium* to support the algal growth was probably due to their cells being so large that *U. americana* was unable to feed on them.

Evidence presented in this chapter indicates that *U. americana* obtains growth potential from bacterial cells and suggests that this alga is phagotrophic. Light and electron microscopic examination of *U. americana* cells are needed to confirm this hypothesis. This was conducted in Chapter 5.

4.5 Summary

An analysis of dependence mechanism of the growth of *U. americana* on bacteria was conducted using temporary axenic culture. Addition of a culture filtrate of bacteria to the algal culture was ineffective. Dialysis cultivation of *U. americana* with bacteria, eliminating cell-to-cell contact, terminated algal growth. Heat-killed bacteria supported algal growth, suggesting phagotrophic feeding of bacteria in this alga (Kimura and Ishida, 1986).

5 Photophagotrophy in *U. americana*

5.1 Introduction

Phagotrophic feeding by flagellates has mostly been investigated in colorless types (Droop, 1953; Storm and Hutner, 1953; Irish, 1979; Spero and Moree, 1981). Little is known about phagotrophy in photosynthetic flagellates. Food vacuoles in some photosynthetic flagellates collected from natural environments have occasionally been reported without any experimental verification (Dodge and Crawford, 1970; Manton, 1972; Wujek, 1976). To our knowledge, *Ochromonas danica* and *O. malhamensis*, Chrysophyceae, are the only photosynthetic flagellates whose phagotrophic nutrition and other nutritional requirements have been studied experimentally (Aaronson and Baker, 1959 & 1973; Stotze *et al.*, 1969; Cole and Wynne, 1974).

In this chapter, microscopic evidence of phagotrophic ingestion of bacteria in *U. americana* is presented and its significance, especially from evolutional aspects in Chrysophyceae, is discussed.

5.2 Materials and methods

5.2.1 Algal cultures and growth conditions

Cultures and growth conditions of *U. americana* were those described in Chapter 2.

5.2.2 Light microscopic study

E. coli cells were stained with tetrazorium chrolide (TTC) using the procedure described by Yamamoto *et al.* (1982). Cultures of *U. americana* were supplied with ca. 1×10^7 cells/m*l* of TTC-stained *E. coli* or a suspension of polystylene latex beads (Difco, 0.81 μm). After 30 min of incubation, light microscopic observations of phagotrophic feeding were performed using a Nikon microscope equipped with Normarski objectives.

5.2.3 Electron microscopic study

U. americana cultures incubated together with *E. coli* cells (unstained cells) or polystylene latex beads for 30 min were fixed for sectioning in 2 % osmium tetraoxide in 0.1 M sodium cacodylate buffer (pH 7) for 20 min at 0 ℃ before rinsing in buffer, dehydrated in an ethanol series, and embedded in an Epon resin. The sections were cut with a diamond knife, stained with uracyl acetate and Reynold's lead citrate, and examined under a JEM Model 100S electron microscope by courtesy of Japan Electronic Optic Laboratory Co., Ltd., Tokyo Japan.

5.3 Results

5.3.1 Light microscopic study

Light microscopic observations were conducted to examine phagotrophic feeding of bacteria in *U. americana*. A suspension of *E. coli* cells stained with TTC was added to the culture of *U. americana*. After 30 min of incubation, *U. americana* cells were observed by light microscopy. Figure 27-A shows that one to five cells of red-stained *E. coli* were engulfed in each of the algal cells. When biochemically inert polystylene latex particles (0.81 μm) were supplied instead of the *E. coli* cells, the algal

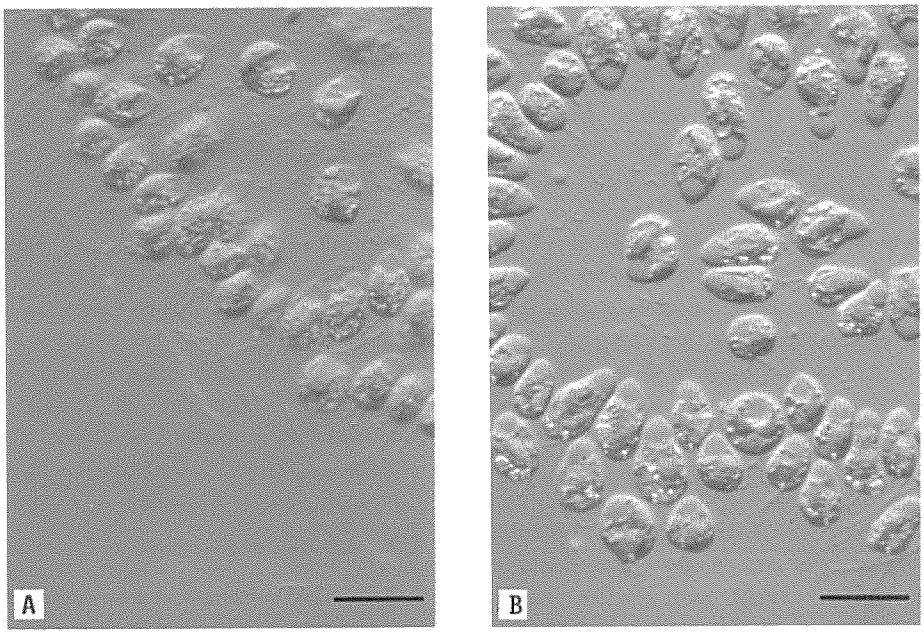

Fig. 27. Microscopic observation on cells of *U. americana* which engulfed TTC-stained cells (red) of *E. coli* (A) and which took up polystylene latex particles (B). Scale bars represent 10 μm.

cells also engulfed the particles (Fig. 27-B), but did not grow in the inert particles.

5.3.2 Electron microscopic study

Electron microscopic observations of *U. americana* cells were conducted. Electron micrographs of the ultrastructure of phagotrophy in *U. americana* which was incubated together with *E. coli* (ca. 1×10^7 cells/m*l*) for 30 min indicated that numerous engulfed *E. coli* cells were present in both the large posterior "leucosin" vacuole and the small digestive vacuoles located around the cell (Fig. 28-A and-B). Several bacteria were often seen within the same vacuoles (Fig. 28-C). Biochemically inert polystyrene latex particles (0.8 μm) supplied instead of *E. coli*. cells were also engulfed as shown in Fig. 28-D. This confirms that their ability to utilize a variety of food bacteria is quite unselective, regardless of the nutrition value of the particles.

5.3.3 Growth of *U. americana* in dark

Considering the fact that *U. americana* has the ability to take up bacteria as foods as described above, the capability of this alga to grow heterotrophically was examined. In dark, *U. americana* (a temporary axenic culture) could not be grown in Ur-1 medium supplimented with 3 μg dry weight/m*l* of heat-killed *E. coli.* cells plus various concentrations of glucose as a carbon source (Fig. 29). Various concentrations of acetate or trypticase were also examined as a carbon source, but the results were the same. From these results, it was suggested that *U. americana* could not grow heterotrophically.

5.4 Discussion

Studies with both light and electron microscopy indicate that *U. americana* exhibits phagocytosis for a wide range of bacteria as well as biochemically inert particles. The feeding mechanism may possibly be the same as that described for *O. danica* (Aaronson, 1973 & 1974; Cole and Wynne, 1974) and *O. malhamensis* (Dubowsky, 1974; Daley *et al.*, 1973) which is morphologically similar to *U. americana* in cell structure (Hibberd, 1976). *U. americana* as well as *O. danica* appear evolutionally adapted to take up particulate foods, as it lacks cell walls that reject phagocytosis on the cell surface (Fig. 30). An interesting comparison between *U. americana* and *O. danica* is as follows, *O. danica* grows not only photosynthetically (Myers and Graham, 1956) but heterotrophically independent of phagotrophy (Aaronson, 1959; Gibbs, 1962), and also grows phagotrophically independent of photosynthesis (Hutner *et al.*, 1953). On the other hand, *U. americana* is an obligate photophagotroph, in which both light and phagotrophic feeding of bacteria are essential for growth. The inability of phagotrophic species to grow heterotrophically has also been reported in Prymnesiophyceae *Chrysochromulina* spp. (Pintner and Provasoli, 1968). As a possible explanation of the photoheterotrophy of this species, Allen (1969) speculated that this organism does not possess the capacity for oxidative phosphorylation and must obtain its ATP from only photosynthetic phosphorylation. Perhaps, the dependence on light for the growth of *U. americana* might be explained in the same way. However, the other interesting feature in *U. americana* is that phagotrophy is also essential for its growth. *Chrysochromulina* can photosynthetically grow without depending on phagotrophy (Pintner and Provasoli, 1968). It is interesting, therefore, that *U. americana* is the first real photophagotrophic species. An explanation why phagotrophy is essential for the growth of *U. americana* is presented in Chapter 6.

Another interesting aspect of phagotrophy in *U. americana* is its evolutionary significance in Chrysophyceae, especially from the point of view of cell morphology. Many algae belonging to the Chrysophyceae, such as *Ochromonas*, expose naked protoplasts, while others have various cell coverings including scales (e.g. *Mallomonas*), loricae (e.g.

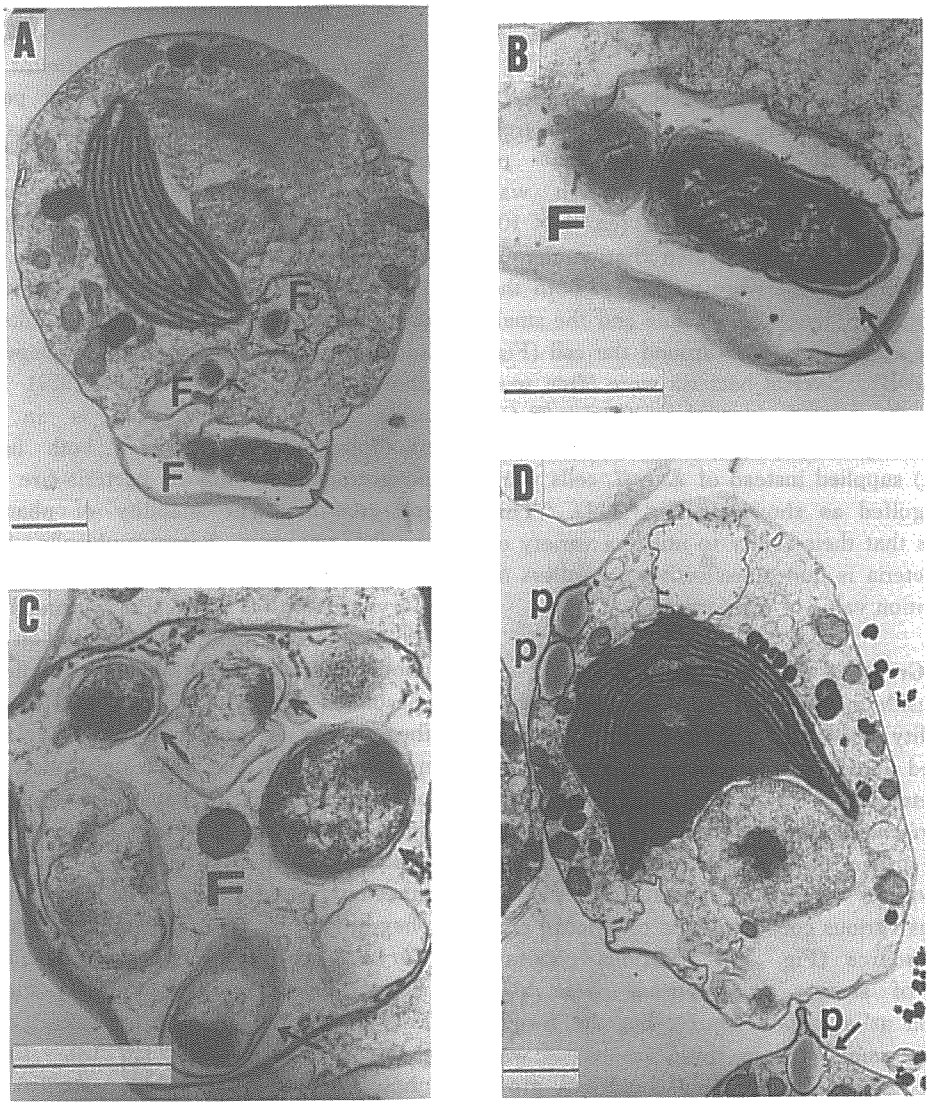

Fig. 28. Electron micrographs of a thin section of *U. americana* grown in Ur-1 medium.
(A); An algal cell incubated for 30 min with *E. coli* suspension. A large posterior food vacuole containing bacteria is present in this profile. Other food vacuole containing bacteria are also shown. Arrow indicates bacterial cells. F shows food vacuole. (B); A larger magnification of a large posterior food vacuole shown in A. (C); Section through a large food vacuole containing several bacterial cells. (D); Two algal cells allowed to feed on 0.8 μm polystyrene latex beads (P) for 30 min. One particle (arrow) appears to be undergoing exocytosis. Scale bars represent 1 μm.

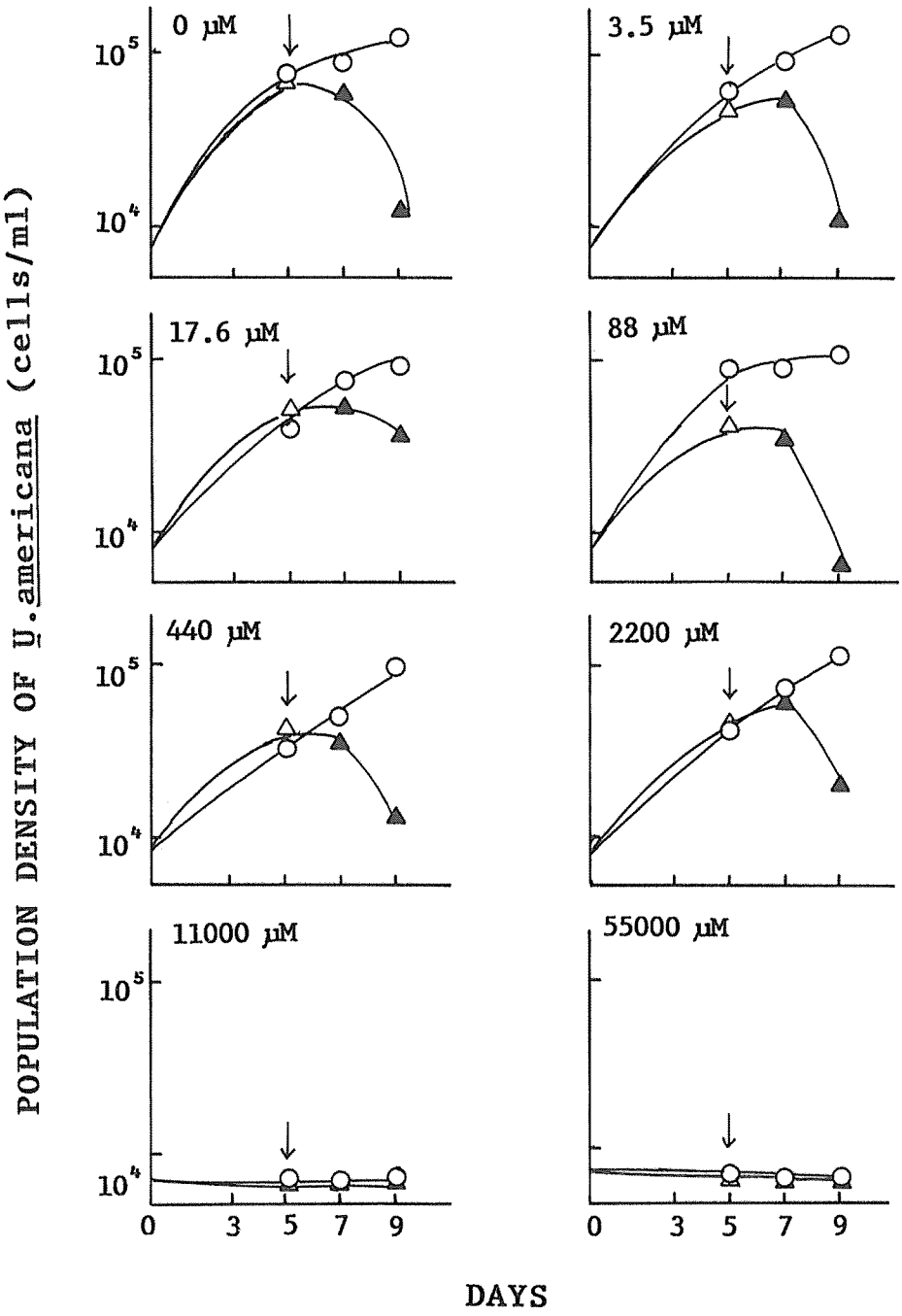

Fig. 29. Growth of *U. americana* in dark with heat-killed bacterial cells and various concentrations of glucose. Symbols: ○, △; light, ▲; dark after the time indicated by the arrow.

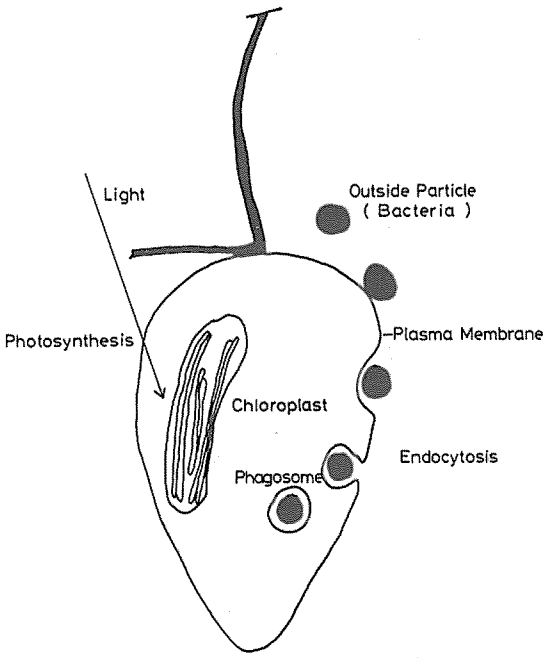

Fig. 30. Phagocytosis in *U. americana*. The passage of a particle through the cell membrane is illustrated.

Dinobryon), and slime (e. g. *Uroglena*) (Lee, 1980; Bold and Wynne, 1978). Evolutional affinities in the Chrysophyceae are based on morphological and colonial elabolation of the basic unicellular *Ochromonas* like cells (Fig. 31).

Ochromonas spp. are the closest to a basic pattern. The order Ochromonadales occupies a central position with the genus Ochromonas at the base of an evolutionary tree leading to the the remainder of the Ochromonadaceae. *Ochromonas* exibits active phagotrophy.

The genera *Dinobryon* and *Uroglena* are morphologically closest to the genus *Ochromonas*, differing only in the following ways. The former has loricae of a rigid cellulosic-pectic composition and form free-swimming arborescent colonies. The cells are actually naked and loosely embedded in loricae (Franke and Herth, 1973). The latter consists of colonial species in which numerous individual cells form a spherical colony by excreating a gelatious matrix around the cells. Although detailed information is laking, there are two reports suggesting phagotrophy in *Dinobryon* and *Uroglena*. Electron micrographs of natural samples taken by Wujek (1969 & 1976) indicate the existence of food vacuoles in *Dinobryon* sp. and *Uroglena* sp.. Recently, Bird (1986) verified Wujek's report by showing that *Dinobryon* sp. from natural waters actively ingested fluorescent beads. The present study is the first report experimentally verifying the active phagocytosis in *U. americana in vitro*.

The *Synura* and *Mallomonas* have a cell membrane covered with delicately sculptured silicaceous scales (Hibberd, 1973; Harris and Bradley, 1957). Electron microscopic studies indicate that these scales do not completely enclose the protoplast (Bradley, 1966). It is the author's opinion, therefore, that the possibility of phagotrophy still remains in *Synura* and *Mallomonas*, although there is no report on phagocytosis in these groups.

After all, the evolution of cell-wall development in these species described above might not be at an advanced stage enough to make phagotrophic feeding impossible on the cell surface. Future studies would reveal more widespread distribution of phagotrophs in other species of this family.

At present it is not known what substance of the bacterial cells is required by *U. americana*. Experiments to determine the nature of this substance are presented in Chapter 6.

5.5 Summary

Light and electron microscopic examination of *U. americana* cells incubated together with *E. coli* cells or polystylene latex beads revealed that *U. americana* actively ingests bacteria or particles. It was confirmed that this alga is a photophagotrophic organism (Kimura and Ishida, 1985). Evolutionary aspects of

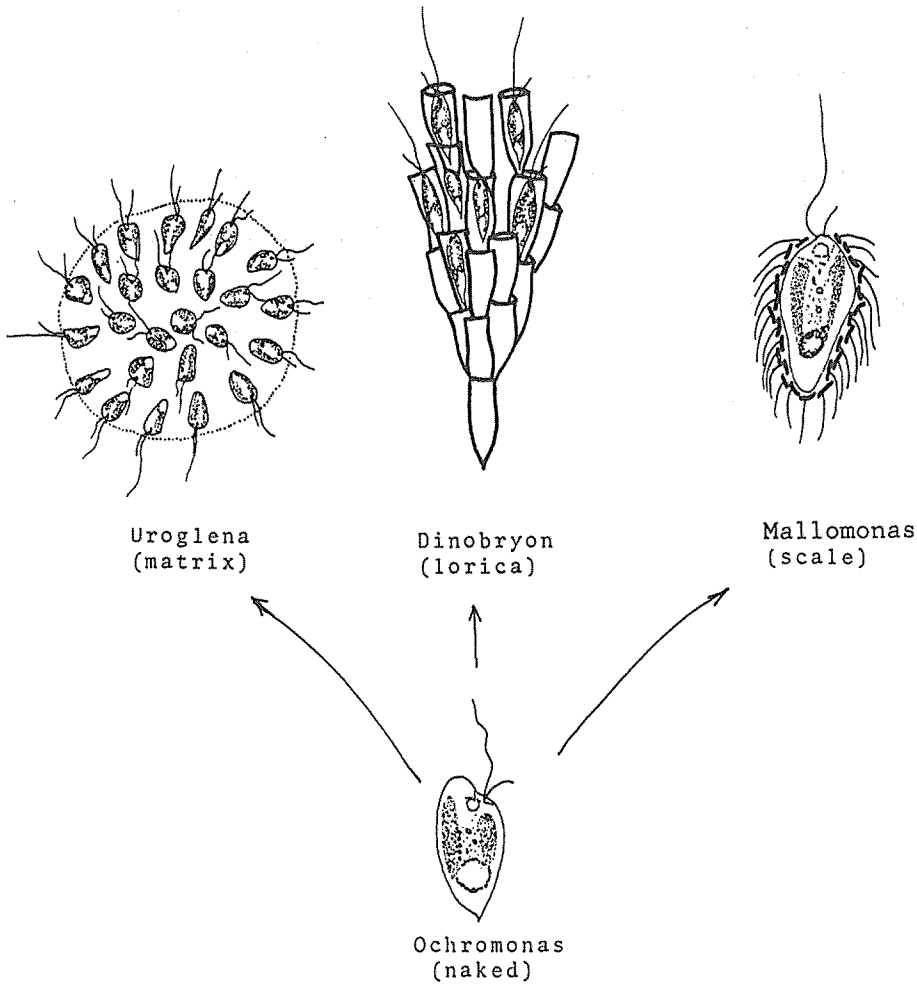

Fig. 31. Diagram of assumed divergent lines relating to the genera *Ochromonas*, *Uroglena*, *Dinobryon* and *Mallomonas*.

phagotrophy in *U. americana* was discussed (Ishida and Kimura, 1986; Kimura and Ishida, 1987).

6 Identification of bacterial factors required for the growth of *U. americana*

6.1 Introduction

In Chapter 5, *U. americana* was shown to exhibit phagotrophic feeding of bacteria. *U. americana* grows photosynthetically, but the presence of bacteria is essential for algal growth in Ur-1 medium. An essential substance (a bacterial factor) must, therefore, be present in bacterial cells.

This chapter describes work by the author to isolate and identify a bacterial factor essential for growth of *U. americana*. The nutritional requirement of phospholipid, identified as a bacterial factor, is also discussed.

6.2 Materials and methods

6.2.1 Algal cultivation and growth conditions

Cultures, growth conditions, and measurements of the alga were described in Chapter 2. A temporary axenic culture of this alga was used as an assay culture for a bacterial factor.

6.2.2 Source of materials

Yeast extract (Difco), proteose peptone (Difco) and trypticase (BBL) were used as organic sources in this experiment. Fatty acids, natural phospholipids, and synthetic phospholipids (dipalmitoyl) were obtained from Sigma Chemical Co.. The triglycerides, diglycerides, and monoglycerides were obtained from the Nakarai Chemical Co.. Tweens were obtained from Wako Chemical Co..

6.2.3 Preparation of a bacterial factor for algal growth

Fractionation steps for the purification of a bacterial factor from *E. coli* cells are illustrated in Fig. 32.

Escherichia coli (ATCC 1105) was

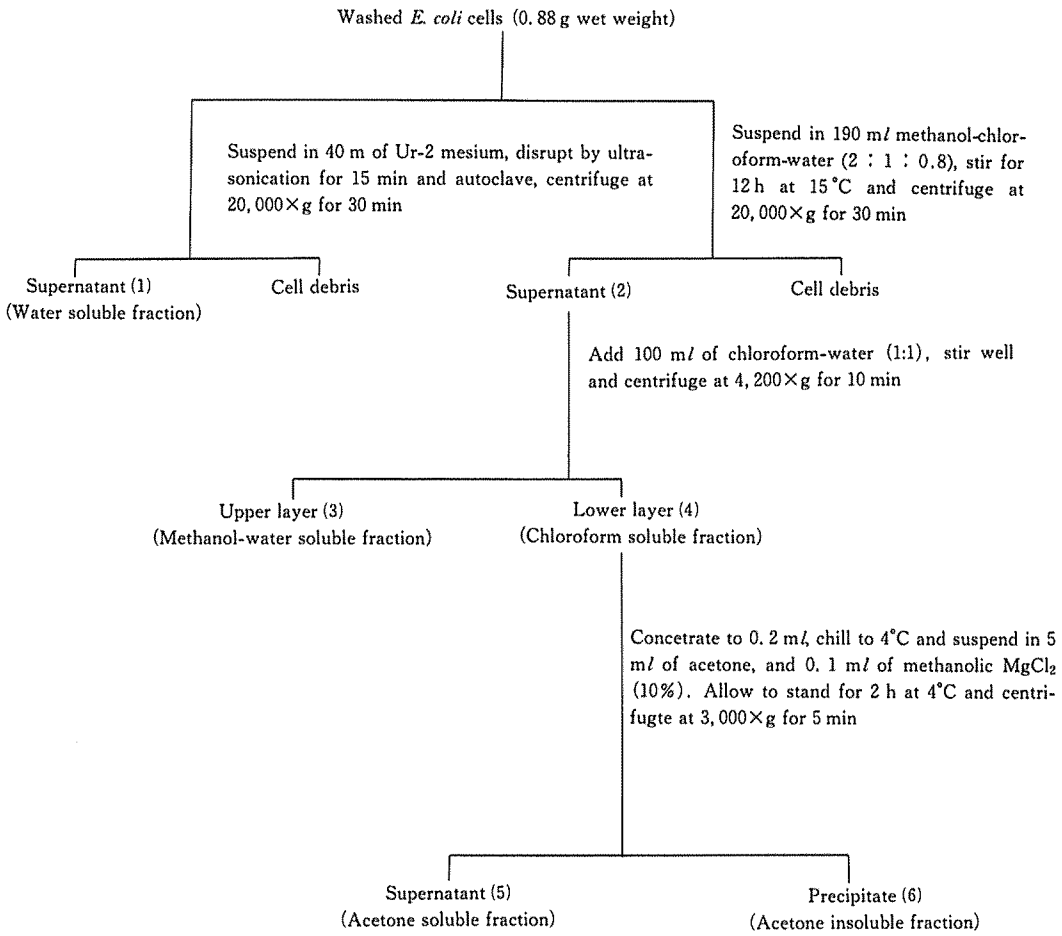

Fig. 32. Scheme for the purification of the growth factors for *U. americana*.

cultivated in nutrient broth at 37 ℃ for 24 h and the cells were routinely harvested by centrifugation at 10, 000 g for 20 min and washed twice with Ur-1 medium. The cells (0.22 g wet weight) were suspended in 40 m*l* of Ur-1 medium and then sonicated for 15 min with a sonic oscillater (Tomy Seiko Co. Ltd, Model UR-20P, 20KC) at 0 ℃. The sonicate was centrifuged at 10,000 × g for 20 min to remove cellular debris and insoluble materials. In this way the cold water extract was prepared and was kept after autoclaving sterilization.

The hot water extract was prepared in the same manner as the cold water extract except that the suspension of disrupted cells was autoclaved before removal of cellular debris and insoluble materials.

The lipid fraction was prepared by extracting cells (0.88 g wet weight) with 190 m*l* of methanol-chloroform-water (2:1:0.8; v/v/v) under stirring overnight at 15 ℃. After removal of cellular debris and insoluble materials, the extract was divided into a chloroform soluble fraction and methanol-water soluble fraction using a phase distribution technique adding 100 m*l* of chloroform-water (1:1). A chloroform soluble fraction was concentrated from 100 m*l* to 0.2 m*l*, chilled to 4 ℃, suspended in 5 m*l* of acetone, and added with 0.1 m*l* of methanolic $MgCl_2$ (10 %) solution. The suspension was allowed to stand for 2 h at 4 ℃ and centrifuged at 3,000 × g for 5 min. The precipitate was suspended in 10 m*l* of chloroform and stored at 5 ℃ as the acetone insoluble fraction until used for assay.

6.2.4 Lipid analysis

For lipid analysis, the acetone insoluble fraction was applied to the thin layer chromatography (TLC) on a silica gel and developed with chloroform-acetic acid (1:2, v/v) as the solvent. After spraying 40 % H_2SO_4 solution on the plate, identification of phospholipids was carried out by comparison of Rf values with standard solutions of phospholipids.

6.2.5 Preparation of assay media

As a basal medium for assay, 4m*l* of Ur-1 medium with 1.5 μg/m*l* of erythromycin was prepared in PP-capped tube (16-25 mm) as described in Chapter 3. The water extracts from bacterial cells and other water-soluble chemicals to be tested were suspended in the basal medium after serial dilutions by using Ur-1 medium. These were then autoclaved. The chloroform-methanol extract and other lipid chemicals were prepared in serial dilutions using choloroform. Aliquots of these solutions were added to the PP-capped tubes, and were evaporated to a drystate under reduced pressure to remove solvents. The Ur-1 medium was added to the tubes and sonicated before autoclaving to obtain stable aquious suspensions.

A 0.1 m*l* preparation of a 7 day old algal preculuture containing an erythromycin-sensitive bacterium NA-1 was used as the inoculum for *U. americana* in each tube.

6.3 Results

6.3.1 Effect of various organic materials on the algal growth

In place of bacteria, various organic materials including proteose peptone, trypicase, yeast extract, and soil extracts were tested for algal growth. As shown in Table 4, none, except the heat-killed bacterial cells, supported algal growth.

6.3.2 Effect of bacterial cell-extracts on the algal growth

The effectiveness of bacterial cell-extracts was examined, as shown in Fig. 33. Neither cold nor hot water extracts supported algal growth, indicating that water extractable constituents of the bacterial cells were not essential. Algal growth could be found only when chloroform-methanol-water extract was supplied. Chloroform-methanol-water extract was then divided into a chloroform-soluble fraction and a methanol-water soluble fraction with a phase distribution technique. Most

Table 4. The nutritional activity of various organic materials for axenic growth of *U. americana* in Ur-1 medium

Meterials added	Concentration (μg/100ml)	Growth response* (% control)
Trypticase	5,000	100
	500	100
	50	100
Yeast extract	500	100
	50	100
	5	100
Trypticase	5,000 + 500	100
+ Yeast extract	500 + 50	100
	50 + 5	100
Proteose peptone	8,000 + 800	100
+ Yeast extract	1,600 + 160	100
	320 + 32	100
Autoclaved *E. coli*	650	1,150

* Population density expressed as % control.

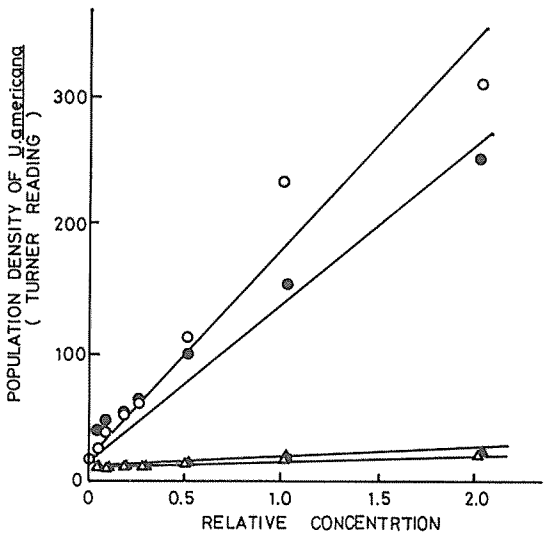

Fig. 33. Growth response of *U. americana* to various amounts of cold water (▲), hot water (△), and chloroform-methanol-water (●) extracts of *E. coli* cells. The algal growth response with intact *E. coli* cells (○) are also shown as control.

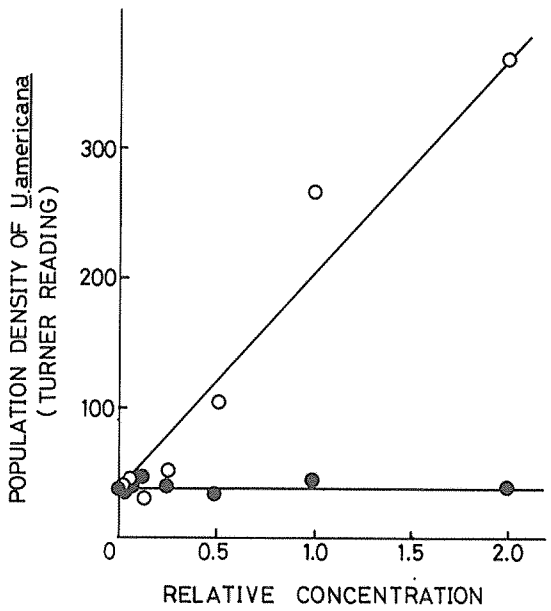

Fig. 34. Growth response of *U. americana* to various amounts of the chlololform soluble (○) and the methanol-water soluble (●) fraction extracted from *E. coli* cells.

activity was present in the chloroform-soluble fraction (Fig. 34). This fraction was further separated into an acetone-soluble fraction (nonpolar lipid fraction) and an acetone-insoluble fraction (polar lipid fraction). The assay experiments with both fractions were conducted, as shown in Fig. 35. No growth of alga was detected with the addition of acetone-soluble fraction, but the acetone-insoluble fraction did support good growth of the alga. It is suggested that the alga essentially requires phospholipids for the replacement of bacterial cells.

6.3.3 Identification of a bacterial factor

Thin layer chromatography of silica gel of the acetone insoluble fraction exhibited three distinct bands as shown in Fig. 36. By applying standard solutions of the phospholipids to the chromatogram, they were identified as phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and cardiolipin (CL), respectively. PE was a major band.

Commercial samples of highly purified PE extracted from *E. coli* were assayed for algal growth response. As shown in Fig. 37, PE supported excellent growth of *U. americana*. Their optimal concentration was 0.7 to 2.5 μg/m*l* for both. The maximum growth yield was similar to that of the acetone-insoluble fraction of bacterial cells.

6.3.4 Phospholipid requirement of the alga

To clarify the specifity of the phospholipid requirement, various commercially-available phosphplipids of natural origin were tested as essential nutritional factors for the alga. Table 5 shows algal growth in a temporary axenic culture in Ur-1 medium added to several phospholipids as compared with growth in a control medium (no addition). All of the phospholipids used—phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidic acid (PA) and cardiolipin (CL)—permitted good growth of *U. americana*,

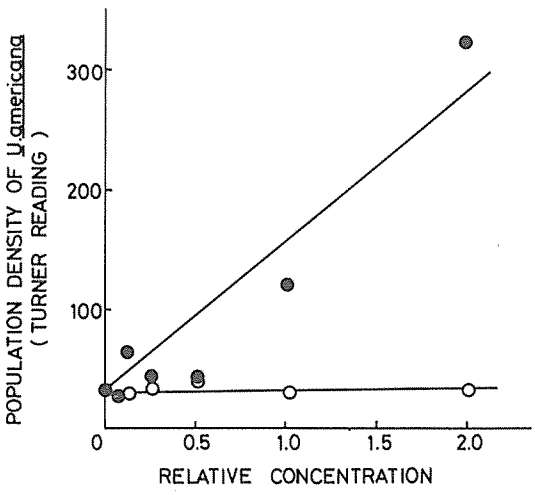

Fig. 35. Growth response of *U. americana* to various amounts of acetone soluble fraction (○) and acetone insoluble fraction (●) of the chloroform soluble fraction extracted from *E. coli* cells.

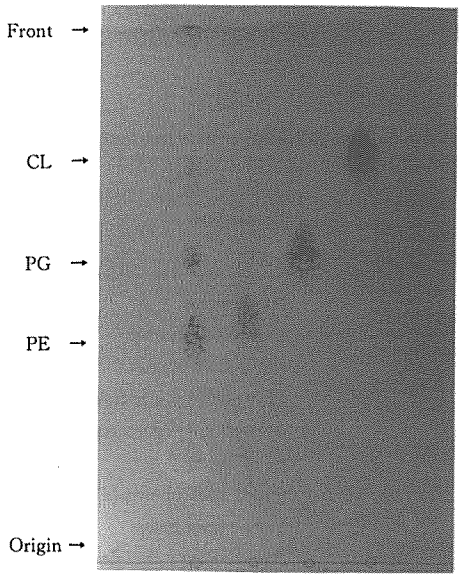

Fig. 36. Thin layer chromatography of the acetone insoluble lipid fraction of *E. coli*.
Solvent: chloroform-methanol-acetic acid (65: 25: 10). Standards: (1); phosphatidyl ethanolamine, (2); phosphatidyl glycerol, (3); cardiolipin. Color reagent: H_2SO_4.

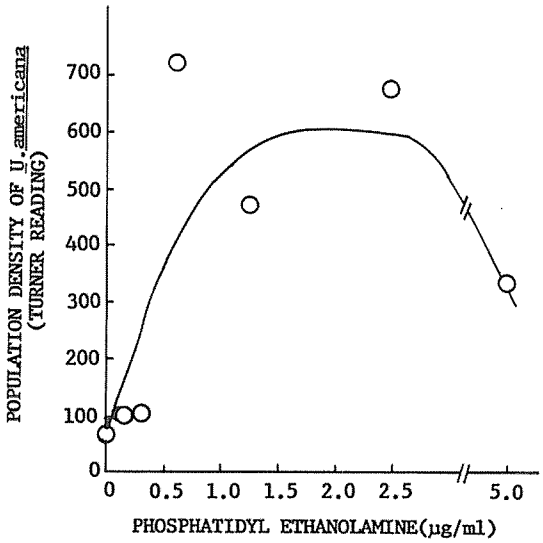

Fig. 37. Growth response of *U. americana* to increasing concentrations of phosphatidyl ethanolamine.

although the maximum population densities of the alga were different depending on the kind of phospholipids added, ranging from 310 to 1641 % of the control medium (Ur-1 medium).

In comparison with PE from egg yolk, a synthetic phospholipid, phosphatidyl ethanolamine dipalmitoyl, was tested for its growth activity. PE dipalmitoyl contains palmitic acid at C_1 and C_2 positions in the glycerol skeleton of PE, while PE from egg yolk contains a variety of saturated as well as unsaturated fatty acids. PE dipalmitoyl supported good growth of alga. The final population of the alga obtained with PE dipalmitoyl were essentially the same as those with PE from egg yolk (Fig. 38).

Various kinds of glycerides containing palmitic acid were then tested in place of PE dipalmitoyl; tripalmitin, dipalmitin, monopalmitin, tween 40, and free palmitic acid. The results are shown in Table 6. The growth

Table 5. The nutritional activity of various phospholipids for *U. americana*

Compound	Optimal concentration (mg/100m*l*)	Growth response* (% control)
No addition		100
Acetone insoluble fraction of *E. coli*	0.30	1330
Phosphatidyl ethanolamine	0.06	1108
Phosphatidyl glycerol	0.16	590
Cardiolipin	0.16	310
Crude phospholipids** from sheep brain	0.31	1057
Phosphatidyl choline	0.16	700
Phosphatidyl serine	0.25	1641
Phosphatidic acid	0.06	1150

* Yield expressed as % of the control medium (no addition).
** A crude extract containing several phospholipids and glycolipids extracted from sheep brain.

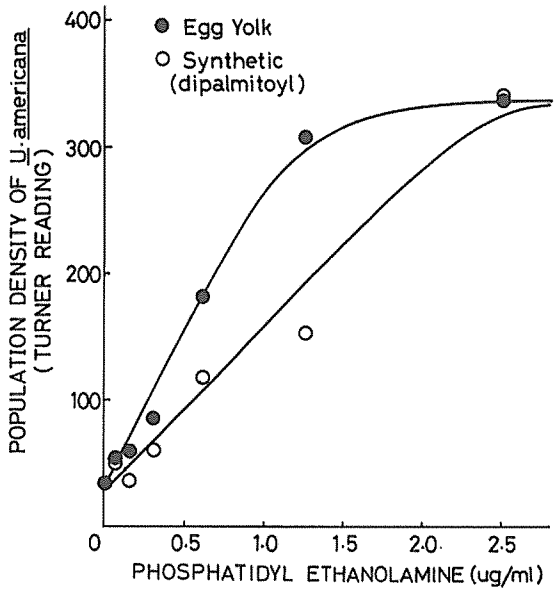

Fig. 38. Growth response of *U. americana* to increasing concentrations of phosphatidyl ethanolamine from egg york (●) and phosphatidyl ethanolamine dipalmitoyl (synthetic) (○).

Table 6. The nutritional activity of various synthetic lipids containing palmitic acid for *U. americana*

Compound	Optimal concentration (mg/100m*l*)	Growth response* (% control)
No addition	—	100
Phosphatidyl ethanolamine dipalmitoyl	0.25	1456
Tripalmitin	1.0	147
Dipalmitin	1.0	188
Monopalmitin	1.0	123
Palmitic acid	0.06	200
Tween 40	0.13	120

* Population density expressed as % control (no addition).

of *U. americana* with these materials was markedly inferior to that obtained with phospholipids.

Further study showed that several other glycerides, tweens, and free fatty acids also were not as nutritionally active for *U. americana* as phospholipids (Table 7). Free fatty acids showed inhibitory effects above the optimum levels which were about two order of magnitude lower than those of phospholipids.

6.4 Discussion

This chapter investigated the identification of a bacterial factor essentially required by *U. americana*. Results indicated that the bacterial factor is phosphatidyl ethanolamine. Phosphatidic acid, the intermediate of various phospholipids synthesis (Fig. 39), also showed excellent growth activity when it was supplied in place of PE, leading to the assumption that *U. americana* doesn't need water soluble

Table 7. Growth response of *U. americana* to various lipids

Lipid tested	Optimal concentration (mg/100m*l*)	Growth response* (% control)
No additon		100
Tripalmitin + Trilinolein	0.016 each	243
Tripalmitin + Triolein	0.03 each	152
Tristearin + Trilinolein	0.008 each	220
Tristearin + Triolein	0.016 each	269
Tristearin + Triolein	0.25 each	214
Dipalmitin + Monopalmitolein	0.004 each	125
Distearin + Diolein	0.016 each	172
Tween 80	0.16	310
Tween 40, 60, 80	0.05 each	230
Tween 40, 60, 80	0.026 each	246
Palmitic acid + Palmitoleic acid	0.008 each	109
Stearic acid + Oleic acid	0.008 each	150

* Population density expressed as % control (no addition).

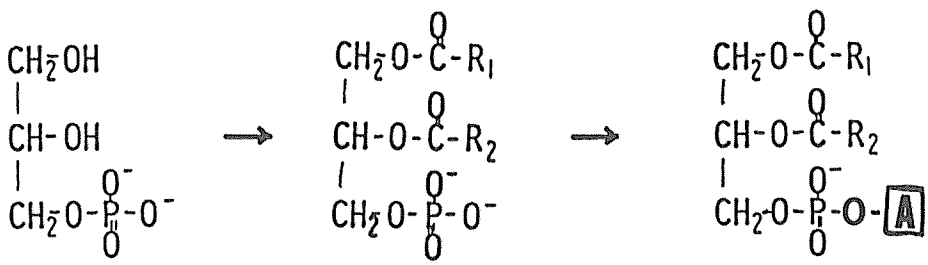

Fig. 39. Synthetic passway of phospholipid. A: head alcohol.

components (ethanolamine, choline, serine etc.) of phospholipids for its growth. The fact that glycerophosphate, the precursor of phosphatidic acids (Fig. 39), did not support algal growth suggests that *U. americana* lacks or is weak in the ability of acylation with acetyl-CoA derivatives and is unable to synthesize the cell membrane. Several researchers (Sharb and Lund, 1959; Soldo and Wagtendonk, 1967), however, reported that the ability of free fatty acids to support growth of the fatty acid requiring protozoa was difficult to demonstrate since fatty acids are often toxic to the test microorganisms. They have suggested that phospholipids are the most convenient source of fatty acids for these organisms. According to this suggestion, *U. americana* may require fatty acids and obtain them through phospholipids which are the most convenient forms for providing fatty acids. At that time, however, it was not possible to offer an accurate explanation for the inability of fatty acids containing glycerides to support good growth of *U. americana*. An enzymatic experiment for phospholipid biosynthesis of this alga is required to clarify these problems in the future.

Another interesting feature in this study is the evolutionary aspect of the lipid requirement in *U. americana*. Since lipids are generally poorly diffusible, it would be difficult for microorgnisms to obtain lipids osmotrophically. Lipid requirement in microorganisms might be interpreted as evidence of a deep-seated adaptation of phagotrophy (Droop, 1959 & 1966). In fact, requirements of lipids such as vitamins A, K, and E, sterols, phospholipids, or fatty acids have been reported in various colorless phagotrophic protists (Storm and Hutner, 1953; Hutner and Holz, 1962; Soldo and Wagtendonk, 1969; Droop and Pennock, 1971). A few examples are given as follows: *Paramecium aurelia* (Ciliata) required fatty acids and sterols for its growth (Soldo and Wagtendonk, 1969); *Oxyrihhis marina* (Dinophyceae) required ubiquinone isolated from lemon rind (Droop and Pennock, 1971); *Peranema trichophorum* (Euglenophyceae) required fatty acids for growth (Storm and Hutner, 1953).

However, to the author's knowledge, this is the first study showing that photosynthetic algae exhibit lipid requirement. *Ochromonas danica*, which also exhibits photoautotrophy and phagotrophy and is the only such organism whose nutritional requirement has been studied experimentally in any great detail, requires vitamin B_{12} and biotin but not lipids in either phototrophic growth or phagotrophic growth without depending on photosynthesis (Aaronson and Baker, 1959). *Ochromonas* belongs to Chrysophyceae and has a close taxonomical relation to *U. americana*. There have been no reports on the requirement of organic growth factor for photosynthetic microalgae except vitamin B_1, vitamin B_{12}, and biotin (Provasoli and Carlucci, 1974). Further study may reveal that phototrophic microalgae with a lipid requirement is more widely distributed. It is presumed that *U. americana* had lost its phospholipid synthesis system during its evolution and this event has obliged this alga to continue with the phagotrophic mode for nutrition as well as photosynthesis.

6.5 Summary

To determine the factor necessary for the growth of *U. americana*, various extracts from cells of *E. coli* were added to a temporary axenic culture of *U. americana* and the effect on algal growth was examined. Water extracts of the bacterial cells were ineffective to promote growth of *U. americana*; however, chloroform-methanol extracts of the bacterial cells were effective in producing algal growth. Furthermore, the acetone insoluble fraction in the extracts had high growth activity for the alga, suggesting that the growth factor is polar lipid. Three phospholipids, among which phosphatidyl ethanolamine was a major component and phosphatidyl glycerol and cardiolipin were minor, were detected in this fraction using thin layer chromatography. The

addition of highly purified, commercial phosphatidyl ethanolamine extracted either from *E. coli* or from egg yolk resulted in excellent growth of *U. americana*. High growth was also sustained by phosphatidyl ethanolamine (synsthetic), phosphatidyl choline, phosphatidyl serine, phosphatidic acid, and other high purity natural phospholipids, indicating no specific requirement for phospholipids by *U. americana*. Phosphatidic acid supported good growth of *U. americana* but glycerophosphate (the precursor of phosphatidic acid) did not, and several glycerides and free fatty acids were not as nutritionally effective as phospholipids. From these results, it is suggested that *U. americana* may lack glycerophosphate acyltransferase for phosphatidic acid synthesis (Kimura and Ishida, 1989).

7 Summary and conclusions

In order to understand the mechanism related to the development of the bloom of *U. americana* in Lake Biwa, some chemical and biological factors influencing algal growth were studied.

The results obtained are summarized as follows:

(1) From the bioassay with or without the addition of nitrogen, phosphorus, iron and/or vitamins to 0.22 μm-filter-sterilized lake water, it was found that phosphorus and iron were the major limiting nutrients for the algal bloom, and that the lake water contained a sufficient quantity of nitrogen.

(2) It was shown that the presence of bacteria and light are required for the growth of *U. americana* in a laboratory culture system. The effect of bacteria on algal growth and the mechanism of the interaction was studied.

All of the bacteria collected from Lake Biwa during the bloom supported algal growth. The addition of bacterial culture filtrate to the algal culture and also dialysis cultivation of *U. americana* and bacteria did not support algal growth, while heat-killed bacteria supported the algal growth. Light and electron microscopic examinations of *U. americana* cells incubated together with *E. coli* cells or with polystylene latex beads revealed that *U. americana* ingests bacteria or particles actively under light. It was confirmed that this algae is a photophagotrophic organism.

(3) To determine the factor (a bacterial factor) required for growth of *U. americana*, an attempt was made to purify and identify the bacterial factor from cells of *E. coli*. Phosphatidyl ethanolamine (PE) was determined to be the bacterial factor. Further study of the nutritional basis of the phospholipid requirement revealed no specific requirement of phospholipids by *U. americana*. Phosphatidic acid supported good growth of *U. americana* but glycerophosphate (the precursor of phosphatidic acid) did not. Several glycerides and free fatty acids were also not nutritionally active as phospholipids. From these results, it is suggested that phospholipids are required as a component of the cell membrane because *U. americana* lacks the ability of a phospholipids synthesis system.

Results presented in this study suggest that the mechanism responsible for the development of the bloom of *U. americana* in Lake Biwa is as follows;

Two different, but not mutually exclusive, factors are involved (Fig. 40).

First, the growth of *U. americana* in Lake Biwa is most largely dependent on the supply of phosphorus and iron among dissolved inorganic nutrients. Since the concentration of phosphorus and utilizable iron in Lake Biwa is extremely low, as described in Chapter 2, it is presumed that the influx of even a slight amount of phosphorus or iron into the lake would stimulate the growth of *U. americana*.

Second, the influx of dissolved organic matter into the lake is equally significant for algal growth in the lake, since the increased amount of organic matter dissolved in the lake water would result in increased bacterial

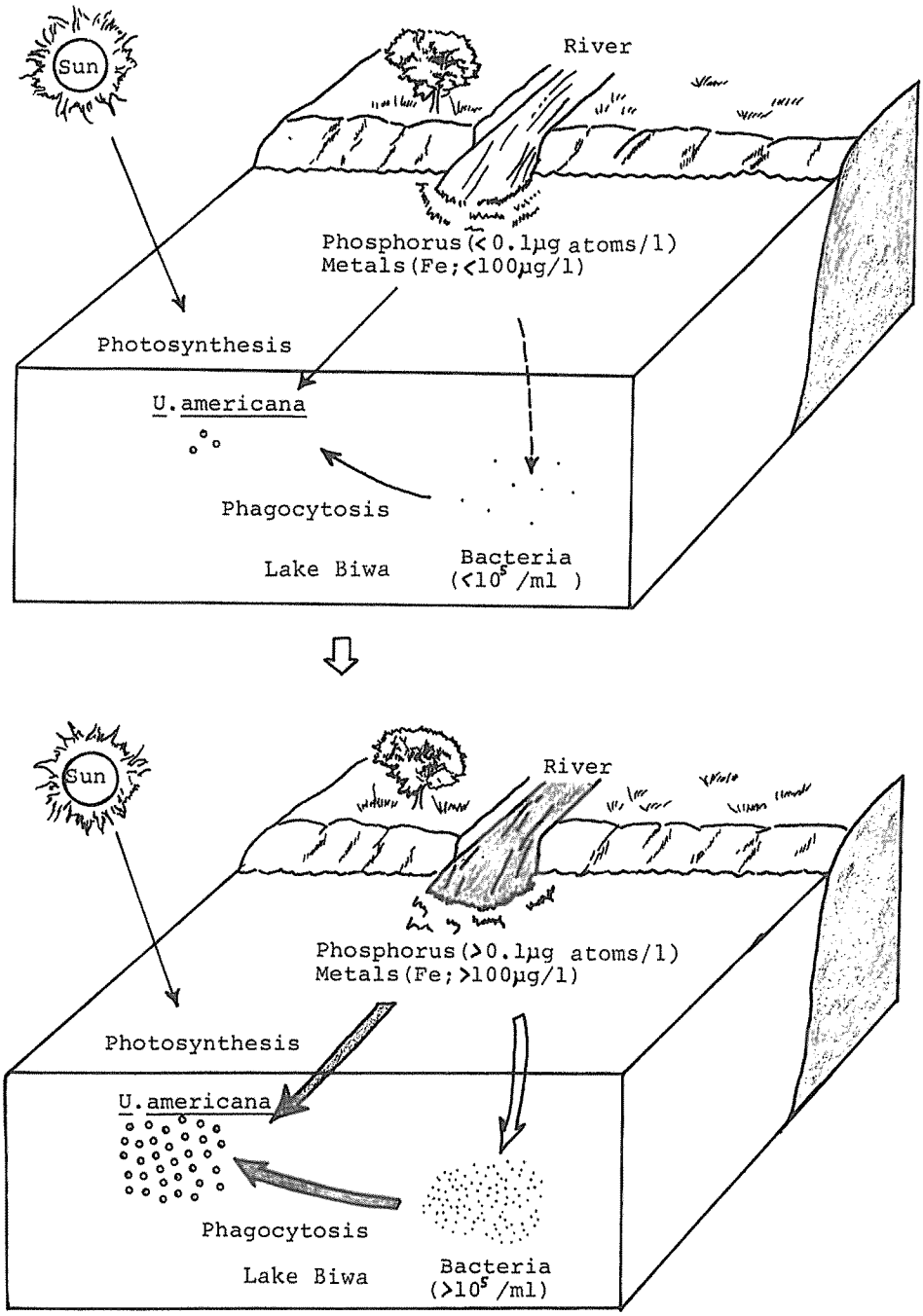

Fig. 40. Possible mechanism responsible for the development of the bloom of *U. americana* in Northern Lake Biwa.

population. This would then promote the growth of *U. americana*, with its growth apparently dependent on ingested bacteria as described by this study. Recently, Bird (1986) also reported that the growth of Chrysophyceae *Dinobryon* sp. is more dependent on ingested bacteria than on photosynthesis in natural water. From both laboratory and field data presented in Chapter 3, it is suggested that 1×10^5 cells/m*l* of bacterial population, the level usually observed in Lake Biwa, is a critical level above which the exponential growth of *U. americana* would be expected. Therefore, even a slight stimulation of bacterial growth caused by the inflow of organic matters would have a significant effect on the growth of *U. americana* in Lake Biwa.

The above two factors are not mutually independent. Perhaps the growth of *U. americana* leading to the bloom in Lake Biwa would be caused by a combination of these factors, if optimum physical conditions are present. To conclude, it is suggested that the control of phosphorus and organic matters are most effective in preventing the future bloom of *U. americana* in this lake.

Acknowledgement

The author wishes to express his thanks to Professor Yuzaburo Ishida of Kyoto University, for consistant kind guidance and encouragement during the course of this investigation.

The author also greatly thanks Dr. Hajime Kadota, Emeritus Professor of Kyoto University, Dr. Aritune Uchida, Assistant Professor of Kyoto University, and Dr. Yoshihiko Sako, instructor of Kyoto University, for consistant critical advice and helpful discussions.

Thanks are also due to Mr. Keizou Nagasaki, Mr. Mikio Tanaka and Mr. Hajime Takamatsu for co-operating in the work of this study and to the other members of the Laboratory of Microbiology, Department of Fisheries, Faculty of Agriculture, Kyoto Universty for their helpful suggestions and discussions. The author thanks Shiga Prefecture who was helpful in collecting water samples.

In addition, the author wishes to thank Professor Hiroaki Fujisawa and Professor Masatada Murakami, Shimonoseki University of Fisheries, for encouragement in preparing the manuscript.

References

1) Aaronson, S. (1973): Digestion in phytoflagellates, in "Lysosomes in Biology and Pathology" (ed. by Dingle J. T.), 18-37, North Holland Pub., Amsterdam.

2) Aaronson, S. (1973): Particle aggregation and phagotrophy by *Ochromonas*, *Archiv. Microbiol.*, **92**, 39-44.

3) Aaronson, S. (1974): The biology and ultrastructure of phagotrophy in *Ochromonas danica* (Chrysophyceae: Chrysomonadida), *J. gen. Microbiol.*, **83**, 21-29.

4) Aaronson, S. and Baker H. (1959): Comparative biochemical study of two species of *Ochromonas*, *J. Phycol.*, **6**, 282-284.

5) Allen, M. B. (1969): Structure, physiology, and biochemistry of the Chrysophyceae, *Ann. Rev. Microbiol.*, **23**, 29-46.

6) Baker, K. H. and Herson, D. S. (1978): Interactions between the Diatom *Thallasiosira pseudonanna* and an associated pseudomonad in a mariculture system, *Appl. Environ. Microbiol.*, **35**, 791-796.

7) Bauld, J and Brock, T. D. (1974): Algal excretion and bacterial assimilation in hot spring algal mats, *J. phycol.*, **10**, 101-106.

8) Bell, W. H. (1980): Bacterial utilization of algal extracellular products 2. A kinetic study of natural populations, *Limnol. Oceanogr.*, **25**, 1021-1033.

9) Bell, W. H. and Mitchell, R. (1972): Chemotactic and growth responses of marine bacteria to algal extracellular products, *Biol. Bull.*, **143**, 265-277.
10) Berland, B. R., Bonnin, D. J., and Maestrini, S. Y. (1970): Study of bacteria associated with marine algae in culture III. Organic substrates supporting growth, *Mar. Biol.*, **5**, 68-76.
11) Bird, D. F. and Kallf, J. (1986): Bacterial grazing by planktonic lake algae, *Science*, **231**, 493-494.
12) Blasco, R. J. (1965): Nature and role of bacterial contaminants in mass cultures of thermophilic *Chlorella pyrenoidosa*, *Appl. Microbiol.*, **13**, 473-477.
13) Bold, H. C. and Wynne, M. J. (1978): Introduction to the Algae, 706 pp, Printice Hall, INC, New Jersey.
14) Bradley, D. E. (1966) Observations on some chrysomonads from Scotland, *J. Phycol.*, **13**, 143-154.
15) Brown, E. J., Button, D. K., and Lang, D. S. (1981): Competition between heterotrophic and autotrophic microplankton for dissolved nutrients, *Microbial. Ecol.*, **7**, 199-206.
16) Calkins, G. N. (1892): On *Uroglena*, a genus of colony building infusoria, observed in certain water supplies of Massacheusettes, 23rd, Ann., Report State Bd. of Health of Mass.
17) Casper, S. J. (1972): Zum Feinbau der Geisseln der Chrysomonaden 1. *Uroglena americana*, Calkins., *Arch. Protistenk.*, **114**, 65-82.
18) Chu, S. P. (1942): The influence of the mineral composition of the medium on the growth of plankton algae I. Methods and culture media, *J. Ecol.*, 30, 284-325.
19) Cole, G. T. and Wynne, W. J. (1974): Endocytosis of *Microcystis aeruginosa* by *Ochromonas danica*, *J. Phycol.*, **10**, 397-410.
20) Collier, A., Wilson W. B., and Borkowski M. (1969): Responses of *Gymnodinium brave* Davis to natural waters of diverse origin, *J. Phycol.*, **5**, 168-172.
21) Daley, R. J., Morris, G. P., and Brown, S. R. (1973): Phagotrophic ingestion of a blue-green alga by *Ochromonas*, *J. Protozool.*, **20**, 58-61.
22) Delucca, R. and McCracken, M. D. (1976): Observation on interactions between naturally-collected bacteria and several species of algae, Hydrobiologial., **55**, 71-75.
23) Dodge, J. D. and Crawford, R. M. (1970): The morphology and fine structure of *Ceratium hirundinella* (Dinophyceae), *J Phycol.*, **6**, 137-149.
24) Droop, M. R. (1953): Phagotrophy in *Oxyrrhis marina* Dujardin, *Nature*, **172**, 250-251.
25) Droop, M. R. (1959): Water-soluble factors in the nutrition of *Oxyrrhis marina*, *J. Mar. Biol. Ass. U. K.*, **38**, 605-620.
26) Droop, M. R. and Doyle, J. (1966): Ubiquinone as a protozoan growth factors, *Nature*, **212**, 1474-1475.
27) Droop, M. R. and Elson, K. G. R. (1966): Are pelagic diatoms free from bacteria ?, *Nature*, **211**, 1096-1097.
28) Droop, M. R. and pennock, J. F. (1971): Terpenoid quinones and steroids in the nutrition of *Oxyrrhis marina*, *J. Mar. Biol. Ass. U. K.*, **51**, 455-470.
29) Dubowsky, N. (1974): Selectivity of ingestion and digestion in the chrysomonad flagellate *Ochromonas malhamensis*, *J. Protozool.*, **21**, 295-298.
30) Franke, W. W. and Herth, W. (1973): Cell and lorica fine structure of the chrysomonad alga, *Dinobryon sertularia* ehr. (Chrysophyceae), *Arch. Mikrobiol.*, **91**, 323-344.
31) Frecrickson, A. G. (1977): Behavior of mixed cultures of microorganisms, *Ann. Rev. Microbiol.*, **31**, 63-87.
32) Frey, B. E. and Small, L. F. (1980): Effects of micronutrients and major nutrients on natural phytoplankton populations, *J. Plankton Res.*, **2**, 1-22.
33) Gibbs, S. P. (1962): Chloroplast

development in *Ochromonas danica*, *J. Cell Biol.*, **15**, 343-361.
34) Gloschenko, W. A. and Curl, H. C., Jr. (1971): Influence of nutrient enrichment on photosynthesis and assimilation ratios in natural North Pacific phytoplankton communities, *J. Fish. Res. Bd. Canada*, **25**, 790-793.
35) Haines, K. C. (1974): Isolation of vitamin B_{12}-requiring marine bacteria on bioassay agar plates containing vitamin B_{12} requiring marine centric diatoms, *J. Phycol.*, **10**, 367-368.
36) Haines, K. C. and Guillard, R. R. L. (1974): Growth of vitamin B_{12} requiring marine diatoms in mixed laboratory cultures with vitamin B_{12}-producing marine bacteria, *J. Phycol.*, **10**, 245-252.
37) Harris, K. and Bradley, D. E. (1957): An examination of the scales and bristles of *Mallomonas* in the electron microscope using carbon replicas, *J. Roy. Microscop. Soc.*, **76**, 37-46.
38) Hibberd, D. J. (1973): Observations on ultrastructure of flagella scales in the genus *Synura* (Chrysophyceae), *Arch. Mikrobiol.*, **89**, 291-304.
39) Hibberd, D. J. (1976): The ultrastructure and taxonomy of the Chrysophyceae Prymnesiophyceae (Haptophyceae); a survey with some new observations on ultrastructure of the Chrysophyceae, *Bot. J. Linn. Soc.*, **72**, 55-80.
40) Hirayama, K., Izuka, S., and Yonegi, T. (1972): On culture of *Gymnodinium* type'65 in the sea water sampled in Omuta Bay during summer, 1971, *Bull. Faculty Fish, Nagasaki Univ.*, No. **33**, 11-20.
41) Hitchcock, G. L. and Smayda, T. J. (1977): Bioassay of lower Narragansett Bay waters during the 1972-1973 winter-spring bloom using the diatom *Skeltonema costatum*, *Limnol. Oceanogr.*, **22**, 132-139.
42) Hutner, S. H. and Holz, G. G. (1962): Lipid requirements of microorganisms, *Ann. Rev. Microbiol.*, **16**, 189-204.
43) Hutner, S. H. , Provasoli, L., and Filfus, J. (1953): Nutrition of some phagotrophic fresh-water chrysomonads, *Annal. N. Y. Acad. Sci.*, **56**, 852-862.
44) Irish, A. E. (1979): *Gymnodinium helveticum* Penard F. Achroum Skuja a case of phagotrophy, *Br. J. Phycol.*, **14**, 11-15.
45) Ishida, Y. and Kadota, H. (1983): Study of the bloom of *Uroglena americana*. in Lake Biwa, *Seikatsu Eisei*, **27**, 236-248 (in Japanese).
46) Ishida, Y. and Kimura, B. (1986): Photosynthetic phagotrophy of Chrysophyceae: evolutionary aspects, *Microbiological Science*, **3**, 132-135.
47) Ishida, Y. and Kimura, B., Nakahara, H., and Kadota, H. (1982): Analysis of major nutrient effecting *Uroglena americana* bloom in the Northern Lake Biwa, by use of algal assay, *Nippon Suisan Gakkaishi*, **48**, 1281-1287.
48) Ishida, Y., Shibahara, K., Uchida, H., and Kadota, H. (1980): Distribution of obligately oligotrophic bacteria in Lake Biwa, *Nippon Suisan Gakkaishi*, **46**, 1151-1158.
49) Jolley, E. T. and Jones, A. K. (1977): The interaction between Navicula muralis Grunow and an associated species of *Flavobacterium*, *Br. Phycol. J.*, **12**, 315-328.
50) Keating, K. I. (1977): Allelopathic influence on bluegreen bloom sequence in a eutrophic lake, *Science*, **196**, 885-886.
51) Kimura, B. (1984): The growth promotion of *Uroglena americana* (Chrysophyceae) by several species of bacteria, *Otsuchi Marine Reserch Center Report*, No. 10, 58-60 (in Japanese).
52) Kimura, B. and Ishida, Y. (1985): Photophagotrophy in *Uroglena americana*, Chrysophyceae, *Japan. J. Limnol.*, **46**, 315-318.
53) Kimura, B. and Ishida, Y. (1986): Possible phagotrophic feeding of bacteria in a freshwater red tide chrysophyceae *Uroglena americana*, *Nippon Suisan*

Gakkaishi, **52**, 697-701.
54) Kimura, B. and Ishida, Y. (1986): Evolutionary aspects of endocytosis in Chrysophyceae *Uroglena americana*, in "Biseibutsu no seitai", vol. 14, Gakkai Shuppan Center, pp. 171 − 182 (in Japanese).
55) Kimura, B. and Ishida, Y. (1989): Phospholipid as a growth factor of *Uroglena americana*, a red tide Chrysophyceae in Lake Biwa, *Nippon Suisan Gakkaishi*, **55**, 799−804.
56) Kimura, B., Ishida, Y., and Kadota, H. (1986): Effect of naturally collected bacteria on growth of *Uroglena americana*, a freshwater red tide Chrysophyceae, *Nippon Suisan Gakkaishi*, **52**, 691−696.
57) Kondo, M. (1933): *Suido Kenkyu shiryo* (Osaka Japan), No. 19, 308 (in Japanese).
58) Lange, W. (1970): Cyanophyta-bacteria systems: effects of carbon compounds or phosphate on algal growth at low nutrient concentrations, *J. Phycol.*, **6**, 230−234.
59) Lange, W. (1971): Enhancement of algal growth in cyanophyta-bacteria systems by carbonaceous compounds, *Can, J. Microbiol.*, **17**, 303−314.
60) Lee, R. E. (1980): *Phycology*, p. 478, Cambridge University Press.
61) Lund J. W. G., Jaworski, G. H. M., and Butterwick (1975): Algal bioassay of water from Blelham tern, English lake district and the growth of plankton diatoms, *Arch. Hydrobiol.*/Suppl., **49**, 49−69.
62) Lorenzen G. J. (1967): Determination of chlorophyll and phaeo-pigments: spectrometric equations, *Limnol. Oceanogr.*, **12**, 343−346.
63) Machilis, L. (1973): The effects of bacteria on the growth and reproduction of *Oedogonium cardiacum*, *J. Phycol.*, **9**, 342−344.
64) Manton, I. (1972): Observation on the biology and micro-anatomy of *Chrysochromulina megacylindra* Leadbeater, *Br. Phycol. J.*, **7**, 235−248.
65) Mayfield, C. I. and Innis, W. E. (1978): Interactions between freshwater bacteria and *Ankistrodesmus braunii* in batch and continuous culture, *Microbial Ecology*, **4**, 331−344.
66) McFeters, G. A., Stuart, S. A., and Olson, S. B (1978): Growth of heterotrophic bacteria and algal extracellular products in oligotrophic waters, *Appl. Environ. Microbiol.*, **35**, 383−391.
67) Myers, J. and Graham, J. R. (1956): The role of photosynthesis in the physiology of *Ochromonas*, *J. Cell. and Comp. Physiol.*, **47**, 397−414.
68) Nakamoto, N. (1977): A AGP method based on the growth of heterotrophic bacteria, *J. Water and Waste*, **19**, 87−94 (in Japanese).
69) Nakamura, H. (1963): Studies on the ecosystem of *Chlorella*, in "Studies on Microalgae and Photosynthetic Bacteria", Univ. Tokyo Press, pp. 197−204.
70) Nalewajko, C., Dunstall, T. G., and Shear H. (1976): Kinetics of extracellular release in axenic algae and in mixed algal-bacterial cultures: significance in estimation of total (gross) phytoplankton excretion rates, *J. Phycol.*, **12**, 1−5.
71) Nalewajko, C., Lee, K., and Fay, P. (1980): Significance of algal extracellular products to bacteria in lakes in cultures, *Microb. Ecol.*, **6**, 199−207.
72) Paerl, H. W. and Keller, P. E. (1978): Significance of bacterial-*Anabaena* (Cyanophyceae) associations with respect to N fixation in freshwater, *J. Phycol.*, **14**, 254−260.
73) Parker, B. C. and Bold H. C. (1961): Biotic relationships between soil algae and other microorganisms, *Amer. J. Bot.*, **48**, 185 − 197.
74) Pintner, I. J. and Provasoli, L. (1968): Heterotrophy in subdued light of 3 *Chrysochromulina* species, *Bull. Misaki Marine Biol. Inst. Kyoto Univ.*, **12**, 25−31.
75) Pringsheim, E. G. (1952): On the nutrition of *Ochromonas*, *Quart. J. Microscop. Sci.*,

93, 71−96.
76) Provasoli, L. (1958): Nutrition and ecology of protozoa and algae, *Ann. Rev. Microbiol.*, **12**, 279−308.
77) Provasoli, L. and Carlucci, A. F. (1974): Vitamins and growth regulators, in "Algal Physiology and Biochemistry" (ed. by Stewart, W. D. P.), pp. 741−787, Blackwell Scientific Publications, Oxford.
78) Provasoli, L. and Pintner, I. J. (1960): Artificial media for fresh-water algae, in "The Ecology of Alga" (ed. by Tryon C. A. and Hartman R.), pp. 84−96, Spec. Publ., No. 2, University of Pittsuburgh Press, Pittsburgh.
79) Provasoli, L. and Pintner, I. J. (1980): Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae), *J. Phycol.*, **16**, 196−201.
80) Richey, J. E. (1979): Patterns of phosphorus supply and utilization in Lake Washington and Findley Lake, *Limnol. Oceanogr.*, **24**, 906−916.
81) Ryther, J. H. and Guillard, R. R. L. (1959): Enrichment experiments as a means of studying nutrients limited to phytoplankton populations, *Deep Sea Res.*, **6**, 65−69.
82) Schindler, D. W. (1978): Factors regulating phytoplankton production and standing crop in the world's freshwaters, *Limnol. Oceanogr.*, **23**, 478−486.
83) Shorb, M. S. and Lund, P. G. (1959): Requirement of *Trichomonads* for unidentified growth factors, saturated and unsaturated fatty acids, *J. Protozool.*, **6**, 122−130.
84) Skulberg O. M. (1964): Algal problems related to the eutrophication of European water supplies, and a bioassay method to asses fertilizing influences of pollution on inland waters, in "Algae and Man" (ed. by Jackson, J.), pp. 262−299, Plenum press, New York.
85) Skulberg, O. M. (1970): The importance of algal cultures for the assessment of the eutrophication of the Oslofjord, *Helgolalnder Wiss. Meeresunters.*, **20**, 111−125.
86) Smayda, T. J. (1970): Growth potential bioassay of water mass using diatom cultures: Phosphorescent Bay (Puerto Rico) and Caribbean waters, *Helgolander wiss. Meeresunters*, **20**, 172−194.
87) Soldo, A. T. and Wagtendonk, W. J. V. (1967): An analysis of the nutritional requirements for fatty acids of *Paramecium aurelia*, *J. Protozool.*, **14**, 596−600.
88) Soldo, A. T. and Wagtendonk, W. J. V. (1969): The nutrition of *Paramecium aurelia*, stock 299, *J. Protozool.*, **16**, 500−506.
89) Spero, H. J. and Moree, M. D. (1981): Phagotrophic feeding and its importance to life cycle of the holozoic Dinoflagellate, *Gymnodinium fungiforme*, *J. Phycol.*, **17**, 43−51.
90) Stoltze, H. J., Lui, N. S. T., Anderson, O. R., and Roels, O. A. (1969): The influence of the mode of nutrition on the digestive system of *Ochromonas malhamensis*, *J. Cell. Biol.*, **43**, 396−409.
91) Storm, J. and Hutner, S. H (1953): Nutrition of *Peranema*, *Ann. N. Y. Acad. Sci.*, **56**, 901−909.
92) Thomas, J. P. (1971): Release of dissolved organic matter from natural populations of marine phytoplankton, *Mar. Biol.*, **11**, 311−323.
93) Ukeles, R. and Bishop, J. (1975): Enhancement of phytoplankton growth by marine bacteria, *J. Phycol.*, **11**, 142−149.
94) Vance, B. D. (1965): Composition and succession of cyanophycean water blooms, *J. Phycol.*, **1**, 81−86.
95) Vance, B. D. (1966): Sensitivity of *Microcystis aeruginosa* and other blue-green algae and associated bacteria to selected antibiotics, *J. Phycol.*, **2**, 125−128.
96) Wujek, D. E. (1969): Ultrastructure of flagellated chrysophyceae I. *Dinobryon*, *Cytologia*, **34**, 71−79.
97) Wujek, D. E. (1976): Ultrastructure of

flagellated Chrysophytes II. *Uroglena* and *Uroglenopsis*, *Cytologia*, **41**, 665−670.
98) Yamamoto, H., Ezura, Y., and Kimura, T. (1982): Evaluation of bacteriological agents affecting on the survival of *Vibrio parahaemoliticus* in seawater, *Nippon Suisan Gakkaishi*, **48**, 1433−1439.
99) Yoshida, Y., Mitamura, O., and Kadota, H. (1983): Studies on a freshwater red tide in Lake Biwa I. Changes in the distribution of phytoplalnkton and nutrients, *Japan J. Limnol.*, **44**, 21−27 (in Japanese).
100) Yoshimura, S. (1976): Kosho-Gaku (Limnology), p. 439, Seisan-gijutsu center, Tokyo.

淡水赤潮藻 *Uroglena americana* の増殖に及ぼす生理・生物的因子に関する研究

木 村 凡

近年淡水赤潮の発生が多く，その発生機構の解明が必要とされている。本研究は，淡水赤潮の原因種 *Uroglena americana* の増殖機構を明らかにする目的で，*U. americana* の異常増殖に影響を与えている生理・生物的因子の解明を試みたものである。その結果，*U. americana* の赤潮発生期の現場においては，生理的因子としては，リンおよび微量金属が同時に本藻の増殖の第一次制限因子になっている可能性を algal assay 実験により証明した。また，本藻は光合成と同時に細菌を摂食し，発育因子を補わなくては増殖することができないという事実を明らかにした。したがって，現場における *U. americana* の異常増殖の前提となる生理・生物的環境は，現場へのリン，微量金属の流入，および有機物の流入に伴う細菌数の増加が，複合的，相乗的に作用して形成されるものと推定できる。