Analysis of Major Nutrient Effecting *Uroglena americana* Bloom in the Northern Lake Biwa, by Use of Algal Bioassay*¹

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In an attempt to discover the major nutrients controlling *Uroglena americana* bloom in the Northern Lake Biwa, the algal bioassay was conducted by using a monoxenic culture of *U. americana* isolated from the bloom. From the bioassay with or without addition of nitrogen, phosphorus, iron and/or vitamins to unenriched lake water, it was discovered that phosphorus and iron were the first limiting nutrients for the algal bloom, and that the lake water contained a sufficient quantity of nitrogen.

The great bloom by *Uroglena americana* occurred at the end of May to the beginning of June, 1977, in the Northern Lake Biwa, which had been believed to be an oligotrophic lake. Since then the freshwater red tide of *U. americana* occurs in late spring to early summer of every year. Historically the presence of *U. americana* in the Lake Biwa was reported by KONDO¹⁾ 1933, but no one had found the bloom of *U. americana* until 1976.

In an attempt to discover the major nutrients controlling this bloom in the Northern Lake Biwa, and algal bioassay was conducted with a monoxenic culture of *U. americana* which was isolated from the 1978-bloom in the Northern Lake Biwa. The algal bioassay used in this study, therefore, is different from the common AAP (Algal Assay Procedure Bottle test)²⁾ which has been usually used for assessment of potential fertility of water. The algal bioassay is the most controversial matter in analysis of algal bloom from the methodical point of view. Hitherto, only several researchers^{3,4,5,6)} examined the potential role of nutrients during algal bloom by the algal bioassay using the bloom algae. They mutually differ in the methods treating water sample. HITCHCOCK and SMAYDA,³⁾ COLLIER *et al.*,⁴⁾ and HIRAYAMA *et al.*⁵⁾ used the water samples filtered through glass fiber filter and frozen before use. LUND *et al.*⁶⁾ also filtrated the water through glass fiber filter without freezing. In both samples

bacteria in part were not removed. It is clear that uncomplete sterilization of water (e.g. GF/C filtration) brings undesirable changes in water sample before and during the test. By algal bioassay experiments in 1978- and 1979-blooms it was observed that in autoclaved water sample the addition of EDTA-Fe to it gave better growth of *U. americana* than that of other elements, and that in the frozen filtered water samples positive effect of vitamin B₁₂ have been remarkable, although effectiveness of the addition of EDTA-Fe or vitamin B₁₂ to the filtered water sample were not observed. Considering these observations it was assumed that the drastic treatments such as autoclaving or freezing of water sample give artificial results. In this study *U. americana*-monoxenic culture was inoculated into the water samples which had been aseptically filtered by using 0.22 μ m membrane filter, immediately after water collection.

Methods and Materials

Sampling Station

Water samples were collected with Van Dorn sampler (5 liter) from 3 m depth at station A from January 1980 to June 1981, from 3 m depth at station C and from 10 m depth at station A during April-December, 1980, from 3 m depth of station N4 during April-September, 1980, and

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Fig. 1. Location of sampling stations of Lake Biwa.

from 3 m depth of stations E and F during April-June, 1980. These stations distribute in the Northern Lake Biwa (Fig. 1). Within 3 h after

the water samples were collected and maintained in sterilized 500 ml Pyrex glass bottle (precombusted for 1 h at 450°C) in ice, the water samples were treated according to the following procedure.

Procedure of the Algal Bioassay

Procedure of algal bioassay was described in Fig. 2.

Process-1: The water samples were aseptically filtered through sterilized 0.22 μ m membrane filter (Millipor filter GS).

Process-2: Four ml aliquots of the filter-sterilized water samples were transferred to pre-rienced and precombusted glass tube (13 ϕ × 105mm) covered by polypropylene cap. Prior to the transfer the tubes were enriched in six different ways (at final concentration) and autoclaved: None, No nutrient added; N, ammonium nitrate (1.75 mg N/l); P, β -Na-glycerophosphate (0.42 mg P/l); V, vitamin mixture (vitamin B₁ 10, vitamin B₁₂ 0.1 and biotin 0.1 μ g/l); M, PIV metal mixture (EDTA-Na₂ 1.0, Fe(as Cl) 0.04, Mn(as Cl) 0.01, Zn (as Cl) 0.005, Co (as Cl) 0.001, Mo (as Na) 0.005 mg/l); All, all four elements added. The concentrations of those four elements are based on chemically defined medium Ur-1 (unpublished data).

Process-3: The cultres of *U. americana** (in which a bacterial strain N1A of *Vibrio-Aeromonas* group exists together with *U. americana*) were

Fig. 2. Diagram of experimental procedure of the algal bioassay. None, no addition; N, NH₄NO₃ added; P, β -glycerophosphate added; V, B₁+B₁₂+biotin added; M, PIV metal added; All, N+P+V+M added.

* *U. americana* cannot grow in Ur-1 medium without some kind of bacteria.

Fig. 3. The algal bioassay of water samples frozen or autoclaved after 0.22 μ m filtration.

Filtration, only 0.22 μ m sterilized water samples; Freezing, water sample frozen after 0.22 μ m filtration; Autoclaving, water sample autoclaving after 0.22 μ m filtration.

maintained in Ur-1 medium. Exponentially growing culture was washed on a sterilized 5 μ m Nylon net (Nytal, Switzerland) with unenriched filtered lake water to eliminate carryover of the culture medium, and was resuspended in filter-sterilized lake water. The population density of the inoculum was approximately 30 colonies ml^{-1} .

Process-4: A 4 ml of unenriched and enriched tubes (in triplicate) was inoculated with 0.2 ml of the inoculum.

Process-5: The tubes were incubated at 15°C, and 4,000 lux on a 14L: 10D photoperiod cycle.

Growth in the tubes was determined as a concentration of chlorophyll *a* at intervals of two days by using modified Turner fluorophotometer (110 type) equipped with photomultiplier RCA-R136 and with Corning 2-64 filter. Growth in unenriched water (none) was estimated as an average in triplicates of Turner reading in stationary phase, and growth in enriched cultures was expressed as a difference in Turner reading (in triplicates) between an unenriched culture and an enriched culture in stationary phase.

If the long period storage of filtered lake waters sterilized by freezing or autoclaving is possible

without undesirable changes in quality, it would be very convenient for us to do bioassay experiments. However, the bioassay values of water samples frozen or autoclaved after 0.22 μ m filtration were lower than that of water samples treated with 0.22 μ m sterile filtration techniques (Fig. 3). Then in this study the filter-sterilized waters were assayed as soon as possible on the day of collection.

The amount of chlorophyll-*a* was determined by Lorenzen method.⁷⁾

Results

Bioassay for Unenriched Water Samples

Mean growth yields of *U. americana* in unenriched filtered sample of lake water collected from 3 m depth of station A were shown in Fig. 4. Algal growth exceeding Turner reading 20 (0.3 $\mu\text{g chl. } a/\text{l}$, which corresponds with approximately twice as high as initial concentrations of *U. americana*.) was regarded as effective growth.

In the period from January, 1980, to June, 1981, the bioassay in unenriched water gave effective growth in five experiments (March, early June, November, 1980, and January, early June, 1981), among which two were just after the bloom (early June). The *in situ* concentrations of DIN, DIP, filtered vitamin B₁₂ and filtered iron in these two early June were not significantly different from those of each element in the other dates; DIN 3–

Fig. 4. Seasonal changes in the algal bioassay values (E) for unenriched filtered lake water sample and the amounts of chlorophyll *a* (D), DIN (C) and DIP (B), and water temperature (A) at 3 m depth of station A in Lake Biwa, 1980–1981.

Fig. 5. Horizontal profiles of the algal bioassay values for unenriched filtered water samples at each station of Lake Biwa.

17 $\mu\text{g atoms/l}$, DIP 0.01–0.14 $\mu\text{g atoms/l}$, vitamin B_{12} 0.1–3.5 ng/l and iron 90–170 $\mu\text{g/l}$ (data of B_{12} and iron were provided by A. KURATA, personal communication).

This result was also nearly similar in stations E and F, where inflow of nutrient would be larger than that at stations A, C, and N4 (Fig. 5).

Effectiveness of the Addition of Each Element 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 an effectiveness of addition of each element on *U. americana* growth. The result in enriched samples of lake water at 3 m depth of station A is shown in Fig. 6. With addition of ammonium nitrate there was no significant stimulation of growth in the bioassay during 1980–1981 (Fig. 6-B). When β -Na-glycerophosphate was added, positive results

Fig. 6. Seasonal changes in the algal bioassay with addition of nitrogen (B), phosphorus (C), vitamins (D), metals (E) and all four elements (F) to unenriched filtered lake water at 3 m depth of station A, and colony number of *U. americana* (A). Growth was expressed as a value which was subtracted Turner reading of unenriched culture from that of enriched culture in the stationary phase.

were obtained on second half of May 1980 (a) and early of May 1981(b), just before *U. americana* was abundant at station A (Fig. 6-C). And other increased growth was in late April, early July, late September and late December, 1980. Effective growth of *U. americana* by phosphate addition was not observed during spring in both years (January-early April).

The addition of metal mixture generally gave better growth than that of other element alone (Fig. 6-E). During the large bloom of *U. americana* in the lake in both 1980 and 1981, however, there were very weak positive results, though the concentration of iron in filtered lake water (100 to 150 $\mu\text{g/l}$) during this period was not higher than that in the other period. The experiment carried out with addition of Na-EDTA alone gave the result similar to that with metal mixture (Fig. 6-F).

If vitamin mixture was added, positive results were sporadically obtained. These good growth did not correspond with a fluctuation in the concentration of vitamin B_{12} essential vitamin for

Fig. 7. Seasonal changes in the algal bioassay with addition of nitrogen (A), phosphorus (B), vitamins (C), metals (D), and all four elements (E) to unenriched filtered lake water at 10 m depth of station A(a), and 3 m depth of stations C(b), N4(c), E(d) and F(e).

Fig. 8. Comparison of the algal bioassay with addition of two or three elements from that with addition of either element alone to filtered lake water at 3 m depth of station A. A) phosphorus, B) vitamins, C) methals, D) all four elements, E) phosphorus+vitamins, F) phosphorus+metals, G) vitamins+metals, and H) phosphorus+vitamins+metals.

growth of *U. americana*, which began to fall in 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. 6-D).

The results obtained in bioassay of water sample from 3 m depth of station A with addition of nitrogen, phosphorus, vitamin, or metal alone was nearly similar in the results with water samples

from 10 m depth of station A, and 3 m depth of stations N4, C, E and F (Fig. 7-A, -B, -C, -D).

Addition of those all four elements (complete enrichment) to unenriched filtered lake water at station A gave generally the high growth, but weak positive result was observed several times, especially in large blooms (late May, 1980, and late May, early June, 1981) (Fig. 6-F). At the

other station also strong positive results were mostly obtained except the water samples collected from station C on middle April, 1980. Some growth inhibitor may have been contained in the river water of Ado river which flowed in Lake Biwa.

Bioassay with Addition of Two or Three Elements

As shown in Fig. 8 the addition of both elements (phosphorus + vitamin, phosphorus + metal, and vitamin + metal) commonly gave better growth than either element alone. Growth was greater with the three elements (phosphorus + vitamin + metal) than with two elements in the period from October 1980 to June 1981, except April 16, 1981.

Discussion

By our laboratory experiment (unpublished data) the nutrients concentrations essential for minimum growth* of *U. americana* (14–55 $\mu\text{g N/l}$ as NH_4^+ or NO_3^- , 10 $\mu\text{g P/l}$ as PO_4 , 40 $\mu\text{g Fe/l}$ as Fe-EDTA, and 10 ng B_{12}/l as cyanocobalamin) are defined. When comparing the concentrations for the minimum growth with the *in situ* concentration of dissolved inorganic phosphate, dissolved inorganic nitrogen ($\text{NO}_3^- + \text{NO}_2^- + \text{NH}_4^+$), filtered vitamin B_{12} and filtered iron in the lake water, it is clear that the lake water contains sufficient quantity of nitrogen to support minimum growth for bloom occurrence (final cell yield corresponds to 6–10 $\mu\text{g chl. a}$ per liter of water). From the result of algal bioassay, however, it seems that the concentrations of phosphorus (less than 3 $\mu\text{g/l}$), vitamin B_{12} (less than 1 ng/l) and iron (less than 200 $\mu\text{g/l}$) 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 aerobic condition. This fact nearly corresponds with the results by bioassay, that phosphorus, vitamin B_{12} and soluble iron are generally the major limiting factors in the Northern Lake Biwa. More detail observation shows that phosphorus is the first limiting factor in the time just before the large bloom of *U. americana* occurs (Fig. 6-A and -B). Considering that the amount of intracellular phosphorus μg per chlorophyll a μg of *U. americana* during bloom determined by our laboratory experiment (unpublished data)

was approximately 2, the concentration of phosphorus in lake water necessary for the bloom was calculated roughly at 12–20 $\mu\text{g P/l}$. This concentration is about 10 times higher than dissolved inorganic phosphorus in lake water. As this period is on stratification, it seems possible that land drainage is the chief point source of dissolved phosphate. Of course the possibility of iron as the first limiting factor as well as phosphate is also not negligible during this period. In the lake water the concentration of filtered iron (not dissolved iron) during April–November, 1980, ranged from 100 to 150 $\mu\text{g Fe}$ per liter. By consulting our laboratory data these values are seemingly enough for *U. americana* to grow. The fact in bioassay, however, that iron was the major limiting factor suggests that the large amount of iron is likely to be in oxidised iron state. The result in Fig. 6-F that the addition of EDTA-Na generally showed the similar results to those of EDTA-Fe (as PIV metals mixture) would be presumptive evidence that the concentration of iron utilizable for *U. americana* in the lake is negligible.

Also from the bioassay it appears that vitamin B_{12} was a major limiting factor at least during April–June and August–January, but not during February–middle April in 1980 and 1981 though 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 NIA contaminated in *U. americana* culture employed as inoculum produces vitamin B_{12} .

In general phosphorus is a major limiting factor for algae in oligotrophic water. According to YOSHIMURA's definition⁹⁾ even now the Northern Lake Biwa is placed under the category of oligotroph in reference of the *in situ* concentration of total phosphorus less than 20 $\mu\text{g/l}$, as well as the lake before 1977. Nevertheless why did the great bloom of *U. americana* suddenly occur in 1977? Now we can not accurately answer to the question. It is probable that phosphorus, soluble iron and vitamin B_{12} has been the major limiting chemical factors of the algal growth until 1977 as well as after 1977. It is assumed that a gradual increase of the load of phosphorus and organic substances from the catchment area of the lake and a increase of bacterial activity which produces

* For the occurrence of the bloom of *U. americana* it is necessary to have a minimum growth of this species and the proper hydrographic and meteorological conditions to allow an accumulation at the surface. The minimum growth means the algal density equivalent to 6–10 $\mu\text{g chl. a/l}$, according to field data.

organic chelators, vitamin B₁₂ etc from organic substances, bring the largest bloom of the algae 1977. On the other hand, the specific physiological characteristics of *U. americana* which can grow well in such extremely low concentration of phosphate-P (3–10 µg per liter) as other unicellular algae⁹⁾ cannot grow (unpublished data) also can not be overlooked.

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