O-1 Development of assessment methods for water pollution and monitoring methods for toxic cyanobacteria in water resource regions in Asia (Abstract of the Final Report)

Contact person Kunimitsu Kaya
Director, Laboratory of Intellectual Fundamentals
for Environmental Studies,
National Institute for Environmental Studies
Onogawa 16-2,Tsukuba, Ibaraki, 305-8506 Japan
Tel:+81-298-50-2428 Fax:+81-298-50-2900
E-mail:kayakuni@nies.go.jp

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1. Introduction

As eutrophication progress, toxic cyanobacteria (blue-green algae) have been detected in freshwater reservoirs throughout the world. In Asia, freshwater in reservoirs have been utilized not only drinking, industry and agriculture but also aquaculture. The aquaculture is one of the most important businesses for supplement of food. However, toxic cyanobacteria have induced the decrease in the aquacultural productivity. The peoples of Asia desire the water quality that it causes a highly plankton productivity, but does not cause the occurrence of toxic cyanobacteria.

2.Research Objective

The objective of this study is development of assessment methods for water pollution and monitoring methods for toxic cyanobacteria in water resource regions in Asia. In order to develop the above methods, we clarify inflows of nitrogen and phosphorus into eutrophicated freshwater reservoirs, and simulate changes in the water quality of the reservoirs in future. To decrease nitrogen and phosphorus in freshwater, a biomanipulation method applied ecosystem are tested. On the other side, toxic cyanobacterial waterblooms are occurred in eutrophic freshwater reservoirs. As actual researches of this project, we focus on: (1) effect of variations in external pollutant loadings on water quality in lakes, (2) restoration of water quality by enforcing food web structure in lakes, (3) predicting the occurrence of cyanobacterial waterbloom, (4) controlling methods of the blooms using natural products, (5) monitoring of toxic cyanobacteria based on toxin genes, and (6) monitoring of cyanobacterial toxins using chemical techniques. At the ending of this project, we perform to develop a new

management method of water quality for freshwater reservoirs in Asian region by systematizing of the developed methods.

3. Results and Discussion

3-1 Effect of Variations in External Pollutant Loadings on Water Quality in Lakes

A physical-ecological coupling model was developed as a mathematical tool to analyze the effect of variations in characteristics of terrestrial pollutant loadings on water quality and species composition of phytoplankton in lakes. Based on the model proposed by Nakata et al.[1], growth rates of diatom and blue-green algae are formulated with different dependency on temperature and salinity. Not only grazing food webs but also microbial loops based on bacteria were incorporated in the model. Calculations were conducted with database in 1998. The model well predicted seasonal variations of water quality and the difference in dominant species of phytoplankton between the Lake Shinji and Lake Nakaumi.

Temporal variations in external loading from Lakes Shinji-Nakaumi basin was estimated by normal unit-loading method since 1986. External loading of phosphorus is estimated to be declining rapidly, whereas COD and nitrogen to the lakes are only slightly decreasing. However, variations in water quality of the lakes showed no clear tendency of recovering. Internal loading, especially for phosphorus release form sediments under anoxic conditions, is attributed to the phenomenon. The model analysis by the physical-ecological model also support the importance of internal loading of phosphorus.

3-2 Restoration of Water Quality by Enforcing Food Web Structure in Lakes

In Lake Shinji, bivalve *Corbicula japonica* inhabited in the littoral sediment of the shallow coastal zone, while its prey phytoplankton predominates in the central pelagic zone. In order to investigate material circulation through food web from primary producer and to higher trophic level, it is necessary to understand the coupling between littoral benthic system and pelagic system. We conducted field survey in a north-south cross section of the lake by employing TDS, chlorophyll, and turbidity sensors and velocimeters. Low temperature water mass often developed in the littoral region, especially in the northern shallower littoral zone of the lake. Associated numerical analysis revealed that heat-induced flow structure was prevailed in the lake and that it played important role in connecting benthic and pelagic systems under relatively calm weather conditions.

So far, little is known about standing shock and migration pattern of commercially important fishes. Variation of fish assemblages was investigated through repeated field surveys to clarify the migration pattern and growth rates of marine, brackish and freshwater species in Lake Shinji and adjacent Lake Nakaumi. In Lake Shinji, two types of brackish fishes were distinguished: one that migrate in to the lake during the

warmer seasons; and the other which stayed during winter for reproduction. Even with the recent acceleration in eutrophication of the lake, fisheries yield during the warmer seasons had not declined, however, the fish fauna have drastically changed. Warmer temperature in winter also has impacts on fish assemblages.

3-3 Study on methods for predicting the occurrence of water bloom (Aoko)

(1)Statistical analyses on relationships between lake environmental factors and the occurrence of water bloom (Aoko)

We have acquired lake environmental data for 40 lakes located in China through Dr. Jin Xiang in Water research Institute of Environmental Sciences, Chinese Research Academy of Environmental Sciences. Relationships between the environmental parameters and the occurrence of water bloom were analyzed statistically and compared with those for lakes in Japan. It was found that in Chinese lakes, green algae were more probable to occur as compared with the lakes in Japan. When dissolved solids are greater than 500 mg/L, or the lake size is small with a water depth less than 2 m, or suspended solids are greater than 200 mg/L, in those cases, typical water-blooming cyanobacteria *Microcystis sp.* are not predominant.

(2) Development of methods for evaluating the environmental factors controlling the occurrence of water bloom (Aoko)

An experimental methodology has been developed for evaluating key environmental factors controlling the occurrence of water bloom in lake waters. In particular, we focused on the presence of dissolved organic matter (DOM), which is likely to influence substantially the occurrence of water bloom. Since the currently available algal-growth-potential (AGP) tests neglect completely the presence of DOM, we developed a new-type of AGP test procedure in which using ultraviolet-ray (UV) radiation, the DOM concentration in the sample solution can be altered without decreasing the concentrations of inorganic and trace-metal constituents. By applying this AGP test to water samples collected from Lake Kasumigaura, we were able to elucidate the effect of DOM on the growth of water-bloom cyanobacterium *Microcystis aeruginosa*. The presence of DOM depressed clearly the growth of *M.aeruginosa*.

Iron is an essential element and very important to the growth of water-bloom cyanobacteria. It is well known that chemical speciation of iron affects its bioavailability to cyanobacteria. In order to evaluate the speciation of iron in lake water, we have developed a polarographic method called competitive adsorptive cathodic stripping voltammetry. Applying this method to water samples of Lake Kasumigaura, we found that more than 99.9% of the dissolved iron was complexed with dissolved organic matter. Bioavailable iron, that is free and hydrolyzed iron concentrations were extremely low in Lake Kasumgiaura.

We also applied this voltammetric method to three water samples (Sts. 1, 2 and 3) collected in an agricultural drain channel where a massive *Microcystis sp.* water bloom

occurred. Drained water flows down St.3, St.2 and St.1, and finally empties into Lake Kasumigaura. The cell concentration of *Microcystis* was increased upstream. No *Microcystis* colonies were found at St.1, a great number of the colonies were present at St.2, and massive algal mats were formed at St.3. The traditional AGP test using *Microcystis aeruginosa* as a test strain produced an interesting result. *M. aeruginosa* was nitrogen- and iron-limited in St.1, nitrogen-limited in St. 2, and iron-limited in St.3. Concentrations of nitrogen, phosphorus and dissolved iron all increased from St.1 to St.3. Thus, the trend in the concentration of dissolved iron along the sampling points was not consistent with the AGP results obtained for the St.3 sample where iron was limiting. However, the free and hydrolyzed iron concentration was found to be greater in St.2 than in St.3. This corresponded to the state of iron limitation at St.3. Therefore, it is very likely that iron speciation is a key factor in controlling the growth and dominance of water-bloom cyanobacteria (Aoko).

Algorithms and prediction models for suspended solids and chlorophyll-a estimation using Landsat TM images were developed and tested with real data collected at Lake Kasumigaura, in order to evaluate the possibility of monitoring the occurrence of algal-bloom in a large lake easily and economically. The model was developed successfully, but deviations between the model predictions and the real data were relatively large. More wide-range concentration data were needed to improve the model accuracy in a eutrophic lake such as Lake Kasumigaura.

3-4 Controlling of Cyanobacterial Waterblooms using Natural Products

Allelochemicals were searched from microalgae. We found a cell growth inhibitor against toxic *Microcystis* from natural waterblooms of *Anabaena spiroides* collected from a pond in Chiang Mai, Thailand(2). The cell growth inhibitor was named as "spiroidesin". Spiroidesin, a novel D-amino acid-containing linear lipopeptide, was isolated from waterblooms of the cyanobacterium. The structure was identified by 2D NMR, HRFABMS and chemical degradation analyses, and was established as [(Hexanoic acid)-(D-Homotyrosine)-(L-Homotyrosine)-(D-Phenylalanine)]. Spiroidesin inhibited cell growth of the toxic cyanobacterium Microcystis aeruginosa (IC_{50}, 1.6 x 10^{-6}M).

To identify the algicidal compounds, yeast extract was fractionated using ion-exchange columns and thin layer chromatography (TLC). Two algicidal compounds were isolated. One was adsorbed to DEAE Sephadex and eluted with the 0.4 M ammonium carbonate-containing solvent. The structure of the compound was elucidated as malonic acid by high resolution fast atom bombardment mass spectrometer (HRFABMS) and 1H and 13C-NMR spectra. Another one was adsorbed to CM-Sephadex and eluted with the 0.2 M ammonium carbonate containing solvent. The compound also purified using TLC. From the spectral data of HRFABMS and NMR, the structure of the compound was identidied as L-lysine. Cells of *M. viridis* were completely killed within 48 hrs by

L-lysine at a concentration of 1.0 ppm. In the case of malonic acid, the minimum lethal concentration was 40 ppm.

In this year, we have examined an enclosure experinent for selective conterol of microcystis blooms using lysine and malonic acid. The enclosure experiments conducted from 28 July to 26 August, 2003, nearby Dianchi Lake, Kunming, PR China. Three enclosures (10 m x 10 m x 1.5-1.3 m depth) were set. The enclosures were filled with the cyanobacteral (*Microcystis aeruginosa*) waterblooms- containing lake water. Moreover, the lake sediment contained seeds of macro-algae and water chestnut was spread all over the bottoms of the enclosures. Initially, 10 g/m^2 of lysine were sprayed to the enclosure B, and both of lysine (10 g /m^2) and malonic acid (10 g/m^2) were sprayed to the enclosure C. Enclosure A remained untreated and was used as a control. Cells of phytoplankton such as cyanobacteria, diatom and euglena were counted and monitored. Also the concentrations of lysine, malonic acid, chlorophyll a and microcystin were monitored. One day after the treatment, Cyanobacterial blooms in enclosures B and C were almost precipitated, while cells of *Microcystis* in the control still formed blooms. On 7 days after the treatment, *Microcystis* cells in enclosure B treated with lysine were grown again, whereas *Microcystis* cells in enclosure C treated with lysine and malonic acid were still disappeared. On 28 days after the treatments, the surface of enclosure B was covered with leaves of water chestnut (*Trapa* sp.). However, *Microcystis* blooms were increased again in enclosure B. In contrast, macroalgae (*Myriophllum spicatum* L. and *Potamogeton crispus* L.) were grown, and no cyanobacterial blooms were observed in enclosure C. Lysine, malonic acid and microcystin in enclosure C were decomposed completely. Furthermore, pH of enclosure C was shifted from 9.2 as the initial pH to 7.8. From the results, we concluded that the treatment with lysine and malonic acid was an effective method for extermination of toxic *Microcystis* waterblooms.

As a conclusion, the treatment with lysine plus malonic acid is an effective method for the controlling of toxic cyanobacterial blooms, and induces that the incorporation cycles of nitrogen and phosphorus in eutrophicated water switch from cyanobacteria to macroalgae.

3-5 Toxin Genes of Toxic Cyanobacteria

Cyanotoxins produced by various waterbloom-forming cyanobacteria are of worldwide concern, because these toxins present a considerable threat to the health of animals and humans. To develop an experimental system for monitoring toxic cyanobacteria, first we focus on the genus *Microcystis*, a most abundant toxic cyanobacterium in waterblooms. Five set of primers targeted to amplify five separate region of a microcystin synthetase gene clusters[(2)] are tested for detections of toxic genes from 20 Japanese isolates of *Microcystis* spp (NIES strains) using PCR experiments. Although there is a little difference as to the relative performance of PCR amplification, all sets of primers successfully recovered corresponding sequences from all toxic strains

tested (4 strains), as confirmed by a direct sequencing of amplified fragments. However, these experiments also identify toxin synthesis genes from two strains that do not produce detectable amounts of toxin. This result suggests that these two strains could produce toxin on certain unknown conditions. We fail to detect considerable amounts of amplicons from other non-toxic strains tested (14 strains), suggesting that these primers can be useful for discriminating toxic strains from non-toxic strains to some extent. In addition, two sets of primers out of five were shown to be more effective in detection, so we employ these two sets of primers for subsequent experiments.

Next, we evaluated the performance of the above-mentioned PCR experiment for DNA extracted directly from mixed environmental samples. Our preliminary experiments indicated that the genomic DNA extraction protocol using NaI and lysozyme could get more DNA with purity. DNA samples from one Chinese and two Thailand environmental samples were tested for the presence or absence of toxin genes by PCR analyses with two selected sets of primers and following sequencing analyses. Although optimum PCR conditions for specific detection of toxin genes remain to be determined, the result indicates that these primers can easily detect the toxin genes.

As a first year conclusion, we developed an experimental system for detecting a toxin gene from cultured *Microcytis* strains as well as from mixed environmental samples. This method can be powerful tool for monitoring toxic *Microcystis* in various water environments.

In Last year, we have developed a PCR detection system to identify toxic *Microcystis* strains. Using a specific DNA extraction protocol and several sets of primers which are directed to amplify parts of microcystin synthetase genes *mcy A, B, D, G, and J*, we can successfully detect toxin genes from Japanese strains as well as environmental water samples containing potential toxic strains. Since no amplified band was observed in nontoxic strains, this method is useful in discriminating toxic strains from nontoxic ones. Subsequent analyses indicated that this protocol is also useful in identifying toxic strains from Thailand and China. Next we have determined the sequences of *cpcB-cpcA* locus (a potential marker for cyanobacterial phylogeny) and *mcyA* (a part of toxin gene) of given strains of *Microcystis*. The result of molecular phylogenetic analyses indicated that *Microcystis* could be intercontinentally transmitted among water environments all over the world. It is also indicated that toxin genes could be transferred laterally among different strains of *Microcystis*. Our result emphasizes the importance of toxin-gene based approach to monitor toxic cyanobacteria in water environments.

In this year, reliable and convenient molecular detection systems to identify or discriminate toxic cyanobacteria occurred in water environments are developed. We designed sets of specific PCR primers that are directed to the various parts of the microcystin synthetase (*mcy*) genes of *Microcystis* spp. PCR experiments and sequence analyses indicated that these primers amplified *mcy* genes only from toxic strains but not from any nontoxic ones. This result suggests the potential utility of this PCR system to the discrimination of toxic *Microcystis*. This PCR system

is also shown to be useful for the detection of *mcy* genes from mixed environmental samples. Phylogenetic analysis of *cpcBA* locus indicates no correlation between the genotype and isolated location, suggesting that toxic *Microcystis* strains frequently dispersed and migrated all over the world. Comparative phylogenetic analyses of *cpcBA* and *mcy* genes indicate many discrepancies between the two phylogenies, suggesting the presence of horizontal transmission of *mcy* genes among strains. In addition, sequence analysis of *mcy* genes identified several mosaic structures probably as a result of past interstrain recombination. These results highlight the role of dynamic population genetic processes in the generation and maintenance of genetic diversity of *mcy* genes. Also, these basic findings provide insights into the future direction for the control of *Microcystis* as well as other toxic cyanobacteria. We also develop a PCR system for the detection of *mcy* genes of another toxic cyanobacteria, *Planktothrix* spp. However, the PCR primers designed to amplify a part of *mcy* genes are shown to be present in all *Planktothrix* strains tested irrespective of toxicity. This result suggests that nontoxic *Planktothrix* harbor the potential for microcystin production.

3-6 Detection of Cyanobacterial Toxins

Toxic cyanobacteria (blue-green algae) have been found in eutrophic lakes, ponds, and drinking water reservoirs, and produce neurotoxins, hepatotoxins and cytotoxins. These toxins are called cyanotoxins. Microcystins, hepatotoxic cyclic peptides, are produced by *Anabaena, Microcystis, Oscillatoria* and *Nostoc*. About 70 microcystin variants have now been isolated and identified. Microcystins are classified four groups according to the amin acid structure at unit 7. Normal microcystins contain Mdha or Dha at unit 7, and command the great part of all microcystins. As unusual microcystin classes, [Dhb7] microcystins, [D- and L-Ala7, or N-MeAla] microcystins and [L-Ser7] microcystins have been found. To determine normal microcystins as hepatotoxic tumor promorters, a selective determination method was developed[3]. Only Mdha or Dha in normal microcystins was reacted with glutathione (GSH). The GSH-normal microcystin conjugate was reacted with trinitrobennzene sulfonate (TNBS). The TNB-GSH-normal microcystin conjugate can be determined as the total normal microcystin by colorimetry. After methanolysis of the conjugate, dimethyl TNB-glutamate from the conjugate was determined by liquid chromatography/ultraviolet detection (LC/UV) and or liquid chromatography/mass spectrometry (LC/MS). The detection limits of the total normal microcystin by colorimetry, LC/UV and/or LC/MS were 1mg, 10 and 0.1 ng, respectively.

To clarify normal microcystin contents in the waterblooms of cyanobacteria isolated from China, Thailand, Japan and Scotland, Contents of total Microcystin[4] and normal microcystin in the freeze-dried waterblooms samples were determined. Microcystins in all the waterblooms from Asian countries were reacted with GSH completely, while microcystins in the wateréblooms from Scotland were not reacted at all. These results showed that microcystins in the cyanobacterial waterblooms from Asian countries were

composed of only the normal microcystin class, whereas those in the waterblooms from Scotland were composed of unusual microcystin classes.

In order to detect normal microcystin found from all over the world, a simple detection method was developed using a nucleophilical reaction of glutathione (GSH). The total normal microcystin was measured by colorimetric method using ninhydrin. The GSH conjugates of individual microcystin variants were sepatrated by thin-layer chromatography, and was visualized with ninhydrin. This simple detection method is useful as a screening method of normal microcystin in biological and/or water samples.

In this year, we developed a new selective adsorbed polymer using the pseudomolecular imprinteing method for a simple detection method of microcystin. The polymer showed selective adsorption effects against microcystins. These results suggest that the polymer can be utilized for clean up of microcystin in environmental water.

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