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Alternative Alert System for Cyanobacterial Bloom, Using Phycocyanin as a Level Determinant

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Chlorophyll *a* concentration and cyanobacterial cell density are regularly employed as dual criteria for determinations of the alert level for cyanobacterial bloom. However, chlorophyll *a* is not confined only to the cyanobacteria, but is found universally in eukaryotic algae. Furthermore, the determination of cyanobacterial cell counts is notoriously difficult, and is unduly dependent on individual variation and trained skill. A cyanobacteria-specific parameter other than the cell count or chlorophyll *a* concentration is, accordingly, required in order to improve the present cyanobacterial bloom alert system. Phycocyanin has been shown to exhibit a strong correlation with a variety of bloom-related factors. This may allow for the current alert system criteria to be replaced by a three-stage alert system based on phycocyanin concentrations of 0.1, 30, and 700 μg/L. This would also be advantageous in that it would become far more simple to conduct measurements without the need for expensive equipment, thereby enabling the monitoring of entire lakes more precisely and frequently. Thus, an alert system with superior predictive ability based on high-throughput phycocyanin measurements appears feasible.

Keywords: alert system, bloom, chlorophyll *a*, cyanobacteria, phycocyanin

Algal bloom is a worldwide environmental issue. In particular, cyanobacterial blooms pose additional health risks due to their production of toxins and malodorous compounds. Livestock deaths and human illnesses associated with cyanobacterial toxins have already been reported in many countries (WHO, 2003). In order to deal with these threats, England and Wales alone spend approximately 200 million dollars each year for damage costs and policy response costs (Pretty *et al.*, 2003).

The World Health Organization (WHO) has established two guidance levels for recreational water. Two alert levels according to cyanobacterial growth in sources of drinking water supply have also been suggested in Australia. The thresholds for Alert Level 1 are 2,000 cyanobacterial cells/ml or 1 μg/L chlorophyll *a* or a biovolume of 0.2 mm^3/L, and the thresholds for Alert Level 2 are 100,000 cyanobacterial cells/ml or 50 μg/L chlorophyll *a* or a biovolume of 10 mm^3/L (Bartram *et al.*, 1999). In Korea in 1997, an "alert system for algal bloom" was established by the Ministry of Environment (Ahn *et al.*, 2003a), in which the Caution, Warning, and Outbreak Levels are determined by cyanobacterial cell densities of 500, 5,000, and 1,000,000 cells/ml and chlorophyll *a* of 15, 25, and 100 μg/L, respectively. All of these alert levels were developed on the basis of the assumption that the principal bloom-formers are cyanobacteria, and that they are toxic.

However, these criteria have intrinsic fatal defects, despite their salience to public health. Most notably, cyanobacterial cell counts can include substantial error, as a microscopic enumeration of cyanobacterial cells can often be hindered by large colonies and entangled long filaments. As *Microcystis* colonies are normally comprised of thousands of cells, they must be disaggregated into small colonies or individual cells for counting (Lawton *et al.*, 1999). The enumeration of filamentous cyanobacteria, such as *Oscillatoria* or *Anabaena*, is performed roughly either by an enumeration of the filaments or by an indirect calculation predicated on the length of the filaments. Consequently, cyanobacterial cell counts can vary significantly, depending on the skillfulness and subjective decisions of each counter, thereby making the results rather unreliable. Thus, a more simple and objective method is required to replace the traditional cell counting technique, to establish a more accurate alert system.

Although cyanobacterial bloom is a general phenomenon in eutrophic freshwaters, diatom or dinoflagellate blooms can also sometimes contribute to high levels of chlorophyll *a*, depending on the season and the relevant lacustrine characteristics. As cyanobacterial blooms and the toxins they generate are the principal concerns in an alert system, chlorophyll *a* data cannot be the sole criterion, but must always be accompanied with cyanobacterial parameters. Furthermore, the chlorophyll *a* concentration does not increase in a linear relationship with the cyanobacterial cell number, even though the current alert system is based on just such an assumption (Bartram *et al.*, 1999).

In order to establish a more accurate and reasonable cyanobacterial bloom alert system, cyanobacteria-specific factors should be developed that are easy to measure, do not require expensive equipment, and can replace the current chlorophyll *a* and cell count criteria. Superior cyanobacteria detection

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techniques could also enable the precise monitoring of entire lakes, thereby significantly improving the predictability of bloom occurrence.

As all algal classes have their own light-harvesting pigment system, a spectral discrimination method was developed via the analysis of *in vivo* fluorescence fingerprints (Beutler *et al.*, 2002). This technique has proven valid in several lakes with different trophic states, using a commercialized device (Gregor *et al.*, 2005), and has also been further refined (Parésys *et al.*, 2005). This study, however, was limited to the cyanobacteria-specific pigment protein, phycocyanin, so as to simplify the monitoring method and to provide the possibility that the current dual-criteria alert system might be replaced.

Materials and Methods

Data acquisition

Data regarding the chlorophyll *a* and cyanobacterial cell counts in the Daechung, Juam, Paldang, and Chungju Reservoirs were acquired from the Korean Ministry of Environment. All data were collected for the Korean algal bloom alert system, primarily between May and November. The Daechung data from three sampling sites obtained from 2002 to 2004 were analyzed in greater detail, as these data contained total algae data that were not reported at the other reservoirs. The Juam data were collected from 2001 to 2004, the Paldang data from two sampling sites were collected from 2002 to 2004, and the Chungju data from three sampling sites were collected only in 2004.

Analyses of water quality and phycocyanin

Water samples for phycocyanin determinations were collected weekly from a floating wharf located approximately 20 m offshore near the Daechung Dam from June 22 to October 19, 2004, and from June 3 to November 23, 2005. Surface water (less than 20 cm depth) was sampled after some mixing. The phycocyanin concentrations were determined using two methods, the first of which assessed the *in vivo* fluorescence of the phycocyanin from the water samples, at an excitation wavelength of 620 nm and an emission wavelength of 645 nm (Lee *et al.*, 1995), after being concentrated on a plankton net. The second method determined the phycocyanin concentration by calculating the absorbance values at 620 and 650 nm, following cell disruption in a 20 mM sodium acetate buffer, at a pH of 5.5 (Tandeau de Marsac and Houmard, 1988). Fluorescence was measured with a PerkinElmer Luminescence Spectrometer (LS 45) and absorbance with a Shimadzu Spectrophotometer (UV-160A). Chlorophyll *a* was extracted with a chloroform-methanol mixture [2:1 (vol/vol)] and measured with a fluorometer (Turner 450, Barnstead/Thermolyne, Dubuque, IA) (Wood, 1985). The samples for the cyanobacterial cell counts were preserved in Lugol's solution and enumerated with a haemocytometer (Fuchs-Rosenthal, Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany) under microscopy (Microphot-FXA, Nikon Corp., Japan).

Statistical analyses

The data distributions for chlorophyll *a*, cyanobacteria, and phycocyanin were positively skewed, i.e. they had long upper tails. As data should be normally distributed for parametric analyses such as the Pearson's correlation, they were log-transformed to meet this requirement (Zar, 1996). Log transformation converts positively skewed distributions into normal distributions. Pearson's correlation coefficients and linear regression equations were obtained using the R software package (ver. 2.0.1, http://www.r-project.org).

Results

Problems of the current alert systems

Fig. 1 illustrates the relationship between chlorophyll *a* and

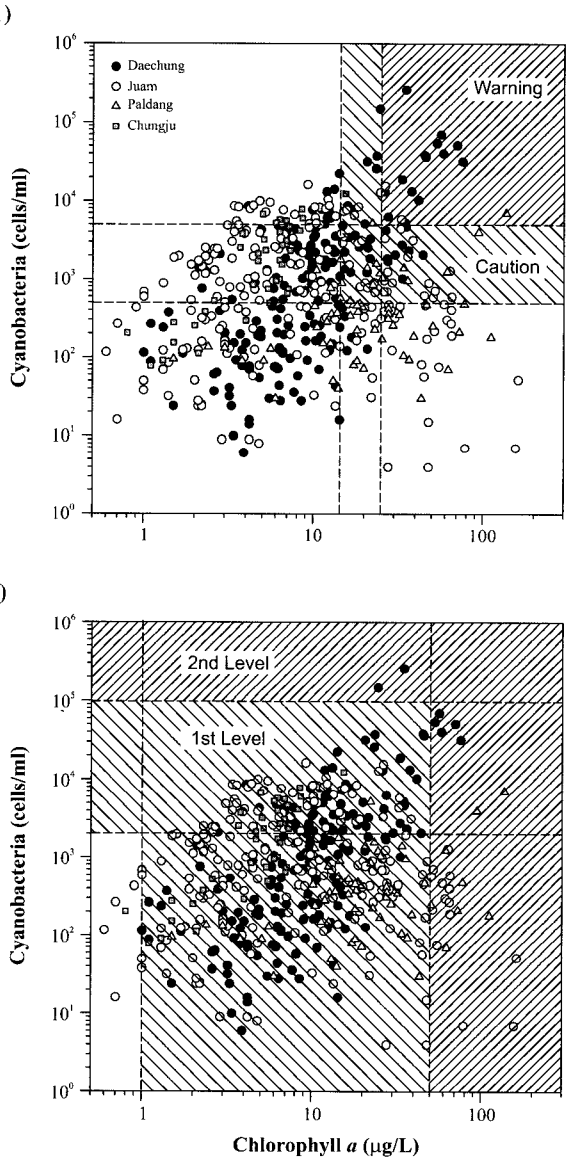

Fig. 1. Relationship between cyanobacteria and chlorophyll *a* in four Korean reservoirs, in terms of the Korean (A) and Australian (B) algal bloom alert systems. The highest alert level in the Korean system, Outbreak, in which cyanobacterial density exceeds 10^6 cells/ml and chlorophyll *a* concentration exceeds 100 μg/L, is not shown. Daechung and Paldang data, 2002-2004; Juam data, 2001-2004; Chungju data, 2004.

cyanobacteria in four representative Korean reservoirs, from spring to autumn. Although the Daechung (r=0.637, P< 0.001), Paldang (r=0.354, P<0.01), and Chungju reservoirs (r=0.813, P<0.001) evidenced strong correlations, the Juam Reservoir (r=0.002, P>0.5) did not manifest a significant relationship. This was attributed to a *Peridinium* (dinoflagellate) bloom occurring in the spring. Even with strong correlations, the distribution patterns of the data points differed among the reservoirs. The slopes for the Daechung and Chungju reservoirs were 1.52 and 1.47, respectively, yet the slope for the Paldang Reservoir was much lower, at 0.48.

The Korean alert system is structured into three levels: Caution, Warning, and Outbreak. It does not assume any linear relationship between cyanobacterial cell density and chlorophyll *a* concentration, as the alert criteria for cyanobacteria (500, 5,000, and 1,000,000 cells/ml) do not increase in a consistent ratio with the chlorophyll *a* criteria (15, 25, and 100 μg/L). As is shown in Fig. 1A, the Caution range for the chlorophyll *a* concentration was too narrow, whereas the Caution range for the cyanobacteria was relatively wide, and the Warning range was even wider in both directions. By way of contrast, the majority of the data points were included under Alert Level 1 in the Australian alert system (Fig. 1B). Another salient difference between the two alert systems is that the Korean system declares an alert only when both criteria exceed the threshold values, whereas the Australian system declares an alert if either threshold is exceeded.

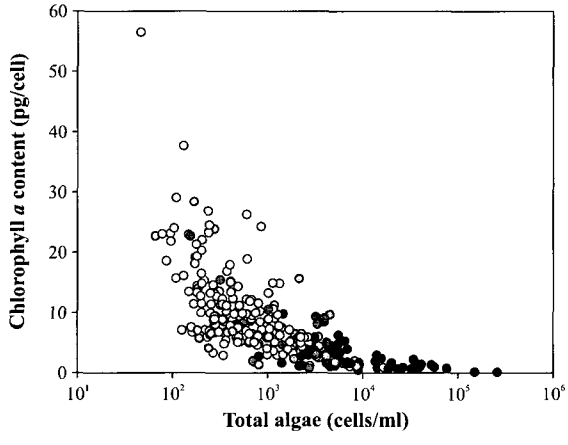

Fig. 2. Chlorophyll *a* content along with total algal cell density in the Daechung reservoir from 2002 to 2004. The darkness of the symbols indicates the proportion of cyanobacteria among the total algae, from 0% (white) to 100% (black).

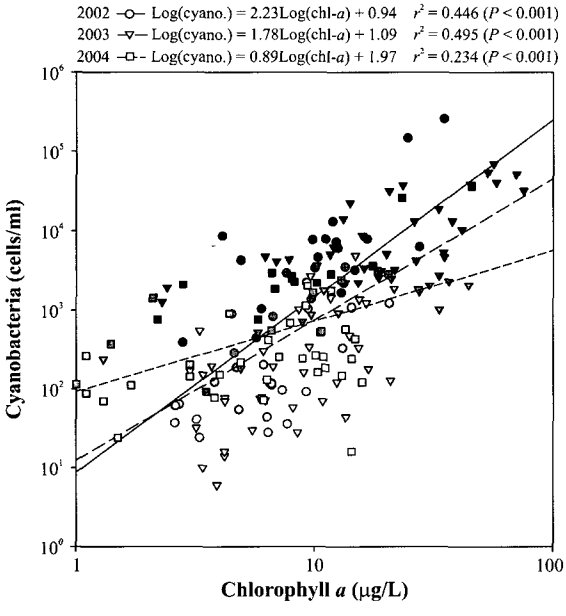

Fig. 3. Yearly patterns of cyanobacteria and chlorophyll *a* in the Daechung reservoir, including the proportion of cyanobacteria among the total algae as indicated by symbol darkness, from 0% (white) to 100% (black).

Nonlinear relationship between cyanobacteria and chlorophyll a

With regard to the present data, the nonlinearity between the cyanobacteria and the chlorophyll *a* concentration was the result of a different cyanobacterial proportion and chlorophyll *a* content, depending on the algal cell density (Fig. 2). When the algal cell density was less than 1,000 cells/ml, the chlorophyll *a* content was dispersed into a higher value. However, the chlorophyll *a* content converged to a lower value, as low as 1 pg/cell, when the total algae density increased above 10,000 cells/ml. This decrease in chlorophyll *a* content was also partially associated with a higher composition of small-sized cyanobacteria, shown by darker symbols in Fig. 2.

Even at the same location, the relationship existing between the two parameters differed from year to year (Fig. 3). The slopes decreased gradually from 2002 to 2004. In 2004, the slope was particularly low and the y-intercept was high, owing to several points at which the cyanobacterial counts were relatively high at around 100 cells/ml, with very

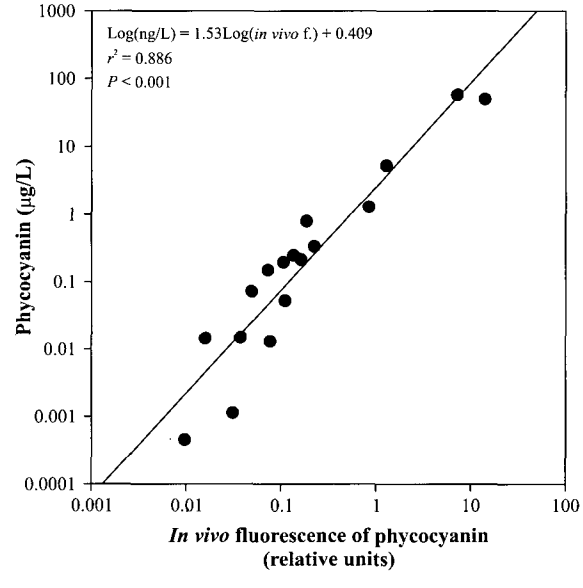

Fig. 4. Comparison of two methods for phycocyanin measurement, extraction and *in vivo* fluorescence.

Table 1. Correlation of phycocyanin with bloom-related factors (n=42)

Factors	Correlation coefficient	
	In vivo fluorescence	Extraction
Secchi depth (m)	- 0.823***	- 0.822***
Turbidity (NTU)	0.531***	0.528***
Chlorophyll *a* (μg/L)	0.786***	0.762***
Cyanobacteria (cells/ml)	0.607***	0.594***
Microcystis spp. (cells/ml)	0.401**	0.413**
Oscillatoria spp. (cells/ml)	0.576***	0.546***

*: P<0.05, **: P<0.01, ***: P<0.001

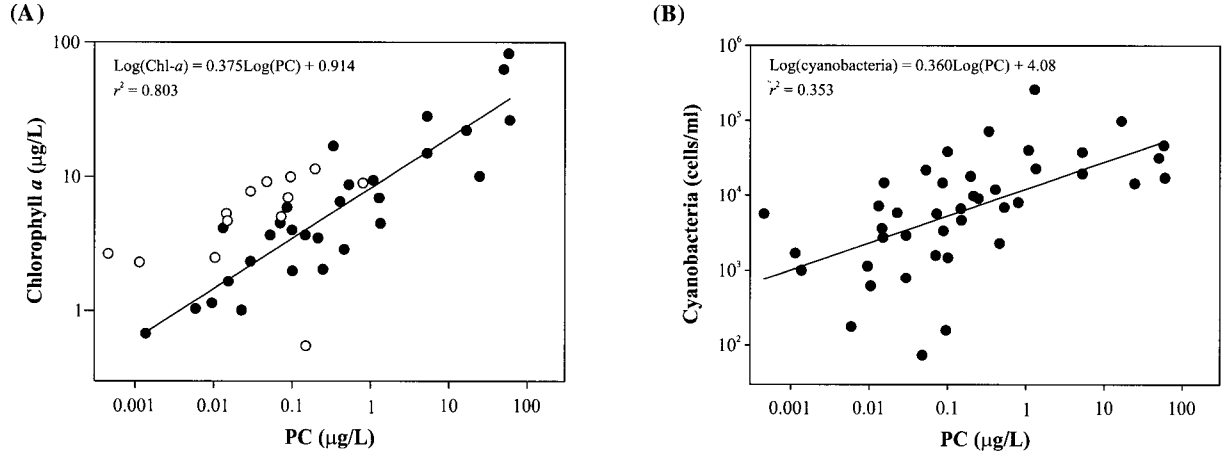

Fig. 5. Relationship between current criteria for alert systems and phycocyanin. The extracted phycocyanin was compared with the chlorophyll *a* concentration (A) and cyanobacterial density (B). The regression line in (A) was acquired using only the data points of cyanobacterial proportion among the total algae above 50% (black symbols), in order to obtain a more reliable relationship between cyanobacterial chlorophyll *a* concentration and phycocyanin.

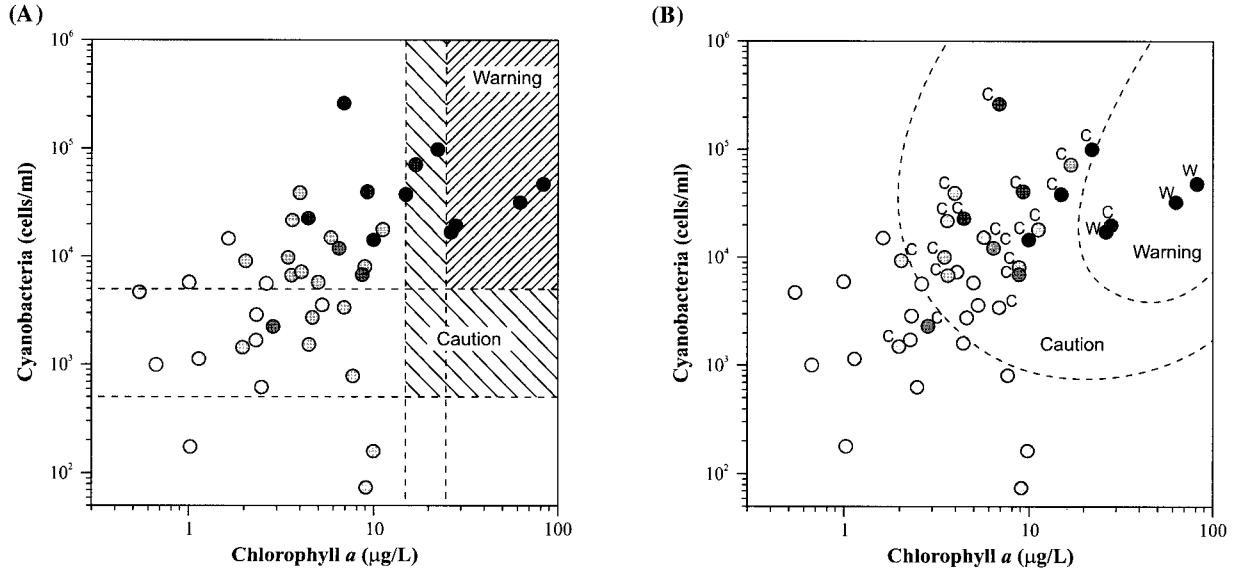

Fig. 6. Comparison of the current (A) and suggested (B) alert systems, applied to the Daechung reservoir in 2004 and 2005. The dotted lines in B indicate the rough boundaries for Caution and Warning based on phycocyanin concentrations. Symbols with the characters, 'C' and 'W', indicate the precise points of the Caution and Warning Levels, respectively. The darkness of the symbols is indicative of the *in vivo* fluorescence of phycocyanin, from 0 (white) to 20 (black).

low chlorophyll *a* concentrations, approximately 1-2 μg/L. At these points, the proportion of cyanobacteria was also quite high, while at other times the proportion was much lower, even in the same cyanobacterial range. The lighter (white) symbols in Fig. 3, occupying the lower parts of the graph, were usually comprised of diatoms and chlorophytes other than cyanobacteria, and their seasonal dominance was limited principally to spring and late autumn. In contrast, the darker symbols in the upper parts of the graph were cyanobacteria-dominant, and this was primarily the case in the summer. In addition to the yearly differences, the three sampling sites in the Daechung reservoir also evidenced shifting relationships between cyanobacterial counts and chlorophyll *a* concentrations (data not shown).

Phycocyanin, a better alert system criterion

As a potential candidate to replace the current criteria for the Korean alert system, phycocyanin concentrations in the Daechung reservoir were determined in 2004 and 2005. The results of the two measurement methods, extraction and *in vivo* fluorescence, yielded an excellent correlation (Fig. 4). The phycocyanin concentrations were strongly correlated with other bloom-related factors, in addition to chlorophyll *a* concentration and cyanobacterial counts (Table 1). Thus, phycocyanin could be employed in the establishment of a new three-level alert system based on the new chlorophyll *a* criteria (3, 30, and 100 μg/L). In the "Discussion" section, this is argued to be a more practical approach. Chlorophyll *a* was correlated more strongly with phycocyanin concentrations than with cyanobacterial cell density (Fig. 5). Thus, phycocyanin concentrations of 0.1, 30, and 700 μg/L might prove useful as levels for a new alert system. Because *in vivo* fluorescence was a simpler and quicker measurement method, the new criteria could also be expressed as *in vivo* phycocyanin fluorescence values of 0.1, 5, and 40, as measured by a PerkinElmer LS 45. Since the *in vivo* fluorescence value may change depending on the instrument used to measure it, these criteria need to be recalibrated for other fluorometers.

The Australian level criteria were not considered in this study, as they are inappropriate for assessments of Korean freshwater. When the phycocyanin alert system was applied to the Daechung data, the problem of the impracticality of the current Korean system was solved (Fig. 6). For example, the points of high cyanobacterial cell density and low chlorophyll *a* concentration that were not included in the Caution Level under the current system could then be assigned to the Caution Level under the phycocyanin alert system.

Discussion

Most current alert levels are based on dual criteria: chlorophyll *a* concentration and cyanobacterial cell density. The rise and fall of these variables have frequently been assumed to occur at a constant ratio (WHO, 2003; Falconer, 2005). However, Figs. 1 and 2 clearly indicate that this was not the case, as the slopes of the cyanobacterial cell density to the chlorophyll *a* concentration deviated from 1.0 in the evaluated reservoirs. Although the Korean alert system does not assume any such relationship, its dual criteria still did not match the field data, thus necessitating a reassessment (Fig. 1A). It was confirmed that the level criteria for the alert system required revision. The Caution Level in the Korean system needs to be wider on the chlorophyll *a* axis and shifted upward on the cyanobacterial density axis. Specifically, more practical criteria can be suggested for use in the Korean alert system. The following criteria are suggested: 3, 30, and 100 μg/L for chlorophyll *a* concentration and 1,000, 10,000, and 100,000 cells/ml for cyanobacterial density. In addition, an alert should be declared if either of these parameters is exceeded, as is the case with the Australian system. Even in cases in which the cyanobacterial cell densities and chlorophyll *a* concentrations were both low, they could still quickly shift to alert levels within 1 or 2 weeks, if the proportion of cyanobacteria was sufficiently high. Therefore, aside from the absolute cyanobacteria and chlorophyll *a* concentrations, the cyanobacterial proportion would appear to have a greater importance with regard to the prediction of an imminent bloom.

These increases in cyanobacterial proportion accompanied declines in chlorophyll *a* content, which was related in part to a higher composition of small-sized cyanobacteria (Fig. 2). The chlorophyll *a* content generally decreases with higher cell density values when the cells enter the stationary phase (Ahn *et al.*, 2003b), thereby disproving the concept of a direct proportionality existing between cyanobacteria and chlorophyll *a*.

The cyanobacteria increased faster than did the chlorophyll *a* concentrations in the Daechung and Chungju reservoirs, yet increased at a slower rate in the Paldang reservoir. In the case of frequent cyanobacterial blooms, in which toxins are of primary concern, chlorophyll *a* plays only a supplementary role to the cyanobacteria, as chlorophyll *a* is commonly detected in eukaryotic algae. In the present study, chlorophyll *a* concentrations showed a decreased correlation with cyanobacteria when the chlorophyll *a* concentration was low. This may be because the cyanobacterial composition decreased with an increased proportion of diatoms and chlorophytes. Consequently, current alert systems, which are predicated on the assumption of a constant linear relationship, would appear to be valid only when the cyanobacterial density and chlorophyll *a* concentration are both relatively high (Fig. 2). Furthermore, if cyanobacterial cell density and chlorophyll *a* concentration were highly correlated, there would be no need to measure both factors, as either one would be sufficient.

In the Daechung reservoir in August 1999, Oh *et al.* (2001) reported that the dominant species were cyanobacteria. They were *Microcystis* spp. (47%), *Anabaena* spp. (39%), and *Oscillatoria* spp. (6%), all of which are known to generate cyanobacterial toxins. Therefore, it appears that the increased phycocyanin concentration during cyanobacterial blooms is reflective of an increased potential for toxicity in water. The alert system based on cyanobacterial biomass, accordingly, can be employed as a primary monitoring technique to obtain information regarding water safety.

Cyanobacterial cell density, the most important alert level determinant, is also to some degree a flawed measure, as an accurate cell count is quite difficult, time-consuming, and unduly dependent on the skills of the individual counter,

making the reliability and effectiveness of cell count data rather unstable. For a more efficient and objective cyanobacteria-specific parameter, phycobiliprotein measurements may prove more effective than cell counts for monitoring and alert systems. Phycocyanin has several attractive characteristics in comparison with cell counts and chlorophyll *a* concentrations: it is easy to measure, does not require a great deal of dexterity or expensive equipment to determine, and faithfully represents the cyanobacterial biomass (Lee *et al.*, 1995; Ahn *et al.*, 2002; Izydorczyk *et al.*, 2005). In the current study, phycocyanin was strongly correlated with a variety of bloom-associated factors, including the Secchi depth, turbidity, chlorophyll *a* concentration, and cyanobacterial count (Table 1). Specifically, *Oscillatoria* and *Microcystis* blooms were severe in the summers of 2004 and 2005, and this was reflected by the phycocyanin concentrations. Although allophycocyanin and phycoerythrin were also tested, their concentrations were far lower than that of phycocyanin. The freshwater cyanobacteria are generally known to be rich in phycocyanin, as compared with allophycocyanin and phycoerythrin. Using the equation in Fig. 5A, new criteria for phycocyanin are suggested, at 0.1, 30, and 700 μg/L, based on the previously suggested chlorophyll *a* criteria of 3, 30, and 100 μg/L. For greater simplicity, the *in vivo* fluorescence of phycocyanin could be alternatively used at 0.1, 5, and 40 (when using a PerkinElmer LS 45) for a three-level alert system. When using *in vivo* fluorescence as level criteria, the values require recalibration for individual fluorometers, as fluorescence values tend to differ between different instruments.

Phycocyanin has several other advantages over cell counts for use in an alert system. The simplicity inherent to *in vivo* fluorescence measurements makes it possible to conduct continuous real-time *in situ* monitoring (Asai *et al.*, 2000; Izydorczyk *et al.*, 2005). Although remote satellite phycocyanin sensing has already been explored, this remains a limited option due to high costs, cloud cover, and a long-term cycle of approximately 16 days between measurements (Vincent *et al.*, 2004). Bloom occurrence and extinction can arise within days, without being detected, if a weekly sampling strategy is utilized. Furthermore, the current use of a few sampling sites does not adequately characterize the horizontal movement of cyanobacteria for an entire lake, particularly in branch-type reservoirs, such as the Daechung reservoir. More frequent and precise monitoring is urgently required, not only for a more accurate alert system, but also for better freshwater management. The current weekly monitoring system conducted at only a few sampling sites provides fairly limited information regarding the real dynamics of cyanobacterial blooms. However, the installation of small phycocyanin sensors throughout a whole lake would allow for the daily collection of data, and would significantly improve predictive ability. A quantitative microarray (Cho and Tiedje, 2002) for the determination of the gene copy numbers of phycocyanin and microcystin synthetase might also be an effective solution for the monitoring of bloom occurrence, as well as toxin production.

In conclusion, the use of phycocyanin measurements as an alert criterion would allow us to circumvent the current disadvantages inherent to the use of chlorophyll *a* and the cyanobacterial cell count as criteria for an alert system, because phycocyanin is a cyanobacteria-specific pigment. Furthermore, its measurement requires no particular experience or skill. Due to the simplicity of phycocyanin measurement, a large number of small fluorescence sensors for phycocyanin detection can be installed throughout entire lakes or reservoirs, thereby making it possible to manage lakes more efficiently and to control cyanobacterial bloom more effectively.

Acknowledgements

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