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Simple Method for a Cell Count of the Colonial Cyanobacterium, *Microcystis* sp.

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The cell counting of colonial *Microcystis* spp. is a rather difficult and error-prone proposition, as this genus forms irregularly-shaped and irregularly-sized colonies, which are packed with cells. Thus, in order to facilitate a cell count, four methods of dividing the colonies into single cells were compared, including vortexing, sonication, TiO_2 treatment, and boiling. As a result, the boiling method was determined to generate the greatest number of single cells from a colony, and all colonies were found to have divided completely after only 6 min of treatment. Furthermore, no significant cell destruction, which might alter the actual cell density, was detected in conjunction with the boiling method (P = 0.158). In order to compute the cell number more simply, the relationship between the colony size and the cell number was determined, via the boiling method. The colony volume, rather than the area or diameter was correlated more closely with the cell number (r^2 = 0.727), thereby suggesting that the cell numbers of colonial *Microcystis* sp. can also be estimated effectively from their volumes.

Keywords: boiling method, cell count, colony volume, cyanobacteria, *Microcystis*

In eutrophic freshwater lakes, some species of cyanobacteria form surface scum, and also generate toxins and malodorous compounds (Falconer, 1994; Soranno, 1997). Thus, bloom-forming cyanobacteria pose a serious environmental problem in many world regions.

Cyanobacterial blooms occur in lakes and reservoirs throughout South Korea. Since the Korean Government established their first "algal bloom alert system" at the Daechung Reservoir in 1997, such systems are now in effect for 10 lakes in 2005. The alert system includes three levels of alert, which can be declared on the basis of two factor values: the concentration of chlorophyll *a* and the number of cyanobacterial cells. Providing an accurate cyanobacterial cell count is an important factor in the effective operation of the alert system.

There is no current consensus on a standard method for establishing a cell counting procedure for colonial *Microcystis* spp., one of the most commonly encountered bloom-forming cyanobacteria (Takamura and Watanabe, 1987). As *Microcystis* spp. form irregularly shaped and irregularly-sized colonies during the bloom season (Dubelaar and van der Reijden, 1995; Oliver and Ganf, 2000; Ishikawa *et al.*, 2004; Welker *et al.*, 2004), accurate cell counts are very difficult, and are subject to significant individulal variations within intact colonies. The majority of studies have applied flow cytometry and sonication for the establishment of a cell count of colonial *Microcystis* spp. (Dubelaar and van der Reijden, 1995; Kurmayer *et al.*, 2003; Lyck, 2004). Flow cytometry using the fluorescence of a single cell is unstable, as *Microcystis* spp. generally exist in colonies (Asai *et al.*, 2000; Lyck, 2004), and sonication can involve different treatment times and intensities, according to the operator. Thus, a standard method is required in order to provide a more accurate *Microcystis* spp. cell count.

The initial objective of this study was to establish an effective method that ameliorates counting errors, via the division of colonial *Microcystis* spp. into single cells. In this study, four methods were compared, and the best one was selected, while ensuring that the

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Microcystis sp. cells were not broken in the execution of the selected method. The second objective of the study was to estimate the number of *Microcystis* sp. cells from the size of the colony, thereby reducing the counting error, and streamlining the process. Therefore, in the current study, we have attempted to establish a convenient, simple, and objective method for the counting of *Microcystis* sp. cells.

Colonial *Microcystis* sp. were collected from the Daechung Reservoir, located at Chuso-ri, Okcheon-gun, Chungcheongbuk-do, South Korea, as *Microcystis* blooms occur frequently at this site. The sampling, which was conducted primarily with the surface water, was conducted during blooming on the 25th of August, 2004 and the collected samples were transported on ice and maintained at 4°C. This study employed only colonial *Microcystis* sp. of the same species from the collected samples. The *Microcystis* sp. cells ranged in size from 4-5 μm, and the colonies ranged in size from 52-200 μm. The identification of the collected samples, colonial *Microcystis* sp., was conducted via sequence analysis from the PCR amplification of the cyanobacterial universal primers, PCβF and PCαR (Neilan *et al.*, 1995). This sample was identified as accession number AY568682, *Microcystis aeruginosa*, from a BLAST search of the NCBI database.

Four methods, including vortexing, sonication, TiO_2 treatment, and boiling, were tested for their efficacy with regard to the dispersal of the colonial *Microcystis* sp. into single cells. All tests used 1 ml of the collected samples, all of which included only colonial *Microcystis* sp. The vortex method was applied using a Vortex-2 Genie (G-560, Scientific Industries Inc., NY) at maximum speed, and sonication was conducted using an ultrasonicator (D300H, Daihan, Korea) at 40 kHz. TiO_2 treatment was also assessed for efficacy, as TiO_2 and UV are known to degrade organic compounds photocatalytically (Whang and So, 1993). As such, TiO_2 (0.1 to 10.0 mg/L) was added to samples harboring *Microcystis* sp. colonies, which were then irradiated using a UV lamp (DVB-913, Daeil Engineering, Korea) at 40 W. Finally, the boiling method was conducted in a double boiler. Each method involved 5 min of treatment, after which the extent of colony shattering and the resultant number of single cells was compared.

Cell numbers were enumerated using a hemocytometer (Fuchs-Rosenthal, Paul Marienfeld GmbH & Co., Lauda-Königshofen, Germany) under an optical microscope (Microphot-FXA, Nikon, Japan). Because *Microcystis* spp. generally form colonies in nature, it was impossible to determine the accurate cell number prior to boiling treatment. Thus, *Microcystis aeruginosa* UTEX 2388, which does not form colonies, was employed in order to determine whether the cells were destroyed when boiled for 6 min. The cell numbers prior to and after boiling were counted with a hemocytometer under an optical microscope, and 20 replicates were compared.

Individual colonies of various sizes were isolated from the collected samples using an inverted microscope (TMS, Nikon, Japan). A total of 47 colonies (AY568682) were separated via a micropipette technique (Oh and Rhee, 1990). The size of each of the colonies was determined under a microscope at a magnification of 100x, and regarded as a sphere. The single colonies were then divided into single cells via the boiling method, and the resultant cell numbers were enumerated with a hemocytometer under an optical microscope. Thereafter, the counted cell numbers were assessed via regression analysis with the diameter, area, and volume of the original colony. The diameter, area, and volume of the colony were calculated from the sphere formula.

The degrees of cell dispersion by four methods, including vortexing, sonication, TiO_2 treatment, and boiling, are provided in Fig. 1. The vortexing, sonication, and TiO_2 treatment revealed uncountable colonies after 5 min of treatment (Fig. 1A, 1B, and 1C), whereas the boiling method left the fewest and smallest unbroken colonies (Fig. 1D). Therefore, the boiling method was determined to be the most effective with regard to the dispersal of the *Microcystis* sp. colony. In several previous studies (Manage *et al.*, 1999; Kurmayer *et al.*, 2003; Tsujimura, 2003), the sonication method has been utilized to generate a cell count of colonial *Microcystis* spp.

In order to determine the optimum boiling time, the

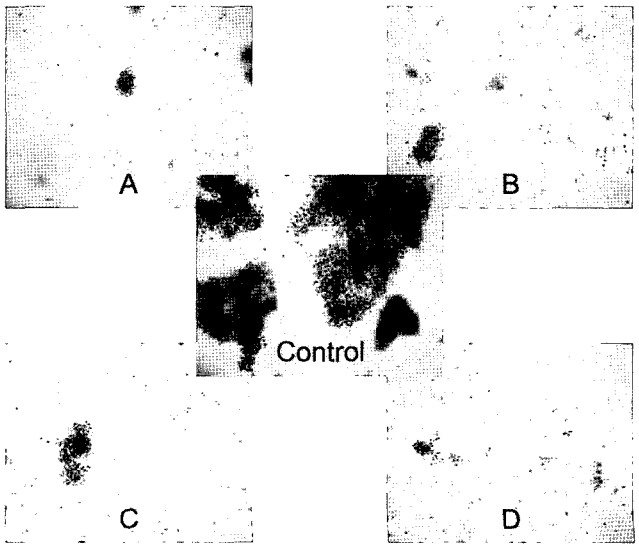

Fig. 1. Comparison of methods for the division of *Microcystis* sp. colonies into single cells after 5 min of treatment. (A) Vortexing, (B) Sonication, (C) TiO_2 + UV, (D) Boiling (Control) no treatment.

Table 1. Cell number of *Microcystis* sp. colonies treated with several boiling times (Average ± SE, n = 20)

Item	Boiling time (min)					
	4	5	6	7	8	9
Average of cell number ($x10^5$ cells/colony)	5.8 (± 0.6)	6.4 (± 0.7)	6.9 (± 0.7)	6.0 (± 0.4)	5.8 (± 0.4)	5.7 (± 0.3)

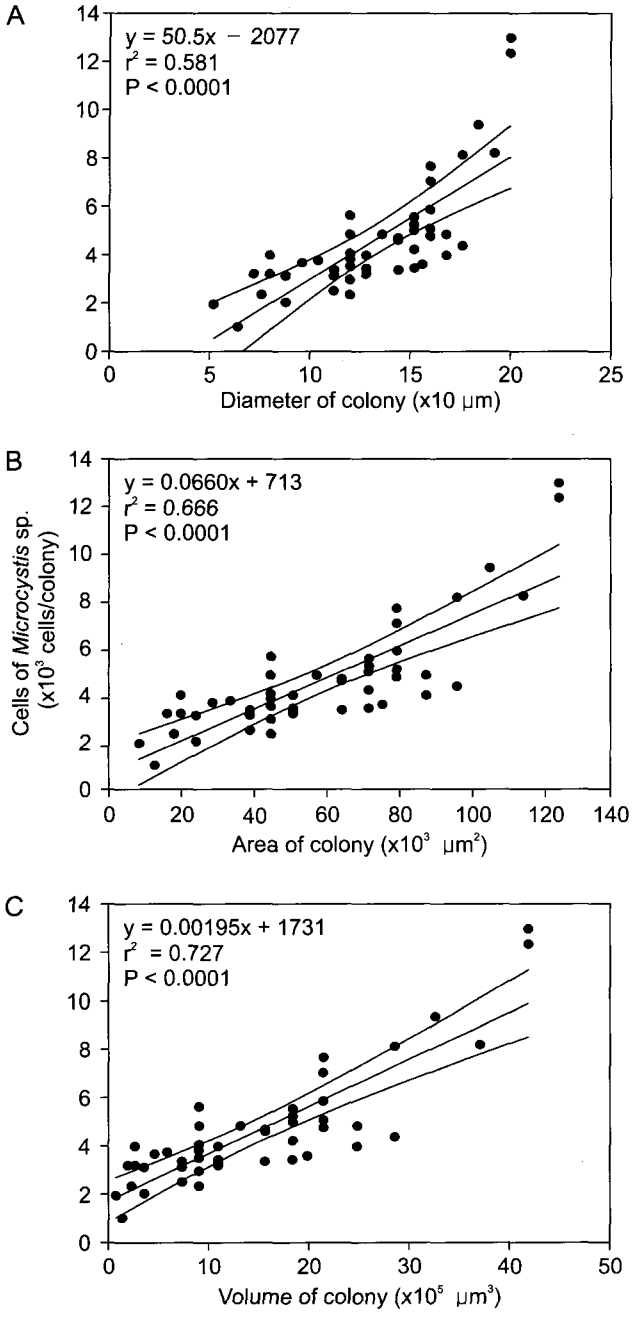

Fig. 2. Relationship between *Microcystis* sp. cell number and diameter (A), area (B), and volume (C) of colony.

effects of boiling times from 4 to 9 min on the dispersion of *Microcystis* sp. colonies were determined. The *Microcystis* sp. colonies were divided completely into single cells after 6 min of boiling, while incompletely broken *Microcystis* sp. colonies were still observed at boiling times of less than 5 min (Table 1). However, the results of ANOVA indicated that the resultant single cell numbers did not significantly differ between the treatment times (P = 0.565). Therefore, 6 min of boiling was chosen as the standard method for a simple, objective, and expedient cell count of colonial *Microcystis* sp. In order to determine whether *Microcystis* sp. cells were destroyed during the boiling process, the cell numbers prior to and after boiling were compared using *Microcystis aeruginosa* UTEX 2388, as this species does not form colonies. The results of the Student's t-test indicated that the boiling method did not significantly reduce the cell numbers (n = 20; P = 0.158). Accordingly, the boiling method was determined to perfectly disperse the colonial *Microcystis* sp. into single cells, after only 6 min of treatment. It also appears that the boiling method can be utilized as an effective standard cell counting method for colonial *Microcystis* sp., due principally to its simplicity, and the absence of any requirements for specialized equipment.

Although boiling can facilitate the cell counting of colonial *Microcystis* sp., the computation of the cell number from the colony size would prove much easier, as direct enumeration would no longer be necessary. The diameter, area, and volume were all determined to be significantly related to the cell number (P < 0.0001) (Fig. 2). However, the volume evidenced the highest correlation coefficient (r^2 = 0.727). This verifies that *Microcystis* spp. colonies have a spherical shape. Reynolds *et al.* (1981) reported previously that colonies of *Microcystis* spp. tend to be globular or semi-spherical, and harbor thousands of cells per colony. Thus, the following formula can be employed for the calculation of the approximate cell number in a colony:

$$Y = 0.00195 \cdot X + 1731$$

In the above equation, Y is the cell number (cells/colony) calculated from the colony volume, X (μm^3). The cell numbers calculated based on the colony volumes were quite similar to the counted cell numbers of single cells dispersed via the boiling

method (data not shown).

In conclusion, we demonstrated in this study that an effective cell count could be facilitated by the boiling method, and that an approximate cell number in a colony could be calculated using the suggested formula, thereby providing convenient and objective methods for the cell counting of colonial *Microcystis* sp. In general, the *Microcystis* genus can be classified into five morphospecies, *Microcystis aeruginosa*, *Microcystis ichthyoblabe*, *Microcystis novacekii*, *Microcystis viridis*, and *Microcystis wesenbergii*, in accordance with morphological characteristics, including cell size, colony form, and sheath characteristics (Otsuka *et al.*, 1998; Via-Ordorika *et al.*, 2004). The current method was adopted for *Microcystis* sp., primarily *M. aeruginosa*, which was known to be most abundant in eutrophic waters, and also comprised the exclusively dominant species in Korean eutrophic freshwater. In cases in which a field sample is comprised of several species of the *Microcystis* genus, the species-specific formula for *Microcystis* spp. other than *M. aeruginosa* might be examined prior to cell counting, in accordance with the procedures introduced in this study.

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