Vidyodaya J. of Sci. (2013) Vol. 18 PP 45-54

Optimization of a standard method for enumeration of total cell counts of colonial *Microcystis aeruginosa* in environmental samples collected from Boralasgamuwa lake, Sri lanka

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Received on : 07-06-2013
Accepted on:18-12-2013

Abstract

Effectiveness of alkaline hydrolysis, heating and regression method for enumeration of cells of *Microcystis aeruginosa* was assessed using water samples collected from Boralasgamuwa lake during the bloom period from 15th February to 15th May 2013. Alkaline hydrolysis was performed using; 0.01, 0.1 and 1.0 M of NaOH with a control. Alkaline hydrolysis at 80°C with NaOH for 15 minutes followed by 30s vortexing produced single cells of all the test samples. A total of $2.34 \pm 0.11 \times 10^5$ cells/ml of single cells were generated during hydrolysis with 0.01 M NaOH. The use of higher NaOH molarities resulted in cell losses. The control sample which contained only distilled water resulted highest numbers of single cells ($2.89 \pm 0.01 \times 10^5$ cells/ml). The heating method was employed by heating *M. aeroginosa* colonies at 40 °C,60 °C,70 °C,80 °C and 90 °C for 15 minutes. Heating at 80°C for 15 min, followed by 30s vortex-mixing produced a suspension of single cells with $2.76 \pm 0.81 \times 10^5$ cells/ml. A standard plot was developed for the direct enumeration of cell number against the area of *M.aeroginosa* colonies using 30 selected colonies. The standard plot slightly overestimated the cell counts during enumeration ($2.980 \pm .51 \times 10^5$ cells/ml).

Key words: Enumeration of cell counts, *Microcystis aeruginosa,* Alkaline hydrolysis, Heating method

Introduction

The genus *Microcystis* (Chroococcales) is one of the most important bloom-forming cyanobacteria responsible for poisonings of domestic livestock, wild animals, fish, and humans (Zurawell et al, 2005). In eutrophic freshwater lakes, some species of cyanobacteria from surface scum, generate toxins and malodorous compounds (Falconer, 1994; Soranno, 1997). In many ecological studies; populations of these organisms are necessarily cited as colonies per unit volume of lake water. Inherent variations in the cell:colony ratio even within individual populations reduce the comparability of colony counts and limit the scope of any data related to the specific biomass of these algae. *Microcystis* spp, one of the most commonly encountered bloom- forming cyanobacteria (Takamura and Watanabe,1987). *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). Thus, enumeration of accurate cell counts of these irregular shaped colonies is very difficult, and subjects to significant individual variations within intact colonies. The majority of studies have applied flow cytometry and sonication for determination of cell counts 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 mostly unstable, as *Microcystis* spp. generally exist in colonies (Asai et al, 2000; Lyck, 2004) while sonication can involve different treatment times and intensities, according to the operator. Thus, a standard method is required to enumerate cell counts of *Microcystis* spp.

There is a need for a precise, simple and cost effective method for enumeration of cells in colonial *M. aeroginosa* for decision making process of water treatment practices. *M. aeroginosa* cell density values are required to determine the mode of operation of chemical treatment methods during water treatment. Pre-treatment of samples with alkali hydrolyses of the mucilage binding colonial algae is sufficient for preparation of individual cells to be microscopically visible (Reynolds, 1973). More recently, disintegration of blue green algal colonies has been achieved by exposing them to ultrasonic waves enabling cell counts to be made (Cronberg et al ,1975; Coveney et al,1997). Furthermore, based on observations on algae collected from lake Crose colony of *M. aeruginosa*, Reynolds (1973) obtained a regression relating the number of cells in a given colony to its average linear diameter. The method was originally designed to quantify developing *Microcystis* populations present in low densities.

The present study discusses the effectiveness of alkaline hydrolysis, heating and regression method in enumerating cell counts of colonial *M.aeroginosa.* Moreover the study aims in developing a calibration plot using cell numbers and colony areas of *M.aeroginosa.* These enumeration methods were validated using water samples collected from Boralasgamuwa lake, Sri Lanka. This is the first study carried out to enumerate the cell counts of colonial *M.aeruginosa* in fresh lake water of a tropical country.

Materials and methods

Sampling

Microsystis populations used in the present study was collected from Boralasgamuwa lake (6050'41"N 79055'5"E) (Fig.1) from 15th February to 15th May 2013 between 7.00-8.00 a.m. Random samples were collected from five sampling locations of the lake (Fig.1) which contained thick scum of the algal bloom. Surface water samples with phytoplankton were collected into clean plastic bottles and samples were transported to the laboratory in an ice box.

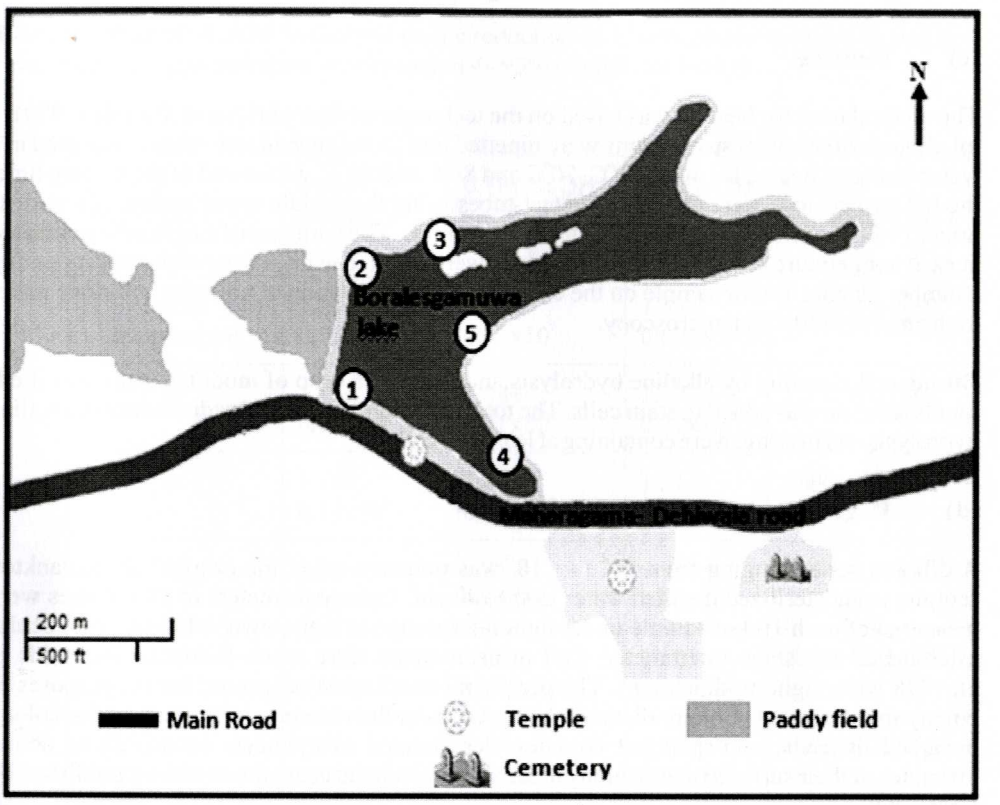

Figure 1: Sampling locations at Boralasgamuwa lake

Estimation of cell density

(a) Natural sedimentation method

Collected fresh plankton samples were concentrated by adding non acidified Lugols' solution at a final concentration of 1%. The acetic component of the Lugols solution was omitted as it was found that acidified lugols disintegrate colonial suspensions (Box.1978). Cell numbers of *M.aeroginosa* was enumerated using a scedwick-rafter counting chamber under light microscopy at (x400) magnification.

(b) Alkaline hydrolysis of mucilage

1ml of algal suspension was added to 4ml of 1M, 0.1M and 0.01M NaOH solutions respectively. Sterile distilled water was used as the control. To ensure complete disruption of the mucilaginous colonies, alkaline suspensions and controls were placed in a water bath for varying time periods from 5-30 minutes at room temperature (29°C), 40°C, 60°C, 70°C, 80°C and 90 °C respectively. Subsequently, the suspensions were neutralized using an equal volume of equimolar HCI to give a final volume of 10 ml. Algal suspensions were subjected to vortexing and the numbers of single cells generated were counted using a Scedwick-rafter counting chamber under high power (x400) light microscopy.

(c) Heating

The method used for heating was based on the technique of Humphries and Widjaja (1979). 1 ml aliquots of the algal suspensions were pipetted into 20 ml capped test-tubes and placed in a water-bath and heated for 40°C, 60°C, 70°C and 80°C and 90 °C. At the end of the heating time, the hot suspensions were mixed in the test-tubes using the middle speed setting of a vortex-mixer (VELP scientific / F20220176) for 5-30 seconds. The numbers of single cells generated at each temperature at 5, 10, 15 and 20 minutes were counted using a Scedwick-rafter counting chamber placing 1ml of sample on the chamber. The observation of colonies was done under high power (x400) light microscopy.

During cell counting by alkaline hydrolysis and heating, a drop of modified (non- acidified) lugols solution was added to stain cells. The total cell counts in normal sedimentation, alkaline hydrolysis and heating, were containing at least 100 cells.

(d) Regression solution

A dilution series ranging from 10^{-1} – 10^{-3} was prepared using the original phytoplankton sample, using sterilized distilled water as the diluent. Colony diameters of 30 colonies were measured of each 1ml of sample under light microscope at high power x400 using an ocular micrometer and stage micrometer. Cell measurements were taken following Reynolds et al.,1978 with slight modifications. The peripheral mucilage was ignored for the purposes of colony measurement. Colony diameter being taken as the diameter of the part of the colony occupied by cells; non-spherical colonies were treated as cylinders or ovoid's to obtain estimates of their surface areas. Colony areas were calculated using the equations published by Carr., 1886.

Spherical colonies: $A = 4 \pi (d/2)^2$

Non- spherical colonies: $A = 2 \pi (d/2)^2 + 2 \pi (d/2) h$

A- Colony area (mm^2)
d- Mean colony diameter (mm)
h- Mean length of colony (mm)

(e) Chlorophyll- a concentration

A serial dilution from 10^{-1} to 10^{-3} was prepared using the colonial suspension of *M.aeroginosa* obtained from Boralasgamuwa Lake. Chlorophyll-a concentration of each of the diluted sample was measured using the standard method (Arvola.,1981) and the cell number of each of the suspension was enumerated using the developed standard plot.

Results

The natural sedimentation method recorded a total *M.aeroginosa* cell number of $1.27\pm0.01x10^2$ cells ml^{-1}. Therefore colonial suspensions were assumed to contain a similar number of cells. Disintegration of colonies hydrolysed in 0.01, 0.1 and 1M NaOH at 29°C of room temperature was found to be incomplete, even after 10 hours. Hydrolysis at 80 °C, however, consistently produced single-cell suspensions after 15 minutes. Cell counts of these suspensions are given in Table 1.

Table 1: Effect of alkaline hydrolysis on the reduction of *M.aeruginosa* colonies to single cells (mean cell concentration of suspension 4- 95% confidence limits)

Heating temperature (°C)	0.01M NaOH	0.1M NaOH	1M NaOH	Control (distilled water)
Room temperature	$1.21\pm 0.07x10^2$	$1.18\pm 0.01x10^2$	$1.57\pm 0.02x10^2$	$1.31\pm 0.34x10^2$
40	$1.11\pm 0.27x10^2$	$1.08\pm 0.01x10^2$	$1.17\pm 0.12x10^2$	$1.27\pm 0.91x10^3$
60	$1.16\pm 0.13x10^3$	$1.04\pm 0.04x10^3$	$1.51\pm 0.16x10^2$	$1.98\pm 0.87x10^3$
70	$1.26\pm 0.02x10^5$	$1.21\pm 0.41x10^3$	$1.91\pm 0.06x10^2$	$1.13\pm 0.04x10^5$
80	$1.34\pm 0.11x10^5$	$1.09\pm x0.09x10^3$	$1.16\pm 0.01x10^3$	$2.89\pm 0.01x10^5$
90	$1.04\pm 0.61x10^5$	$1.17\pm x0.52x10^2$	$1.03\pm 0.41x10^3$	$1.79\pm 0.32x10^5$

1ml aliquots of the same colonial suspension was heated at temperatures ranging from 40-90°C for 5,15 and 20 minutes following vortexing for 15 seconds. Complete reduction of colonial suspension for single cells required at least 15 minutes heating at 80°C, while temperatures below 80°C did not completely disintegrate the Colonies. Temperature above 80°C resulted in cell loss (Table II).

Table II: The influence of heating on the reduction of Colonies of *M. aeruginosa* to Single Cells (Mean Cell Concentration of Suspension 95% Confidence limits)

Heating temperature(C^0)	5 minutes	10 minutes	15 miniutes	20 mimutes
40	1.13±0.12x10	1.03±0.21x10^3	1.27±0.03x10^3	1.15±0.47x10^3
60	1.91±0.11x10	1.21±0.14x10^2	1.51±0.13x10^2	1.98±0.47x10^3
70	1.31±0.12x10	1.47±0.42x10^3	2.91±0.16x10^4	1.13±0.44x10^3
80	1.79±0.11x10	2.09±x0.09 x10^4	2.76±0.81x10^5	1.09±0.11x
90	1.09±0.33x10	1.69±x0.86 x10^3	1.76±0.91x10^4	2.09±0.11x

The standard plot developed for the algal sample using the colony area (mm^2) of *M. aeroginosa* and the cell number per colony (Fig 2) was used to enumerate the *M. aeroginosa* cell count of the sample. The resulted value gave the highest density of 2.98±0.5x 10^5 cells ml^{-1} for the

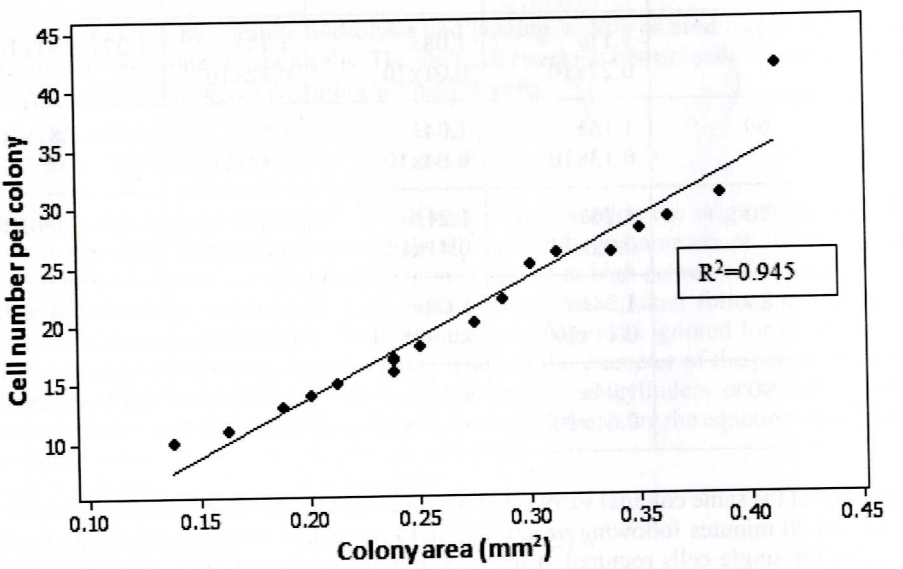

Figure 2: The relationship between the mean number of cells per *Microcystis* colony and mean colony area

Table III gives the values for chlorophyll-a concentration and the *M.aeroginosa* cell counts in diluted suspension and the figure given above was used to count the cell numbers of each colony. The relationship between the chlorophyll-a concentrations and the *M.aeroginosa* cell counts is indicated in the fig.3.

Table III: Chlorophyll-a concentrations and cell Numbers of the diluted colonial suspensions.

Dilution of samples	Chlorophyll - a Concentration	Cell Number
1	18.81±0.01	$2.98\pm0.5 \times 10^5$
0.1	9.34±0.12	$1.26\pm0.02 \times 10^4$
0.01	4.767±0.92	$1.27\pm0.65 \times 10^3$
0.001	1.87±0.31	$2.167\pm0.01 \times 10^2$

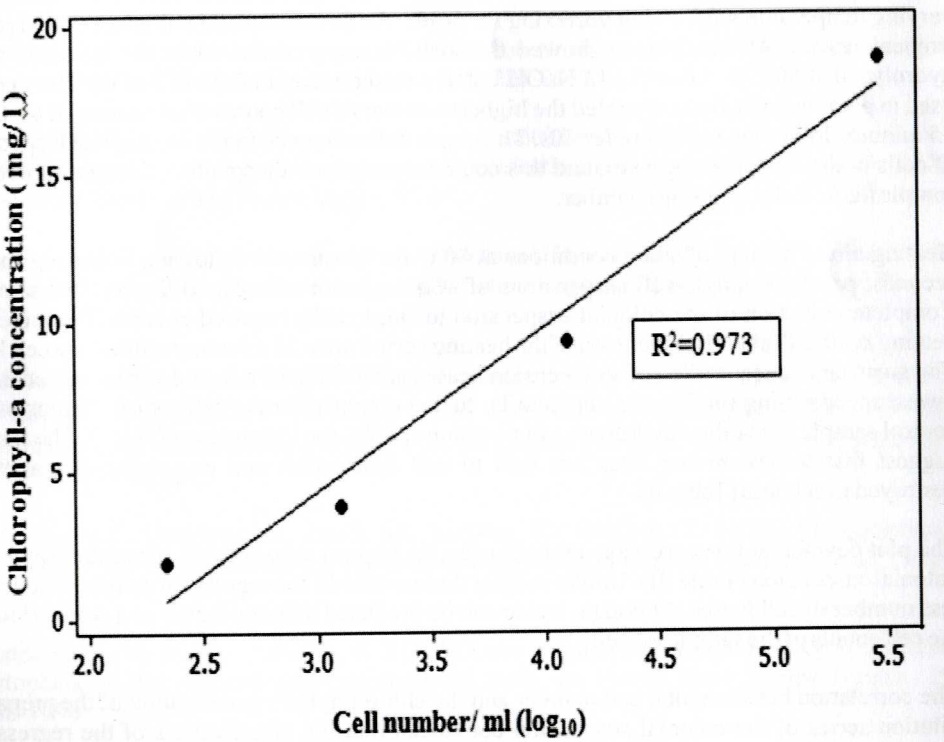

Figure 3. The relationship between the cell concentration of dominant *Microcystis* populations in Boralasgamuwa lake and the chlorophyll-al content of corresponding water samples

Discussion

All three experimental methods provided estimates of natural, colonial, *Microcystis* populations which were mutually similar, or at least, whose differences do not exceed the confidence limits of the counting methods that was used in the study. The natural sedimentation method, which was used to enumerate the cell numbers, was an approximation to estimate the cell numbers of the original colonial suspension. However, the addition of Lugol's iodine to suspensions of *M. aeruginosa* at the rate of 1:100 was found to be sufficient to produce single-cell suspensions in water sample. This was found to be due to the acetic acid component of Lugol's iodine, as the colonies were unaffected or only slightly affected by Lugol's iodine made up without the acid (Box.,1978)

Alkaline hydrolysis of the mucilage of *M. aeruginosa* has been achieved by Reynolds (1973) using KOH [the actual effective concentration is given by Reynolds and Jaworski (1978) as 2.5 by weight, which would give 0.45 M KOH]. Reynolds and Jaworski (1978) used NaOH or KOH at 0.3 g per 100 ml (which would produce, 0.075 M NaOH and 0.05 M KOH solutions respectively) in conjunction with heating at 90°C for 30 min. The present study was carried out based on the above findings with certain modifications with the use of 0.01, 0.1 and 1M NaOH at varying temperatures following vortexing for 5-30s, for environmental colonial microalgae in tropical waters. *M. aeruginosa* showed that cell losses occurred when the colonies were hydrolyzed at 80°C in 1.0 or 0.1 M NaOH but at a lesser extent in 0.01 M NaOH. The control used in alkaline hydrolysis recorded the highest number of cell counts after heating at 80°C for 15 minutes following vortexing for 30s. The major defect may lie in the incomplete dispersion of cells in the treated suspension; and this could be overcome by counting a larger number of sample fields in the counting chamber.

Heating alone without alkaline conditions at 80°C for 15 minutes following vortexing for 30 seconds, produced single-cell suspensions of *M.aeruginosa* colonies in the original sample. Complete reduction of the colonial suspension to single cells required at least 10 minutes of heating at 80°C (Table II). Extension of the heating period up to 20 minutes resulted in a cell loss. The mean cell concentration of a suspension heated at 80°C for 15 min and vortex-mixed at the lowest speed setting for 30s was very similar to that obtained for the cell concentrations of the control sample of alkaline hydrolysis after heating at 80°C for 15 minutes (Table I). The results suggest that vortex-mixing does not lead to cell destruction and excess heating at 90°C destroyed in cell loss (Table II).

The plot developed for regression analysis gave the highest value of cell concentration in the colonial suspension (Table III). Unlike in other three methods, the regression method resulted in less number of cell losses. Although, it also can be predicted that regression plot overestimates the cell counts of the sample as well.

The correlation between total cell number and the chlorophyll-a concentration of the prepared dilution series of the colonial suspension convinced that the effectiveness of the regression analysis. It is necessary to employ a method which does not lead to loss of cells.

The results of the study suggest that the effects of alkaline hydrolysis of some colonial microalgae causes cell losses, while regression solution overestimates the cell counts for a lesser extent. Moreover, regression method can be a cumbersome procedure as it requires stage and ocular micrometers which are not readily accessible in all laboratories. Therefore it can be concluded that the most appropriate method to be employed for the enumeration of single cells of colonial *M.aeruginosa* in an environmental sample is by heating the sample at 80^0C for 15 minutes following by vortexing for 30s without any treatment.

Acknowledgements

The authors would like to extend their heartiest thanks to the co-workers of Algae and water quality laboratory of university of Sri Jayewardenepura for their cooperation given during the study period. Moreover the facilities provided by the University of Sri Jayewardenepura are also highly appreciated in completing this study.

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