Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 2012, 24(9) 1709–1716
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

A flow cytometer based protocol for quantitative analysis of bloom-forming cyanobacteria (*Microcystis*) in lake sediments

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Received 06 November 2011; revised 20 March 2012; accepted 28 April 2012

Abstract

A quantitative protocol for the rapid analysis of *Microcystis* cells and colonies in lake sediment was developed using a modified flow cytometer, the CytoSense. For cell enumeration, diluted sediment samples containing *Microcystis* were processed with sonication to disintegrate colonies into single cells. An optimized procedure suggested that 5 mg dw (dry weight)/mL dilution combined with 200 W × 2 min sonication yielded the highest counting efficiency. Under the optimized determination conditions, the quantification limit of this protocol was 3.3×10^4 cells/g dw. For colony analysis, *Microcystis* were isolated from the sediment by filtration. Colony lengths measured by flow cytometry were similar to those measured by microscopy for the size range of one single cell to almost 400 μm in length. Moreover, the relationship between colony size and cell number was determined for three *Microcystis* species, including *Microcystis flos-aquae*, *M. aeruginosa* and *M. wessenbergii*. Regression formulas were used to calculate the cell numbers in different-sized colonies. The developed protocol was applied to field sediment samples from Lake Taihu. The results indicated the potential and applicability of flow cytometry as a tool for the rapid analysis of benthic *Microcystis*. This study provided a new capability for the high frequency monitoring of benthic overwintering and population dynamics of this bloom-forming cyanobacterium.

Key words: flow cytometry; benthic *Microcystis*; cell enumeration; colony size measurement

DOI: 10.1016/S1001-0742(11)60993-5

Introduction

The occurrence of toxic cyanobacterial blooms in eutrophic freshwaters has increased in both intensity and extensity in many countries (Carmichael, 1997; Chen et al., 2008). Toxic cyanobacteria in freshwater reservoirs and lakes jeopardize water quality and reduce water availability, therefore the problem has received much attention from both scientists and the public in China (Chen et al., 2006; Song et al., 2007). Cyanobacterial blooms often consist of *Microcystis*, a widely distributed cyanobacterium which has two main stages in its life cycle (Reynolds, 1984): (1) the pelagic stage from summer through to early autumn (Reynolds et al., 1981); and (2) the benthic stage as a physiological rest period during winter (Takamura et al., 1984; Brunberg and Boström, 1992; Tsujimura et al., 2000). The overwintering colonies, which accumulate in the lake sediment, constitute a large number of inocula for the next pelagic life stage, which reinvade the water columns in the coming spring (Preston et al., 1980). Consequently, the benthic *Microcystis* population has been considered closely related to the scale and outbreak of *Microcystis* blooms (Reynolds, 1994). Nevertheless, understanding the status of benthic *Microcystis* has been limited to a certain degree due to the lack of an efficient and convenient quantitative method.

Traditionally, optical microscopy has been used as a routine facility for quantitative analysis of benthic *Microcystis* (Brunberg and Blomqvist, 2003; Latour and Giraudet, 2004; Misson et al., 2011). However, there are two recognized shortcomings with this conventional method: (1) it is time-consuming and dependent on skilled personnel; and (2) the number of small colonies and single cells can be underestimated (Tsujimura et al., 2000). Due to such limitations, optical microscopy is considered somewhat inefficient for benthic *Microcystis* studies, especially for high frequency monitoring. Applications of the quantitative polymerase chain reaction (qPCR) technique and pigment analysis to investigate phytoplankton abundance are more straightforward, but they cannot provide direct analysis at the single cell level and have limitations in colony size measuring.

In the past few decades, flow cytometry has been

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universally accepted as a method for phytoplankton analysis (Trask et al., 1982; Olson et al., 1985; Veldhuis and Kraay, 2000). This technique allows discrimination and fast quantification of target cells in complex samples, based on auto-fluorescence emitted by intracellular pigments such as chlorophyll and phycobilin. Traditionally, the application of flow cytometry was restricted to unicellular samples since the flow system allows only narrow-sized particles to pass through (Dubelaar and Gerritzen, 2000). Dubelaar et al. (1999, 2003) introduced a modified instrument, the CytoBuoy series flow cytometer, to extend the flow cytometric analysis to large, asymmetrical cells or colonies (*Microcystis* colonies, for example). Another improvement on CytoBuoy is the applicability for natural, untreated field samples (Dubelaar et al., 2003), including sediment-rich samples in which non-living particles constitute more than 90% of the total number of particles (Dubelaar et al., 1999; Dubelaar and Gerritzen, 2000). In previous studies, the CytoBuoy series flow cytometer was successfully used for phytoplankton analysis in water columns, but to our knowledge, its application in benthic *Microcystis* analysis has never been reported.

This study aimed to develop an optimized protocol for the counts of living benthic *Microcystis* cells via CytoSense (a benchtop type of the CytoBuoy). The impact of sediment particles on the counting efficiency was evaluated by enumerating sediment-rich laboratory *Microcystis* cultures; and the optimal conditions for colony dispersion based on sonication were examined to facilitate the cell count. Furthermore, the percentage contribution of different-sized colonies (calculated as cell number) in the total population was estimated. Species-specific formulas were developed via regression analysis using three *Microcystis* species to help the fast calculation of approximate cell number of different-sized colonies. To test the potential and applicability of this protocol, the changes of benthic *Microcystis* in Lake Taihu was investigated by CytoSense seasonally from 2009 to 2010.

1 Materials and methods

1.1 Field site description

Lake Taihu (30°56′N–31°33′N, 119°54′E–120°36′E) is the third largest freshwater lake in China, and is located in Wuxi, Jiangsu Province. The water depth of the lake ranges from 1 to 2.5 m (average 1.89 m), with an approximate total surface area of 2338 km^2 and a mean water volume of 4.43×10^9 m^3. The sampling site (31°32′31.8″N, 120°13′21.2″E) of this study, located in Meiliang Bay, has been considered the most eutrophic area in Lake Taihu. Samples were seasonally collected at the sampling site from June 2009 to March 2010. The colonial *Microcystis* samples in the water column were taken at the site from 0 to 0.5 m depth using a glass vertical sampler. The sediment samples were collected using an acrylic rod core sampler topped with PVC tubing. The 0 to 5 cm upper layers of the core samples (including three replicates) were collected. All samples were stored in an icebox before returning to the laboratory.

1.2 Culture of single celled *Microcystis*

Microcystis aeruginosa Kütz FACHB 905 was obtained from the Freshwater Algae Culture Collections of the Institute of Hydrobiology (FACHB Collection, Wuhan, China). The strain was cultured with BG11 medium in a 1000 mL flask for one week. The light intensity and temperature were set at 25 μE/(m^2·sec) (12 hr light/12 hr dark cycle) and (25 ± 1)°C, respectively. The alga was centrifuged when it reached the exponential phase of its growth stage. Residuals were washed three times by fresh BG11 medium, and then diluted into different cell concentrations for detection.

1.3 Microscopic inspection

The enumeration of *Microcystis* cells was conducted with a plankton counting chamber under an optical microscope (YS100, Nikon, Japan), as described by Bowe (2002). When possible, more than 400 cells were enumerated. For morphological observation and colony size measurements, a biomedical microscope (Eclipse 90i, Nikon, Japan) was employed. Photography and measurements were carried out via the NIS-Elements B.R. software (Version 3.0, Nikon, Japan).

1.4 Flow cytometry

A CytoSense flow cytometer (CytoBuoy b.v., Nieuwerbrug, The Netherlands) was used throughout. This instrument accepts large phytoplankton cells/colonies (1 to 800 μm) and allows flow cytometry of natural, untreated samples. The instrument was equipped with a bare solid-state laser (488 nm, 20 mW), with five detectors for forward scatter (FWS), side scatter (SWS), red fluorescence (> 655 nm, FLR), orange fluorescence (585 to 655 nm, FLO) and yellow fluorescence (518 to 548 nm, FLY). Data recording was triggered by the FWS signal. Controlled by a peristaltic pump, a flow rate ranging from 120 to 600 μL/min was chosen. The injection time of each sample varied from 3 to 10 min, depending on particle concentrations. An external sheath fluid system was employed to reduce sediment recycling, using distilled water working at a flow rate of 80 mL/min. The tip of the sampling tube was enlarged by a 1500 μm nozzle to facilitate the sampling of large colonies. The performance of the instrument was tested with fluorescent beads (Micron, Polysciences Inc., USA).

The Cytoclus software (Cytoclus3, CytoBuoy, b.v., The Netherlands) was used for data analysis. The *Microcystis* cells (and colonies) were selected by considering the amplitude and shape of the different signals (FWS, SWS, FLR, FLO and FLY). Since there was no objective standard to provide an absolute cluster resolution, the selection of cells (and colonies) sometimes needed the experience of the operator. In addition, colony lengths (the length along the direction of the flow) were calculated with built-in models based on 50% of the peak value of the FWS signal (CytoClus3 manual book).

1.5 Optimization of cell counting in sediment-rich samples

To evaluate the influence of sediment on flow cytometric analysis, different amounts of sediment (with the final concentration of 0 to 25 mg dw/mL) were artificially mixed with certain amounts of single *Microcystis* cells. The mixtures were shaken for 10 min, left to stand for a while, and enumerated by CytoSense. Then to test the detection limit of Cytosense, *Microcystis* cells that ranged from 10^2 to 10^7 cells/mL were mixed with certain amounts of sediment. Before mixing, the sediment (taken from Lake Taihu) was dried at 105°C for 24 hr to destroy the indigenous cells.

Microcystis colonies collected from Lake Taihu were dispersed using an ultrasonicator (JY88, Scientz, China). The sonications were conducted at 25 kHz with cyclic operation of 3 sec and 5 sec intervals. To examine whether cell losses had been caused during the sonication, both treated and untreated colonies were preserved in 1% Lugol's solution for over 48 hr, then microscopically examined.

1.6 Validation of length estimation

Natural *Microcystis* colonies from a bloom water sample (from Lake Taibu) were employed to test the accuracy of the length estimation. The colonies were fractionated by different mesh size sieves, and the mean length of each size-group was determined by CytoSense. Each sample was shaken during the counts to prevent heterogeneity caused by buoyancy. Microscopic measurements of the same colonial samples were conducted simultaneously.

1.7 Determination of the relationship between colony size and cell number

The relationships between colony size and cell number of three dominant *Microcystis* species were determined. Approximately 60 individual colonies were picked by glass Pasteur Pipettes for each species, colony length was microscopically measured, and colony volume was calculated with the sphere formula (using the length instead of diameter). Thereafter, each colony was stored in 1% Lugol's solution until the colony disintegrated into single cells (normally 2 days), then enumerated microscopically. The cell number and the size (i.e., volume) of the original colonies were assessed via regression analysis to obtain species-specific formulas.

1.8 Sediment sample analysis

The seasonal sediment samples from Lake Taihu (described in Section 1.1) were well-mixed before being analyzed. For cell enumeration, a small part of the sediment was dried at 105°C to determine the water content, thereafter, the cell concentration of benthic *Microcystis* was determined with CytoSense following the optimized protocol. For colony analysis, the sediment was filtered with 50 μm mesh sieves to isolate *Microcystis* colonies (Latour and Giraudet, 2004). In some cases, more than several hundred milliliters of wet sediment were required to obtain a sufficient amount of colonies. The lengths of the preconcentrated colonies were flow cytometrically determined, then the cell numbers in different-sized colonies were calculated by species-specific formulas.

2 Results

2.1 Dilution of sediment

Changes in efficiency of CytoSense counts as a function of sediment volume are shown in Fig. 1. The detected cell concentration was 1.88×10^5 cells/mL (> 10,000 cells were recorded) for the phytoplankton sample with 0 mg/mL sediment (control group). Compared with the control group, nearly 99% of the *Microcystis* cells were recorded (1.86×10^5 cells/mL) when 5 mg dw/mL sediment was present. The detected cell concentration decreased significantly (independent samples *t*-test, $P < 0.05$, vs. 0 mg/mL) in phytoplankton and sediment mixtures with more than 5 mg dw/mL sediment. This indicates that the minimal dilution rate of sediment should be 5 mg dw/mL.

2.2 Detection limit of *Microcystis* cells in sediment-rich sample

CytoSense was applied for the estimation of samples containing different densities of *Microcystis* cells mixed with 5 mg dw/mL sediment. Data measured by CytoSense and microscope are listed in Table 1. When the cell concentration was between 10^3 to 10^6 cells/mL, both methods gave similar data, although the CytoSense counts showed a slight underestimation. For Sample F (10^7 cells/mL), however, data obtained from CytoSense was one third of that from microscopy, which indicated that the high concentration of total particles had caused unwanted particle coincidence. On the contrary, the cell concentration in Sample A (10^2 cells/mL) was too low to provide statistical analysis, and the reproducibility of CytoSense counts only achieved a coefficient of variation (CV) of 8.5%. Therefore, the detection range of *Microcystis* cells in 5 mg dw/mL sediment-rich samples was between 10^3

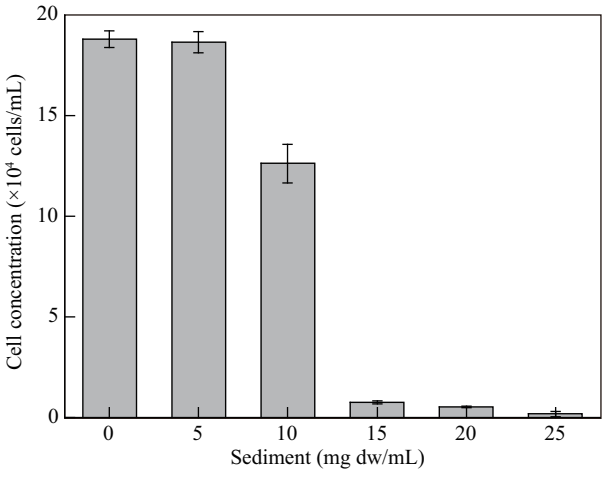

Fig. 1 Effect of sediment content on the counting efficiency of CytoSense. The phytoplankton and sediment mixtures had identical cell concentrations of single celled *Microcystis*. Data are the means with standard deviations of five replicates.

Table 1 *Microcystis* cultures with different cell densities enumerated by CytoSense (FCM) and microscope (M)

	Sample A		Sample B		Sample C		Sample D		Sample E		Sample F	
	FCM	M	FCM	M	FCM	M	FCM	M	FCM	M	FCM	M
$\bar{u}$ (cells/mL)	9.84×10^1	/	1.10×10^3	1.18×10^3	1.13×10^4	1.25×10^4	1.06×10^5	1.15×10^5	9.98×10^5	1.09×10^6	3.56×10^6	1.10×10^7
SD (± cells/mL)	8.38×10^0	/	4.93×10^1	8.82×10^1	4.42×10^2	8.80×10^2	2.83×10^3	7.42×10^3	3.02×10^4	8.70×10^4	2.85×10^5	8.17×10^5
CV (%)	8.5	/	4.5	7.5	3.9	7.0	2.7	6.5	3.0	8.0	8.0	7.4
RE (%)	/		7.2		9.2		7.8		8.3		67.6	

Samples A to E were diluted from Sample F by 10 times increased dilution rate (that is $E = F \times 10^{-1}$; $D = F \times 10^{-2}$; $C = F \times 10^{-3}$; $B = F \times 10^{-4}$; $A = F \times 10^{-5}$). For the CytoSense counts, each sample was artificially mixed with 5 mg dw/mL sediment. The arithmetic mean ($\bar{u}$) and standard deviation (SD) are derived from five replicates. The coefficient of variance (CV) is calculated by: $CV = (SD/\bar{u}) \times 100\%$. The relative error (RE) between these two methods is calculated by: $RE = | (\bar{u}_{FCM} - \bar{u}_M) / \bar{u}_M | \times 100\%$.

and 10^6 cells/mL.

2.3 Optimization of sonication time and intensity

The effect of different sonication time and intensities on colony dispersion is shown in Fig. 2. For 200 and 300 W sonication, the resultant cell number (including the number of incompletely broken colonies) increased with the length of treatment time, and reached the maximum value at 2 min. Thereafter, cell number decreased with the prolonging of treatment time. For 100 and 400 W sonication, the intensities were either too low or too high to provide an appropriate disintegration of the colonies.

Microscopic inspection showed that the resultant single cell numbers did not significantly differ before and after the treatments of 200 W × 2 min sonication and the 300 W × 2 min sonication (one-way ANOVA, $P > 0.05$). The 200 W × 2 min sonication resulted in the highest cell number and was therefore considered as the optimal dispersion condition in this study.

2.4 Validation of length estimation

Based on the pre-experiment, *Microcystis* colonies up to about 400 μm in length could be nonselectively loaded by CytoSense (data not shown). As shown in Fig. 3, data obtained by both methods were similar, with a regression formula as: $Y = (0.87 \pm 0.04)X + (10.21 \pm 6.42)$, $R^2 = 0.98$, $P < 0.01$. The standard deviations in colony lengths

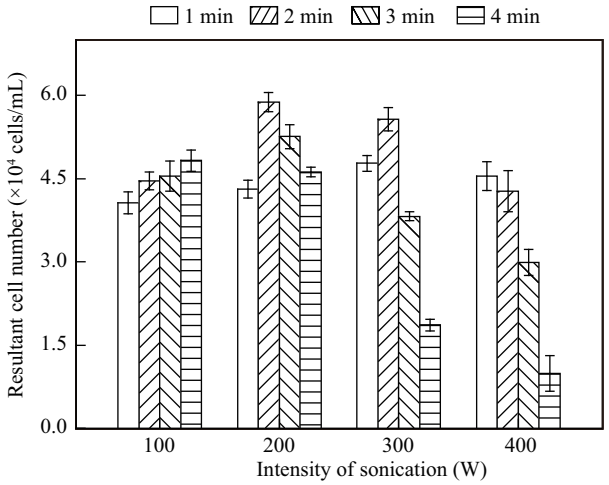

Fig. 2 Effect of different sonication time and intensity on colony dispersion. The *Microcystis* colonies were collected from the water column of Lake Taihu. For each sonication treatment, 10 mL subsample (artificially mixed with 5 mg dw/mL sediment) from a well-mixed sample was involved. Data are expressed as the means with standard deviations of five replicates.

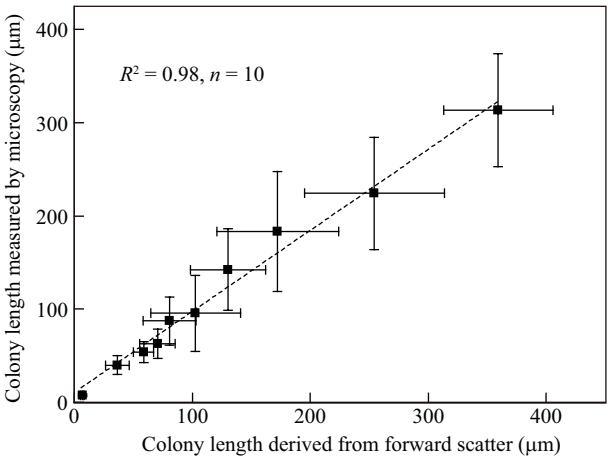

Fig. 3 Comparison between the colony lengths determined with CytoSense and microscope for nine colonial samples and one single celled sample. The fractionating of colonies was conducted with different mesh size sieves. Data are the means and deviations from more than 1000 colonies (CytoSense) and 50 colonies (microscope).

were mainly dependent on the uncertainties during fractionating and error in the different mesh size sieves. The consistency between the manually and flow cytometrically measured lengths suggested that most colonies underwent re-orientation during the flow system, enabling the real estimation of colony length.

2.5 Estimation of cell number via colony size

The relationships between colony volume and cell number of *Microcystis flos-aquae*, *M. aeruginosa* and *M. wessenbergii* are shown in Fig. 4. The colony size was determined to be significantly related to the cell number for all species ($P < 0.01$). The highest correlation coefficient was found in *M. flos-aquae* ($R^2 = 0.817$, $n = 58$), while the other two species yielded 0.742 (*M. aeruginosa,* $n = 61$) and 0.760 (*M. wessenbergii,* $n = 63$), respectively. Therefore, cell number in a colony could be estimated through the following equations:

$$Y = 0.0022X + 517 \quad (M.\ flos\text{-}aquae) \tag{1}$$

$$Y = 0.0015X + 1122 \quad (M.\ aeruginosa) \tag{2}$$

$$Y = 0.0013X + 1489 \quad (M.\ wessenbergii) \tag{3}$$

where, Y is the approximate cell number in one colony; and X is the colony size calculated from colony length. The species-specific formulas demonstrate the apparent difference of colony structure between these three species, which was also found in morphological observations.

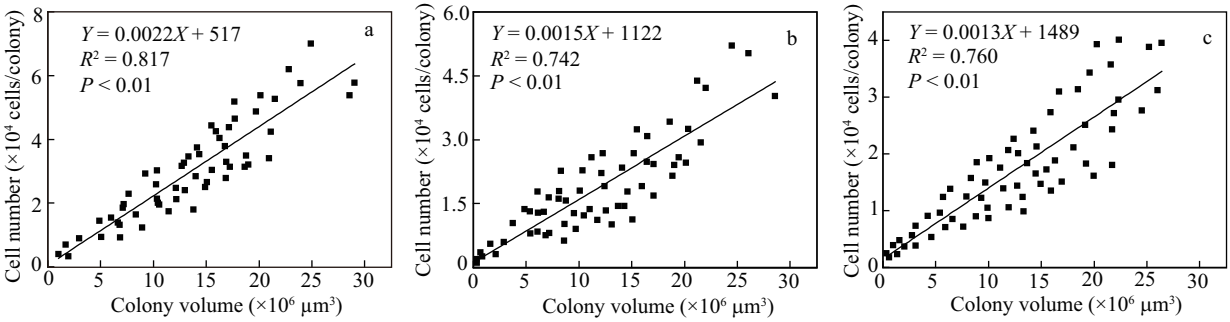

Fig. 4 Relationship between cell number and colony volume of *M. flos-aquae* (a), *M. aeruginosa* (b), and *M. wessenbergii* (c). The colony volume was calculated by the sphere formula, using the colony length instead of diameter.

2.6 Comparison of CytoSense-based protocol with microscopic method

A sediment sample from a bloom-occurring pond was used to verify the applicability of the protocol for benthic *Microcystis* analysis based on CytoSense. The average cell concentrations of 7.33×10^6 and 6.85×10^6 cells/g dw were obtained by CytoSense and conventional microscopy, respectively (Fig. 5a). No significant difference was detected between the results (independent samples *t*-test, $P > 0.05$).

Microcystis colonies from 300 mL wet sediment were isolated and pre-concentrated (dominated by *M. aeruginosa*) to flow cytometrically determine the colony length. Then the cell number in different-sized colonies was calculated through the species-specific formula of *M. aeruginosa*. The number of cells in small colonies (< 50 μm in length) was simply obtained by deducting the cell number of colonies > 50 μm from the total benthic population.

2.7 Measurement of benthic *Microcystis* by Cytosense in Lake Taihu

Data from the CytoSense-based protocols revealed a seasonal change in both the cell concentration and the colony size distribution of benthic *Microcystis* (Fig. 6). From June to November, the cell concentration in the sediment increased to almost 1×10^7 cells/g dw, probably due to the sedimentation of overwintering colonies from the water column. Thereafter, the cell concentration began decreasing, and dropped to one third of the maximum value at the last sampling date (21 March, 2010).

The dominant species of benthic *Microcystis* also varied throughout the samplings. In June and November, the benthic population was dominated by *M. wessenbergii* and *M. aeruginosa*, respectively. In January and March, the dominant species was *M. flos-aquae*. As shown in Fig. 6, the percentage contribution of larger colonies (> 150 μm in length) in late autumn (November) was higher than the other three seasons. In winter (January), cells from small colonies (< 150 μm in length) constituted the main part of the benthic *Microcystis* population. An increase of cell contribution from larger colonies was found in March.

3 Discussion

3.1 Enumeration of benthic *Microcystis* via CytoSense

The presence of excessive amounts of sediment in the sample is expected to result in particle coincidence or/and obscuring of the target particles, causing breakdown of the flow cytometry (Marie et al., 2005; Dubelaar et al., 1999). Dilution of the sediment is a routine pretreatment

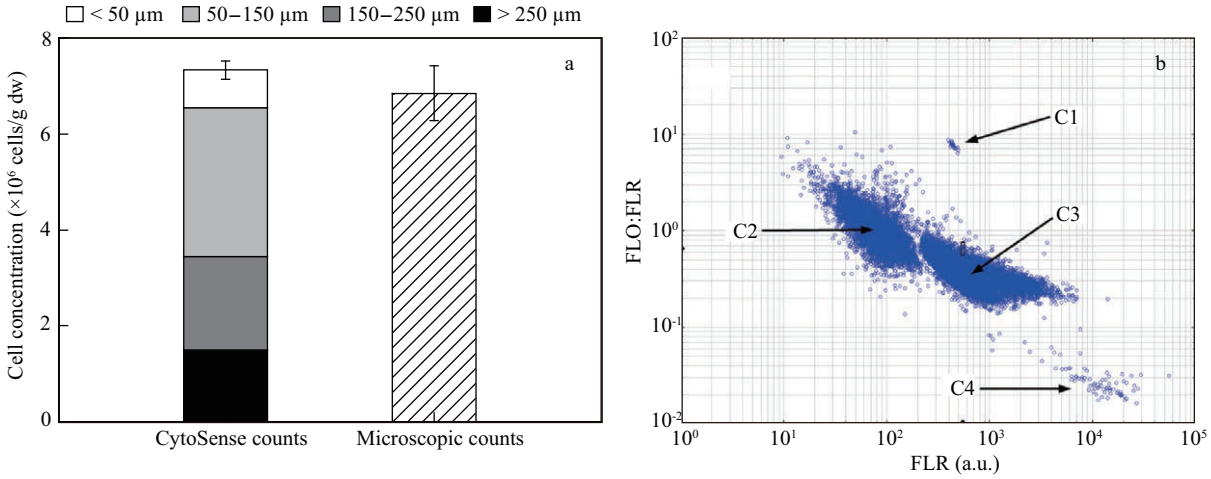

Fig. 5 (a) Comparison between CytoSense counts and conventional microscopic counts for the enumeration of benthic *Microcystis* cells. Data are the means with standard deviations of five replicates. The sediment sample was collected from a *Microcystis* bloom-occurring pond. (b) Cytometric dots depicted by the fluorescence signal of four main clusters. The ratio of Fluorescence orange (FLO) to Fluorescence red (FLR) vs. FLR (a.u.) leading to the resolution of fluorescence beads (C1), noise (C2), targeted *Microcystis* cells (C3) and non-targeted phytoplankton cells (C4). FLR and FLO refer to, respectively, chlorophyll *a* and phycocyanin.

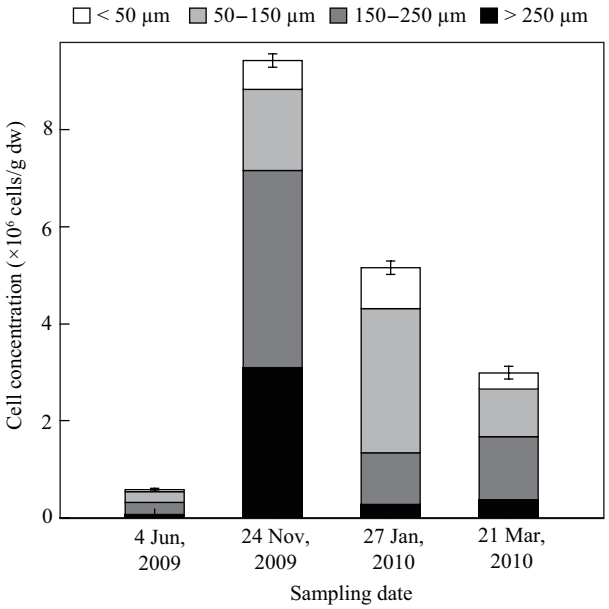

Fig. 6 *Microcystis* cell concentration in the lake sediment of Meiliang Bay (Lake Taihu) determined by CytoSense counts with the optimized protocol. Data are expressed as the means and standard deviations of five replicates.

to diminish the disturbance of sediment when enumerating benthic *Microcystis* with epifluorescence microscopy (Boström et al., 1989; Ihle et al., 2005), but its effectiveness as well as the required degree of dilution (of the sediment) for flow cytometric enumeration were still uncertain. Our pre-experiment showed that in a cell-sediment mixture, the heavy particles (such as sand) sank rapidly to the bottom of the flask within less than 30 sec, while the feathery particles (such as clay, silt and *Microcystis* cells) remained suspended a relatively long time. Therefore, the influence caused by lake sediment on flow cytometry mainly depends on the proportion of the small and light particles (such as clay and silt) that are suspended together with *Microcystis* cells. This in turn depends on the texture (or character) of the lake sediment. In the present study, clay and silt accounted for over 70% (V/V) of the total sediment (Zhong et al., 2010). Correspondingly, the living cells could be effectively discriminated and accounted for by CytoSense when sediment was below 5 mg dw/mL (Fig. 1). Although this parameter is subject to change based on sediment properties, it could be used as a reference for flow cytometrically enumerating benthic *Microcystis*.

According to Thyssen et al. (2008), the results of CytoSub (a submersible type of CytoBuoy) counts are reliable when $> 10^3$ target particles are counted. Our measurements with CytoSense are in agreement with this conclusion (Table 1). Additionally, microscopic inspection showed that the *Microcystis* cells could be easily detached from lake sediment by shaking or other actions. Similar phenomena were also found by Oliver et al. (1985) and Verspagen et al. (2004). In theory, the lower detection limit of the reported protocol for benthic *Microcystis* is 3.3×10^4 cells/g dw (as up to 30 mg of dry sediment could be analyzed per run). This suggests that this protocol may be applicable in the analysis of benthic *Microcystis* in similar eutrophic freshwaters (Boström et al., 1989; Tsujimura et al., 2000; Latour and Giraudet, 2004; Misson et al., 2011).

For the dispersion of *Microcystis* colonies, sonication was selected because it retains the fluorescence properties of living cells (Ihle et al., 2005). However, an insufficient sonication may lead to redundancy of partially divided colonies, while an overpowered sonication may destroy too many cells. The most efficient condition in our experiment was chosen as a 200 W × 2 min combination (Fig. 2).

3.2 Determination of percentage contribution of different sized colonies

The estimation of cell diameter through flow cytometry is limited by several factors, such as the particle size dependency of the scatter signals, the cell shape, and the refractive index (Toepel et al., 2005). These limiting factors would also influence the estimation of larger particles' diameter (i.e., *Microcystis* colonies). In this study, the length (rather than diameter) estimations conducted with natural *Microcystis* colonies showed a high correlation between the results from CytoSense and microscope (Fig. 3), which supports the accuracy of this instrument for colony length measuring. However, further description of the percentage contribution of different-sized colonies on the basis of biomass will help obtain more valuable information about the status of benthic *Microcystis*. Joung et al. (2006) have reported a linear relationship between the colony volume and the cell number for *M. aeruginosa*, and subsequently the approximate cell number in a colony could be calculated using a regression formula. The determination of colony volume directly by CytoSense is impractical at the current time, therefore, a globular shape of the *Microcystis* colony was assumed (Reynolds et al., 1981). However, *Microcystis* colonies belonging to different morphospecies often vary in characteristics such as compactness, thickness and cell size, which would cause great differences in the cell number among colonies of different species/strains with the same dimension (Chen et al., 2009). To facilitate a relatively accurate calculation, we investigated the three most dominant species in Lake Taihu, including *M. flos-aquae*, *M. aeruginosa* and *M. wessenbergii*. The linear regression analysis supported good correlations between colony size and cell number among all three species (Fig. 4). *M. flos-aquae* yielded the highest correlation coefficient ($R^2 = 0.817$), and contained the largest number of cells per unit volume. This indicates that *M. flos-aquae* might form more regular and thicker (or more compact) colonies than the other two species. Using the suggested formulas, colony lengths measured by CytoSense could be conveniently transformed to the cell number, then the percentage contribution of different-sized colonies to the total population could be estimated.

3.3 Examples of field application

Little information is available regarding determination of the absolute cell numbers of benthic *Microcystis* using conventional light microscopy. In field application with sediment samples from a bloom-occurring pond, the results showed no significant difference between the Cy-

toSense and microscopic counts based on the mean values (Fig. 5a); the coefficient of variance (2.5%) substantiated the low variability of CytoSense. This further suggested the efficiency of the flow cytometric protocol. The similarity of the cell concentration obtained by both methods also showed that the interference of other cyanobacteria cells (such as *Anabaena* cells) for CytoSense counts was negligible (which was in agreement with the microscopic pre-examination). As shown in Fig. 5b, four main clusters (abbreviated as C1 to C4) were identified in the cytometric graph. Cluster C3 was defined as *Microcystis* cells owing to its strong emission of phycocyanin fluorescence. The observed phenomenon that the boundary between C3 (*Microcystis* cells) and C2 (noise, including non-living particles) was not very distinct may be due to the presence of some dormant *Microcystis* cells in the lake sediment (i.e., low in vitality), which showed little intracellular fluorescence during flow cytometric detection. The particles in C4 contained a substantial amount of chlorophyll *a*, but only trace phycocyanin as inferred by the very low FLO:FLR, therefore, C4 stands for other phytoplankton such as Chlorophyta, Diatom, etc.

Field applications during the year 2009 to 2010 (Fig. 6) revealed a visible seasonal change of benthic *Microcystis* in Lake Taihu, although the sediment samples were collected infrequently. The high efficiency of flow cytometry allowed the analysis of one sample in less than 10 min. This is an advantage when high frequency monitoring is required. Due to the high abundance of benthic *Microcystis* cells during our investigation, the statistical significance of CytoSense counts could be ensured in most cases. For sediment samples in which the abundance of the target cluster is too low to provide sufficient cells (or colonies), the combined counts of several parallel analyses would be needed (Thyssen et al., 2008).

Nevertheless, for the application of this protocol, factors such as the sediment properties, abundance of *Microcystis* and interference of other cyanobacterial species should be taken into consideration in order to match the practical situation.

4 Conclusions

Many studies have suggested the role of benthic cyanobacteria in the early outbreak of bloom. For this reason, the demand for an analysis method for benthic living cyanobacteria is increasing. The present study established an enumeration protocol of benthic *Microcystis* based on a modified flow cytometer, the CytoBuoy. According to our experimental results, *Microcystis* cells could be effectively enumerated in diluted sediment samples (5 mg dry sediment/mL) after colony dispersion (200 W sonication with treatment time of 2 min). Furthermore, the cell number in different-sized colonies (up to 400 μm in length) could be calculated from colony length through species-specific formulas. The proposed protocol was successfully applied to the quantitative analysis of benthic *Microcystis* in Lake Taihu, and therefore can be of aid in understanding the early stage of freshwater phytoplankton blooms.

Acknowledgments

This work was supported by the National Basic Research Program (973) of China (No. 2008CB418006), the National Special Program of Water Environment (No. 2009ZX07106-001-002), the National Natural Science Foundation of China (No. 31070355) and the National Major Science and Technology Program for Water Pollution Control and Treatment (No. 2009ZX07101-013).

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