Dynamics of bacterial abundance and the related environmental factors in large shallow eutrophic Lake Taihu

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ABSTRACT
In aquatic ecosystems, estimation of bacterial abundance (BA) is intimately connected to the development of an understanding of the role of bacteria in microbial food webs. Flow cytometry (FC) can rapidly calculate BA, but to date mostly has been applied in marine waters with limited use in shallow eutrophic lakes because of the abundant organic and inorganic matter. To seek the best compromise between precision and the clarity for the differentiation of bacteria from other submicron-sized particles and colloids in eutrophic lakes, we optimized FC instrument settings, fixatives and storage conditions, ultrasonic pre-treatment and staining protocols. Then, BA in Lake Taihu was investigated by the optimized FC procedures. Regression analysis showed good correspondence ($R^2 = 0.72$, $p < 0.001$, $n = 128$) of BA counted by FC and epifluorescence microscopy. The developed protocol was successfully applied to monitor the dynamics of BA in the large shallow eutrophic Lake Taihu. A distinct spatio-temporal shift of BA in Lake Taihu was observed. The shift was found to be mainly related to dissolved organic carbon, total suspended solids, water temperature and chlorophyll-a. Our results highlight the effect of sediment re-suspension and algal biomass on BA in eutrophic shallow lakes.

KEYWORDS
Flow cytometry; bacterial abundance; generalized additive models (GAM); Lake Taihu; sediment re-suspension

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Introduction

Bacteria are a key component of microbial food webs and have crucial roles in biogeochemical cycling and energy influx in aquatic ecosystems (Cotner & Biddanda 2002; Newton et al. 2011). They not only mineralize organic materials and convert them into their inorganic constituents (Cole et al. 1988), but also transform dissolved organic matter (Marie et al. 1997) into particulate organic matter which can be consumed by high trophic levels; thus, they have a profound impact on ecosystem metabolism and function (Giorgio et al. 2011; Attermeyer et al. 2013). Hence, the quantification of bacteria is essential for understanding the ecological role of bacteria in aquatic environments.

Different methods have been used to enumerate bacteria, including traditional agar plating, direct cell counting, optical density measurement as well as by the concentration of protein or cell wall constituents (Senjarini et al. 2013). Epifluorescence microscopy (EpiM) and flow cytometry (FC) have recently been the common method of determining bacterial abundance (BA) in aquatic microbial ecology (Khan et al. 2010; Tang et al. 2010; Li et al. 2011). Compared to EpiM, FC has many
advantages. FC permits the reliable and rapid detection of free-living microbes and can provide information about their distribution within cell populations. For example, FC was reported to be four times faster and more accurate (<5% standard deviation compared to >10% for EpiM) than EpiM (Wang et al. 2010). Moreover, it is possible to directly count by FC when the concentrations of bacteria are as low as $10^3$–$10^4$ cells/mL, whereas EpiM requires sample concentration (Wang et al. 2007; Hammes et al. 2008).

FC has become a valuable tool for determining the abundance of autotrophic and heterotrophic bacteria rapidly and directly in oceanography (Fuchs et al. 2000; Jiao et al. 2005; Nelson et al. 2011). This method, however, is rarely used in enumerating microorganisms in shallow eutrophic lakes. There are two major problems that have hindered the use of FC in this kind of ecosystem. First, these lakes have high particulate and solid phase content, especially numerous abiotic particles (such as inorganic and organic complexes, clay and detritus, etc.) which could interact with fluorochromes, limiting the effectiveness of cell binding. Second, disaggregating attached bacteria from suspended particles, which can possibly lead to underestimating the bacteria densities, is a challenge.

In this paper, we compare the effects of sample fixation, storage and staining conditions on BA using FC, and optimize the pre-treatment procedures. In particular, ultrasonication was used to disaggregate samples in order to obtain a single-cell suspension and facilitate FC analysis. The main objective of this paper was to establish a rapid FC method for the accurate enumeration of heterotrophic bacteria in shallow eutrophic lakes. Then, the method was verified by comparing the results with EpiM. Furthermore, the spatio-temporal patterns of BA in Lake Taihu were explored using the optimized procedures for FC analysis. In addition, the main environmental factors related with the shifts of BA were also revealed by generalized additive models (GAM).

Materials and methods

Counting BA by FC

BA was counted by a FACSJazz cell sorter (Becton Dickinson) equipped with an air-cooled argon laser (488 nm, 80 mW). The instrument was configured for linear signal amplification for forward scatter light (FSC) detectors and logarithmic (log) amplification for side scatter light (SSC) and fluorescence detectors. All water samples were filtered through a 40-μm mesh sieve before analysis to avoid clogging the instrument’s fluidics system. Bacteria were detected using a combination of SSC (related to cell size) versus green fluorescence (FL1, 530/40 nm, due to SYBR Green staining of nucleic acids), because FSC provided less resolution than side light scatter (SSC) (Sherr et al. 2001). Due to photosynthetic pigments, phytoplanktonic cells (mainly Microcystis) in water samples showed distinct red fluorescence. Therefore, red fluorescence (FL3, 692/40 nm) and green fluorescence were used to discriminate heterotrophic bacteria from autotrophic phytoplankton. 1.0 μm yellow-green beads (Polyscience Inc.) were added into samples as an internal standard that was used to normalize the cell fluorescence and light scatter emissions. The rate of sample injection was maintained at <1000 events/s, because high event rates greatly raise the possibility of having double events and cell coincidence. When the sample pressure offset was about 1.0 psi and the data acquisition was maintained at below 1000 events/s, the coefficient of variation of bead-fluorescence was below 5% that was regarded as the highest limit acceptable. In this experiment, threshold values at 1.35 and 320 volts on the FL1 photomultiplier tube were applied to exclude unstained particles.

A precise volume of each sample was added to BD Trucount tubes (BD-Biosciences) and incubated for 15 min in the dark at room temperature, which released a known number of fluorescent beads. Absolute counts of bacteria were determined by comparing cellular events to bead events. Gates were drawn around the beads and bacteria populations, and the number of events in each
gate recorded. BA was calculated using the formula,

\[ C_t = \frac{N}{V} \times \frac{N_t}{N_0} \]

where \( C_t \) is the sample’s concentration (cells/mL), \( N \) is a known number of fluorescent beads (cells), \( V \) is a volume of the sample added to BD Trucount tubes (mL), \( N_t \) is the number of samples tested by FC (events) and \( N_0 \) is the number of the fluorescent beads tested by FC (events).

**Assessing the effect of fixatives and storage conditions**

**Effect of different fixatives**

To find the optimum fixative for lake samples, three common fixatives were selected: formaldehyde (FA), paraformaldehyde (PFA) and glutaraldehyde (GA). They were all purchased from Sigma-Aldrich Inc., USA. Ten samples were selected for preliminary experiments that allowed checking of the effectiveness of different fixatives. All treatments in this experiment were conducted in replicates.

Particle-free neutralized FA was filtered through a 0.2-μm-pore-diameter cellulose ester filter using borax-buffered 37% FA and stored in the dark at room temperature. To count BA, a subsample aliquot (10 mL) of each sample was fixed using freshly prepared FA with a final concentration of 2% and incubated for 15 min at room temperature, then stored at 4°C in the dark until analysis.

PFA was prepared from 20% PFA in phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]). It was heated to 60°C in a water bath kettle until dissolved completely (about 40 min). The cooled solution was filtered through a 0.2-μm-pore-diameter polypropylene filter and was divided into small aliquots kept frozen directly upon preparation. For the fixation of water samples, second subsample aliquots (10 mL) were fixed immediately with the prepared aliquot PFA (1% final concentration), and incubated for 15 min at room temperature.

GA was filtered with 0.2 μm filters and stored in individual 10 mL sealed glass tube in order to ensure the best storage conditions. A third aliquot (10 mL) was fixed with GA (1% final concentration) for 15 min at room temperature.

**Effect of storage temperature and time**

Ten samples from Lake Taihu were chosen to compare the effects of sample storage time and storage temperature. Individual subsamples in three replicates were stored either in liquid nitrogen (N₂), −80 or 4°C after fixation, and BA was measured by FC within a week.

To explore the effect of storage time on BA counting, 10 samples (fixed by FA and stored at 4°C) were tested after 0.5, 1, 3, 6, 12 months’ storage. Loss ratios (%) of bacterial cells were counted using fresh water samples as control.

**Optimization of ultrasonic pre-treatment**

One of the major problems associated with bacterial enumeration with FC analyses is the association of bacteria with aggregates, inorganic particles and detritus in shallow eutrophic lakes, which may affect the enumerating accuracy. Sonication was used to separate particles into single cells in order to facilitate FC analyses (Foladori et al. 2007). In this study, sonicator (XO-1000D, Sino Tech Inc.) with a horn tip and a temperature probe was used to pre-treat samples. The tip was placed in the center of 10 mL glass tubes on ice that contained 5 mL samples. In order to compare the results from different sonicators, reference parameter Es here was used to calculate the transferred specific energy (Foladori et al. 2010). It was expressed in kJ/L (\( Es = P \times t/V \)) (\( P \), transferred power; \( t \), time; \( V \), treated volume). In this research, two samples (Site 4 and Site 22) from Lake Taihu were selected to analyze. Site 4 was located in Meiliang Bay, suffering from intensive blooms of algae during summer and autumn. It is one of the most eutrophic regions in the north part of Lake Taihu (Qin 2009). Site 22 was located in the southern part of the lake that is less eutrophic. The efficiency of
ultrasonication was optimized when the transferred power was fixed at 25 W. After ultrasound 2, 4, 6, 8, 10, 20 and 30 min (a fixed time interval of 5 × 5 s), the intensities of green fluorescence and BA were recorded by FC analysis.

**Optimization of staining procedures**

SYBR Green I is a high-affinity nucleic acid stain which is commonly used to stain electrophoretic gels and supplied in a 10,000-fold concentrate of the typical ‘stock’ solution. Stock SYBR Green I solution was diluted with dimethyl sulfoxide (DMSO) buffer (Molecular Probes Inc.), and the best dye concentration for staining on FC was defined by testing five concentrations (0.1×, 0.5×, 1×, 5×, 10×) of the ‘working’ solution (1/10,000 of the stock solution). The staining time was compared by recording the intensities of cellular fluorescence with FC after 5, 10, 20, 30, 40 and 60 min incubation.

**Shift of BA in Lake Taihu**

Lake Taihu (30°55′40″–31°32′58″ N, 119°52′32″–120°36′10″ E) is a large and shallow freshwater lake with an area of 2338 km² and an average depth of 1.9 m, which is suffering from eutrophication and heavy cyanobacterial blooms (Qin et al. 2007). In this study, 32 surface water samples (top 50 cm) (Figure S1 in the supplemental materials) were collected with a 5 L Schindler sampler from Lake Taihu in February, May, August and November 2013. Three replicates were collected on each station and then mixed.

**Measurement of environmental parameters**

Electrical conductivity (EC), pH, dissolved oxygen (DO) and water temperature (WT) were measured in situ using a multi-parameter water quality sonde (YSI 6600 V2, USA). For each water sample, about 100 mL were filtered (glass-fiber filter (GF/F), nominal pore size 0.7 μm) in the field for measuring ammonium nitrogen (NH₄⁺–N), nitrate nitrogen (NO₃⁻–N) and dissolved organic carbon (DOC). For chlorophyll-a (Chl-a) analysis, 100–500 water samples were filtered (GF/F) and frozen prior to analysis. Another unfiltered water sample was frozen for total nitrogen (TN), total phosphorus (TP) and total suspended solids (TSS) analysis.

NH₄⁺–N concentrations were analyzed by Nessler’s reagent colorimetry, and NO₃⁻–N concentrations were measured by the ultraviolet spectrophotometry. DOC was analyzed using a Torch TOC Analyzer (Teledyne Tekmar, USA) by high-temperature catalytic oxidation. To determine Chl-a, thawed filters were grinded and extracted by ethanol. Chl-a concentrations were determined by testing the extract at wavelengths of 665 and 750 nm (Jin & Tu 1990). TN and TP were determined by per sulfate oxidation and spectrophotometry. Samples analyzed for TSS were filtered with GF/F filter membrane and oven-dried the filtering residua over 4 h at 105 °C (Jin & Tu 1990).

**Counting BA**

Water samples were fixed by FA (2% final concentration) and stored at 4 °C. BA was counted within one week by FC using the optimized procedures as described (i.e. 5 min of ultrasonic pre-treatment, staining 20 min with 1× SYBR Green I working solution).

To compare with the counting results of FC, EpiM was also performed to enumerate BA fixed by FA as described previously (Porter & Feig 1980; Tang et al. 2010). Natural water samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) (final concentration 2 μg/mL) for 10 min and then filtered onto black polycarbonate filters (0.2 μm pore size, 25 mm diameter; Poretics™). Bacteria were counted using a Zeiss Axioskop 2 plus epifluorescence microscope, equipped with a HBO 100 W mercury lamp. At least 20 fields and 400 cells were enumerated per sample.
Statistical analysis

All statistical analyses were performed in R environment (version 3.2.2, http://www.r-project.org). GAM was used to explore nonparametric relationships between BA and environmental factors in Lake Taihu using the package ‘mixed GAM computation vehicle’ (mgcv) (Wood 2006; Wood 2016). All environmental parameters except WT, EC, DO and DOC were square root transformed before analysis to reduce the effect of outliers on GAM.

Results and discussion

Assessing the effect of fixatives and storage conditions

The effects of fixation were tested using 10 samples fixed with the three fixatives and counted using FC. Our result showed that the BA of 9 out of 10 samples fixed by PFA was much lower than the abundances fixed by FA and GA (Figure 1(a)). Analysis of variance (ANOVA) results demonstrated that samples fixed by FA and GA yielded significant (p < 0.01) higher BA than those fixed by PFA (Figure 1(b)), indicating the potential damage of bacterial cells fixed by PFA. This is consistent with a report that showed PFA prepared in phosphate-buffered saline usually precipitates in the presence of salts and consequently produces colloids interfering with the FC analysis (Christaki et al. 2011). In aquatic microbiological study, cell fixation is an obligatory preliminary step before analysis using FC. It can provide many advantages: (1) fixation facilitates the penetration of certain fluorochrome into the cell; (2) fixed samples may be stored and it is not necessary to test samples in real time; (3) all samples can be analyzed in one run for better comparison (Vives-Rego et al. 2000; Hyka et al. 2013).

Due to better performance (Figure 1), FA and GA fixation was performed further to test the effect of storage temperature and storage time. Our results indicated that BA of samples stored in liquid-N was slightly higher than samples frozen directly at −80 and 4 °C and tested within a week for both

Figure 1. Effects of fixative on heterotrophic bacterial counting. (a) Bacterial counts fixed with formaldehyde (FA), glutaraldehyde (GA) and paraformaldehyde (PFA) using different samples from Lake Taihu. (b) Comparison of the three selected fixatives. ANOVA demonstrated that the use of PFA yielded significant lower bacterial abundance (p = 0.007).
FA and GA fixation (Figure 2(a,b)). It is consistent with the previous reported from the coastal water (Troussellier et al. 1995). However, no significant differences were observed under the three storage temperatures ($p > 0.05$). Due to the easier accessibility, the effect of storage time on the loss of BA was carried out with the fixative FA (2% final concentration) and stored at 4 °C. Our results demonstrated that the mean loss ratios of bacteria were about 8%, 20%, 30%, 40% and 60% after 0.5, 1, 3, 6 and 12 months' storage, respectively (Figure 2(c)). It is well known that the bacteria counts were getting less and less during storage period (Troussellier et al. 1995; Shibata et al. 2006). The results were similar to those of Troussellier et al. (1995) who checked BA (about 70%) in marine samples fixed with FA after four weeks' storage at 5 °C.

**Optimization of ultrasonic pre-treatment**

In contrast to marine systems, shallow lakes are limited in the application of counting bacterial cells by FC. The major reason is the large amounts of particulate and solid phase content that exist in shallow eutrophic lakes, including bacteria aggregates, organic aggregate-attached bacteria and so on (Tang et al. 2010). The more complicated aggregates the samples contained, the more difficult is the FC analyzed. The crux of successful quantification is how to maximize the abundances of bacteria converted into free single cells into solution. Ultrasonication is an effective method to detach bacterial cells from surfaces or substrate in lake sediment environment (Schallenberg & Kalff 1993; Duhamel & Jacquet 2006; Amalitano & Fazi 2008). In the present investigation, ultrasonication was adopted to obtain a suspension of free single cells from lake water in order to facilitate FC analysis. The result showed a progressive increase of green fluorescence intensity and BA with the length of treatment time in ultrasonication, and reached the maximum value at 4–6 min (Figure 3). Thereafter, BA decreased with the prolonging of ultrasonic treatment time. This result was consistent with previously reported for wastewater and activated sludge from wastewater treatment plants (Foladori et al. 2007). However, activated sludge flocs required Es around 80 kJ/L which is quite smaller than the value used in this research (Es = 1500 kJ/L). A possible reason might arise from the fixation. We fixed samples with FA while no fixation was used by Foladori et al. (2007). It is reported that fixation with FA or GA can reinforce the cellular layer in order to reduce the damage from sonication.

![Figure 2](image_url)

**Figure 2.** Effects of storage temperature and storage time on bacterial counting. (a) Samples fixed by FA and preserved in liquid nitrogen (Liquid-N), in −80 and in 4 °C. (b) Samples fixed by GA and preserved in liquid nitrogen (Liquid-N), in −80 and in 4 °C. ANOVA demonstrated that storage temperature did not have significant effect on bacterial counting ($p = 0.75$ for FA fixed samples; $p = 0.32$ for GA fixed samples). (c) Loss ratio (%) of bacterial cells fixed by FA after 0.5, 1, 3, 5, 12 months' storage under 4 °C.
As a result, 5 min of ultrasonic treatment time (i.e. $E_s = 1500 \text{ kJ/L}$) seemed to be optimal pre-treatment for FA fixed eutrophic lake samples.

**Optimization of staining procedures**

The nucleic acid stain SYBR Green I has been used with great success for total cells enumeration in marine environment (Broadaway et al. 2003; Kamiya et al. 2007; Merwe et al. 2014) and proven to clearly differentiate between bacteria and nonspecifically dyed bacteria-like particles (Senjarini et al. 2013). In the present study, a distinct increase of the green fluorescence intensity was observed with...
the increasing working concentration of commercial SYBR Green I solution from 0.1-fold to 1-fold (Figure 4(a)). Then, with the increasing concentration of SYBR Green I, the green fluorescence intensity decreased obviously. SYBR Green I was supplied as stock solution (10,000-fold concentration) in DMSO. In order to avoid repeated freezing, we prepared an intermediate 1:100 dilution of the SYBR Green stain in high-quality anhydrous DMSO.

As for incubation time, the green fluorescence intensity increased rapidly during the first 20 min, and then stabilized for the rest of 40 min (Figure 4(b)). Therefore, 1-fold concentration working concentration of SYBR Green I and 20 min’s incubation were the optimal staining concentration and staining time.

**Comparison between FC counts and EpiM counts**

BA in Lake Taihu was investigated by FC using the optimized procedures as described previously (Table S1 in the supplemental materials). In addition, EpiM was also performed to compare the counting result with FC. Regression analysis showed that BA counted by FC significantly \( p < 0.001 \) correlated with those counted by EpiM (Figure 5(a)). Although paired \( t \)-test indicated that there were no significant differences between the two counting methods \( p > 0.05, \) Figure 5(b)), the mean BA counted by FC was 1.22 times than that counted by EpiM, indicating higher fluorescence intensity yield by SYBR Green I than DAPI (Lebaron et al. 1998; Troussellier et al. 1999; Kamiya et al. 2007).

**Shift of BA in Lake Taihu and the driven factors**

As shown in Figure 6, we observed a distinct spatio-temporal shift of BA in Lake Taihu. BA had the highest value in summer \( \left(8.46 \times 10^6 \text{ cells/mL}\right) \) and the lowest value in spring \( \left(2.68 \times 10^6 \text{ cells/mL}\right) \). Spatially, western and northern Taihu had higher BA than that in eastern and southern Taihu except in winter (Figure 6(a)). Environmental parameters in different seasons were listed in Table S2 in the supplemental materials. GAM was used to investigate the relationship between the shift of BA and the related environmental factors. The best GAM contain four environmental factors, i.e. WT, TSS,
Chl-α and DOC. They were all significantly \((p < 0.001\) for WT and TSS; \(p < 0.05\) for Chl-α and DOC) related to BA and explained 77.5% of the variance of BA totally (Figure 7).

Degrees of freedom in smoothing function revealed that DOC was the most important factor, followed by TSS, WT and Chl-α (Figure 7). DOC showed a strong non-linear relation with BA, but the general trend indicates a positive effect at higher concentrations. DOC is one of the most important carbon resources for aquatic heterotrophic bacteria (Wetzel 2001). Therefore, the positive relationship between BA and DOC is well understood.

TSS also showed a strong positive non-linear relation with BA, indicating the importance of sediment re-suspension on BA. Because of the shallowness (mean depth < 2.0 m), Lake Taihu experiences frequent wind-induced sediment re-suspension. The much higher number of bacteria in sediments was easily entered into water column passively by wind, which can increase BA in the water column in a short time. According to long observation (Wu et al. 2015), the main wind direction over Lake Taihu is east-southeast from spring to summer and east-northeast from autumn to winter. In the present study, the high abundance of bacteria that occurred at south Taihu in winter was coincident with the high TSS concentration pattern in Lake Taihu (Figure 6(b)). This result confirmed that wind-induced sediment re-suspension is an important factor on BA pattern in shallow Lake Taihu.

Square root transformed Chl-α had a positive linear relation with BA. In addition, the distribution pattern of Chl-α (an indicator of algal biomass) in Lake Taihu was similar to that of BA, especially in summer and autumn (Figure 6(c)). This result indicated a strong interaction between autotrophic algae and heterotrophic bacteria. Previous researches (Sigee 2005; Prieto et al. 2016) have demonstrated that algae can release DOC to the water column during their growth period, which can be utilized by algal attached or free-living bacteria.

Normally, BA was positively related with WT and nutrient level. In the present study, however, there were no significant differences of BA among winter, spring and autumn with the mean WT of 9, 24 and 15 °C, respectively. The possible reason for such inconsistency is that the relative higher BA in winter may be caused mainly by wind-induced sediment re-suspension (i.e. high TSS, Figure (6)), while the relative lower BA in spring may be related to calm weather and low algal density in typical 'clear water' spring period (Figure 6, Wetzel 2001). In addition, we found no significant
relationship between BA and nutrient level, which indicated that nutrient is not limited factor for BA in the eutrophic shallow Lake Taihu. It is notable that the patterns of BA in Lake Taihu were just based on samplings once a season. Considering the large heterogeneity and the effect of frequent wind in Lake Taihu, cautions should be taken when evaluating BA response to environmental variables.

Conclusions

The present study established a rapid protocol using FC to enumerate heterotrophic bacteria in large shallow eutrophic lakes (summarized in Table S1 in the supplemental materials). The protocol was verified by comparing the results with EpiM and was successfully applied to count BA in Lake Taihu. Using the optimized FC counting procedures, we observed a distinct spatio-temporal dynamics of BA in Lake Taihu. GAM revealed that TSS (an indicator of sediment re-suspension), DOC and Chl-\(a\) (related to algal biomass) and temperature were the main environmental factors, which explained 77.5% of the shift of BA. The results highlighted the potential release of sediment bacteria to the water column and the interaction between autotrophic algae and heterotrophic bacteria in large, shallow eutrophic lakes.
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Disclosure statement

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