

Computerized Enumeration and Bio-volume Estimation of the Cyanobacteria *Anabaena flos-aquae*

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Abstract

One of the most noticeable consequences of global climate change is the increased occurrence of algae and cyanobacteria blooms in surface waters. Some of these organisms may release hazardous toxins which represent a threat for human and animal health worldwide. Accordingly, the identification of threshold levels of toxic cyanobacteria cells has become common practice to ensure successful water management. The majority of current methods for cyanobacteria enumeration and bio-volume assessment are very time-consuming and costly. Furthermore, when dealing with multicellular organisms (i.e., filaments, colonies, agglomerates etc.), none of the existing enumeration methods can achieve good accuracy and all tend to underestimate cell concentrations and bio-volume. In this study, we introduce an integrated method for automated enumeration and bio-volume estimation of *Anabaena flos-aquae*, a common filamentous species of cyanobacteria often present in water blooms. Since *Anabaena* filaments are often long and tangled, a sample of its culture was first sonicated to isolate individual cells, and then imaged while being excited by a 546nm light source to considerably improve contrast. A probabilistic unsupervised classification was introduced to detect the target cells, and the size distribution of the cells was used for model calibration. Using this learned cell model, subsequent samples with natural *Anabaena* filaments were automatically enumerated and the bio-volume estimated. Compared to traditional manual enumeration using a hemacytometer, the developed method achieved equivalent accuracy in much less time, with less resources, and provided additional bio-volume information. These preliminary results demonstrate the potential of the developed method as a robust tool for water quality monitoring.

1. Introduction

Global climate change has become one of the driving factors to enhance toxic blooms in surface waters worldwide [1]. Some cyanobacteria presents in blooms can produce and release of hazardous hepatotoxic, neurotoxin and genotoxic toxins and taste/odor compounds (i.e., Geosmin, 2-methylisoborneol)[2-3]. To effectively improve water management strategies, identification of threshold levels of cyanobacteria in water sources has become common practice. For example, two alert levels of algae cell concentrations, 2000 and 100,000 cells/ml were suggested by the WHO for drinking water sources [2]. Therefore, accurate measurement of cell concentration and volume have become key factors in the implementation of successful water management practices, not only in drinking water production but also in source and recreation water monitoring and in water re-use.

Accurate, effective and cost efficient quantification of unicellular algae and cyanobacteria concentration in water is a long standing problem. Enumeration of organisms with complex morphology is even more challenging. And bio-volume (or biomass) is commonly used as an indirect estimation of cell concentration[4]. Numerous methodologies have been developed and implemented for this purpose, including flow cytometry [5], qPCR[6], fluorometric probes[7] and microscopical image analysis[8]. None of these technologies are however, effective and accurate enough to evaluate cell concentrations and bio-volumes especially when cells are aggregated as clusters, filaments or other colonial forms. For example, *Anabaena* sp. is one of the most common cyanotoxin producers in natural waters. Its filaments are 4-6 μm wide and can reach 50 μm to more than 2000 μm in length [1,4]. During bloom events, filaments often entangle forming complex agglomerates. Other challenges that prevent a more wide application of these methods are: a) biomass and community composition varies in time and space; c) direct estimation methods such as microscopic enumeration and bio-volume estimation are labor intensive and very variable; d) most current enumeration methods are indirect and do not reliably to distinguish target cells from unwanted background (i.e. lysed cells and abiotic particles that can jeopardize the quality of the measurements); e) the detection limits are narrow for most of the methods proposed so far. For example, Lzydorczyk et al (2003) noticed that good correlation between the fluorescent signal and cell concentration for *Mycrocystis* sp. was only valid at cell concentrations below 15,000 $\mu\text{g/L}$ and when the dominating cells were those being enumerated[9]. These difficulties prevent the use of existing methods in routine monitoring of water quality.

Automated enumeration with computer-assisted image analysis is a promising solution for low-cost, rapid water quality monitoring.. There are still several challenges to be overcome before automated enumeration can be routinely used for accurate and robust analysis. First, cells present in water environments are mostly transparent and un-uniform in color which results insufficient contrast between them and other background particles. Staining is often used to enhance the contrast between cells and background during the image acquisition process[2]. Second, effective separation of cells in multicellular structures of complex morphology (i.e. entangled filaments, clustered cells, clumps of colonies etc.) is necessary for reliable identification and quantification[4,8]. Third, cells in filaments or overlapping cells can often be out of focus since they occupy more than one vertical plane of view. Variability during the image acquisition process, the underlying microscopy system characteristics (e.g. light source) require manual tuning for specific observation. Last but not least, the readily available automated algorithm mostly accounts for the total projected area (in 2 dimensions) of wanted

cells and then back calculates volumes assuming the 3D structure of cells. If information on individual cells such as shape and morphology are not quantitatively assessed this can lead to significant over estimation or sometimes underestimation of cell numbers and volumes.

In this work, we introduce a two-phase model-driven auto enumeration and biovolume analysis for determining the concentration of *Anabaena flos-aquae* and other filamentous organisms with complex morphology. Integrated with sonication as pretreatment, an imaging-based method was developed using isolated *Anabaena* cells for model calibration. Enumeration and bio-volume estimation were conducted and validated by comparison with manual counted *Anabaena* filaments.

2. Materials and Method

To ensure accurate cell enumeration and bio-volume estimation experiments were conducted in two phases. In a first phase the filamentous *Anabaena* culture was pretreated by sonication to break up the filaments. Images of the sonicated culture (mostly isolated cells) were captured under fluorescent light (546 nm). These images were analyzed aiming to obtain quantitative information on mean cell size and deviations to be used for model calibration purposes. In a second phase the concentration and bio-volume of a non-sonicated filamentous *Anabaena* culture was analyzed based on the quantitative information obtained during the first stage.

2.1 Algae cells preparation

The *Anabaena flos-aquae* culture was obtained from the Canadian Phycological Culture Centre (CPCC), University of Waterloo. This cyanobacteria has been selected for use in this study as a representative of an aquatic multicellular organism which cells are difficult to enumerate due to its filamentous nature and tendency to present tangled agglomerates (Fig 2-c). BG-11 was used as growth medium [10] and the culture was grown in glass flasks exposed to a full spectrum of white light (~1200 lumens), in a Percival growth cabinet (John's Scientific Inc., Canada) at a temperature between 19 and 22 °C.

2.2 Cell pre-treatment (Sonication)

One of the key steps to quantify individual cells in multicellular organisms, filamentous or other, is measuring individual cells including shape, size and size distribution. Thus, cultured cells were diluted to 10 times using the original suspension and submitted to sonication to break the filaments into isolated cells. To ensure we were just breaking the connection between cells but not lysing the cells the diluted suspension was sonicated for 10, 20 and 30 mins, respectively and observed under the microscope. The 20 mins sonication was selected for further use since it has shown to achieve the most isolated cells without causing cell lysis. The prepared cells were mounted on pre-cleaned quartz slides (3'x1') previously rinsed 3 times with acetone and surfactant and air dried. Then 10 µl of each prepared cell suspensions were carefully placed on the slides for image acquisition.

2.3 Image acquisition

As mentioned above, poor contrast between *Anabaena* cells and background in natural water samples is one of the biggest obstacles for use of automated imaging-based enumeration. Even when examined at higher microscopic magnifications (i.e.400X), factors such as deviations in morphology, overlapping cells, tangling filaments and out of focus targets could lead to significant errors, making routine monitoring difficult. Fluorescent light at a wavelength of 546 nm (FluorAcr epifluorescent light source via a FITC/AO filter cube), was used to illuminate the samples, exciting natural pigments to enhance the contrast between target cells and background. Fluorescent imaging signal acquisitions were conducted using an AXIOSKOP 2 Plus microscope (Zeiss, Germany) at 200X and 400X magnification with a CCD camera (QImaging Retiga EXi Mono 12 bit, 1600x1200 pixels, Fast 1394).

2.4 Model Calibration

The sonicated *Anabaena* suspensions consisting mostly of isolated cells were analyzed first. Given the previous fluorescent imaging signal acquisitions, a probabilistic unsupervised classification approach was introduced to detect isolated cells from the surrounding background based on the fluorescent signal. Using the probabilistic cell and background models, a Maximum Likelihood (ML) classifier was used to automatically delineate between the target cell pixels and the background pixels. Individual cells were identified based on results from the ML classifier for a given map. Quantitative information regarding the mean size μ , distribution σ was automatically computed for further analysis. Assuming that the isolated *Anabaena* cells are spherical in shape, the projected area per cell unit in 2 dimension images can be approximated as $S_{ISO} = \frac{\pi}{4}(\mu \pm \sigma)^2$.

2.5 Enumeration and Bio-volume estimation

In a second Phase images of un-sonicated *Anabaena* cell culture presenting tangled filaments exposed to fluorescent light (546nm) were collected and analyzed. Based on the total area identified using the ML classifier, the total surface area S_{Total} of the fluorescent zone considered as *Anabaena* cells was recognized and calculated. The number of cells was determined as $N_{Cell} = S_{Total} / S_{ISO}$ and the bio-volume expressed as $N_{cell} * V_{ISO}$.

3. Results and discussion

As illustrated in Fig 1a, the contrast between un-sonicated *Anabaena* cells and the background is very poor making it challenging to distinguish the target cells from the background and other particles. This fact makes it almost impossible to identify and enumerate cells in this preparation using a computer-assisted program. Fig 1b shows the same sample exposed to 546 nm fluorescent light which significantly increases the contrast between the cells and the background making it feasible to track each cell contour using connect component analysis for identification.

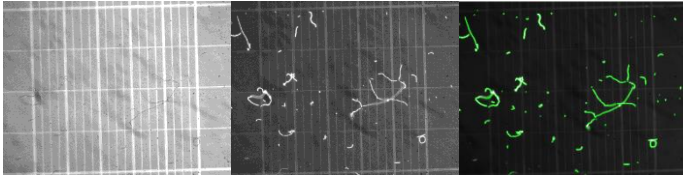


Figure 1. Laboratory cultured cells of *Anabaena flos-aquae* a) exposed to white light b) exposed to fluorescent light and c) enumerated results

Fig 2a shows the cell samples after 20 min of sonication and Fig 2b shows the enumeration results where the labelled contour marks the boundary of each isolated *Anabaena* cell against the background. The cells are intact and mostly isolated after sonication. The mean area of isolated cells is $28 \pm 1 \mu\text{m}^2$, which shows that in laboratory grown cultures that are grown under the same conditions, there is no significant variation in individual cell size and shape. To validate the concentration, chained cells were manually and automatically enumerated 3 times using a Hauser Scientific Bright-Line Hemacytometer under 200X magnification. The enumerated number of *Anabaena* cells (Fig 2d) is 12~14 cells and manual counted number is 12 cells (Fig 2c) for this specific images; thus, the estimated bio-volume is $\sim 112 \mu\text{m}^3$. The agreement between of these counts demonstrates that the developed method is robust and effective to enumerate the cell numbers and evaluate the bio-volume of cells present in a cell culture with complex morphology.

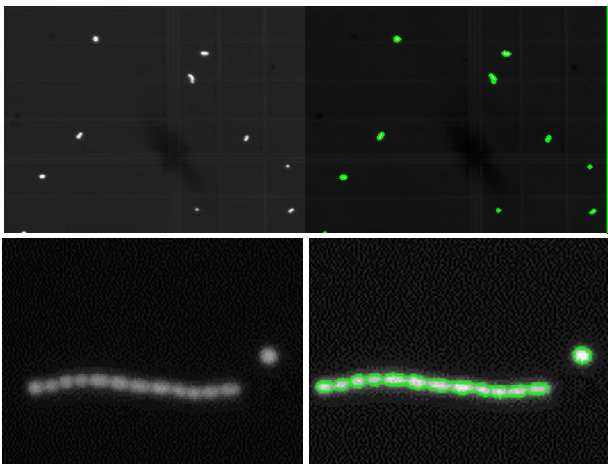


Figure 2. Cells of *Anabaena flos-aquae* a) sonicated and exposed to white light, b) sonicated and exposed to 546nm fluorescent light and chained filaments under c) white light and d) 546nm fluorescent light

Fig 1c presents the image used for enumeration of filamentous *Anabaena* cells under fluorescent light. In contrast to Fig 1a, only the target *Anabaena* cells were identified and labeled for enumeration. The total surface area of labeled cells (in green) is $22487 \mu\text{m}^2$. Based on the quantitative information obtained from Phase 1, the enumerated cell concentration is estimated as 775-833 cells and the determined bio-volume is $8.59-9.23 \times 10^4 \mu\text{m}^3$. It should be noticed that the automated enumeration process took an untrained person less than 2 mins whereas the manual enumeration by an experienced technician took 30 to 40 mins.

5. Conclusions

In this study, we developed an automated integrated method for the quantitative evaluation of the cyanobacteria *Anabaena*

flos-aquae concentration in water samples. When compared with the commonly used manual enumeration method, the newly developed method is equally accurate but saves significant time and resources making it a potential very useful tool for routine water quality monitoring.

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References

- [1] Heil, Cynthia A., Patricia M. Glibert, and Chunlei Fan. "Prorocentrum minimum (Pavillard) Schiller: A review of a harmful algal bloom species of growing worldwide importance." *Harmful Algae* 4.3 (2005): 449-470.
- [2] I. Chorus, J. Bartram, *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*, World Organization Health (WHO), London, United Kingdom, 1999.
- [3] H.W. Paerl, V.J. Paul, Climate change: links to global expansion of harmful cyanobacteria, *Water Res.* 46 (2012) 1349–1363.
- [4] Zamyadi, Arash, et al. "A review of monitoring technologies for real-time management of cyanobacteria: Recent advances and future direction." *TrAC Trends in Analytical Chemistry* (2016).
- [5] Steen, H. B. "Flow cytometric studies of microorganisms." *Flow cytometry and sorting*, 2nd ed. Wiley-Liss Inc., New York (1990): 605-622. [6]
- [6] Popels, Linda C., et al. "The use of quantitative polymerase chain reaction for the detection and enumeration of the harmful alga *Aureococcus anophagefferens* in environmental samples along the United States East Coast." *Limnology and Oceanography: methods* 1.1 (2003): 92-102.
- [7] Beutler, M., et al. "A fluorometric method for the differentiation of algal populations in vivo and in situ." *Photosynthesis Research* 72.1 (2002): 39-53.
- [8] Gray, A. J., et al. "Cell identification and sizing using digital image analysis for estimation of cell biomass in High Rate Algal Ponds." *Journal of applied phycology* 14.3 (2002): 193-204.
- [9] Izydorczyk, Katarzyna, et al. "Measurement of phycocyanin fluorescence as an online early warning system for cyanobacteria in reservoir intake water." *Environmental Toxicology* 20.4 (2005): 425-430.
- [10] Rippka, Rosmarie, et al. "Generic assignments, strain histories and properties of pure cultures of cyanobacteria." *Microbiology* 111.1 (1979): 1-61.