Automated enumeration and size distribution analysis of Microcystis aeruginosa via fluorescence imaging

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Abstract

Due to climate change, toxic cyanobacteria and algae blooms and the associated exposure risk to humans has become a global issue. As a result, routine monitoring to evaluate cell concentrations is increasingly required to ensure safe water supplies. Current methods for cyanobacteria and algae cells enumeration are time consuming and costly -intensive due to the need for manual labor, which prevents their widespread adoption for routine water monitoring.. Automated enumeration with computer-assisted image analysis has strong potential to become a viable solution for continuous routine monitoring; however, the design of such automated systems is challenging due to: a) poor contrast between the target cells and the background, b) presence of confounding cells and abiotic particles and b) image quality variability depending on factors such as the underlying microscopy system in use and the sample condition. In this study, we introduce a novel integrated imaging-based method for automated enumeration and size distribution of *Microcystis aeruginosa*, a species of freshwater cyanobacteria that can originate harmful blooms. The target cells were excited using a 546nm light source and the resulting fluorescent imaging signal was acquired. A probabilistic unsupervised classification approach was taken to detect *Microcystis* cells from the surrounding background based on the fluorescent signal. A Gaussian mixture model was learned from the fluorescent imaging signal. The detected *Microcystis* cells were then enumerated and statistics regarding their size distribution automatically computed. When compared to the manual enumeration data using an hemacytometer, the developed method achieved higher accuracy using much less time and resources, without cell staining. These preliminary results demonstrate the potential of the proposed method as a powerful and robust tool for water quality monitoring and safe water quality control when used alongside gold standard methods.

1. Introduction

Toxic algae and cyanobacteria blooms are increasingly prominent in drinking water supplies worldwide due not only to high nutrient loads at the source but also to global climate change and the resulting extreme weather events (i.e. drought, excessive rain, intense storms, higher ,water body temperatures, etc.)[1-3] During blooms, exposure even to low concentrations (~ 1~2 ppb) of toxin associated with *Anabaena*, *Microcystis*, *Planktothrix*, *Nostoc*, and other cyanobacteria may represent a risk for animal and human consumers. The World Health Organization (WHO) recommends a maximum acceptable concentration of Microcystin-LR toxin of 1 µg/L in drinking water supplies[1] and Health Canada's Guidelines for Drinking Water Quality require a maximum level of 1.5µg/L for the same toxin[4].

To overcome the health-related challenges introduced by the presence of blooms, alert levels based on the number of cyanobacteria cells present have been recommended for drinking water treatment processes. According to the WHO guidance, when the cyanobacteria concentration is above 100,000 cells /ml, the relative probability of acute health effects could be high [1]. Health Canada recommends that "drinking water supplies suspected or know to be susceptible to blooms should be routinely monitored for the presence of cyanobacteria (at the sites of) raw water intakes, reservoir and various stages in the water treatment process.''. Accordingly, the enumeration of cyanobacteria in water supplies has become increasingly required as part of water quality monitoring programs [4].

While a number of methods are available to conduct cyanobacteria and algae cells enumeration, none is applicable for routine monitoring. The gold-standard cell enumeration method consists of using an hemocytometer, (i.e. a small chamber with a microscopic grid that can be filled with a known volume of water sample and is used for cell enumeration using an optical microscope)[5]. Although accurate, this method is time consuming and labor intensive, making it ill-suited for continuous routine monitoring. Techniques such as flow cytometry[6],laser granulometry[7], antibody immunofluorescent technique[5], PCR-fluorescent fragment detection[5,8], and qPCR have also been usedto conduct cell enumeration. However, flow cytometry and laser granulometry are not robust enough to distinguish cells from contaminants, while molecular-biology based methods are very costly to perform. Furthermore, non-specific binding often occurs with polyclonal techniques, resulting in overestimation of cell counts[9]. In contrast, imaging-based enumeration methods are very promising as potential alternatives to the aforementioned methods, as they can be: a) significantly more cost effective and time efficient, b) more flexible in identifying different types of target cell based on their size, shape, and, color, c) more easily integrated into the current monitoring pipeline, and d) more easily applicable requiring less intensive training[10].

While automated enumeration with computer-assisted image analysis has strong potential to be a viable solution for enabling continuous routine monitoring, there are a number of critical challenges that need to be overcome before it can be used for widespread accurate cell enumeration. First, accurate delineation between contaminants in water samples and target cells is critical for reliable enumeration. Due to the natural transparency of live cells the contrast between background contaminants and

target cells is very poor under white light, making it very difficult to distinguish them visually.. Second, the quality of the acquired images can exhibit significant variability depending on factors such as the underlying microscopy system, the imaging parameters (e.g., focus), the light source, and the condition of the sample being imaged. Therefore, it is necessary to investigate and explore new automated image-based enumeration methodologies that allow for not only for reliable and accurate delineation between target cells and background contaminants, but also to ensure that are flexible enough to be used in a wide range of conditions without requiring significant manual tuning.

In this study, we introduce a novel integrated imaging-based method for automated enumeration and size distribution evaluation of a laboratory culture of *Microcystis aeruginosa*, a species of freshwater cyanobacteria that can form harmful algae blooms. The proposed method allows for reliable and accurate detection, enumeration, and size analysis of *Microcystis* cells, and automatically adapts to the underlying imaging conditions. For method validation, *Microcystis* culture with different concentrations were enumerated using both the new automated method and traditional method using a hemacytometer.

2. Materials and Method

2.1 Algae cells preparation

Microcystis aeruginosa was obtained from the Canadian Physiological Culture Centre (CPCC), University of Waterloo were used as representative algae cells for enumeration evaluation. The BG-11 growth medium[11] was used to culture the cells in Erlenmeyer flasks inside a Percival growth cabinet (John's Scientific Inc., Canada) maintained at a temperature of 19~ 22℃ and a light intensity of ~ 1100 lumens with full spectrum of white light. The cultured cells were diluted to 5 different concentrations (initial concentration and diluted by 10, 50, 100 and 500 times).

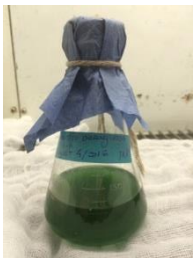

Figure 1. Cultured cells of *Microcystis aeruginosa*

The prepared cells were mounted on pre-cleaned quartz slides (3'x1') which were pre-rinsed 3 times with acetone and surfactant and air dried, 10 µl of each cell suspension were carefully placed on the slides for image acquisition.

2.2 Image acquisition

As mentioned earlier, one of the biggest hurdles with achieving automated imaging-based enumeration is the poor contrast between the target cell and the background as well as background contaminants found in real-world water samples, with even well-trained technicians taken substantial time performing manual enumeration under higher magnifications (i.e. 400X). Factors such as deviations in particle morphology(i.e. size and shape) lead to further errors, making continuous routine monitoring intractable in practice. An example of the difficulty in delineating *Microcystis* cells under white light is shown in Fig. 2a, where its transparent nature makes it very difficult to identify.

To remedy this issue, we illuminated the sample using a fluorescent light source, causing the *Microcystis* aeruginosa cells to be excited and emit a strong fluorescent signal. Through empirical testing with light sources at different wavelengths, it was found that only light at a 546 nm wavelength resulted in a strong excitation of the cells pigment , leading to a significant fluorescent signal.. This is likely due to the presence of chemicals such as photosensitizers such as chlorophyll excited by the light source at 546nm. After the sample exposure to a FluorAcr epifluorescent light source at 546nm via a FITC/AO filter cube (Set 15), fluorescent imaging signal acquisitions were conducted using a AXIOSKOP 2 Plus microscope (Zeiss, Germany) at 200X and 400X magnification using a CCD camera (QImaging Retiga EXi Mono 12 bit, 1600x1200 pixels, Fast 1394). To validate the new methodology the same samples were also manually enumerated using a Hausser Scientific Bright-Line Hemacytometer (VWR International, Mississauga, Canada) and counted under 200X and 400X magnification under both white light and fluorescent light at the wavelength of 546 nm.

2.3 Image analysis

Given the fluorescent imaging signal acquisitions, a probabilistic unsupervised classification approach was introduced to detect *Microcystis* cells from the surrounding background based on the fluorescent signal. Such a probabilistic approach enables dynamic adaptation to handle a wide range of imaging and sample conditions without requiring significant manual tuning, and can be described as follows. Given a fluorescent imaging signal *f*, an expectation maximization method was used to learn a Gaussian mixture model (resulting in a cell model $P(f|c_1)$ and a background model $P(f|c_2)$) for the imaging signal intensities. Given 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 in the fluorescent imaging signal *f*: $C = \arg_c \max P(f|c)$ where *C* is the target cell map. Given the target cell map obtained using the ML classifier, connected component analysis was used to identify all the individual cells. These cells were then enumerated automatically and statistics regarding the size distribution of the cells was also automatically computed.

3. Results and discussion

Laboratory cultured *Microcystis aeruginosa* was selected for this study for a number of reasons: a) it is one of the most common cyanobacteria in natural water bodies, b) is unicellular and spherical in shape with relatively uniform cell size that is easy to identify, and c) when grown under laboratory conditions its cells are well isolated from each other rather than aggregated allowing for more reliable counts with less false positive error.

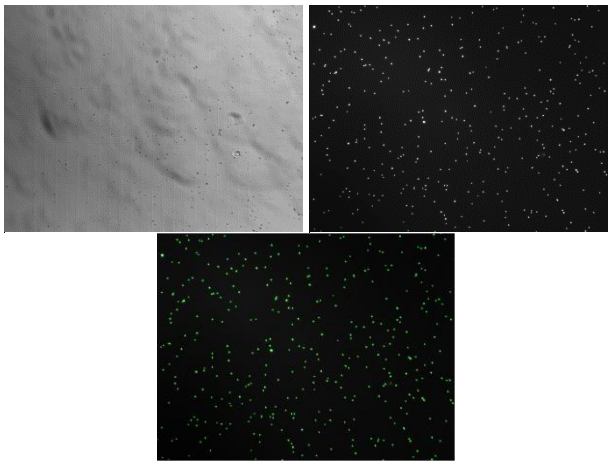

Figure 2. Acquired images of cultured *Microcystis aeruginosa* cells under 200X magnification: under a) normal white light, b) light source at 546nm, and c) enumerated results

As shown in Figure 2a, under normal white light commonly used in traditional enumeration, the contrast between target cells and background contaminants is very poor, making it extremely challenging to distinguish cells from other particles. Figure 2b shows the acquired image when a 546 nm light source was used to illuminate the sample causing *Microcystis* cells to emit a very strong fluorescent signal compared to background contaminants, making it significantly easier to perform automated enumeration and size distribution analysis. Figure 2c shows the enumerated results. The counts for 5 initial concentrations with 3 replicates obtained by the proposed method and the manual counted results are shown for comparison. The numbers of *Microcystis* cells obtained using the new method are very close to the values counted manually, which shows the efficacy of the proposed methodology. In Figure 3, the cell counts obtained using the proposed method was plotted against the manual counts for various initial concentrations. For ease of comparison, a line of y=x is also plotted representing a perfect match between manual counts and enumerated results. As demonstrated in Figure 3, the proposed method has been successfully applied to enumerate the fluorescent cells under different scenarios. The raw suspension of *Microcystis* without dilution was not included into Fig 3 because the cell number is too many to count manually. Enumerated values are all centered around the y=0.975x line yielding a coefficient of determination of 0.995 with an intercept of 37.7.

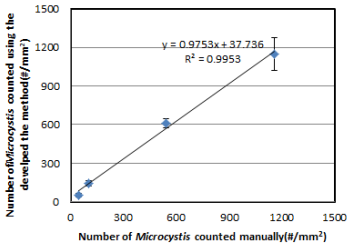

Fig 3. Comparison of cell numbers enumerated manually and by the developed method

4. Conclusion

In this study, a novel integrated imaging-based method for automated enumeration and size distribution of *Microcystis aeruginosa* is presented. The rigorous analysis herein conducted has demonstrated the efficacy of the proposed method, and its potential usefulness as a tool for continuous routine water monitoring to ensure public health safety.

Acknowledgements

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