




Monitoring of cyanobacteria using derivative spectrophotometry and improvement of the method detection limit by changing pathlength

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ABSTRACT

Effective monitoring tools and methods are needed for the early detection and management of cyanobacteria in water bodies to minimize their harmful impacts on the environment and public health. This research investigated changing the cuvette pathlength (10-, 50-, and 100-mm) to improve the detection of cyanobacteria using UV-Vis spectrophotometry with subsequent application of derivative spectrophotometry and Savitzky-Golay (S-G) transformation. A non-toxicogenic strain of blue-green cyanobacteria, *Microcystis aeruginosa* (CPC 632), and a green algae strain for comparison, *Chlorella vulgaris* (CPC 90), were studied in a wide range of concentrations (955,000 to 1855 cells/mL). In each concentration range, method detection limits were established with absorbance measurements and S-G first derivative of absorbance using 10-, 50-, and 100-mm cuvette pathlengths. Increasing the cuvette pathlength from 10 to 100 mm resulted in a 15-fold improvement in sensitivity with absorbance and a 13-fold improvement with S-G first derivative of absorbance for *M. aeruginosa*. Overall, adoption of 100 mm pathlength and application of S-G derivative spectra improved the method detection limit for *M. aeruginosa* from 337,398 to 4916 cells/mL, which is below the WHO guideline for low probability of adverse health effects (<20,000 cells/mL). Similarly, the detection limit for *C. vulgaris* was improved from 650,414 to 11,661 cells/mL. The results also showed that spectrophotometry could differentiate *M. aeruginosa* from *C. vulgaris* based on the variations in their pigment absorbance peaks.

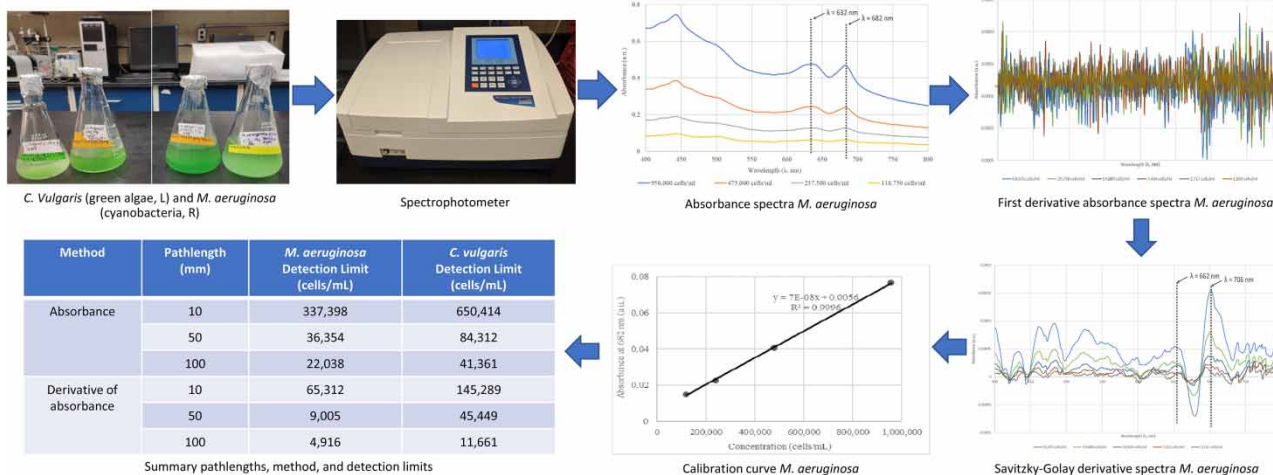
Key words: *C. vulgaris*, cyanobacteria, derivative spectrophotometry, microcystis aeruginosa, monitoring, water

HIGHLIGHTS

- UV-Vis spectrophotometry was studied for early detection of cyanobacteria.
- Increasing cuvette pathlength (10, 50, 100 mm) improved the method detection limit.
- Applying S-G derivative spectrophotometry improved the method detection limit.
- MDL was improved from 337,398 to 4916 cells/mL for *M. aeruginosa*.
- Spectrophotometry could differentiate *M. aeruginosa* from *C. vulgaris*.

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GRAPHICAL ABSTRACT



1. INTRODUCTION

The need for better, simple, and affordable early detection systems is imperative with increasing harmful algal blooms (HABs) in water sources worldwide (Hudnell 2008; Zamyadi *et al.* 2016; Liu *et al.* 2020). The presence of HABs creates nuisance from poor water quality, surface scum production and can lead to problems with drinking water treatment such as clogging of filters, increased disinfectant requirements, and in the case of extreme events, the need for alternate water sources (Oren 2014). Cyanobacteria can be toxigenic or non-toxigenic in nature; the toxigenic cyanobacteria can produce highly potent cyanotoxins stored inside the cell walls, which are released upon cell lysis. This poses a potential risk to human health, wildlife, livestock, and the aquatic environment (Srivastava *et al.* 2013; Al-Sammak *et al.* 2014; Bukaveckas 2018). *Anabaena*, *Aphanizomenon*, and *Microcystis* are the three genera of cyanobacteria prominently found in freshwater sources, of which *Microcystis aeruginosa* is the most common toxin-producing species, and microcystins (hepatotoxins) are the most widespread toxins (Stefanelli *et al.* 2014; Mohamed *et al.* 2015). Studies have shown that microcystins intoxication can lead to gastroenteritis, liver damage, cancer, and acute poisoning death (Chorus & Bartram 1999; Hudnell 2008; Wilson *et al.* 2008; Pelaez *et al.* 2010). In addition to toxin production, some cyanobacterial species generate compounds such as geosmin and 2-methylisoborneol that produce unpleasant tastes and odors, causing water quality deterioration (Graham *et al.* 2010).

It should be noted that the toxins released when the algal cells are still alive and healthy in freshwater are negligible compared to the toxins released on cell lysis (Bartram & Rees 2000). Toxins released in freshwater bodies quickly dissolve in water and rapidly dilute and degrade with time. Therefore, cell-bound cyanotoxins are a bigger concern when it comes to drinking water supply and recreational waters due to cell lysis and increased concentration of localized toxins in the conformed area (EPA 2005; Graham *et al.* 2010). Therefore, there is an increasing need for monitoring tools and technologies that are sensitive, easy to use, and implementable for the early detection of cyanobacteria before bloom formation.

Due to the harmful effects of cyanobacteria, early detection is crucial and heavily relies upon monitoring programs established by water authorities (EPA 2005). Several water authorities have developed guidelines based on the guidance values recommended by the World Health Organization (WHO) (Bartram & Rees 2000; Giddings *et al.* 2012; WHO 2017). Examples of national regulations or recommendations based on an alert threshold for relatively mild and/or low probability of adverse health effects of cyanobacteria in drinking and recreational waters are provided in Table 1. The ability to monitor cyanobacteria quickly and sensitively prior to bloom formation is a key factor in a successful HAB management strategy and is of utmost importance to water quality managers (Le *et al.* 2010; Altenburger *et al.* 2015; EPA 2015a). This allows authorities to manage blooms and prevent potential harm to the water source.

Detection technologies for cyanobacteria monitoring are broadly classified into two categories: (1) methods without extraction such as direct counting methods using a microscope, remote sensing, and spectrophotometric, and (2) methods relying

Table 1 | Examples of national regulations for managing cyanobacteria/microcystin in water sources

Country/ Source	Drinking water	Recreational water	Reference
WHO	1 µg/L	≤20,000 cells/mL or 10 µg/L Chl-a	WHO (2003)
Canada	1.5 µg/L	≤100,000 cells/mL or 20 µg/L Chl-a	Health Canada (2016)
USEPA	1.6 µg/L	≤20,000 cells/mL or 10 µg/L	EPA (2015b)
Brazil	10,000–20,000 cells/mL	Not declared	Chorus (2012)
France	1 µg/L	≤20,000 cells/mL	Chorus (2012)
Cuba	<20,000 cells/mL	20,000–100,000 cells/mL	WHO (2003)

on quantification after pigment extraction such as quantitative polymerase chain reaction (qPCR), chromatography based techniques, and biochemical methods/screening assays (UNESCO *et al.* 2004; Moreira *et al.* 2011, 2014; Lane *et al.* 2012; Altenburger *et al.* 2015; Wang *et al.* 2015; AlMomani & Örmeci 2018). However, many of these technologies are not suitable for real-time or near real-time monitoring and often require extensive sample preparation making the methods labor- and time-intensive. For example, real-time qPCR is a promising technology but requires highly skilled personnel for operation and relies on intrusive pigment extraction (Zhang *et al.* 2014). Methods like remote sensing enable rapid observation but heavily rely on weather conditions being favorable for monitoring purposes and have a low overall accuracy of detecting a bloom (Wang *et al.* 2015).

The working principle for estimating cyanobacteria biomass relies on determining primary photopigments present in an algal sample (Bertone *et al.* 2018). Six different types of chlorophyll (Chl-a, b, d, f, and divinyl-chl a and b) occur naturally in cyanobacteria, but Chl-a predominates accompanied by accessory phycobilin pigments such as phycocyanin and phycoerythrin (Gray 2010; Shin *et al.* 2018a, 2018b). Chlorophyll-a is an excellent photoreceptor and thus is the primary pigment for cyanobacteria detection in water sources (Tran Khac *et al.* 2018). There are some in-situ methods used for real-time monitoring, which mainly rely on fluorometry or infra-red spectral reflectance technology for cyanobacterial quantification (Zamyadi *et al.* 2016; Bertone *et al.* 2018; Liu *et al.* 2020). Fluorometry is the most commonly used method for sensitive in-situ monitoring of cyanobacteria and generally relies on the detection of chlorophyll-a (Chl-a) (Garrido *et al.* 2019). Studies have reported that the sensitivity of fluorescence measurements for cyanobacterial estimations is affected by factors like varying cells size, cell agglomeration, prior light exposure, presence of other phytoplankton containing Chl-a or phaeopigments, and water turbidity (Bowling *et al.* 2016; Zamyadi *et al.* 2016; Liu *et al.* 2020).

Spectrophotometric analysis is the most well-documented method used to estimate chlorophyll concentrations in the laboratory (Gray 2010). Spectrophotometry is less sensitive than fluorometry and shares some of the limitations of fluorometry; therefore, it has not been widely employed for cyanobacteria monitoring. The main advantage of the spectrophotometric method lies in its simplicity, accuracy, rapidness, flexibility to measure multiple pigments with no pigment extraction or reagent requirement. More importantly, spectrophotometry can measure various water quality parameters (such as organic carbon, nitrate, UV absorbance) (Burgess & Thomas 2017) and transmit data in real-time. Studies have shown that several water quality parameters determine the growth rate and characteristics of bloom formation (Pelaez *et al.* 2010; Health Canada 2016), and UV-vis spectrophotometry can measure both cyanobacteria concentrations and water quality parameters simultaneously and offers an advantage in this regard over the other technologies.

Agberien & Örmeci (2019) used derivative spectrophotometry to measure cyanobacteria concentrations in water and reported detection limits of 90,200 cells/mL in surface water and 41,800 cells/mL in deionized water for *M. aeruginosa*, which indicated that the derivative spectrophotometry could be used for the monitoring of cyanobacteria, but the method may not be sensitive enough for early detection (Table 1). The objective of this research was to improve the sensitivity of UV-Vis spectrophotometry for early detection and monitoring of cyanobacteria by increasing cuvette pathlength (10-, 50-, and 100-mm) and applying derivative spectrophotometry. The first derivative of absorbance and complementary statistical tools were investigated with changing pathlengths, and new method detection limits were established that showed substantial improvements. A strain of *Chlorella vulgaris* was used for comparative purposes as green algae are the most prevalent bio-spheric inhibitors and *Chlorella* is the most common species found in nutrient-rich waters (Naselli-Flores & Barone 2009; Borowitzka 2018).

2. MATERIALS AND METHODS

2.1. Cultivation of microalgae

A non-toxicogenic strain of cyanobacteria, *Microcystis aeruginosa* (CPCC 632) and *Chlorella vulgaris* (CPCC 90), for comparison, were used in this study. *M. aeruginosa* was cultured in sterile 3N-BBM medium while *C. vulgaris* was cultured in sterile BBM medium (CPCC 2013). *M. aeruginosa*, *C. vulgaris* and the growth mediums were purchased from Canadian Phycological Culture Centre (CPCC) at the University of Waterloo (Ontario, Canada). Both strains of microalgae were cultured in separate 500 mL Erlenmeyer flasks. To prevent contamination, the flasks were rinsed in deionized (D.I.) water, the flask mouths were covered using aluminum foil paper and sterilized using an autoclave for 30 min at 15 psi and 121 °C. *M. aeruginosa* and *C. vulgaris* were inoculated in the growth medium using 1:2 and 1:4 dilution ratios, respectively (CPCC 2013). The cultivation flasks were placed inside a temperature-controlled incubator (24 °C) equipped with two daylight fluorescent tube lights. The cultures were maintained under a 24-hour light photoperiod at an intensity of 1000 lux for *M. aeruginosa* and 1800 lux for *C. vulgaris*, respectively. Higher intensity for *C. vulgaris* was attained by elevating the Erlenmeyer flask using a stand inside the incubator. Higher light intensity was provided to *C. vulgaris* to improve the growth rate (KBGAWG 2009). No supplementary carbon dioxide CO₂ was supplied to the cultures for growth purposes other than the diffused CO₂ present in ambient air. The flasks were agitated gently and manually twice a day.

2.2. Preparation of samples

M. aeruginosa and *C. vulgaris* cultures were separated from their growth medium by centrifugation at 8000×g for 5 min each using 50 mL tubes. Following centrifugation, the growth medium was discarded, and the collected microalgae were re-suspended in 50 mL D.I. water (Hellebust *et al.* 1973). The samples were then gently inverted to ensure a homogenous concentration of algae over the entire volume. The cultures were subsequently enumerated under a Leitz Laborlux 12 light microscope and quantified using an improved Neubauer hemocytometer (Bastidas 2013). Following quantification of both algal strains, respective dilutions samples were prepared in D.I. water. The same dilution ratios were used for both samples to ensure that the concentration of the samples remained approximately equal to each other. This was done in order to enable meaningful and representative comparisons between both algal cultures. The concentration of samples ranged approximately from 7,612,903 cells/mL to 1855 cells/mL for *M. aeruginosa*, and 7,627,628 cells/mL to 1855 cells/mL for *C. vulgaris*.

2.3. UV-Visible spectrophotometry

Analysis was performed using a Cary 100 Bio UV-Vis Spectrophotometer (Varian) with 10-, 50-, and 100-mm pathlength quartz cuvettes. The spectral absorbance analysis was carried out between 200 and 800 nm wavelength range, and the results were reported in absorbance units (a.u.). For each concentration level, eight technical replicates were analyzed for both algal strains to avoid photobleaching of the algal photopigments present in the sample. The resultant absorbance spectra were subtracted by absorption blanks (D.I. water) to obtain the final absorbance measurements for both *M. aeruginosa* and *C. vulgaris*. Higher and lower concentration ranges for *M. aeruginosa* were 118,750–955,000 and 1855–59,687 cells/mL, and for *C. vulgaris* were 119118–953,333 and 1855–59,686 cells/mL respectively. To ensure representativeness of the samples, near-similar concentration ranges were maintained for every experiment, and the sample volume for 10-, 50-, and 100-mm cuvettes was kept at 3-, 17.5-, and 35-mL, respectively, for both algal strains.

2.4. First derivative of absorbance

The first-order derivative of absorbance represents the rate of change in absorbance with respect to wavelength ($dA/d\lambda$) (Owen 1998). This technique was used in pursuit of improving the detection limit of microalgae in water. First-order derivatives were calculated by taking a difference between each successive absorbance values and dividing it by the wavelength interval separating them. The interval used for change in absorbance values was taken at 1 nm and resulted in $d\lambda$ value of 1.

2.5. Savitzky-Golay first derivative of absorbance

Savitzky-Golay (S-G) first order derivative was used to smoothen the plot of first order derivative and improve the signal to noise ratio using the following correlation (Savitzky & Golay 1964):

$$a_j = \frac{\sum_{i=-\frac{m-1}{2}}^{\frac{m-1}{2}} C_i F_{j+i}}{N} \quad \frac{m+1}{2} \leq j \leq n - \frac{m-1}{2}$$

where a_j = Savitzky-Golay first derivative of absorbance; m = number of data points used; C_i = Savitzky-Golay filter coefficient; F = absorbance value measured at a specific wavelength; j = smoothened data point; N = standardization factor.

Each measured absorbance value was smoothened using twenty-three data points such that $i = -11, -10, -9, \dots, 9, 10, 11$; $m = 23$; $N = 1,012$; $C_i = -11, -10, -9, \dots, 9, 10, 11$; following S-G first derivative of absorbance (Savitzky & Golay 1964).

2.6. Method detection limit

The minimum concentration of a component measured using an analytical procedure with 99% confidence, where the concentration of the component is greater than zero, is known as the method detection limit (MDL) (WDNR 1996). The MDLs were calculated according to the Hubaux and Vos method, using a tool developed by Chemiasoft, with a minimum of eight technical spike replicates for MDL calculation of individual algal strain (Hubaux & Vos 1970).

2.7. Statistical analysis

Statistical analysis of the measured algal concentration was carried out using Microsoft Excel software. Absorbance graphs, S-G first derivative of absorbance graphs and standard calibration curves were plotted for higher and lower concentration ranges (for both algae) using the average values of the eight technical replicates. Standard calibration curves were used to validate whether the absorbance measurements agreed with the Beer-Lambert Law. Coefficient of determination (R^2) and slopes with their individual standard deviations were obtained for both *M. aeruginosa* and *C. vulgaris* at their individual wavelengths of interest.

3. RESULTS

3.1. Absorbance measurements

Experiments were carried out in D.I. water to establish interference-free spectral lines for cyanobacterial analysis. At a higher concentration range, *M. aeruginosa* resulted in three peaks at approximately 442, 632, and 682 nm, with 682 nm being the most prominent peak, which was used for analysis (Figure 1). The absorbance peaks near 440 and 680 nm are the primary characteristics of the photopigment chlorophyll-a, while the peak at 632 nm corresponds to phycocyanin. This explains the peaks observed for *M. aeruginosa* as cyanobacteria carry both Chl-a and phycocyanin photopigments (LeBlanc Renaud *et al.* 2011). Considering the aforementioned peaks, the primary analysis focused on wavelengths between 400–800 nm.

Comparison of absorbance spectra of *M. aeruginosa*, indicates an increase in absorbance with growing cyanobacterial concentration for each individual pathlength used (Figure 1). With longer cuvette pathlength, higher absorbance measurements were observed, which is expected as more light is absorbed by a larger photosensitive sample volume (Burgess & Thomas 2017). For 10 mm pathlength analysis, no significant peak was observed for the lower concentration range test (Figure 1(b)), but peaks corresponding to Chl-a can be observed for the higher concentration (Figure 1(a)). For 50- and 100-mm pathlength tests, solely Chl-a peaks were clearly observed at lower concentration range (Figure 1(d) and (f)); meanwhile, at higher concentration range (Figure 1(c) and (e)), both Chl-a and phycocyanin peaks were prominently visible. It was observed that the sensitivity from 10 to 50 mm pathlength increased approximately 9-fold, with the detection limit improving from 337,398 to 36,354 cells/mL for 50 mm pathlength. On the other hand, with 100 mm pathlength, the MDL improved further to 22,038 cells/mL for cyanobacterial detection, but the improvement was not as significant as the change from 10 to 50 mm pathlength. From the results obtained, 100 mm pathlength cuvette displayed the highest sensitivity.

To validate the analysis, standard calibration curves at 682 nm were generated for higher and lower concentration range tests to ensure that the data followed the Beer-Bouguer-Lambert Law (refer to supplementary information Figure S.1). All higher concentration range results conformed with Beer-Bouguer-Lambert Law, validating the analysis. As the focus of

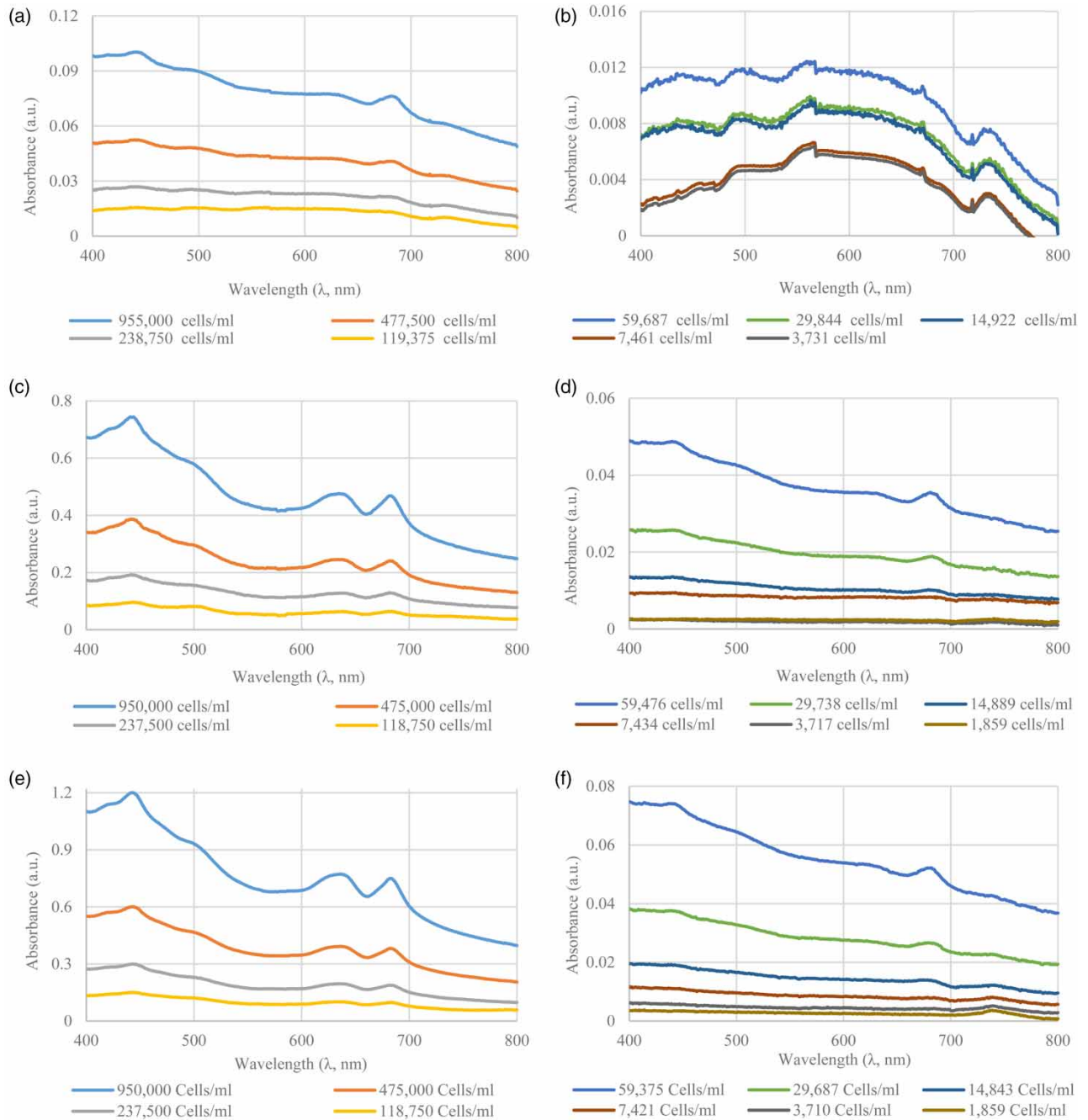


Figure 1 | Absorbance spectra at higher and lower concentration ranges of *M. aeruginosa* for 10 mm (a, b), 50 mm (c, d), and 100 mm (e, f) cuvette pathlengths, respectively.

this study is for sensitive detection of cyanobacteria, the discussion from here on focuses on the lower concentration results, and the high concentration results are also presented. For all low concentration range results, a strong correlation between cyanobacterial concentration and absorbance was observed ($R^2 = 0.9137, 0.9491, \text{ and } 0.9995$, for 10-, 50-, and 100-mm pathlengths, respectively). There was a positive linear trend for the entire concentration range of absorbance measurements ranging from 1855 to 955,000 cells/mL. Further, the slopes obtained at 632 and 682 nm were approximately the same $8.6637 \times 10^{-07} \pm 1.1741 \times 10^{-08}$ and $8.6234 \times 10^{-07} \pm 1.0036 \times 10^{-08}$ au/(cells/mL), respectively, with a strong correlation maintained between concentration and absorbance ($R^2 > 0.99$) at 632 nm. However, even with higher pathlengths, it is difficult to distinguish the peaks at 632 nm from background noise at the lower concentration range. Despite that, for 50- and 100-mm

pathlengths, the peaks at 682 nm can be readily observed at a concentration of approximately 60,000 cells/mL, with a diminishing peak at around 30,000 cells/mL.

3.2. First derivative of absorbance

The first derivative corresponds to the slope of the traditional spectrum, where the maxima correlate with the increase in absorbance with wavelength, while the minima appear after maxima of the normal spectrum. Additionally, the maxima of the normal spectrum correspond to the zero value on the derivative spectra (Burgess & Thomas 2017). First-order derivative of absorbance was implemented in a study to identify key wavelengths to improve the signal-to-noise ratio and reduce background noise while reducing issues that arise from shifting water baseline and overlapping spectral peaks (AlMamani & Örmeci 2018). A similar technique was applied in this study to investigate the effect of the first derivative on the detection of cyanobacteria. Figure 2 illustrates a plot of the first derivative of absorbance versus concentration for *M. aeruginosa* at higher and lower concentrations. For higher concentration tests, a peak close to 670 nm can be observed for 50- and 100-mm pathlengths (Figure 2(c) and (e)), while no peak is observed for 10 mm pathlength test (Figure 2(a)). At the same time, the signal cannot be readily differentiated from background noise at lower concentrations (as seen from Figure 2(b) and (d)), and an increase in noise can be observed. Moreover, even with increasing cuvette pathlength, no significant peak was observed at lower concentration making further analysis and detection of cyanobacteria difficult (Figure 2(f)).

Upon comparison of the normal absorbance spectra to the first-order derivative of absorbance spectra, it was observed that the number of peaks increased drastically (increasing noise), but there were no prominent peaks observed unlike the peaks readily observed in the normal absorbance spectra. Besides, the absorbance values observed upon application of the first derivative of absorbance were lower than the normal absorbance spectra, which is an attribute of the first derivative of absorbance, as it plots the rate of change in absorbance against wavelength, instead of a plot of absorbance versus wavelength. An unwanted effect of the first-order derivative is that as the signal-to-noise ratio decreases, it results in sharper noise features in the spectrum and a higher number of peaks due to rapid changes in amplitude under the presence of random noise (Kus *et al.* 1996). This can be reduced using alternatives techniques such as the Savitzky-Golay derivative as discussed below.

3.3. Savitzky-Golay first derivative of absorbance

Savitzky-Golay (S-G) first derivative of absorbance was applied to simultaneously obtain first-order derivative and smoothen the plot obtained by derivative absorbance, which reduces the enhanced background noise obtained due to the first derivative of absorbance (Ruffin *et al.* 2008). This resulted in sharp and distinct peaks for *M. aeruginosa* at higher as well as lower concentrations for 50 and 100 mm pathlengths (lower concentration illustrated in Figure 3(c) and (e)). Conversely, for *C. vulgaris*, 50 mm pathlength tests revealed sharp peaks at higher concentrations (supplementary information Figure S.3), while lower concentrations displayed peaks accompanied with added noise features (Figure 3(d)). Nonetheless, distinct peaks can be observed for 100 mm pathlength tests at higher as well as lower concentration ranges (lower concentration illustrated in Figure 3(f)) for *C. vulgaris*. However, similar to the normal absorbance spectra, it is difficult to differentiate between signal and noise on the Savitzky-Golay spectral scan using 10 mm pathlength for both cyanobacterial and non-cyanobacterial algae strain at lower concentrations (as illustrated in Figure 3(a) and (b)).

Upon application of the derivative spectrophotometry method, a shift in the primary peak of interest (at 706 nm) was observed for *M. aeruginosa* when compared to the normal absorbance spectra (at 682 nm). This is an attribute of the derivative spectrophotometry as it plots the rate of change of absorbance against wavelength (Agberien & Örmeci 2019). Both 50- and 100-mm pathlength results revealed analogous spectral patterns at higher and lower concentration ranges. The standard calibration curves (illustrated in Figure S.2), plotted at 706 nm showed a linear trend and a strong positive correlation between the S-G first derivative of absorbance and concentration ($R^2 = 0.8381, 0.9981, \text{ and } 0.9943$, for 10, 50, and 100 mm pathlength, respectively). The MDL using S-G first derivative of absorbance for 10, 50, and 100 mm pathlength was calculated to be 65,312, 9005 and 4916 cells/mL, respectively. An approximately 7-fold improvement in detection was observed between 10 and 50 mm pathlength; at the same time, a 13-fold improvement was observed between 10 and 100 mm pathlength. A summary of the results obtained for both algal strains from the above experiments, including pathlength and detection limits, are shown in Table 2.

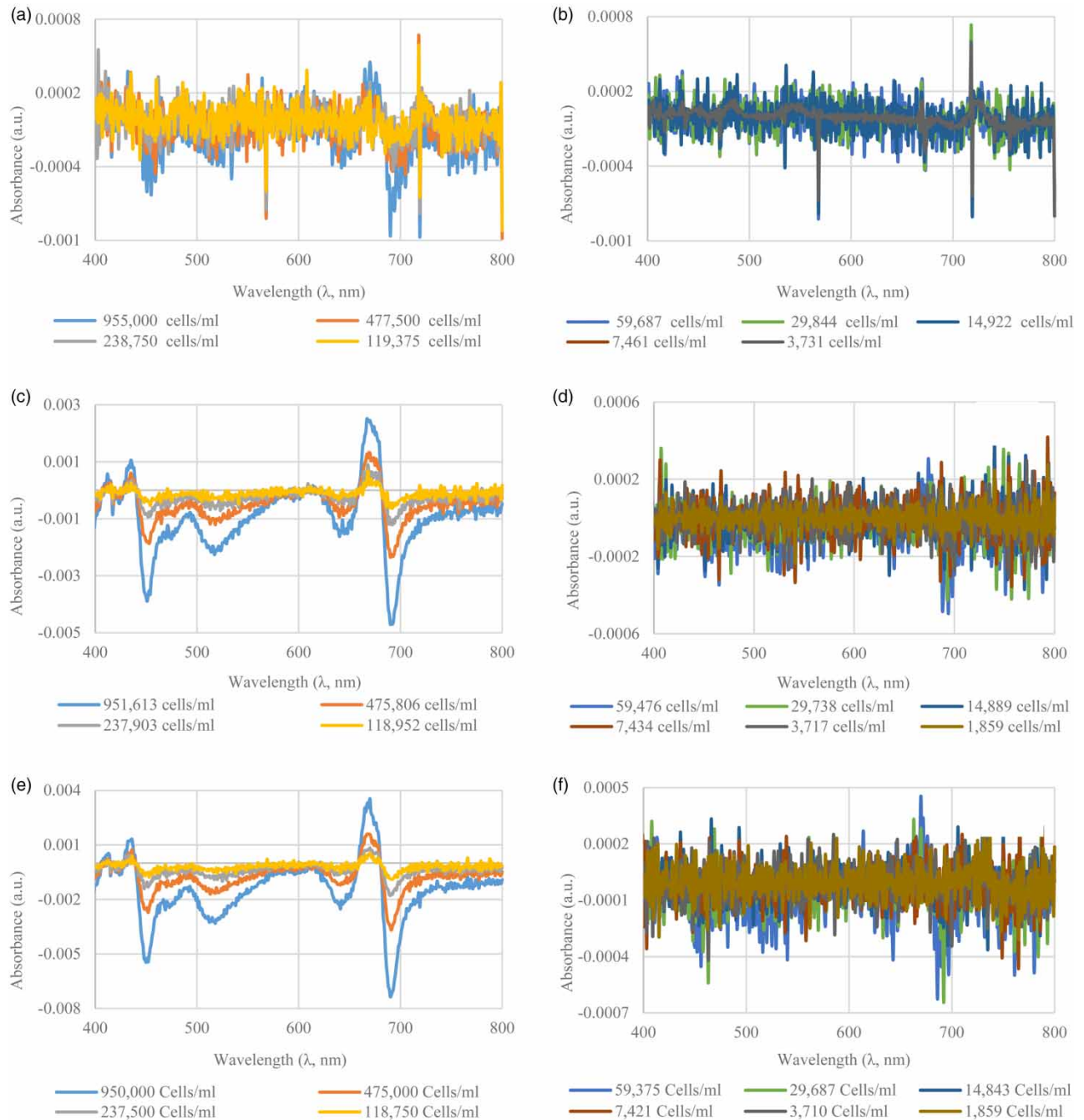


Figure 2 | First derivative of absorbance spectra of *M. aeruginosa* at higher and lower concentrations for 10 mm (a, b), 50 mm (c, d), and 100 mm (e, f) cuvette pathlength, respectively.

3.4. Spectral comparison of *M. aeruginosa* and *C. vulgaris*

Investigating the potential differences between the absorbance spectra of a blue-green cyanobacteria (*M. aeruginosa*) and a non-cyanobacterial green algae (*C. vulgaris*) is critical to determine whether cyanobacteria can be differentiated from non-cyanobacterial algae for detection purposes. *M. aeruginosa* and *C. vulgaris* have similar structural morphologies where cells are spherical or ovoidal in shape and can be present in the form of single cells and/or mucilaginous colonies (Safi 2014; Álvarez *et al.* 2020). However, the organisms have varying cell sizes with *M. aeruginosa* ranging from 3 to 5 μm in diameter, while *C. vulgaris* cells range between 3 and 10 μm in diameter, which may potentially impact the absorbance measurements due to physical characteristics (Wilson *et al.* 2006; Safi 2014; Bañares-España *et al.* 2016; Li *et al.* 2016).

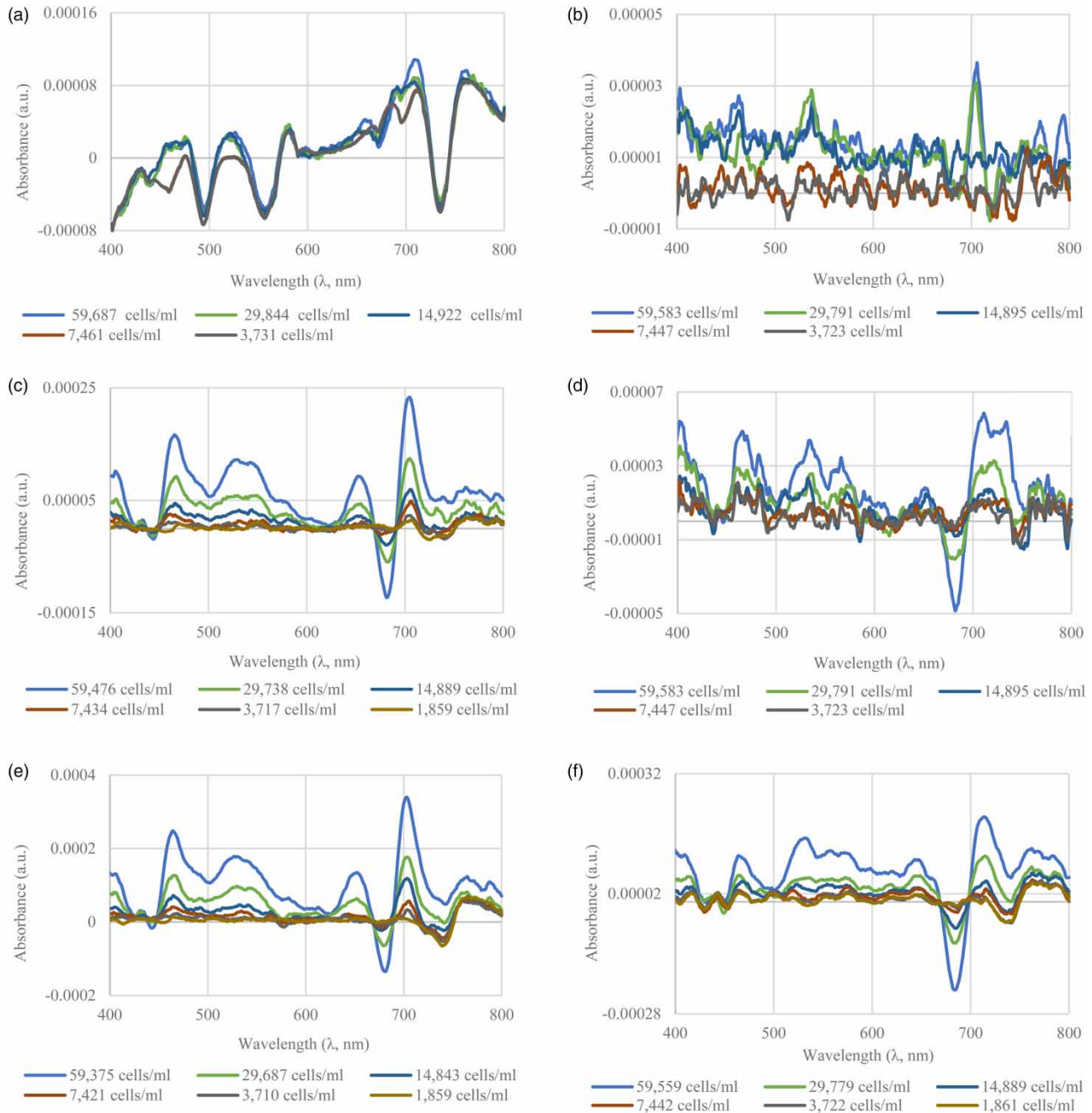


Figure 3 | Savitzky-Golay first derivative of absorbance spectra of *M. aeruginosa* and *C. vulgaris* at lower concentrations for 10 mm (a, b), 50 mm (c, d), and 100 mm (e, f) cuvette pathlength, respectively.

In addition, *M. aeruginosa* is known to form colonies of variable size (10–1000 μm) under natural conditions (as a response to prevailing environmental conditions), and studies have reported that the size of the colonies can increase by a factor of 2.7 in less than a week (Kurmayyer *et al.* 2003; Yang *et al.* 2008; Gan *et al.* 2012). This aggregation behavior can potentially impact the normal absorbance spectra.

For comparison purposes, the absorbance spectra using 50 mm pathlength of *M. aeruginosa* and *C. vulgaris* at similar concentrations are illustrated in Figure 4 (a,b, respectively). *C. vulgaris* absorbance spectra resulted in two peaks at 444 and 684 nm. These peaks are characteristic of *C. vulgaris* as it is known to carry photopigments chlorophyll-a and chlorophyll-b (Chl-b) (Miazek *et al.* 2015). On the other hand, as discussed before, *M. aeruginosa* carry both Chl-a and

Table 2 | Summary of experimental results for *Microcystis aeruginosa* and *Chlorella vulgaris* for higher and lower concentration ranges

Test	Pathlength (mm)	Concentration range (cells/mL)	Slope ± standard deviation	R ²	MDL (cells/mL)
<i>M. aeruginosa</i>					
Absorbance at 682 nm	10	3731–59,687	$1.0843 \times 10^{-07} \pm 1.9245 \times 10^{-08}$	0.9136	337,398
		119,375–955,000	$7.8861 \times 10^{-08} \pm 2.27084 \times 10^{-09}$	0.9981	
	50	1859–59,476	$5.4483 \times 10^{-07} \pm 6.3111 \times 10^{-08}$	0.9491	36,354
		118,952–951,613	$5.7188 \times 10^{-07} \pm 6.6346 \times 10^{-09}$	0.9997	
	100	1859–59,375	$8.6234 \times 10^{-07} \pm 1.0036 \times 10^{-08}$	0.9995	22,038
		118,750–950,000	$9.1702 \times 10^{-07} \pm 1.2518 \times 10^{-09}$	0.9999	
S-G first derivative of absorbance at 706 nm	10	3731–59,687	$7.5988 \times 10^{-10} \pm 1.9284 \times 10^{-10}$	0.8381	65,312
		119,375–955,000	$5.0784 \times 10^{-10} \pm 6.5253 \times 10^{-12}$	0.9951	
	50	1859–59,476	$3.6577 \times 10^{-09} \pm 7.9187 \times 10^{-11}$	0.9981	9,005
		118,952–951,613	$3.8234 \times 10^{-09} \pm 8.5669 \times 10^{-12}$	0.9999	
	100	1859–59,375	$5.5873 \times 10^{-09} \pm 2.1122 \times 10^{-10}$	0.9943	4,916
		118,750–950,000	$6.0763 \times 10^{-09} \pm 1.6246 \times 10^{-11}$	0.9999	
<i>C. vulgaris</i>					
Absorbance at 684 nm	10	3723–59,583	$1.1652 \times 10^{-07} \pm 4.6621 \times 10^{-08}$	0.6756	650,414
		119,166–953,333	$2.6319 \times 10^{-08} \pm 1.6184 \times 10^{-09}$	0.9924	
	50	3723–59,583	$1.8628 \times 10^{-07} \pm 1.3855 \times 10^{-09}$	0.9931	84,312
		119,166–953,333	$1.8497 \times 10^{-07} \pm 1.4197 \times 10^{-09}$	0.9998	
	100	1861–59,559	$9.6102 \times 10^{-07} \pm 1.2770 \times 10^{-08}$	0.9992	41,361
		119,118–952,941	$8.1156 \times 10^{-07} \pm 8.3307 \times 10^{-08}$	0.9997	
S-G first derivative of absorbance at 708 nm	10	3723–59,583	$5.6416 \times 10^{-10} \pm 7.9760 \times 10^{-11}$	0.9434	145,289
		119,166–953,333	$1.6681 \times 10^{-10} \pm 9.7520 \times 10^{-12}$	0.9932	
	50	3723–59,583	$8.3759 \times 10^{-10} \pm 3.6126 \times 10^{-11}$	0.9945	45,449
		119,166–953,333	$9.0879 \times 10^{-10} \pm 2.2396 \times 10^{-11}$	0.9987	
	100	1861–59,559	$3.6620 \times 10^{-09} \pm 1.0246 \times 10^{-10}$	0.9969	11,661
		119,118–952,941	$3.8160 \times 10^{-09} \pm 7.6739 \times 10^{-11}$	0.9991	

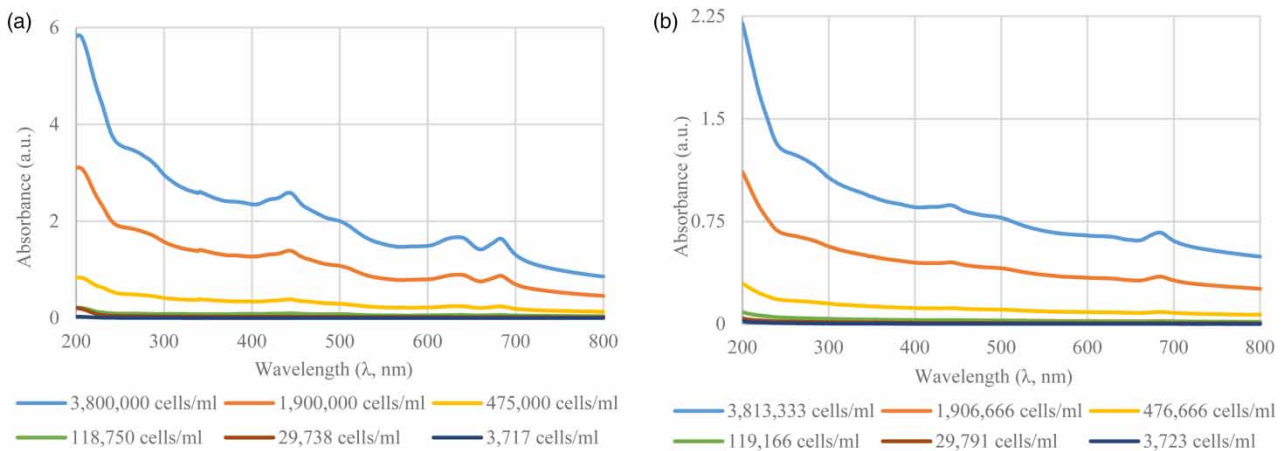


Figure 4 | Absorbance spectra of *M. aeruginosa* (a) and *C. vulgaris* (b) using 50 mm pathlength cuvette.

phycocyanin photopigments, which resulted in three peaks on the absorption spectra. Phycocyanin is an accessory pigment of chlorophyll and is predominantly found in cyanobacteria, which allows differentiating cyanobacteria from other algae for detection (Cotterill *et al.* 2019). Additionally, experimental results indicate a significant difference in the absorbance value between *M. aeruginosa* and *C. vulgaris* at the chlorophyll peaks (near 440 and 680 nm). For example, at the peak close to 680 nm and approximately 1,900,000 cells/mL concentration: *M. aeruginosa* exhibits an absorbance value of 0.8740 (a.u.), while *C. vulgaris* has an absorbance value of 0.3492 (a.u.). The likely reason *C. vulgaris* displays a lower overall absorbance

when compared to *M. aeruginosa* is because of the presence of photopigments chlorophyll-a and chlorophyll-b. While Chl-a is known to absorb light strongly, Chl-b on the other hand absorbs light weakly, which results in lower overall absorbance (Gray 2010). This indicates that the cyanobacterial and non-cyanobacterial algae could be differentiated based on the differences of their absorbance spectra, and the application of mathematical and statistical tools can increase the specificity of detection further.

4. DISCUSSION

The presented results indicate that spectrophotometry, when employed in tandem with mathematical and statistical tools such as Savitzky-Golay first derivative of absorbance, can be used for sensitive detection and early-monitoring of cyanobacteria in source waters. By increasing the cuvette pathlength and implementing derivative spectrophotometry, a significant improvement in the detection limit for *M. aeruginosa* and *C. vulgaris* was observed. Comparing the detection limit obtained by normal absorbance spectra to S-G derivative spectra for *M. aeruginosa* (cyanobacteria), 10 mm pathlength showed a 5-fold approximate increase in sensitivity (from 337,398 to 65,312 cells/mL); while a 4-fold improvement was observed for the 50-, and 100-mm pathlength (from 36,354 to 9005 cells/mL, and 22,038 to 4916 cells/mL, respectively). In addition, *C. vulgaris* resulted in an approximate 4-fold improvement in detection for the 10 mm pathlength (from 650,414 to 145,289 cells/mL); while 50- and 100-mm pathlength followed a 2-fold (from 84,312 to 45,449 cells/mL), and 3-fold (from 41,361 to 11,661 cells/mL) improvement in detection between the normal absorbance spectra and S-G derivative spectra. A higher increase in sensitivity was found with increasing pathlength for *C. vulgaris* in comparison to *M. aeruginosa*, as *C. vulgaris* does not absorb light as strongly due to the presence of Chl-b (Gray 2010). For all the tests conducted, 100 mm pathlength results showed the highest sensitivity for detection. Compared to the 10 mm pathlength conventionally used for absorbance measurements in the field, adoption of 100 mm pathlength and application of S-G derivative spectra improved the detection limits from 337,398 to 4916 cells/mL for *M. aeruginosa*, and from 650,414 to 11,661 cells/mL for *C. vulgaris*.

A previous study conducted by Agberien & Örmeci (2019) reported an approximately 4-fold improvement in detection using derivative spectrophotometry, but the method was not sensitive enough to lower the detection limit to the WHO guidelines. The method presented in this study successfully improved the method detection limit to approximately 5000 cells/mL for monitoring cyanobacteria, which is well below the WHO and USEPA established guidelines (<20,000 cells/mL) for low probability of adverse health effects in recreational waters (WHO 2003; EPA 2015b). Further research is recommended to test the detection limits in surface waters under realistic conditions and determine its applicability to the real-time monitoring of cyanobacteria. Future testing utilizing different signal processing and mathematical tools should also be investigated to improve the sensitivity of detection further.

The primary advantage of using spectrophotometry is the readiness of the technology for real-time water monitoring applications. In-line and real-time spectrophotometers are widely used for water monitoring applications, such as at water and wastewater treatment plants, and they can also transmit data in real-time. A wide range of water quality parameters can either be directly measured or calculated (i.e. UVT, UV254, dissolved and total organic carbon, biochemical and chemical oxygen demand, nitrate, nitrite, total suspended solids), and the results of this study show that cyanobacteria can be added to these parameters. Simultaneously measuring multiple parameters is critical for monitoring cyanobacteria as nutrient concentrations and water quality have a considerable impact on cyanobacteria dominance in source waters (Ha *et al.* 2009; Srivastava *et al.* 2013). In addition, the method presented in this study requires no sample processing, reagent use, or pigment extraction required by traditional approaches for cyanobacterial monitoring. The results of this study also indicate that spectrophotometry could differentiate the cyanobacterial algae from non-cyanobacterial algae based on the variations in their pigment absorbance peaks, and this could be a useful tool. Previously, Agberien & Örmeci (2019) reported that spectrophotometry was not able to differentiate between a toxigenic and non-toxigenic strain of *M. aeruginosa*, which were morphologically identical and carry the same photopigments.

Spectrophotometry also carries some limitations. It can overestimate chlorophyll concentration in the presence of other interfering pigments such as phaeopigments, which are the degradation products of algal chlorophyll pigments (Dos Santos *et al.* 2003). Furthermore, green-sulfur bacteria present in freshwater and marine systems can cause interference in the estimation and interpretation of algae chlorophyll concentration (Gray 2010). Spectrophotometry is also susceptible to background noise in the presence of other constituents such as turbidity and baseline shift due to changing water characteristics (Burgess & Thomas 2017). However, the derivative of absorbance can be used to identify critical wavelengths while

improving the signal-to-noise ratio and the interference caused due to baseline shift (Demetriades-Shah *et al.* 1990; Wiggins *et al.* 2007). Finally, spectrophotometry does not have the sensitivity or specificity of fluorometry. In fluorometric scans, concentrations are estimated via excitation and emission characteristics, and each phytoplankton compound has a distinct signal in fluorescence (Liu *et al.* 2020). Fluorometry is capable of monitoring cyanobacterial concentrations within the WHO established guidelines; however, the maximum operational capability is limited to 200,000 cells/mL (Zamyadi *et al.* 2016). Furthermore, fluorometric sensors have to be calibrated to a known biological entity and temperature to be able to measure a specific bloom of interest and do not quantify data on cyanobacterial biomass or concentration but rather provide relative phytoplankton concentration (Bowling *et al.* 2016). This can potentially lead to over- or under-estimation of algal concentration. Contrary to this, spectrophotometry has a wider working range, which allows it to be employed at highly eutrophic lakes (Gray 2010).

5. CONCLUSION

This study improved the sensitivity of UV-Vis spectrophotometry for early detection and monitoring of cyanobacteria by increasing cuvette pathlength from conventional 10-mm to 50- and 100-mm and applying S-G derivative spectrophotometry. The first derivative of absorbance and complementary statistical tools were investigated with changing pathlengths in lower and higher concentration ranges, and new method detection limits were established that showed substantial improvements. Compared to the 10 mm pathlength conventionally used for absorbance measurements in the field, adoption of 100 mm pathlength and application of S-G derivative spectra improved the detection limit from 337,398 to 4916 cells/mL for *M. aeruginosa*, successfully lowering the detection limit below the WHO guideline (<20,000 cells/mL) for low probability of adverse health effects. *C. vulgaris* was also studied to compare against *M. aeruginosa*, and similarly the detection limit was improved from 650,414 to 11,661 cells/mL. The results also showed that spectrophotometry could differentiate *M. aeruginosa* from *C. vulgaris* based on the variations in their pigment absorbance peaks, which could provide a useful tool in identifying blooms of interest.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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