ZIBELINE INTERNATIONAL™ PUBLISHING

Water Conservation & Management (WCM)

DOI: http://doi.org/10.26480/wcm.01.2024.20.23

ISSN: 2523-5664 (Print)
ISSN: 2523-5672 (Online)
CODEN: WCMABD

RESEARCH ARTICLE

A COMPARISON OF CHLOROPHYLL-A MEASUREMENT IN TROPICAL URBAN POND WATERS USING *IN VIVO* AND *IN VITRO* METHODS

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ARTICLE DETAILS

Article History:

Received 26 July 2023
Revised 29 August 2023
Accepted 02 October 2023
Available online 18 October 2023

ABSTRACT

The concentration of chlorophyll-a in waters is an important indicator in determining the trophic status. *In vivo* and *in vitro* methods are the available options for measuring the concentration of chlorophyll-a. Frequently the *in vivo* and *in vitro* methods for determining chlorophyll-a concentrations are used separately, so there is less opportunity to compare the measurement results of the two methods. This study aims to compare the results of chlorophyll-a measurements with *in vivo* and *in vitro* methods using tropical urban pond water as the media. Water media was collected from two ponds, and then prepared into three different concentrations before the measurements. The results of this study showed that the results of the chlorophyll-a measurement values in one of the two media water sources showed a significant difference (*p* value = *7.94E-12*), while in the other media water sources there was no significant difference. The measurement of overall water sources showed significant differences *(p* value = *1.00E-07*) between the two methods. As a standard method, the *in vitro* method was proven to be more stable in measuring chlorophyll-a concentrations, but this method has issues related to the need for sample handling and preparation so it requires a longer time to obtain results. The *in vivo* method can be an alternative method of measuring chlorophyll-a concentrations, especially in situations where monitoring needs to be carried out quickly and intensively.

KEYWORDS

Water pollution control, phytoplankton, algae, fluorometer, eutrophication, trophic state, freshwater

1. INTRODUCTION

In waters, chlorophyll-a is a color pigment contained by various types of algae, including microalgae. A high concentration of chlorophyll-a (>10µg/L) is one of the markers of eutrophication in tropical/subtropical reservoirs (Cunha et al., 2013). The parameter of chlorophyll-a concentration is also an essential factor in determining the trophic status index of waters of the lentic system, in addition to the total phosphorus and water clarity, measured by Secchi disk (Carlson, 1977; Cunha et al., 2013). When controlling eutrophication, monitoring the concentration of chlorophyll-a in water is one of the essential indicators to determine the necessity of further measures in handling water pollution issues related to the management of eutrophication (Kalaji et al., 2016; Mozafari et al., 2023). However, measuring chlorophyll-a concentration in water is relatively challenging.

The standard method often applied is the *in vitro* measurement method (referred to the United States Environmental Protection Agency (US EPA) 445.0 Method). This method involves a destructive process and requires complex and relatively time-consuming treatments. The complexity of this method is related to specific treatments during the transfer of samples from the field to the laboratory, filtration, and destruction processes using acetone before eventually being inserted into a fluorometer instrument for measurement. Meanwhile, there is also an *in vivo* measurement method for chlorophyll-a, which allows direct measurement in the field without the need for sample processing as required in the previous method (Salonen et al., 1999; Ghadouani and Smith, 2005; Lu et al., 2020). Commercial *in vivo* instruments became widely available by the mid-1970s (Suggett and Prášil, 2010; Zavafer et al., 2020). Recently, instruments in this method are portable and work simply by dipping the probe directly into the water on the field, the result will be displayed instantly (*in situ*).

The destructive method is considered the standard method because it produces higher precision (Gregor and Maršálek, 2004). However, this method requires sample transport and relatively longer sample processing time, as well as the potential for failure in the destruction process. As a result, there can be doubts about its role in representing the actual concentration in the field, considering that algae growth (represented by chlorophyll-a concentration) is highly dynamic over time (Almomani and Örmeci, 2018; Hamdhani et al., 2021). Improper sample storage can also lead to the growth or death of microalgae during the transportation process to the laboratory.

Frequently, the *in vitro* and *in vivo* methods for determining chlorophyll-a concentration are used independently, limiting the opportunity to compare their measurement results. Hence, the fundamental question arising is whether there are differences in the measurement results between the *in vivo* and *in vitro* methods. This question underpins the motivation for conducting this research. Specifically, this study aims to compare the measurement results of chlorophyll-a using the *in vivo* and *in vitro* methods in water media from ponds in tropical areas.

2. METHODOLOGY

2.1 Description of *In Vivo* and *In Vitro* Instrument

In this study, the *in vivo* chlorophyll-a measurement instrument utilized a handheld fluorometer produced by Turner Designs (Sunnyvale, CA, USA). Instruments in this method work by producing certain light emissions that

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	Website: www.watconman.org	**DOI:** 10.26480/wcm.01.2024.20.23

Cite The Article: Hamdhani Hamdhani (2024). A Comparison of Chlorophyll-A Measurement in Tropical Urban Pond Waters Using in Vivo and in Vitro Methods. *Water Conservation & Management, 8(1): 20-23.*

cause the chlorophyll-a pigments within the microalgae cells to fluoresce and this fluorescence is then received by a fluorometer sensor, which transforms it into a value representing the concentration of chlorophyll-a (Gregor and Maršálek, 2004; Hamdhani et al., 2021). It works by simply dipping into the water and then pressing the measure button. The instrument is equipped with a little screen to display the result. On the other hand, for *in vitro* measurements, a benchtop fluorometer version TD-700, also manufactured by Turner Designs (Sunnyvale, CA, USA) was used. In this method, sample processing following the US EPA 445.0 Method is required before the measurement (Arar and Collins, 1997).

Figure 1: Handheld fluorometer (*in vivo method*) (A) and benchtop TD-700 fluorometer (*in vitro method*) (B)

2.2 Description of Site and Water Collection

The water used as the media in this experiment was collected in the 2022 rainy season from two tropical urban ponds located within the Mulawarman University campus area in Samarinda City, East Kalimantan. The first pond is situated near the main entrance of the university complex, referred to as "Pond 1," while the second pond is located within the Mulawarman University area, referred to as "Pond 2." Pond 1 has an irregular shape for its perimeter, while Pond 2 has a rectangular shape. Both receive surface runoff from the surrounding urban area. However, the depth is unknown. The estimation of the pond perimeter and area was conducted using the Arc-GIS, and is shown in Table 1.

On a daily basis, the water in both ponds has a brownish-green color, indicating a high concentration of chlorophyll-a. This characteristic forms the basis for selecting water from both ponds as the comparison media for chlorophyll-a concentration measurements using *in vivo* and *in vitro* methods. To collect water for the experiment, one 15-liter water tank was filled with pond water from each pond. The filled water tanks were then immediately transported to the Water Quality Laboratory at Mulawarman University for further processing as the measurement media using the two compared methods.

Table 1: Pond Perimeter and Area Size

Pond\Dimension	Perimeter (m)	Area (m^2)
Pond 1	310	2.880
Pond 2	232	2.811

On a daily basis, the water in both ponds has a brownish-green color, indicating a high concentration of chlorophyll-a. This characteristic forms the basis for selecting water from both ponds as the comparison media for chlorophyll-a concentration measurements using *in vivo* and *in vitro* methods. To collect water for the experiment, one 15-liter water tank was filled with pond water from each pond. The filled water tanks were then immediately transported to the Water Quality Laboratory at Mulawarman University for further processing as the measurement media using the two compared methods.

2.3 Water Media Processing and Chlorophyll-A Measurement

Initially, a rapid measurement was performed to obtain an overview of the original chlorophyll-a concentration using a handheld fluorometer. The water samples were then diluted to obtain three concentration levels, ensuring that the highest concentration did not exceed the maximum measuring capacity of the instruments. The handheld fluorometer used for measurements specifically has a concentration measurement range from 0 to 199 µg/L only. Concentration 1 represents the highest concentration, where no dilution was applied to the water from the source. Concentration 2 was obtained by mixing 2/3 of the water from the source with 1/3 distilled water, while Concentration 3 representing the lowest concentrations, was achieved by mixing 1/3 of the water from the source with 2/3 distilled water.

The water samples with the three concentration levels were then measured using the handheld fluorometer (*in vivo*) and TD-700 (*in vitro*) instruments, which were initially factory-calibrated according to the manual instructions of each instrument. For each concentration level, 10 sub-samples of the media were taken to obtain chlorophyll-a concentration values for comparison. In the *in vivo* method, the handheld fluorometer was simply immersed up to the dip line indicated on the instrument, and the results were displayed on the instrument's screen after pressing the measurement button. For the *in vitro* method, for each of the three concentration levels, 10 sub-samples of 20 ml were taken. Each sub-sample was then filtered using special filter paper that could dissolve in 90% acetone with the aid of a mechanical tissue grinder to allow complete extraction of chlorophyll-a. The filter paper along with the filtered contents was transferred to a glass test tube containing 10 ml of acetone. After shaking until all the filter paper dissolved, the test tube was inserted into the TD-700 fluorometer instrument for measurement and calculation according to the instrument's manual.

2.5 Data Analysis

The paired t-test analysis of the mean of two samples was used to determine the level of difference in measurements between the *in vivo* and *in vitro* methods. The t-test was performed using the Statistical Software of Stata Version 15.1 (StataCorp, College Station, TX, USA). Firstly, the statistical test was conducted separately for Pond 1 and Pond 2, and then a similar test was performed for the overall data from both ponds. A two-tailed p-value <0.05 indicates that the paired data differ significantly. To visually assess the proximity of the measurement results between the two methods, scatter plots are presented. The scatter plot helps visualize how close the values from both methods are to each other. The closer the points align to the one-to-one line (1 by 1), the more similar the values are between the two methods.

3. Results and Discussion

From the three prepared concentrations, the scatter plot visualizes that in Pond 1, the *in vivo* method shows higher chlorophyll-a concentration measurement results compared to the results obtained by the *in vitro* method. This trend was observed for concentrations 1 (high), 2 (medium), and 3 (low). On the other hand, in Pond 2, the chlorophyll-a measurement results between the two methods show almost the same values. In more detail, for Pond 2, the range of chlorophyll-a measurement results at all concentrations obtained from the *in vitro* method falls within the range of values obtained from the *in vivo* method. Figure 2 illustrates that the *in vivo* method exhibits a wider range of variation in measurement results compared to the *in vitro* method.

The results of the paired t-test comparison of the mean of two samples (Table 2) indicate a difference in statistical results between Pond 1 and Pond 2. In Pond 1, there was a significant difference (*p* value = *7.94E-12*) between the chlorophyll-a concentration measurements obtained with the *in vitro* method compared to the *in vivo* method. On the other hand, in Pond 2, there was no significant difference between the two methods (*p* value > *0.05*). These results align with the visual findings from the scatter plot (Figure 2), which show that the regression line of chlorophyll-a concentration in Pond 2 is closer and intersects with the one-to-one line (1 by 1). In contrast, in Pond 1, the regression line of measurement results is farther away and does not intersect with the one-to-one line.

The exact reason for the differences in the measurements using both methods in the two-water media used is unknown, as this falls outside the scope of our research. Therefore, further research is required to address this question. We suspect that future investigation should include observing the types and quantities of phytoplankton that may have an impact on the measurement outcomes obtained through the two compared methods. Falkowski & Kolber conducted a study on natural phytoplankton communities, investigating the changes in the quantum yield of chlorophyll fluorescence over both space and time (Falkowski and Kolber, 1995). These phytoplankton communities are highly diverse, comprising a wide range of prokaryotic and eukaryotic oxygenic photoautotrophs, belonging to at least 11 different classes. The sizes of these organisms vary significantly, ranging from approximately 0.6 µm for prokaryotic Prochlorophytes to over 1 mm for certain diatoms and dinoflagellates. They found, there are notable differences in the abundance of light-harvesting pigments among these organisms.

In the overall measurements from both Pond 1 and Pond 2, the t-test shows a significant difference between the two methods in measuring chlorophyll-a concentration (*p* value = *1.00E-07*). This indicates that, in general, there was a difference (discrepancy) in chlorophyll-a measurement results between the *in vivo* and *in vitro* methods. Frequently, the choice between the two methods often considers the availability of instruments and how quickly the measurement data needs to be obtained (Hamdhani et al., 2021). The use of the *in vitro* method might be ideal as it is more stable and considered a standard method (Arar and Collins, 1997). However, it comes with the consequence of more complex sample processing and a preparation longer time compared to the *in vivo* method (Gregor and Maršálek, 2004; Hamdhani et al., 2021). Another important concern is that longer processing time may reduce the accuracy of representing the actual conditions in the field. This is due to the highly dynamic nature of photosynthesis in microalgae (phytoplankton) over time, influenced by the availability of sunlight, nutrient factors, especially total phosphorus and water turbidity (Carlson, 1977; Cunha et al., 2013).

Regardless of the slight discrepancy between the two methods, the use of the *in vivo* method can address the limitations of the *in vitro* method, as measurement results can be obtained quickly in the field (*in situ*), even though it is not a standard method. In reality, the speed of chlorophyll-a measurement is crucial to support routine water quality monitoring activities, which are highly dynamic. Early knowledge of chlorophyll-a concentrations exceeding the water quality threshold in water bodies serves as a vital signal for rapid water quality management efforts to prevent and control eutrophication in aquatic environments.

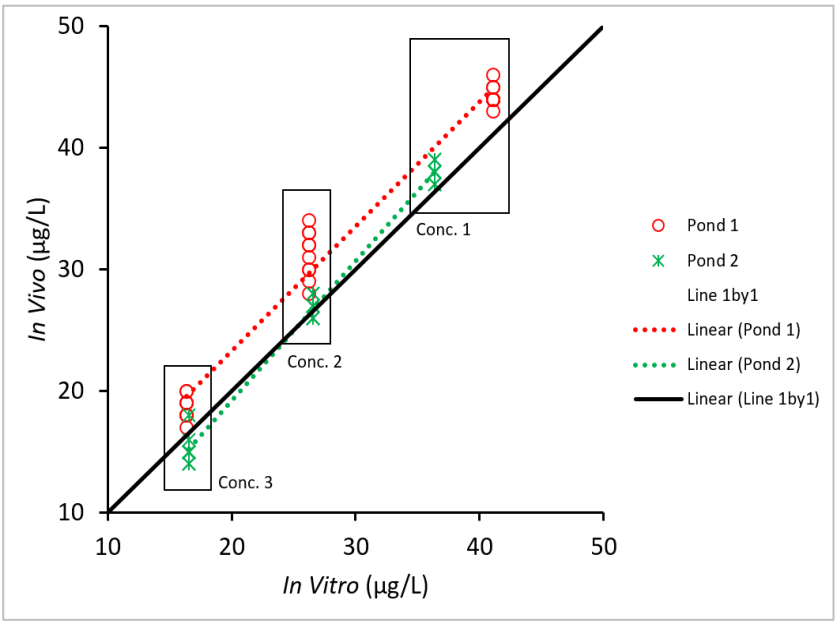

Figure 2: Scatter plot of measurement results using *in vitro* and *in vivo* methods at three different concentration levels of chlorophyll-a

Table 2: The T-Test Results of Two Paired Samples For Measuring Chlorophyll-A in Water Media from Pond 1, Pond 2 And Overall Measurements from Pond 1 And 2

Results	Pond 1		Pond 2		Pond 1 and 2	
	In Vitro	***In Vivo***	***In Vitro***	***In Vivo***	***In Vitro***	***In Vivo***
Mean	27,91	31,37	26,50	26,67	27,20	29,02
Variance	106,80	115,56	67,57	90,37	86,21	106,83
Observations	30	30	30	30	60	60
Pearson Correlation	0.987		0.996		0.978	
df	29		29		59	
t Stat	-10,96		-0,59		-6,07	
P(T<=t) two-tail	**7,94E-12**		0,56		**1,00E-07**	
t Critical two-tail	2,045		2,045		2,001	

4. Conclusion

In conclusion, this study documents a difference or discrepancy in chlorophyll-a concentration measurement results between the *in vivo* and *in vitro* methods. However, both methods still exhibit similar trends. As a standard method, the *in vitro* method has proven to be more stable in measuring chlorophyll-a concentration. Nevertheless, it does face issues related to sample handling and preparation, leading to a longer time required to obtain final measurement results. Consequently, the capability of producing measurements that reflect the field conditions becomes questionable with the use of the *in vitro* method. The *in vivo* method can serve as an alternative for measuring chlorophyll-a concentration, especially in situations where monitoring needs to be conducted intensively, and rapidly. Ideally, this method is employed as the frontline in routine water quality monitoring. If there are indications of concentrations exceeding the water quality threshold, the *in vitro* method can then be used as a follow-up to ensure the accuracy of those conditions.

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