The Characterization of Chlorophyll-A and Microalgae Isolation Process of Wastewater Collected at Sembrong Dam

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Abstract. Recently, there has been an increasing number of river water quality deterioration that has brought into water quality disruptions that entering dams including in Johor and one of them is occurred in Sembrong Dam in Johor. Sembrong Dam is a major water source for some 120,000 people in the districts of Kluang and parts of Batu Pahat. The quality of water in Sembrong should be well-monitored in ensuring the continuous distribution of clean and safe water supply to peoples. Based on the news reported by The Star news dated on 11 May 2015, the water bodies in Sembrong Dam are polluted by the algae blooms which has started to cause problems in treating water phase by clogging up the filters and causing the production to be reduced and finally resulting in frequent water disruptions to residents. Therefore, there is a need to study the water quality of the dam water prior to further water treatment. One of important characterizations is by measuring chlorophyll-a and the isolation of the dominant microalgae species in the water body in which they are able to indicate the level of water pollution. This paper presents the determination of chlorophyll-a and the isolation of microalgae strains collected from Sembrong Dam. Chlorophyll-a is a photosynthetic pigment present in all species of phytoplankton, including algae and in some photosynthetic bacteria, known as cyanobacteria. The method used in measuring the chlorophyll-a is based on the standard method of ISO 10 260. The average chlorophyll-a concentration measured at Sembrong Dam is 175.9 μg L⁻¹ and it is responsible for the appearance of green color in the sample and it is categorized into hypereutrophic state which is highly polluted. The technique used for isolation of microalgae strains is traditional method which is by spreading the sample on agar. The pure isolate indicated that the genus Botryococcus is the dominant algae species which is characterized morphologically. Both chlorophyll-a and microalgae isolation are good biological indicator that indicate the pollution of Sembrong Dam. The pure culture is very important that it can be used for further studies with series of different tests to understand its properties and character for sustainability approach towards environmentally friendly as well as for microalgae removal formula.

Keywords: Microalgae, isolation, chlorophyll-a.
1. Introduction
Sembrong Dam was previously built for flood mitigation in 1984 and due to the demand for clean water supply to peoples, it has been providing water for human consumption since 1990. It covers up to 775ha and supplies 55 million litres of treated water via its Sembrong Barat water treatment plant daily [1]. Since that, the water quality control in Sembrong Dam has always been monitored to prevent from water quality deterioration that can affect the treatment water process as well as to provide clean water to peoples.

Unfortunately the water bodies in Sembrong Dam has been degraded by two general type of pollution which is point sources and nonpoint sources. Point sources is a single, identifiable source of pollution while the nonpoint source is those inputs and impacts which occur over a wide area and are not easily attributed to a single source. Based on the study by [2], the nonpoint sources that contribute to the increasing pollution are coming from indiscriminate farming activities around the dam, agriculture activities, husbandry and planting of oil palm trees which cover at least 87% of the dam’s 130 sq km catchment area.

The Star news dated on 11 May 2015, in this year had reported that the dam is slowly dying due to the eutrophication or better known as algae bloom and had caused 244 disruptions at the Sembrong water treatment. This phenomena has turned the dam water into green water which indicates the present of high concentration of nutrients in the dam water such as nitrogen and phosphorus [3]. The source of the nutrients, mainly due to the excessive fertilizer used in oil palm plantation, animal waste and discharges from agriculture and sewerage. The Star news described that the algae bloom has started to cause problems when treating water, as they clog up the filters, causing the production to be reduced or halted and finally resulting in frequent water disruptions to residents.

The pollution by microalgal blooms is concerned to affect the process of water treatment in Sembrong Dam by carrying high loading of microalgae that would make the treatment process more costly. Normally, high cost will be needed in the primary separation of alum flocculation to reduce the sediment of microalgae and more chlorine is required to fully kill the microalgae which would cause the high concentration of Trihalomethanes (THMs). THMs constitutes the major category of chlorinated disinfection byproducts, formed by the reaction between residual chlorine and natural organic matter in water such as algae, river weeds, and decaying leaves. They are tasteless and odourless, but harmful and potentially toxic [4]. Lifetime exposure to THMs may pose risks to human health. Several epidemiologic studies have suggested a link between THMs exposure and risk of bladder, colon, and rectum cancers [5]. However, knowledge on characterization of the water body in Sembrong Dam will help in monitoring the level of eutrophication by measuring the concentration of chlorophyll-a in the water. According to Robinson et al [6], chlorophyll-a is probably the most-often used estimator in North America of algal biomass in lakes and streams by providing an estimate for measuring algal weight and volume. More than that, chlorophyll-a able to relate the relationship between nutrient concentration and other biological phenomena in aquatic ecosystem as well as effective measure for trophic status for pollution classification [7]. The results of chlorophyll-a with average reading 175.9 μg L−1 in this study has indicated that the dam water is polluted and classified under hypereutrophic state.

Algae is a good indicators of water quality and many lakes are characterized based on their dominant phytoplankton groups. The microalgal species found in the Sembrong dam can be further studied in enhancing the water treatment process as well as can be utilized in many field such as in medicine, foods, agriculture, chemical, drugs and treatment for wastewater. More than that, the disadvantages of the microalgal is when excessive blooms occurs in which they will produce scums and lead to the discoloration of water and when left untreated, these alage could suffocate fish and affect the entire marine environment [8]. The high loading of microalgae also will cause the cost of treatment water to be increased due to the extra amount of chemical and additional treatment system needed to remove the alage.

The most dominant microalgae species can be identified by observing the samples under the microscope on Phytoplankton chamber and by isolation process. The overall chlorophyll-a
concentration comes from the pigment of dominant species in which it becomes important indicator of pollution as well will be useful if studied further in reducing them in Sembrong Dam and the isolated alage can be studied further for the optimization for biodiesel production.

2. Materials & Method

2.1 Field collection
Water samples with visible microalgal population were collected from River sembrong dam located about 10km from Air Hitam in the state of Johor on the Air Hitam-Kluang road. The sampling was conducted during dry season for ensuring undiluted concentration of contaminant in the water. Fourth times of sampling were conducted within 4 consecutive weeks in which one sampling for microalgae isolation and Chlorophyll-a and the other for chlorophyll-a. All the samples locations were taken at the same location which is at the water intake of water treatment plant. The samples were collected by using two of 4 L bottle sampling for each time sampling. The bottles containing sample were wrapped with aluminium foil and placed into a polystyrene box containing ice cubes to be carried to laboratory for about 15 minutes travel time using a car. The standard method used for sampling is based on [9].

2.2 The Determination of Chlorophyll-a
In determining the chlorophyll-a ESS Method 150.1 [10] and IS0 10260 are adapted [11]. This experiment is conducted immediately after the samples arrived in the laboratory. In this experiment, samples of 100 m and 2.5 mL of MgCO₃ were directly filtered through a 47 mm Whatman GF/C filter. After that, the filter paper then was folded by using tweezer and placed into a graduated boiling tube and pushed down. Then, 90 % of methanol is added into the graduated boiling tubes up to 10 mL mark and capped very loosely and the tubes are placed into the water bath with 60 °C for 2 minutes. The resultant solutions are filtered through a 25mm GF/C filter and the filtrate was collected. The absorbance of both extracts was measured at 665 and 750 nm. The concentration of chlorophyll-a was determined according to [11]. The steps were repeated for another replicates.

2.3 Microalgae isolation
Alga isolation in this study involved the combination of dilution techniques and with standard plating agar methods to separate algal populations. Once the samples arrived in Lab, 200 ml of sample was centrifuged at 4000 rpm with 28 °C and washed by the addition of autoclaved distilled water for three times centrifugation to remove the mucilage or other contaminant. Right after the sample was rinsed, a simple dilution was conducted by using four 10 mL test tubes with the addition of 1mL of every test tube to aid the isolation process. Sterilized plastic petri dishes (100 × 15 mm) were used for the preparation of agar plates. The media growth used is the modified bold’s basal medium that is used for many of the green algae. The 50 agar plates were made by mixing the 1000 mL of distilled water, 20 mL of BBM, and 23 g of pure agar powder altogether in the beaker and autoclaved for 121o C for 15 minutes. After that, two agar plating techniques have been applied which are by using spreading and streaking method. In spreading procedure, a drop of sample from the test tube is put on the agar plate by using a disposal Pasteur pipettes and the glass hockey stick is used in spreading a drop of sample on agar thoroughly until the spreading became sticky. After that, the petri dish is covered with its lid and wrapped it with parafilm to avoid from contamination. The sample is stacked upside down inside the incubator and left for 14 days and steps were repeated for the other three diluted samples. In streaking, the wire loop is sterilized by putting the loop in flame using Bunsen burner inside the fume cabinet to sterilize it and the sterilized wire loop is cooled down for few seconds and dipped into the sample. The wire loop then pulled out and the sample was streaked back and forth across the surface of the agar. After that, the petri dish is covered with its lid and wrapped it with parafilm to avoid from contamination. The sample is stacked upside down inside the incubator and left for 14 days and the steps were Repeated for the other three diluted sample.
On the 14th day, there were number of algal colonies in form of green dots observed inside the agar plates. Once the algae colonies formed, the next step to proceed is to culture the colonies into the conical flasks. In transferring the algal colonies, a green dot is taken out by using sterilized wire loop and placed it into the conical flask containing BBM. There were 10 green dots of algae colonies transferred and cultured into the 10 conical flasks. Culturing the algae is based on the natural condition by covering the conical flask’s mouth with cotton wool, have it shaken twice per day and exposed it with the sunlight. After the 14 days passed, the algae cultures turned green and ready to be checked under the inverted microscope. By observing the 10 algae cultures from different conical flasks the result showed that there were still two and three species of algae living in the culture. As the pure culture is still have not yet obtained, the streaking and spreading have been conducted once again as well as the transferring the colonies into 12 conical flasks. Finally, the pure culture is obtained. Figure-1(a) is the colonies formed on the surface of the agar and Figure-1(b) is the culturing of algae colonies into the conical flasks.

![Figure 1(a). Colonies formed.](image1.png)

![Figure 1(b). Culturing algae colonies.](image2.png)

3. Results and discussion

3.1 Concentration of Chlorophyll-a
One of the easy and efficient ways to monitor the quality of water in Sembrong Dam is by measuring the level of chlorophyll-a. As all phytoplankton have chlorophyll-a, then chlorophyll-a is an important parameter to be monitored. In addition, it can be used for continuous or long-term monitoring. Lakes are commonly classified according to their trophic state, a term that describes how “green” the lake is as measured by the amount of algae biomass in the water. Three trophic state categories that are used
to describe lakes as they grow progressively greener are oligotrophic, mesotrophic, and eutrophic. Lakes with extreme trophic indices may also be considered hyperoligotrophic or hypereutrophic.

Since the the classification of trophic based on chlorophyll-a is still not established by NWQS, the classification of the trophic status was conducted based on Us Trophic Status Index [12]. This Index was developed as a part of of The National Eutrophication Survey (NES), comparing the work of some investigator on chlorophyll-a vs tropic state as show in in Table-1 [13]. More than that, the comparison with the Carlson Trophic State index [14] was made as in Table-2.

The average value of chlorophyll-a for four weeks of Sembrong Dam is 162.78 µgL-1 as shown in Figure 2. By comparing the result with Table 1, the trophic status of Sembrong Dam is belong to eutrophic state which is following the range given by Academy, Dobson and EPA-NES but is not valid for Sakamoto. Therefore, when the result is compared with Table 2 which stated that the chlorophyll-a ranged from 56 – 155+ and secchi depth ranged from 0.25 - 0.5 is belong to hypereutrophic state. By comparison, the average chlorophyll-a of sembrong dam is 162.78 µgL-1 and the depth of sembrong dam ranged from 0m to 7m have classified Sembrong Dam as in hypereutrophic state [2].

The result also shows that the river quality is in the range considered problematic by the New Hampshire Department of Environmental Services which provides the chlorophyll guidelines for river quality. Chlorophyll-a measurement below 7 µg/l is within a desirable range. 7-15 µg/l is less than desirable, while over 15 µg/l is considered problematic. Furthermore, Based on the study of 5 lakes conducted by [15] one of the lakes named Feldberga Haus with the depth 6m has the highest measurement of chlorophyll-a which is 175.9 µg L−1 and with that value he categorized the lake is belong to hypereutrophic state.

According to Dietrich [16] hypereutrophic lakes are very nutrient-rich lakes characterized by frequent and severe nuisance algal blooms and low transparency. More than that, hypereutrophic lakes have a visibility depth of less than 3 feet, they have greater than 40 micrograms/litre total chlorophyll and greater than 100 micrograms/liter phosphorus.

Table 1. Trophic state vs. chlorophyll-a [13].

| Trophic Condition | Chlorophyll-a (µgL⁻¹) | Sakamoto | Academy | Dobson | EPA-NES |
|-------------------|----------------------|----------|---------|--------|---------|
| Oligotrophic      | 0.3-2.5              | 0-4      | 0-4.3   | < 7    |         |
| Mesotrophic       | 1-15                 | 4-10     | 4.3-8.8 | 7-12   |         |
| Eutrophic         | 5-140                | >10      | 8.8     | > 12   |         |

Table 2. Trophic status [14].

| Secchi Depth, SD (Metres) | Chlorophyll-a | Tropic class µgL⁻¹ |
|---------------------------|---------------|--------------------|
| >8 - 4                    | 0—2.6         | Oligotrophic       |
| 4 - 2                     | 2.6—20        | Mesotrophic        |
| 2 – 0.5                   | 20—56         | Eutrophic          |
| 0.5 -0.25                 | 56—155+       | Hypereutrophic     |
3.2 Morphological Identification

The water from Sembrong Dam was sampled onto agar plates. Green microalgal clones were picked up after a few cycles of agar plate spreading. This general classification method was only used to distinguish isolates on the most basic level. Identification of these isolates to the genus level was based on the morphology of the individual cells following microscopic examination. The colony characteristics and morphological features of the Sembrong Dam water isolate have demonstrated it is close similarity with the genus Botryococcus. The individual cells of the colonies were in the range of 3-11 µm. Cells are spherical in shape and the variation in colonial size is depending upon the daughter colonies which remain attached to one another. More than that, cells are generally green to yellowish and some of them are in dark green, they also show orange yellow colouration as well.

Based on the study by Dayananda et al. [17] the Botryococcus bunaii were collected from freshwater ponds in Mahabalipuram, Tamil Nadu, India. Specimens were isolated and examined for morphological features using microscopic and was found to be genus Botryococcus and after further after 18S rRNA sequence analysis was carried out the analysis has shown more than 90% similarities with genus Botryococcus and in particular to the species B. braunii. In the morphological, the species of the genus Botrococcus were mainly distinguished based on colony size, colour and the cell shape. Based on Dayananda morphological result, it almost same with the algae isolate from Sembrong Dam and the colonies shape, colour and size is observed and distinguished as shown in Figure-2(a) and Figure-2(b). By comparison, both are spherical and oval-shaped cells. More than that, both isolates also structured irregularly and the cells are attached to each other that links two and more distinct clumps of cell. The colour of both algae isolate also the same which are generally green to yellowish green, some of the cells are dark green colour. The size of individual cell for both isolate are in the range between 3-11 µm.

According to Metzger and Largeau [18], the Botryococcus braunii cells are green color, and together by a lipid matrix under microscopic, whereas some of the strains have dark green color with irregular colonies consisting of hundreds of elliptical cells interconnected by strand of though mucilage. More than that, sometimes Botryococcus braunii cells are attached to each other by a refringent material that sometimes links two or more distinct clumps of cell. It has been reported that, Botryococcus braunii exists in the form of blooms in fresh water bodies like ponds, lakes and reservoirs and the existence of Botryococcus braunii in USA, Ivory Coast, Portugal, Bolivia, Morocco, India, Philippines, Thailand, France and West Indies has confirmed its wide distribution [18].

**Figure 2.** Chlorophyll-a sample reading within 4 consecutive weeks.
4. Conclusion
As conclusion, the concentration of chlorophyll-a of Sembrong Dam has proved that the water body is in hypereutrophic state which is heavily polluted by the microalgae blooms and one of the dominant species of microalgae is botryococcus braunii. The further study can be done is to use the alage as the indicator for pollution and the alage also can be studied for the potential of biodiesel production.
References
[1] Sharip, Z. & Jusoh, J., 2010. Integrated lake basin management and its importance for Lake Chini and other lakes in Malaysia. Research and Management, (15), pp.41–51.
[2] Daud, Z., Awang, H., Zainuri, M and Hatta, M. (2015). Hydrology Properties and Water Quality Assessment of the Sembrong Dam, Johor, Malaysia. Social and Behavioral Sciences 195, pp.2868 – 2873.
[3] Chislock, M. F., Doster, E., Zitomer, R. A. & Wilson, A. E. (2013) Eutrophication: Causes, Consequences, and Controls in Aquatic Ecosystems. Nature Education Knowledge 4(4), pp.10.
[4] Uza, M., R. LeCraw, L. Fortin, H. Broomer, and A. Edmonds. 1997. “Optimization of WTPs for the Control of Trihalomethanes.” Ontario Water and Wastewater Division Conference.
[5] Chowdhury, M. J. Rodriguez, and R. Sadiq. (2011). “Disinfection byproducts in Canadian provinces: associated cancer risks and medical expenses,” Journal of Hazardous Materials, vol. 187, no. 1–3, pp. 574–584.
[6] Robinson, K.W., S.M. Flanagan, J.D. Ayotte, K.W. Campo, A. Chalmers, J. F. Coles, and T.F. Cuffney. 2004. Water Quality in the New England Coastal Basins: Maine, New Hampshire, Massachusetts, and Rhode Island, 1999–2001. U.S. Geological Survey Circular 1226, pp.38.
[7] Wickliff, J.L. and Aronoff, S.(2015). Quantitative Measurement of Leaf Chlorophylls by Spectrophotometry of Their Pheophytins in Aqueous Alcoholic Extracts. Department of Biochemistry & Biophysics, & the Institute for Atomic Research, Iowa State University, Ames.
[8] Duong, V.T., Yan, L., Nowak, E and Peer, M.S. (2012). Microalgae Isolation and selection for prospective Biodiesel Production. Energies 2012, (5), pp.1835-1849.
[9] APHA (2005). Standard Methods for the Examination of Water and Wastewater 21st ed., American Public Health Association, US.
[10] ESS Method 150.1:Chlorophyll-a Spectrophotometric, Environmental Sciences Section, Inorganic Chemistry Unit, Wisconsin State Lab of Hygiene, 465 Henry Mall, Madison, WI 53706. Equation for chlorophyll a from Jeffrey and Humphrey (1975).
[11] ISO 10260:1992. Water quality measurement of biochemical parameters spectrometric determination of the chlorophyll-a concentration. International Organization for Standardization, Geneva, Switzerland, 1992.
[12] Trophic state index”. Wikipedia: The Free Encucpledia. Wikimedia Foundatio, Inc., 29 Sept. 2015. <https://en.wikipedia.org/wiki/Trophicstate/index>.
[13] Premazzi and Chiaudani. (1992). Ecological Quality of Surface Waters. Quality Assessment Schemes for European Community Lakes Commission of the European Communities, Joint Research Centre, Ispra (Varese).
[14] Carlson R.E. and J. Simpson (1996) A Coordinator's Guide to Volunteer Lake Monitoring Methods. North American Lake Management Society.pp.96.
[15] Peter, K., Judit, P., Riner, K., Koschel, L. K. and Frank, G.(2008) Chlorophyll a concentration across a trophic gradient of lakes: An estimator of phytoplankton biomass?.Ecology and Management of Inland Waters, (38), pp.327-338.
[16] Dietrich, U.(1980).Stability and multiple steady states of hypereutrophic ecosystem Development in hydrobiology. Hypertrophic Ecosystem, (2), pp.235-247.
[17] Dayananda, C., Kumudha, A., Sarada, R. and Ravishankar, G. A. (2010). Isolation, characterization and outdoor cultivation of green microalgae Botryococcus sp. Scientific Research and Essays. 5(17), pp. 2497-2505.
[18] Metzger P, Largeau C (2005). Botryococcus braunii: a rich source for hydrocarbons and related ether lipids. Apply Microbiol Biotechnol 66, pp. 486-496.