Bloom of a freshwater green alga *Botryococcus braunii* (Botryococcaceae, Trebouxiophyceae) and the associated mass fish mortality in a man-made lake, Sarawak, Malaysia

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**Abstract:** Mass mortality of fish (∼8,500 fishes), mainly *Oreochromis placidus*, was noted in a man-made lake located at Kuching, Sarawak (Malaysia). A field investigation was conducted to collect water samples and fishes. Patches of discoloration in brick red were observed in the lake and clear oil layer was found on the surface of the water. Microscopic observation and enumeration of the water samples showed that the plankton composition was dominated by a green algal species *Botryococcus* sp., with the colony densities ranging 1.2×10³–7.4×10⁶ colonies L⁻¹. Detailed morphological assessment by light microscopy revealed the dominant species as *Botryococcus braunii* Kützing. Molecular characterization using an rDNA marker further supported the species identity as *B. braunii* in the L race. Fish gill observation showed that cells of *B. braunii* and the oily substances were found in the dead fish gills. The race-L *B. braunii* bloom was reported, for the first time, to be associated with a fish kill event in a freshwater lake in Malaysia and confirmed the species as one of the algal types causing harmful effects to the environment.

**Key words:** Black tilapia, *Botryococcus braunii*, fish kill, harmful algal bloom, hydrocarbon

**Introduction**

The green alga *Botryococcus braunii* Kützing is widely distributed in freshwater system worldwide. This species has been considered a slow-growing species (Banerjee et al. 2002, Melis 2013) but the species has also been reported to form massive blooms (Mitchell 1975, Labib et al. 2012). *Botryococcus braunii* has attracted the world’s attention in the last decade due to its ability to produce hydrocarbon compounds (Metzger et al. 1991, Banerjee et al. 2002).

The alga *Botryococcus* was first described by Kützing in 1849, with *B. braunii* as the type species (Kützing 1849). Up to this date, there are a total of 14 taxonomically accepted species in the genus worldwide, including one fossil species, *B. balkachicus* Zalessky (Guiry and Guiry 2020). Komárek & Marvan (1992) described the species of *Botryococcus*, including five new species, from environmental populations (Komárek and Marvan 1992); however, species delineation in this genus was not clearly unravelled, as the morphology of single isolates could resemble several described species depending on the growth stage (Plain et al. 1993). Notwithstanding this confusion, the type species *B. braunii* has often referred to in many studies related to this green alga (Senousy et al. 2004).

As morphological differences to delimit the species of *Botryococcus* are debatable, several studies have adopted molecular techniques to classify the species of *Botryococcus* and to infer the relationships among the species (Kawachi et al. 2012, Hegedüs et al. 2015). The gene marker in the region of the small subunit ribosomal RNA gene (SSU rDNA) has been commonly used to infer phylogenetic relationships between the species of *Botryococcus* (Sawayama...
et al. 1995, Senousy et al. 2004). The SSU rDNA phylogeny of the species has revealed three distinct evolutionary lineages in *B. braunii* (Senousy et al. 2004, Kawachi et al. 2012). The three lineages of *B. braunii* correspond to four chemical races based on the hydrocarbon profiles, these being race-A, B, and L/S (Kawachi et al. 2012). Another region of the rDNA marker, the second internal transcribed spacer (ITS2), has recently been used to infer the phylogeny of *B. braunii*, demonstrating congruent inferences as in the SSU rDNA phylogeny (Hegedűs et al. 2015).

The species *B. braunii* has been reported to form massive blooms in Tomahawk Lagoon, New Zealand (Mitchell 1975) and the Darwin River Reservoir, Australia (Wake and Hillen 1980) since the 1970s. Lately, blooms of *B. braunii* have been observed in Liyu Lake, Taiwan (Chiang et al. 2004). The species was later reported to cause a massive bloom in Paoay Lake, Philippines (Papa et al. 2008). Recently, blooms of *B. braunii* have been observed in Nozha Lake, Egypt and Tilda Viljoen Dam, Namibia (Labib et al. 2012, Janse van Vuuren and Levanets 2019). Nonetheless, only blooms in Nozha Lake, Egypt and Liyu Lake, Taiwan have been associated with fish kill events (Chiang et al. 2004, Labib et al. 2012). The harmful effects and fish-killing mechanism of *B. braunii* remained unknown.

The distribution and occurrence of *Botryococcus* are not well-studied in Malaysia. Most of the studies that have been conducted focus on research and development of biofuel, mainly on the growth potential, as this is one of the important factors to boost the production of sustainable biofuel (Joan Iye et al. 2014, Asma et al. 2015, Zakariah et al. 2015, Gani et al. 2017). To our knowledge, there was no record of its distribution nor bloom events in Malaysian freshwater systems. In January 2018, we encountered a bloom of *B. braunii* in a man-made lake in Sarawak, Malaysia, that accompanied massive fish kills. A rapid sampling response was undertaken, and the plankton samples were examined. The microalga was characterized based on its morphology and through a molecular phylogenetic approach. Hereby, we report, for the first time, the green algal species *B. braunii* race-L bloom and its association with fish kills.

### Materials and Methods

#### Sampling sites and sample processing

The study site was located at Kota Samarahan, Sarawak (1.4672°N, 1104313°E; Fig. 1). The lake was constructed in 2003, covering an area of approximately 15 hectares with an average depth of 3.5 m (Leong 2010). The lake is in the middle of the Universiti Malaysia Sarawak campus and surrounded by university buildings, including residential colleges and cafeterias.

One-litre water samples were collected using a Van Dorn water sampler a day after the massive fish kill event on 11 January 2018. A total of ten sampling sites were selected in this study, three of these sites were located at inflow stations, three at cafeterias, two at outflow stations and two in other areas (Fig. 1).

Physical water parameters of salinity, temperature, pH, and DO were measured using a Hanna HI 96822 salinometer (Hanna, Romania), Hanna HI 98127 pH meter, and YSI multiprobe (6820 V2, YSI, USA), respectively. Dead fish were collected and brought back to the laboratory for further analysis. Fish samples were identified morphologically. The fish samples were dissected, and fish gills were directly observed under an Olympus IX51 light and fluorescence microscope (Olympus, Tokyo, Japan).

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**Fig. 1.** A map of Malaysian Borneo showing the sampling location of Kota Samarahan, Sarawak. Inset: A diagram showing the sampling sites of the man-made freshwater lake, with inflow stations, cafeterias, and outflow stations.
Fish kill associated with *Botryococcus*

Table 1. Primer sets targeting the SSU rDNA of *Botryococcus* designed and used in this study.

| Primer name   | sense | Primer sequence (5‘–3’) | SSU rDNA region |
|---------------|-------|-------------------------|-----------------|
| Botryo18SF1   | forward | GCTTGTCTCAAAGATTAAGCCATG | V1–V5           |
| Botryo18SR1   | reverse  | GGTGAGAGCTAGGACGGGTATC  | V1–V5           |
| Botryo18SF2   | forward  | GATACCGTCTAGTCTCAACC    | V5–V9           |
| Botryo18SR2   | reverse  | CTTGTACGCTTCTCCTTCC     | V5–V9           |

**Plankton identification**

One litre of water samples was sieved through a 20-µm mesh sieve and the retained materials were transferred to a 50 mL tube. One mL of the concentrated sample was enumerated on a Sedgewick Rafter by direct observation under an Olympus CX23 light microscope (Olympus, Tokyo, Japan) with a magnification of 40×. Detailed cell morphology was examined under an Olympus BX51 or IX51 fluorescence microscope (Olympus, Tokyo, Japan).

**DNA isolation, gene amplification and sequencing**

A single colony of the dominant plankton morphotype was isolated for genomic DNA extraction (Lim et al. 2014). The small subunit of the ribosomal RNA gene (SSU rDNA) was amplified using two sets of primer pairs that were designed in this study (Table 1) based on the SSU rDNA sequences of *Botryococcus* in the NCBI nucleotide database. Gene amplification was performed in a total volume of 25 µL PCR cocktail, containing 1 × PCR buffer (Promega, Madison, WI, USA), 1.5 mM MgCl₂, 0.2 mM of each dNTP (QIAGEN, GmbH, Hilden, Germany), 0.02 µM of each primer, 2.5 U Taq DNA polymerase (Promega), and ca. 80 ng µL⁻¹ DNA template. Amplification was performed using a Thermo Scientific Arkitik Thermal cycler (Thermo Fisher Scientific, Finland) with amplification conditions as follows: Initial denaturation for 4 min at 94°C followed by 35 cycles of 35 s denaturation at 94°C, 50 s annealing at 53.5°C and 35 s extension at 72°C, plus a final extension of 7 min at 72°C for 35 s.

**Sequence analysis and phylogenetic reconstruction**

In this study, four new SSU rDNA sequences (Table 2) were obtained from single colonies of field bloom samples. The sequences, together with another 95 sequences from the Genbank nucleotide database (NCBI), were used to infer the phylogeny. The sequences of *Choricystis minor* (Skuja) Fott, *Neochloris aquatica* Starr, and *Chlorella sorokiniana* Shihira & Krauss were used as in-groups, and *Ulva compressa* Linnaeus served as the outgroup. Multiple sequence alignment was performed using MUSCLE (Edgar 2004). The aligned dataset consisted of 2034 characters, of which 1583 were constant and 181 were parsimony informative.

Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were performed using PAUP* ver. 4.0b.10 (Swofford 2001). Bayesian Inference (BI) was investigated using MrBayes (ver. 3.2.7) (Ronquist et al. 2012). MP was performed using heuristic searches with 1000 random-addition replications, and 1000 bootstrap replicates (as detailed in Teng et al. 2014, Teng et al. 2015).

For ML and BI, the best evolutionary model was calculated by the Akaike information criterion (AIC) in jModelTest 2.1.3 (Darriba et al. 2012). ML was performed using the best fit model, with 100 random-addition replications; heuristic searches with branch-swapping, and TBR as detailed in Teng et al. (2014, 2015). Bayesian analysis (BI) was performed using MrBayes 3.2.2 (Ronquist et al. 2012) based on the best fit model calculated by Bayesian information criterion (BIC) using jModelTest 2.1.3. A four-chain run for 10,000,000 generations was used and trees were sampled every 100 generations; posterior probabilities (PP) were estimated with 20,000 generations burn-in (as detailed in Teng et al. 2014, Teng et al. 2015).

**Results and Discussion**

**Fish kills and the green algal bloom**

Massive fish kills were observed in a man-made lake located at Kota Samarahan, Sarawak, between January 17–30, 2018 (Fig. 2). The dead fish were identified as *Oreochromis placidus* (Trewavas, 1941). The water was discolored with a brick-red colour (Figs. 2D, E). Patchy discoloration of the surface water was observed during the fish kill event. An oily substance was found on the surface water of the lake (Figs. 2C, E).

Under light microscopic observation, oily substances were detected in the fish gills (Figs. 3A, B). Brownish colonies were found clogging the gills (Figs. 3C, E). These brownish colonies emitted red autofluorescence when observed under a fluorescence microscope (Fig. 3D, F), indicating the presence of chloroplasts.

The colony densities of green algae in the lake were observed in the range of 4×10²–1.9×10⁴ colonies L⁻¹. Station

Table 2. *Botryococcus* isolates with the SSU rDNA sequences obtained in this study.

| Isolate | Genbank accession number |
|---------|--------------------------|
| B18S1BF | MT809176                 |
| B18S1AF | MT809178                 |
| B18S1CF | MT809177                 |
| B18S1DF | MT809175                 |
S9 showed the highest colony density while S3 recorded the lowest (Fig. 4A). Bloom events of *B. braunii* have been commonly reported in freshwater systems such as lakes and reservoirs (Wake and Hillen 1980, Papa et al. 2008, Labib et al. 2012, Janse van Vuuren and Levanets 2019). This study reported, for the first time, blooms of *B. braunii* from a freshwater system in Malaysia that were also associated with massive fish kills. The maximum colony densities of *B. braunii* in this study (∼2×10^4 colonies L⁻¹) are comparable to those reported elsewhere [the Philippines, 4.4×10^4 colonies L⁻¹ (Papa et al. 2008); Egypt 5.5×10^4 colonies L⁻¹ (Labib et al. 2012); Australia (8.3×10^4 colo-
Fish kill associated with *Botryococcus*

Fish kill associated with *Botryococcus* (Wake and Hillen 1980). In this study, the water pH of the lake ranged from 6.5–8.0 (Fig. 4B). Station S6 recorded the highest pH of 8.0 while S1 and S2 recorded the lowest pH of 6.5. The range of pH in the lake was slightly higher, compared to previous studies (6.13 to 7.55; Tukau 2007, Nordin 2009, Leong 2010). This was likely due to high photosynthesis rates during the bloom events observed during the study period.

The surface water temperature of the lake ranged from 28.6°C to 30.6°C (Fig. 4C). The salinity of the waters was constantly measured as zero at each sampling site. The range of dissolved oxygen (DO) levels (7.64 to 9.31 mg/L; Fig. 4D) was within the range reported in previous studies (4.96 to 9.3 mg/L; Tukau 2007, Nordin 2009, Leong 2010).

*Botryococcus braunii* is a highly adaptable species, found in a wide spectrum of environmental conditions (Komárek and Marvan 1992). Qin et al. (2005) demonstrated that *B. braunii* grew optimally in a laboratory setting of 23–33°C, a salinity of 8.8, and under a 12:12 h light:dark photocycle. In the natural environment, highly eutrophic conditions are common, leading to high nutrient levels and, consequently, high *Botryococcus* bloom events. These blooms can cause fish kills due to the release of toxic substances, such as hydrogen sulfide, and the suffocation of fish due to oxygen depletion. The images (Fig. 3A–F) illustrate the physical and chemical characteristics associated with these bloom events.

Fig. 3. (A–B) Oily droplets (arrows in white) found in the fish gills. (C–F) Colonies of *Botryococcus braunii* clogged in the fish gills, cells with chlorophyll auto-fluorescence observed under fluorescence microscope (D, F).
and alkaline conditions were observed during most of the blooms (Ariyadej et al. 2004, Chiang et al. 2004, Papa et al. 2008, Labib et al. 2012). In this study, temperature and pH observed in the lake were within the ranges of the species growth tolerance. The species was found to tolerate zero salinity, which is different from previous reports (Qin et al. 2005, Rao et al. 2007). This physiological adaptation of *B. braunii* could be race-specific, as demonstrated in *B. braunii* race-A, which can tolerate high salinity (Rao et al. 2007).

**Identification of the algal bloom samples**

Colonies of a dark red colour are observable with the naked eye. The colonies are approximately 60 to 150 µm (Figs. 5A, B). The colony sizes are bigger than those reported in previous studies (Komárek and Marvan 1992, Rai et al. 2007, Hegedűs et al. 2015). The cell sizes of *B. braunii* were in the range of 13 to 15 µm, which is similar to that reported in other studies (Komárek and Marvan 1992, Rai et al. 2007, Hegedűs et al. 2015).

Cells assembled radially and peripherally in irregular grape-like colonies (Figs. 5A, B). The sub-colonies were connected with colorless mucilage (Fig. 5A). Chloroplasts were observed under fluorescence microscopy (Fig. 5C, D).

Cells were ovoid and enveloped by a cup-shaped, mucilaginous sheath (Fig. 5B). Cells were always covered with transparent mucilage and basal cups, and the mucilaginous matrix was firm, irregularly layered and striate (Fig. 5B). The morphology of *B. braunii* examined in this study was congruent with that described by Komárek & Marvan (1992).

Molecular characterization of the bloom samples based on the SSU rDNA revealed that the green algal colonies in this study were identical to *Botryococcus braunii*. The phylogenetic trees inferred from MP, ML, and BI showed congruent tree topology, with three main monophyletic clades (Fig. 6): Clade I represented *B. braunii* of race-A, Clade II race-B, and Clade III race-L/S, which was congruent with the results of Senousy et al. (2004), Kawachi et al. (2012), and Hegedűs et al. (2015). The colonies from the bloom samples were grouped with other *B. braunii* race-L/S in Clade III (Fig. 6) and were closely related to a *B. braunii* strain reported from Okinawa, Japan (Demura et al. 2014).

**Potential fish kill mechanism by B. braunii**

Reports of fish kills associated with *Botryococcus braunii* bloom events are scarce, with only a few cases reported...
Fish kill associated with *Botryococcus*

in Liyu Lake, Taiwan (Chiang et al. 2004) and Egypt (Labib et al. 2012). The threshold of *B. braunii* bloom density that causes fish kills, therefore, could not be deduced from the previously reported figures as high variation has been observed; also, some high biomass blooms have showed no effect on fish (Wake and Hillen 1980, Papa et al. 2008, Labib et al. 2012).

In this study, fish that were killed were mostly the black tilapia *Oreochromis placidus*. Blooms of *B. braunii* in Egypt also caused mortality of the same fish species (Labib et al. 2012). This fish species is known to tolerate a wide variety of environmental conditions, growing at water salinities ranging from 0 to 19 (freshwater to brackish), from low DO levels of 0.1–0.5 mg/L to 400% oxygen supersaturation, and pH of 3.5–12.0 (El-Sayed 2006, 2020). Also, the fish can tolerate ammonia concentrations up to 2.4 mg/L (without acclimatization) and 3.4 mg/L (with acclimatization to the sublethal level of ammonia) (Redner and Stickney 1979, El-Shafey 1998, El-sayed 2006). The environmental conditions in this study (Fig. 4) were within the tolerance levels of *O. placidus*, and therefore fish mortality due to hypoxia was unlikely to have been the cause of the fish kills. Although the nutrient level in the lake (ammonia concentration [0.25 mg/l]; unpublished data) was higher than usual (Tukau 2007), the reading was within the tolerance level of the fish.

In our investigation, colonies of *B. braunii* were observed in the fish gills, clogging them (see Fig. 3). This was likely to have caused the fish to suffocate and eventually die. Nonetheless, the effect of toxic fatty acid produced by *B. braunii* cells could not be ruled out. Earlier studies have shown that the species produces toxic fatty acids that inhibit the growth of other plankton species (Chiang et al. 2004, Wu et al. 2006) and cause zooplankton death (Chiang et al. 2004). Likewise, the production of toxic fatty acids in *B. braunii* may be strain-dependent or race-specific, and this may explain why not all *B. braunii* blooms are associated with fish kill events. More studies should be conducted to investigate this issue. Fatty acids that exist as free forms in alkaline water have been known to be more toxic to aquatic organisms (Proctor 1957). In this study, slightly higher alkalinity was observed at some stations in the lake. The fatty acids produced by *B. braunii* likely existed in a free form. However, we have no direct evidence to show that toxic free-form fatty acids were present in this study. Further study is needed to prove the presence of toxic fatty acids in this species.

In conclusion, we report, for the first time, blooms of

![Fig. 5. Colonies of *Botryococcus braunii* connected with colorless mucilage (A). Colonies are of an irregular grape-like shape, with chlorophyll auto-fluorescence observed under a fluorescence microscope (B–C).](image)
B. braunii in a man-made lake in Malaysia and that these were associated with a massive fish kill. We observed high cell densities of B. braunii that clogged the fish gills, which was likely the cause of suffocation and eventually death. The oily substance found in the gills might also have suffocated the fish. The environmental data provided preliminary evidence that hypoxia and ammonia intoxication is unlikely the cause of this fish kill event. Our findings, however, are not generalizable beyond the case studied. Hence, future work will have to explore and identify the fish kill mechanism by this species, such as doing fish assays with exposure to various B. braunii concentrations.

Fig. 6. Bayesian tree of Botryococcus braunii based on the SSU rDNA dataset. Nodal supports are bootstrap value for Maximum likelihood (ML) and Bayesian inference (BI), with only >50% support shown.
and examining the fatty acid profiles of the species.

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**References**

Ariyadej C, Tansakul R, Tansakul P, Angsupanich S (2004) Phytoplankton diversity and its relationships to the physico-chemical environment in the Banglang Reservoir, Yala Province. Songklanakarin J Sci Technol 26(5): 595–607.

Asma J, Yusoff FM, Srikanth RM (2015) Growth rate assessment of high lipid producing microalga *Botryococcus braunii* in different culture media. Iran J Fish Sci 14(2): 436–445.

Banerjee A, Sharma R, Chisti Y, Banerjee UC (2002) *Botryococcus braunii*: A sustainable source of hydrocarbons and other chemicals. Crit Rev Biotechnol 22(3): 245–279.

Chiang I-Z, Huang W-Y, Wu J-T (2004) Allelochemicals of *Botryococcus braunii* (Chlorophyceae). J Phycol 40(3): 474–480.

Darriba D, Taboada GL, Doallo R, Posada D (2012) *jModelTest* 2: more models, new heuristics and parallel computing. Nat Methods 9(8): 772–772.

Demura M, Ioki M, Kawachi M, Nakajima N, Watanabe MM (2014) Desiccation tolerance of *Botryococcus braunii* (Trebouxiophyceae, Chlorophyta) and extreme temperature tolerance of dehydrated cells. J Appl Phycol 26(1): 49–53.

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5(1): 113.

El-Shafey AAM (1998) Effect of ammonia on respiratory functions of blood of *Tilapia zillii*. Comp Biochem Physiol 121A: 305–313.

El-Sayed A-FM (2006) *Tilapia* culture. CABI Publishing, Wallingford.

El-Sayed A-FM (2020) Environmental requirements, In: El-Sayed, A-FM (Ed.) *Tilapia Culture (Second Edition)*. Academic Press, pp. 47–67.

Gani P, Sunar NM, Matias-Peralta H, Mohamed RMSR, Latiff AAA, Parjo UK (2017) Extraction of hydrocarbons from freshwater green microalgae (*Botryococcus* sp.) biomass after phycorremediation of domestic wastewater. Int J Phytoremediat 19(7): 679–685.

Guiry MD, Guiry GM, (2020) AlgaeBase. World-wide electronic publication. National University of Ireland, Galway, Galway.

Hegedüs A, Mocan A, Barbu-Tudoran L, Coman C, Drăguţ B, Sicora C, Dragoș N (2015) Morphological, biochemical, and phylogenetic assessments of eight *Botryococcus terribilis* strains collected from freshwaters of Transylvania. J Appl Phycol 27(2): 865–878.

Janse van Vuuren S, Levanets A (2019) First record of *Botryococcus braunii* Kützing from Namibia. Afr Biodivers Conserv 49(1): 1–5.

Joan Iye O, Hazel Monica Matias P, Norshuhaila Mohamed S (2014) Growth of freshwater microalga, *Botryococcus* sp. in heavy metal contaminated industrial wastewater. J Sci Technol 6(2): 29–40.

Kawachi M, Tanoi T, Demura M, Kaya K, Watanabe MM (2012) Relationship between hydrocarbons and molecular phylogeny of *Botryococcus braunii*. Algal Res 1(2): 114–119.

Komárek J, Marvan P (1992) Morphological differences in natural populations of the genus *Botryococcus* (Chlorophyceae). Arch Protistenkd 141(1): 65–100.

Kützing FT (1849) Species algarum. Auctore Fridrico Traug. Kützing. F. A. Brockhaus, Lipsiae.

Labib W, Mikhail SK, Kassem AM, Kassas ME, Ahmed MM (2012) Blooms of the colonial green algae *Botryococcus braunii* Kützing associated with massive fish mortality in Nozha Lake, Alexandria, Egypt. In: Kim HG, Reguera B, Hallegraeff GM, Lee CK, Han MS, Choi JK (ed.), Harmful Algae, The 15th International Conference on Harmful Algae, CECO, Changwon, Gyeongnam, Korea, pp. 188–191.

Leong CF (2010) Water quality of the UNIMAS main lake, Resource Chemistry. BSc thesis. Universiti Malaysia Sarawak, Sarawak, Malaysia. p. 43.

Lim HC, Leaw CP, Tan TH, Kon NF, Yek LH, Hii KS, Teng ST, Razali RM, Usup G, Iwataki M, Lim PT (2014) A bloom of *Karldinium australe* (Gymnodiniales, Dinophyceae) associated with mass mortality of cage-cultured fishes in West Johor Strait, Malaysia. Harmful Algae 40: 51–62.

Melis A (2013) Carbon partitioning in photosynthesis. Curr Opin Chem Biol 17(3): 453–456.

Mettzger P, Villareal-Rosales E, Casadevall E (1991) Methyl-branched fatty aldehydes and fatty acids in *Botryococcus braunii*. Phytochemistry 30(1): 185–191.

Mitchell SF (1975) Phosphate, nitrate, and chloride in a eutrophic coastal lake in New Zealand. New Zeal J Mar Freshwater Res 9(2): 183–198.

Nordin MS, (2009) Water quality study on UNIMAS lake, Civil Engineering. BSc thesis. Universiti Malaysia Sarawak, Sarawak, Malaysia. p. 92.

Papa DR, Wu J-T, Baldia S, Cho C, Cruz MA, Saguiguit A, Aquino R (2008) Blooms of the colonial green algae, *Botryococcus braunii* Kützing, in Paoay Lake, Luzon Island, Philippines. Philipp J Syst Biol 2: 13–20.

Plain N, Largeau C, Derenne S, Couté A (1993) Variabilité morphologique de *Botryococcus braunii* (Chlorococcales, Chlorophyta): corrélations avec les conditions de croissance et la teneur en lipides. Phycologia 32(4): 259–265.

Proctor VW (1957) Some Controlling Factors in the Distribution of *Haematococcus Pluvialis*. Ecology 38(3): 457–462.

Qin J, (2005) Bio-hydrocarbons from algae: impacts of temperature, light and salinity on algae growth. Rural Industries Research and Development Corporation, Australian Government. p. 18

Rai UN, Dwivedi S, Baghel VS, Tripathi RD, Shukla OP, Shukla MK (2007) Morphology and cultural behavior of *Botryococcus protuberans* with notes on the genus *Botryococcus* (Chlorophyceae). Arch Protistenkd 141(1): 1–5.
Redner BD, Stickney RR (1979) Acclimation of ammonia by *Tilapia aurea*. Tans Am Fish Soc 108: 383–388.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61(3): 539–542.

Sawayama S, Inoue S, Yokoyama S (1995) Phylogenetic position of *Botryococcus braunii* (Chlorophyceae) based on small subunit ribosomal RNA sequence data. J Phycol 31(3): 419–420.

Senousy HH, Beakes GW, Hack E (2004) Phylogenetic placement of *Botryococcus braunii* (Trebouxiophyceae) and *Botryococcus sudeticus* isolatee UTX 2629 (Chlorophyceae). J Phycol 40(2): 412–423.

Swofford DL, (2001) PAUP* Phylogenetic Analysis Using Parsimony (*and other methods) version 4.04beta. Sinauer Associates, Sunderland, MA.

Teng ST, Lim HC, Lim PT, Dao VH, Bates SS, Leaw CP (2014) *Pseudo-nitzschia kodamae* sp. nov. (Bacillariophyceae), a toxigenic species from the Strait of Malacca, Malaysia. Harmful Algae 34: 17–28.

Teng ST, Lim PT, Lim HC, Rivera-Vilarelle M, Quijano-Scheggia S, Takata Y, Quilliam MA, Wolf M, Bates SS, Leaw CP (2015) A non-toxigenic but morphologically and phylogenetically distinct new species of *Pseudo-nitzschia*, *P. sabit* sp. nov. (Bacillariophyceae). J Phycol 51(4): 706–725.

Tukau F (2007) Water quality study of man-made lake in UNIMA new campus. Aquatic Resource Science and Management. BSc thesis. Universiti Malaysia Sarawak, Sarawak, Malaysia p. 61.

Wake LV, Hillen LW (1980) Study of a "bloom" of the oil-rich alga *Botryococcus braunii* in the Darwin River Reservoir. New Zeal J Mar Fresh 22(8): 1637–1656.

Wu J-T, Chiang Y-R, Huang W-Y, Jane W-N (2006) Cytotoxic effects of free fatty acids on phytoplankton algae and cyanobacteria. Aquat Toxicol 80(4): 338–345.

Zakariah NA, Rahman NA, Hamzah F, Jahi TM (2015) Cultivation system of green microalgae, *Botryococcus braunii*: A review. J Teknol 76(5): 1–5.