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Research Article
Isolation and Potential Culture of Phytoplankton Live Feed for Freshwater Mussels *Sinanodonta woodiana* (Lea, 1834)

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Abstract

Background and Objective: Gastropod and Bivalves are widely known as filter feeders which used to feed the phytoplankton and other micro creatures. This study was conducted to identify, isolate and determine the potential culture of phytoplankton species for mussel culture. **Materials and Methods:** The phytoplankton identification and the culture of phytoplankton in ponds in UPMKB, Sarawak, Malaysia were studied for a period of 3 months from February 2019 to May 2019. **Results:** Three genera were recorded from the ponds namely *Selenastrum* sp. followed by *Licmophora* sp. and *Gloeocapsa* sp. The highest abundant genus was *Licmophora* sp. due to their presence in every pond while the highest composition in culture condition was *Selenastrum* sp. because every treatment had this genus. The impact of physicochemical parameters on phytoplankton compositions and abundances in four ponds in UPMKB was assessed. Water quality parameters, such as temperature, dissolved oxygen, pH and conductivity were measured *in situ* from the ponds. Phytoplankton compositions and abundances were analyzed in the laboratory. ANOVA result of the physicochemical parameters showed the presence of significant difference among pH and temperature between ponds. **Conclusion:** The study concluded that the presence of the *Selenastrum* sp. genus could be the biological indicator of the water quality ponds. The best culture of phytoplankton shown by using the fertilizer treatment which was NPK fertilizer that improves the distribution of the culture of the phytoplankton.

Key words: Phytoplankton, physiochemical parameters, phytoplankton abundance, *Selenastrum* sp., Water quality parameters

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The largest bivalve mollusk family of freshwater mussels is Unionidae which is consisted about 800 species worldwide[1]. Other than that, the freshwater mussel families belong to two different evolutionary families which are freshwater mussels and freshwater clams and these two groups are not closely related. Bivalve food consists of not only a variety of suspended particles such as bacteria, phytoplankton, micro-zooplankton and detritus, but also Dissolved Organic Material (DOM) such as amino acids and sugars[2-5].

Algae can be found in every aquatic ecosystem which can be referred as phytoplankton or macrophytic that is underwater vegetation in marine and freshwaters, but also worldwide distributed in terrestrial habitat[6]. Phytoplankton is the single-celled micro-phyte that accumulate in the water[7]. Sometimes this aquatic micro-phyte can be colonized into a large amount that can be seen by naked eyes[8]. It is also known as microalgae which are similar to plants due to containing chlorophyll and require sunlight for existence and growth as well as reform carbon dioxide to release oxygen. Phytoplankton requires inorganic nutrients such as nitrates, phosphates and sulfur which they convert into proteins, fats and carbohydrates[9]. Phytoplankton is enabled to produce their food like plants which is photosynthetic[10]. Phytoplankton contributes as the essential component for bivalve mollusk feeding[11].

The phytoplankton is fed by zooplankton and other microscopic sea creatures since they are filter feeders. *Sinanodonta woodiana* a most large-sized in of the family Unionidae belongs to East and South-East Asia[12]. Some authors classify this species as belonging to the genus *Anodonta*, but according to the latest taxonomy, it should be more appropriately assigned to the genus *Sinanodonta*[13]. Among freshwater bivalve species recently introduced to Europe, *Sinanodonta woodiana* can assemblage rapidly[14,15].

Mussel plays an important role of protein source of the communities and had been a filter-feeder of phytoplankton. It contributes as a converter of nutrients and organic matter into nutritious animal protein[16]. Aquaculture of mussels can provide an additional and alternative food source for the rapidly growing human population. In east Malaysia, some investigation on freshwater gastropod and bivalves were conducted such as, the taxonomic study of edible bivalve by Hamli *et al.*[17], diversity of edible Mollusc (Gastropoda and Bivalvia) by Hamli *et al.*[18], checklist and habitat descriptions of edible Gastropods by Hamli *et al.*[19], fisheries assessment, gametogenesis and culture practice of local bivalve investigated by Hamli *et al.*[20], diversity and habitat characteristics of local freshwater Gastropoda by Hamli *et al.*[21]. Some studies on mangrove as well as marine gastropod and bivalves study were also performed[22-27]. But no other study did not reveal the planktonic food source and production of plankton for mussel from East Malaysia which is generally known as Sarawak.

In order to culture the mussel species, phytoplankton acts as an important food source for the cultured mussel. Considering above mentioned aquaculture prospect of freshwater mussel, live feed production and feed optimization is necessary to enhance the aquaculture possibility of this species. The aquaculture prospect and economic potentiality lead this investigation to certain objectives and those were, to identify the phytoplankton species from different ponds; to isolate phytoplankton species from the water fish pond and to determine the potential culture of phytoplankton species from a different treatment.

MATERIALS AND METHODS

Study area: The study was conducted at the four sampling sites located at UPMKB (Fig. 1). Three fish pond and one abandoned pond were taken into consideration for phytoplankton collection. The total duration of the study is from February-May, 2019.

Sample collection: Phytoplankton samples were collected horizontally from four selected areas using 90 µm phytoplankton net. After samples were collected samples were preserved in 250 mL plastic bottles with two conditions; in which, first containing 10% buffered formalin and second containing nutrient solution (N:P:K = 1:1:1). Samples with 10% formalin were used for species identification in the laboratory. Meanwhile, samples with nutrient solution to keep them alive for isolation and culture process. The water physical parameters form every study area such as pH, dissolved oxygen, turbidity and temperature were measured *in situ* using multi-parameter equipment (Model WQC-24; DKK-TOA Corporation, Tokyo, Japan)[28].

Identification and count of phytoplankton: After collection of samples, these samples were brought to the Aquatic Ecology Laboratory of Universiti Putra Malaysia Bintulu Sarawak Campus for further analysis and identification. The identification was performed by a compound microscope (LEICA CWE compound microscope; 40×10 magnification) following the method described by Newell and Newell[29] and Sukhanova[30].

Fig. 1: The sampling sites at UPMKB

Table 1: Different mediums of phytoplankton culture in laboratory conditions

Treatment	Ingredients	Concentration	Number of replications
T_0	Control (distilled water)	0	3
T_1	Urea	1 g L^{-1}	3
T_2	Standard fertilizer (NPK fertilizer)	1 g L^{-1}	3
T_3	Waste (banana peel)	1 g L^{-1} (equivalent)	3

Collection and isolation of phytoplankton: Collection and isolation of phytoplankton were carried out by several procedures which are preparation of agar, centrifuge washing technique, streak plating technique based on Phang and Chu[31] and the culture of phytoplankton.

Preparation of agar: Agar plates were prepared by dissolving nutrient agar in Schott bottle and were autoclaved at 126°C for 15 min. Then the nutrient agar poured in plates to cool down. These plates were allowed to cool, kept in an inverted position for not drying and at least 72 h before streaking.

Centrifuge washing technique (Purification of algal samples): A volume of 12 mL phytoplankton samples was taken especially from enrichment culture in each of at least four centrifuge tubes. These tubes were centrifuged at 3000 rpm for 15 min. After removing the supernatant, the cells were suspended in fresh sterile water in each tube using a vortex mixer (rotated at 1000-1500 rpm up to homogeneous suspension). About 500 mL sterile water was prepared using an autoclave (at 126°C for 15 min) to complete the centrifuge-washing process. Centrifugation and washing were repeated six times to expel the microorganisms presented in the algal sample and the cells were then streaked on to agar plates.

Streak plating technique: The streak plating technique was performed based on Phang and Chu[31] and Parvin *et al.*[32]. Washed microalgae allowed to streak through the loop in plates in axenic conditions and to keep for at least seven days to grow microalgae. Repeated streak-plating was carried out to peak up a single colony from earlier streaked plates and to make free from bacteria. From last streaked plates, the single colonies were picked up by loop and allowed to grow in tubes and vials. Before putting in the tubes and vials, the single-cell growth and purity of single species were confirmed after observing under a compound microscope. Then, the pure culture of isolated phytoplankton was maintained in a volumetric flask in the aquatic ecology laboratory for further use. Serial dilution in 0.9% sterilized distilled water of phytoplankton suspension was spread on nutrient agar with bold basal medium plates using pour plate technique which is the method for counting the number of colonies forming phytoplankton present in a liquid specimen. The 1 mL of inoculum from a phytoplankton sample was placed in the petri dish using the pipette and molten cooled nutrient agar (NA) that was added with 2% of Bold Basal Medium (BBM) was poured into the petri dish that contains the inoculum. The agar plate was inverted for 48 hrs after the solidification of agar at room temperature with adequate light in order to obtain a successful single colony of phytoplankton.

Culture of phytoplankton: The experimental design was set up based on Completely Randomized Design (CRD) which consists of three treatments each contains three replications

including one control treatment of *Selenastrum* sp. The culture media of phytoplankton was prepared by washing 500 mL of Schott bottles and sterilized by using autoclave for 15 min at 121°C. After that, the bottles were filled with 250 mL of distilled water. There have four treatments which were urea, standard medium (NPK fertilizer), controlled and waste (banana peel) (Table 1).

The treatments were put in each of the Schott bottles with three replications. The single culture of treatment was pipette 100 mL into the treatment bottle for culturing. The bottles were capped with cork. Blowers and lights were installed and operated for 24 hrs every day for 14 days (two weeks). After successful culture of phytoplankton, 100 mL water had been filtered by using filter paper (Whiteman cellulose nitrate membrane filter paper; pore size, 0.45 µm; diameter, 47 mm) and vacuum pump (Rocker 300). Then the filter paper was oven (Memmert; Loading Modell 100-800) dried with 103°C for 2 hrs, after these, the biomass was calculated. The biomass of phytoplankton was calculated using the formula:

$$\text{Phytoplankton biomass (mg)} = \text{Final weight of filter paper (mg)} - \text{Initial weight of filter paper (mg)}$$

Statistical analysis: Analysis of Variance (ANOVA) and Duncan's test was performed to determine mean differences the number of phytoplankton colonies in nutrient agar and the growth performance of phytoplankton measured based on biomass between treatments. Furthermore, in order to determine to mean differences in water quality parameters between ponds, Analysis of Variance (ANOVA) and Tukey test was performed by using SAS 9.3.

RESULTS

Distribution of phytoplankton species: The three different genera were identified from selected ponds in UPMKB, namely *Selenastrum, Gloeocapsa* and *Licmophora*. The *Selenastrum* sp. and *Gloeocapsa* sp. had occurred in Station 1 and Station 3 while the *Licmophora* sp. was found distributed at the Station 1, 2 and Station 4 (Table 2).

Gloeocapsa sp. were usually spherical in shape and surrounded by gelatinous sheaths that were easily identified by its bright colour. *Gloeocapsa* sp. had colourless sheaths. *Licmophora* sp. was distinct triangular or fan-shaped cells, grow on a common stalk that is attached to rocks or algae. *Selenastrum* sp. has strongly curved and often slightly sigmoid, lunate to sub-circular with pointed tips shape and widely found in freshwater lakes ponds and rivers (Fig. 2a-c).

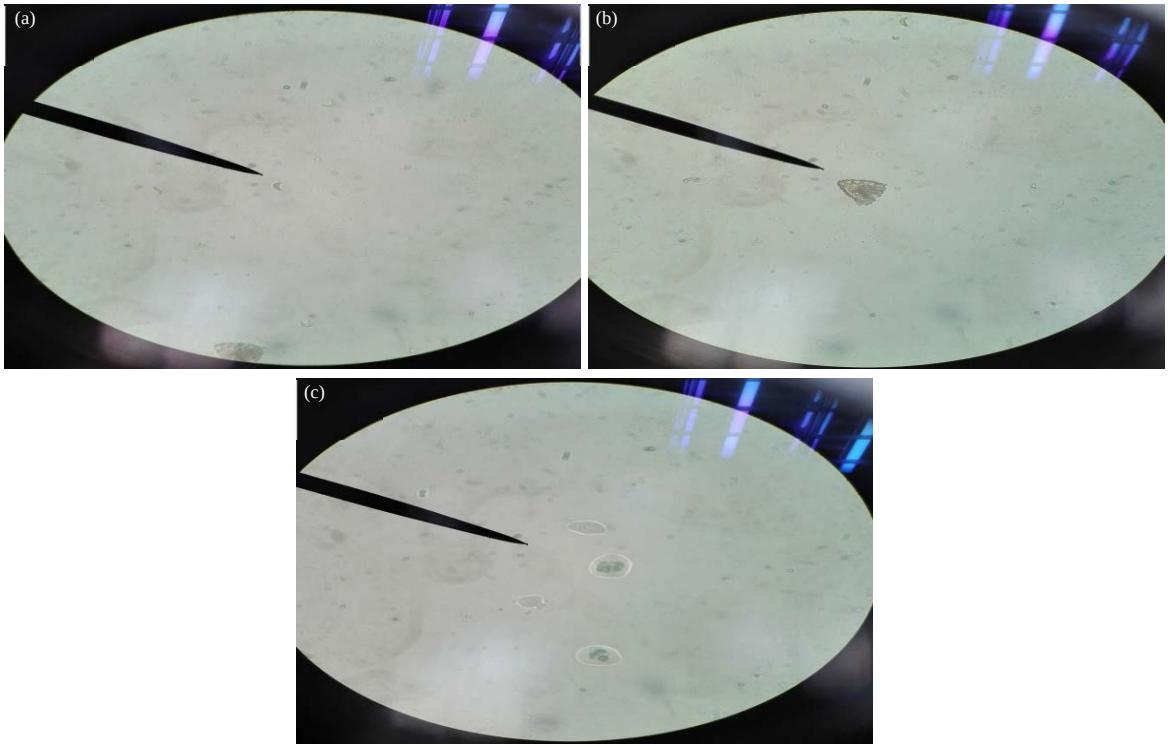

Fig. 2(a-c): The three genus *Selenastrum* sp. (a) *Licmophora* sp. (b) and *Gleocapsa* sp. (c) has been observed under microscope 40×0.65 X magnification

Water quality parameter: The water quality parameters, namely pH, temperature, conductivity and dissolved oxygen were measured *in situ* from three fish ponds and an abandoned pond located in UPMKB (Table 3). There was no significant difference at level ($p>0.05$) on environmental variables which is conductivity and dissolved oxygen. Besides, only Station 4 showed a significant difference for environmental variables for both pH and temperature while the other three stations did not show significant differences. In station 1, the value of pH (7.67±0.10) and temperature (30.2±0.10°C) was highest amongst all stations and parameters. The lowest result of pH (6.65±0.17) and temperature (29.2±0.20°C) was found in Station 4.

Isolation of phytoplankton: The isolation of phytoplankton was carried out with using two medium which are Nutrient Agar (NA) (Fig. 3a-c) and nutrient agar (NA) combined with 2% of Bold Basal Media (BBM) (Fig. 4a-c). The isolation on nutrient agar showing that no growth of phytoplankton's colony. Phytoplankton grown well in Nutrient Agar (NA) and BBM combination.

Samples were streaking until a single colony and the inoculum serially diluted with sterile water spread onto nutrient agar that was added with a modified medium which is bold basal medium agar plates, by using pour plate method until identifiable colonies appeared after two days.

Table 2: Species distribution of phytoplankton at selected ponds in UPMKB

	Stations			
Phytoplankton	1	2	3	4
Selenastrum sp.	+	-	+	-
Gloeocapsa sp.	+	-	+	-
Licmophora sp.	+	+	-	+

+: Present, -: Absent

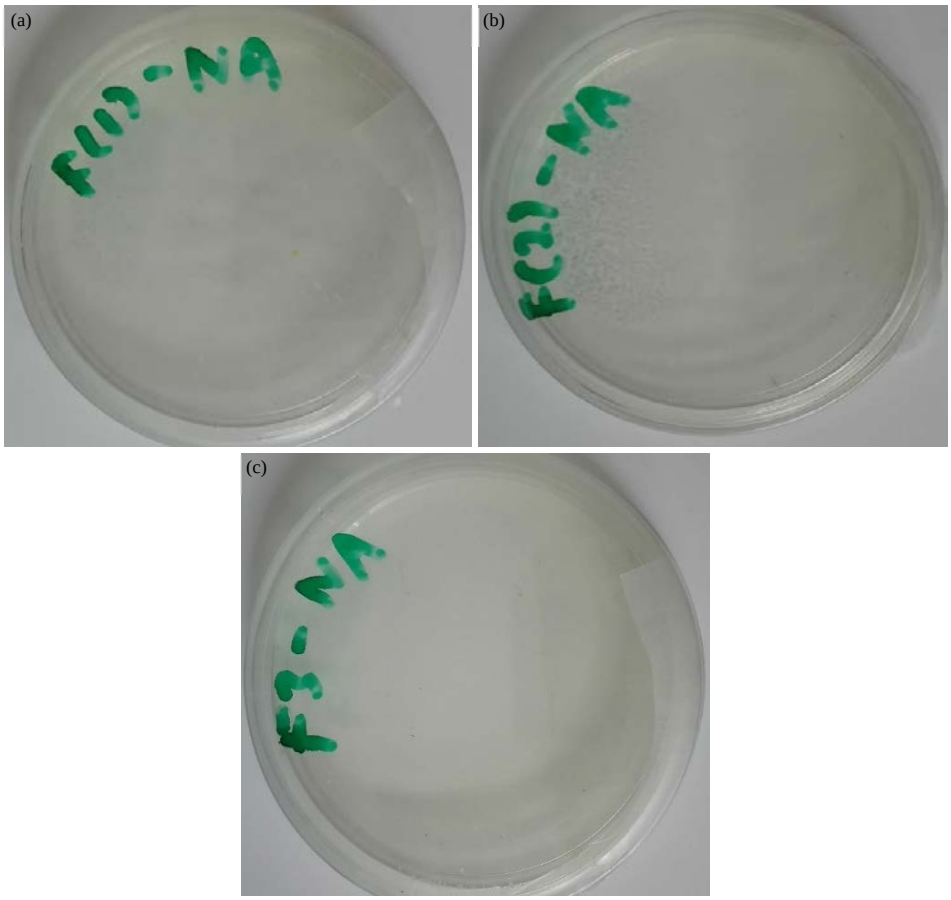

Fig. 3(a-c): The isolation of phytoplankton on nutrient agar (NA)

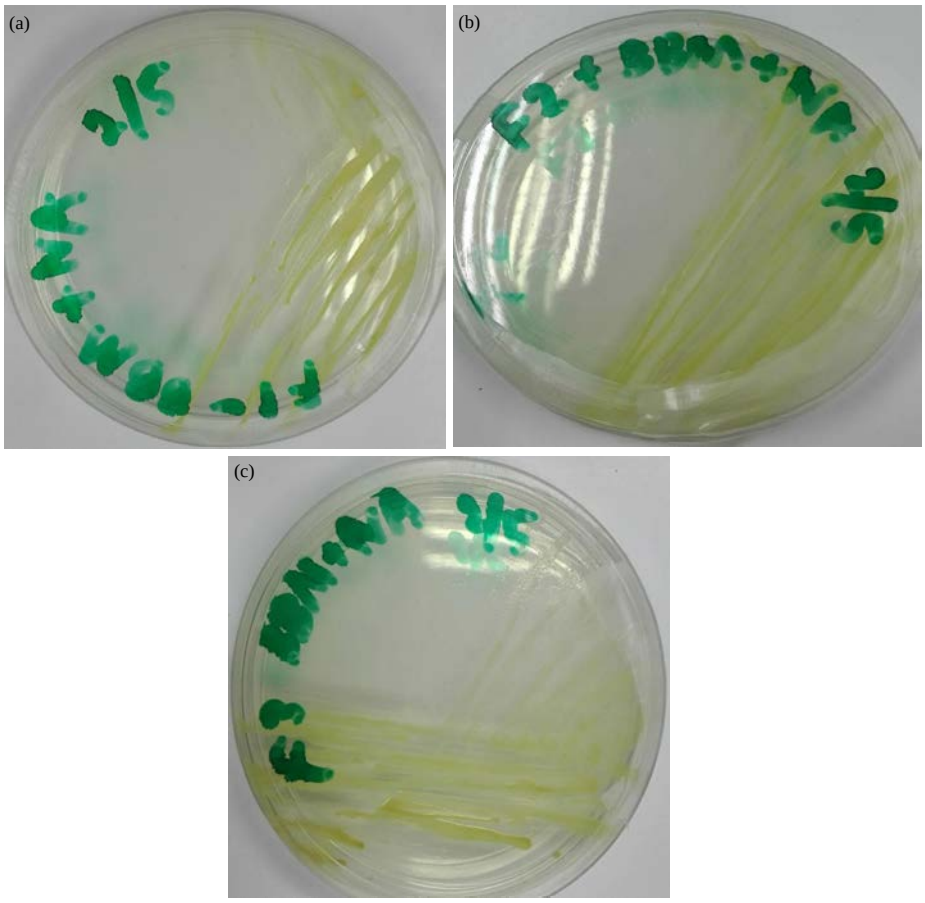

Fig. 4(a-c): The isolation of phytoplankton on combination of NA and BBM[33]

Table 3: Environmental parameters (Mean±SD) of each station at selected ponds in UPMKB

	Parameters			
Stations	pH	Temperature (°C)	Conductivity (μS cm^{-1})	DO (mg L^{-1})
1	7.67±0.10[a]	30.2±0.10[a]	4.5±0.55[a]	2.60±0.24[a]
2	7.63±0.09[a]	30.5±0.03[a]	2.6±0.32[a]	3.06±0.46[a]
3	7.21±0.10[ab]	30.5±0.00[a]	3.5±0.72[a]	1.30±0.33[a]
4	6.65±0.17[b]	29.2±0.20[b]	2.9±0.23[a]	1.72±0.69[a]

Means in the same column followed by the same letter do not differ significantly according to the Tukey's test ($p<0.05$)

Table 4: Biomass of the phytoplankton in four different treatments

Treatment	Mean dry weight (mg)
Controlled	0.052±0.023[a]
Urea	0.027±0.011[a]
Fertilizer	0.067±0.028[a]
Waste	0.026±0.009[a]

Means in the same column followed by the same letter do not differ significantly according to the Tukey's test ($p<0.05$)

Culture of phytoplankton: The standard fertilizer (NPK fertilizer) medium produced phytoplankton showed the highest (0.067±0.028) biomass among all other treatments. On the other hand, waste medium treatment showed the lowest biomass value (0.026±0.009). Meanwhile, the urea (0.027 mg) and waste (0.026 mg) treatment had almost similar value (Table 4).

DISCUSSION

The three genera had identified from the collected samples from different ponds comprised *Licmophora* sp., *Gloeocapsa* sp. and *Selenastrum* sp. All of three genera were found at Station 1. *Licmophora* sp. was only noticed from Station 2 and 4. Station 2 had two genera namely,

Selenastrum sp. and *Gloeocapsa* sp. The research team found the *Licmophora* sp. was the dominant genus compared with the others due to their presence in three stations out of four. *Selenastrum* sp. is freshwater algae or phytoplankton and this genus is cosmopolitan in freshwater lakes, ponds and rivers[34,35]. Cells found spherical with strongly curved and often slightly sigmoid with a pointed tip. The cells of *Selenastrum* sp. used to reproduce by autospore formation and colony fragmentation. Furthermore, *Gloeocapsa* is a genus from the cyanobacteria group. The cells secrete individual gelatinous sheaths which can often be seen as sheaths around recently divided cells within outer sheaths. Komárek[36] stated that the *Gloeocapsa* sp. is a unicellular-colonial species that can form small colony while irregular aggregations. Most of the species known from wet or dry, periodically moistened, inhabits on stony and rocky walls and from rocks with streaming water, distributed all over the world. Lastly, *Licmophora* sp. form fan-shaped colonies attached valve to valve, with wedge-shaped girdles[33].

The collected samples from four stations were put the nutrient medium (NPK fertilizer)to enhance phytoplankton growth. We found that, station 1 had more green color which indicated more phytoplankton abundance compared to the other three stations. Station 1 showed the higher composition of phytoplankton caused the higher possibilities to the isolation of phytoplankton growing on two types of media; nutrient agar and the combination of nutrient agar with a bold basal medium. We figured out that the combination of the nutrient agar with a bold basal medium showed the positive growth performance of phytoplankton. Which was the evidence that the bold basal medium was the good nutrient for freshwater algae that helps to the growth performance of phytoplankton on the agar. We had carried out the Colony-forming Units (CFU) technique to determine the number of colony-forming units. Therefore, often only parts of a plate are analyzed and used to estimate the whole plate count after extrapolation[37]. Furthermore high numbers of CFUs on a plate can lead to false results due to overcrowding of bacteria[38].

The urea and banana waste phytoplankton culture medium showed the lowest (0.027±0.011 and 0.026±0.011) weight among the treatment, while the highest (0.067±0.028) biomass was obtained from NPK fertilizer medium, which was 0.041g higher than the banana waste treatment. In relation to, biomass value of urea and waste medium, the urea medium has almost a similar value with the waste medium phytoplankton biomass where the difference was 0.001. Although the biomass of phytoplankton in NPK fertilizer medium seemed to be the highest among other treatments there was no statistically significant difference among four treatment mediums, the species in the sample probably contribute in a high degree to the total biomass of the phytoplankton in the culture of phytoplankton. Different concentrations of fertilizer can be taken into consideration for further study that which concentration would better for phytoplankton growth and biomass production. Mia *et al.*[39] and Mia *et al.*[40] did similar research used liquid rice starch and rotten apple concentration respectively.

Most of the physicochemical parameters were very suitable for phytoplankton growth. The ANOVA result showed that there were significant differences in temperature, pH, while Dissolved Oxygen (DO) and conductivity had no significant differences. The Tukey test also showed a similar scenario. Specifically, the temperature of the station's water ranged from 29.2 (Station 4) to 30.5 (Station 1). This registered temperature was not optimum for planktons but good for the growth of fish as suggested[41,42,43] which is between 22 and 31. pH of the stations extended from 6.65. (Station 4) to 7.67 (Station 1). These pH values were optimum for aquatic life including fish[44] within the EPA Redbook recommended pH range for freshwater (6.5-9.0)[45] and recommended by others[46,47]. The range of dissolved Oxygen (DO) was from 1.3 to 3.06 mg L^{-1}; meanwhile obtained DO concentration did not satisfy the minimum recommended standard (5 mg L^{-1}) set by EPA Redbook and others[48,49] which suggested that value was good for fish aquaculture and planktons. Dissolved Oxygen (DO) is one of the most important factors used in determining the quality of water[50]. Among four stations conductivity level ranged from 1.3 to 3.06 µS cm^{-1} which was low in comparison to the standards for the World Health Organization of 250 µS cm^{-1}.

The result showed that phytoplankton was highly distributed in fertilizer treatment medium comparison with the other three treatments based on the calculation of growth performance of the phytoplankton measured based on biomass. Phytoplankton analysis, which includes species count and biomass determination, could be used as an indicator of water quality[51]. The results indicated *Selenastrum* sp. was the most abundant species among the phytoplankton composition and stations. The dominance of *Selenastrum* sp. could be related to the abundant quantity of biomass in treatment (NPK fertilizer) because of the mean dry weight of the phytoplankton biomass compared to the other three treatments.

The outcome of this present study will help to accelerate the initiation of freshwater mussel culture research; as live phytoplankton is the primary food source of these filter feeder mussels. Previously no other evidence noticed in Sarawak, Malaysia of phytoplankton culture research which referred for mussel culture; so the present investigation can be the baseline study of phytoplankton culture for different freshwater mussel aquaculture. However, evaluation of dose optimization of daily feed application on freshwater mussel aquaculture is highly recommended for further study.

CONCLUSION

Three different genera of phytoplankton has been recorded from four different ponds in UPMKB, Sarawak, Malaysia which indicate that the overall health of these pond water was not bad during study periods. The genus *Licmophora* sp. found as most distributed species which found at three ponds compared to other species. However, *Selenastrum* sp. was the most abundant genus compared to another genus due to its existence growing on agar (nutrient agar + bold basal medium). Laboratory culture of phytoplankton showed NPK fertilizer treatment improves the distribution and biomass of phytoplankton species. Phytoplankton feeding dose optimization for freshwater mussel species can be taken under consideration for further study by using this present study data.

SIGNIFICANCE STATEMENT

This study was the first approach to isolate and find the potential candidate species of live feed culture for freshwater mussel species *Sinanodonta woodiana* (Lea, 1834) in Malaysia. This present finding will help the related researcher to select and culture of appropriate phytoplankton species to ensure live feed production for freshwater mussel species *Sinanodonta woodiana*. Thus a new phytoplankton species candidate for mussel aquaculture may be arrived at.

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