**N and P removal by free and immobilised cells of *Scenedesmus bijugatus* (Kützing) from the Pinang River estuary, Penang, Malaysia.**

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Acknowledgements

The authors wish to acknowledge the Fundamental Research Grant Scheme (203/PBIOLOGI/6711459) for the financial support provided for the research conducted, and anonymous reviewers for constructive suggestions for improving the manuscript.

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This research studied the effects of inorganic nutrient removal by free and immobilised *S. bijugatus* cells, measured by algal growth (i.e., the chlorophyll *a* concentration) and the efficiency of the uptake of inorganic nutrients by the cells (uptake rate (*b*) and removal percentage) in water samples from the organically polluted Pinang River estuary (PRE). Water samples from the PRE were collected during low and high tide. *S. bijugatus* cells had a higher growth rate when incubated in low tide PRE water samples compared to high tide PRE samples, with a growth rate of 0.29 µg/mL/d and 0.06 µg/mL/d for free and immobilised cells, respectively. *S. bijugatus* were able to more efficiently remove nitrogen, especially ammonium (81-94%), compared to phosphate (62-88%) from both low and high tide water samples. The highest phosphate (0.36 mg/L/d and 0.25 mg/L/d for free and immobilised cells, respectively) and ammonium uptake rates (0.44 mg/L/d and 0.29 mg/L/d for free and immobilised cells respectively) of *S.bijugatus* cells were both in low tide PRE water samples. Both inorganic nutrient removal and microalgal cell growth were not significantly different between free and immobilised *S. bijugatus* (p>0.05). The data obtained indicated that the removal of nutrients by microalgae was affected by salinity, and the immobilisation technique applied may have good potential for bioremediation.

Keywords: *Scenedesmus bijugatus;* free cells; immobilised cells; inorganic nutrients; polluted rivers.

# Introduction

Rivers are exploited in Malaysia as a source for the generation of hydropower (Shafie *et al.*, 2011), while the floodplains around the rivers provide fertile land for the development of agriculture, industry and even housing (Ngai Weng, 2005). Uncontrolled human activity, such as waste discharge from agriculture, industry and domestic dwellings, has caused river water quality to rapidly deteriorate (Abdullah & Mahmood, 1998).

In 2004, the Department of Environment (DOE) Malaysia found that the Pinang River estuary (PRE), the principal river of the Pinang River Basin that is formed from six tributaries (i.e., the Air Terjun River, the Kecil River, the Air Putih River, the Air Itam River, the Dondang River and the Jelutong River), was a Class IV river (DOE, 2004). Ten years later, the condition of the PRE had improved to become a Class III river (DOE, 2014). Most of the pollution sources affecting the Pinang River Basin are from household discharge, waste from animal husbandry and industrial wastes, as well as through soil erosion from construction sites and urban runoff (Farah Naemah & Nik Norulaini, 2000).

The potential of microalgae for bioremediation has been extensively studied for the treatment of agricultural, industrial, and municipal wastewater, as well as in the examination of their potential as feedstock and for biofuel production (e.g. Giorgos & Dimitris, 2011; Cai *et al.*, 2013). However, to date, the study of the application of microalgal bioremediation for wastewater treatment in Malaysia is very limited. As a country that relies on agro-industrial production, Malaysia faces water pollution problems caused by rubber and palm oil effluent discharges, even though most treatment plants comply with the current national standards (Mitra *et al.*, 2010; Mohd Fadhil *et al.*, 2014). Examples of bioremediation studies within Malaysia include that of textile wastewater (Sing-Lai *et al.*, 2010), algae cultivation in palm oil mill effluent water using *Chlorella pyrenoidosa* (Kamyab *et al.*, 2014), and the use of a mixture of micro andmacroalgae for treating palm oil mill effluent discharge, as well as its further enhancement as animal feed stock (Mohd Fadhil *et al.*, 2014).

The removal of the microalgal biomass from culture media can be a problem due to the small size of the algal cells. Currently available harvesting methods require large amounts of chemicals and energy. Immobilisation techniques have been developed to solve this problem (Cai *et al.*, 2013). An immobilisation technique that is frequently used involves alginate, a natural polymer (Jimenez-Perez *et al.*, 2004; de-Bashan & Bashan, 2010). The objective of this study was to explore the potential of free and immobilised microalgal cells for removing inorganic nutrients from the organically polluted PRE, in Penang, Malaysia.

**Materials and Methods**

 ***Microalgae Isolation, Cultivation and Maintenance***

*Scenedesmus bijugatus* was isolated from a retention pond (Tasik Harapan in Universiti Sains Malaysia, Main Campus, Penang), using standard agar plate streaking and serial dilution methods. Algae were cultivated and maintained in enriched Bold’s Basal Medium (Andersen, 2005) under 24 h continuous light (33.75 µmol m-2 s-1) and manually agitated once a day at a constant temperature of 25±2°C. Light (Olympus CX21) and Scanning Electron Microscopy (Carl Zeiss Leo Supra 50 VP Field Emission equipped with Oxford INCA – X energy dispersive microanalysis system) were used to identify the species. Species confirmation was also made by sending the microalgal samples to Biotech International Research & Development (BIRD) Centre, Egypt. The species identified has cell width and length of 2.6-5.3 µm and 6-11 µm, respectively. Individual cells are solitary ellipsoid-cylindrical in shape and usually appear in colonies with cells arranged in groups of two.

***Water sample collections***

For this study, *Scenedesmus bijugatus* was cultivated from water samples collected by grab sampling from the Pinang River estuary (N05º 24.251’ E100 º 19.702’) (Figure 1) during low and high tides. Triplicates of surface water samples were collected using a 40 L bucket attached to a rope. Polyethylene bottles were rinsed with the collected water and were slowly filled to the brim to remove air bubbles, then stored in an ice chest on ice for the duration of sampling. In the laboratory, the water samples were stored in a refrigerator at 4 ºC and under dark conditions prior to analysis**.**

[Figure 1: Sampling site in the Pinang River estuary, Penang Island, Malaysia.](Figure1%20%28Wong%20Swe%20Cheng%29.tif)

 **1km**

***Experimental Design***

*Preparation of free algal cell cultures and immobilised algal bead cultures*

A total of 500 mL of *Scenedesmus bijugatus* cell culture from the initial stock was harvested during the stationary growth phase (23 d after inoculation), concentrated by centrifugation (3000 rpm for 10 mins) and re-suspended in 100 mL of distilled water. The number of cells was estimated using haemocytometer; a cell density of approximately 2.835 x 107 cells mL-1 was used to ensure that the amount of cells inoculated was the same in all experiments.

*Free cell cultures*

Free cell cultures were prepared by inoculating approximately 6.5 mL of the previously prepared algal suspension into 125 mL of river water, to a final volume of 130 ± 1.5 mL culture (approximately 5% inoculum of the 130 mL river water culture). Three replicate free cell cultures for each two day experimental interval (2, 4, 6, 8, and 10 days) were prepared. Replicates without algal inoculum were also prepared as a control.

*Immobilisation of algal cells with sodium alginate*

The immobilisation of microalgal cells was based on the method used by Chen (2001) and Ruiz-Marin *et al.* (2010). The microalgal cells were immobilised by mixing approximately 3.25 mL of a 4% sodium alginate solution (Sigma-Aldrich alginic acid sodium salt from brown algae) with approximately 6.5 mL of a concentrated microalgal cell suspension to give a 1:2 (v/v) alginate to algal cell suspension ratio. The 2% sodium alginate-algal cell mixture was stirred vigorously to ensure that the solution was well mixed. The mixture was taken up in a syringe, that was then held at a height of 8-10 cm above a beaker of a 2% CaCl2∙2H2O solution and added dropwise at a controlled speed. Spherical beads of approximately 2.0±0.5 mm diameter were obtained and allowed to harden in the calcium chloride solution for more than 4 h, then rinsed a few times with sterilised distilled water. The spherical beads had a light green colour. Blank alginate beads (without algae) were prepared the same way but distilled water was used instead of the concentrated microalgal cell suspension. Three replicates for each two-day experimental interval (2, 4, 6, 8, and 10 days) were prepared for the immobilised algal cells and immobilised blank beads (control).

***Analytical Methods***

*Determination of nutrient and chlorophyll a concentrations*

The river water samples were filtered through Whatman GF/C 0.45 µm glass fibres, followed by autoclaving prior determination of the initial concentrations of inorganic nutrients. The filtered water samples were also inoculated with free and immobilised *Scenesdesmus**bijugatus*cellstoremove nutrients under standard lab conditions (24 h continuous light (33.75 µmolm-2s-1) and manually agitated once a day at a constant temperature of 25±2°C; relative humidity of 53.3%). The water samples were analysed for phosphate, ammonium, nitrite and nitrate prior to and after the two day experimental intervals (2, 4, 6, 8, and 10 days). The nutrients were determined following Strickland and Parson (1972). After each two day interval, the bead solution was filtered through a Whatman Cellulose Nitrate membrane filter (0.45 µm) after the beads had been dissolved in a test tube filled with 7 mL of 5% sodium hexametaphosphate solution. The chlorophyll *a* concentration of free and immobilised cells was determined following Lobban *et al.* (1988) and the following formula: Chlorophyll *a* = 11.93A664 – 1.93 A647.

*Logistic growth model for algal growth and nutrient uptake rates*

The algal growth rate and the nutrient uptake rate were calculated based on a logistic growth model (Jiménez-Pérez *et al.*, 2004; Xin *et al.*, 2010), which is used to describe the relationship between the growth of a microorganism and its density in limiting environmental conditions. In this case, it was used to describe the algal growth:

N= [K/ (1+e1-*r*t)], where N (cells mL-1) is the algal density at time *t* (days), and K (cells mL-1) is the carrying capacity (i.e., the maximum algal density achieved by the culture).

This equation can be transformed into a linear form as:

ln [(K/N) – 1] = *a* – *rt*, where *a* is a constant in the logistic model which indicates the relative position from the origin, and *r* (d-1) is the specific growth rate. The data obtained were linearised in the form of a logistic growth model to assist in comparison of the growth rates. A regression line with a slope equal to the specific growth rate (*r*) was obtained.

The same linear equation was also used to calculate the nutrient uptake rate. For the phosphate uptake rate, N (PO43- mg L-1) is the phosphate concentration at time *t* (days), K (PO43- mg L-1) is the maximum phosphate concentration at the end of the experiment, and *r* (d-1) is the phosphate uptake rate. The same procedures were used to calculate the ammonium, nitrite and nitrate uptake rates.

*Nutrient removal percentage*

The percentage of nutrient removed from the medium after treatment was determined according to the following formula:

%N = ((N0 – N) x 100)/N0, where %N is the percentage of nutrient elimination at the end of the treatment for phosphate, ammonium, nitrite, or nitrate, N0 is the initial nutrient concentration, and N = nutrient concentration in mg L-1.

*Statistical Analysis*

Statistical analyses were performed using IBM SPSS Statistics 20. The Mann-Whitney U test was used to determine significant differences in the orthophosphate, ammonium, nitrite and nitrate removal and algal growth between free and immobilised cell cultures. Comparisons of all the dependent variables between the low tide PRE and high tide PRE water samples were analysed using the Kruskal Wallis H test. All the experiments were conducted in triplicate, and all data are presented as the mean ± standard error.

### **Results**

***Algal growth***

Table 1 shows the initial concentrations of phosphate, ammonium, nitrite and nitrate in water samples from the Pinang River estuary (PRE) at low and high tide. The growth rate of *S. bijugatus* free cells was significantly different compared to immobilised cells when incubated in low tide PRE water samples (p < 0.05), whereas no significant difference was evident when incubated in high tide PRE water samples (p > 0.05) (Figure 2). The maximum biomass of *S. bijugatus* achieved for both free and immobilised cells was higher for low tide PRE than high tide PRE (Table 2). The growth rates of *S. bijugatus* in low tide PRE for free cells and immobilised cells were 0.285±0.003 µg/mL/d and 0.062±0.158 µg/mL/d, respectively.

[Table 1 Initial nutrient concentration and salinity of water samples during sampling](Table1%20%28Wong%20Swe%20Cheng%29.docx)

[Figure 2: (A) Growth curves of *S. bijugatus* free cells () and immobilised cells () incubated in low tide PRE water; (B) Growth curves of *S. bijugatus* free cells () and immobilised cells () incubated in high tide PRE water. The bars show standard error; PRE: Pinang River estuary](Figure2%20%28Wong%20Swe%20Cheng%29.tif)

[Table 2 Linearised logistic growth model for the growth, phosphate, ammonium, nitrite and nitrate uptake of *S. bijugatus* free and immobilised cells](Table2%20%28Wong%20Swe%20Cheng%29.docx)

***Phosphate, ammonium, nitrite and nitrate uptake***

*S. bijugatus* free cells showed a higher phosphate uptake rate (0.359±0.003 µg/mL/d) compared to immobilised cells (0.251±0.002 µg/mL/d) when incubated in low tide PRE water (p>0.05) (Table 2). The removal was quite similar (60-80%) between the free and immobilised *S. bijugatus* cells incubated in both low and high tide PRE water (Table 3). Immobilised blank beads showed significant phosphate removal (80%) due to the chelating effects of the alginate beads towards phosphate ions (Table 3).

[Figure 3: (A) Phosphate concentration during ten days in a *S. bijugatus* free cell culture () and an immobilised cell culture () incubated in low tide PRE water. (B) Phosphate concentration during the ten-day experimental study with *S. bijugatus* free cell cultures () and immobilised cell cultures () incubated in high tide PRE water. The bars show standard error. PRE: Pinang River estuary](Figure3%20%28Wong%20Swe%20Cheng%29.tif)

[Table 3 Phosphate, ammonium, nitrite and nitrate removal percentages from the Pinang River estuary under the four experimental treatments](Table3%20%28Wong%20Swe%20Cheng%29.docx)

Free and immobilised cells of *S. bijugatus* incubated in low tide PRE water had a higher ammonium uptake than when incubated in high tide PRE water (Table 2). However, no significant difference in ammonium uptake was evident between the free and immobilised cells in both low and high tide PRE water (p>0.05) (Figure 4). The removal of ammonium was between 80-90% for both free and immobilised cells in both low and high tide PRE water (Table 3). Both free and immobilised cells in low and high tide PRE water showed a negative nitrite uptake rate, and both water treatments were not statistically significantly different (Figure 4). The nitrite removal for low and high tide PRE water was only between 20-50% (Table 3). This was the same for the nitrate uptake rate for both free and immobilised cells in high tide PRE water (p > 0.05) (Table 2 and Figure 4). The nitrate removal was 45% for free cells (high tide PRE water only), whereas it was approximately 80% for immobilised cells for both low and high tide PRE water. The data that are designated with a “-” removal could not be determined. Figure 5 shows the freely suspended *S. bijugatus* cells and immobilised cells beads.

[Figure 4: Ammonium (), nitrite (), and nitrate () concentration during the ten-day incubation of *S. bijugatus* (A) free cells and (B) immobilised cells in low tide PRE water. Ammonium (), nitrite (), and nitrate () concentration during the ten-day incubation of *S. bijugatus* (C)free cells and (D) immobilised cells in high tide PRE water. The bars show the standard error; PRE: Pinang River estuary](Figure4%20%28Wong%20Swe%20Cheng%29.tif)

Figure 5: Illustration of freely suspended cells and immobilised algal beads during the treatment duration

**Discussion**

***Algal growth***

The chlorophyll a concentration of *S. bijugatus* cultured in high tide PRE water in this study was lower compared to low tide water, indicating that the microalgae could not adapt to the saline conditions present at high tide (Figure 2). Higher salinity can decrease the photosynthetic rate resulting in a lower chlorophyll concentration due to salt, osmotic, and toxic ionic stress (Moradi and Ismail, 2007). Anita *et al.* (2011) showed that the biomass of *Scenedesmus quadricauda* increased when incubated in an NaCl concentration of 0.0 - 0.2 mM, and then decreased as the NaCl concentration was increased further. The reason was the same for the undetermined growth rate of *S. bijugatus* in high tide PRE due to erratic data (Table 2).

Nutrient uptake by immobilised microalgal cells may be inhibited by restricted nutrient diffusion through the immobilising matrix of beads when the nutrient concentration is low in the media (Garbayo *et al.,* 2000; Moreno-Garrido, 2008). This could be the reason that *S. bijugatus* free cells had a higher growth rate (Table 2) and chlorophyll *a* concentration (Figure 2) compared to the immobilised cells when incubated in low tide PRE water. The initial nutrient concentration in low and high tide PRE water could be limiting and not diffuse efficiently through the immobilising beads matrix.

A low initial nitrate concentration in low and high tide PRE water samples could be the reason for the low and erratic growth rates found in this study. In a study of *Nannochloropsis oculata* and *Chlorella vulgaris*, Attilio *et al.* (2009) found that decreasing the nitrate concentration in the medium resulted in an overall constant growth rate but enhanced the lipid fraction of the microalgae. The low initial nitrate concentration might have inhibited the growth of *S. bijugatus* after the ammonium concentration (favoured nutrient of the algae) had decreased (Maestrini *et al.*, 1986), but might have contributed to an increase in the lipid content of the *S. bijugatus* cells.

***Phosphate, ammonium, nitrite and nitrate removal***

Table 4 Nitrogen and phosphorus removal by the Chlorophyte genera.

Based on the data presented in Table 4, various studies on the uptake rates of nitrogen and phosphate have been publishedusing *Chlorella* sp. and *Scenedesmus* sp. Various concentrations of nitrogen and phosphate have been used in these studies, and different uptake rates have been recorded. The efficiencies of nitrogen and phosphate removal depend on factors such as the medium composition, environmental conditions, light intensity, nitrogen/phosphorus ratio, light/dark cycle and algal species (Aslan & Kapdan, 2006).

Immobilised cells in low tide PRE water had a lower removal percentage than free cells, which was possibly a reflection of the high standard error among the replicates on day 8 and day 10. Lau *et al.* (1997) mentioned that immobilised cells had a better phosphate removal rate because the calcium ions in the alginate beads have a tendency to bind with the phosphate to form a white precipitate. This would explain the overall higher phosphate removal in immobilised cells and the blank beads treatment in the water samples compared to free cells and control experiments. Phosphate uptake rate increased with an increase in the initial phosphate concentration to a certain extent, from 0.2 mg/mg chl *a*/d to approximately 0.52 mg/mg chl *a*/d for an initial concentration of phosphate between 7.7 mg/L and 149 mg/L (Aslan & Kapdan, 2006). This suggests that the low uptake rate found in this study was caused by the initial phosphate concentration being low.

A higher ammonium uptake rate was evident in low tide PRE water compared to high tide PRE water because *S. bijugatus* was not able to adapt to the saline conditions of high tide PRE water (Anita *et al.* 2011). In addition, the ammonium uptake rate increased with an increase in the initial ammonium concentration (Aslan & Kapdan, 2006). However, if the initial ammonium concentration was greater than 40 mg/L, the ammonium decreased gradually with time (Tam & Wong, 1996). In this study, the initial ammonium concentration in both types of water sample was in the range that could be efficiently removed within the experimental period, in agreement with data presented by Tam & Wong (1996). A high ammonium uptake rate for immobilised cells and immobilised blank beads could also be the effect of ionic interactions between ammonium (cation) and alginate gel (anionic group). This effect could help to concentrate the ammonium ions from the medium onto the alginate gel for assimilation by the immobilised algal cells (Lau *et al.*, 1997).

Ammonium has been proven to inhibit the uptake of nitrite and nitrate in many studies (Maestrini *et al.*, 1986; Vilchez & Vega, 1994), which will only take place after ammonium has been depleted in a water sample (Maestrini *et al.*, 1986). Both free and immobilised cells in low and high tide PRE water showed negative nitrite and nitrate uptake rates, suggesting that uptake by either free or immobilised cells was inefficient due to inhibition by a high initial ammonium concentration in the water (Maestrini *et al.*, 1986; Vilchez & Vega, 1994). Based on the findings of this study, ammonium decreased from day 0 until day 10, while the nitrite and nitrate concentrations were somewhat erratic and inconsistent in terms of depletion trates as assimilation by *S. bijugatus* cells occurred. Ammonium levels underlie the low removal percentage of nitrite and nitrate from PRE water samples for both free and immobilised cells. The higher nitrate and nitrite removal observed in immobilised cells compared to free cells could be due to the adsorption of nitrate and nitrite onto the alginate bead matrix in addition to assimilation by *S. bijugatus* cells (Aslan & Kapdan, 2006).

The assimilation of nitrate by microalgae depends on the availability of light, because microalgae require light energy as a reductant and to produce ATP required for the assimilation process (Guerrero & Lara, 1987; Lara & Romero, 1986). The nitrate uptake efficiency was higher at a light intensity of 100 µmol m-2 s-1 than at 35 µmol m-2 s-1 (Garbisu *et al.*, 1991). In this study, the light intensity was fixed at 33.75 µmol m-2s -1 throughout the experimental duration would therefore have reduced the nitrate uptake rate achievable by *S. bijugatus* cells. Both uptake and nitrate removal rates might be more consistent at a higher light intensity.

Low nitrate and nitrite removal were observed in both free cell control and immobilised blank bead treatments, and might indicate the oxidation or reduction of nitrate or nitrite to other forms of nitrogen during the period of the treatment (de-Bashan & Bashan, 2010).

**Conclusion**

This study was carried out to determine the potential of the free and immobilised green microalgae, *Scenedesmus bijugatus,* to remove inorganic nutrients (phosphate, ammonium, nitrite and nitrate) from organically-polluted PRE water. Salinity affects algal growth, and therefore *S. bijugatus* cellsin high tide PRE water showed poor growth compared to that in low tide water. The efficiency of *S. bijugatus* for phosphate and ammonium removal was high (62-88% and 81-94% efficiency, respectively), compared to values for nitrite and nitrate, supporting previous observations that ammonium is a preferred nutrient for algal assimilation. Although no significant differences were evident between free and immobilised cells, the results overall indicated that cell immobilisation helped in nutrient removal, especially for phosphate via chemical precipitation. However, nitrogen removal (ammonium, nitrite and nitrate) by immobilised cells occurred only to a certain extent through adsorption onto the bead matrix. Microalgal cell immobilisation in wastewater treatment is promising and more sustainable in the long run because the by-product (i.e., the microalgal biomass) can more easily be removed after treatment.

**References**

Abdullah K. and Mahmood, M.F. River Management – The Way Forward. Workshop on River Management, Kuala Selangor. 1999; 30–31 March.

Andersen, R.A., (2005), Algal Culturing Techniques, Elsevier Inc.

Anita, K., Narsi, R.B., and Namita, S. 2011. “Salinity as a factor affecting the physiological and biochemical traits of *Scenedesmus quadricauda*.” Journal of Algal Biomass Utilization. 2(4): 28-34.

Aslan, S., and Kapdan, I.K. 2006. “Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae.” Ecological Engineering. 28:64-70.

Attilio, C., Alessandro, A.C., Erika, Y.O., Perego, P., and Del Borghi, M. 2009. “Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production.” Chemical Engineering and Processing. 48:1146-1151.

Cai, T., Stephen YP, and Yebo, L. 2013. “Nutrient recovery from wastewater streams by microalgae: Status and prospects.” Renewable and Sustainable Energy Reviews. 19:360–369.

Chen, Y. 2001. “Immobilized microalgae *Scenedesmus quadriqauda*, for long-term storage and for application in fish culture water quality control.” Aquaculture. 195:71-80.

de-Bashan, L.E., and Bashan, Y. 2010. “Immobilized microalgae for removing pollutants: Review of practical aspects.” Bioresource Technology. 101:1611-1627.

DOE (Department of Environment). Malaysia Environmental Quality Report 2004, Department of Environment Malaysia, Ministry of Natural Resources and Environment, Malaysia; 2004. 18-33.

DOE (Department of Environment). 2014. “Malaysia Environmental Quality Report 2014.” Department of Environment Malaysia, Ministry of Natural Resources and Environment, Malaysia. pp44-81.

Farah Naemah, M.S., Nik Norulaini, N.A.R, Mohd Omar, A.K., and Fatehah, M.O. 2000. “Identification of Pollution Sources within the Sungai Pinang River Basin.” Project Report. Universiti Sains Malaysia, Penang. 478-485.

Garbayo, I., Javier Vigara, A., Conchon, V., Martins Dos Santos, V.A.P., and Vilchez C. 2000. Nitrate consumption alterations induced by alginate entrapment of Chlamydomonas *reindhartii* cells. Process Biochem. 36:459-466.

Garbisu, C., Gil, J.M., Bazin, M.J., Hall D.O. and Serra J.L. 1991. “Removal of nitrate from water by foam-immobilized *Phormidium laminosum* in batch and continuous-flow bioreactors.” Journal of Applied Phycology. 3:221-234.

Giorgos, M., and Dimitris, G. “Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: A review.” Applied Energy. 2011; 88:3389-3401.

Gonzalez, L.E., Canizares, R.O., Baena, S. 1997. Efficiency of ammonia and phosphorus removal from a Colombian agroindustrial wastewater by the microalgae *Chlorella vulgaris* and *Scenedesmus dimorphus*. Bioresource Technology; 60:259-62

Guarrero, M.G., and Lara, C. 1987. “Assimilation of inorganic nitrogen. In: Fay P, van Baalen C (eds), The Cyanobacteria.” Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam. 163-186.

Hernandez, J.P., de-Bashan, L.E., Basahn, Y. 2006. Starvation enhances phosphorus removal from wastewater by the microalga Chlorella spp. co-immobilized with *Azospirillum brasilense*. Enzyme and Microbial Technology, 38: 190-8.

Hongyang, S., Yalei, Z., Chunmin, W., Xuefei, Z., Jinpeng, L. 2011. Cultivation of *Chlorella pyrenoidosa* in soybean processing wastewater. Bioresource Technology; 102:9884-90.

Jiménez-Pérez, M.V., Sanchez-Castillo, P., Romera, O., Fernandez-Moreno, D., and Pérez-Martinez, C. 2004. “Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure.” Enzyme and Microbial Technology. 34:392-398.

Kamyab, H., Md Din, M.F., Chew, T.L., Ali, K., Arezou, S., Muhd Zaimi A.M., Mohanadoss P. and Xiao Yun T. 2015. “Lipid Production by microalgae *Chlorella pyrenoidosa* cultivated in Palm Oil Mill Effluent (POME) using Hybrid Photo Bioreactor (HPBR).” Desalination and Water Treatment. 1–13.

Khan, M., Yoshida, N. 2008. Effect of L-glutamic acid on the growth and ammonium removal from ammonium solution and natural wastewater by *Chlorella vulgaris* NTM06. Bioresource Technology; 99:575-82

Kong, Q.X., Lil Martinez, B., Chen, P., Ruan, R. 2010. Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. Applied Biochemistry and Biotechnology; 160: 9-18

Lara, C., and Romero, J.M. 1986. “Distinctive light and CO2 fixation requirements of nitrate and ammonium utilization by the cyanobacterium *Ancystis nidulans*.” Plant Physiology. 81: 686-688.

Lau, P.S., Tam, N.F.Y., and Wong, Y.S. 1997. “Wastewater nutrients (N and P) removal by carrageenan and alginate immobilized *Chlorella vulgaris*.” Environmental Technology. 18(9):945-951.

Lee, K., and Lee, C.G. 2001. “Effect of light/dark cycles on wastewater treatments by microalgae.” Biotechnology and Bioprocess Engineering; 6:194–9.

Lobban, C.S., Chapman, D.J., and Kremer, B.P. 1988. Experimental Phycology A Laboratory Manual, Cambridge University Press. pp.35-41.

Maestrini, S.Y., Robert, J.M., Leftley, J.W., and Collos, Y. 1986. “Ammonium thresholds for simultaneous uptake of ammonium and nitrate by oyster-pond algae.” Journal of Experimental Marine Biology and Ecology. 102:75-98.

Mitra, M., Hasfalina, C.M., Mohd, A.H., and Phang, L.Y. 2010. “Treatment of wastewater from rubber industry in Malaysia.” African Journal of Biotechnology. 9(38):6233-6243.

Mohd Fadhil, M.D., Kamyab, H., Chew, L.T., Mohanadoss, P., Mohammad, S., Shaza, E.M., and Anahita, M.R. 2014. “Micro-Macro Algal Mixture as a Promising Agent for Treating POME Discharge and its Potential Use as Animal Feed Stock Enhancer.” Jurnal Teknology (Sciences & Engineering). 68(5):1-4.

Moradi, M., and Ismail, A.M. 2007. “Responses of photosynthesis, chlorophyll fluorescence and ROS – Scavenging systems to salt stress. During seedling and reproductive stages of Rice.” Ann. Botany. 99:1161-1173.

Moreno-Garrido, I. 2008. “Microalgae immobilization: Current techniques and uses.” Bioresource Technology. 99:3949-3964.

Ngai Weng, C. 2005. “Sustainable management of rivers in Malaysia: Involving all stakeholders.” International Journal of River Basin Management. 3(3):147-162.

Ruiz-Marin, A., Mendoza-Espinosa, L.G., and Stephenson, T. 2010. “Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater.” Bioresource Technology. 101:58-64.

Shafie, S.M., Mahlia, T.M.l., Masjuki, H.H., and Andriyana A. 2011. “Current energy usage and sustainable energy in Malaysia: A review.” Renewable and Sustainable Energy Reviews. 15(9):4370-4377.

Sing-Lai, L., Wan-Loy, C., Siew-Moi, P. 2010. “Use of *Chlorella vulgaris* for bioremediation of textile wastewater.” Bioresource Technology. 101:7314-7322.

Strickland, J.D.H., and Parsons, T.R. 1972. A Practical Handbook of Seawater Analysis. Canada: Fisheries Research Board of Canada. pp.49-52.

Tam, N.F.Y., and Wong, Y.S. 1996. “Effect of ammonia concentrations on growth of *Chlorella vulgaris* and nitrogen removal from media.” Bioresource Technology. 57:45-50.

Vı´lchez, C., and Vega, J.M. 1994. “Nitrite uptake by *Chlamydomonas reinhardtii* cells immobilized in calcium alginate.” Appl. Microbiol. Biotechnol. 41:137–141.

Xin, L., Hong-Ying, H., Ke, G., and Sun, Y.X. 2010. “Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake and lipid accumulation of a freshwater microalga *Scenedesmus* sp.” Bioresource Technology. 101:5494-5500.

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Table 1: Initial nutrient concentrations and salinity of water samples obtained during sampling.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Sampling sites | Phosphate (mg/L) | Ammonium (mg/L) | Nitrite (mg/L) | Nitrate (mg/L) | Salinity(ppt) |
| low tide PRE  | 1.016±0.039 | 27.990±1.902 | 0.012±0.004 | 0.007±0.004 | 6.750±0.001 |
| high tide PRE  | 0.609±0.002 | 18.430±1.499 | 0.014±0.004 | 0.008±0.001 | 7.930±0.001 |

PRE= Pinang River estuary; Triplicates of water samples were used for each analysis (n = 24 water samples)

Table 2: Linearised logistic growth model for the growth, phosphate, ammonium, nitrite and nitrate uptake of *S. bijugatus* as free or immobilised cell cultures.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | *K*±SE | *r*±SE | R2 |
| Growth |  |  |  |  |
| low tide PRE  | (free) | 2.646±1.153a | 0.285±0.003a | 0.909 |
|  | (immobilised) | 1.105±0.067b | 0.062±0.158b | 0.421 |
| high tide PRE  | (free) | 0.508a | - | - |
|  | (immobilised) | 0.760±0.265a | - | - |
| Phosphate uptake |  |  |  |  |
| low tide PRE  | (free) | 1.016±0.039a | 0.359±0.003a | 0.946 |
|  | (immobilised) | 1.016±0.039a | 0.251±0.002a | 0.943 |
| high tide PRE  | (free) | 0.609±0.002a | 0.271±0.047a | 0.689 |
|  | (immobilised) | 0.609±0.002a | 0.233±0.099a | 0.784 |
| Ammonium uptake |  |  |  |  |
| low tide PRE  | (free) | 27.990±1.902a | 0.439±0.001a | 0.956 |
| (immobilised) | 27.990±1.902a | 0.297±0.023a | 0.703 |
| high tide PRE  | (free) | 18.430±1.499a | 0.154±0.163a | 0.404 |
| (immobilised) | 18.430±1.499a | 0.110±0.183a | 0.219 |
| Nitrite uptake |  |  |  |  |
| low tide PRE  | (free) | 0.012±0.004a | -0.126 ±0.106a | 0.709 |
|  | (immobilised) | 0.012±0.004a | -0.133±0.098a | 0.418 |
| high tide PRE  | (free) | 0.014±0.004a | - | - |
|  | (immobilised) | 0.014±0.004a | -0.178±0.067 | 0.869 |
| Nitrate uptake |  |  |  |  |
| low tide PRE  | (free) | 0.007±0.004a | - | - |
|  | (immobilised) | 0.007±0.004a | 0.134±0.167 | 0.883 |
| high tide PRE  | (free) | 0.008±0.001a | -0.053±0.167a | 0.335 |
|  | (immobilised) | 0.008±0.001a | -0.340±0.056a | 0.685 |

-*K*: the maximum biomass achieved for *S. bijugatus*(µg/mL); initial phosphate/ ammonium/ nitrite / nitrate concentration in the water sample (mg/ L); *r*: the *S. bijugatus* growth rate (µg/mL/d); phosphate/ ammonium / nitrite / nitrate uptake rate (mg / L /d); R2 is the graph coefficient; - : data cannot be determined; PRE: Pinang River estuary; The values presented are means of three replicates; Means were compared using the Mann-Whitney U test; differences were not significant for groups with the same letter

Table 3: Phosphate, ammonium, nitrite and nitrate removal percentages under the four experimental treatments.

|  |  |
| --- | --- |
| Stations | Removal percentage |
| Free cells | Control(without algae) | Immobilised algal cells | Immobilisedblank beads (without algae) |
| Phosphate |  |  |  |  |
| low tide PRE  | 87.75a | 56.37 | 62.80a | 81.87 |
| high tide PRE  | 64.27a | 62.43 | 82.87a | 81.08 |
| Ammonium |  |  |  |  |
| low tide PRE  | 91.09a | 47.86 | 94.42a | 96.38 |
| high tide PRE  | 88.18a | 44.69 | 81.45a | 89.45 |
| Nitrite |  |  |  |  |
| low tide PRE  | 20.55a | - | 52.51a | - |
| high tide PRE  | 40.31a | - | 42.63a | 1.167 |
| Nitrate |  |  |  |  |
| low tide PRE  | - | 66.65 | 80.74 | - |
| high tide PRE  | 45.59a | 53.07 | 87.75a | 6.134 |

- : data cannot be determined due to fluctuation of the nutrient concentration; PRE= Pinang River estuary; The values presented are means of three replicates; Means were compared using the Mann-Whitney U test; differences were not significant for groups with the same letter

Table 4: Nitrogen and phosphorus removal by different chlorophyte taxa.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Types of media | Genus and species | Ammonia-N/Nitrate-N/ Total Nitrogen | O-phosphate/ Total Phosphate | References |
| Initial conc. (mg L-1) | Removal efficiency  | Initial conc. (mg L-1) | Removal efficiency (%) |
| Artificial medium | *C.kessleri* | f | 168 | 8-19 | 10-12 | 8-20 | Lee & Lee, 2001 |
| i | - | - | - | - |
| Industrial wastewater | *C.pyrenoidosa* | f | 267 | 87-89 | 56 | 70 | Hongyang et al. 2011 |
| i | - | - | - | - |
| Municipal wastewater | *C. sorokiniana* | f | - | - | 22-45 | 45-72 | Hernandez et al. 2006 |
| i | - | - | 22-45 | 71-93 |
| Artificial medium | *C.vulgaris* | f | 13-410 | 23-100 | 5-8 | 46-94 | Aslan & Kapdan, 2006 |
| i | - | - | - | - |
| Agroindustrial wastewater | *C.vulgaris* | f | 3-36 | 30-95 | 112 | 20-55 | Gonzalez *et al.* 1997 |
| i |  |  |  |  |
| Municipal wastewater | *C.vulgaris* | f | 48-1550 | 55-88 | 4-42 | 12-100 | Khan & Yoshida, 2008; Ruiz-Marin et al. 2010 |
| i | - | - | - | - |
| Artificial medium | *C.reinhardtii* | f | 129 | 42-83 | 120 | 13-14 | Kong et al. 2010 |
| i | - | - | - | - |
| KNO3 medium | *S. obliquus* | f | 50 | 90 % | - | - | Urrutia *et al.* 1995 |
| i | 50 | 90 % | - | - |
| Agroindustrial wastewater | *S. dimorphus* | f | 36.3 | 95 % | 111.8 | 20-50 % | Gonzalez *et al.* 1997 |
| i | - | - | - | - |
| Bold Basal Medium | *S. intermedius* | f |  | 0.022 mg/h |  | 0.014 mg/h | Jimenez-Perez et al. 2004 |
| i |  | 0.009 mg/h |  | 0.012 mg/h |

f = freely suspended cells;

i = immobilised cells

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Figure 1: Sampling site in the Pinang River estuary, Penang Island, Malaysia.

Figure 2: (A) Growth curves of *S. bijugatus* free cells () and immobilised cells () incubated in low tide PRE water; (B) Growth curves of *S. bijugatus* free cells () and immobilised cells () incubated in high tide PRE water. The bars show standard error; PRE: Pinang River estuary

Figure 3: (A) Phosphate concentration during ten days in a *S. bijugatus* free cell culture () and an immobilised cell culture () incubated in low tide PRE water. (B) Phosphate concentration during the ten-day duration with *S. bijugatus* free cell cultures () and immobilised cell cultures () incubated in high tide PRE water. The bars show standard error. PRE: Pinang River estuary

Figure 4: Ammonium (), nitrite (), and nitrate () concentration during the ten-day incubation of *S. bijugatus* (A) free cells and (B) immobilised cells in low tide PRE water. Ammonium (), nitrite (), and nitrate () concentration during the ten-day incubation of *S. bijugatus* (C)free cells and (D) immobilised cells in high tide PRE water. The bars show the standard error; PRE: Pinang River estuary