Evaluation of *Ulva reticulata* for the bioremediation of nutrients from Kelung River, Penang, Malaysia

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Abstract

Urban, agriculture, aquaculture and industrial wastewater are rich in nitrogen and phosphorus, and it is necessary to reduce the concentrations of these nutrients in the effluent before wastewater can be discharged into the environment. This study examined the use of *Ulva reticulata* to remove nutrients from water that collected from Kelung River in the Bayan Lepas Free Industrial Zone, Penang, Malaysia. *Ulva reticulata* was exposed to water, and its nutrient uptake was measured at different times. The maximum ammonium and phosphate uptake rates occurred during the first hour of exposure ($V_{300\mu M}^{0-1\,h}$ = 32 and $V_{50\,\mu M}^{0-1\,h}$ = 4 μmol g^{-1} fw h^{-1}, respectively). *U. reticulata* had removed 92.4% of the ammonium and 90% of the phosphate from water by 24 and 48 h, respectively. The growth rate of *U. reticulata* ranged from 3.24 to 4.0% day-1. Our results revealed that *U. reticulata* is an effective biofilter for ammonium and phosphate and will grow quickly in water that contaminated with nitrogen and phosphorus.

Keywords: Nutrient removal, *Ulva reticulata,* Bioremediation, Uptake rate

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Introduction

Among the methods available for treating wastewater effluents, plants are a potential candidate for nutrient removal (Ansari *et al*., 2014; Fan *et al*., 2019; Kambey *et al*., 2020; Nguyen *et al*., 2022). Aquatic plants have been used in constructed wetlands for nutrient removal from domestic sewage and in agriculture wastewater treatment (Zhang *et al*., 2014; Jabłońska *et al*., 2021).The application of artificial wetlands has also been extended to treat other types of wastewater, such as industrial wastewater (Chen *et al*., 2006; Maine *et al*., 2006). Macroalgae are widely used in integrated aquaculture systems (Cohen and Neori, 1991; Chung *et al*., 2002; Neori *et al*., 2004; Marinho-Soriano *et al*., 2009; Al-Hafedh *et al*., 2012; Azman *et al*., 2014; Biswas *et al*., 2020; Kang *et al*., 2021). The excess nutrients from the uneaten food and feces of fish and shrimp are taken up by seaweed. The use of integrated systems balances nutrients and also produces aquatic animals and seaweed (Wu *et al*., 2015). Thus, it is an efficient and economically beneficial method that contributes to sustainable aquaculture (Mao *et al*., 2009). Among the different types of seaweed tested, the macroalgae of the *Ulva* genera have shown high nutrient-removal capabilities. *Ulva* have flat sheet morphotypes, which are associated with high nitrogen contents and growth rates. Such characteristics make these macroalgae excellent candidates for remediation (Cohen and Neori, 1991; Sode *et al*., 2013; Zhang *et al*., 2023).

Industrial activity, urbanization and agriculture have led to the increased production of wastewater and the release of different pollutants into aquatic systems (Abdolali *et al*., 2014; Azman *et al*., 2014; Garg *et al*., 2022). The effluent from wastewater includes nutrients that have been identified as the main causes of eutrophication in natural waters (Rasoul-Amini *et al*., 2014). Penang, in the northwest peninsula of Malaysia, has faced rapid development in both urban and industrial areas over the past 30 years. The area is industrialized and urbanized, and it is a well-known shipping route and a center of aquaculture and tourism (Sakari *et al*., 2008). Because these activities can cause negative environmental impacts, sustainable industrial development has been strongly emphasized (Wurts, 2000; Neori *et al*., 2004). The Free Industrial Zone (FIZ) in Bayan Lepas was established in southeastern Penang Island in 1972. The FIZ houses manufacturers of electronics, fabricated metal products, machinery and precision tools. Wastewater produced by the electronics industry often consists of high organic nitrogen (Chen *et al*., 2003a, b). Ammonium is the inorganic form of nitrogen found in industrial wastewater (Maine *et al*., 2006; Huang *et al*., 2018), and it can also arise from the decomposition of organic nitrogen (Zheng and Li, 1998). High nitrogen and phosphorus contents have been reported In metallurgic wastewater (Maine *et al*., 2006; Ji *et al*., 2022). The results obtained from a water assessment of the Bayan Lepas FIZ showed that among the nutrients analyzed, the ammonium and

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phosphate concentrations were very high in the factory area. Therefore, the concentrations of ammonium and phosphate in the effluent should be reduced before this wastewater can be discharged off the coast of Penang.

There is a lack of information regarding the use of seaweed for the removal of nutrients from wastewater in Malaysia. As *U. reticulata* is a local seaweed in Penang, it was assessed for its ability to act as a bioremediation agent for nutrient removal. The bioremediation potential of *U. reticulata* was investigated by determining the uptake rates of NH_4^+, NO_3^- and PO_4^{3-} from contaminated water and by studying the specific growth rate of *U. reticulata* in wastewater.

Materials and methods

Ulva reticulata cultivation

U. reticulata was collected at the shore of Penang Island (5º 21' N, 100º 20' E) during low tide. The collected samples were then placed in a cool box and transported to the Centre for Marine and Coastal Studies (CEMACS), Universiti Sains Malaysia. To obtain uniform and readily available samples, *U. reticulata* was cultured at the CEMACS, which is located far from sites of industrial activity. Upon arrival at the CEMACS, the samples were washed with seawater until the seaweed was free from sand and epiphytic organisms. The washed samples were cultured in seawater for several months in 5 m (L)×2 m (W)×1 m (H) fiberglass tanks that were covered with one layer of black netting. After the cultivation period, the washed *Ulva* were then transported to a laboratory for further experimentation. In the laboratory, several 1 m (L)×0.60 m (W)×0.45 m (H) aquaria were filled with 30% artificial seawater. The seawater in the laboratory was prepared by dissolving tap water with nutrient-free artificial salt (France). Prior to the nutrient uptake experiments, different light intensities (4, 10, 20 µmol photons m^{-2} s^{-1}) were selected on the basis of preliminary experiments. Maintains of *U. reticulata* under a light intensity more than 4 µmol photons m^{-2} s^{-1} was caused to color changes of *U. reticulata*. Then, *U. reticulata* were maintained in aerated aquaria under a light intensity of 4 µmol photons m^{-2} s^{-1} and a 12-h light/dark photoperiod and acclimatized for five days. These *Ulva* cultures were used for subsequent experiments.

Wastewater sampling

Effluents from the Bayan Lepas FIZ are discharged into several drainage canals and the Keluang River, where they are finally released into the open sea across from Jerjak Island (Fig. 1); the Keluang River is the largest of the canals in the Bayan Lepas FIZ. To determine the uptake rates of the nutrients NH_4^+, NO_3^- and PO_4^{3-} by *U. reticulata*, water was collected at the end of the Keluang River (in the factory region) during low tide. The water samples were transferred into a 10-L plastic jerry can at -4°C before transport to the laboratory. During sample collection, physicochemical parameters such as temperature, pH, dissolved oxygen and salinity were measured *in situ* using a multi-parameter probe, model YSI Pro Plus.

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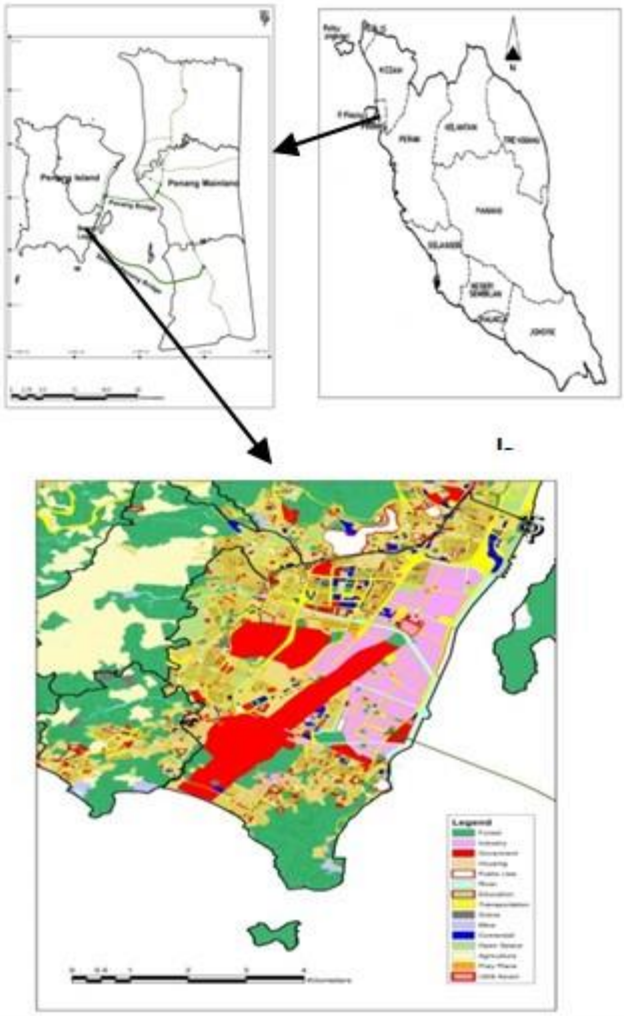

Figure 1: Map of the Peninsular Malaysia (a), Penang state (b), and Bayan Lepas FIZ (c).

Nutrient removal

The water was filtered through glass wool to remove dye and dregs. Preliminary experiments showed that the salinity ranged from 0 to 30% in industrial wastewater from the shore of Penang Island. Additionally, more than 90% of the water ammonium and phosphate was removed by *U. reticulata* after 48 and 72 h, respectively; however, nitrate was not removed during the experiment. Hence, the ammonium and phosphate removal and uptake rate experiments were performed at different salinities (0, 10, 20 and 30%) after 48 and 72 h, respectively. Artificial salt was added to wastewater in 500-mL conical flasks. Two grams of fresh *U. reticulata* was added to the experimental flasks. The ammonium and nitrate uptake rates were measured after 1, 3, 6, 12, 24 and 48 h, and the phosphate uptake rate was determined after 1, 12, 24, 48 and 72 h. The *U. reticulata* in the experimental flasks were exposed to a light intensity of 40 µmol photon m^{-2} s^{-1} under a 12-h light/dark photoperiod. Nutrient concentrations were measured using a spectrophotometer (Hach DR/ 2800) for fresh water, wastewater (Adams, 1991; APHA, 2005) and seawater (Grasshoff *et al*., 2009) samples. The uptake rate

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and the removal of ammonium, nitrate and phosphate were calculated as follows:

$$Uptake = \frac{(C_i - C_t) \times v}{m \times t}$$

Where, uptake is (µmol g^{-1} fw h^{-1}), C_i and C_t are the concentrations (µmol L^{-1}) at the beginning and end of the uptake, respectively, v is the volume (L), m is the fresh weight of *Ulva* (g), and t is the time interval.

The removal of nutrients was calculated using the following formula:

$$Removal\ of\ nutrient\ (\%) = \frac{(C_0 - C_e)}{C_0} \times 100$$

Where, C_0 is the initial nutrient concentration, and C_e is the residual nutrient concentration.

Results

The *U. reticulata* ammonium uptake rates in wastewater at different salinities (0, 10, 20, and 30%) over 48 hours are shown in Figure 2. The ammonium uptake rate increased rapidly during the first hour and then more gradually until 48 hours for all of the salinities tested.

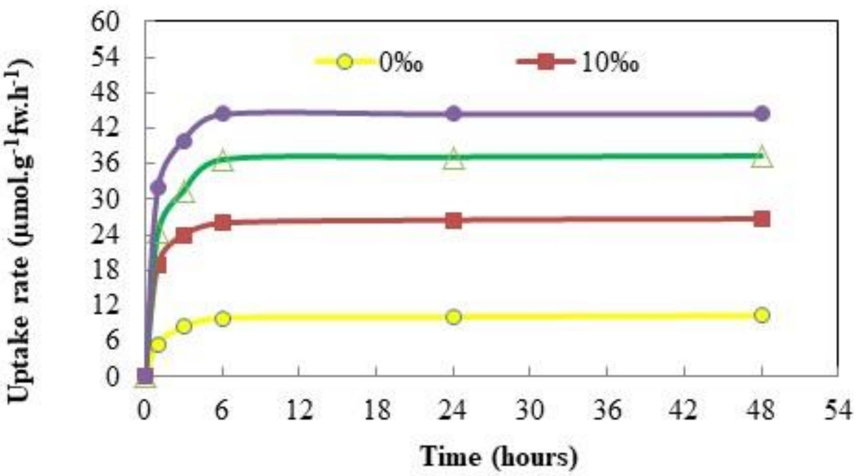

Figure 2: ***Ulva reticulata*** **ammonium uptake rates in wastewater with different salinities (0, 10, 20, and 30%) measured at 1, 3, 6, 12, 24 and 48 h (n=5).**

Figure 3 presents the removal of ammonium (%) from wastewater by *U. reticulata* at different salinities (0, 10, 20, or 30%) over 48 hours. Ammonium removal increased from 1 to 48 hours but was much higher in the first 6 hours. The ammonium removal rates at different salinities showed similar fluctuations, but the highest value was attained at a salinity of 30%. The ammonium uptake increased as salinity increased. Evaluation of the ammonium removal by *U. reticulata* from wastewater at different salinities revealed that the maximum removal occurred after 1 hour of exposure. The rate of ammonium removal (%) was significantly different between 1 and 24 h (one-way ANOVA, $p<0.05$), and approximately 90% of the ammonium was removed by 24 h at all salinities tested. After 24 h, the removal percentages were not significantly different ($p>0.05$).

Our results showed that there was no nitrate uptake by *U. reticulata* from the medium containing 300 µM NH^{4+}, 50 µM PO_4^{3-} and 15 µM NO_3^- over the course of the experiment (48 h).

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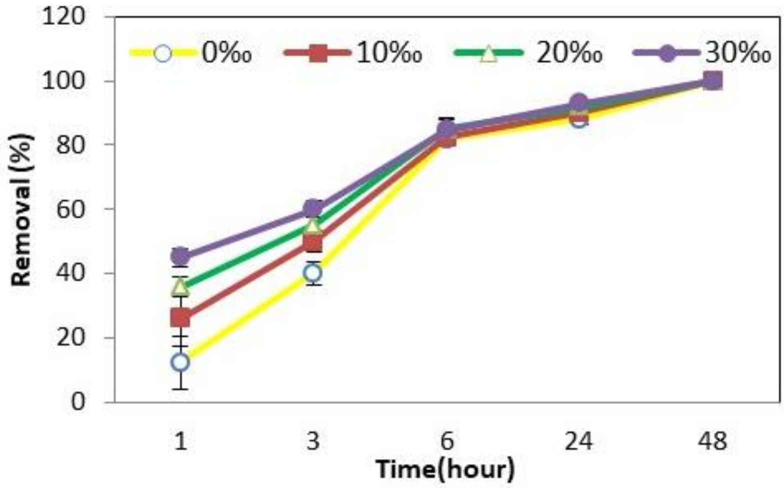

Figure 3: Ammonium removal (%) from wastewater by *Ulva reticulata* at different salinities (0, 10, 20, and 30%) over 48 hours (n=5).

Figure 3 shows *the U. reticulata* phosphate uptake rate from wastewater at different salinities (0, 10, 20, and 30%) over 72 hours. The phosphate uptake rate decreased drastically from 1 to 24 h and then further decreased until 72 h. The phosphate uptake rate trends were nearly identical under different salinities. The highest phosphate uptake rate for each salinity (30, 20, 10 and 0%) was observed after 1 hour with values of 4.05±0.01, 3.1±0.02, 2.81± 0.02 and 2.54±0.03 µmol g^{-1} fw h^{-1}, respectively. The uptake rate increased with increasing salinity from 0 to 30%. The phosphate uptake rate at 30% salinity with initial wastewater nutrient concentrations of 300 µM NH_4^+, 50 µM PO_4^{3-} and 15 µM NO_3^- was 4.05±0.01 µmol g^{-1} fw h^{-1}. The percentage of phosphate removal by *U. reticulata* from wastewater at different salinities (0, 10, 20, or 30%) over 72 h is shown in Figure 4. The percentage of phosphate removal increased gradually from approximately 20% in the first hours to 90% after 48 h, and then the removal slowed from 48 to 72 h. In this study, wastewater with different salinities and an initial phosphate concentration of 50 µM exhibited an approximately 90% reduction in the level of phosphate after 48 h, and the phosphate removal percentages at all salinities tested were similar. Phosphate removal was significantly different (one-way ANOVA, $p<0.05$) at different salinities after 1 hour; however, this difference was not significant ($p>0.05$) at different salinities after 48 h.

Discussion

The water was collected at 26ºC and had 0% salinity, 2 mg L^{-1} dissolved oxygen and a pH of 7.10. The ammonium, nitrate and phosphate concentrations were 300, 15 and 50 µM, respectively. The ammonium concentration was high, whereas the dissolved oxygen level was very low. Sode *et al*. (2013) stated that ammonium is the dominant form of nitrogen in urban, aquacultural and agricultural wastewaters.

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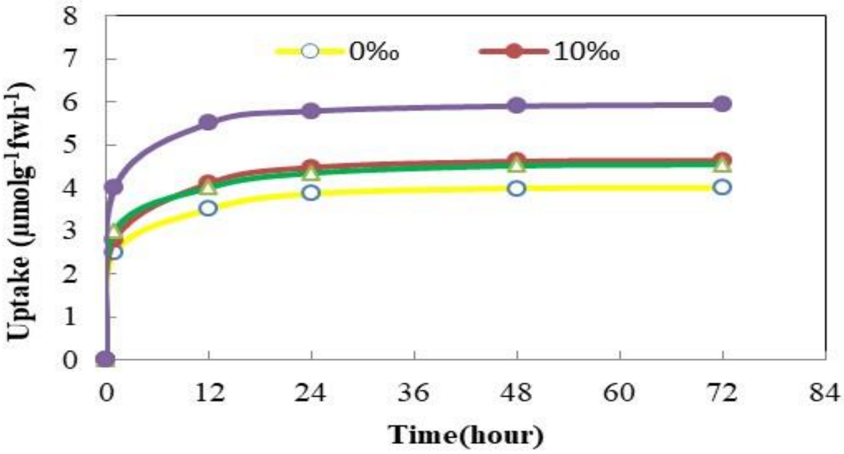

Figure 4: ***Ulva reticulata*** **phosphate uptake rates from wastewater at different salinities (0, 10, 20, and 30%) measured at 1, 12, 24, 48 and 72 h (n=5).**

Most macroalgae can take up nutrients over their surface area. *Ulva* has a foliated morphology and is able to take up nutrients to support its growth (Sode *et al*., 2013). Therefore, the high ammonium and phosphate concentrations of the Bayan Lepas FIZ wastewater were suitable for the growth of *U. reticulate.* Our results showed that the maximum ammonium uptake occurred in the first hour (31.97±1.84, 24.42±2.09, 19.01±2.16 and 5.47±1.44 µmol g^{-1} fw h^{-1} in 30, 20, 10 and 0% salinities, respectively). Similar results were observed by Luo *et al*. (2012), who reported that the maximum nutrient uptake rate occurred during the first hour of exposure. The ammonium uptake rates of *Ulva linza* and *Ulva prolifera* decreased over time, and their maximum uptake rates occurred within the first 30 minutes of exposure (Luo *et al*., 2012). The results obtained by comparing the ammonium uptake rates indicated that there was a significant difference (p<0.05) before 24 hours; however, no significant difference was observed after 24 hours (one-way ANOVA). Moreover, the results showed that the uptake was significantly different between different salinities (one-way ANOVA, p<0.05). Nutrient uptake rates can be affected by various factors and should therefore be compared with caution. Our results showed that the ammonium uptake rate of *U. reticulata* in industrial wastewater containing initial concentrations of 300 µM NH_4^+, 50 µM PO_4^{3-} and 15 µM NO_3^- was 31.97 µmol g^{-1} fw h^{-1} (5.33 µmol g^{-1} dw min^{-1}). Ammonium uptake rates at an initial concentration of 150 µM NH_4^+ were reported for *Gracilaria textorii* Harriot (0.28 µmoles g^{-1} dw min^{-1}), *Ulva pertusa* Kjellman (1.91 µmoles g^{-1} dw min^{-1}) and *Porphyra tenera* Kjellman (3.44 µmoles g^{-1} dw min^{-1}) (Chung *et al*., 2002). In addition, the ammonium uptake rates at an initial concentration of 2 µM NH_4^+ were reported in *Xiphophora 83eucosti* (0.3 µmoles g^{-1} dw h^{-1}) and *Stichosiphonia arbuscula* (2.4 µmoles g^{-1} dw h^{-1}) (Phillips and Hurd, 2004). Consequently, a comparison of the ammonium uptake rates of other macroalgae with our results indicates that *U. reticulata* is a good candidate for

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ammonium uptake in industrial wastewater.

In this study, *Ulva* was maintained at a salinity of 30%; when the salinity was changed from 30% to 20, 10 or 0 %, the ammonium removal efficiency decreased. The relatively low *Ulva* uptake rates at salinities less than 30% may be due to the stress endured by *Ulva* upon salinity change, and the ammonium uptake rate and removal percentage after 1 hour were significantly different among salinities ($p<0.05$). However, after 24 hours, these differences disappeared ($p>0.05$). When exposed to changes in salinity, most intertidal algae use a complex set of physiological and biochemical mechanisms to acclimate to fluctuating salinities in their habitats (Lobban, 1994). One typical tolerance mechanism in marine algae is the maintenance of constant cell turgor pressure with the use of changing osmotic potentials (Kirst, 1990). Therefore, it appears that *U. reticulata* uses a tolerance mechanism, given that the ammonium removal was nearly identical at all salinities after 24 hours.

The biofiltration capacity of NH_4^+ by *U. reticulata* in this study was 60, 84.6, 92.8 and 100% within 3, 6, 24 and 48 h, respectively. Cohen and Neori (1991) stated that various studies have reported nitrogen removal efficiencies in the range of 10 to 90% in polyculture systems. Ammonium removal by *Gracilaria caudata* was 59.5% within 4 h (Marinho-Soriano *et al*., 2009), while *Gracilaria lemaneiformis* removed 60% within 8 days (Zhou *et al*., 2006). *Ulva 84eucost* removed 45% and *Gracilaria edulis* removed 70% after 14 days (Azman *et al*., 2014), and *Ulva 84eucost* removed 40-90% depending on the NH_4^+ supply (Cohen and Neori, 1991).

The nitrate concentration in the experimental flasks also increased within 48 h. The ammonium concentration was 20-fold higher than the nitrate concentration. Nitrogen and phosphorus uptake depends on the media composition and environmental parameters, such as the initial nutrient concentrations, the ratio of nitrogen to phosphorus and light intensity (Aslan and Kapdan, 2006). Ammonium uptake rates with an initial NH_4^+ concentration of 150 µM were reported in *Gracilaria verrucosa* Papenfuss and *Hypnea charoides* Lamouroux (0.48 µmoles g^{-1} dw min^{-1}) and *Porphyra serriata* Kjellman (2.89 µmoles g^{-1} dw min^{-1}) (Chung *et al*., 2002), whereas ammonium uptake rates with an initial NH_4^+ concentration of 2 µM were recorded in *Apophlaea lyallii* (0.6 µmoles g^{-1} dw h^{-1}) and *Stichosiphonia arbuscula* (2.4 µmoles g^{-1} dw h^{-1}) (Phillips and Hurd, 2004). In addition, Harrison and Hurd (2001) stated that when algae take up nitrate, it can be stored in the cytoplasm or a vacuole or it can be enzymatically reduced to nitrite (and subsequently to ammonium). Similarly, urea can be stored in the cytoplasm or a vacuole or consumed via the enzyme urease, thus reducing it to ammonium. Ammonium is assimilated or formed from nitrate or urea and is subsequently converted to an amino acid. Therefore, higher ammonium

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uptake may be related to the lower energy required to assimilate NH_4^+, as NO_3^- needs to be reduced to NH_4^+ by the enzyme nitrate reductase before assimilation can occur (Abreu *et al*., 2011; Cai *et al*., 2013).

The maximum and minimum rates of phosphate uptake by *Ulva pertusa* with an initial concentration of 8 µM PO_4^{3-} were reported to be 0.157 µmol g^{-1} dw h^{-1} and 0.04 µmol g^{-1} dw h^{-1} at salinities of 25 and 15%, respectively (Choi *et al*., 2010). The highest phosphate uptake rates with an initial PO_4^{3-} concentration of 30 µM were reported in *Porphyra 85eucosticte* (16 µmol g^{-1} dw h^{-1}) and *Porphyra rosengurttii* (6 µmol g^{-1} dw h^{-1}). The phosphate uptake rates of *Porphyra 85eucosticte*, *Porphyra rosengurttii*, *Porphyra suborbiculata* and *Porphyra purpurea* in a medium containing 30 µM NO_3^- and 3 µM PO_4^{3-} were higher than in a medium with 3 µM NO_3^- and 0.5 µM PO_4^{3-}; therefore, an increased initial phosphate concentration increased the uptake rate (Pedersen *et al*., 2004). Comparison of the *U. reticulata* phosphate uptake rate in this study with those of other macroalgae reported elsewhere should be undertaken with caution. The phosphate uptake rate depends on the initial nutrient concentrations in the medium, the duration of exposure, and the previous nutritional history of the algae; directly comparing these values can be misleading (Chopin *et al*., 1990). In addition, if researchers using fresh or dry seaweed do not report the ratio between fresh and dry macroalgae, a comparison of their results is not feasible. However, the rate of phosphate uptake by *U. reticulata* was close to that of *U. pertusa,* as reported by Choi and Lee (2012).

A comparison between the phosphate and ammonium uptake rates showed similar trends and removal efficiencies at different salinities. Nevertheless, the ammonium uptake rate was higher than the phosphate uptake rate. The average N:P ratio for seaweeds is 30N:1P, ranging from 10:1 to 80:1 (Atkinson and Smith, 1983). These ratios indicate that seaweed requires nitrogen more than phosphorus, which explains the lower phosphate uptake rate compared to ammonium uptake. A comparison of ammonium removal with phosphate removal (Figs. 3 and 5) indicates that approximately 80% of the phosphate was removed by *U. reticulata* after 48 h. The rate of phosphate uptake was less than that of ammonium, and *U. reticulata* also required a longer exposure time to remove phosphate. The PO_4^{3-} biofiltration capacity of *U. reticulata* was 40, 70, 94 and 100% after 12, 24, 48 and 72 h, respectively (Fig. 5). A study on the PO_4^{3-} biofiltration capacity of *Gracilaria caudate* reported 12.3% phosphate removal from shrimp-farm wastewater (Marinho-Soriano *et al*., 2009), whereas *Gracilaria chilensis* removed 27% of the phosphate from salmon-farm effluents (Troell *et al*., 1997).

Our results revealed that *U. reticulata* removed ammonium (92.4%) and phosphate (90%) from wastewater within 24 and 48 h, respectively. Therefore, *U. reticulata* can be considered an effective biofilter for ammonium and phosphate in industrial wastewater.

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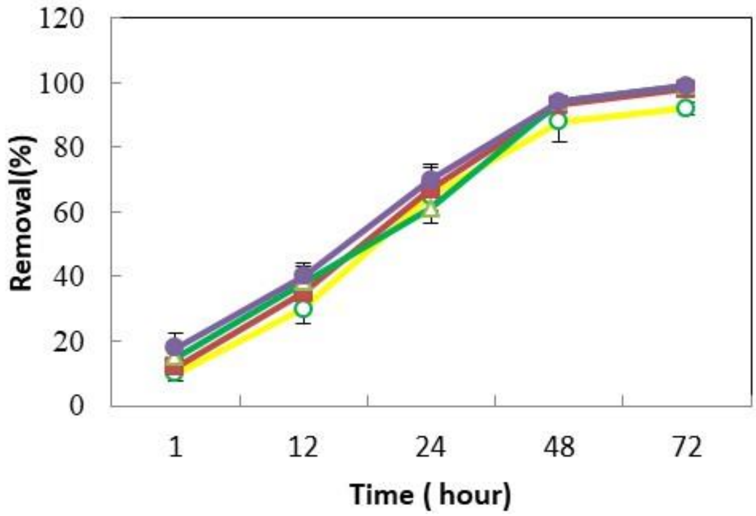

Figure 5: Phosphate removal (%) from wastewater by *Ulva reticulata* at different salinities (0, 10, 20, 30%) over 48 hours (n=5).

In summary, Kelung River in the Bayan Lepas FIZ exhibited high concentrations of ammonium and phosphate, which were suitable as nutrient resources for the growth of *U. reticulata*. This organism showed various nutrient uptake rates and preferred ammonium to nitrate; this organism also exhibited a broad salinity tolerance. These results reveal that *U. reticulata* has the potential to reduce ammonium and phosphate loading within 48 and 72 h, respectively, at different salinities (0, 10, 20, and 30%). These highly efficient uptake rates at different salinities, demonstrate that *U. reticulata* can remove high levels of ammonium and phosphate in seawater as well as contaminated water.

Acknowledgments

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