

Immobilized periphytic cyanobacteria for removal of nitrogenous compounds and phosphorus from shrimp farm wastewater

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Abstract: Cyanobacteria can be used to remove nitrogenous compounds from wastewater, but a major bottleneck in the process is the separation of cyanobacterial biomass from the treated water discharge, which may cause eutrophication. The current study assessed the suitability of three periphytic cyanobacteria (*Geitlerinema* sp., *Gloeotrichia* sp., and *Lyngbya* sp.) isolated from shrimp ponds. These cyanobacteria were immobilized by self-adhesion to polyvinyl chloride sheets, forming mats, and were screened for their efficacy to reduce nitrogenous compounds and phosphorus. Among the three isolates, *Geitlerinema* sp. showed the highest reduction rate (98%) and was further tested using shrimp pond wastewater. The results showed that initial wastewater concentrations (mg L^{-1}) of total ammonia nitrogen (5.0), nitrite nitrogen (2.9), and soluble reactive phosphorus (2.5) were significantly ($P < 0.05$) reduced within 7 days in tanks containing wastewater + *Geitlerinema* sp. mats (0.1, 0.2, and 0.8) compared to wastewater + substrate only (4.3, 2.7, and 2.3) or wastewater only (4.4, 2.8, and 2.4). In addition, *Geitlerinema* sp. was found to be nontoxic to *Artemia*. Fast and effective sequestration of nutrients from wastewater by the *Geitlerinema* mats, as well as ease of harvesting from the mats, make this treatment an attractive alternative for wastewater treatment.

Key words: Cyanobacteria, wastewater, ammonia, nitrite, phosphorus, shrimp

1. Introduction

Aquaculture has grown rapidly over the last couple of decades due to significant increases in the demand for fish and seafood throughout the world. This intense development has resulted in the generation of large quantities of wastewater, which includes dissolved inorganic nitrogen and phosphorus (Kamilya et al., 2006). Nitrogenous compounds and phosphorus are major contaminants in aquaculture wastewater and their release into surrounding water bodies can cause eutrophication (Kamilya et al., 2006). It is imperative that this wastewater be treated by employing environmentally friendly and cost-effective methods.

A number of techniques have been developed to control nitrogenous substances in aquaculture systems (Yusoff et al., 2011; Devaraja et al., 2013). Some of these processes tend to be either highly sophisticated or extremely complicated and expensive (Hoffmann, 1998). Several authors have demonstrated the use of cyanobacteria in treating

wastewater from dairy (Boominathan and Manoharan, 2008), dye (Vijayakumar et al., 2005), and sewage (Manoharan and Subramanian, 1992), indicating that it may be a feasible option. This may be due to the fact that cyanobacteria have simple growth requirements and do not need energy-rich compounds like other nonphotosynthetic microorganisms. In addition, many cyanobacteria combine photosynthesis and nitrogen fixation, which is an advantage over other eukaryotic photosynthetic organisms (Vijayakumar, 2012).

Most wastewater treatments describe microalgae growing in suspension, and there are reports of planktonic microalgae being used for removal of nitrogenous compounds from wastewater (Shelknanloymilan et al., 2012; Sriram and Seenivasan, 2012). However, one of the major limitations of this technology is the difficulty encountered in separating suspended biomass from the treated water discharge. As such, there has been an increased interest in using immobilized cyanobacteria to

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treat wastewater since it is possible to avoid harvesting costs. Immobilized cells are also found to be more efficient in removing nitrogen and phosphorus compared to their free-living counterparts (Chevalier and de la Noüe, 1985). Furthermore, immobilization can retain high-value nutrient-rich biomass for further processing into biofuel or other products.

A common immobilization technique involves passively immobilizing organisms onto a synthetic or natural polymeric matrix (substrate), such as polyurethane foam (Garbisu et al., 1991), polyvinyl foam (Urrutia et al., 1995), or loofa sponge (Liu et al., 1998). This technique is based on the fact that many cyanobacteria have a natural tendency to attach to surfaces and grow on them (Khatoon et al., 2007b). Immobilization can be done by simply submerging a particular substrate into a microalgae suspension, with aeration, for a certain period of time (Travieso et al., 1996).

In aquaculture, most farmers usually discharge their wastewater directly into water bodies without proper treatment or a sufficient storage period for nutrient removal. Given the brief withholding time of aquaculture wastewater and the problems associated with the separation of biomass, the present work investigates the use of cyanobacterial mats inoculated with selected periphytic cyanobacteria to treat water in a short period of time. The use of the selected periphytic cyanobacteria that were screened for their efficacy to remove nitrogenous compounds and phosphorus prior to treatment of aquaculture wastewater has not been previously studied. Wastewater treatment using periphytic cyanobacterial mats is an environmentally friendly treatment alternative. In the present study, the use of the periphytic cyanobacterium *Geitlerinema* sp., immobilized by self-adhesion to polyvinyl chloride (PVC) sheets, was evaluated for the removal of nitrogenous compounds and phosphorus from shrimp pond wastewater. In addition, the toxicity of *Geitlerinema* sp. was also assessed.

2. Materials and methods

2.1. Isolation and identification of cyanobacteria

Cyanobacteria growing on inlet pipes and feeding trays were isolated from a shrimp pond in the coastal area of the state of Selangor, Malaysia. Samples were brought back to the Aquatic Animal Health Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia, and then centrifuged and washed with 1 mL of filtered water (0.2 µm, Millipore, UK) and autoclaved seawater. Next, 0.1 mL of the concentrate was spread evenly onto Conway agar plates (Tompkins et al., 1995) and kept in an incubator (Sanyo Versatile Environmental Test Chamber, Sanyo, Japan) at 28 ± 2 °C for 10 days. The Conway medium was chosen to culture the periphytic cyanobacteria on the basis of

Khatoon et al. (2007b). After 10 days, colonies were isolated and inoculated in a 50-mL flask containing Conway broth medium. The flask was stored in an incubator shaker for 14 days at 28 ± 2 °C. Successive subculturing was done and three pure isolates, which were easier to grow, were selected for further experiments. The three selected pure isolates were maintained in Conway medium at 28 °C under a 12/12 photoperiod, with light provided at an intensity of $31.9 \mu\text{E m}^{-2} \text{ s}^{-1}$. Identification of the three isolated species was done to genus level according to Bellinger (1992).

Molecular identification was done for cyanobacteria that had higher growth on PVC sheets and the greatest ability to reduce total ammonia nitrogen (TAN, $\text{NH}_3 + \text{NH}_4^+$), nitrite nitrogen ($\text{NO}_2\text{-N}$), and soluble reactive phosphorus (SRP, $\text{PO}_4\text{-P}$) concentrations. DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Germany). Extracted genomic DNA was used as a template for PCR amplification to identify cyanobacteria based on 16S rRNA detection. PCR was carried out according to the method of Nübel et al. (1997) with minor modifications. A total of 25 µL of PCR reaction mixture was prepared, containing 5 µL of 1X PCR buffer (Promega), 2 mM MgCl_2 (Promega), 0.2 mM dNTPs (Promega) each, 1.25 U of Taq DNA polymerase (Promega), 1.0 mM forward primer CYA106F (CGG ACG GGT GAG TAA CGC GTG A), 1.0 mM equimolar mixture of reverse primers CYA781R(a) and CYA781R(b) (GAC TAC TGG GGT ATC TAA TCC CAT T; GAC TAC AGG GGT ATC TAA TCC CTT T), 2 µL of genomic DNA as a template, and 10.25 µL of sterile Milli-Q water. The PCR reaction was carried out in a thermal cycler (Eppendorf, Germany), beginning with initial denaturation at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min, and termination by an extension step at 72 °C for 5 min. Amplified products were analyzed on a 1.5% agarose gel. PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and sequenced by First Base Laboratories (Malaysia). Partial sequences of the 16S rRNA gene were compared to the NCBI GenBank database using a basic local alignment search tool (BLAST).

2.2. Formation of cyanobacterial mats on PVC sheets

Based on a previous study by Khatoon et al. (2007a), widely available and low-cost PVC sheets were chosen as the substrate for periphytic cyanobacteria to form mats by self-adhesion. To generate mats, pure cultures of the three periphytic cyanobacterial isolates (100 mL L^{-1}) were inoculated in tanks (50 L) containing autoclaved seawater (28 ppt, pH 8.0), Conway medium, and 10 pieces of PVC sheet ($15 \times 11 \text{ cm}$). The substrate was submerged below the surface of the water. Tanks were kept in the open under shade and provided with continuous aeration using an air compressor and natural light at a 12/12 light/

dark photoperiod. Cyanobacterial biomass in terms of chlorophyll *a* on the PVC sheet was measured on alternate days. All experiments were performed in triplicate.

2.3. Selection of cyanobacterial isolates for maximum reduction of TAN, NO₂-N, and SRP

To test the efficacy of the three different isolates in reducing TAN, NO₂-N, and SRP, 10 cyanobacteria-coated PVC sheets of each isolate were placed in individual rectangular tanks containing 40 L of seawater. Known amounts of ammonium sulfate, sodium nitrite, and potassium dihydrogen phosphate were used to obtain final concentrations of TAN, NO₂-N, and SRP to 5 mg L⁻¹, 3 mg L⁻¹, and 3 mg L⁻¹, respectively. TAN, NO₂-N, and SRP were analyzed on alternate days as described by Parsons et al. (1984). All measurements were conducted in triplicate.

2.4. Shrimp pond wastewater collection

Wastewater was collected 1 day after shrimp were harvested from ponds located in Banting, Malaysia, and brought to the Aquatic Animal Health Unit at Universiti Putra Malaysia. Wastewater was analyzed for initial concentrations of TAN, NO₂-N, and SRP and then filtered using a filter bag (5 µm) prior to use.

2.5. Experimental design

In the current study, only one isolate was selected based on maximum ability to reduce TAN, NO₂-N, and SRP and mat formation. Two treatments were included in the study: 1) wastewater + periphytic cyanobacterial mat and 2) wastewater + substrate only (PVC sheet without cyanobacterial mat). A tank with wastewater only was also included as a control. Nine 40-L rectangular plastic tanks, each containing 30 L of filtered (5 µm) wastewater (28 ppt) were used in the experiment. Substrate was introduced into the tanks 2 weeks after the surface was fully coated with periphytic cyanobacteria. An air compressor was used to provide continuous aeration to each tank. The treatment and control were performed in triplicate, and the water was not exchanged or refreshed during the experimental period.

2.6. Physicochemical and biological analyses

Temperature, salinity, pH, and dissolved oxygen in the culture tanks were measured daily using YSI 551 MPS (YSI, USA). TAN, NO₂-N, and SRP were analyzed on alternate days as described by Parsons et al. (1984). Cyanobacterial biomass accumulation on substrate, in terms of chlorophyll *a*, was determined following the standard methods of APHA (1992).

2.7. Cytotoxicity bioassay

Cytotoxic bioassay was conducted as previously described by Lincoln et al. (1996). Briefly, *Geitlerinema* sp. was harvested by centrifugation for 15 min at 4000 × *g*. The aqueous supernatant was retained and used for cytotoxicity study with *Artemia* (Golden Dolphin, Malaysia). *Artemia*

were hatched and 20 nauplii were placed in glass tubes containing 5 mL of seawater and 100 µL of the aqueous supernatant. Surviving nauplii were counted after 24 h. Medium used for culturing *Geitlerinema* sp. was used as the control. The presence of toxic compounds was indicated by the ability of cell supernatants to kill *Artemia* nauplii 24 h after addition of the cyanobacterial supernatant.

2.8. Statistical analysis

Collected data were analyzed using one-way analysis of variance (ANOVA). Significant differences among the different substrates and treatments were determined using Duncan's multiple range test at the 0.05 level of probability. All the data that were expressed in percentages were arcsine-transformed to satisfy the condition of homogeneity of variance. All statistical analyses were done using Statistical Analysis System software.

3. Results

3.1. Identification of cyanobacteria

The isolate that grew the fastest on PVC sheets and had the greatest ability to reduce TAN, NO₂-N, and SRP concentrations was identified as *Geitlerinema* sp. Based on BLAST comparison, the sequence from the isolated marine periphytic cyanobacteria possessed a 99% sequence identity to *Geitlerinema* sp. (accession number: HQ197684). The second isolate was identified as *Gloeotrichia* sp. and the third as *Lyngbya* sp.

3.2. Formation of cyanobacterial mat by different cyanobacteria on PVC sheets

PVC sheets inoculated with *Geitlerinema* sp. exhibited a significantly greater ($P < 0.05$) accumulation of biomass, in terms of chlorophyll *a*, among the three cyanobacterial isolates (Figure 1). However, no significant difference ($P > 0.05$) in accumulation of biomass in terms of chlorophyll *a* was observed between *Gloeotrichia* sp. and *Lyngbya* sp. (Figure 1).

3.3. Selection of cyanobacterial isolate for maximum reduction of TAN, NO₂-N, and SRP

Among the three isolates, *Geitlerinema* sp. significantly reduced ($P < 0.05$) TAN by $98 \pm 0.05\%$, NO₂-N by

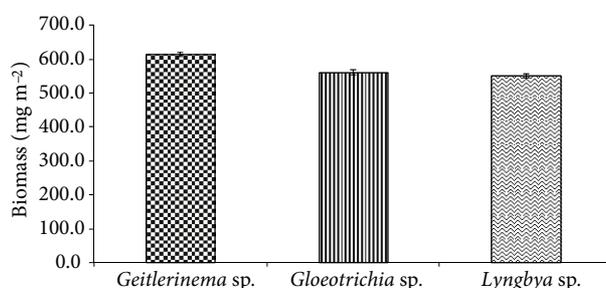


Figure 1. Biomass (chlorophyll *a*) of three different periphyton isolates grown on PVC substrate.

93 ± 0.04%, and SRP by 74 ± 0.03% as compared to *Gloeotrichia* sp. (93 ± 0.02%, 83 ± 0.05%, 56 ± 0.02%, respectively) and *Lyngbya* sp. (94 ± 0.01%, 80 ± 0.04%, 59 ± 0.01%, respectively) (Table 1). *Geitlerinema* sp. also had significantly higher ($P < 0.05$) removal rates of TAN, NO₂-N, and SRP compared to the other isolates (Figure 2).

3.4. Reduction of TAN, NO₂-N, and SRP of shrimp farm wastewater using *Geitlerinema* sp. grown on PVC sheets

Concentrations (mg L⁻¹) of TAN, NO₂-N, and SRP were significantly reduced ($P < 0.05$) from initial concentrations of 5 ± 0.02, 2.9 ± 0.02, and 2.5 ± 0.01 to 0.1 ± 0.02, 0.2 ± 0.09, and 0.8 ± 0.09, respectively, in tanks containing wastewater + *Geitlerinema* sp. mats compared to wastewater + substrate (4.3 ± 0.07, 2.7 ± 0.08, and 2.3 ± 0.09, respectively) or wastewater only (4.4 ± 0.09, 2.8 ± 0.08, and 2.4 ± 0.09, respectively) (Figure 3). No significant difference in water quality parameters, such as water temperature, dissolved oxygen, salinity, and pH, was observed among the different treatments (Table 2).

3.5. Cytotoxicity bioassay

No toxic effect on *Artemia* nauplii was detected in the bioassay with the aqueous supernatant of *Geitlerinema* sp. All the nauplii were found to be alive after 24 h.

4. Discussion

Intensive development in the aquaculture industry has led to considerable production of waste, including dissolved inorganic nitrogen and phosphorus. Nitrogenous compounds and phosphorus are considered major contaminants in aquaculture wastewater and, when released into surrounding water bodies, may cause eutrophication in rivers and coastal waters (Ziemann et al., 1992). For the treatment of wastewater, cyanobacteria have been a subject of research and development (de la Noüe and Proulx, 1988; de la Noüe et al., 1990). However, a major drawback of bioremediation using cyanobacteria is the requirement to separate or harvest the cyanobacterial biomass from the treated water before discharge. Therefore,

Table 1. Total reduction (%) of total ammonia nitrogen (TAN), nitrite nitrogen (NO₂-N), and soluble reactive phosphorous (SRP) in water by different periphytic microalgal isolates. Data are means ± standard errors. Means in a column with different superscripts are significantly different at $P < 0.05$.

Isolates	TAN	NO ₂ -N	SRP
<i>Geitlerinema</i> sp.	98 ± 0.05 ^a	93 ± 0.04 ^a	74 ± 0.03 ^a
<i>Gloeotrichia</i> sp.	93 ± 0.02 ^b	83 ± 0.05 ^b	56 ± 0.02 ^b
<i>Lyngbya</i> sp.	94 ± 0.01 ^b	80 ± 0.04 ^b	59 ± 0.01 ^b

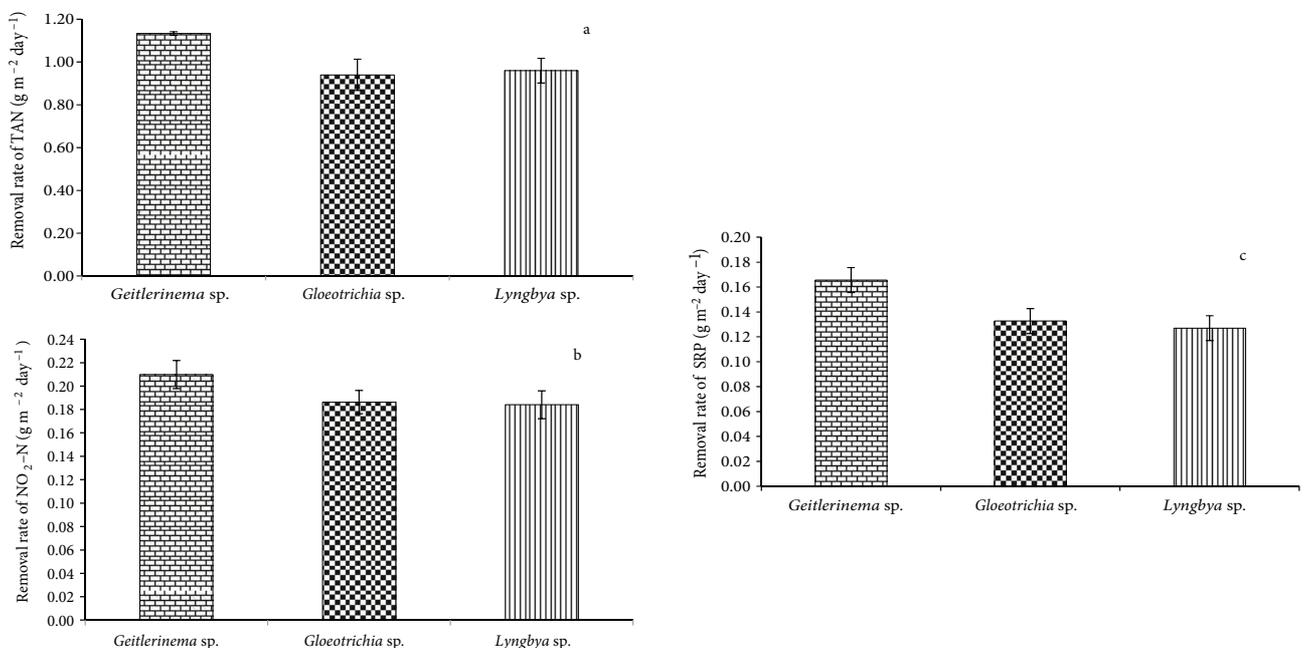


Figure 2. Removal rate (g m⁻² day⁻¹) of (a) TAN, (b) NO₂-N, and (c) SRP in water with different periphytic isolates.

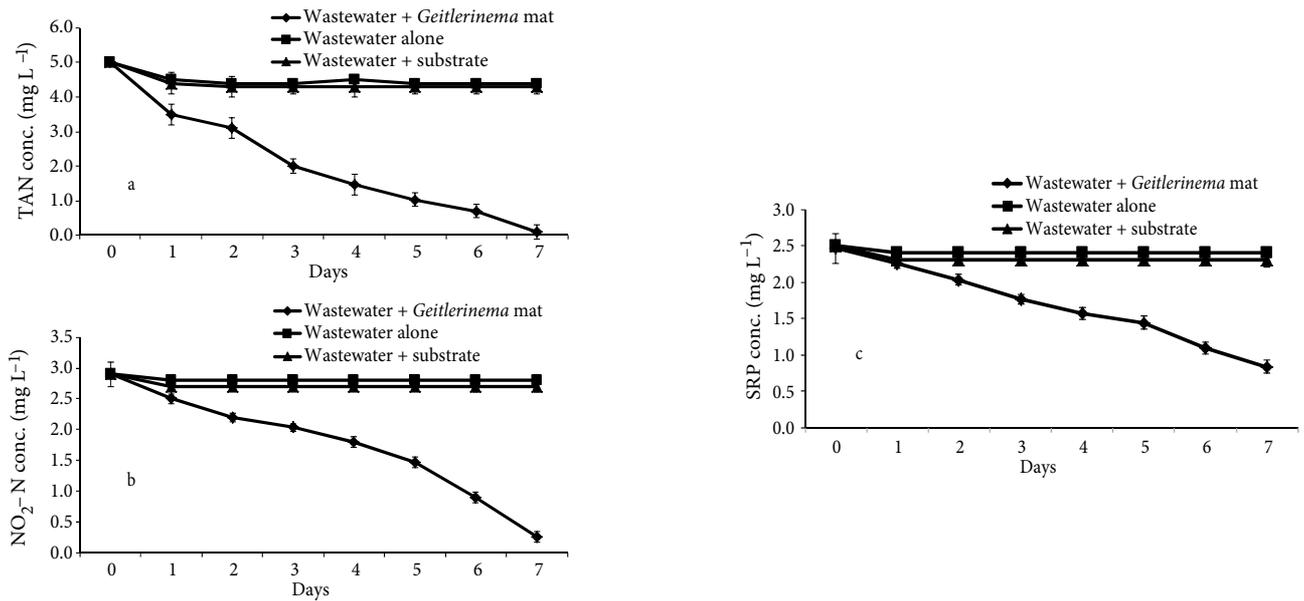


Figure 3. Concentration (mg L^{-1}) of (a) TAN, (b) $\text{NO}_2\text{-N}$, and (c) SRP among the three different treatments during experimental period.

Table 2. Temperature, dissolved oxygen, salinity, and pH range in control and treated tanks. Data are means \pm standard errors. Means in a column with different superscripts are significantly different at $P < 0.05$.

Treatments	Temperature ($^{\circ}\text{C}$)	Dissolved oxygen (mg L^{-1})	Salinity (ppt)	pH
Wastewater + <i>Geitlerinema</i> sp. mat	29.04 ± 0.71^a	6.59 ± 0.06^a	30.43 ± 0.34^a	7.54 ± 0.12^a
Wastewater alone	28.74 ± 0.50^a	6.63 ± 0.07^a	30.58 ± 0.35^a	7.62 ± 0.13^a
Wastewater + substrate	29.02 ± 0.54^a	6.60 ± 0.06^a	30.23 ± 0.13^a	7.75 ± 0.12^a

the present study investigated the use of periphytic *Geitlerinema* sp. immobilized on PVC sheets to form mats, since there has been no report on this for treatment of shrimp wastewater. Advantages of immobilizing the cells include faster removal rates of nutrients and cell-free effluent (Hoffman, 1998). Bender et al. (2004), in one of their studies, used a combination of microbial mats and fluidized sand filters for the removal of ammonia in a black sea bass (*Centropristis striata*) recycled-water mariculture system. They found that total ammonia concentrations were 0.30 and 0.55 mg L^{-1} with and without the sand filter, respectively. Cañizares et al. (1994) showed 92% removal of $\text{NH}_4\text{-N}$ in 7 days when K-carrageenan-immobilized *Spirulina maxima* was used to treat 25% diluted swine waste. In a study by Lezama-Cervantes and Paniagua-Michel (2010), the lowest ammonia levels were observed in tanks containing microbial mats (1.55 mg L^{-1}) compared to control tanks without mats, which were reported to have increased accumulation of ammonia at the end of 33 days. However, in the present experiment, the reduction rates were markedly higher and the ammonia levels were

lower than in previous studies for wastewater treatment (Cañizares et al., 1994; Lezama-Cervantes and Paniagua-Michel, 2010). This may be due to the selection process of the cyanobacterial performance for maximum uptake efficacy prior to the experiment. In addition, the use of filamentous cyanobacteria in this study is responsible for the greatest uptake of ammonia from water, as has been previously reported by Thompson et al. (2002). Khatoun et al. (2007b) also reported that the cyanobacterium *Oscillatoria* sp. was able to maintain low levels of TAN, $\text{NO}_2\text{-N}$, and SRP in a shrimp hatchery tank without water exchange.

The selection of cyanobacteria for treatment of wastewater is dependent on their robustness against wastewater as well as their ability to grow under such conditions while absorbing nutrients from the wastewater (Olguín, 2003). In the present study, the cyanobacterium *Geitlerinema* sp. was isolated from a shrimp farm, its natural habitat, and hence had better ability to uptake TAN, $\text{NO}_2\text{-N}$, and SRP for growth and in turn reduced nitrogenous and phosphorus compounds. As indicated in the present

study, to obtain good results, the selection and source of isolates used should be from a similar environment for its ultimate utilization. Although the three cyanobacteria were isolated from the same environment and were easy to grow under laboratory conditions, *Geitlerinema* sp. was chosen based on its ability to form more biomass in terms of chlorophyll *a* compared to *Gloeotrichia* sp. and *Lyngbya* sp. The formation of more biomass helped in the rapid sequestration of nutrients. Similarly, Jiménez-Pérez et al. (2004) reported that *Scenedesmus intermedius* and *Nannochloris* sp. isolated from pig manure showed higher phosphorus and nitrogen removal rates than commercial species. Therefore, autochthonous species should be utilized for wastewater treatment whenever possible to increase nutrient removal efficiency.

In nature, microbial mats consist of vertically stratified, interdependent layers of polymicrobial communities (Stolz, 2000). Different microalgae genera commonly found in such mats include *Oscillatoria*, *Navicula*, *Nitzschia*, *Scenedesmus*, *Stigeoclonium*, and *Phormidium* (Sládečková, 1994), with many being cyanobacteria. However, due to the production of compounds toxic to certain aquatic animals, cyanobacteria are reported to have a negative effect on water quality and aquatic animal life (Pearl and Tucker, 1995; Ju et al., 2008). Interestingly, *Geitlerinema* sp. in the current study did not show negative effects when tested on *Artemia*. *Artemia* assay is a convenient, rapid, cheap, simple, and useful test to screen for microalgae, including cyanobacterial toxins (Lincoln et al., 1996; Sökmen, 2001). According to Lincoln et al. (1996), the aqueous supernatant of *Oscillatoria agardhii*, which is a cyanobacterium, was found to be nontoxic when tested on *Artemia* nauplii. The aqueous supernatant (Lincoln et al., 1996) as well as the methanol extract (Hrouzek et al., 2005) of another cyanobacterium, *Calothrix parietina*, also did not cause any mortality in *Artemia* nauplii. In contrast, Mohamed et al. (2006) reported toxic effects of *Calothrix parietina*, which was isolated from benthic mats, on *Artemia salina*. Therefore, to reduce the risk of developing harmful organisms in naturally developing periphyton, mats can be developed with selected species of cyanobacteria. These selected cyanobacteria can also reduce nutrient loads and improve water quality in

hatchery rearing tanks without having to exchange water during the culture period (Khatoun et al., 2007b).

Since the substrate has a significant influence on microbial growth, previous studies have explored the possibilities of using different materials for the construction of microbial mats for pollutant removal (Bender et al., 1989; Phillips and Bender, 1995). Bender and Phillips (2004) reported the use of silage for the treatment of fish culture effluent and organic contaminants. Franco-Rivera et al. (2007) reported that low-density polyester can be used for immobilization of nitrifying biofilms. In aquaculture, various types of materials, such as halved plastic bottles, bamboo, firewood, water hyacinth, bamboo mats, nylon netting (Huchette and Beveridge, 2005), PVC pipes (Keshavanath et al., 2001), plastic sheets (Shrestha and Knud-Hansen, 1994; Tidwell et al., 1998), and custom-designed materials such as Aquamats (Bratvold and Browdy, 2001), have been used as substrates to improve water quality. Studies by Khatoun et al. (2007a) have shown that formation of periphyton biofilm was significantly higher on PVC compared to bamboo, ceramic tiles, plastic, and fibrous scrubbers in brackish water. The choice of support material is often based on practicality and cost-effectiveness. The present study demonstrated that the use of cyanobacteria on PVC sheets effectively sequestered ammonia, nitrite, and phosphorus from shrimp wastewater. The immobilization process of *Geitlerinema* sp. on PVC sheets was relatively simple and used diffused sunlight, making the process cost-efficient and easy to execute. In addition, after uptake of nutrients from wastewater, the nutrient rich biomass attached on the surface of the PVC sheet could be easily harvested by scraping and can be used as a fertilizer or soil conditioner (de la Noüe et al., 1992; Mallick, 2002; Mulbry et al., 2005). The positive results of the study warrant further research for treatment of commercial-scale shrimp wastewater.

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