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Microcystins in Cyanobacterial Blooms from Two Freshwater Prawn (*Macrobrachium rosenbergii*) Ponds in Northern Thailand

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ABSTRACT: The presence of cyanobacterially-produced microcystins (cyclic peptide hepatotoxins) was determined by analysis of Microcystis spp. in scum and water samples collected from a surface cyanobacterial bloom at a giant freshwater prawn (*Macrobrachium rosenbergii*) farm in Teung District, Chiang Rai Province, in northern Thailand during March to August 2004. *M. aeruginasa* and *M. wesenbergii* were the dominant species of cyanobacteria at concentrations between 850,000±190,000 and 302,000±73,000 colonies l^{-1}. Microcystins were present at 0.44±0.020 g kg^{-1} dry weight with microcystin-LR and microcystin-RR as the dominant microcystin types, accounting for 45% and 48% of the total microcystins detected, respectively. Microcystins in pond water were present at between 2.2±3.0 μg l^{-1} and 9.4±2.0 μg l^{-1}. Total microcystin concentrations in water seemed to be positively correlated with the number of Microcystis colonies. A decrease of microcystins in water was observed from April to August 2004, which may have resulted from removal by mechanisms not examined in this study. The total microcystin in water was slightly negatively correlated with total culturable bacteria numbers. Microcystis spp. colony number showed a significant negative correlation with soluble reactive phosphorus ($r = -0.98$, $p<0.05$). Nitrate-N, ammonium-N and soluble reactive phosphorus concentrations were between 1.2-1.9, 0.85-1.15 and 0.9-1.1 mg l^{-1}, respectively. Phosphorus concentrations were higher than the permitted limit for waste water from a fishery farm (less than 0.4 mg l^{-1}). This study suggested that surface blooms of Microcystis species in cultivation ponds may present a risk for microcystin bioaccumulation in prawns, either directly or via other organisms in the food web.

KEYWORDS: Prawn cultivation, *Macrobrachium rosenbergii*, *Microcystis*, Microcystins, toxic cyanobacteria.

INTRODUCTION

At present, prawn production is important to the economy of Thailand for domestic consumption and for export to many countries. The giant freshwater prawn (*Macrobrachium rosenbergii*) is indigenous to most Southeast Asian and South Pacific countries[1,2]. Since its successful domestication in the late 1960s[3], the culture of giant freshwater prawns has gained great popularity worldwide, mostly in tropical and subtropical regions[4]. In recent years the global production of freshwater prawns has increased steadily with intensive production in East and South Asian countries, including China, India, Indonesia, Bangladesh, Thailand and the Philippines. Consisting primarily of *M. rosenbergii*, freshwater crustacean production in the region reached 0.5 million tonnes. In Thailand, annual production averaged about 8,300 tonnes during 1989-1998, with peaks in 1992 (10,306 tonnes) and 1994 (10,124 tonnes),[5] although freshwater prawn production has tended to increase gradually. In 2001, the production of freshwater prawns was 13,300 tonnes[6] and domestic consumption was 70 % of total production[7].

Many scientific reports[8,9,10,11] have shown that the proliferation of cyanobacteria in water bodies as blooms is associated with enrichment with nutrients including nitrate, ammonium and phosphate. High concentrations of these nutrients that are degradation products from organic waste and uneaten food during prawn cultivation may promote the rapid growth of cyanobacteria[12]. This may result in the production of cyanobacterial toxins, both cell-associated and

dissolved in the water, with the potential for accumulation in prawns.

Toxic cyanobacterial blooms, found throughout much of the world have the potential to cause harm to animal and human health. The most frequently reported toxin-producing cyanobacterial genus causing blooms in freshwater is *Microcystis*[13], capable of producing cyclic peptide hepatotoxins and tumor promoters named microcystins[14]. Microcystins have also been characterized from other cyanobacterial genera such as *Anabaena*, *Planktothrix*, *Nostoc* and *Anabaenopsis*[8]. A number of incidents involving cyanobacterial toxins have occured throughout the world, including deaths of haemodialysis patients inCaruaru, Brazil, that were attributed to microcystin contamination in water used for haemodialysis[15]. In addition, human populations in Australia have suffered from acute toxicity associated with cyanobacterial blooms[16]. With animals, for example, mortalities of three species of flamingoes were observed where cyanobacterial toxins were indicated as a primary or major contributory cause[17]. Furthermore, microcystins can accumulate in fish tissues destined for human consumption[18].

In Thailand, *Microcystis* species are common members of toxic cyanobacterial blooms found in many water bodies in several regions, including drinking water sources and fisheries. These waterbodies were found to contain toxic cyanobacteria and associated toxins were identified,[9,19] but so far there have been no reports describing contamination by cyanobacteria and their toxins in freshwater prawn ponds in Thailand.

The purpose of this study was to monitor surface cyanobacterial blooms in prawn cultivation ponds for microcystins with the aim of contributing to risk assessment of potential microcystin contamination of water and bioaccumulation in prawns.

Materials and Methods

Freshwater Prawn Farm Location, Cyanobacteria and Water Sampling

The two prawn cultivation ponds with cyanobacterial blooms were located at a farm in Teung District, Chiang Rai Province in northern Thailand. Samples were taken on four separate occasions from March to August 2004.

Cyanobacterial scum samples were lyophilised and stored at –20 °C for microcystin analysis. Enumeration of *Microcystis* spp. and other phytoplankton in water samples was performed on samples obtained using a plankton net (mesh size, 10 micrometers). The water samples were filtered from 10 litre water samples to give about 100 ml, and preserved with 6-7 drops of Lugol's iodine solution for phytoplankton identification and counting.

Water samples were randomly collected at six sampling points from two ponds (3 sampling points in each pond). These mixed samples were kept in an ice container during field work.

Nutrient Analysis

Two-litre water samples collected in polyethylene containers were filtered through GF/C filter discs and the concentrations of nitrate nitrogen, ammonium nitrogen and soluble reactive phosphorus (SRP) were measured using colorimetric methods[20] with a spectrophotometer (model DR2010; HACH company).

Bacterial Counts

One milliliter of water, collected aseptically in a sterile bottle, was added to tryptic soil agar at 60 °C. The plates were incubated at 37 °C for 24 hours and the numbers of colonies then counted (CFU ml^{-1}).

Identification and Counting of *Microcystis* spp. and Other Phytoplankton

The identification of *Microcystis* colonies and other phytoplankton was carried out using a microscope and identification keys[21,22].

Microcystin Analysis

Microcystin extraction from lyophilised cyanobacterial scum was performed with methanol. The material was resuspended in 70% methanol and vortexed for 3 minutes, followed by ultrasonication at 90 Hz for 5 minutes. The suspension was then centrifuged at 6,000 rpm for 10 minutes. The methanolic supernatant containing extracted microcystins was analysed by HPLC with photodiode array detection[23].

Ten litres of water were filtered through GF/C filter discs. Water which had passed through the filters was applied to pre-conditioned C18 Sep-Paks, after which the cartridges were washed with 10 ml of 20% methanol and microcystins were eluted with 3 ml of 70% methanol. The 70% methanol solution containing microcystins was dried at 40 °C under nitrogen gas. The microcystin residue was resuspended in Milli-Q water before analysis by Enzyme-Linked Immunosorbent Assay (ELISA)[24].

This research used microcystin-LR and –RR standards which were purified from *Microcystis* PCC7813 (Pasteur Culture Collection, Paris)[23] for analysis by HPLC and ELISA.

Results and Discussion

Cyanobacterial and Phytoplankton Identification and Numbers

Many phytoplankton genera were found in water samples taken from the prawn cultivation ponds on the

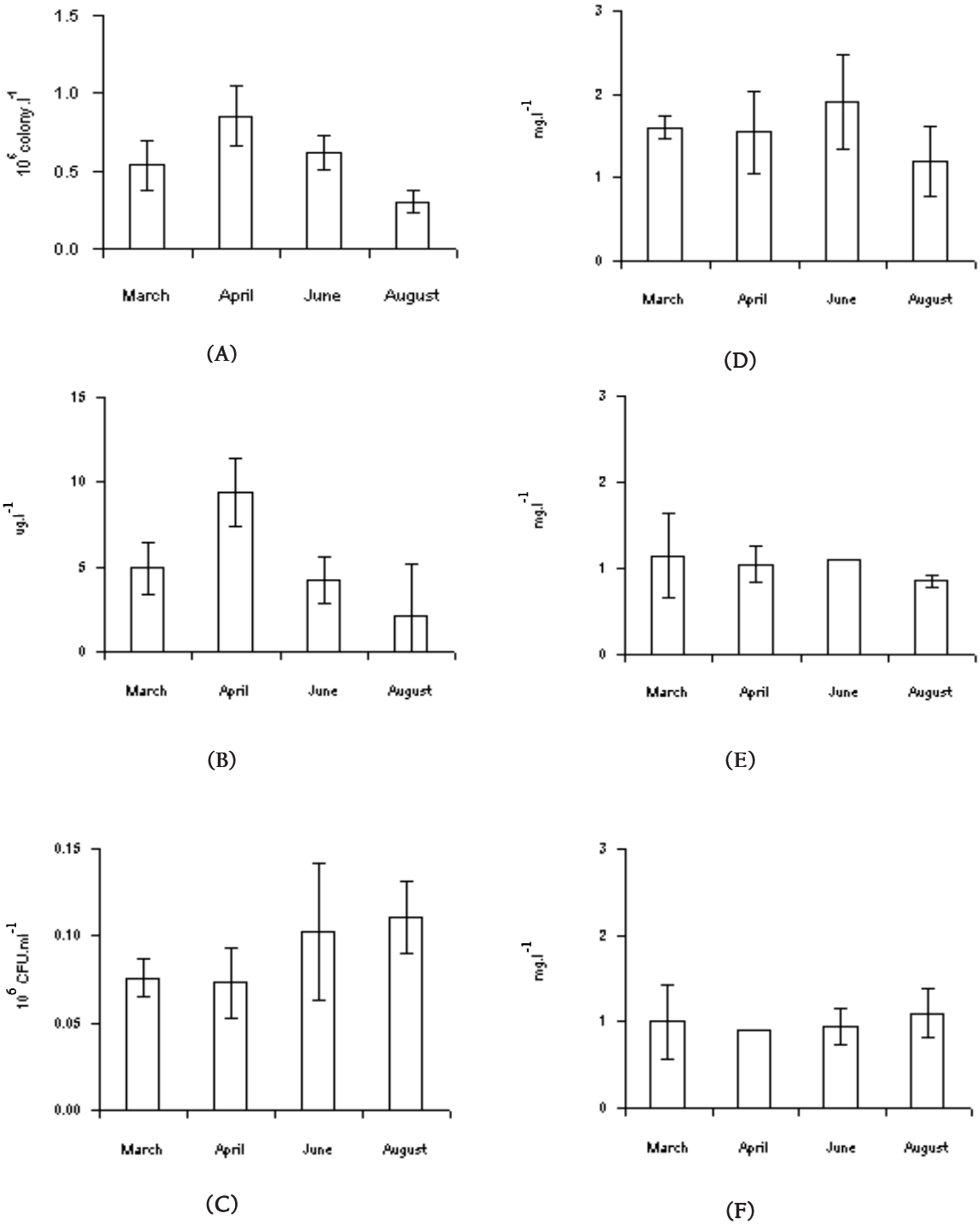

Fig 1. Comparison of *Microcystis spp.* colony numbers, total microcystins, total culturable bacteria, nitrate nitrogen, ammonium nitrogen and soluble reactive phosphorus in water collected from prawn cultivation ponds during March – August 2004. Points are the means of three determinations, with vertical bar showing standard deviation of the mean.
(A) Colony number of *Microcystis* spp.
(B) Total microcystin.
(C) Culturable bacteria (plate count).
(D) Nitrate nitrogen.
(E) Ammonium nitrogen.
(F) Soluble reactive phosphorus.

four sampling occasions from March to August 2004. These included *Microcystis* spp., *Merismopedia* spp., *Pseudoanabaena* spp. and *Euglena* spp. The dominant genus was *Microcystis*, which comprised two species, namely *Microcystis aeruginosa* and *Microcystis wesenbergii*. *Microcystis* accounted for almost 100% of the phytoplankton population in the green surface scum collected during the study. The highest number was observed in the middle of the summer in April, 2004 at 85,000±190,000 colonies l^{-1}. The lowest number was observed in the middle of the rainy season in August, 2004 at 302,000±73,000 colonies l^{-1} (Fig 1). The dominance of *Microcystis* in the prawn cultivation ponds of Thailand agrees with its occurrence as the most common bloom-forming freshwater cyanobacterial genus worldwide and with reports on *Microcystis* as the dominant genus in some reservoirs used for water supplies and fisheries. Microcystins were also detected in those waterbodies[9,19]. In Thailand, the amounts of *Microcystis* found during the summer were higher than in the rainy season by a factor of three, and it was found more often in summer than in other seasons. The occurrence of several other phytoplankton genera could be used as a bioindicator of the trophic status of the waterbody. *Microcystis* spp., *Oscillatoria* (*Planktothrix*) spp. and *Euglena* spp. can often be found as the dominant genera in eutrophic water bodies containing high concentrations of inorganic nutrients[10].

Nutrient Analysis

The concentrations of nitrate-N, ammonium-N and soluble reactive phosphorus in the water did not differ much from March to August, 2004 (Fig 1). Nitrate-N was present between 1.2-1.9 mg l^{-1} and ammonium-N concentrations were between 0.85-1.15 mg l^{-1}. Soluble reactive phosphorus was present at concentrations between 0.9-1.1 mg l^{-1} and these were higher than the limits allowed for discharge water from the fishery farm (less than 0.4 mg l^{-1})[25]. *Microcystis* colony number had a significantly negative correlation with soluble reactive phosphorus ($r=-0.98$, $p<0.05$), even though the concentration of soluble reactive phosphorus in the water did not change significantly during the study. Although several studies have found N and P to be associated with phytoplankton biomass, neither P nor N are consistently the only limiting factors for phytoplankton, including cyanobacterial growth[26]. Moreover, N and P concentrations did not seem to be related to summer cyanobacterial biomass, because the concentrations of TN and TP were always high. TP and dissolved inorganic nitrogen concentrations during the bloom may be lower than those in the non-blooming period[11]. It is not only the amount and ratio of nutrients that influence cyanobacterial growth, but also environmental factors such as temperature and light. These may influence the rapid proliferation of cyanobacteria in the natural environment. We found the highest *Microcystis* biomass in the middle of summer and the lowest in the mid rainy season. Maximum growth rates are attained by most cyanobacteria at temperatures above 25 °C. These optimum temperatures are higher than those for green algae and diatoms. This could explain why most *Microcystis* species bloom during summer[27].

Bacterial Counts

Bacterial plate counts were determined, with counts of between 73,000 and 110,500 CFU ml^{-1}. However, a slight increase occurred by the end of the study (Fig 1). An increase in culturable bacterial plate count number may be promoted by products from *Microcystis* bloom decay available for bacterial proliferation.

Microcystins in Lyophilised Scum

Microcystis scum was found to contain microcystins at a total concentration of 0.44±0.020 g kg^{-1} dry weight (Fig 2). Although three microcystins showing typical UV absorption spectra for microcystins were found, the most abundant variants were microcystin-LR and microcystin-RR at concentrations of 0.20±0.001 and 0.21±0.021 g kg^{-1} dry weight respectively. Thus, in the *Microcystis* scum microcystin-LR accounted for 45% and Microcystin-RR accounted for 48% of total microcystins. In addition, from toxicity studies, microcystin-LR is one of the most toxic microcystins with an LD_{50} of 50 µg kg^{-1}, which is approximately 10 times more toxic than microcystin-RR[8].

Microcystins in Water Samples

Microcystin concentrations in filtered water

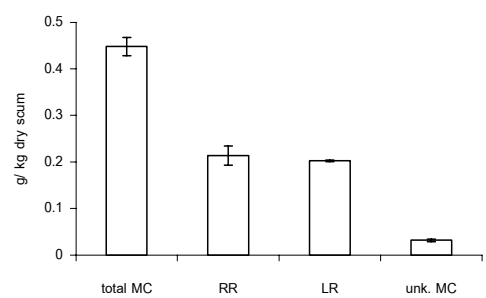

Fig 2. Amount of total microcystin, microcystin-LR (MC-LR) microcystin-RR (MC-RR) and unknown microcystin (unk. MC) extracted from the lyophilised *Microcystis* scum collected from prawn cultivation ponds. Points are the means of three determinations, with vertical bar showing standard deviation of the mean.

samples (i.e. dissolved microcystins) collected from *Microcystis* surface blooms at the prawn cultivation ponds are given in microcystin-LR equivalents in Fig 1B. The highest concentration of total dissolved microcystins was found in the middle of the Thai summer in April at 9.43±2.02 μg l^{-1}, while the lowest concentration was found in the middle of the rainy season in August at 2.15±3.04 μg l^{-1}. It appeared to decrease towards the end of the study.

Thirteen species within ten genera of cyanobacteria were found and these included potential microcystin producing genera such as *Anabaena*, *Phormidium* and *Oscillatoria*. However, they were found in small amounts and were not candidates for significant microcystin production in the pond. The principal source was obviously genus *Microcystis*, since it occurred as the dominant genus in the massive scum at the water surface. However, not all species within a genus or strains within a species produce a particular toxin[28]. It is also possible that other genera of cyanobacteria in ponds could be sources of cyanotoxins in addition to microcystins. Our demonstration of microcystin in predominantly *Microcystis* scums substantiated their principle source in the pond water and verified that the *Microcystis* species included toxic strains. The lack of direct connection between microcystins in scums and the amount of dissolved microcystins in water can be affected by various factors, including mixing due to wind action or currents[29]. Furthermore, microcystins may be removed by many factors in the natural environmental condition, for example, biodegradation by bacteria in water and sediments[30], photochemical breakdown and isomerisation[31], and sorption onto sediment particles[32]. This study did not analyse microcystins extracted from *Microcystis* scum in a manner parallel to the microcystins in water and *Microcystis* amount because of many variances in the natural environmental condition mentioned previously. Studies over prolonged periods usually show that toxin concentration per gram dry weight of scum may vary substantially over a time scale of weeks to months, but rarely from day to day as is sometimes reported. In any case, the time of toxin concentration maximum and biomass maximum are not necessarily coincident[8], and there can be significant variation in the amount of toxin per mass of cyanobacteria over time. The determination of microcystin accumulation in the scum remains an interesting subject for the further study. This may help establish the relationship between microcystins in the scum and in the water.

Microcystins are produced in algal cells by multi-enzyme complexes, including enzymes produced from peptide synthetase genes[8]. They are not actively secreted into the surrounding water. Studies with laboratory cultures of cyanobacterial strains have shown that most (>80%) of the toxin is intracellular in healthy growing cells, and that the release of toxin occurs during senescence of the cultures and the shift from growth to stationary phase and cell death[33]. The release of toxins from cells is enhanced by many physico-chemical factors in the natural environment. Chemical treatments for the eradication of cyanobacteria, especially the use of algicides (eg. copper based or organic herbicides) may lead to complete lysis of the bloom population within three days and release of all the toxins into the surrounding water[8]. This study found a trend of positive correlation between colony number of *Microcystis* spp. and total microcystin dissolved in water (Figs 1(A) and 1(B)). The highest concentration of total microcystin in water was found in the summer of 2004 and coincided with the period of highest number of *Microcystis* colonies. The decrease of *Microcystis* spp. numbers from April to August 2004 (Fig 1(A)) would theoretically provide higher concentrations of microcystins in water due to cell lysis, but the total microcystin in water did not increase from April to August (Fig 1(B)). Perhaps the released microcystins were removed by factors not examined in this study but of interest for further investigation.

The presence of microcystins in aquatic animal cultivation ponds entails the potential risk of bioaccumulation and biomagnification in food chains. The WHO (World Health Organisation) has recommended Guideline Values for a tolerable daily intake (TDI) of microcystin at 0.04 μg kg^{-1} body weight day^{-1} [8]. According to published research, microcystins have been found in a wide variety of organisms and tissues, such as fish muscle, shrimp and crab. The highest value was found in crab samples (103.3 μg kg^{-1}). It is also possible for microcystins to be adsorbed by sediment particles and consequently the contamination in crabs could have occurred via particle feeding[18]. Laboratory experiments indicated that prawn hepatopancreas, heart and brain were also primary organs for hepatotoxin bioaccumulation. Toxin concentrations in other organs, including muscle was lower[12].

The present study has suggested that *Microcystis* species and microcystins in prawn cultivation ponds may pose a hazard to aquatic organisms and to humans through food webs. Although some research has shown that relatively low microcystin accumulation occurs in prawn muscle (the most commonly consumed part of prawns) and that dissolved microcystins can be degraded by certain bacterial strains and be adsorbed by sediment particles in cultivation ponds, the long-term effect of persistent low microcystin concentrations in prawn cultivation water has not yet been properly assessed.

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