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Effect of dietary cyanobacteria on growth and accumulation of microcystins in Nile tilapia (*Oreochromis niloticus*)

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Abstract

A 12-week growth trial was conducted in a flow-through system to investigate the chronic toxic effect of dietary intake of cyanobacteria on growth, feed utilization and microcystins accumulation in Nile tilapia (*Oreochromis niloticus* L.) (initial body weight: 5.6 g). Six isonitrogenous and isocaloric diets were formulated to include different contents of cyanobacteria with the dietary microcystins increasing from 0 to 5460.06 ng/g diet. The results showed that dietary intake of cyanobacteria could increase the growth of tilapia while there are no impacts on feed conversion efficiency or mortality. Feeding rate was higher for the diets containing highest cyanobacteria. Microcystins were mostly accumulated in fish liver. The relationship between microcystins contents in muscle, liver, spleen and dietary intake could be described by quadratic equations.

Microcystins content in the muscle of Nile tilapia in present study exceeded the upper limit of the tolerable daily intake (TDI) of microcystins suggested by the WHO (0.04 µg/kg body weight/d). It is suggested that Nile tilapia fed on toxic cyanobacteria is not suitable for human food.
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1. Introduction

Water blooms of toxic cyanobacteria in fresh water bodies have been reported all over the world (Bouvy et al., 2000; Magalhães et al., 2001; Haider et al., 2003; Soares et al., 2004; Dionisio Pires et al., 2004) and it is often dominated by *Microcystis aeruginosa* (Orr et al., 2001; Park et al., 2001). *M. aeruginosa* and some other freshwater cyanobacteria often produce hepatotoxic cyclic heptapeptides microcystins (Kaya, 1996). The ingestion of intact *M. aeruginosa* cells or microcystins is reported to be toxic to aquatic organisms, domestic animals, wildlife and humans (Miura et al., 1989; Žegura et al., 2003). Fish mortality was reported to be associated with toxic cyanobacterial water blooms (Andersen et al., 1993; Rodger et al., 1994; Tencalla et al., 1994; Zimba

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Table 1
Formulation and chemical composition of experiment diets (% in dry matter)

Diet	D1	D2	D3	D4	D5	D6
Soybean meal (oil-extracted)	69.47	68.12	66.82	65.49	64.17	62.84
Algae meal	0.00	1.19	2.34	3.51	4.68	5.85
White fish meal (USA)	15.00	15.00	15.00	15.00	15.00	15.00
Fish oil	4.81	4.76	4.72	4.68	4.64	4.59
Corn starch	1.06	1.26	1.46	1.66	1.85	2.05
Carboxymethyl cellulose	3.00	3.00	3.00	3.00	3.00	3.00
Vitamin premix[1]	0.55	0.55	0.55	0.55	0.55	0.55
Vitamin C	0.01	0.01	0.01	0.01	0.01	0.01
Mineral premix[2]	5.00	5.00	5.00	5.00	5.00	5.00
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Cr_2O_3	1.00	1.00	1.00	1.00	1.00	1.00
Chemical composition (in dry matter)						
Crude protein (%)	40.18	40.46	40.87	40.99	40.41	40.31
Crude lipid (%)	8.14	6.65	7.03	7.57	7.48	7.63
Ash (%)	12.51	12.34	12.42	12.51	12.67	12.59
Water (%)	6.66	6.84	6.76	6.24	6.91	6.50
Gross energy (kJ/g)	17.03	17.18	17.18	17.14	17.42	17.02
Microcystins (ng/g)	0.00	1253.52	2210.07	3363.02	4440.64	5460.06

[1]Vitamin premix (mg kg^{-1}): thiamin, 20; riboflavin, 20; pyridoxine, 20; vitamin B_{12}, 2; vitamin A, 1.83; vitamin D, 0.5; vitamin K, 10; folic acid, 5; Ca-pantothenate, 50; inositol, 100; vitamin E, 10.
[2]Mineral premix (g kg^{-1}): NaCl, 0.8; $MgSO_4 \cdot 7H_2O$, 12; $NaH_2PO_4 \cdot 2H_2O$, 20; KH_2PO_4, 25.6; $Ca(PO_4)_2 \cdot H_2O$, 16; $FeSO_4$, 2; $(CH_2CHCOO)_2$-Ca·$5H_2O$, 2.8; $ZnSO_4 \cdot 7H_2O$, 0.028; $MnSO_4 \cdot 4H_2O$, 0.013; $CuSO_4 \cdot 5H_2O$, 0.0025; $CoCl_2 \cdot 6H_2O$, 0.0008; $KIO_3 \cdot 6H_2O$, 0.0024; Cellulose, 0.36.

et al., 2001). The acute effects on fish exposed to *M. aeruginosa* or microcystin have been confirmed (Carbis et al., 1996; Bury et al., 1997). But in most situations, the impacts of toxic cyanobacteria on fish could be at sublethal or chronic levels. But it is still lack of research data on chronic toxic effects of cyanobacteria.

Microcystins can be accumulated in the tissues of fish (Tencalla et al., 1994; Soares et al., 2004), mussels (Amorim and Vasconcelos, 1999; Vasconcelos, 1995; Williams et al., 1997b; Dionisio Pires et al., 2004) and aquatic macrophytes (Pflugmacher et al., 1998) and also transferred from crab larva to salmons through food chain (Williams et al., 1997a). Many fishes are not able to avoid the ingestion of these toxic organisms through food in the waters such as small eutrophicated lakes and aquaculture ponds (Tencalla and Dietrich, 1997; Magalhães et al., 2001). The microcystins could be accumulated in fish through food chain and could be a threat to human food safety.

Microcystins are seldom ingested by human at the amount high up to acute lethal dose, but long term exposure could lead to some problems (Magalhães et al., 2001, 2003). The eutrophication in fresh water bodies normally resulted in frequent cyanobacterial water blooms and aquatic product can be contaminated by microcystins. On the other hand, filtering-feeding fishes such as silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Aristichthys nobilis*) are used to intake and

control these blooming algae (Xie, 2003). However, there is few research about the accumulation of microcystins in fish (Soares et al., 2004) and there are no reports on the relationship between body accumulation and dietary intake of microcystins. Data is not enough to provide the evidence or the prediction of the food safety for those fishes from cyanobacteria blooming waters.

The purpose of the present study is to find dietary intake of cyanobacteria on the growth performance of Nile tilapia (*Oreochromis niloticus* L.) and the quantitative relationship between dietary intake and fish tissues accumulation of microcystins in fish. And the effect of chronic microcystins exposure to fish and the potential harm of the microcystins contaminated fish to human is evaluated.

2. Materials and methods

2.1. Fish and experimental diets

Nile tilapia used in the experiment were obtained from Puqi hatchery farm (Hubei, China). The fish were stocked in experimental tanks for acclimatization 2 weeks before experiments. During this period, the fish were fed practical diets (protein content: 40%) two times daily (9:00 and 15:00).

The cyanobacteria (blue-green algae) were collected in Dianchi Lake, Yunnan, China. The fresh algae were
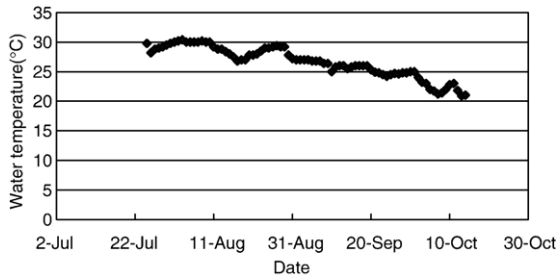
Fig. 1. Water temperature variation during the experiment.

sun-dried. 90% of the algae were *M. aeruginosa*. The microcystins (MC) content of dry algae meal was 0.14%.

Six isoenergetic (17 kJ g DM^{-1}) and isonitrogenous (40% protein in DM) experimental diets were formulated. Diet 1, containing soybean meal as the main protein source, was used as the control diet. The dry algae meal content of Diet 2 to 6 was 1.19%, 2.34%, 3.51%, 4.68% and 5.85%, respectively. 1% Cr_2O_3 was added in the diets as indicator for digestibility determination. Diet formulation and chemical composition of the experiment diets are shown in Table 1. All diets were made into 1 mm pellet by extrusion machine, air-dried and stored at 4 °C.

2.2. Growth trial

The growth trial was conducted in an indoor flow-through system consisting of 30 fiberglass tanks (cylindrical, volume 98 L). Nile tilapia were weighed and randomly assigned to 30 tanks after deprivation of food for 1 day. There were 30 fish in each tank. The initial total biomass was 197.3 ± 1 g/tank. Thirty tanks were randomly used for six experimental diets. Five replicates were assigned to each diet. At the beginning of the growth trial, three samples (6 fish for each) were randomly selected from the stock and frozen at −20 °C for the analysis of initial body composition.

Tap water was supplied to each tank at a rate of 10 mL/ min after aeration and dechlorination in a reservoir tank. During the experiment, continuous aeration was supplied

to each tank and water temperature varied between 20.9 °C and 30.4 °C (26.6 ± 2.6 °C) (Fig. 1). The photoperiod was 12D:12L with the light period from 8:00 to 20:00 by 3 fluorescent lamps. Dissolved oxygen, ammonia nitrogen and residual chlorine in water were measured once a week, which was >5 mg/L, <0.5mg/L and <0.05 mg/L, respectively. Water pH was around 7.0.

During the experiment, fish were fed to apparent satiation twice daily (9:00 and 15:00), 7 days a week for 12 weeks. At each feeding, an excess quantity of the diet was provided and uneaten diet was collected 1.5 h after feeding.

At the end of the experiment, fish were batch weighted after 1 day food deprivation. Three fish from each tank were sampled and killed by a heavy blow on the head and dissected on an ice pan. Muscle, liver, gallbladder and spleen of these fish were taken for microcystins analysis. The other fish in each tank were sampled for final body composition analysis.

3. Chemical analysis

The initial and final fish samples were autoclaved at 120 °C, homogenized and oven-dried at 70 °C. Crude protein, fat, ash and energy contents were analyzed for fish and diets.

Crude protein content was determined by Kjeldahl method (AOAC, 1984). Fat content was measured by chloroform–methanol extraction (Lambert and Dehnel, 1974). Ash content was measured after 6 h at 550 °C in a muffle furnace. Gross energy content was measured by combustion in a micro bomb calorimeter (Phillipson microbomb calorimeter, Gentry Instruments Inc., Aiken, South Carolina, USA).

For each analyse, at least duplicate samples were measured.

4. Microcystins analysis

Fish muscle, liver, gallbladder and spleen samples were ice-dried and ground. Dried cells after lyophilization

Table 2
Mean initial and final wet weight, SGR, mortality, FCE and FR of Nile tilapia fed diets containing different level of algae meal (means ± SE)*

Diet	Initial body weight (g)	Final body weight (g)	SGR (% d^{-1})	Mortality (%)	FCE (%)	FR (%)
Diet 1	5.64 ± 0.06	16.25 ± 0.98a	1.26 ± 0.08a	10.83 ± 6.97	55.97 ± 4.83	2.21 ± 0.12a
Diet 2	5.63 ± 0.05	19.16 ± 1.57b	1.46 ± 0.10b	4.17 ± 4.17	62.70 ± 3.65	2.12 ± 0.13a
Diet 3	5.63 ± 0.06	19.06 ± 1.70b	1.45 ± 0.12b	6.67 ± 10.46	59.03 ± 4.83	2.12 ± 0.13a
Diet 4	5.64 ± 0.04	19.39 ± 3.08b	1.46 ± 0.19b	10.00 ± 8.64	62.65 ± 7.07	2.24 ± 0.07a
Diet 5	5.68 ± 0.04	18.81 ± 1.06b	1.43 ± 0.07b	5.83 ± 6.97	59.36 ± 4.05	2.23 ± 0.09a
Diet 6	5.64 ± 0.07	18.30 ± 0.51ab	1.40 ± 0.03ab	13.33 ± 13.94	57.19 ± 3.77	2.41 ± 0.11b

Means with different superscripts are significantly different (P<0.05).

Table 3
Microcystin intake and microcystin-LR equivalents found in tissues of Nile tilapia fed different diets (means±SE)*

Diet	Mean microcystin intake (ng/fish/d)	Muscle (ng/g)	Liver (ng/g)	Spleen (ng/g)	Gall bladder (ng/g)
Diet 1	0.00±0.00	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
Diet 2	327.83±10.99	0.77±0.43ab	423.55±123.77b	22.85±4.22b	17.18±2.71b
Diet 3	630.67±74.85	1.69±0.26ab	538.44±11.62b	25.58±3.39b	28.26±4.58c
Diet 4	884.06±83.20	2.59±0.26b	784.13±45.23b	51.52±12.42c	36.22±2.08d
Diet 5	1259.64±93.05	6.47±2.46c	1596.14±300.12c	53.91±3.84c	41.21±4.16d
Diet 6	1570.98±75.59	14.62±2.54d	3007.33±631.54d	54.6±4.19c	38.66±6.38d

*Means with different superscripts are significantly different ($p < 0.05$).

were extracted twice with 100% methanol for 30 min with sufficient mixing by a magnetic stirrer. The methanol extract was diluted and redissolved in deionized water, and then passed through Sep-pak C18 cartridge (Waters). The cartridge was rinsed with water and 20% methanol solution. The microcystins were eluted with 90% methanol in water, and the methanol extract was dried and redissolved in deionized water. Monoclonal anti-

bodies (MAB) against microcystin were provided by Prof. Ueno (Department of Toxicology and Microbial Chemistry Faculty of Pharmaceutical Sciences, Science University of Tokyo, Ichigaya, Shinjuku-ku, Tokyo 162-0826, Japan). Microtiter plates (Costar, USA) were coated with MAB (4.0 µg/mL) and incubated overnight at 4 °C, then blocked with blocking buffer 170 µL (0.5% (w/v) gelatin in phosphate-buffered saline (PBS) for 2 h in the
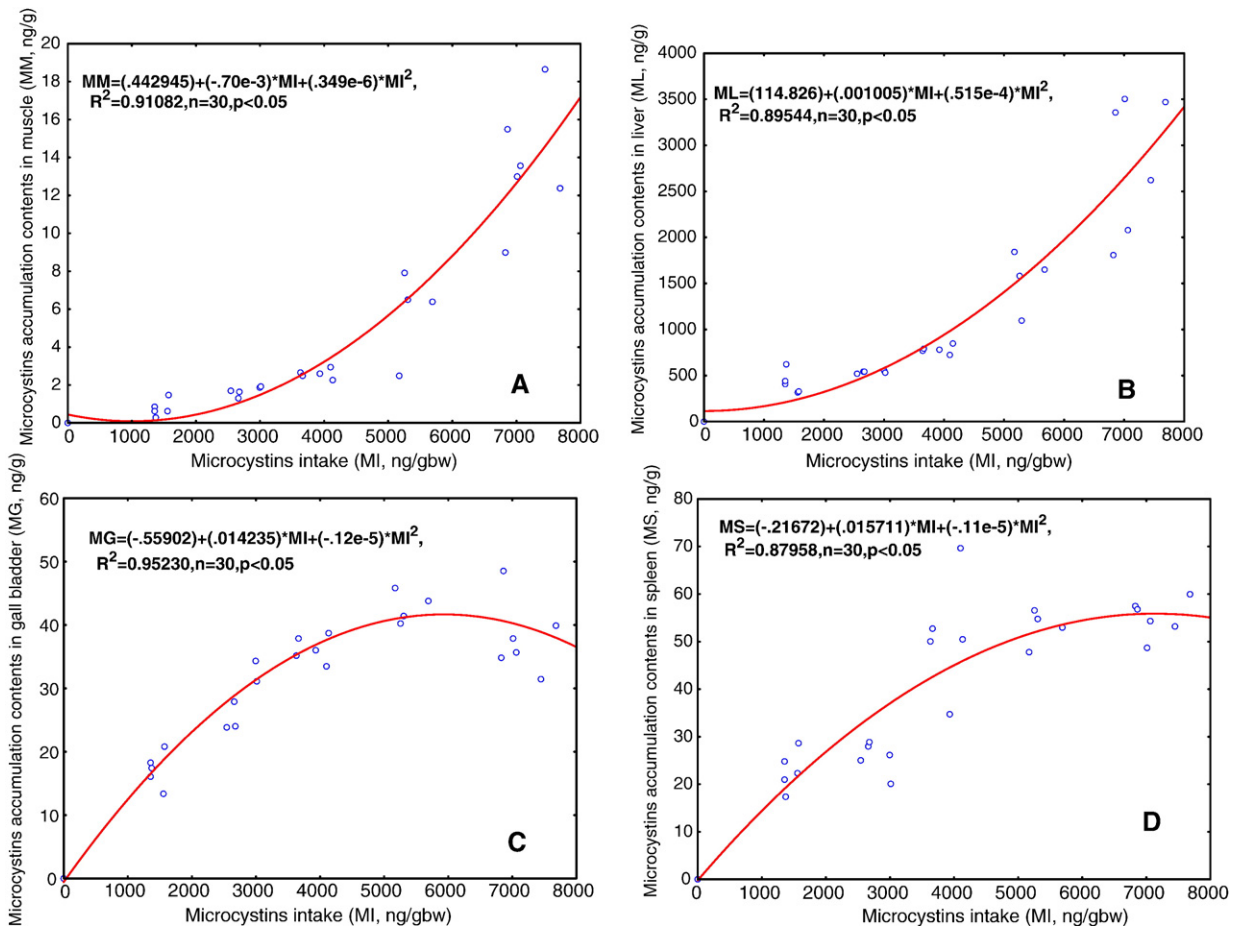

Fig. 2. The relationship between microcystin-LR equivalents content in muscle (A, MM), liver (B, ML), spleen (C, MS), gallbladder (D, MG) (ng/g) and microcystins intake of Nile tilapia (MI, ng/gbw).

Table 4
Initial and final body composition of Nile tilapia fed different diets (means±SE)*

Diet	Dry matter (%)	Crude protein (%)	Crude lipid (%)	Ash (%)	Energy (kJ/g)
Initial	17.45±1.39a	11.45±1.67a	0.86±0.28a	3.40±0.28ab	2.91±0.20a
Final					
Diet 1	22.46±0.32a	11.42±0.16a	3.88±0.10a	4.43±0.10a	4.42±0.13
Diet 2	25.52±1.04b	13.81±0.43b	4.31±0.49ab	5.28±0.28b	4.75±0.55
Diet 3	25.93±1.81b	13.82±1.19b	4.81±0.73b	5.25±0.30b	4.86±0.60
Diet 4	25.91±0.64b	13.71±0.85b	4.6±0.68ab	5.31±0.24b	4.67±0.27
Diet 5	25.43±2.22b	13.47±1.30b	4.67±0.38ab	5.12±0.61b	4.77±0.53
Diet 6	25.46±0.72b	13.90±0.44b	4.33±0.28ab	5.26±0.19b	4.58±0.18

model 237 microplate incubator (Bio-rad, USA) at 37 °C or overnight at 4 °C. Various concentrations of MC-LR were preincubated 70 µL at 37 °C for 30 min, then an equal volume of biotinylated MC MAB (25 ng/mL) was added to the coated wells for 30 min. Plates were washed thoroughly with PBS-T three times with a model 1575 immun wash apparatus (Bio-rad, USA). HRP-streptavidin (Sigma) diluted 1:10,000 in dilution buffer (PBS containing 0.5% (w/v) gelatin and 0.05% (v/v)) was added and incubated 30 min at 37 °C. The enzyme reaction was started by adding the substrate solution (0.1 M sodium acetate buffer (pH 5.0) containing 100 µg/mL of TMBZ and 0.005% (v/v) H_2O_2) and stopped with 1 M H_2SO_4. The absorbance at 450 nm was measured with a model 550 microtiter plate reader (Bio-Rad, USA).

5. Calculations and statistical analysis

Specific growth rate (SGR, %/day) = 100
  × [ln final body weight (wet, g)
  − ln initial body weight (wet, g)]/time (days)

Feed conversion efficiency (FCR, %) = 100
  × [final body weight (wet, g)
  − initial body weight (wet, g)]
  / feed consumed (in dry matter, g)

Feeding rate (FR, %/day) = 200
  × total feed intake (dry matter, g)
  /[initial body weight (wet, g)
  + final body weight (wet, g)]/time (days)

Mortality (%) = 100 × dead fish in a tank/40

Duncan's multiple range test was used to compare the difference between means after one-way analysis of variance. Quadratic equations were derived from the regression between tissue accumulation and dietary intake of microcystins.

6. Results

6.1. Growth and feed utilization

Table 2 shows that specific growth rate (SGR) of the fish fed algae meal diets (Diet 2–5) was higher than that fed the control diet ($p < 0.05$) except that the highest inclusion of algae (Diet 6) showed no significant difference than that of the control ($p > 0.05$). There were no significant differences in mortality and feed conversion efficiency between the fish fed different diets ($p > 0.05$). Feeding rate was significantly higher for the diet with the highest algae meal inclusion (Diet 6) ($p < 0.05$).

6.2. Microcystins contents in tissue

The microcystins intake (MI) by tilapia and the contents in liver (ML), muscle (MM), gall bladder (MG) and spleen (MS) of fish are shown in Table 3. The microcystins contents increased with increased dietary intake ($p < 0.05$). The relationship between the microcystins contents in four tissues and dietary intake by fish is shown in Fig. 2 and the equations are as the follows:

Fig. 2 A: $MM = (.442945) + (-.70e-3) * MI + (.349e-6) * MI^2$, $R^2 = 0.91082$, $n = 30$, $p < 0.05$
Fig. 2 B: $ML = (114.826) + (.001005) * MI + (.515e-4) * MI^2$, $R^2 = 0.89544$, $n = 30$, $p < 0.05$
Fig. 2 C: $MG = (-.55902) + (.014235) * MI + (-.12e-5) * MI^2$, $R^2 = 0.95230$, $n = 30$, $p < 0.05$
Fig. 2 D: $MS = (-.21672) + (.015711) * MI + (-.11e-5) * MI^2$, $R^2 = 0.87958$, $n = 30$, $p < 0.05$

6.3. Fish body composition

Table 4 showed that dry matter and crude protein contents of the fish fed the control diet were significantly lower than that of the fish fed algae meal

diets (Diets 2–6) ($p < 0.05$). The crude lipid content of fish fed Diet 3 was significantly higher than that of fish fed the control diet ($p < 0.05$). No significant difference was found in energy and ash contents between different groups ($p > 0.05$).

7. Discussion

In the present study, the accumulation of microcystins in Nile tilapia was observed in liver, muscle, gall bladder and spleen and about 90% microcystins were accumulated in the liver. The results were in agreement with the previous studies (Bury et al., 1998; Soares et al., 2004; Zhao et al., 2005).

Normally, toxic effect of oral administration is approximately 10 times lower than that of intraperitoneal application (Carbis et al., 1996). In the present experiment, the dietary intake of microcystins by tilapia was 1500–6000 μg/kg body weight and it could show the similar impact on fish as 150–600 μg/kg body weight through intraperitoneal injection. Råbergh et al. (1991) injected purified microcystin-LR into common carp and found the consistently lethal dose to this fish was 550 μg/kg body weight. In the present study, however, the intake of microcystin didn't affect the survival of Nile tilapia. In gibel carp (*Carassius auratus gibelio*), high mortality was observed when fish were fed low microcystins content feed (1.02–10.76 μg/kg body weight) (Zhao et al., 2005). It suggested that Nile tilapia was more tolerant to microcysins than common carp and gibel carp.

Soares et al. (2004) reported that, when Tilapia *rendalli* (Cichlidae) was fed microcystins at 29.2 μg/fish/d, on the 42nd day, the accumulation contents of microcystins were 1.7 μg/g in the liver and 0.1 μg/g in the muscle. In the present study, the accumulation rate is much higher than that (Table 3 and Fig. 2). In previous study with gibel carp, when fish were fed diets containing microcystins (3.2–34.2 ng/fish/d) for 12 weeks, the accumulation contents of microcystins was 1.7–40.8 ng/g in the liver and 0.02–0.17 ng/g in the muscle (Zhao et al., 2005). The accumulation rate in muscle and liver to dietary intake is similar to the present study. It might suggest that the longer time dietary intake (84 days compared to 42 days) leads to higher accumulation rate (accumulation compared to dietary intake) and there could be not too much related to fish species. Soares et al. (2004) reported that, the ratio of microcystins accumulation in liver (1.7 μg/g) to muscle (0.1 μg/g) is 17. However, the ratio is about 800–200 in the present study (Diet 2–Diet 6) (Table 3). Therefore, it showed that the ratio of liver accumulation to muscle accumulation decreased with the increase in dietary microcystins. The other reason for the different accumulation rate might be due to the availability of other feeding sources in fish feed besides toxic cells (Soares et al., 2004).

The microcystins content in tilapia muscle is high in the present study. If a human weighing 60 kg ingested 300 g muscle, the ingestion of microcystins is 0.004–0.075 μg/kg body weight/d. The tolerable daily intake (TDI) of microcystins suggested by the WHO was 0.04 μg/kg body weight/d (Chorus and Bartram, 1999). Many fish species living in environments such as small lakes and aquaculture ponds can't avoid the intake of toxic algae during water blooms (Tencalla and Dietrich, 1997). In the present experiment, the daily intake of microcystins of Nile tilapia is very low (300–1500 ng/d/fish), but it is still not safe to human. Therefore, the fish in toxic cyanobacterial blooming ponds should be checked before being sent to the market and the relationship between the muscle accumulation and dietary intake in the present study could provide the predicted evaluation for food safety. It has also been reported that, some filter-feeding fish such as tilapia (Turker et al., 2003), silver carp and bighead carp (Liu and Xie, 2002) were used to control cyanobacterial bloom. The food safety of those fish is still a big problem. Some researches found some fishes could depurate microcystins accumulated in the liver through biliary excretion (Sahin et al., 1996) and some fishes can degrade microcystins (Xie et al., 2004). Expanded researches should be focusing on the effect of chronic microcystins exposure on different fishes and food safety warrantee.

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