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IMPACT OF MICROCYSTIN CONTAINING DIETS ON PHYSIOLOGICAL PERFORMANCE OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*) CONCERNING STRESS AND GROWTH

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Abstract—Diets containing *Microcystis* with considerable amounts of the cyanotoxin microcystin-LR (MC-LR) were fed to determine their impact on the physiological performance of the omnivorous Nile tilapia (*Oreochromis niloticus*) with regard to stress and growth performance. Four different diets were prepared based on a commercial diet (control, MC-5% [containing 5% dried *Microcystis* biomass], MC-20% [containing 20% dried *Microcystis* biomass], and Arthrospira-20% [containing 20% dried *Arthrospira* sp. biomass without toxin]) and fed to female Nile tilapia. Blood and tissue samples were taken after 1, 7, and 28 d, and MC-LR was quantified in gills, muscle, and liver by using high-performance liquid chromatography (HPLC). Only in the liver were moderate concentrations of MC-LR detected. The stress hormone cortisol and glucose were analyzed from plasma, suggesting that all modified diets caused only minor to moderate stress, which was confirmed by analyses of hepatic glycogen. In addition, the effects of the different diets on growth performance were investigated by determining gene expression of hypophyseal growth hormone (GH) and hepatic insulin-like growth factor-I (IGF-I). For all diets, quantitative reverse transcription-polymerase chain reaction (RT-qPCR) demonstrated no significant effect on gene expression of the major endocrine hormones of the growth axis, whereas classical growth data, including growth and feed conversion ratio, displayed slight inhibitory effects of all modified diets independent of their MC-LR content. However, no significant change was found in condition or hepatosomatic index among the various diets, so it seems feasible that dried cyanobacterial biomass might be even used as a component in fish diet for Nile tilapia, which requires further research in more detail. Environ. Toxicol. Chem. 2010;29:561–568. © 2009 SETAC

Keywords—Microcystin-LR Fish diet Tilapia Stress Growth

INTRODUCTION

Blooming of photosynthetic prokaryotic organisms generally named ''cyanobacteria'' in freshwater and marine habitats has recently become a worldwide issue [1–3]. Increase in these primary producers is due to nutrient input, especially nitrogen and phosphorus, caused by human activities such as agriculture and sewage effluents as well as climate change. In addition to the negative effects caused by oxygen depletion and the foul odor from decaying cyanobacterial biomass, many cyanobacteria species are able to produce a wide range of harmful bioactive compounds [4]. These substances are called ''cyanotoxins'' and can be classified into three groups based on their chemical structure: cyclic peptides, e.g., microcystins and nodularins; alkaloids, e.g., anatoxins and saxitoxins; and lipopolysaccharides. These compounds are produced by several cyanobacteria species [5] and have severe but differential effects on vertebrates with regard to their chemical structure. Cyclic peptides are associated mainly with hepatotoxicity, whereas alkaloids are known to be neurotoxic, and lipopolysaccharides are potentially considered as irritants. Microcystins (MCs), the most common cyanotoxins in freshwater, are a family of toxins produced primarily by *Microcystis aeruginosa* but also by other *Microcystis* species and other genera, namely, *Anabaena*, *Oscillatoria,* and *Nostoc* [6]. By far the main microcystin occurring is MC—LR, a hepatotoxin that can threaten the health of animals and humans. Several cases have been reported concerning acute poisoning events [7–12], including humans, for whom exposure to MCs resulted in acute sickness and death in dialysis patients in Brazil [13]. Furthermore, it has been suggested that MCs might play a role in the high incidences of hepatocellular carcinoma in specific regions of China [14]. The most common MCs are also reported to cause severe hepatotoxicity in a variety of mammalian species [15–19].

However, cyanobacterial water blooms are occurring in sea and surface water, so aquatic animals, especially fish, are intensely exposed to different cyanotoxins. Freshwater ecosystems are used for human food production by inland fisheries and extensive and intensive aquaculture. Therefore, there is an urgent need to assess the potential impact of cyanobacteria and their metabolites on fish physiology.

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Although primarily fish are exposed to cyanobacteria in their natural environment, most studies concerning cyanotoxins, especially MC-LR, have been performed in mammals. However, the rare studies conducted in fish focus mainly on the exposure of MC-LR via intraperitoneal injection affecting hematological and biochemical parameters associated with detoxification [20–24]. Therefore, to further our knowledge concerning MC-LR given via natural exposure, the aim of the current study was to determine the potential impact of an MC-containing diet on the physiology of an omnivorous aquaculture species, the Nile tilapia (*Oreochromis niloticus*), addressing stress and growth parameters. Several tissues such as gills, muscle, and liver were considered, and the effect on growth performance was studied by classical growth parameters such as growth and feed conversion ratio and by the gene expression of the major hormones of the growth axis, growth hormone (GH) and insulin-like growth factor-I (IGF-I). For the first time, whether MC-LR might cause stress response in fish leading to elevated cortisol levels was investigated.

MATERIALS AND METHODS

Fish and experimental setup

Female Nile tilapia (*Oreochromis niloticus*) weighing between 30 and 50 g at the beginning of the experiment were obtained from the fish farm České rybářství Mariánské Lázně s.r.o. (Czech Republic). Four experimental diets were formulated as 1.5-mm pellets using a commercial diet (DanEx 1344; Danafeed): control without any alteration; MC-5% in which 5% of the commercial diet was replaced by dried cyanobacterial biomass consisting of 90% *Microcystis aeruginosa*, 5% *Microcystis flos-aquae*, 5% *Microcystis ichthyoblabe* as determined by light microscopic inspection and thus supposed to contain considerable amounts of MC-LR; MC-20% in which 20% of the commercial diet was replaced by dried cyanobacterial biomass as described; and Arthrospira-20% in which 20% of the commercial diet was replaced by dried biomass of the nontoxic cyanobacterium *Arthrospira* sp. and thus considered as positive control supposed to contain no MC-LR. Dry matter, ash, crude protein, crude lipid, crude fiber, nitrogen-free extract (NFE), and energy contents [25] of the experimental diets are summarized in Table 1.

Fish were randomly distributed to the experimental groups, namely, control, MC-5%, MC-20%, and Arthrospira-20%. For each treatment, 24 individuals were placed into three 60-L aquaria each maintaining eight fish, and these aquaria were connected to a separate, closed-recirculation system for each experimental diet. Fish were kept at a temperature of $26^{\circ}C \pm 0.5^{\circ}C$ under natural light conditions. Fish were allowed to acclimate for two weeks prior to the start of the experiment and were fed twice per day at 9 AM and 3 PM, resulting in a feeding rate of 1.5% of the whole body weight per day using the control diet. Water parameters such as pH, dissolved oxygen, temperature, $N\text{-}NH_4^+$, and $N\text{-}NO_2^-$ were measured daily at 10 AM [26]. Every second day, 50% of the water was replaced to maintain stable water conditions.

Sampling took place after 1, 7, and 28 d starting at 9 AM, using at every time point for each diet one aquarium with eight individuals. After sacrificing the animals by a blow on the head and transection of the spinal cord, fish were weighed and blood was drawn immediately from the caudal vein by a syringe within 1 min and centrifuged for 1 min at 2,000 *g* to obtain plasma, which was snap frozen in liquid nitrogen and stored at $-80^{\circ}C$ until further analysis. Whole body length was measured to the nearest millimeter, and the complete liver was removed and weighed to calculate the hepatosomatic index. For the determination of MC-LR by high-performance liquid chromatography (HPLC), approximately 1 g tissue was taken from the gills, muscle, and liver of fish from all diets as well as approximately 1 g from the filter scum of each closed-recirculation system. Furthermore 1 g liver was prepared for biochemical determinations, including glycogen measurements. For gene expression analyses, whole pituitary and approximately 50 mg liver were separately taken from each individual. All samples were collected in Eppendorff vials, immediately snap frozen in liquid nitrogen, and stored at $-80^{\circ}C$ until further processing. The growth of the four experimental groups sacrificed after 28 d was measured in intervals of 7, 14, 21, and 28 d by weighing the total in each group.

Analyses

Microcystin-LR determination. The cyanotoxin MC-LR was determined in tissue samples derived from gills, muscle, and liver and in scum of all closed-recirculation systems after 1, 7, and 28 d and, in addition, in the four experimental diets using the HPLC method [27] with a detection limit of 3 ng/g fresh weight.

Stress parameters. The stress hormone cortisol was determined in plasma by radioimmunoassay [28,29]. Stress parameters [30,31], plasma glucose, [32] and hepatic glycogen levels [33] were also determined.

Analyses of growth parameters. The general status of fish was determined by condition index (CI) and hepatosomatic index (HSI) [25] at all sampling points for each individual. The CI was calculated as percentage of final body weight/final body length^3 $\times$ 100, and HIS was determined as percentage of liver weight/final body weight. Growth was determined by measuring the total weight of all eight individuals per experimental group sampled at day 28 after 0, 7, 14, 21, and 28 d. In parallel

Table 1. Composition of the four different experimental diets

Diet	Dry matter (%)	Ash (% of dry matter)	Crude protein (% of dry matter)	Crude lipid (% of dry matter)	Crude fiber (% of dry matter)	Nitrogen-free extract (% of dry matter)	Energy (MJ/kg)
Control	92.74	6.50	32.35	10.46	2.60	48.09	18.8786
MC-5%[a]	91.89	6.55	34.17	10.80	2.60	45.88	18.4083
MC-20%	91.66	6.52	38.15	9.04	2.20	44.09	18.5064
Arthrospira-20%	91.82	6.99	40.25	9.70	2.00	41.05	18.8217

[a] MC = microcystis biomass.

with growth, the feed conversion ratio (FCR) was determined as feed consumed (dry, g)/(final body wt − initial body wt) [25].

Gene expression of hypophyseal GH and hepatic IGF-I. Gene expression was determined by two-step RT-qPCR. The RNA extractions were carried out with approximately 10 mg liver samples using phenolic extraction by Trizol (Gibco BRL) [34] and whole pituitaries by RNAeasy Mini kit according to the manufacturer's protocol (Qiagen), with an additional DNAse digestion [35]. The RNA integrity of samples was evaluated with a RNA 6000 Nano Lab Chip using the Agilent 2100 bioanalyzer (Agilent Technologies) for a subset of randomly chosen samples per experimental group and sampling point ($n = 2$ samples from a total of eight). The RNA integrity numbers [36] were >9.0, confirming the high quality of RNA. Reverse transcription of liver mRNA was performed using avian myeloblastosis virus reverse transcriptase (AMV-RT; Finnzymes; conditions followed a protocol described elsewhere [34]), whereas hypophyseal mRNA was reversely transcribed using affinity script (Boehringer) according to the manufacturer's protocol. A control RT-PCR (–RT) was included in which the enzyme RT was replaced by distilled water to exclude any potential DNA contamination during extraction. Primers for GH and IGF-I were designed on the tilapia sequences submitted to the National Center for Biotechnology Information (NCBI; Table 2). For use of polymerase α (pol) as housekeeping gene, selected sequence information from teleosts was used to calculate a multiple alignment with BioEdit 7.0.0 [35] and to design consensus primers with Oligo 5.0 (National Biosciences). After PCR [95°C for 3 min, 35 cycles: 95°C for 30 s, Ta for 30 s, 72°C for 1 min, 72°C for 5 min; 1.5 mM $MgCl_2$, 1 × PCR buffer, 0.3 μM primer, 0.2 mM dNTP, 0.7 U GoTaq, 3 μl cDNA], sequencing was carried out with a capillary sequencer CEQ 8800 (Beckman Coulter) according to the manufacturer's protocol. Sequences were analyzed by the BLAST search tool [35] and by multiple alignment comparison in ClustalX (BioEdit 7.0.0).

Real-time quantification

With hot-start platinum Taq polymerase (Invitrogen), qPCR was carried out with SYBR Green in a Stratagene MX3000P cycler under the following conditions: 94°C initial denaturation for 5 min 40 s, followed by 40 cycles of 94°C denaturation for 30 s, 58°C primer annealing for 30 s, and 72°C polymerization for 40 s (final concentration: 3 mM $MgCl_2$, 1× PCR buffer, 1× SYBR Green, 0.4 μM primer, 0.3 mM dNTP, 1 U Taq, 4 μl cDNA). For each run, relative quantification was calculated from a coamplified standard dilution series (cDNA obtained from a pooled liver or pituitary sample, respectively), compensating for lot-to-lot and run-to-run variations [37]. For normalization, pol was used as housekeeping gene [35]. All samples were performed in duplicate.

Statistical analysis

Results are presented as the mean ± standard deviation of eight individuals. Data were analyzed for normal distribution by Kolmogorov–Smirnov and equal variance by Kruskal–Wallis one-way analysis of variance (passed if $p < 0.05$) in SigmaStat 2.0 software (Jandel Scientific). Statistical comparisons were carried out by nonparametric Mann–Whitney rank sum test or by parametric Tukey's multiple-comparisons test.

RESULTS

Throughout the experiments, water parameters were successfully maintained in the optimal range for oxygen (89% ± 5%), pH (7.79 ± 0.19), water temperature (26°C ± 0.5°C), N-NH_4^+ (0.1 ± 0.1 mg/L), and N-NO_2^- (0.25 ± 0.15 mg/L). Good husbandry was also indicated by the 100% survival rate. However, it was obvious at the start of the experiment that only control fish ate as usual, whereas all groups receiving modified diets had difficulties for the first couple of days in eating the altered food composition. A further noteworthy observation was that swimming behavior was altered in the last MC-20% group after 21 d, when individuals started to group in a corner and to swim in a more horizontally oriented manner.

MC-LR determinations in diets, fish, and filter scums

Microcystin-LR was not detected in the control diet or Arthrospira-20%, whereas MC-5% and MC-20% contained considerable amounts of MC-LR, with 4.92 μg/g and 19.54 μg/g, respectively. In tissue samples MC-LR was found only in liver after 1, 7, and 28 d, having mean values ± SD of 0.433 ± 0.421 after 1 d, 0.494 ± 0.362 after 7 d, and 0.223 ± 0.069 μg/g after 28 d of exposure in the MC-5% group and of 0.118 ± 0.069 after 1 d, 0.443 ± 0.445 after 7 d, and 0.162 ± 0.153 μg/g after 28 d of exposure in the MC-20% group. In both gill and muscle samples, the MC-5% and MC-20% groups did not indicate any presence of MC-LR by HPLC having a quantification detection limit of 3 ng/g sample weight. Furthermore, no accumulation of MC-LR was detected in the scums of all closed-recirculation systems, not even in the MC-5% and MC-20% groups at any sampling time, indicating that MC-LR provided by the diets is not released or excreted into the water of the closed-recirculation system in measurable amounts.

Stress parameters

Mean levels of the stress hormone cortisol were elevated after day 1 in fish that consumed all diets other than the control diet (Fig. 1A). After day 7, all groups including control had

Table 2. Primer sequences for Nile tilapia (*Oreochromis niloticus*)

Target	Primer	Sequence	Position	T (°C)	International access number (ACCN)
Polymerase α (pol)	pol forward (f)	GACACAGGATGAATTGGACCAG	60		
	pol reverse (r)	ACTGTTTTGCTGAGGGTGTCA	326	58	EU878756
Insulin-like growth factor I (IGF-I)	IGF-1 f	AGTTTGTCTGTGGAGAGCGAG	176		
	IGF-1 r	GTGTGCCGCTGTGAACG	362	58	EU272149.1
Growth hormone (GH)	GH f	CTGCTGATCAGGGCCAATC	394		M97765.1
	GH r	TCGACATTTAGCTACCGTCAGG	585	58	M97766.1

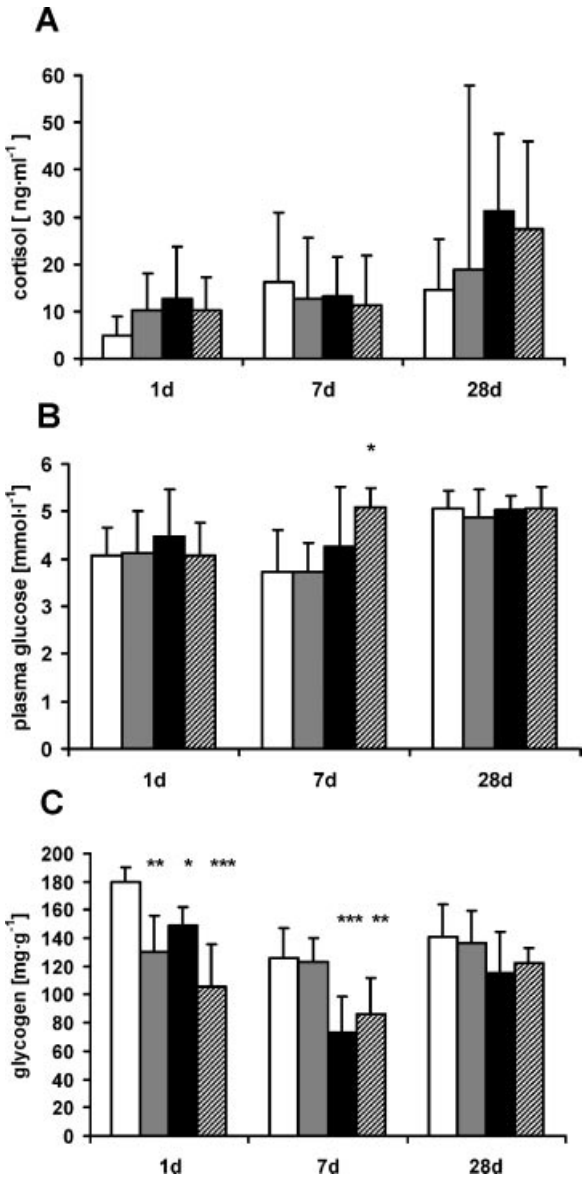

Fig. 1. Impacts of microcystin-LR (MC-LR)-containing diets on plasma cortisol (**A**), plasma glucose (**B**), and hepatic glycogen (**C**) levels in female Nile tilapia. Fish were fed with diets based on a commercial diet (control, open bars), and three variants by which the control diet was in part replaced by 5% (MC-5%, shaded bars) or 20% (MC-20%, solid bars) dried biomass of *Microcystis sp*. containing considerable amounts of MC-LR and by 20% dried biomass of the nontoxic cyanobacterium *Arthrospira* sp. (Arthr-20%, hatched bars). Data represent mean values + standard deviation ($n = 8$) sampled after 1, 7, and 28 d after the start of the feeding experiment. Statistical comparison was performed by one-way analysis of variance, followed by Tukey's multiple-comparisons test, and significant differences compared with controls are marked by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

higher cortisol mean values at a very similar level, whereas, at the end of the experiment at day 28, the control remained at that level, but MC-5% and MC-20% demonstrated an increasing trend, and Arthrospira-20% also had a higher level, reaching nearly the cortisol concentration of the MC-20% group. However, no significant differences concerning cortisol among any groups were detected at any sampling time.

Plasma glucose values (Fig. 1B) displayed a somewhat distinct pattern compared with cortisol. After day 1, only the MC-20% group showed a tendency to elevate glucose levels, whereas, after 7 d, especially the Arthrospira-20% group showed the highest glucose values, being significantly higher compared with control and MC-5%, whereas MC-20% resulted in values ranging between these values. After 28 d, glucose levels were similar in all four diet groups, without any noticeable difference.

Hepatic glycogen values (Fig. 1C) displayed a somewhat opposite trend and a more sensitive pattern compared with plasma glucose levels. After day 1, fish in all the experimental diets had significant lower values compared with the control. However, after 7 d, only fish in the 20% cyanobacteria replacement groups, MC-20% and Arthrospira-20%, were significantly lower than the controls. Finally, although both 20% groups were still less than controls at 28 d, the differences were not statistically significant from controls.

Growth parameters

No statistically significant differences in measures of the general status of fish as determined by CI and HSI were observed at any sampling point among the four different diets, suggesting only minor or negligible impacts on general physiological performance. Measurement of the total weights of all four diet groups revealed growth values that could be subdivided into three phases during the time course (Fig. 2A). In the first phase during the first 7 d, control diet demonstrated the biggest increase, whereas all diets resulted in similarly decreased growth. In the second phase after one week until day 21, the growth with all four diets had a very similar pattern, whereas, in the last week from day 21 to day 28, the growth of control, MC-5%, and Arthrospira-20% were in the same range, whereas MC-20% showed a decreasing tendency. The FCR after 7 d displayed the lowest value for control, followed by Arthrospira-20%, and at a marked distance by MC-5% and MC-20%. For days 14 and 21, the FCR values were in a similar range, and, after 28 d, FCR values had a ranking of control $<$ MC-5% $<$ Arthrospira-20% $<$ MC-20% (Fig. 2B), implying that the MC-20% group requires the highest FCR during longer term feeding.

Gene expression of growth parameters

Measurement of hypophyseal GH gene expression (Fig. 3A) by real-time RT-qPCR indicated no significant changes between any of the treatments at any of the sampling points. The only difference observed was after 7 d, when the mean value of GH mRNA in the control was markedly elevated compared with the other treatments, but this difference was not statistically significant. A similar pattern was observed for hepatic IGF-I mRNA expression (Fig. 3B), in which no significant differences between treated diets and controls were observed at any sampling points.

DISCUSSION

Cyanotoxins derived from cyanobacteria are well known to cause deleterious effects in many organisms. Microcystins, especially MC-LR, that are produced by several species of *Microcystis* in considerable amounts during cyanobacterial water blooms are well characterized concerning their hepatotoxic effects in mammals, and it is noteworthy that the toxic impacts of MC-LR varying greatly as a result of the chosen exposure route [38]. In rodents, the greatest toxic potency has

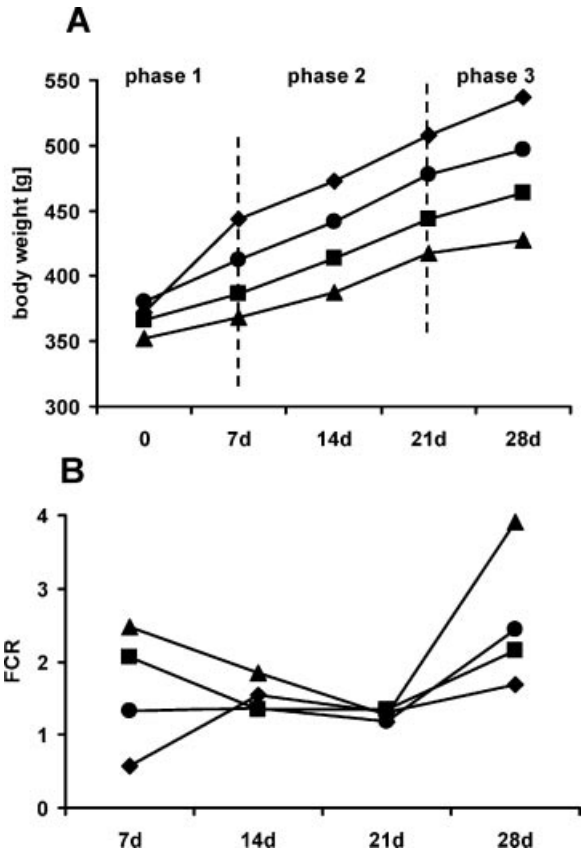

Fig. 2. Growth indicated by total weight (**A**) and feed conversion ratio (FCR; **B**) of the feeding experiment in which the impacts of control (diamonds), microcystin (MC)-5% (squares), MC-20% (triangles), and Arthr-20% (circles) diets were recorded as total weight of eight individuals only for the last sampled groups after 7, 14, 21, and 28 d.

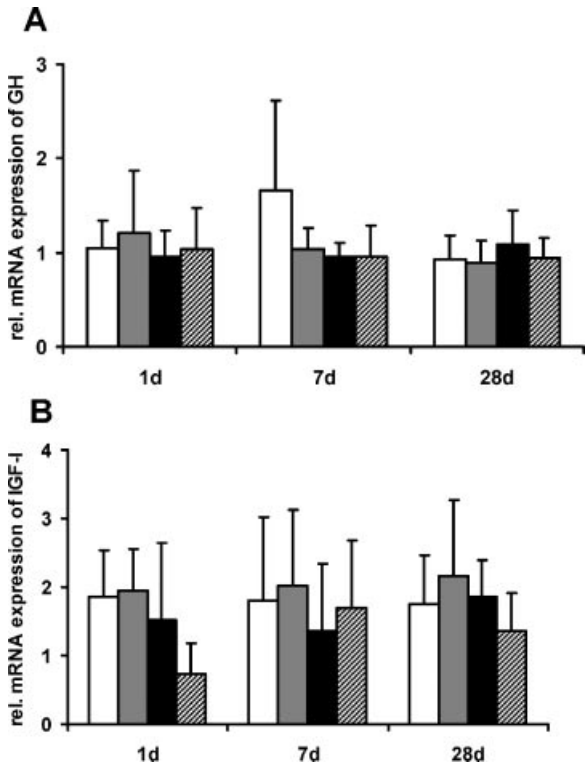

Fig. 3. The values corresponding to Figure 1 for gene expression of the target genes, hypophyseal growth hormone (GH; **A**) and hepatic insulin-like growth factor-I (IGF-I; **B**) normalized to the housekeeping gene polymerase α are shown and analyzed according to the stress parameters indicated in Figure 1 (mean + standard deviation, $n = 8$). Control (open bars), microcystin (MC)-5% (shaded bars), MC-20% (solid bars), and Arthr-20% (hatched bars).

been observed following intraperitoneal injections of MC-LR, whereas exposure to MC-LR via inhalation or especially ingestion results in a dramatic decrease in toxicity. However, whereas the primary targets of naturally occurring MCs that are the result of cyanobacterial water blooms are aquatic organisms such as fish, only a few studies have been conducted to investigate the impact of MCs on fish. Furthermore, most of these studies considered exposure to MC-LR only via intraperitoneal injection [20]. Information on effects of MC via the exposure routes such as water [39] or by ingestion [40] is rather limited in fish. However, a pattern of MC toxicity similar to that in mammals might also exist in fish, suggesting that the natural exposure routes might be much less harmful compared with intraperitoneal application. Thus, the question arises of the extent to which fish might be able to cope with MCs during natural exposure routes, especially via ingestion. To date, only one study has addressed the issue of fish diets containing MCs and their effect on growth. In this study, feed containing MC resulted in higher growth rates in Nile tilapia compared with the control diet, suggesting that cyanobacteria dry mass could become an efficient supplementary alternative for fish meal in fish diets [40]. However, the authors measured relatively high concentrations of MC in several tissues, including muscle by enzyme-linked immunosorbent assay (ELISA), suggesting that the MC contents in muscles of MC-fed tilapia are too high for human consumption.

The current investigation sought to determine whether fish diets containing MC affect the physiological performance of Nile tilapia as evaluated by stress and growth endpoints. To our knowledge, this is the first study in fish that has assessed the stress response to MC by measuring the stress hormone cortisol, which serves as general indicator for stress conditions in fish [32,33]. In addition, the general performance of growth by classical parameters such as growth and feed conversion ratio has been determined, but the current aim was to investigate for the first time the potential impacts of MC on the molecular mechanisms underlying the growth axis by determining gene expression of hypophyseal GH and hepatic IGF-I by RT-qPCR.

The HPLC determinations revealed that only in the two groups receiving *Microcystis*-containing diets, MC-LR could be detected in liver after 1, 7, and 28 d. Surprisingly, only low MC-LR concentrations without a dose response of MC-LR accumulation has been observed between MC-5% and MC-20% diets. It seems that the highest values are obtained after one week, whereas hepatic MC-LR contents decreased at the end of the experiment, suggesting a metabolic adaptation to cope with MC-LR uptake via ingestion. It would be of major interest to investigate the relevant detoxification enzymes associated with oxidative stress [22] and glutathione-*S*-transferase [24] to prove that the low accumulation of MC-LR in Nile tilapia is due to adapted metabolism rather than different digestibility of cyanobacteria. The current results concerning feed conversion ratio suggest that the cyanobacteria-supplemented diets have

slightly lower digestibility. Nevertheless, such low changes in digestibility cannot cause such a low accumulation of MC-LR, so this seems to favor the hypothesis that metabolic adoption is the main trigger to reduce MC-LR accumulation in Nile tilapia. Neither in gill nor in muscle could MC-LR be determined, indicating that in these tissues MC-LR was always clearly below the detection limit of 3 ng/g tissue. However, considering the World Health Organization recommendation for daily MC-LR uptake of humans of 0.04 μg/kg/day in the worst-case scenario, calculating that the muscles would contain 3 ng/g, a daily uptake of more than a 900-g filet would be allowed per person weighing 70 kg. If there might still be found some considerable amount of cyanotoxins and metabolites, then it might be interesting to determine how long it might take to depurate MC-LR, its metabolites, or further cyanotoxins from different tissues by rearing fish in clear water without MC-LR to allow later consumption without any risk for humans. An extensive study by Chinese colleagues [40] determined much higher concentrations of MCs in several tissues, including muscle, as determined by ELISA, following feeding of diets containing lower amounts of MCs than those used in the current study, and their conclusion was that these fish cannot be eaten by humans. The differences between the two studies might be due to the use of the different methods determining MCs as well as metabolites by ELISA versus MC-LR by HPLC, to the physiological status potentially varying because of different age and size of the tilapias used, and/or to the duration of exposure to the MC-containing diet.

At the beginning of the experiment, the fish fed the prepared diets supplemented with dry cyanobacterial biomass (MC-5%, MC-20%, Arthrospira-20%) did not feed as well as the control group, which might indicate that the change in the diet resulted in stress that inhibited feed uptake [41]. However, the cortisol data indicate only a minor or moderate stress level in tilapia, similar to that in controls, because no statistical differences were observed during any sampling points between any of the groups (Fig. 1A). Furthermore, a general increase in cortisol levels in the controls was observed, which might have been caused by higher rearing densities resulting from fish growth throughout the experiment. However, at study termination, higher mean values of cortisol in fish fed both diets supplemented with 20% dried cyanobacterial biomass relative to control or 5% MC diets were observed. Thus it seems that the different feed supplementations are more important concerning potential stress by affecting taste or visual preference by different color instead of toxicity stress caused by MC-LR.

Plasma glucose was also used to evaluate stress in fish. Plasma glucose has been measured as an indirect indicator of stress, because it is known that glucose levels are increased by the stress hormones, corticosteroids such as cortisol, and especially by the catecholamines such as adrenaline and noradrenaline [42]. The pattern of blood glucose (Fig. 1B) was also similar to that of cortisol (Fig. 1A), but plasma glucose seemed to be a more sensitive biomarker, because significant elevations were obtained in both groups with diets supplemented by 20% dried biomass of cyanobacteria. At the end of the experiment, glucose levels were similarly high in all four groups, supporting our interpretation that the high supplementation by 20% of dried cyanobacterial biomass might be the most important stressor regardless of the contents of MC-LR, and finally all four groups seem to be under elevated stress conditions because of higher rearing densities.

Liver glycogen revealed a pattern somewhat opposite to that of plasma glucose but was the most sensitive parameter following change of diets, resulting in decreased glycogen levels already after 1 d with all diet variants. Glycogen depletion might have been caused in particular by short-term stress inducing high values of the catecholamines, epinephrine and norepinephrine, inducing very efficiently glycogenolysis in teleost liver [38], to provide enough glucose in blood circulation to scope with any kind of stressor. The fact that the MC-20% groups after 7 and 28 d reached the lowest values suggests not only that the marked feed supplementation by cyanobacteria biomass has an impact as a stressor but also that the content of the cyanotoxin MC-LR at least at the higher concentration used here might require a more pronounced energy consumption to deal with metabolism and detoxification of that toxic compound.

Fish growth was divided into three phases: adaptation to changed diets, growth at a comparable feeding rate, and decrease of growth rates resulting from limited space in the aquaria. At the beginning of the experiment, the fish fed the prepared diets supplemented with dry cyanobacterial biomass did not feed as well as the control group, and it took approximately one week for the fish to adapt their feeding behavior to that of the controls. This change in feed consumption seems to be the reason for the differences in growth observed after one week (Fig. 2). However, after one week, feed consumption became similar in fish from all four diets, resulting in comparable growth rates from week 1 to week 3. Only during the last week were growth rates decreased in all groups, probably because of limited space in the aquaria. In addition, the MC-20% group displayed a slightly lower growth rate for the last week, suggesting that high supplementation of diet with *Microcystis* biomass might inhibit growth by additional mechanisms, such as loss of energy for enhanced hepatic metabolism to cope with MC-LR and further metabolites by detoxification. During the last phase, analyses of the feed conversion ratio resulted in a clear ranking of control < MC-5% < Arthrospira-20% < MC-20%, indicating that the different supplemented diets impact feeding behavior adversely, which in turn also leads to higher feed conversion ratios. During the last week of experimentation, the MC-20% group displayed unusual swimming behavior when individuals started to group in a corner of the experimental aquarium and to swim in a more horizontally oriented way, indicating adverse impacts.

To evaluate potential impacts of fish diets on mechanisms underlying growth regulation, gene expression of the two main endocrine factors associated with growth, hypophyseal GH and hepatic IGF-I [39], were determined. Real time RT-qPCR demonstrated neither for GH nor for IGF-I any significant changes concerning mRNA compared with controls. Even correlations of gene expression between GH and IGF-I (not shown) did not result in significant differences among the fish diets at any sampling point. To our knowledge, it is the first study aiming to determine gene expression of the growth axis of fish related to MC exposure. However, it seems obvious that, despite the fact that the established methodology is very sensitive concerning quantification of mRNAs, the individually existing variations concomitant with high standard deviation

concerning gene expression do not allow the most sensitive determination of impacts on the growth axis. Therefore, surprisingly, the classical methodology of weighing the animals for calculation of growth and feed conversion ratios had more relevance for determining impacts of different diets on growth, so potential impacts on gene expression of hepatic IGF-I might be analyzed in a better way by using in vitro approaches such as the use of primary hepatocytes [43]. In contrast [41], we could not detect any growth-promoting effect of food supplementation by *Microcystis,* and even a slightly reversed impact at least for the MC-20% group was observed, which it can be expected was due to the relative toxicity of the MC-LR ingested.

In summary, mainly the feed supplementation by dried cyanobacterial biomass causes minor to moderate stress, surprisingly in a manner nearly independent of any MC-LR content of the diet as revealed by plasma cortisol, glucose, and hepatic glycogen. Only hepatic glycogen suggests that the potential toxic impacts of MC-LR at the higher concentration require more energy to cope with its detoxification. The concentrations of MC-LR found in tilapia after feeding with MC-supplemented diets seem to be relatively low, but this requires further detailed investigation of whether tilapia can adapt their metabolic pathways in such a way that MC-LR concentrations in muscle might be below the limit of the World Health Organization recommendation for human food. Another possibility would be to clear potentially accumulated MC by rearing the fish for some time in clear water before it becomes consumable, without any risk for humans [39]. The impacts on growth, leading to lower feed conversion ratios by all supplemented diets, were due mainly to behavioral changes induced by switching to different diets, which might affect taste. Only the MC-20% group displayed at the end of the experiment a strange swimming behavior, suggesting additional adverse effects caused potentially by toxic effects of MC-LR or other cyanotoxins. However, the MC-5% as well as Arthrospira-20% groups seemed to be adapted similarly to the diet changes after one week revealing growth similar to that in the control. Thus it seems feasible that MC-LR containing dried cyanobacterial biomass might be used in fish diets for Nile tilapia, which requires further research in greater detail to address very carefully this important issue for aquaculture.

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