Fish tapeworm *Khawia sinensis*: an indicator of environmental microcystins?

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Abstract

OBJECTIVES: Parasites have recently been recognized as accumulation indicators that take up and bio-concentrate substances from environmental pollution. Interestingly, helminths of fish are known to accumulate metals from the ambient environment and to contain several orders of magnitude higher concentrations than hosts. While the majority of reports mention inorganic toxin accumulation in parasites, studies concerning effects of organic pollution are infrequent and little is known about the potential of parasites to bio-accumulate microcystins.
METHODS: The parasite-host system of tapeworm *Khawia sinensis* and common carp (*Cyprinus carpio*) was used to address this issue. Both the tapeworms and livers were dissected from experimental carps orally exposed to cyanobacterial biomass for 20 days. The total dose of microcystins amounted to 27 mg/kg of feed, i.e., 0.4 mg/kg of fish mass a day. Microcystin concentrations in tapeworms and carp liver tissues were measured using the LC-MS/MS method.
RESULTS: Considering the three measured microcystin variants LR, YR and RR, only MC-RR was detected and its concentrations in tapeworms and carp liver tissue amounted to 5.78±3.78 ng/g and 2.11±0.74 ng/g fresh weight ($p<0.05$), respectively.
CONCLUSIONS: Here we show accumulation of microcystin MC-RR in the tapeworm *Khawia sinensis*, a parasite of common carp (*Cyprinus carpio*). As this is the first report addressing this issue, further studies will be necessary to examine this specific parasite-host system.

ORIGINAL ARTICLE

Abbreviations:

Adda	- 3-amino-9-methoxy-2,6,8-trimethyl-10-fenyldeka-4,6-dien acid
ANOVA	- analysis of variance
BE	- brutto energy
ESI	- electrospray ionization
HPLC	- high performance liquid chromatography
LC-DAD	- liquid chromatography with diode array detector
LC-MS/MS	- liquid chromatography with double mass spectrometry
LSD test	- the least significant difference test
MC	- microcystin
MC-RR	- microcystin RR
MC-YR	- microcystin YR
MC-LR	- microcystin LR
MDL	- method detection limit
MRM	- multiple reaction monitoring mode
NFE	- nitrogen free extract

INTRODUCTION

It has recently been shown that parasites can be indicators of ecosystem health. Considering their biology and life histories, environmental degradation can be measured by changes in parasite populations and communities (Marcogliese 2005). As wildlife can be exposed to natural toxins, anthropogenic pollutants and infectious agents at the same time, the issue of multiple stressors has drawn attention of ecotoxicologists (Pikula *et al.* 2010; Bandouchova *et al.* 2011; Ondracek *et al.* 2012; Osickova *et al.* 2012). A combination of effects of environmental stressors and parasites often results in an increased mortality and animal health reduction (Marcogliese & Pietrock 2011). For example, experiments with amphibians demonstrate that two stressors are deadlier than one and more realistic under natural conditions (Relyea & Mills 2001; Sih *et al.* 2004).

On the other hand, studies of effects and interactions between parasites and the presence and concentration of various pollutants and/or environmental stressors suggest that parasites can be useful effect or accumulation bio-indicators of environmental impact (Sures 2004; Vidal-Martinez *et al.* 2010). There are numerous reports on the accumulation of heavy metals in fish parasites such as nematodes, cestodes and acanthocephalans (Sures 2004). Orders of magnitude higher concentrations of heavy metals in the parasites provide evidence that these parasite-host systems might be valuable for environmental pollution monitoring (Sures *et al.* 1999; Barus *et al.* 2007). However, metal and parasite-host system specific interactions and mechanisms have to be considered (Oyoo-Okoth *et al.* 2010).

While the majority of reports mention inorganic toxin accumulation in parasites, studies concerning organic pollutants of sentinel parasites are infrequent (Sures 2004). To the best of our knowledge, the potential of parasites to bio-accumulate microcystins, potent toxins produced worldwide by cyanobacteria during bloom events (Dawson 1998; Amado & Monserrat 2010), has not yet been examined. For this purpose, therefore, we used the parasite-host system of tapeworm *Khawia sinensis* and common carp (*Cyprinus carpio)* and compared microcystin concentrations in tapeworms and carp liver tissue.

MATERIAL AND METHODS

Experimental fish and design

The common carp (*Cyprinus carpio*) with an average body mass of 233±66 g were obtained from the Pohorelice Fishery, placed into laminated circular tanks equipped with independent water recirculation of 1 m^3 volume, and allowed to adapt for experimental conditions for 14 days. Commercial granulated diet Dibaq Carpio Plus (Spain, 35% proteins, 9% fat, 29.5% NFE, BE 24.4 MJ/kg) was provided during this period. After the adaptation period they were randomly divided into 2 groups of 40 specimens (i.e., control and experimental ones). While the control fish were given the commercial feed, the experimental carp were fed the same diet with the addition of 1% of lyophilised toxic cyanobacterial biomass. A plankton net (25 μm) was used to collect the cyanobacterial biomass from the Musovska reservoir (Czech Republic) in summer 2007. The biomass included *Microcystis aeruginosa* (90%) and *M. ichthyoblabe* (10%). The biomass was frozen after collection and stored at –20 °C. It was lyophilized using Christ Gamma 1-16 LSC Freeze dryer (Osterode am Harz, Germany) prior to the experiment. The total amount of microcystins was 27 mg/kg of feed, i.e. 0.4 mg/kg of fish weight and day. Microcystin structural variants in the above dose of 27 mg included 11.64 mg MC-RR, 3.34 mg MC-YR and 12.02 mg MC-LR. Microcystin contents in biomass were analysed using LC-DAD (Agilent 1100 Series, Agilent Technologies, Japan) on a Supelcosil ABZ+Plus column, 150×4.6 mm, 5 μm according to Babica *et al.* (2006) with detection limit of 15 μg/kg dry matter. Feeds were supplied twice a day and amounted to 1.5% of the fish stock. The ration was changed on a weekly basis to take actual fish weights into account. Common carp were killed after the 20-day period of exposure to cyanobacterial biomass, underwent a necropsy and samples of liver tissue were collected. These were stored at –20 °C until analysis. Tapeworms isolated from the gastrointestinal tract were identified as *Khawia sinensis* and also stored for toxicological analysis. Prior to storage, tapeworms were washed by surplus physiological saline to remove external contamination.

The above-experiment was performed in compliance with the laws for the protection of animals against cruelty as approved by the Ethical Committee of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

Laboratory analyses

Microcystin concentrations in samples of liver tissue and tapeworms were analysed by LC-MS/MS method according to Kohoutek *et al.* (2010). Tissues (frozen sample; 0.5 g fresh weight) were homogenised thrice with methanol (3 mL), sonicated in an ultrasonic bath

for 30 min, and centrifuged at 2 900 *g* for 10 min. Supernatants were pooled and extracted repeatedly (3 times) with hexane (1 mL) to remove lipids. Extract was evaporated at 50 °C, and the residue was dissolved in 300 µL of 50% aqueous methanol (v/v) and used for LC-MS/MS analyses. Analyses were based on Liquid Chromatography Mass Spectrometry (MS/MS) with MRM (multiple reaction monitoring mode) using the HPLC apparatus Agilent 1200 series (Agilent Technologies, Waldbronn, Germany), which consisted of a vacuum degasser, a binary pump, an autosampler, and a thermostated column compartment kept at 30 °C. The column was a Supelcosil ABZ+Plus RP-18 endcapped (5 µm) 150×4.6 mm i.d. (Supelco). A SecureGuard C18 (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM ammonium acetate in water, pH 4 (A) and acetonitrile (B). The binary pump gradient was linear (increase from 20% B at 0 min to 59% B at 30 min, then 90% B for 15 min); the flow rate was 0.4 mL/min. A total of 20 µL of individual sample were injected for the analyses. The mass spectrometer was an Agilent 6410 Triple Quad mass spectrometer (Agilent Technologies, Waldbronn, Germany) with electrospray ionization (ESI). Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350 °C; desolvation gas flow, 11 L/min. The transitions from the protonated molecular ion to a fragment of amino acid Adda (unusual aminoacid present only in microcystins and related nodularins – (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) at m/z 135.2 and fragment at m/z 127.1 were monitored in multiple reaction monitoring (MRM) mode. Collision energies (CE, V) used for fragmentation were 50 V for MC-RR and respective conjugates, and 40 V for MC-YR and -LR and respective conjugates). Quantification of analytes was based on external standards of MC-RR, MC-YR, MC-LR in matrix (final extract of microcystin-free fish tissue). Method detection limit (MDL; per gram of tissue, fresh weight) was 1.20 ng/g fw for MC-RR and 5.4 ng/g fw for MC-LR and MC-YR.

Statistical analysis

Statistica for Windows® 10 (StatSoft, Inc., Tulsa, OK, USA) was used to compare different experimental groups using procedures such as one-way analysis of variance (ANOVA), post-hoc analysis of means by the LSD test, Levene's method to test for the homogeneity of variances, log-transformation of non-homogenous parameters prior to analysis and comparison with the non-parametric Kruskal-Wallis test. The levels of significance used were either $p<0.05$ or $p<0.01$.

RESULTS AND DISCUSSION

As shown in Figure 1, microcystin RR concentrations in tapeworms *Khawia sinensis* were nearly thrice higher than in liver tissues of carps exposed to cyanobacterial biomass for 20 days. Values of MCs in liver and tapeworms of control group fish were under detection limit. The prevalence of infection by tapeworms was 7.5% and 30.0% in control and cyanobacterial-biomass-exposed groups, respectively. While the cyanobacterial biomass added to the fish diet contained three microcystin variants and the contents of MC-LR and MC-RR were nearly equal, only MC-RR was detected both in the fish tissue and tapeworms. This fact may be due to differences in the detection limits of these toxin structural variants.

The parasite-host system included common carp (*Cyprinus carpio)* infected naturally with tapeworms *Khawia sinensis.* This was recognised during the necropsy of experimental carp used in another study (Palikova *et al.* 2012). In general, cestodes are widespread, prevalent in their fish host populations, large-sized to provide sufficient material for chemical analysis and can be associated with environmental pollution (Oros & Hanzelova 2009; Oros *et al.* 2009).

Higher microcystin levels detected in this gastrointestinal parasite are in agreement with the earlier described phenomenon of heavy metal bio-concentration linked to parasites with an intestinal location (Sures *et al.* 1999). This is also understandable in toxins entering the organism via oral route. Unfortunately, concentrations of microcystins in the intestinal wall were not analysed to provide another aspect of toxin distribution in the tapeworm and host tissues.

While environmentally relevant doses of cyanobacterial biomass containing microcystins were employed in this experiment (Pikula *et al.* 2010), responses to high-dose exposure might provide a more pronounced insight into the effects, accumulation and distribution of microcystins both in the parasite and its host (Kral *et al.* 2012).

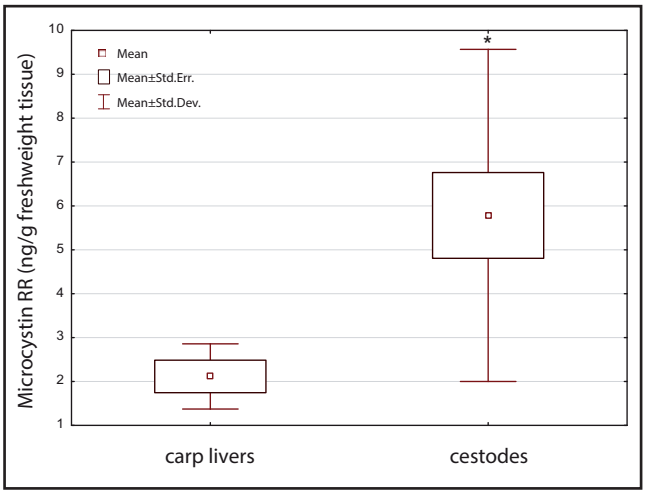

Fig. 1. Concentrations of microcystin RR in samples of liver tissue of common carp exposed to cynobacterial biomass for 20 days compared with those measured in tapeworms *Khawia sinensis* from their gastrointestinal tract. *$p<0.05$, n=40 common carps and 12 tapeworm samples.

On the other hand, cyanobacteria contain a great number of compounds of potential biological activity with various mechanisms of action and some studies have documented significant biological effects independent of the known cyanotoxins content (Damkova *et al.* 2009). Their impact on the parasite-host system is largely not known.

A sentinel parasite, when an ideal accumulation indicator, must be efficient in the uptake and bio-concentration of substances from environmental pollution without showing signs of toxicity (Sures 2004; Vidal-Martinez *et al.* 2010). Adverse effects or responses of tapeworms to exposure by microcystins, however, were not part of the present study. As documented by the data showing only a threefold magnitude of bioconcentration of microcystin RR, tapeworms *Khawia sinensis* cannot be considered an effective indicator.

CONCLUSIONS

As little is known about the potential of parasites to bioaccumulate microcystins, this is the first report addressing this issue. Further studies, however, are necessary to examine the effects of microcystins in parasites as well as dose-dependent responses and to promote this new field of environmental parasitology.

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