

Original Article

Are little brown bats (*Myotis lucifugus*) impacted by dietary exposure to microcystin? [☆]

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ABSTRACT

The cyanobacterium, *Microcystis aeruginosa*, can produce the hepatotoxin microcystin. When toxic *M. aeruginosa* overwinters in the sediments of lakes, it may be ingested by aquatic insects and bioaccumulate in nymphs of *Hexagenia* mayflies. When volant *Hexagenia* emerge from lakes to reproduce, they provide an abundant, albeit temporary, food source for many terrestrial organisms including bats. Little brown bats, *Myotis lucifugus*, feed opportunistically on aquatic insects including *Hexagenia*. To determine if microcystin moves from aquatic to terrestrial ecosystems via trophic transfer, we combined a dietary analysis with the quantification of microcystin in bat livers and feces. In June 2014, coincident with the local *Hexagenia* emergence, bat feces were collected from underneath a maternity roost near Little Traverse Lake (Leelanau County, Michigan, USA). Insects in the diet were identified via molecular analyses of fecal pellets from the roost and from individual bats. Livers and feces were collected from 19 female *M. lucifugus*, and the concentrations of microcystin in these liver tissues and feces were measured using an enzyme-linked immunosorbent assay (ELISA) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). We show that the majority of the bats' diets consisted of aquatic insects and that microcystin was detected in high concentrations (up to 129.9 µg/kg dw) in the bat feces by ELISA. Histopathological examination of three bat livers with the highest concentrations of microcystin showed no evidence of phycotoxicosis, indicating that *M. lucifugus* may not be immediately affected by the ingestion of microcystin. Future work could examine whether bats suffer delayed physiological effects from ingestion of microcystin.

1. Introduction

As blooms of cyanobacteria become more common in freshwater ecosystems due to factors such as global climate change (Paerl and Huisman, 2008), eutrophication (Huisman et al., 2005; Paerl and Fulton, 2006), and invasive species (e.g., zebra mussels, Vanderploeg et al., 2001), harmful algal blooms pose a threat to both aquatic and terrestrial ecosystems. Several genera of cyanobacteria produce neurotoxins and hepatotoxins that have resulted in poisonings of both humans and animals (Carmichael and Boyer, 2016; Kuiper-Goodman et al., 1999). *Microcystis aeruginosa*, a common bloom-forming cyanobacterium, is typically found in phosphorus-rich (eutrophic) fresh water (Woller-Skar, 2009). However, research conducted in Michigan lakes has shown

that invasive zebra mussels can promote the growth of *M. aeruginosa* (Vanderploeg et al., 2001), particularly in nutrient-poor (oligotrophic) basins (Raikow et al., 2004). Zebra mussels may selectively reject toxic *Microcystis* due to its large colonial size and poor nutritional quality, giving *Microcystis* spp. a competitive advantage (Vanderploeg et al., 2001). *Microcystis* also has an array of nutrient uptake strategies that allow it to use multiple forms of nitrogen and phosphorus (Marinho and de Oliveira e Azevedo, 2007; Wan et al., 2019). As a result, *M. aeruginosa* can form unsightly blooms that reflect rapid increases in their population density and result in decreases in water transparency (Woller-Skar, 2009).

In addition to forming blooms, some strains of *M. aeruginosa* produce hepatotoxins called microcystins. Microcystins can cause vomiting, skin

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irritation, liver cancer, and death in humans, pets, livestock, and various aquatic organisms (Carmichael and Boyer, 2016; Chorus and Welker, 2021; Kuiper-Goodman et al., 1999; Silvonen and Jones, 1999). Evidence exists to support both bioaccumulation of microcystins in many aquatic organisms, including zooplankton, crustaceans, mussels, and zooplanktivorous fish (Falconer et al., 1992; Kotak et al., 1996; Kozłowsky-Suzuki et al., 2012; Mohamed, 2001; Vasconcelos, 1995), and biodilution (Ferrão-Filho and Kozłowsky-Suzuki, 2011; Ibelings et al., 2005), including decapods, molluscs, some fishes, turtles, and birds. High concentrations of microcystins were found in nymphs of *Hexagenia*, a genus of burrowing mayfly (Woller-Skar, 2009), as well as in subimago (subadult) and imago (adult) life stages (Woller-Skar et al., 2015, 2020). Mayfly nymphs are extremely resistant to microcystin toxicity (Smith et al., 2008a) and thus provide an excellent pathway for food web transfer of microcystins.

After eggs hatch, newly emerged *Hexagenia* nymphs burrow in the sediments of freshwater sources (Hunt, 1953). The nymphs overwinter in the lake sediments where they consume detritus and algae (Hunt, 1953). There, the nymphs likely ingest *M. aeruginosa*, which also overwinters in lake sediments (Reynolds et al., 1981). *Hexagenia* spend 1–2 years as nymphs, then move to the surface of the water and, within a few days, undergo their first molt and enter terrestrial systems as winged subimagos (Edsall, 2001). Shortly thereafter, the subimagos undergo a second molt to become imagos (Hunt, 1953). The imagos mate during flight and the females lay their eggs in the water to produce a new generation (cohort) and then die (Edsall, 2001). Subimagos and imagos enter the terrestrial food web as potential prey for organisms such as birds and bats. Although the emergence of subimagos and imagos is brief, lasting only a few weeks, it provides copious amounts of food for insectivorous animals. Thus, *Hexagenia* serves as a link between aquatic and terrestrial food webs and may transfer aquatic toxins such as microcystin to terrestrial insectivores including bats.

One of the most common bats in Michigan is the little brown bat, *Myotis lucifugus*. Little brown bats are predators that feed opportunistically on aquatic insects such as mayflies and chironomid flies (Anthony and Kunz, 1977; Belwood and Fenton, 1976; Clare et al., 2011). A dietary analysis in southwestern Ontario found that 66% of the diet of little brown bats consisted of mayflies (Ephemeroptera) during the summer maternity season (Clare et al., 2011). Due to their reliance on aquatic insects, little brown bats may be at a heightened risk of exposure to aquatic ecosystem toxins. Furthermore, the timing of parturition in *M. lucifugus* coincides with the mass emergences of many aquatic insects. Lactating female bats consume more insects than non-reproductive females or males (Anthony and Kunz, 1977), further increasing the likelihood of toxin bioaccumulation.

To date, only two studies have implicated algal toxins in the death of bats (Isidoro-Ayza et al., 2019; Pybus et al., 1986). Both studies recovered bats from bodies of water with distinct blooms of cyanobacteria. Pybus et al. (1986) were unable to confirm algal poisoning, but given the circumstances around the mortality event (i.e., presence of a visible algal bloom and several dead bats floating in the water), algal toxicity was concluded to be the most likely cause of death. Isidoro-Ayza et al. (2019) confirmed high concentrations of microcystins in the gastrointestinal tracts of bat carcasses as well as in the body of water from which the dead bats were collected. These two studies indicate that bats may be directly exposed to algal toxins by drinking contaminated water, given the concurrent presence of algal blooms. However, neither of these studies demonstrate the extent to which indirect exposure to microcystins through diet may affect bat health. By contrast, Woller-Skar et al. (2015) detected microcystins in bat feces collected during an emergence of *Hexagenia*, suggesting that bats may be exposed to microcystins via their diet.

Here, we use chemical and histopathological analyses of *M. lucifugus* livers and feces to determine whether the bats are exposed to or experiencing sublethal effects from microcystin by consuming *Hexagenia* mayflies. We hypothesized that (1) bats are feeding heavily on emerging

Hexagenia, (2) microcystins are present in both bat livers and feces, and (3) increased concentrations of microcystins are associated with liver damage in wild bat populations. This is the first study to attempt to demonstrate a mechanism of bat exposure to microcystins. From these results, we can more fully understand the health risks faced by little brown bats and determine whether new management strategies are warranted to address these risks.

2. Methods

2.1. Study site and sample collection

All samples were collected at a maternity roost in a barn near Little Traverse Lake (Leelanau County, Michigan, USA, Fig. 1). Zebra mussels were introduced into this lake in 1998 and have been established since 2001 (Woller-Skar, 2009). Since the establishment of zebra mussels, *M. aeruginosa* has become the dominant cyanobacterial species in Little Traverse Lake, with blooms occurring each year in August–September (Woller-Skar, 2009). During and after bloom events, this lake (Woller-Skar, 2009) has had concentrations of total microcystin (MC_{tot}) exceeding the U.S. Environmental Protection Agency's (2015) health advisory guideline for safe drinking water at or below 0.3 µg/L or 0.3 ppb. Furthermore, *Hexagenia* emerging from this lake have high concentrations of microcystin at both the nymph (Woller-Skar, 2009) and volant (subimago and imago, Woller-Skar et al., 2015, 2020) stages.

A total of 19 *M. lucifugus* were collected on June 20 (*N* = 9) and 27 (*N* = 10), 2014. After adult bats left the roost, all exits to the barn were blocked and a harp trap was placed in the open doorway to catch bats returning from foraging. Captured *M. lucifugus* were identified to species and placed individually in cotton bags. Individuals were euthanized with isoflurane followed by cervical dislocation. Approximately half of each liver was placed in foil and stored at –20 °C for microcystin analysis. The other halves of the sampled livers were placed in RNAlater and stored at –20 °C. Feces from all individual bats were collected from the cotton bags as defecated material. Approximately 10% of the feces from each bat were placed in 1.5-mL tubes with silica gel desiccant for dietary analysis. The remaining feces were placed in individual foil packets for analyses of microcystins. Fresh feces were also collected from roosting bats by placing foil directly underneath the roosting colony each trap night. Feces that had accumulated on the foil were collected the next day. Approximately half of the feces from the roosting colony were used for microcystin analyses and half were used for dietary analysis.

2.2. Ethics statement

Bats were captured under Michigan Scientific Collector Permit #SC1498. Captured individuals were humanely euthanized with isoflurane followed by cervical dislocation, a protocol approved by the Grand Valley State University Institutional Animal Care and Use Committee (IACUC Approval #14–08-A).

2.3. Dietary analysis

For the fecal samples reserved for dietary analysis, DNA was extracted using a PowerFecal® DNA Isolation Kit (MOBIO) according to the manufacturer's protocol. After extraction, a portion of the mitochondrial cytochrome oxidase I gene was amplified via polymerase chain reaction (PCR) using primers LCO1490 and HCO2198 (Folmer et al., 1994) that yielded a fragment of approximately 650 bp. Each PCR was carried out using illustra PuReTaq Ready-to-go PCR beads (GE Healthcare Life Sciences) with 5 mM forward primer, 5 mM reverse primer, and 1 µL of template DNA in a 25-µL reaction. The thermal cycling conditions of this PCR were as follows: 10 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 48 °C, and 1.5 min at 72 °C with a final extension step at 72 °C for 7 min. All PCRs that showed visible

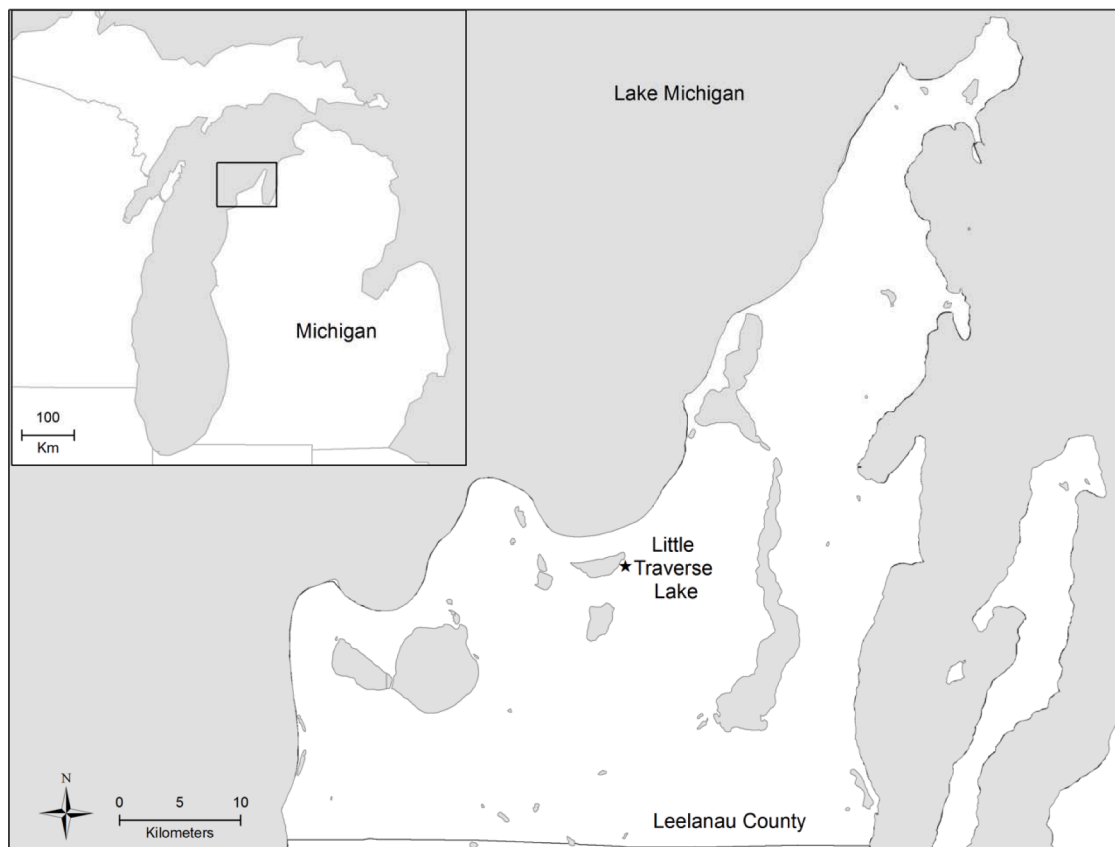


Fig. 1. Map of Little Traverse Lake in Leelanau County, Michigan, USA. The site of the sampled *Myotis lucifugus* roost is indicated with a star next to Little Traverse Lake.

bands of the expected size were used as template DNA in a second PCR with the primers ZBJ-ArtF1c and ZBJ-ArtR2c (Zeale et al., 2011), using the same PCR profile as above and yielding a product of approximately 157 bp. Each PCR was carried out using 1 μ L of product from the first PCR as template with 5 mM of each Zeale et al. (2011) primer in a 25- μ L reaction. Samples that successfully amplified were cleaned using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol to excise fragments of approximately 157 bp.

The PCR products from the second reaction were cloned using the TOPO TA Cloning Kit (Life Technologies). Ligation was performed according to the manufacturer's protocol and using 4 μ L of the cleaned Zeale PCR product with a 3'-A overhang. Transformations were carried out using half the manufacturer's recommended amount of competent cells and SOC media. Colonies were selected using β -galactosidase blue/white screening, and picked colonies were suspended in 20 μ L of dH₂O. Ligated plasmids were liberated by incubating the suspended cells at 95 °C for 10 min. The cell lysate (2 μ L) was used directly as template in a 24- μ L PCR that contained 4.8 μ L of 10X PCR buffer with MgCl₂ (Empirical Bioscience), 1.21 mM additional MgCl₂, 0.2 mM dNTPs, 0.42 mM of each primer (Zeale et al., 2011), 2 U Taq (Empirical Bioscience), and 1 μ L of 100X BSA (New England Biolabs), and followed the thermal profile of Zeale et al. (2011). Reactions yielding only one band at the target size (~157 bp) were cleaned using ExoSAP-IT (Affymetrix) according to the manufacturer's instructions. Appropriately sized products from PCRs with multiple bands were excised using a QIAquick Gel Extraction Kit (QIAGEN). Cleaned products were sent to the University of Arizona Genetics Core for unidirectional sequencing with either the forward or reverse primer. Following Zeale et al. (2011) and Clare et al. (2009), 16 clones were sequenced for each sample (data available on Dryad at doi:10.5061/dryad.cfxpvnv75).

Sequence data were compiled, aligned, and edited in Sequencher

v.5.1 (GeneCodes). Query sequences were then compared to reference sequences in the Barcode of Life Data Systems (BOLD, Ratnasingham and Hebert, 2007). If a match could not be made in BOLD, we identified the sequence using BLAST in GenBank. Due to the short length of the edited fragments (~130 bp after primer sequences were excised), some molecular operational taxonomic units (MOTUs) had $\geq 98.5\%$ matches to multiple species or genera. Following Krüger et al. (2014), we categorized database matches as 1a = true species match ($\geq 99\%$); 1b = good species match ($\geq 98\%$); 2 = match to multiple species or genera, only one of which is located in the sampling region; 3 = match ($\geq 98\%$) to multiple species or genera, most conservative taxonomy kept. Because prey cannot be directly quantified as the number of individuals of each insect species consumed from our data (Clare, 2014; Deagle et al., 2005), we defined the occurrence of prey species by its presence in an individual sample. The frequency of occurrence of a prey taxon in the diet was calculated by dividing the number of occurrences of a taxon by the total number of occurrences of all prey items detected in a sample.

2.4. Microcystin analysis

Frozen livers and feces from the 19 individual *M. lucifugus* and fresh feces from the roosting bats were freeze-dried (Labco Lyophilizer) for 24–48 hrs at –53 °C (pressure = 0.002 mbar). Liver tissue and fecal samples for microcystin analysis were ground with a mortar and pestle and approximately 100 mg was subsampled. Because most of the samples weighed less than 100 mg, livers and feces from 2 to 4 individuals were pooled by tissue type to decrease the probability of false negatives for microcystin analysis. Nine fecal samples and two pooled liver samples were spiked with 0.2 μ g of nodularin after initial processing to serve as positive controls and to determine recovery rates after extraction method. Approximately 100 mg of tissue or feces were added to a

centrifuge tube with 5 mL of 80% methanol (MeOH) and sonicated on ice for two 30-second pulses with 30 s between pulses. Samples were stored at -20°C for 30 min, then centrifuged for 15 min at 14,000 rpm and -5°C . The supernatant was decanted into an 8-mL glass tube using a glass pipet and speed vacuumed until dry. Samples were reconstituted in 1 mL of 80% MeOH, vortexed for 20 s, then stored at -20°C for 30 min. After cold storage, samples were centrifuged for 10 min at 3000 rpm at room temperature. The supernatant was transferred to a 1-mL autosampler vial and kept at -20°C until analysis.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to quantify MC-LR, a toxic and commonly reported variant in the Great Lakes region (Carmichael and Boyer, 2016; Miura et al., 1989; Silvonen and Jones, 1999; Taranu et al., 2019), in our bat liver and fecal samples. MC-LR (retention time 13.35 min) was measured in the samples using the transitions m/z 995.2 \rightarrow 134.5 as the quantitation transition, and m/z 995.2 \rightarrow 107.0 and 155.0 as two separate confirmation transitions. Positive controls for nodularin (retention time 12.6 min) were measured using the m/z 825.2 \rightarrow 135.4 transition as the quantitation transition and m/z 825.2 \rightarrow 163.4 and 226.4 as separate confirmation transitions. The instrument detection limit (Water's 2695 Alliance coupled to a TQD mass spectrometer) for MC-LR was 50 pg on column, which corresponded to a method detection limit of 25 $\mu\text{g}/\text{kg}$ dry weight based on a 200-mg sample. Actual individual method detection limits varied depending on the amount of material available for extraction and ranged from 6.7 to 770 $\mu\text{g}/\text{kg}$. Recoveries of the internal standard nodularin averaged 106.9% (\pm 3.7%) across fecal samples and livers. As the internal standard was not incorporated into all samples, individual samples were not corrected for recovery of the internal standard.

In addition to LC-MS/MS, enzyme-linked immunosorbent assay (ELISA) was used to quantify MC_{tot} concentrations in bat liver and fecal samples with the QuantiPlate Kit for Microcystins (Envirologix EP-022). Optical density was determined at 450 nm on an iMark microplate reader (BioRAD). In contrast to other detection methods like LC-MS/MS, ELISA responds to a wider variety of toxin congeners and metabolites and may detect lower concentrations of microcystin, especially if they have been metabolized or if congeners other than MC-LR are present. The increased sensitivity protocol was used for all bat liver and fecal sample extractions. Extractions were diluted 1:16 to decrease the MeOH concentrations from 80% to 5%. The detection limit for the ELISA assay was 0.96 $\mu\text{g}/\text{L}$, which corresponds to a method detection limit of 8 $\mu\text{g}/\text{kg}$ for a 200 mg sample. Samples not spiked with nodularin were run in duplicate, triplicate, or quadruplicate to test the replicability of the ELISA technique, and the mean of these readings was reported as the final concentration for each sample. Replicates that yielded results differing by an order of magnitude or more were excluded from the calculation of the mean for that sample. To ensure that methanol was not inhibiting the ELISA assay, we ran three positive controls of 0.5 $\mu\text{g}/\text{L}$ MC-LR standard in 5% MeOH for this assay.

2.5. Negative controls

Although ELISA is useful in detecting low concentrations of MC, samples extracted from tissues (as opposed to water or algal samples) can have matrix effects (components of the sample other than the analyte of interest) that may lead to false positives for samples (Geis-As-teggiante et al., 2011; Moreno et al., 2011). To avoid false positives due to matrix effects, bat liver and fecal samples that were attained opportunistically were used for negative controls. Because livers from captive-bred *M. lucifugus* were not available, we used livers from two North American vespertilionid bats (*Eptesicus fuscus* and *Nycticeius humeralis*) that were raised in captivity and therefore not exposed to MC. Feces were also obtained from a colony of *M. lucifugus* kept in captivity for approximately 8 months prior to sample collection. All bats used in the negative controls consumed mealworms and/or wax worms and drank tap or deionized water while in captivity, minimizing chance

exposure to MC.

2.6. Statistical analyses

To test for significant differences among the concentrations of MC_{tot} in each sample type, the data were tested for homoscedasticity and normality using Bartlett's (Bartlett, 1937) and Shapiro-Wilk's tests (Shapiro and Wilk, 1965), respectively. Data were log-transformed to make them normal with equal variance, thereby allowing the use of parametric tests. Analysis of Variance (ANOVA; Rosner, 2010) was used to test for significant differences among the concentrations of MC in each sample type, then a Tukey's honest significant difference (HSD; Rosner, 2010) post-hoc test was used to determine which groups differed from each other. A Mann-Whitney U test (Mann and Whitney, 1947) was used to test for differences in MC_{tot} between feces from the roost and individuals. In all tests, we used $\alpha=0.05$ as the threshold for statistical significance.

2.7. Histopathology

Due to the small amount of tissue available for MC measurement, liver samples from two to four individuals were pooled for LC-MS/MS and ELISA tests. One pooled sample representing three individuals had the highest MC_{tot} level for liver tissue, measured via ELISA. Halves of livers from these three individuals were examined by light microscopy to assess for signs of architectural or cellular damage consistent with microcystin toxicosis. Liver tissue that had been stored in RNAlater was thawed and washed with PBS, then fixed in 10% buffered formalin before processing for histopathological analysis using Luna's (1968) standard protocol. Briefly, tissue samples were dehydrated, embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin for light microscopic examination.

3. Results

3.1. Diet of *Myotis lucifugus*

From the 33 fecal samples collected over two sampling dates (19 individual and 14 roost samples), we identified a total of 41 MOTUs (Fig. 2, Table S1). Detectable prey items in the *M. lucifugus* diet primarily included members of the orders Diptera (42.4% on June 20 and 26.1% on June 27), Ephemeroptera (42.4% on June 20 and 21.7% on June 27), and Lepidoptera (15.2% on June 20 and 45.7% on June 27). Coleoptera and Trichoptera constituted a minor component of the diet, being present at low numbers (2.2% and 4.3%, respectively) only on the second sampling date. Overall, insects with aquatic life stages were well represented in the diet of *M. lucifugus*, with at least 8 identifiable aquatic dipteran species and 3 identifiable species of Ephemeroptera, including 6 samples containing *Hexagenia limbata* on the first sampling date.

3.2. Microcystin concentrations

3.2.1. Microcystin positive controls

Mean extraction efficiencies of nodularin as measured by LC-MS/MS from the samples spiked with nodularin were 107.3% (\pm 3.4 standard error, SE) for feces only and 106.0% (\pm 9.4 SE) for livers only and therefore did not warrant correction of MC concentrations for recovery or matrix effects. Positive controls of MC-LR yielded a mean recovery rate of 94.8% (\pm 26.4 SE) as determined by ELISA and therefore also did not support correction for extraction efficiency in either the LC-MS/MS or ELISA results.

3.2.2. Microcystin levels from LC-MS/MS

All bat liver and fecal samples contained concentrations of MC-LR below the detection limit of LC-MS/MS except for one set of three pooled livers. These livers yielded a concentration of 93.6 $\mu\text{g}/\text{kg}$ dw MC-

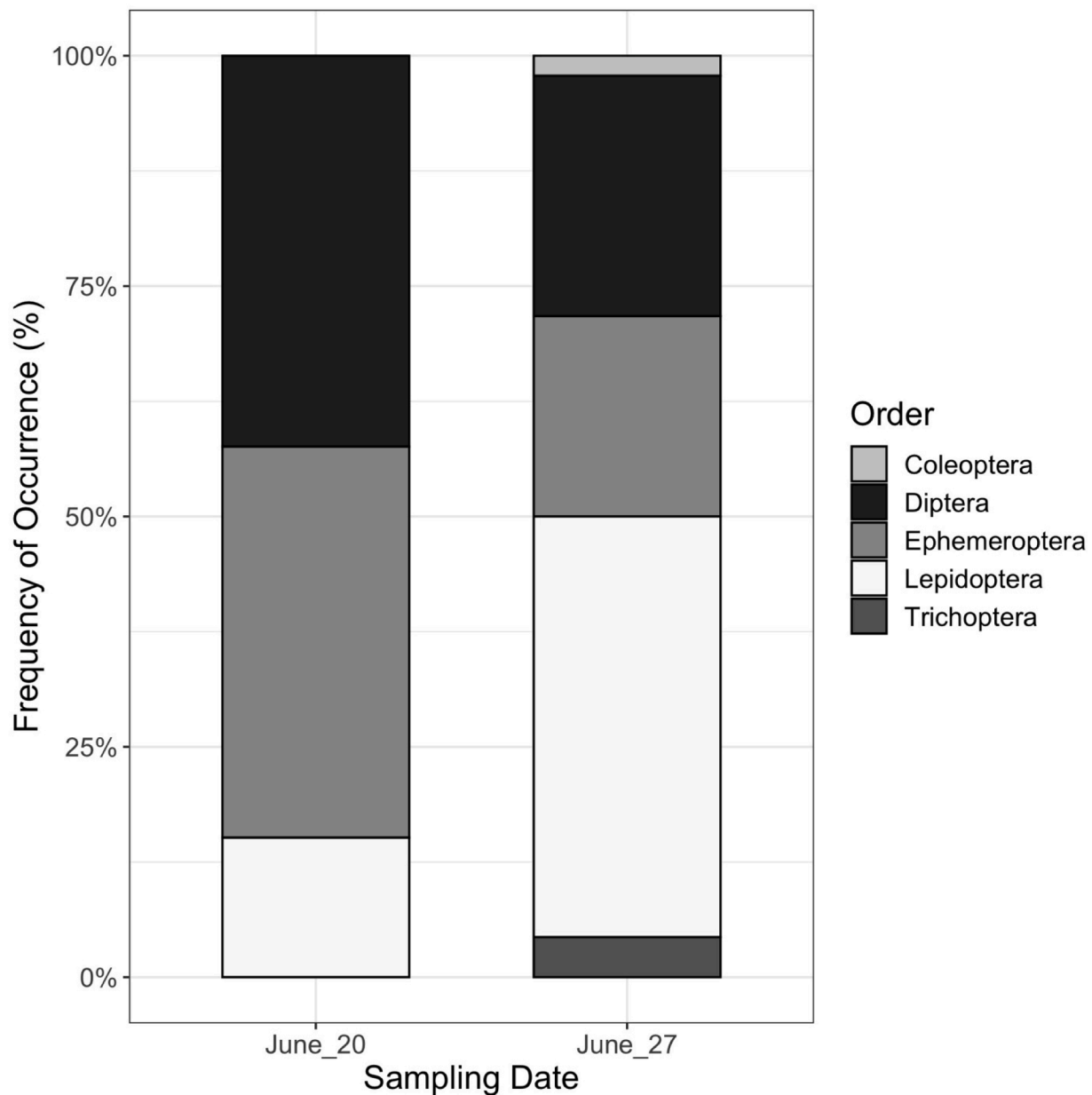


Fig. 2. Frequency of occurrence of each insect order found in the diet of *Myotis lucifugus* on each sampling day as determined by our barcoding analysis.

LR. However, while the quantitation ion of MC-LR was present in this single pooled liver sample, neither confirmation ion for MC-LR was present; thus, this result could not be confirmed as positive.

3.2.3. Microcystin levels from ELISA

All samples, including the negative controls, registered detectable levels of MC within the method limit of detection of ELISA (Fig. 3). To the best of our knowledge, the negative control samples did not contain MC; positive results for these samples (control feces: mean, $\mu = 8.2 \pm 1.8$ SE $\mu\text{g/kg dw}$, control livers: $\mu = 19.6 \pm 3.7$ $\mu\text{g/kg dw}$) are likely due to matrix effects alone, and thus represent an effective zero level for bat liver and fecal samples. Results from field-collected samples are presented as measured (uncorrected) values. Feces collected from each individual bat contained a mean MC_{tot} concentration of $107.7 \mu\text{g/kg dw}$ (± 9.7 SE) and the feces collected from underneath the roost contained a mean of $61.7 \mu\text{g/kg dw}$ MC_{tot} (± 6.6 SE). However, livers from *M. lucifugus* contained a much lower concentration of MC_{tot} than the feces with a mean of $14.1 \mu\text{g/kg dw}$ MC_{tot} (± 2.8 SE). Concentrations of

MC in volant *Hexagenia* captured at Little Traverse Lake at the same time as the bats reported here ranged from $31.2 - 636.4 \mu\text{g/kg dw}$ with a mean concentration of $149.2 \mu\text{g/kg dw}$ (± 23.7 SE, Woller-Skar et al., 2020).

An ANOVA revealed significant differences in MC_{tot} concentrations among sample types ($F = 44.3$, $P < 2 \times 10^{-16}$). Tukey's HSD (Table 1) showed no difference in MC_{tot} concentrations between the feces collected from individuals and feces collected from underneath the roosting colony ($P = 0.534$). Additionally, there is no evidence for a difference in MC_{tot} concentrations of feces from the roost between the two sampling dates ($W = 23$, $P = 0.1$). Concentrations of MC_{tot} in *Hexagenia* were not different from those found in the feces collected from individuals ($P = 0.999$), but were significantly higher than all other sample types, including feces collected from underneath the roosting colony ($P = 4.17 \times 10^{-3}$). Concentrations of MC_{tot} in feces collected from underneath the roost and feces from individuals were significantly greater than concentrations of MC_{tot} detected in the livers of *M. lucifugus* ($P = 1.4 \times 10^{-6}$ and $P = 5.6 \times 10^{-6}$, respectively). Furthermore, there was no evidence of a difference between the concentrations of MC_{tot} in

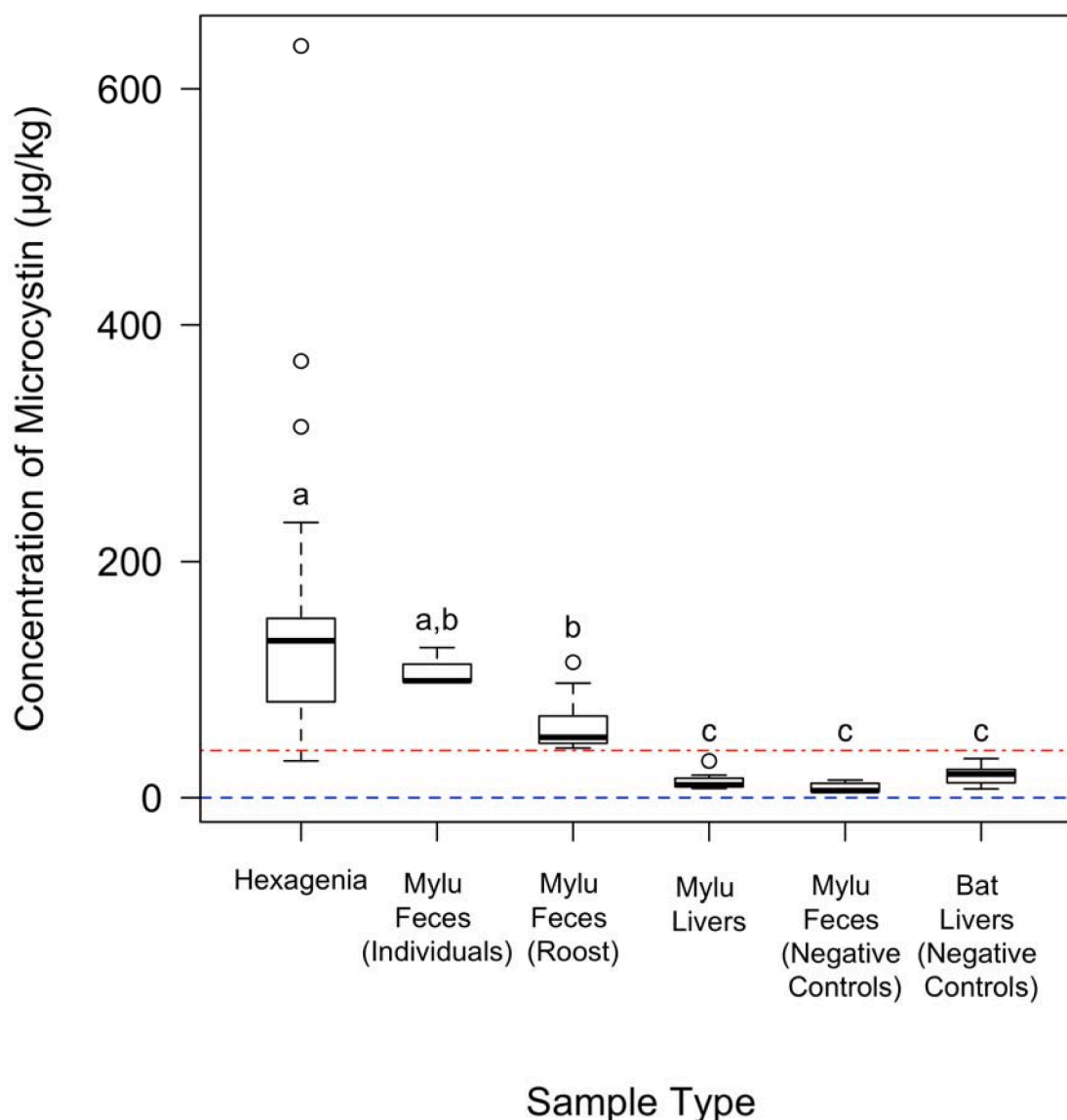


Fig. 3. Boxplot showing the concentrations of total microcystins in each sample type as measured with enzyme-linked immunosorbent assay (ELISA). Boxes show the interquartile range of the ELISA results for each sample type. Bold lines within the boxes show the median concentration of MC_{tot} for each sample type. Whiskers for each box indicate the minimum and maximum MC_{tot} concentrations. Outliers for each sample type are shown with unfilled circles. Significant differences in mean concentrations are denoted by letters above boxes. Sample types with the same letters were not significantly different, whereas different letters indicate a significant difference between sample types. Red dashed line represents the No Observed Adverse Effect Level (NOAEL) for mice (40 µg/kg dw, [World Health Organization, 1998](#)) and the dashed blue line represents the tolerable daily intake (TDI) for humans (0.04 µg/kg dw, [Fawell et al., 1999](#)). Samples from *Myotis lucifugus* are denoted “Mylu”. Microcystin concentrations in *Hexagenia* are from [Woller-Skar et al. \(2020\)](#).

Table 1

P-values from Tukey’s honest significant difference (HSD) pairwise comparisons of mean concentrations of total microcystin (MC_{tot}) among sample types (i.e., feces and livers) collected from *Myotis lucifugus*.

	Feces (Roost)	Feces (Individuals)	<i>M. lucifugus</i> Livers	Livers (Negative Control)	Feces (Negative Control)
<i>Hexagenia</i>	4.17×10^{-3}	0.999	$< 1.0 \times 10^{-7}$	$< 1.0 \times 10^{-7}$	$< 1.0 \times 10^{-7}$
Feces (Roost)	–	0.534	1.4×10^{-6}	7.21×10^{-4}	$< 1.0 \times 10^{-7}$
Feces (Individuals)	–	–	5.6×10^{-6}	2.87×10^{-4}	1.0×10^{-7}
<i>M. lucifugus</i> Livers	–	–	–	0.867	0.452
Livers (Negative Control)	–	–	–	–	0.0774

the livers collected from *M. lucifugus* and either the negative control livers ($P = 0.867$) or the negative control feces ($P = 0.452$). The negative controls (livers and feces) were also not significantly different from each other ($P = 7.74 \times 10^{-2}$).

3.3. Histopathology of livers

Histopathology of the livers ([Lankton et al., 2022](#)) revealed mild, non-specific changes, including centrilobular congestion (3/3), periportal to midzonal hepatocellular vacuolation (3/3), and low numbers

of portal lymphocytes, plasma cells, eosinophils, and neutrophils (1/3). These changes are consistent with background or incidental lesions in wild bats. There were no lesions of microcystin-LR intoxication as reported in other mammals (Cullen and Stalker, 2016).

4. Discussion

Our hypotheses were that (1) bats are feeding heavily on emerging *Hexagenia*, (2) microcystin is present in both bat livers and feces, and (3) high levels of microcystin ingestion are associated with liver damage in wild bat populations. Dietary analyses confirm that *Hexagenia*, as well as other aquatic insects, were a substantial component of the bats' diet. *Hexagenia* spp. collected from this area on the same days as this study had high levels of microcystin in their tissues (Fig. 3; Woller-Skar et al., 2020). However, the presence of MC_{tot} in bat feces but not their livers indicated that *M. lucifugus*, when exposed to this toxin, were not accumulating the toxin in the liver. Consequently, it was not unexpected that histopathology failed to show lesions of microcystin toxicosis in the livers. Whether this lack of accumulation was due to detoxification of the microcystins in the source *Hexagenia*, or detoxification by the bats is unknown.

4.1. ELISA vs LC-MS/MS

All samples included in our analyses contained concentrations of microcystin within the method limit of detection of the ELISA, but no samples, even those with high concentrations of microcystins, were within the detection limit of the LC-MS/MS. A possible explanation for this discrepancy is the fact that only one congener, MC-LR, was measured with LC-MS/MS, whereas ELISA responded to all congeners of microcystin (MC_{tot}), including protein-bound toxin and possible metabolite products. Free MC-LR may be present in these samples, but not in high enough concentrations to be detected with the LC-MS/MS method. Other congeners might have constituted the majority of microcystins present in these samples and would not have been detected using the LC-MS/MS method used here. Given that *Hexagenia* is very resistant to high levels of microcystins (Smith et al., 2008a), it is also possible that the *Hexagenia* or bats rapidly metabolized the MC via the glutathione or other pathway as part of a detoxication approach (see Schmidt et al., 2014). These metabolites would also not have been detected using the LC-MS/MS protocols used here, but may have contributed to the positive ELISA results.

4.2. Are *M. lucifugus* bioaccumulating microcystin?

The difference between the concentrations of MC_{tot} in the livers collected from *M. lucifugus* and the negative control liver was not significant; thus, little to no free microcystin was likely in the *M. lucifugus* livers, and the non-zero values measured by ELISA were likely a result of matrix effects from the liver tissue itself. In contrast, the feces collected from individual *M. lucifugus* and from underneath the roosting colony potentially exposed to microcystins contained significantly higher concentrations of MC_{tot} than the negative control feces. The low positive results from the negative control feces indicated the presence of matrix effects with this sample type, but the significant differences between the negative control feces and the feces from *M. lucifugus* indicate that these bats were exposed to microcystins via their diet. However, the low concentrations of MC_{tot} in the *M. lucifugus* livers (that are not significantly different from the negative controls) would indicate that *M. lucifugus* did not accumulate microcystins from their diet but rather passed them through the intestinal tract into the feces. The nonspecific nature of the liver lesions found in the histopathology of the livers is consistent with the hypothesis that this population of *M. lucifugus*, although this population was exposed to MC, did not accumulate high concentrations of the toxin in the liver and are unlikely to be experiencing clinical toxicity. Furthermore, these findings are consistent with

Isidoro-Ayza et al. (2019) who found no major hepatic lesions in bats with significant MC exposure.

4.3. Route of microcystin exposure

Our dietary analyses showed that *Hexagenia* are a common prey item of *M. lucifugus* during this insect's emergence, and Woller-Skar et al. (2020) confirmed that volant *Hexagenia* contain high concentrations of MC_{tot}. We can infer that *Hexagenia* was a likely source of MC_{tot} in *M. lucifugus* and that *M. lucifugus* were exposed to MC through trophic transfer. The concentrations of MC_{tot} found in *Hexagenia* (Woller-Skar et al., 2020) and in *M. lucifugus* feces (this study) are similar to but lower than those found by Woller-Skar et al. (2015), where MC_{tot} ranged from 40.5 to 1127.7 µg/kg dw for *Hexagenia* and from 81.8 to 627.5 µg/kg dw for bat feces. This decrease in MC_{tot} concentrations in these two sample types may be attributable to different extraction methods used, or to natural inter-annual variation in MC production.

Hexagenia may not be the only route of MC exposure for *M. lucifugus*. We detected significant levels of MC_{tot} in fecal samples of *M. lucifugus* in which no *Hexagenia* were detected. This could be attributable to false negatives in the dietary analysis (i.e., these bats were consuming *Hexagenia*, but this prey item was not detected during the dietary analysis). Alternatively, the presence of MC_{tot} without *Hexagenia* might indicate that *M. lucifugus* were consuming other prey items that contain microcystins such as chironomid flies that can also bioaccumulate MC_{tot}, but to a lesser extent than *Hexagenia* mayflies (Woller-Skar, 2009). Although MC has not yet been confirmed in adult chironomids, *M. lucifugus* did consume at least three genera of these non-biting midges. Furthermore, over half of the diet of *M. lucifugus* consisted of aquatic insects. Not all aquatic insects have been tested for MC, but benthic insects are more likely to contain MC because *M. aeruginosa* overwinters in lake sediments (Reynolds et al., 1981). Future research on the movement of microcystins through trophic interactions might focus on insects with benthic life stages.

Hexagenia were one of the most frequently occurring prey items in the diet of *M. lucifugus* overall, but this taxon was not detected in feces collected on the second sampling date (27 June). This absence could be due to decreasing numbers of *Hexagenia* in the environment causing bats to find alternative prey items, or it could be due to conditioned taste aversion of *M. lucifugus* to *Hexagenia*. If the consumption of toxic *Hexagenia* mayflies made *M. lucifugus* exhibit symptoms of illness, as microcystin does in other mammals, these bats may avoid or decrease consumption of microcystin-laden insects; such conditioned taste aversion has been documented in Seba's short-tailed bat, *Carollia perspicillata* (Terk and Green, 1980). However, the lack of change in MC_{tot} concentrations between sampling dates indicates that bats are not decreasing their intake of MC_{tot} despite a shift in diet.

Although we did not test the water column for the presence of MC_{tot}, it is unlikely that *M. lucifugus* ingested this toxin through drinking water. The concentrations of microcystins at the surface of Little Traverse Lake (where bats would be skimming water to drink) are extremely low before blooms of *M. aeruginosa* (Woller-Skar, 2009) and could not have accounted for the microcystin concentrations observed in the fecal samples.

4.4. How toxic are *Hexagenia* to *M. lucifugus*?

During a 13-week study using mice, Fawell et al. (1999) determined that daily oral dosages of 40 µg MC-LR per kg of bodyweight was the No Observed Adverse Effect Level (NOAEL; maximum dose at which there are no signs of pathological changes in the liver). This study formed the basis of the World Health Organization (WHO) guideline for a tolerable daily intake (TDI) of 0.04 µg MC-LR per kg of bodyweight (0.04 µg/kg) for humans. Almost all *Hexagenia* individuals contained concentrations of MC (mean 152 µg/kg dw; Woller-Skar et al., 2020) that exceeded the NOAEL in mice. This observation is consistent with prior work by Smith

et al. (2008b) who found *Hexagenia* was resistant to very high concentrations (ca. 50–1000 µg/L) of purified microcystins. However, the techniques used in our dietary analyses could not estimate the number of individual *Hexagenia* consumed by each bat; therefore, extrapolating the amount of microcystin consumed by these bats was not possible. The WHO guidelines for microcystins are based on a lifetime exposure and should not be construed as an acute limit. Fawell et al. (1999) showed that exposure to 200 µg/kg over a short period of time (weeks) resulted in minor observed effects in the liver that were detected in only a few animals. This minimal-effect more closely mimics the exposure that *M. lucifugus* may have experienced if they ingested one *Hexagenia* per night that contained a mean concentration of 100 µg/kg dw of microcystins. In reality, an individual *M. lucifugus* likely consumed more than one *Hexagenia* per night and may additionally consume MC in other aquatic insects, leading to a higher exposure over a shorter time period. Lactating *M. lucifugus* may eat more than their body mass in insects each night to cope with the metabolic demand of reproduction. Thus, during this time of increased energy demands, *M. lucifugus* in this study likely consumed large quantities of *Hexagenia* and other aquatic insects. However, as *Hexagenia* were not found in all fecal samples, this consumption of *Hexagenia*, the bats' corresponding exposure to microcystins, and the level of exposure to microcystins through other aquatic insects, may have varied considerably among bats and across time.

4.5. Impact of microcystin on bats

The degree of susceptibility, or even resistance, to MC varies highly among species as well as by MC congener (Schmidt et al., 2014). Bats have shorter intestines and less intestinal mass relative to non-flying mammals (Caviedes-Vidal et al., 2007). Thus, rapid transit times of ingested food may explain the increased concentration of microcystins in feces compared to the lack of accumulation in the liver. However, bats also rely on increased rates of paracellular absorption of water-soluble molecules to compensate for decreased absorptive surface area in their intestines (Caviedes-Vidal et al., 2008). Because microcystins are also water soluble, we would expect greater concentrations of microcystins in bats' livers relative to fecal samples. The presence of microcystin in the feces but not the livers of bats indicates that *M. lucifugus* may preferentially excrete the toxin rather than absorb it. Microcystins are absorbed from the intestine through organic anion transport polypeptides (OATP, Fischer et al., 2005). Microcystins can also be rapidly metabolized in some species via the glutathione detoxification pathway (Schmidt et al., 2014). The lack of any apparent liver damage in bats could indicate that bats were not exposed to a high enough dose, not exposed to a hepatotoxic congener, or that they have modified OATPs or other rapid detoxification method for microcystins. Given the high levels of microcystins observed in *Hexagenia*, consistent exposure to elevated levels of microcystins through their diet may have favored the evolution of glutathione-based detoxification methods. These were not measured here and are a subject for future research.

Currently, North American bats are experiencing several threats of mortality. The spread of white-nose syndrome and an increase in wind turbine facilities has led to population declines in many bat species (Arnett and Baerwald, 2013; Coleman and Reichard, 2014). In addition, insectivorous bats are more susceptible to ingesting environmental toxins than other organisms due to their longevity relative to mammals of similar size and higher metabolic rates associated with flying (Clark and Shore, 2001). Bats have been shown to be exposed to environmental contaminants such as mercury and polychlorinated biphenyls (PCBs) through the consumption of aquatic insects (Baron et al., 1999). The bioaccumulation of synthetic environmental contaminants such as polychlorinated biphenyl ethers (PBDEs) and the insecticide DDT has been documented in bats (Kannan et al., 2010; O'Shea and Johnston, 2009; Secord et al., 2015), but less effort has focused on the bioaccumulation of naturally occurring toxins (Pybus et al., 1986). Recently, MC-LR was implicated as the cause of mortality of 27 *M. lucifugus* in Utah

(Isidoro-Ayza et al., 2019). High concentrations of MC-LR were detected in the bats' intestinal contents, but histopathology of the livers did not reveal signs of damage expected from microcystin toxicity (Isidoro-Ayza et al., 2019). As in our results, the greater concentrations of MC_{tot} in the bat gut contents than livers and lack of hepatic damage found by Isidoro-Ayza et al. (2019) indicate that the bats are excreting more MC_{tot} than they are bioaccumulating. Although mass mortalities of bats from microcystin have not been confirmed, this toxin may impart some physiological stress that may increase vulnerability of bats to predators, parasites, or disease (Smith et al., 2008b). Secondary metabolites of cyanobacterial toxins may bioaccumulate in aquatic organisms that serve as prey items for bats. Water quality can also affect the health of bats either directly or indirectly and therefore it would be beneficial to monitor for both inorganic and organic toxins.

4.6. Future work

Other emerging aquatic insects, particularly those with benthic life stages, could be tested for microcystin as they may demonstrate an alternative route of exposure to this toxin. In this study, feces were collected before a bloom event. Microcystin concentrations in lakes are the highest during and right after blooms (Woller-Skar, 2009). Thus, bats may be ingesting more microcystin later in the year. Alternatively, exposure may occur during relatively short time periods through major insect emergences such as those of *Hexagenia*. Because North American bats are presently facing many threats, toxicological analyses of bat feces collected over an entire season would further improve our understanding of the potential effects of microcystin on bat health.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2022.102221.

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