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Biotransport of Algal Toxins to Riparian Food Webs

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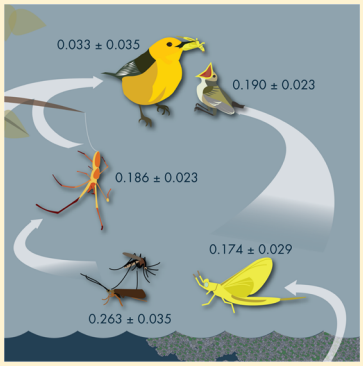
Supporting Information

ABSTRACT: The occurrence of harmful algal blooms has resulted in growing worldwide concern about threats to aquatic life and human health. Microcystin (MC), a cyanotoxin, is the most widely reported algal toxin in freshwaters. Prior studies have documented its presence in aquatic food webs including commercially important fish and shellfish. In this paper we present the first evidence that algal toxins propagate into riparian food webs. We show that MC is present in emerging aquatic insects (*Hexagenia* mayflies) from the James River Estuary and their consumers (*Tetragnathidae* spiders and Prothonotary Warblers, *Protonotaria citrea*). MC levels in Prothonotary Warblers varied by age class, with nestlings having the highest levels. At the site where nestlings received a higher proportion of aquatic prey (i.e., mayflies) in their diet, we observed higher MC concentrations in liver tissue and fecal matter. Warbler body condition and growth rate were not related to liver MC levels, suggesting that aquatic prey may provide dietary benefits that offset potential deleterious effects of the toxin. This study provides evidence that threats posed by algal toxins extend beyond the aquatic environments in which blooms occur.



INTRODUCTION

Emerging aquatic insects are an important food source for bats, reptiles, amphibians, spiders, and, in riparian birds, can account for 50−90% of the monthly energy budget.[1,2] As emerging aquatic insects cross habitat boundaries, this food subsidy can be shadowed by the movement of pollutants (e.g., mercury, PCBs).[3,4] The export of aquatic contaminants to consumers outside of the aquatic realm has been referred to as the "dark side of subsidies", whereby benefits of greater prey availability are offset by exposure to potentially toxic contaminants.[4] For example, higher mercury levels in insectivorous birds were linked to a diet consisting mainly of emerged aquatic insects.[5,6] Prior studies on this topic have focused on persistent and bioaccumulative contaminants such as mercury and organic chemicals delivered to aquatic systems and exported to terrestrial food webs. In this study, we expand on the concept of the "dark side of subsidies" to assess the exposure of riparian consumers to algal-derived toxins produced in aquatic systems.

Algal blooms are associated with a range of deleterious effects including the proliferation of harmful algae which produce toxic secondary metabolites.[7] The occurrence of harmful algal blooms (HABs) has been increasing worldwide raising concerns for aquatic life and human health.[8−10] Some cyanobacteria, including the genus *Microcystis*, produce microcystins (MC), a class of monocyclic heptapeptide hepatotoxins.[11] These toxins inhibit the activity of protein phosphatases which are important in many cell cycles.[7,12,13] MC accumulates in a variety of aquatic organisms including zooplankton, bivalves, insects, wild and farmed fishes, sea otters, turtles, and water birds.[14−22] MC can be transported through food webs via consumption; however, there is no evidence of biomagnification.[12] The extent to which MC can be transported out of the aquatic realm has only recently been documented and with limited scope. Takahashi et al.[23] found low levels of MC in midge-flies and dragonflies as aquatic-stage juveniles, as well as in a riparian predator (*Tetragnathidae* spiders).

In this study we describe the movement of algal toxins from an aquatic food web into a riparian food web by measuring MC concentrations in adult (emergent) aquatic insects (*Hexagenia* mayfly, *Chironomidae* nidges, and *Trichoptera* caddisflies), an invertebrate riparian predator (*Tetragnathidae* spider), and a vertebrate riparian predator (Prothonotary Warbler; *Protonotaria citrea*). We also analyze variation in MC concentrations among nestling warblers in relation to diet (aquatic vs terrestrial prey) to determine whether body condition and growth rates are affected by MC exposure.

MATERIALS AND METHODS

Study Site. The James River Estuary is a freshwater-dominated subestuary of Chesapeake Bay with low salinity zones (tidal fresh and oligohaline; salinity <5 ppt) comprising more than half of its surface area. The tidal freshwater segment of the James has similarities with other systems experiencing harmful algal blooms including large anthropogenic nutrient loads and elevated chlorophyll *a*.[24−28] Cyanobacteria contribute a small proportion of phytoplankton biomass (~10%) but their presence results in low levels of MC in the water column during summer (typically 0.5−1.5 μg L^{-1}) and widespread occurrence in tissues of fish and benthic macroinvertebrates.[21] Highest

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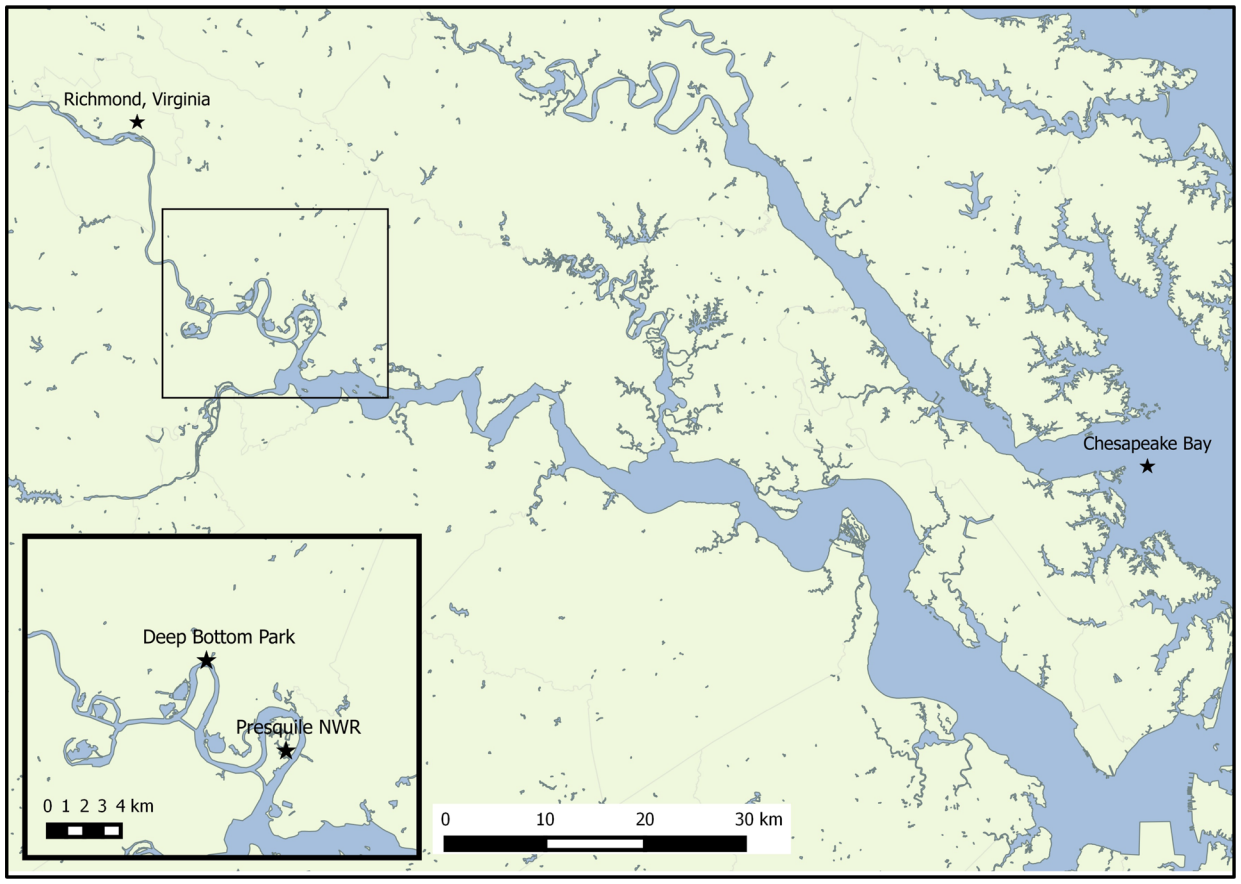

Figure 1. Locations of the two study sites (Deep Bottom Park and Presquile National Wildlife Refuge) along the James River Estuary where emerging insects and riparian consumers were analyzed for the presence of the algal toxin microcystin. This map was created in QGIS using an opensource basemap.[59,60]

levels of MC in consumers are observed in late summer coinciding with peak values in the water.

Sample Collection. Emerging insects and riparian consumers were sampled along two tidal freshwater creeks located ~10 km apart at their confluence with the James River: Four Mile Creek, which is located in Deep Bottom Park (DB, Henrico, Virginia), and an unnamed creek at Presquile National Wildlife Refuge (PNWR; Henrico, Virginia; Figure 1). We sampled emergent aquatic insects, including mayflies (*Hexagenia* spp., Ephemeroptera: Ephemeridae), Chironomid midges (Diptera; Chironomidae), and caddisflies (Trichoptera), as potential vectors of MC transport from aquatic to terrestrial realms. *Hexagenia* nymphs are aquatic benthic macroinvertebrates that build burrows through which they pump water and feed on suspended particulate matter.[29] MC exposure occurs through ingestion of suspended materials.[14,20,30] These insects typically spend 1−2 years as nymphs and emerge synchronously in large swarms. Emergence events occur in May through July where large numbers of nymphs swim to the surface of the water and molt into subimagos. Subimagos are winged subadults that fly to land for 1−3 days before molting into reproductive adults. During this life stage mayflies have atrophied mouthparts and do not feed in the terrestrial environment.[31]

We sampled Long-jawed Spiders (Araneae: *Tetragnathidae*) and Prothonotary Warblers (*Protonotaria citrea*) to assess MC exposure for insectivorous consumers. *Tetragnathid* spiders build webs on vegetated river banks, prey on emerging aquatic insects, and are increasingly used as sentinels for aquatic contamination.[4,32,33] Prothonotary Warblers are migratory riparian songbirds that breed in bottomland hardwood forests throughout the southeastern United States and overwinter in Central America and northern South America.[34] Mayflies and other emerging aquatic insects make up a significant portion of their diet, along with terrestrial caterpillars. Our study population breeds in man-made nest boxes and is part of a long-term monitoring project.[35,36] Because they nest in artificial boxes, the birds are accessible for quantifying nestling diet, survivorship and growth. Females lay 4−6 eggs per clutch, and commonly raise two broods per season.[36,37]

Water, insect, spider and bird samples were collected during (May−July 2014) and after (August−October 2014) the warbler breeding season. Water samples (near-surface) were collected every other week near the mouth of the two creeks. Mayflies and other emerging aquatic insects were sampled from the shore using Pennsylvania-style light traps[38,39] and from the water's surface using emergence traps.[40] One light trap and four emergence traps were deployed at two locations along each creek (near confluence and upstream). Samples were sorted to obtain mayflies, Chironomid midges and caddisflies, which together comprised 30−100% of the trap contents. For each taxa, individuals from several collection dates were pooled (~20/sample) to obtain a monthly composite for each site. *Tetragnathidae* spiders were obtained opportunistically from structures and vegetation adjacent to the water. A pooled sample of ~30 individuals was obtained monthly at each site.

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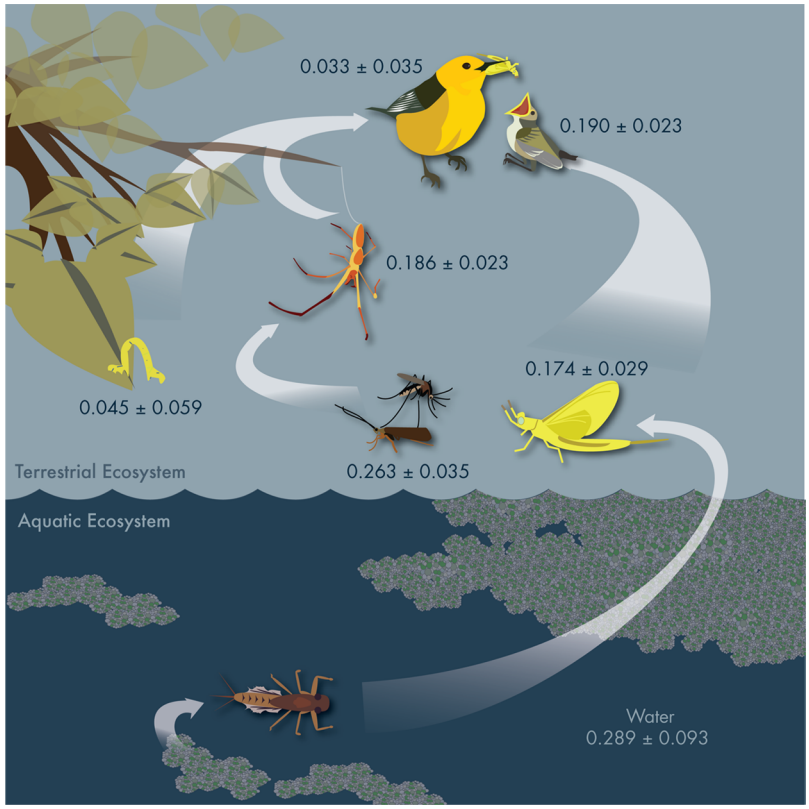

Figure 2. Concentrations of the algal toxin microcystin among terrestrial insects (caterpillars), aquatic emerging insects (caddisflies, midges and mayflies) and riparian consumers (spiders; nestling and adult warblers) from the James River Estuary (Virginia). Values shown are mean ± standard error for whole-body concentrations (insects and spiders) and liver concentrations (warblers) as μg g^{-1} DM. Water values are volumetric concentrations (μg L^{-1}).

On three occasions we collected terrestrial caterpillars (*Geometridae*) to determine whether microcystin was present in nonaquatic prey. Three samples comprised of ~25 individuals were obtained at both sites.

Microcystin has been shown to accumulate in a variety of tissues in vertebrates including stomach, spleen, and intestine, although highest levels are found in the liver.[13,41] We collected Prothonotary Warbler samples by sacrificing individuals and extracting liver tissue post-mortem. All birds were collected and sacrificed using thoracic compression as described by the Ornithological Council[42] and under approved protocols (VCU IACUC #AM10230, Federal Scientific Collection Permit #MB29235B, State Scientific Collection Permit #050784, USGS Federal Bird Banding Lab Permit # 23486). Nestlings were taken from the nest (one per brood), and hatch-year fledglings and adults were captured using target mist-netting techniques and playback. Nestlings were sacrificed when 9−10 days old (fledging typically occurs between day 10−12) and mist-netted birds were aged as fledglings or adults using skull pneumatization techniques.[43] Sacrificed chicks were chosen randomly from the nest in order to control for the presence of dominant or subordinate individuals. We also analyzed fecal-sac samples provided by nestlings during banding activities (at 7−8 days) to determine whether this was a viable nonlethal method for assessing MC exposure.

Nestling diet was quantified using video observations to record provisioning of aquatic and terrestrial insects by adults.[44,45] Video data from a total of 104 nest boxes (263 h of observation) were used to identify and quantify food items brought to the nest.[46] A subset of these were for boxes containing nestlings that were analyzed for liver MC (23 nests monitored for 57 h). A Canon FS400 camera was placed outside of the nest with a clear view of the nest box for 1.6−3.2 h. All video observations were conducted in the morning (6:40−9:40 a.m.) when the nestlings were between 6 and 9 days old. For each adult visit, we recorded the type of food and the number of food items brought. All observers were trained by watching the same video to ensure consistent identification of prey items. Mayflies (aquatic) and caterpillars (terrestrial) were the most common food being provisioned and were easily identified (71% of all prey items were identified). Based on the number of nestlings and length of monitoring, the provisioning data were expressed as number of prey items $chick^{-1}$ h^{-1}.

To determine body condition and growth rate, nestling mass (g) and tarsus length (mm) were measured at 5/6 days and again at 7/8 days. Growth rate was calculated as the change in body mass day^{-1} between these two measurements. A body condition index was calculated for nestlings and adults as the residuals from a least-squares regression of mass (g) by tarsus length (mm). This index of relative condition[47] is correlated with stronger immune function and higher survival.[48] For nestlings, these residuals were calculated separately by age (days). To check for sampling bias, a two-tailed t test was used to confirm that growth rate and body condition of sacrificed nestlings was not different from that of nestlings that were not sacrificed (p = 0.58 and p = 0.23, respectively).

Microcystin Analysis. Water and tissue MC concentrations were determined using a commercial ELISA kit (Abraxis; Warminster, PA). The assay measures numerous forms of MC using polyclonal antibodies with concentrations reported in

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MC-LR equivalents. To release MC from cells, water samples were thawed and refrozen two times (as recommended by the manufacturer), and then microwaved and sonicated to improve extraction efficiency.[49] To extract MC from tissues, we used methods described by Wilson et al.[15] and Garcia et al.[17] Samples were dried at 80 °C for 48 h, ground with a mortar and pestle, and extracted in 75% aqueous methanol for 24 h. Extracts were centrifuged and supernatant collected. Subsamples were diluted with deionized water such that samples to be run on the ELISA plate contained <5% methanol. For each 96-well plate, six standards were used to derive plate-specific standard curves. Samples were run in duplicate and plates were read on an ELISA plate reader at 450 nm. The mean standard error among duplicate water samples was 0.03 μg L^{-1} (equivalent to 9% of the mean); the mean standard error for duplicate tissue samples was 0.014 μg g DM^{-1} (equivalent to 12% of the mean). Average recovery from positive internal controls was 104 ± 4%. A subset of the emergent insect samples were analyzed by multiple reaction monitoring mass spectrometry and found to contain two isoforms: DAsp[3] microcystin-LR and microcystin YR (P. Zimba, Pers. comm.).

Statistical Analysis. Differences in MC concentrations across sample type (e.g., mayflies, spiders, warbler age groups) were compared using one-way analysis of variance. Backward stepwise multiple linear regression was used to determine the effects of site (Deep Bottom vs Presquile NWR), date, nestling diet, and age on variation in the microcystin content of adult birds, fledglings, nestlings, all birds and fecal-sacs. Two-way ANOVA including site and date were used to partition variation in the microcystin content of mayflies and spiders. Due to the inherent non-normal distribution of MC concentrations in organisms (many low values and few high values), data were log-transformed for statistical analysis. Diet was calculated as proportion mayfly foodscore and was arcsine square-root transformed due to non-normal distribution. Means were backtransformed for figures. All analyses were completed using JMP 11.0 statistical package.[50]

RESULTS

Microcystin was detected among aquatic emerging insects and riparian consumers collected at two sites along the James River Estuary, Virginia (Figure 2). Among consumers, highest toxin concentrations were found in spiders (mean = 0.186 ± 0.023 μg g^{-1}) and the livers of nestling warblers (mean = 0.190 ± 0.023 μg g^{-1}). High concentrations were also observed in fecal sacs obtained from nestling warblers (mean = 0.091 ± 0.022 μg g^{-1}). Microcystin levels in livers from fledgling (mean = 0.038 ± 0.015 μg g^{-1}) and adult warblers (mean = 0.033 ± 0.035 μg g^{-1}) were 5-fold lower than that found in nestling livers. Differences in liver MC concentrations among warbler growth stages were statistically significant ($p < 0.005$; Figure 3). Among insects, we observed high levels of microcystin in aquatic emergent forms (caddisflies and midges = 0.263 ± 0.035 μg g^{-1}; mayflies = 0.174 ± 0.029 μg g^{-1}) and low levels in terrestrial caterpillars (mean =0.045 ± 0.059 μg g^{-1}). Microcystin concentrations in water averaged 0.29 (±0.09) μg L^{-1} during the period of study. Highest concentrations were observed at the Deep Bottom site in mid-July (1.34 μg L^{-1}), though average values were not significantly different between the two sites (DB = 0.35 ± 0.17 μg L^{-1}; PNWR = 0.22 ± 0.07 μg L^{-1}; $p = 0.35$).

We found statistically significant differences between the two study sites in the provisioning of aquatic prey to warbler nestlings (Figure 4, see also Supporting Information (SI)). At Deep Bottom, mayflies accounted for 79.9% of food provided to nestlings, whereas at Presquile NWR, the mayfly proportion was lower (1.7%) due to greater contributions from terrestrial caterpillars and unidentified insects. Microcystin concentrations in nestling fecal sacs were significantly higher at the site where mayflies accounted for a greater proportion of nestling diet (Deep Bottom = 0.128 ± 0.030 μg g^{-1}) relative to the low-mayfly site (Presquile NWR = 0.043 ± 0.030 μg g^{-1}; $p = 0.036$). We did not observe significant differences in liver concentrations between nestlings from the two locations (Deep Bottom = 0.222 ± 0.060 μg g^{-1}; Presquile NWR = 0.174 ± 0.040 μg g^{-1}; $p = 0.53$). Nestling growth rates were found to be significantly higher at Deep Bottom (mean = 1.28 ± 0.05 g d^{-1}) relative to Presquile NWR (mean = 1.07 ± 0.06 g d^{-1}; $p = 0.008$).

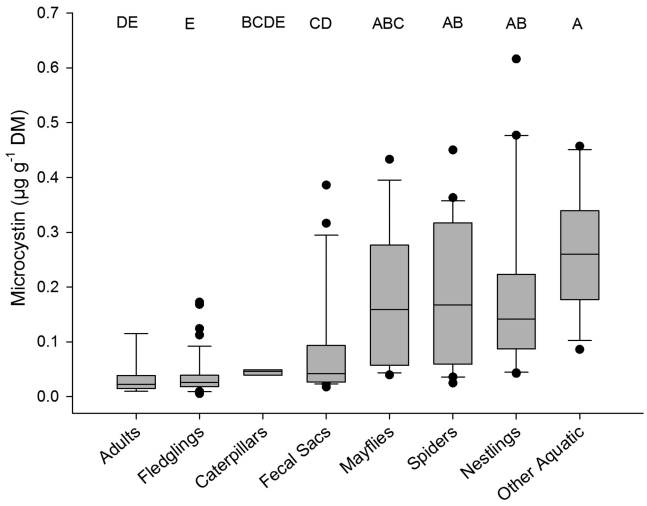

Figure 3. Microcystin concentrations (μg g^{-1} DM) of Prothonotary Warblers (adults, fledglings, nestlings, nestling fecal sacs), terrestrial insects (caterpillars), aquatic insects (mayflies, other) and spiders collected from two sites along the James River Estuary. Spider and insect values are whole body concentrations; Prothonotary Warbler values are liver concentrations. Measures of variability are among individuals (warblers) or pooled samples of individuals (spiders and insects). The line within each box represents the median, box boundaries are 25th and 75th percentiles, whiskers are 10th and 90th percentiles, and points are outliers. Letters across the top indicate statistical significance: categories that share the same letter are not significantly different.

We derived univariate regression models to test for relationships among diet, liver MC and body condition using data for individual nestlings. At the site where mayflies constituted a greater proportion of nestling diet (Deep Bottom), we found that mayfly provisioning rates (mayflies $chick^{-1}$ h^{-1}) were significantly correlated with liver microcystin concentrations in nestlings ($R^2 = 0.58$; $p = 0.006$; see SI). There was no relationship between mayfly provisioning and liver MC at the site where terrestrial caterpillars were the larger component of diet (Presquile NWR). Body condition was not correlated with liver microcystin levels in fledglings ($n = 42$, $p = 0.98$) or nestlings ($n = 16$, $p = 0.66$).

DISCUSSION

We documented the presence of a cyanotoxin, Microcystin, in the riparian food web of the James River Estuary, Virginia. Toxin concentrations measured in riparian consumers (spiders,

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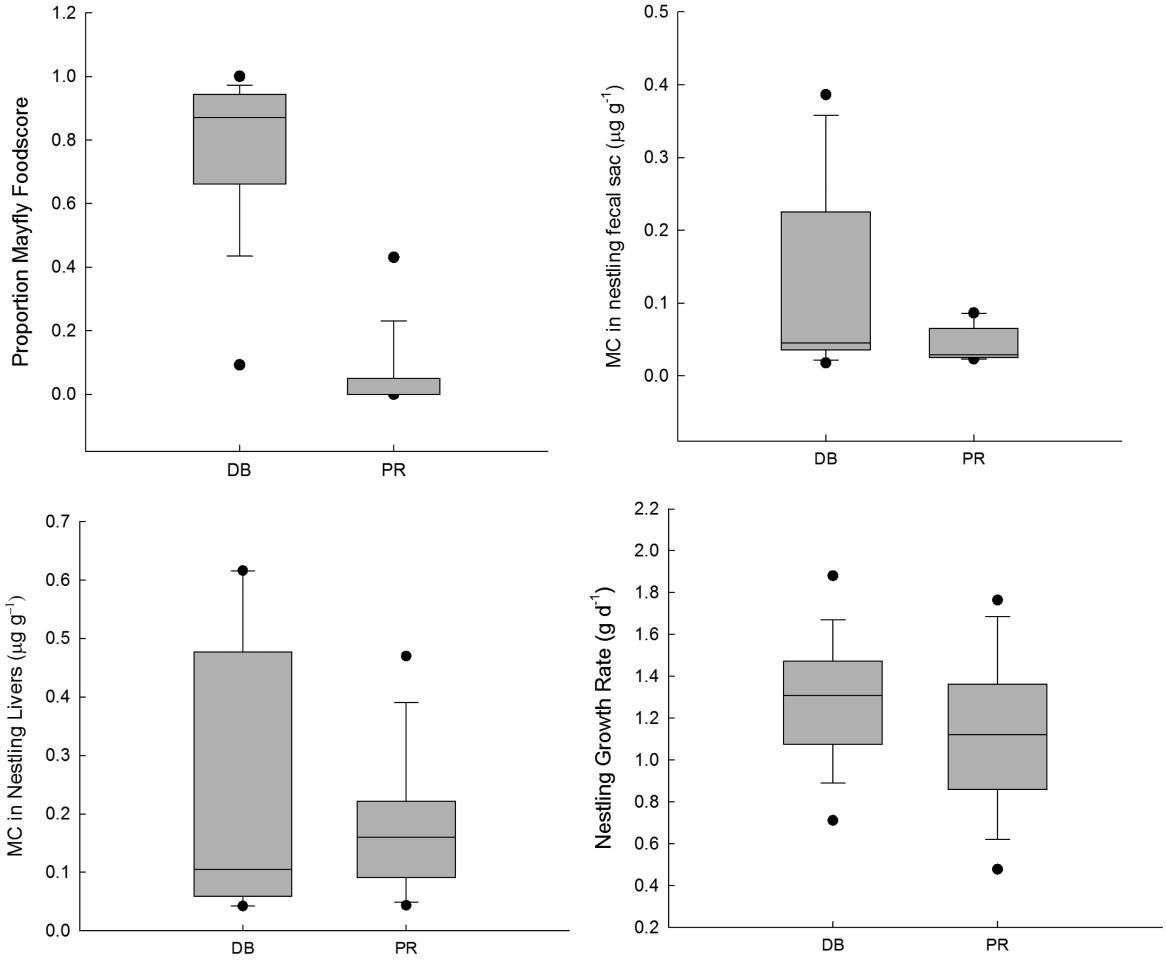

Figure 4. Intersite differences in (a) proportion of mayfly prey in nestling diet ($p < 0.0001$), (b) MC levels in nestling fecal-sacs ($p = 0.031$), (c) liver MC concentrations in nestlings ($p = 0.66$), and (d) nestling growth rates ($p = 0.008$). *t* tests were performed on arcsin squareroot transformed values for proportion mayflies and on log-transformed MC values. Untransformed values are shown here. The line within each box represents the median, box boundaries are 25th and 75th percentiles, whiskers are 10th and 90th percentiles, and points are outliers.

warblers) were comparable to values reported for aquatic consumers (planktivorous fish and benthic bivalves) from the James Estuary.[21] The likely mechanism of exposure for riparian consumers is via toxins in emergent aquatic insects, which contained 20-fold higher levels of microcystin than terrestrial prey (caterpillars). Toxin concentrations in nestlings were similar to those of their aquatic prey, but it should be noted that the former are based on an analysis of liver tissues, where microcystin levels are highest. Microcystin was detected in some terrestrial caterpillars, though the mechanism accounting for its presence is unknown. We cannot discount the possibility of false positives from using ELISA to measure microcystin in complex matrices (i.e., tissues).[51] However, microcystin has been reported in terrestrial plants grown in agricultural settings where the toxin is present in water sources for irrigation.[11,52,53] At both Presquile NWR and Deep Bottom Park, the riparian zone is flooded up to 50 m from the shoreline during high tide. Because all caterpillars were sampled from this zone, it is possible that caterpillars were exposed to toxin contained in floodplain vegetation. Further testing of caterpillar and leaf tissue from this and other riparian habitats is warranted as it provides an additional mechanism for transport of an algal toxin from the aquatic to the terrestrial ecosystem.

Age class was a significant predictor of microcystin in Prothonotary Warblers with nestlings having higher levels compared to older birds. We also observed that fledglings caught later in the season had lower microcystin levels than those caught earlier (see SI). These findings indicate a reduction in toxin levels with age, particularly after birds leave the nest. Lower body burdens of microcystin may occur as fledglings shift their diet to terrestrial prey as aquatic prey become less abundant.[1,46] Other factors contributing to age-specific differences in toxin levels may include high consumption rates of nestlings (i.e., greater toxin ingestion per unit body weight) and a lower capacity of nestlings to depurate the toxin.

At the site where mayflies constituted a greater proportion of nestling diet (Deep Bottom), we found that mayfly provisioning rates were significantly correlated with liver microcystin concentrations in nestlings. Microcystin in nestling fecal sacs was also significantly higher at this site where the proportion of aquatic prey in the nestling diet was greater. Intersite differences in nestling diet were attributed to aquatic vs terrestrial prey availability at these sites,[46] which may explain differences in the amount of toxin being passed in fecal sacs. These findings suggest that microcystin elimination, as indicated by excretion, follows trends in exposure, as indicated by mayfly provisioning rates. Analysis of fecal sacs can therefore provide a useful and nonlethal means for assessing microcystin exposure in riparian birds. We lack paired observations that

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would allow us to determine whether microcystin concentrations in fecal sacs are correlated with tissue concentrations, but suggest that this may be an interesting area for further research. We did not find intersite differences in liver microcystin concentrations of nestlings. At our low mayfly abundance site (Presquile NWR), there was a higher proportion of food items provisioned that were too small to identify. A portion of these may have been aquatic prey such as diptera and trichoptera, which exhibited microcystin levels similar to mayflies. Regardless, the increase in nestling liver MC with an increase in mayfly provisioning at one of our sites indicates an important connection between a riparian predator and an aquatic toxin.

MC levels were not correlated with warbler body condition or nestling growth rate, suggesting that these consumers do not suffer deleterious effects detectable at the organismal level. The liver has important metabolic functions and aids in fat deposition—a critical process prior to and during migration. However, it has been shown that migratory insectivores may have greater tolerance to environmental toxins than other passerine species due to their evolutionary history of exposure to a more diverse array of toxins.[54] Our assessment of health effects on the migratory Prothonotary Warbler may therefore be conservative in predicting effects of algal toxins on other insectivorous songbirds. We found that growth rates among nestlings receiving a greater proportion of mayflies in their diet was significantly higher than those feeding predominantly on caterpillars. We hypothesize that the benefits of an aquatic insect-based diet may outweigh potential deleterious effects of greater exposure to algal toxins. While provisioning rates were similar at the two sites,[46] mayflies were on average larger (~24 mg ind^{-1}) than caterpillars (~14 mg ind^{-1}) suggesting that nestlings at the high-mayfly site may have benefitted from greater food resources. Further study is needed on algal and terrestrial plant toxins and other dietary factors (e.g., protein and lipid content) to better understand the nutritional benefits of aquatic vs terrestrial prey for riparian consumers.

In summary, this study provides evidence that the presence of algal toxins in food webs is not limited to the aquatic realm. The presence of microcystin in emerged mayflies, caddisflies and midges has implications for the diverse assemblage of insectivorous organisms found in riparian habitats including bats, reptiles, amphibians and birds. As many of these are species of management concern, it is important to assess threats that may arise from the presence of toxins in their prey. Our results are from a system with relatively low cyanobacteria abundance and toxin concentrations;[55] riparian communities adjacent to cyanobacteria—dominated waters are likely to be at greater risk. These findings support recent studies documenting biotransport of contaminants via emerging aquatic insects.[56,57] There are however a number of considerations in extending the "dark side of subsidies" concept[4] to algal toxins. First, for contaminants such as PCBs, their capacity for bioaccumulation, coupled with their widespread occurrence in streams, creates the potential for quantitatively significant fluxes via emerging insects. As algal toxins are not known to bioaccumulate, their fate is linked to fluxes of the algae themselves (e.g., sedimentation, downstream transport) and it is unlikely that export via emerging insects would be a quantitatively important loss mechanism from aquatic systems. Second, our data suggest low persistence of algal toxins in riparian consumers such as Prothonotary Warblers, possibly due to age-related dietary shifts to terrestrial prey. The utility of algal toxins as tracers of aquatic subsidies to riparian habitats may therefore be more limited than for persistent contaminants such as mercury and PCBs. However, the presence of microcystin among diverse consumers provides evidence that despite known mechanisms of feeding avoidance, cyanobacteria directly support secondary production of higher trophic levels in aquatic and riparian food webs.

Additional studies are needed to characterize algal toxins in food webs and facilitate cross-system comparisons that will improve our understanding of risks to humans and biota. Technical difficulties in measuring microcystin in tissues pose a challenge to synthesis efforts. ELISA, the widely used method for measuring microcystin, has been shown to yield reliable results in simple matrices, such as water, but determinations from complex matrices, such as tissues, result in variable recoveries. While some studies have shown good correspondence between ELISA-based and other methods of analysis, some have not, thereby complicating cross-system comparisons where different methods are used.[51,58] Further advances in analytical procedures that are applicable to monitoring efforts are needed to improve our understanding of the presence of microcystin in food webs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02760.

A table showing results of backward stepwise multiple linear regression used to determine significant factors predicting the microcystin content of mayflies, spiders, all birds, adult birds, fledglings, nestlings, and fecal-sacs, a figure showing the relationship between liver microcystin concentrations in Prothonotary Warbler fledglings by date, and a figure showing the relationship between liver microcystin concentrations and provisioning of aquatic prey for Prothonotary Warbler nestlings at Deep Bottom Park (PDF)

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Notes

The authors declare no competing financial interest.

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