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Bacterial Microbiota Response in *Graptemys pseudogeographica* to Captivity and Roundup® Exposure

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Understanding how environmental factors influence various aspects of freshwater turtle health remains an important yet understudied topic within the context of individual–environment interactions. This is particularly true of host-associated bacterial microbiota, which are being increasingly recognized as a significant and understudied topic in the context of individual turtle health. While this area of work has expanded in certain areas, research efforts remain limited with regard to host–microbiota interactions in the context of habitat contaminants. Specifically, the commonly used herbicide, glyphosate, is of interest due to its massive worldwide use and known effects on various organisms. Effects of captivity on host-associated microbial community structure also remain largely unknown in various non-model organisms. To address these unknown effects of Roundup® and captivity on host-associated microbiomes, we examined the effects of low-level Roundup® exposure and captivity on the cloacal microbiota of the False Map Turtle, *Graptemys pseudogeographica*. We determined the effect of glyphosate by taking cloacal swabs pre- (0 h) and post-exposure (72 h) and examined microbial community beta- and alpha-diversity through 16S rRNA gene high-throughput sequencing. The results of this study indicate that low-level, short-term glyphosate exposure does not significantly alter the microbiota structure of *G. pseudogeographica*. However, there was a significant decrease in microbial community beta-diversity over time, confirming a trend that has been observed to a limited extent in other non-model organisms when put in laboratory conditions. These results are useful in understanding the baseline cloaca microbial community structure of *G. pseudogeographica*, as well as the implications and limitations of laboratory-based microbiota studies. Furthermore, this work suggests that low-level and short-term glyphosate exposure does not have a significant effect on the cloacal microbial community structure in wild-caught *G. pseudogeographica*.

FRESHWATER turtles have a global distribution and are an important component of various aspects of ecosystem health and function (Bakker et al., 2016; Rodrigues et al., 2017; Ali et al., 2018). With mounting pressures on their habitat and health, many freshwater turtle populations are experiencing declines with multiple species now threatened (Quesnelle et al., 2013; Gibbons et al., 2017). Nonetheless, freshwater turtle conservation remains relatively understudied. Freshwater turtle habitats face increasing threats globally, and understanding the effects of these threats remains an increasing and important challenge for turtle conservation biology. Of the many aspects of these threats to be studied, those at the interface of individual turtles and their environment are of key concern.

One area that has recently become of interest is the role animal-associated microbiota have on both host and ecosystem health (Marchesi et al., 2015; Hird, 2017; McKenney et al., 2018). The maintenance of a functionally diverse microbial community structure has an important role in host health, which includes the prevention and amelioration of disease, the breakdown of contaminants, and the generation of organic molecules used in host function (Silbergeld, 2016). Within turtles, the current understanding of host–microbiota dynamics is especially narrow, being restricted to bacterial microbiota from wild-caught, marine turtles (Price et al., 2017; Ahasan et al., 2018; Kittle et al., 2018). Virtually nothing is known regarding the host-associated bacterial community of turtles or how it is altered via captivity or various environmental factors. Understanding the effects of captivity could have profound effects on laboratory-based experimental results. Additionally, while numerous environmental factors can be accounted for, agricultural activities and their associated environmental contaminants are increasingly the subject of intense scrutiny vis-à-vis their effects on host-associated microbiota (Kakumanu et al., 2016; Krynak et al., 2017). Examinations of contaminant effects on turtle microbiota remains a largely unexplored frontier.

Turtles possess many structures capable of maintaining a diverse microbiota by interaction and seeding with the external environment. This includes the skin, gastrointestinal tract, oral cavity, and cloaca. The cloaca is of exceptional interest due to its unique position connecting the immediate external environment with the more isolated regions of the gastrointestinal tract. This structure is likely rich in microbial diversity due to its interface between the host gut and external environment. Moreover, some turtle species are known to respire via their cloacal bursae, but this function is not confirmed in *G. pseudogeographica* and may vary with location and time of year (Gonzalez, 2017). The cloacal bursae in many cryptodiran turtle species may also be used for discharging water to soften soil during nest building (Ehrenfeld, 1979). Transfer of microbiota from the nesting female to the hatchlings might occur through bacterial seeding of the nest site. Because of these factors as well as the ease of sampling which precludes the need to euthanize turtles, the cloaca deserves special attention for initial work on turtle-associated microbiota.

External environmental factors influencing cloacal bacteria communities are diverse and include both natural and anthropogenic sources. One major contaminant that has been recognized within aquatic environments is the herbicide glyphosate, often marketed under the commercial name Roundup® (Monsanto Company, Marysville, OH). Glyphosate is the most common herbicide used worldwide (Giesy et al., 2000; Annett et al., 2014; Benbrook, 2016). The active ingredient of glyphosate, 2-(phosphonomethylamino)acetic acid, is a broad spectrum, non-selective herbicide. Glyph-

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osate functions by inhibiting a key enzyme, 5-enolpyruvyl-shikimate-3-phophate (EPSP) synthase, in the shikimate pathway. The shikimate pathway is responsible for the biosynthesis of aromatic amino acids (phenylaline, tyrosine, and tryptophan), which are necessary for peptide, secondary metabolite, and vitamin synthesis, and has been recorded in plants, fungi, and bacteria (Franz et al., 1997). Glyphosate is frequently detected in aquatic systems near corn and soybean fields, and increasing levels of glyphosate have been reported in monitored streams in the Midwestern United States since 1989 (Battaglin et al., 2005). Our own laboratory has found glyphosate present in river water at similar concentrations (2.0 μg/L±factor of 2) to previous studies looking at glyphosate levels within the Missouri River and its tributaries (Battaglin et al., 2005; Kolpin et al., 2006; JLK, unpubl. data). Therefore, if glyphosate is present in aquatic environments, it is hypothesized that it may disrupt microbiota compositions of non-target organisms and have the potential to influence host health and fitness (Samsel and Seneff, 2013).

Considerable effort has sought to elucidate the effects of short-term glyphosate-based herbicide exposure on amphibians (Relyea, 2012), invertebrates (Folmar et al., 1979), and fishes (Mitchell et al., 1987; Guilherme et al., 2012); however, few studies have examined effects of glyphosate on reptiles such as turtles. Recent work has also been completed on Red-eared Sliders (*Trachemys scripta elegans*) with Héritier et al. (2017) showing glyphosate exposure induces stress in *T. s. elegans* and Sparling et al. (2006) finding that glyphosate exposure reduces hatching success and inhibits hatchling growth. Even fewer studies have examined the effects of glyphosate on microbiota composition in turtles. An in vitro experiment on mixed bacterial communities and microbial isolates from the gastrointestinal tracts of freshly euthanized Green Sea Turtles (*Chelonia mydas*) found that glyphosate inhibited the growth of bacteria (Kittle et al., 2018). Though a few studies have characterized the microbiota of turtles, to our knowledge, there have been no such studies examining the effects of glyphosate on the microbiota of field-collected, freshwater turtles.

Here, we present data examining the aforementioned effects in the False Map Turtle (*Graptemys pseudogeographica*), a widespread riverine, emydid turtle that is primarily found in the Mississippi and Missouri river drainages as well as several smaller river drainages in Louisiana and east Texas (Lindeman, 2013). In the upper Missouri River, *G. pseudogeographica* is listed as a "Species of Conservation Priority" in North Dakota (Dyke et al., 2015) and state-threatened in South Dakota (Ashton and Dowd, 2008). The primary cause for population declines in the upper Missouri River are poorly understood, though are potentially the result of extensive habitat modification and contaminants from agricultural areas that enter the river.

To better understand both microbial community taxonomy dynamics in freshwater turtles as well as the potential risk faced by remaining populations of *G. pseudogeographica* in agriculturally dominated landscapes, we examined the effects of acute Roundup® exposure on the cloacal microbiota of *G. pseudogeographica*. We hypothesized that Roundup® exposure may alter the bacterial diversity and community composition within the cloaca of *G. pseudogeographica*. We also examined the effects of captivity on the host-associated microbiota over time. This work provides novel insights into how environmental contaminants and captivity conditions disrupt cloacal bacteria community structure. Such insights are important for informing future study design and management decisions regarding turtles in freshwater systems with adjacent agricultural landscapes.

MATERIALS AND METHODS

Animal capture, treatment, and sample collection.—*Graptemys pseudogeographica* were collected along Goat Island, in the 59-mile stretch of the Missouri National Recreational River between Yankton and Vermillion, South Dakota, USA on 27 July 2017. Turtles were captured using partially submerged hoop traps that were baited with sardines, which were set near basking surfaces (e.g., downed trees) along the shoreline of Goat Island. After 24 h, the traps were removed and ten adult female turtles were collected. Mass, carapace length, and plastron length were recorded for each turtle ($\bar{x}$±SEM; mass: 556.5±104.6 g; carapace: 161.3±12.5 mm; plastron: 147.9±10.6 mm) to ensure nonsignificant differences of these metrics when separating individuals into experimental groups. Only females were included in this study as previously collected data suggest female-biased sex ratios of *G. pseudogeographica* in this stretch of the Missouri River (Lindeman, 2013; DRD, unpubl. data) and acquiring comparable numbers of males was not possible. Upon removal of individuals from traps, a cloacal microbiota swab of each turtle was immediately collected using a sterile cotton swab (#MWE113, Medical Wire & Equipment, Corsham, Wiltshire, UK) representing the pre-exposure microbiota sample. Sterile cotton swabs were inserted into the cloaca of turtles, fully rotated three times, and then gently removed. Following collection of the microbiota sample, individual turtles were immediately transported to the University of South Dakota. Each turtle was placed into separate HDPE plastic tubs containing 20 L of reconstituted RO water and was randomly subjected to either RO water (control) or 10 μg/L glyphosate in RO water (n = 5/treatment). Glyphosate was dosed by dilution of a standardized Roundup® solution containing 2.0% w/v glyphosate (Roundup® Ready-to-Use Weed & Grass Killer®, Monsanto Company, Marysville, OH: 100 μL of 2 g/L glyphosate stock solution was added to 20 L of water for a final concentration of 10 μg/L). Turtles remained in these containers for 72 h, at which point they were removed from the water and a post-exposure microbiota swab was taken following the same methods described above. All swabs were kept in sterile microcentrifuge tubes and immediately stored at –20°C until extraction. Turtles were not fed during the experiment to prevent the addition of any foreign microbiota. Animal handling in both the lab and the field as well as all swabbing was completed with gloves using standard sterile technique.

DNA extraction and purification.—DNA extractions were conducted using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Overnight digestion for all samples was done using standard protocols for tissue with a proteinase K digestion at 56°C. After extraction, DNA was cleaned and concentrated using the ZR-9 Genomic DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA) following provided protocols.

Library preparation and high-throughput sequencing.—DNA from each sample was quantified on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) using the Qubit dsDNA HS Assay Kit (quantitation range: 0.2–100 ng). Up to 15 ng of DNA was used to prepare a library for high-

throughput sequencing (HTS) using a modified dual indexing protocol proposed by Illumina (Illumina 16S Metagenomic Sequencing Protocol [15044223 Rev. B]). Primers used in the first round of amplification targeted the V4 region of the 16S rRNA gene. Illumina overhang adapters were added to 515F and 806R primers (V4_515F: 5′–TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **GTG YCA GCM GCC GCG GTA A**–3′ and V4_806R: 5′–GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GG ACT ACH VGG GTW TCT AAT**–3′; 515F and 806R base primer in bold). Primary amplification was done in duplicate for each sample using 2x KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, MA) following the cycling protocol: initial denaturation at 95°C for 3 min, followed by 25 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min on a Veriti Thermal Cycler (Thermo Fisher Scientific). All reactions were purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) prior to indexing. A bead solution/PCR product ratio of 0.8 (20 μL of bead solution with 25 μL of PCR product) was utilized for this and all subsequent purifications. Secondary amplification of each sample was done using 2x KAPA HiFi HotStart Ready Mix and a combination of two unique Nextera XT Index primers (N7xx and S5xx). Amplification was completed on a Veriti Thermal Cycler (Thermo Fisher Scientific) using the following cycling protocol: initial denaturation at 95°C for 3 min, followed by 8 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The libraries were purified with Agencourt AMPure XP beads and quantified on a Qubit 2.0 Fluorometer, using Qubit dsDNA HS Assay Kit, normalized, and pulled together. The final library was gel re-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI). Paired-end sequencing was performed on a MiSeq instrument using the MiSeq Reagent Kit v3 600 cycles (Illumina Inc., San Diego, CA).

Sequence data and statistical analysis.—Initial processing and analysis of HTS data was completed using the Mothur bioinformatics software tool (version 1.39.5; Schloss et al., 2009). Briefly, the standard MiSeq SOP was followed starting with generating contigs from paired-end reads, clean-up steps (including screening, filtering, and chimera removal), alignment to the Silva database (1.32), and generation of OTUs for statistical analysis (Kozich et al., 2013; corresponding webpage accessed 29 January 2018). Community composition, alpha-diversity, beta-diversity, and two-sample t-test metrics were all calculated and visualized with the R statistical language (version 3.4.2; R Core Team, 2017). R packages used included Phyloseq, ggplot2, Vegan, dplyr, Scales, Grid, and Reshape2. All scripts used have been deposited in GitHub (for supplementary information, see Data Accessibility).

RESULTS

Microbial community composition of *G. pseudogeographica* varied with time, but not between herbicide treatments (Fig. 1). Non-metric multidimensional scaling was used to visualize ordinal distances of community beta-diversity using the Bray-Curtis distance in two-dimensional Euclidean space with a square root transformation and Wisconsin double-standardization (Fig. 2, stress of fit = 0.103). There was found to be no significant difference in the microbial community structure between control and glyphosate treatments (PERMANOVA: pseudo-F = 1.1053, $R^2 = 0.05785$, $P = 0.31$; Figs. 1, 2). However, we did observe a significant difference between 0 h and 72 h treatments (PERMANOVA: pseudo-F = 5.0592, $R^2 = 0.2194$, $P = 0.001$; Figs. 1, 2). This significance, however, does not account for all of the variation seen in the data with other factors possibly being involved (e.g., individual variation, age of turtle). In addition to PERMANOVA tests, a permutation test for homogeneity of multivariate dispersions was completed with no significant differences between groups (F = 0.1028, number of permutations = 999, $P = 0.732$), allowing an additional level of confidence in the PERMANOVA results in that the differences seen are not the result of differences in group dispersions.

Overall, Proteobacteria dominated the cloacal communities under all conditions tested. Notable dynamics included emergence of the Verrucomicrobia after 72 h in captivity (Mean proportion±SEM; 72 h: 0.008±0.006). There were also significant proportional changes (Welch two-sample *t*-test, $P < 0.05$) of microbial phyla between time treatments in the phylum level representation of Actinobacteria (0 h: 0.064±0.009; 72 h: 0.028±0.009), Cyanobacteria (0 h: 0.064±0.012; 72 h: 0), and Proteobacteria (0 h: 0.495±0.025; 72 h: 0.643±0.020). Changes seen in other represented phyla, however, are not fully understood. In the numerous rare phyla identified in the generated OTUs, the presence of the Chlamydiae in 12 OTUs (0.19% of all OTUs generated) is noted here due to its unique lifecycle involving intracellular growth in eukaryotic cells.

Average and changes in alpha-diversity are also reported (Fig. 3). Both within community richness and inverse Simpson is compared with operational taxonomic units (OTUs) as the unit of analysis. A general trend is seen of decreasing alpha-diversity over time. However, this qualitative trend cannot be statistically verified due to limited biological replication.

DISCUSSION

This study represents the first effort examining changes in freshwater turtle microbiota community composition in response to captivity and glyphosate. Previous work in marine turtles by Kittle et al. (2018) showed significant effects on microbial isolates grown in vitro as well as in bacterial culture density (OD600) when exposed to varying concentrations of glyphosate in vitro. Prior to glyphosate exposure, however, these bacterial isolates were allowed to grow in vitro in nutrient broth, likely altering the initial community abundance and composition of the gut sample. While our results with glyphosate exposure to the cloacal microbiota showing no significant effects disagree with the in vitro work of gut-derived bacteria done by Kittle et al. (2018), it should be noted that in vitro growth methods are likely to have significant differences from direct in vivo sampling and that host-associated microbial communities are possibly more robust in resisting changes to environmental conditions, as suggested by our work. Kittle et al. (2018) also used culture density for statistical comparisons while our work directly examined community diversity. To what extent bacterial culture density can be used as a proxy for host-associated microbiota disruption by glyphosate-based solutions remains unclear. Previous studies have also looked at the general effects of glyphosate on bacteria on cow or plant root-based microbiota. Schlatter et al. (2017) found no significant effect of glyphosate on the root-associated microbiota. Additionally, the effects of glyphosate on the

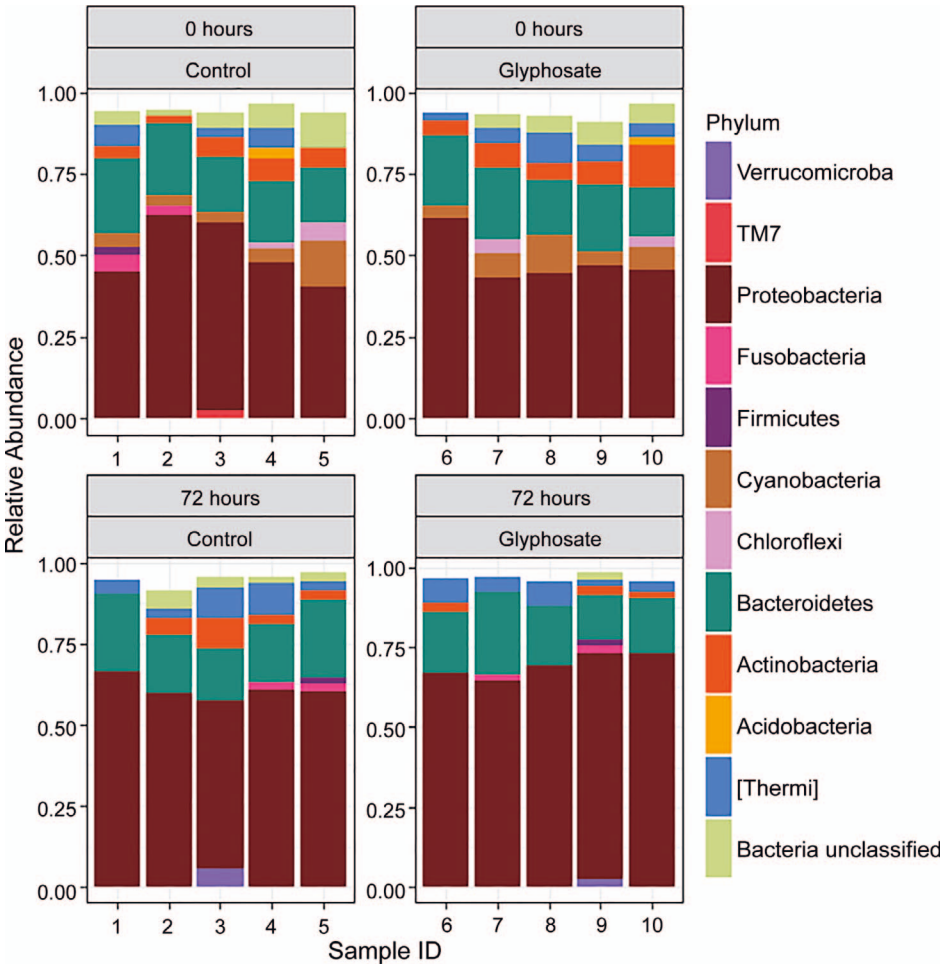

Fig. 1. Gut microbiota composition shown with each bar representing one sample. Samples are separated into groups by time (0 h, 72 h) and treatment (control, glyphosate). Each color is representative of a corresponding phylum (or in the case of "Bacteria unclassified," unidentified members of the Bacteria). Bars do not fully reach 1.00 as low representation groups (<2% of total abundance) were excluded from the analysis for ease of interpretation.

ruminal microbiota of cows has also been examined with only small shifts in microbial community structure (Riede et al., 2016). These studies, along with the presented work, suggest that low-level glyphosate exposure may have no or only a small effect on bacterial community structure in turtles.

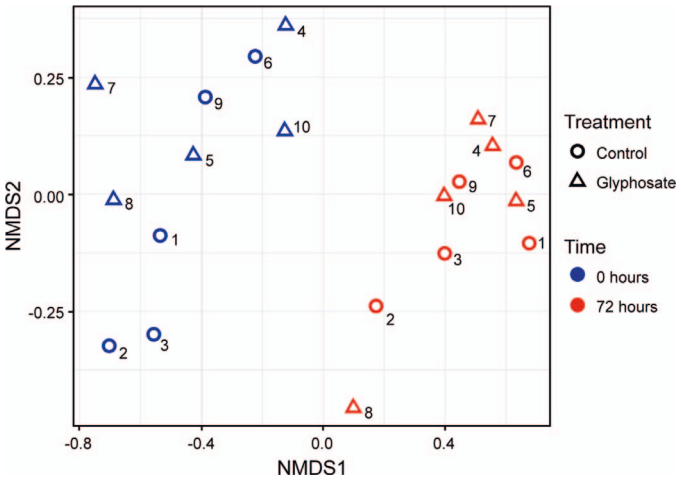

Fig. 2. Non-metric multidimensional scaling (NMDS) calculated with the Bray-Curtis distance metric using a square root transformation and Wisconsin double-standardization. Treatments and time are represented by shape and color, respectively. Stress of fit for the ordination is reported at 0.103. Axis titles represent the two dimensions to which the data have been ordinated.

In this work, we also present the first dataset taxonomically characterizing the microbiota of the cloaca of *G. pseudogeographica*. While changes in microbial beta-diversity among samples by treatment were not observed, we did see a significant change in beta-diversity that can be at least partly attributed to time. While this is not necessarily a function of being in captivity, all individuals were in captivity, which is a likely explanatory factor. A decrease in beta-diversity over time is not uncommon and has been seen in other animal microbiota studies completed in captivity with wild-caught organisms such as woodrats and salamanders (Kohl et al., 2014; Loudon et al., 2014). However, an examination of the cloacal microbial community structure as a function of captivity in marine turtles was completed by Ahasan et al. (2018) showing no differences in community structure pre- and post-captivity over time and thus in disagreement with the results presented from our study. This apparent divergence of results can be attributed to many factors including different species of turtles, time in captivity, marine versus freshwater environments, and also the conditions of captivity. Ahasan et al. (2018) kept turtles with recirculated seawater which likely better simulated natural microbial conditions than captivity in RO water only. Regular feeding by Ahasan et al. (2018) over the course of captivity with seafood is also a possible source of seeding and maintenance of the cloacal microbiota which was absent from our short-term study. We therefore suggest that our results are useful in showing the effects of different types of captivity when housing turtles whether it be for experimental or rehabilitation purposes. Indeed, a general decrease in both alpha- and

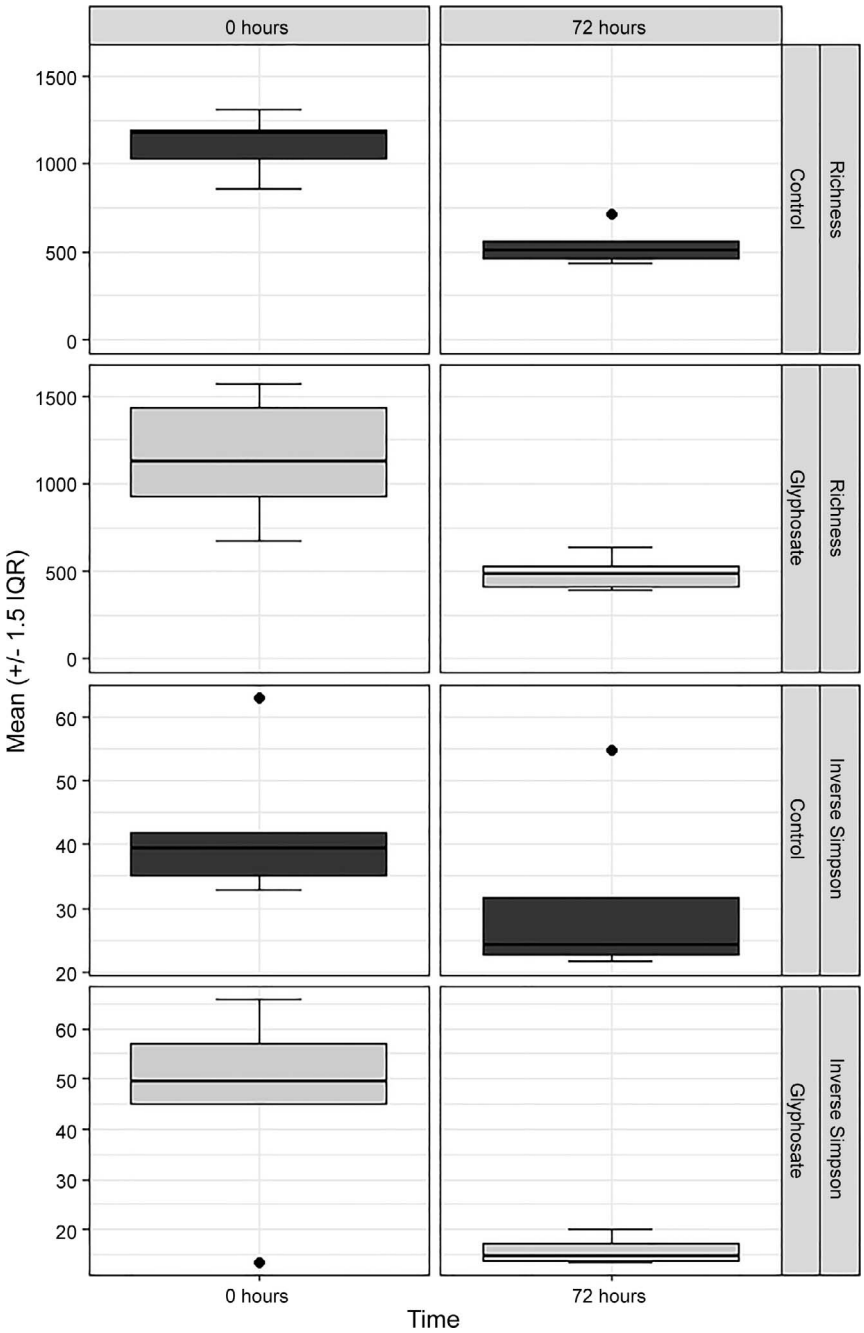

Fig. 3. Comparison of richness and alpha-diversity (Inverse Simpson) between treatments. Box plots are given with whiskers representing 1.5× the interquartile range (IQR). Outliers are given as single dots. Each bar subset represents $n = 5$ samples.

beta-diversity is an important consideration when doing microbiota studies on herpetofauna which aim to answer ecological questions pertaining to the environment of interest. These changes in community structure pre- and post-captivity over time are likely caused by a variety of factors including differing water conditions, temperatures (more fluctuations in a non-laboratory environment), and potential light differences. The artificial nature of a laboratory setting also limits the ability of continuous seeding of the host gut microbiota from the environment, which is probably an important component to the maintenance of bacterial diversity and retention of varied community functionality in natural settings.

In addition to general community shifts, dynamics of specific bacterial phyla are also of interest in these turtles. The Proteobacteria constituted the most represented phylum in cloacal microbiota samples of *G. pseudogeographica* under all treatment conditions. The dominance of the Proteobacteria in the host-associated microbiota of other taxa is not uncommon, and in the case of the amphibian cutaneous microbiota, is also known to harbor the largest number of anti-fungal isolates (Woodhams et al., 2015). Many members of the Proteobacteria are also ubiquitous in the environment (Spain et al., 2009; Tomczyk-Żak and Zielenkiewicz, 2016). The significant increase of the Proteobacteria from 0 h to 72 h is likely due to stabilization of the microbiota under captive conditions. These results suggest that the Proteobacteria are more resilient to the experimental conditions and thus tend to dominate the overall microbial community over time. This is in agreement with the experimental results of Ahasan et al. (2018). The decreased presence of the Cyanobacteria in this study can also be viewed as part of a community stabilization trend over time under captivity conditions. Reduction of Cyanobacteria in the cloaca for turtles brought into laboratory conditions is possibly due to a lack of seeding from the external environment in which conditions for cyanobacterial

growth may be better due to sunlight. The lack of sunlight in the cloaca, however, does not preclude species of Cyanobacteria from being part of the cloacal microbial community with many species having the ability to undergo chemoheterotrophic growth (Stebegg et al., 2012). Lack of a competitive advantage (photosynthesis), however, may be enough to exclude members of the Cyanobacteria when not being maintained through constant environmental seeding. Other conditions may have also contributed to the reduction in Cyanobacteria such as a lack of iron which may have been limiting under the experimental conditions used. Iron is known to be an important requirement in Cyanobacteria for the maintenance of various metabolic processes including photosystem function, nitrogen fixation, and overall growth rates (Berman-Frank et al., 2007). Cyanobacteria are also known to produce a number of compounds with various anti-bacterial, anti-fungal, and anti-viral properties (Volk and Furkert, 2006; Abed et al., 2009). Thus, the significant decrease seen in the Cyanobacteria after 72 h in captivity is of interest for future in vitro work with turtles. Such studies should take note of the potentially reduced anti-microbial properties of the cloacal microbiota of *G. pseudogeographica* when kept in laboratory conditions and should be cautious in explaining any resulting data.

A significant decrease in representation of Actinobacteria was also seen over time with a strong potential for laboratory conditions affecting community dynamics. Specifically, the Gram-positive Actinobacteria are often found in soil and freshwater habitats making the possibility of environmental seeding of this phylum likely and a possible explanatory factor in its decrease under laboratory conditions (Fierer et al., 2007; Liu et al., 2012). Some members of the Actinobacteria are also known for their spore forming ability under adverse growth conditions, of which laboratory conditions may be considered (Barka et al., 2016). Thus, it is also reasonable that the decrease of the Actinobacteria seen was due to a subset of Actinobacteria ending active replication and switching to the spore stage of their life cycle.

Other phyla comprising a large component of the samples examined were Acidobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Fusobacteria, Verrucomicrobia, Thermi (Deinococcus-Thermus), and candidate phylum TM7. These phyla all represent ubiquitous bacteria that are commonly found in environmentally sourced microbial datasets (Costello and Schmidt, 2006; Fierer et al., 2007; Tian et al., 2009; Bergmann et al., 2011; Zhao et al., 2012; Santhanam et al., 2017). Many other phyla were also represented within the overall community in very low abundance. Unique among the rare phyla present were OTUs representing members of the Chlamydiae. This is of interest due to the known tendency of many members of this phylum to be intracellular and disease-causing (Taylor-Brown et al., 2015). However, it is unclear if intracellular infection of turtle cells by members of the Chlamydiae is occurring or if these are merely transitory non-infectious bacteria amplified during HTS. The identification of *Salmonella* and *Shigella* spp. by Ahasan et al. (2018) would suggest that turtles may generally harbor bacteria pathogenic to humans. The ability to generalize this trend among turtles and environs, however, is not fully understood and merits further consideration. While the bacterial phyla presented here are diverse and have various explanations for their dynamics over time, these data should nonetheless be found useful in understanding the microbial communities associated with freshwater turtles. The taxonomic descriptions of the dominating bacterial phyla will also complement future work examining turtles and other aquatic reptiles.

Based on low-level glyphosate exposure (10 μg/L), the effects on microbial community structure were not significant when compared to the control group. While higher glyphosate concentrations could have been used, the concentrations used in this study reflect the more general concentrations seen in many watersheds in the United States (Battaglin et al., 2005; Relyea, 2005, 2012). The long-term effects of low-level glyphosate exposure on host microbial community structure in turtles, however, are at this point not well understood. We suggest that understanding the short-term effects of low-level glyphosate exposure is nonetheless important for understanding the effects on riverine turtle species due to the short-term exposure associated with pulse events (i.e., heavy rainfall) over land area which glyphosate has been recently applied. The relatively short half-life of glyphosate in freshwater environments also merits the study of short-term effects on host-associated microbiota.

The lack of effects from glyphosate-containing Roundup® has multiple potential explanations with regard to this study. One plausible explanation is that herbicide effects from previous rainfall events have already influenced bacterial community structure and that these effects may have a prolonged or even permanent impact. The sampling location used in this study avoided the mouths of large rivers (James, Vermillion, and Big Sioux rivers), which may have had disproportionately high concentrations of glyphosate where they entered the Missouri River. Another plausible explanation for the lack of effects seen by glyphosate-containing Roundup® could be due to a masking effect from loss of diversity over time. As no environmental seeding was occurring in captivity, and based on the significance seen over time in captivity, it is plausible that small or similar effects that would be attributable to glyphosate were masked by this factor. One final explanation is that glyphosate is presently not having significant effects on the microbiota community structure. As all microbial communities are from *G. pseudogeographica* that were captured in an agricultural landscape, it is likely that the microbial communities associated with these turtles have been exposed to glyphosate for long periods of time. This long-term exposure could have had stabilizing effects on the community. It is also plausible that previously glyphosate-sensitive bacteria species that were constituents of the community evolved resistance, thereby making the community resilient to fluctuating glyphosate effects.

We have therefore shown that low-level glyphosate exposure over short time periods in a laboratory setting had no significant effect on bacterial community structure in wild-caught *G. pseudogeographica* and that time does, however, have a significant effect. The reasons for acute glyphosate exposure not affecting the microbial community of the cloaca remain unclear, with the extent of lifetime environmental exposure to glyphosate uncertain and likely varying over time and place. While we would suggest the same experiment with *G. pseudogeographica* which have never been exposed to glyphosate, it is doubtful such a population exists outside of captivity. Rearing a population in captivity, however (as we have shown), will also have challenges in maintaining a diverse microbial community equivalent to what would be found in wild-caught *G. pseudogeographica* never exposed to glyphosate or glyphosate-containing solutions (e.g., Roundup®). The significance of the time effect on the cloacal microbiota is likely due to captivity under

laboratory conditions. Continued work is needed in understanding the role of glyphosate and other environmental contaminants on host microbiota under natural conditions and with varying time durations. Due to the emerging understanding of the importance of host-microbiota associations and the subsequent impact on host health, research on the effects of contaminants on host-microbiota associations both short and long-term is a critically important frontier with many unresolved questions that remain to be addressed.

DATA ACCESSIBILITY

All raw sequencing data used in this study can be accessed through the NCBI database in the Sequence Read Archive (SRA) under accession number SRP132267 (BioProject: PRJNA433062). All R scripts used in data analysis and figure generation can be found on GitHub (kvasir7/turtle_glyphosate /R_script).

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