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SHORT TERM MICROBIAL COLONIZATION OF REPTILE ROADKILL

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ABSTRACT: Little is known about how microbes such as bacteria and fungi in the environment temporally colonize common roadkill reptile carcasses (turtles and snakes). We opportunistically collected and deployed a variety of reptile carcasses often found deceased near roads, including an Eastern Ratsnake (*Pantherophis alleghaniensis*), an Eastern Box Turtle (*Terrapene carolina*), and a Snapping Turtle (*Chelydra serpentina*). We sampled bacteria communities of these carcasses daily for five consecutive days. We enumerated the number of colony-forming units (CFUs) and characterized microbial distinct colonies using morphology and identification of dominant colonies using 16S rRNA sequencing across carcasses. Several ecologically relevant bacterial phyla were successfully identified colonizing and dominating carcasses differentially, including members of Actinobacteria, Firmicutes, and Proteobacteria. We observed higher bacterial colonization (CFUs) for both terrestrial and aquatic turtles, *T. carolina* and *C. serpentina*, compared to *P. alleghaniensis*. This study provides baseline data on the temporal microbiology of deceased reptiles found on roads in the piedmont of North Carolina.

Key Words: Anthropogenic Mortality, Microbiology, Scavenging, Bacteria, Turtle, Snake

INTRODUCTION

Each year many reptiles are observed deceased on roads (Dodd et al., 1989), often in urban areas when they are either basking or crossing roads and fall victim to vehicular encounters (Smith and Dodd, 2003; Degregorio et al., 2011). This includes representatives from Squamata and Testudines (Quintero-Angel et al., 2012; Gibbs and Shriver, 2002). Once these host organisms die, their own native microflora combine with other soil microbes present in the environment which begin to differentially colonize a decomposing carcass (Howard et al., 2010). Among microorganisms, both fungi and bacteria are known to colonize different surfaces of both living and decomposing animals (carcasses) albeit, at different stages (Carter et al., 2007). While all types of reptile carcasses are scavenged by a variety of vertebrates and invertebrates, often within hours after death (Abernathy et al., 2017), very little is known regarding dominant microbial communities which sequentially colonize reptile roadkill (Cooper, 2012), especially within the first few days of mortality.

The microorganisms of terrestrial and aquatic habitats are ubiquitous and abundant, comprising a diverse array of microbial communities in the environment. These varied bacterial and fungal microbes have many different roles on living reptiles, with some being beneficial (Bouchard and Bjorndal, 2005), some detrimental (Jho

et al., 2011), and others likely omnipresent animal, soil, and environmental microbes. Bacterial and fungal communities in the soil where mortalities occur are incredibly rich and extremely diverse (Dunbar et al., 2002). They thrive and change in composition temporally, depending on myriad of factors including soil composition, nutrient presence, location, pH, temperature, and humidity (Fierer and Jackson, 2006). Cadaver studies in mammals have found vast differences in microbial communities between initial deployment (day 0) and day 5, with early communities dominated by the phylum Proteobacteria (largely aerobic bacteria) and later stages dominated by the phylum Firmicutes or anaerobic bacteria (Pechal et al., 2014). However, there is a dearth of information on dominant microbial communities of herpetofauna.

In this study we used standard microbiological techniques (enumerating cultured bacteria on selective media and characterization of colony morphology) and 16S rRNA sequencing (for identification confirmation) to examine the diversity of microbial flora present on decaying reptile carcasses over a period of four days. Our experiment simulated a turtle or snake being run over near a road with the carcass coming to rest in a nearby forested roadside area. We report on the relative abundance and short-term bacterial growth as a component of the microbial ecology of a reptile carcass.

METHODS

Field Sampling — A total of three reptile carcasses were deployed in a forest area on Wingate University, Wingate, North Carolina: a Snapping Turtle (*Chelydra serpentina*) on 27 March 2018, and on 10 April 2018, an Eastern Ratsnake (*Pantherophis alleghaniensis*), and an Eastern Box Turtle (*Terrapene carolina*). Carcasses were obtained in coordination with local state North Carolina Wildlife Resources Commission biologists and kept frozen until deployed. Areas of the mixed Oak-Hickory hardwood forest floor, comprised of a combination of Willow Oak (*Quercus phellos*), Shagbark Hickory (*Carya ovata*), Sweetgum (*Liquidambar styraciflua*), and Shortleaf Pine (*Pinus echinata*), were cleared of leaves and debris prior to deployment. Carcasses were carefully placed in covered forested areas near a paved highway (~ 5 meters) to simulate natural road mortality but include surrounding roadside soil microbes. We sampled for microbes using Pur-Wraps® sterile collection swabs (Puritan Medical, Maine). Gloves and foot covers were worn when sampling to prevent contamination or introduction of additional microorganisms. All carcasses were swabbed externally across all surfaces (shell, head, body, and cloaca) immediately upon initial deployment for a total of ten seconds. Each carcass was swabbed twice using this procedure, and a randomly selected swab was used for microbial plating on media. Swabs selected were placed in sterile 10 ml collection tubes. This procedure was repeated for 4 more days (with Day 0 = first deployment day).

Laboratory Methodology — Swabs were taken each day to the laboratory and agar plates were made in triplicate for each carcass and for two different agar media, Brain Heart Infusion (BHI) and Potato-Dextrose Agar (PDA) following standard gel preparation guidelines (Cappuccino and Welsh, 2017). To select for bacteria and limit overall fungal growth, we added 1ml of cycloheximide per liter to the BHI mix. A total of 450 plates were used at 5 different ten-fold serial dilutions (10^0 , 10^1 , 10^2 , 10^3 , 10^4), for 5 days (Day 0, 1, 2, 3, and 4), across carcasses (Eastern Ratsnake, Eastern Box Turtle, and Snapping Turtle), for both media (PDA and BHI) in triplicate. Samples were serially diluted in 100 μ l increments, up to 10^4 based on initial dilution ratios following standard protocols (Cappuccino and Welsh, 2017) and injected on to agar gels (plates) in 100 μ l increments and spread using a sterilized metal rod. The plates were then incubated at 20 °C for approximately 48 hours to ensure proper growth of mesophilic bacteria (Lagier et al., 2015).

Following incubation, we assessed microbial growth by counting individual colonies on plate lids (Cappuccino and Welsh, 2017). We enumerated the amount of CFUs (colony-forming units) per ml for all dilution plates exhibiting 30 to 300 colonies. The average number of colony-forming units was determined across media and averages were compiled for both media. We further characterized colony characteristics and morphology (shape, color, and other defining characteristics) for each plate. Distinct colonies which we observed to be dominant were individually grown using four-way streaking methods to ensure purity (Seeley and Vandemark, 1962; Cappuccino and Welsh, 2017). Of these, we selected a subset of 11 dominant colonies for identification via 16S rRNA sequencing. A Gram stain was performed on these same colonies to aid in identification (Sanders and Miller, 2010). We then ran a standard colony PCR (Lane, 1991) and gel electrophoresis to confirm PCR product. Colony

PCR was performed in Bio Rad thermocyclers and consisted of 50 μ l reactions: 25 GoTaq® G2 master mix, 2 μ l forward primer, 2 μ l reverse primer, and 21 μ l of water using 16SrRNA gene universal ReadyMade™ Primers (Integrated DNA Technologies). The thermal profile consisted of 10 minutes at 94°C, then 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute and 50 seconds, ending with 72°C for 10 minutes. Standard 16S rRNA sequencing was performed by Eton Bioscience (www.etonbio.com). Gene sequences were subject to Basic Local Alignment Search Tool or BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to determine probable identity of isolates with 99% sequence identity.

RESULTS

Across carcass types, days sampled, and serially diluted plates, 67.3% of *C. serpentina* plates cultured bacterial colonies, whereas 80% of *T. carolina* and 48.7% of *P. alleghaniensis* plates successfully cultured bacterial and fungal colonies (Figure 1). Across all plates, we detected ten distinct fungal types, representing only ~12 non-yeast fungal colonies, confirmed via microscopic examination using a compound light microscope at 40X magnification, therefore we report mainly on bacterial

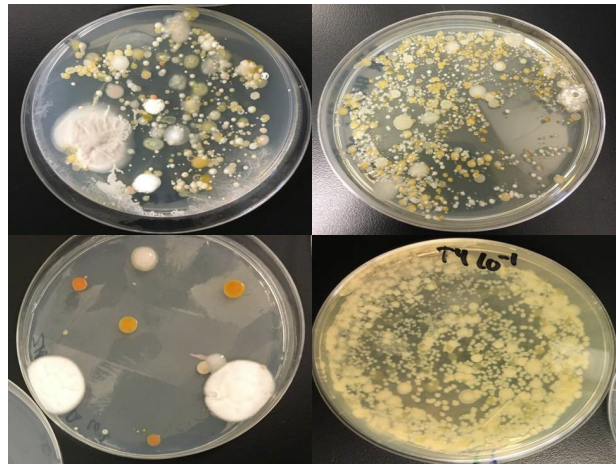


Figure 1. Representative bacterial agar plates showing distinct colony morphology and numbers in cultured media of *T. carolina*: Day 0 (top left), *C. serpentina*: Day 0 (top right), *P. alleghaniensis* Day 0 (bottom left), and *T. carolina*: Day 4 (bottom right). Note higher number of CFUs for both turtles.

colonies. Nine of the 11 dominant isolates, which were comprised largely of bacteria, were positively identified and confirmed according to 16S rRNA sequencing, Gram staining, and colony morphology (Table 1). Bacteria cultures for number of colonies varied across media and dilution factor, with both BHI and PDA media exhibiting bacterial growth at most dilutions (10^0 , 10^1 , 10^2 , 10^3) with minimal growth observed for 10^4 dilution. We observed a greater number of CFU's in both turtle carcasses across the experiment compared to the snake carcass (Figure 2). Overall CFUs increased each day the carcass was in the field, with higher CFU values observed on day 2 for *T. carolina*. Bacteria that colonized carcasses included representatives from phyla Actinobacteria, Firmicutes, and Proteobacteria, with representative genera *Staphylococcus*, *Gordonia*, *Bacillus*, *Serratia*, *Streptomyces*, *Chryseobacterium*, and *Pantoea* (Figure 3).

Table 1. 16S rRNA probable identity of isolates with host carcass, day sampled, dilution, gram staining, and basic morphology of microbial communities in our study. 16S rRNA identification of highest probable identification of genus based on BLAST alignment.

Isolate	16 S rRNA Identification	Host Carcass	Day Sampled	Dilution	Gram (+/-)	Colony Morphology, Shape, and Arrangement
A	<i>Staphylococcus</i>	Ratsnake	3	10 ⁰	+	Dull, yellow, circular, entire: Staphylococci
B	<i>Gordonia</i>	Ratsnake	2	10 ²	+	Tan, irregular, raised: Bacillus
C	Unknown	Box Turtle	4	10 ²	-	Circular, raised, convex, Bacillus
D	<i>Bacillus</i>	Box Turtle	2	10 ²	+	Irregular, rough, raised: Streptobacillus
E	<i>Serratia</i>	Box Turtle	2	10 ¹	-	Circular, off-white, entire: Bacillus
F	<i>Bacillus</i>	Snapping Turtle	0	10 ⁰	+	Raised, volcano-shaped: Streptobacillus
G	<i>Streptomyces</i>	Box Turtle	0	10 ³	+	Circular, entire, raised, powdery: Filamentous
H	<i>Chryseobacterium</i>	Snapping Turtle	0	10 ²	-	Bright, orange, iridescent, flat, circular: Bacillus
I	<i>Pantoea</i>	Box Turtle	3	10 ³	+	Dull, yellow, flat, oval: Bacillus
J	Unknown	Ratsnake	4	10 ⁰	-	White, flat, circular: Yeast
K	<i>Pantoea</i>	Box Turtle	2	10 ²	+	Dull, yellow, circular: Bacillus

DISCUSSION

While the study of reptile carcass microbiology is limited, much of the focus in bacterial communities in reptiles has focused on detection and prevalence of the *Salmonella* bacteria (McCoy and Seidler, 1973) in turtles, which we did not detect. Out of the eleven isolates selected for sequencing, nine of them were able to be identified with some degree of certainty using 16S rRNA sequencing. The majority of plates and isolates included several

types of environmental bacteria, including *Gordonia*, and the non-pathogenic *Bacillus*. In this study, we also observed *Serratia*, a soil bacterium which can be pathogenic in some nematodes and insects (Kwak et al., 2015), an opportunistic pathogen in humans (Hejazi and Falkiner, 1997), and previously detected in the Red-eared Slider, *Trachemys scripta elegans* (McCoy and Seidler, 1973) and Green Sea Turtles, *Chelonia mydas* (Santoro et al., 2006). Another isolate we identified, *Chryseobacterium*,

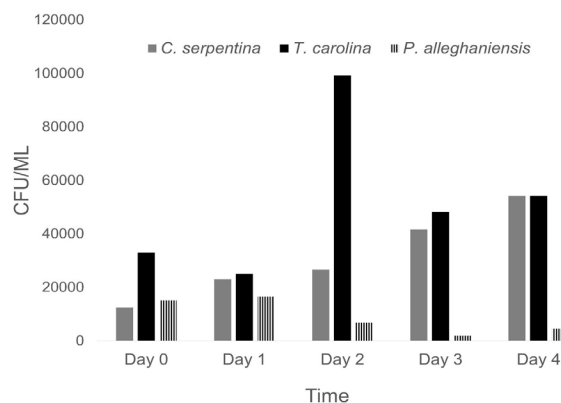


Figure 2. Bacterial growth measured by colony-forming units (CFUs) per milliliter observed across carcasses for Day 0 (carcass deployment) to Day 4. Note both turtle carcasses (*Chelydra serpentina* and *Terrapene carolina*) with higher CFU values than the Eastern Ratsnake (*Pantherophis alleghaniensis*).

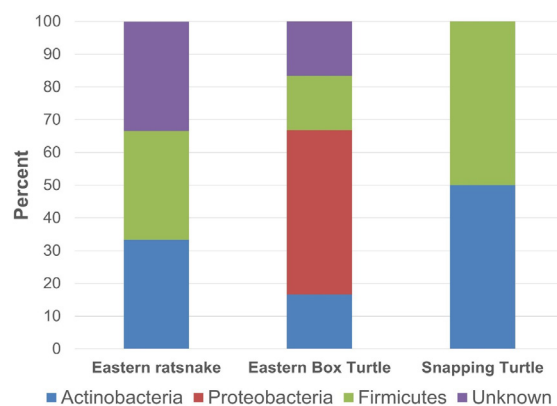


Figure 3. Relative abundances (%) of Bacteria phyla identified across carcass type (Eastern Ratsnake, *P. alleghaniensis*, Eastern Box Turtle, *T. carolina*, and Snapping Turtle, *C. serpentina*), with Actinobacteria and Firmicutes present in all reptile carcasses.

is a pathogenic and prolific soil bacterium which may be a health risk for immune-compromised individuals (Bhalla et al., 2018). Little is known regarding another isolate, *Pantoea*, which is a diverse group of bacteria both ubiquitous in the soil environment and with some varieties potentially pathogenic to humans, plants, or both (Deletoile et al., 2018). *Staphylococcus* has previously been documented in the microbiota of reptiles (Walther et al., 2008).

Based on our findings, it appears that of the bacteria observed and identified, almost all of them are common in the soil or the environment. It is unknown to what extent these bacteria affected decomposition rates and if they are common on live reptiles or simply common soil bacteria which colonized carcasses. We successfully observed several dominant types of microbial communities across carcasses. However, given our limited sample size (N = 3) and time frame (Day 0 to Day 4), it is likely we preferentially observed the most ubiquitous bacteria present on carcasses at one time. Alternatively, major groups of bacteria may have outcompeted other bacteria groups present but in smaller overall colonies, resulting in the specific groups we observed being the most prominent species present during sampling or successfully grown on a variety of media plates. Decomposition of animal carcasses, and thus colonization by both insects and bacteria, is strongly affected by temperature (Howard et al., 2010) and season (Pechal et al., 2013). Future studies could focus on increased sampling post mortality and in a variety of environmental and temporal conditions (i.e. spring vs. summer, or at varying humidity and temperatures).

The higher counts noted for both turtle species compared to the snake may be due to increased bacterial colonization of overall greater surface area (shell) or alternatively a more diverse surface morphology (plastron, head, and limbs) for bacteria to colonize than the snake carcass, mainly elongate body and keeled scales of the snake carcass. Further research and sampling could examine specific anatomical regions across carcass types as well as internal versus external bacteria as decomposition progresses in reptiles. Moreover, the microorganisms inherent in soil communities should also be studied to compare bacteria already present at time of death to differentiate the role of environmental versus bacteria present or common on living reptiles. Lastly, the role of various insect scavengers we occasionally observed visiting the decomposing carcasses should be further examined, as each may harbor their own unique microbial communities, and potentially transfer them to decomposing reptile carcasses.

In conclusion, there remains much work to be done regarding microbial communities of both wild reptiles and amphibians, including the diverse microflora which colonize herpetofauna which die from either natural causes, disease, or anthropogenic roadkill mortality. The microbiome of herpetofauna remains a vastly understudied area of research, particularly as it relates to decomposition and the ecology of visiting invertebrate and vertebrate scavengers, which may further alter carcass microbial communities. Therefore, we recommend increased attention with further experimental variables considered in future research and possibly incorporation of emerging technologies, such as next-generation sequencing approaches in the study of the "living dead" of reptile and amphibian carcass microbiology.

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