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LOYOLA UNIVERSITY CHICAGO

THE EFFECT OF ENVIRONMENTAL POLLUTANTS ON THE SPOTTED TURTLE GUT MICROBIOTA

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN BIOINFORMATICS

BY

ROZA GAWIN

CHICAGO, IL

AUGUST 2024

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I would like to thank all the people who made this thesis possible, starting with my wonderful committee members Dr. Micheal Burns, Dr. Joseph Milanovich, and Dr. Timothy O'Brien. I would also like to thank Jess Lindberg for sharing her study sites and data, allowing me to further expand her spotted turtle study to include analysis on the microbiota of the gut as well as Dr. McLaughlin for providing the raw painted turtle data for a comparison. Finally, thank you also to my friends and family for their endless support and encouragement.

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ABSTRACT

Heavy metals are a common pollutant from many industrial processes. In addition to the various diseases that these pollutants cause, they can also affect the composition and structure of the gut microbiome of many organisms which is in many cases associated with overall organism health. The effect of these pollutants on the gut microbiota of the spotted turtle is unknown. Here we characterize the gut microbiome of the spotted turtle and explore the effect of various environmental pollutants on its composition. We found significant differences in the composition of the gut microbiome based on turtle sampling location (geographic), turtle sampling time (season), and turtle sex as well as many significantly differentially associated taxa for each of those comparisons. Heavy metal analysis revealed much less significant changes and associations. Although this work does not present many significant differences in the gut microbiome based on heavy metal contaminants, it helps characterize the gut microbiome of the spotted turtle and can hopefully be used as a starting point for further work and analysis of the gut microbiome of at-risk reptiles and how it is affected by various environmental pollutants. This will also help provide more concrete evidence and impetus for the importance of ecological rehabilitation and restoration of natural areas, especially those on or near prior or current industrial operations.

Introduction

There are a variety of pollutants from various anthropogenic sources that affect the environment. This includes air, water and soil pollutants as well as pesticides, PAHs (polycyclic aromatic hydrocarbons), and heavy metals (Özkara & Akyıl, 2019). Many of these pollutants are released during industrial processes. For example, heavy metal contamination often occurs as a result of mining and smelting operations and the burning of fossil fuels (ATSDR, 2015, 2023; Chen et al., 2012; EPA, 2024a; Fishbein, 1981; NIH, 2024). Many of these pollutants also persist and can accumulate in the environment, requiring remediation which can be both expensive and time consuming. Previous research has also shown that these pollutants can have many different negative effects on wildlife. Heavy metals in particular are known to affect survival, development, body weight, and even behavior of certain species (Table 1). This heavy metal accumulation in any vertebrate can occur through consumption of contaminated food sources, environmental exposure to mucous membranes, and inhalation (Engwa et al., 2019). Some heavy metals of concern include arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), and thallium (Tl). Although all these metals are naturally occurring, environmental contamination with excess heavy metals often coincides or directly results from a variety of industrial operations. Arsenic, for example, is often a byproduct of mining and fracking, coal-fired power plants, arsenic-treated lumber, and arsenic-containing pesticides (NIH, 2024). Cadmium similarly can be released through mining, smelting, use of fossil fuels and certain fertilizers, and improper waste disposal (ATSDR, 2023). Chromium is released through

processes such as ore refining, chemical and refractory processing, cement-producing plants, automobile brake lining and catalytic converters for automobiles, leather tanneries, and use of chrome pigments (Fishbein, 1981).

SPECIES	CHEMICAL	OBSERVED EFFECT(S)	REFERENCE
White-footed mouse (*Peromyscus leucopus*)	PCB (Polychlorinated biphenyls)	reduced testis size	Batty *et al*. (1990)
Seagull (*Larus californicus*)	DDT (dichlorodiphenyltrichloroethane)	feminization of embryos	Fry & Toone (1981)
Alligator (*Alligator mississippiensis*)	dicofol, DDT	abnormal testes, phalli and testosterone	Guillette *et al*. (1994)
Flathead minnow (*Pimephales promelas*)	methyl mercury	expression of multiple genes	Klaper *et al*. (2006)
Dogwhelk (*Nucella lapillus*)	tributyltin	imposex	Gibbs & Bryan (1987)
Mayfly (*Cloeon dipterum*)	esfenvalerate	reduced survival	Beketov & Liess (2005)
Annelids	toxic metals	survival and reproductive development	Spurgeon *et al*. (1994)
Nematodes (*Caenorhabditus elegans*)	many (individually)	reduced fecundity	Hoss & Weltje (2007)
Bacteria	mixture (sewage sludge)	altered communities	Kuntz *et al*. (2008)
Fence lizards (*Sceloporus occidentalis*)	lead (Pb)	altered body weight, food consumption, behavior	Salice et al. (2009)
Ferret badger (*Melogale moschata*)	lead (Pb)	higher mean corpuscular hemoglobin concentration and lower mean corpuscular volume	Liu et al. (2020)
Small Indian mongoose (*Herpestes javanicus*)	chromium (Cr)	testicular dysfunction	Andleeb et al. (2018)

Table 1. Various observed effects of common environmental pollutants on organisms adapted and expanded from "Anthropogenic pollutants: a threat to ecosystem sustainability?" (Rhind, 2009)

Lead is also released through a variety of industrial processes, use of fossil fuels, and use of various lead containing products (EPA, 2024a). Pollution with mercury occurs from processes such as agriculture, municipal wastewater discharges, mining, incineration, and discharges of industrial wastewater (Chen et al., 2012). Finally, thallium is released from coal-burning power plants, cement factories, and smelting operations (ATSDR, 2015). Toxicity from exposure to these metals can occur chronically or acutely and can lower energy levels and damage the functioning of the brain, lungs, kidney, liver, blood composition and other important organs. Chronic exposure to these metals can also cause diseases and certain cancers in both humans and animals (Jaishankar et al., 2014).

Specifically focusing on turtles, it has been found that turtles do accumulate heavy metal contaminants from their environments in their tissues at levels that correspond with the levels of these metals present in their environments (Smith et al., 2016). Accumulation of mercury has been shown to correlate with lower reproductive and hatchling success in some turtle species, with the heavy metal contaminants even maternally transferred to hatchlings (Hopkins et al., 2013). There is also a trend of lower turtle population densities in ponds with higher heavy metal contamination (Yu et al., 2013), all suggesting that heavy metals negatively impact turtle survival and fitness. Previous research has also shown that many different heavy metals can disrupt the structure of the gut microbiome in other eukaryotes. Arsenic (Brabec et al., 2020), cadmium (Liu et al., 2014), chromium (Yan et al., 2023) mercury (Tian et al., 2023), lead (Gao et al., 2017), and thallium (D. Li et al., 2022) have each been found to change the gut microbiome in eukaryotes along with other health effects. Although we are gaining an understanding of how heavy metals impact turtle populations, more research is needed specifically for wild populations of freshwater turtles. One method to gain a more complete

picture is characterizing the gut microbiome of these turtle populations and examining the correlations between heavy metal contamination and microbial dysbiosis.

Microbiome research can help provide insight into both environmental and organism health. Chemicals in the environment can affect the microbiomes in water, sediment and soils, and therefore affect the microbiomes which colonize the host organism in that environment (Handy et al., 2023). Heavy metals can also affect the microbial communities in the gastrointestinal tract, which are often essential for digestion and other vital body processes. For instance, it was found that exposure to cadmium significantly changed the gut microbiome and resulted in a significantly lower microbial diversity in an inbred strain of laboratory mice (C57BL/6), whereas the decrease in microbiome diversity from arsenic exposure was not significant (X. Li et al., 2019). This suggests that different heavy metals affect the gut microbiome in different ways. In other vertebrates, in this case Seychelles Warblers (*Acrocephalus sechellensis*), gut microbiome structure was significantly different among animals that survived and those that died by the next breeding season (Worsley et al., 2021), suggesting that the composition of the microbiome potentially influences survival and fitness of some animal species. In terms of the turtle gut microbiome, there are limited studies on wild freshwater populations. Much research focuses on saltwater turtle species or various farmed freshwater species in Asia, however, not many wild North American freshwater species have been studied. There is also very limited to no research exploring the gut microbiome of turtles and how that is affected by heavy metal pollutants. Some preliminary preprint data suggest no significant correlation between the gut microbiota of sea turtles and the environmental heavy metal concentrations (C. X. Li et al., 2024). Overall, the interactions between gut microbiome

composition and heavy metal contamination are not yet well understood in turtles but can potentially be utilized to assess turtle health and fitness in the future.

With this project we hope to characterize the spotted turtle gut microbiome, how it is affected by factors including geographic collection site, season, and turtle sex and ultimately describe the effect of heavy metal contaminants on the spotted turtle gut microbiota. We hypothesized that turtles with high levels of heavy metal contamination will have significantly different gut microbiome composition relative to animals that are less contaminated. We will also include a comparison to the painted turtle gut microbiome as there is no prior spotted turtle gut microbiome data.

Methods

Study Species

The spotted turtle, *Clemmys guttata*, is a small turtle species that resides in wetlands along the east coast and great lake regions in the United States (Figure 1). Individual's carapace lengths are generally 9 to 11.5 cm with adult turtles weighing anywhere from 120-200 grams. The turtles have dark shells and bodies with spots along their carapaces and lighter coloration towards the tips and undersides of their heads and limbs (Figure 2). The species is threatened by habitat loss and fragmentation as well as poaching (COSEWIC, 2014). It is on the International Union for the Conservation of Nature's (IUCN) Red List of Threatened Species as endangered (van Dijk, 2011), listed as endangered in Canada (COSEWIC, 2014) and currently under review for a federal listing in the United States (USFWS, n.d.).

Figure 1. Range map of the spotted turtle (*Spotted Turtle (Clemmys Guttata) rSPTUx_CONUS_2001v1 Range Map: U.S. Geological Survey Data Release*, 2018)

Although it is not yet listed as federally endangered, it is listed as endangered in many states, including Indiana (Indiana Division of Fish & Wildlife, 2020), where this study takes place. Another potential threat for spotted turtles is environmental heavy metal contamination, especially for those near current or historical industrial areas, including mining or smelting operations (Tchounwou et al., 2012).

Figure 2. Images of spotted turtles. (L) Spotted turtle held facing camera showing body and carapace pattern and coloration. (R) Spotted turtle plastron coloration. Photo credits: R. Gawin

Sample Collection

The study was carried out at two locations in northern Indiana (Lake County), Calumet Nature Preserve and Pine Station Nature Preserve. Both sites are on or near current and past industrial operations, with varying levels of heavy metal contamination due to steel production and other industry (City-Data, n.d.). The two sites contained 30 adult radio tagged turtles (21 males and 9 females) (10 at Calumet (9 males, 1 female) and 20 at Pine Station (12 males and 8 females)) for an ongoing spotted turtle morphology, behavior, and range study (Lindberg, n.d.). For that study, starting on 5 April 2022, ground-based telemetry was used to track each turtle during the active season (April – November) using a 3-yagi antenna attached to a receiver (Advanced Telemetry Systems, Model R410). At various timepoints, individuals were captured for morphometric data including carapace length (mm), carapace width (mm), plastron length (mm), shell height (mm), and mass (g). In April and October 2022, heavy metal data was collected from soil, vegetation, macroinvertebrates, and spotted turtles using blood samples. Heavy metal quantification was performed by Rutgers University Environmental and Occupational Health Sciences Institute looking at chromium (Cr), arsenic (As), cadmium (Cd), thallium (Tl), lead (Pb), and mercury (Hg). For this study, only the fall 2022 metal values were used as no microbiome samples were collected during spring 2022. Limits of detection for each heavy metal in blood samples were as follows for fall 2022 in parts per billion (ppb): 0.7 for Cr; 6.4 for As; 0.3 for Cd; 0.9 for Hg; 0.6 for Tl; and 0.6 for Pb.

Microbiome samples were collected during the routine capture of radio tagged turtles in October 2022 and April 2023. Gut microbiome samples of untagged adult turtles that were encountered at the sites were also collected, recording turtle sex based on dimorphic traits.

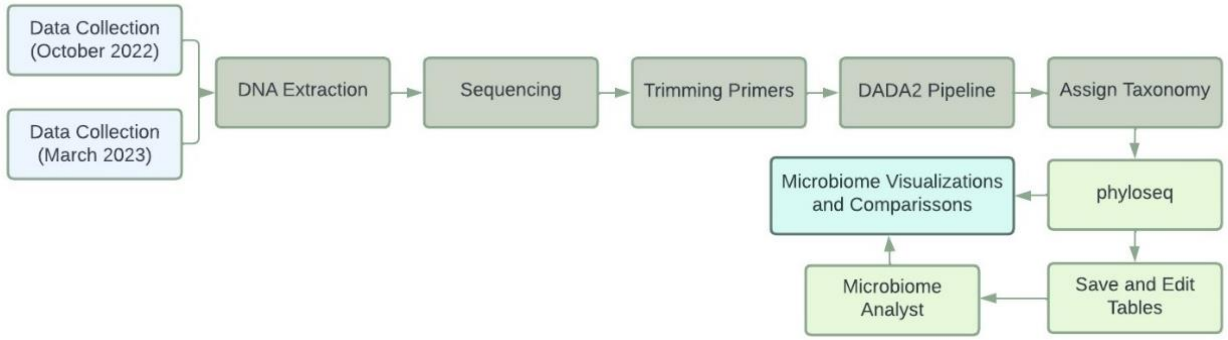


Figure 3. General workflow of the data collection, processing, and analysis for the characterization of the gut microbiome of the spotted turtle and exploring the effect of environmental pollutants on the spotted turtle gut microbiota

Gut microbiome data was collected through the use of cloacal swabs (nylon flocked FLOQSwabs, COPAN Corporation). The majority of the turtles were found in or near water or while basking. For any turtles that were dry, a sterile, normal saline solution was used to wet the swab before swabbing the cloaca. Two fecal samples were also collected as comparisons for turtles which also had cloacal swabs collected. Swabs of the saline solution, processing table, and collection technician glove and skin were collected as controls. Environmental samples such as water and submerged soil were collected where the turtles were found. Finally blanks from the site were collected by waving swabs open in the air for ten seconds. All the samples were kept in a cooler after collection and moved to a -80°C freezer at the end of the day. The samples were stored there, only being removed to thaw for the DNA extraction process.

Overall, 58 cloacal swabs were extracted and sequenced, including 20 from Calumet Nature Preserve (7 from fall 2022, 13 from spring 2023) and 38 from Pine Station Nature Preserve (15 from fall 2022, 23 from spring 2023). This included 17 female, 35 male, 6 unknown turtles (not radio tagged, and sex not recorded at time of capture) with nine radio tagged turtles having repeat sampling in both the fall and spring. Heavy metal data from October 2022 includes

17 radio tagged turtles for which cloacal samples were also collected (11 males and 6 females with 5 turtles from Calumet and 12 from Pine Station).

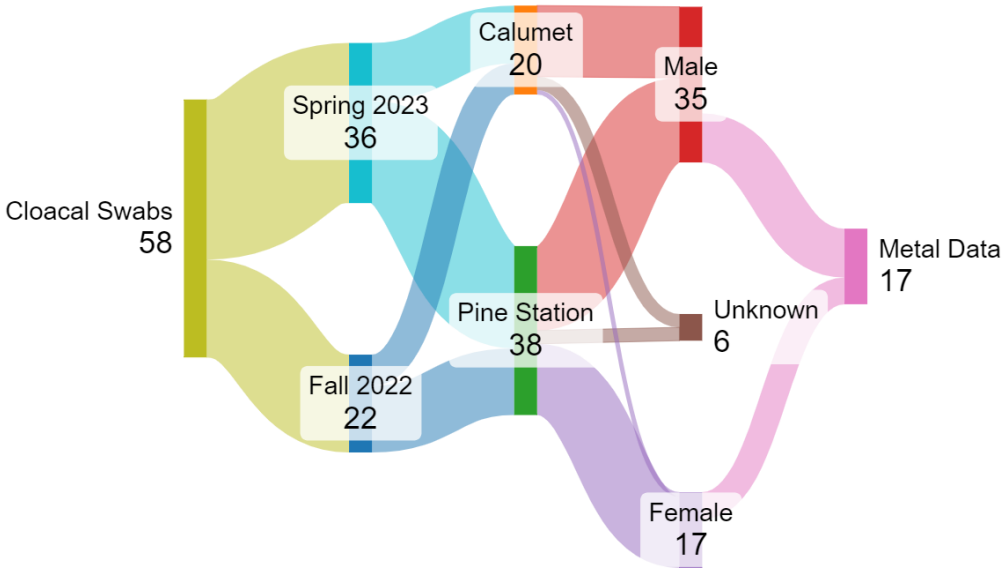


Figure 4. Sankey diagram generated using SankeyMATIC to visualize the gut microbiome samples collected from the spotted turtles throughout the course of this study.

DNA Extraction

The DNA was extracted from the swab, environmental, and control samples using the ZymoBIOMICS DNA Microprep Kit in small batches of 6-8 samples per extraction including one method blank per batch. Soil samples were massed with 80-90 mg used for DNA extraction. For the cell lysis step, the QIAGEN TissueLyser bead beater was used at 30 Hz continuously for 8 minutes. For low biomass samples (swabs) the first filtering step was skipped as suggested in the ZymoBIOMICS extraction procedure. All the remaining steps were performed following the ZymoBIOMICS procedure. After DNA extraction, the samples were assessed looking at the nucleic acid values and 260/280 nm absorbance using a Thermo Scientific NanoDrop 2000c Spectrophotometer. Generally, ratios close to 2 for the 260/280 nm absorbance indicate that

DNA was extracted successfully and with adequate purity. PCR and agarose gel electrophoresis were also performed to confirm DNA extraction and determine if sufficient microbial DNA was extracted for sequencing purposes in some low yield samples. Samples were then sent off in two batches for V3/V4 16S gene amplicon sequencing to SEQCENTER using the 341F and 806R PCR primers where, following clean up and normalization, samples were sequenced on a P1 600cyc NextSeq2000 Flowcell to generate 2x301bp paired end (PE) reads.

Sequencing Data Processing

Sequencing data was received in the fasta.gz file format. First the files were uncompressed to the fasta file format. Then sequences were trimmed, removing primers using Cutadapt (Martin, 2011). The raw sequences were then quality control processed and denoised using the dada2 pipeline (Callahan et al., 2016). Finally taxonomy was assigned to the sequence variants using the SILVA reference database (Quast et al., 2013) and taxa were agglomerated at the genus level. The results were then visualized using both phyloseq (McMurdie & Holmes, 2013) and MicrobiomeAnalyst (Dhariwal et al., 2017). For visualization with MicrobiomeAnalyst, each of the phyloseq objects of interest were downloaded by saving the sequence table, taxonomy table, and meta data which were then modified into the correct format before being uploaded to the webpage. The uploaded raw sample data was then filtered using a low count filter (minimum count: 4, prevalence in samples: 10%) and a low variance filter (percentage to remove: 10% based on the inter-quantile range). The data were not rarefied (Figure 8) or scaled but were transformed using the centered log ratio for multi-factor analysis. The data were analyzed using alpha diversity, beta diversity, and multi-factor analysis metrics. For alpha diversity profiling, the Shannon Diversity Index was used with t-tests for pairwise comparisons. For beta diversity profiling, the PCoA ordination method was used, using the Bray-

Curtis index and PERMANOVA tests for pairwise comparisons. Finally, for multi-factor analysis, MaAsLin2 (Mallick et al., 2021) was used to identify significantly differently associated taxa between various experimental factors.

In addition to the spotted turtle cloacal samples, raw sequencing data from painted turtles (*Chrysemys picta*) (Fugate et al., 2020) were assessed from a prior publication. As there is no prior gut microbiome data for spotted turtles, these samples were included as a visual comparison. Both spotted and painted turtles are freshwater turtle species found in North America with our data being from wild turtles in Northwest Indiana and the painted turtle data from a wild population in south Wisconsin. However, the two turtle species occupy different ecosystem niches and have different diets. The painted turtle samples included fecal samples from 10 individuals from August 2017. These raw sequencing files were processed using the same pipeline as the spotted turtle data. Both were then corrected for batch effects using the percentile-normalization method of Gibbons et al (Gibbons et al., 2018). This method requires normalizing the data in reference to the controls of a study. As this experimental design does not have true turtle controls, two of the average individuals within each study when assessing alpha diversity from each species were used for the normalization process. This then allows for a comparison between the gut microbiota of the two freshwater turtle species.

Results

Characterization of the Spotted Turtle Gut Microbiome- Painted Turtle Comparison

First, the gut microbiota of the spotted and painted turtles was compared and visualized, merging on turtle species. The relative abundances of the two species by phylum and genus can be seen in figures 5 and 6.

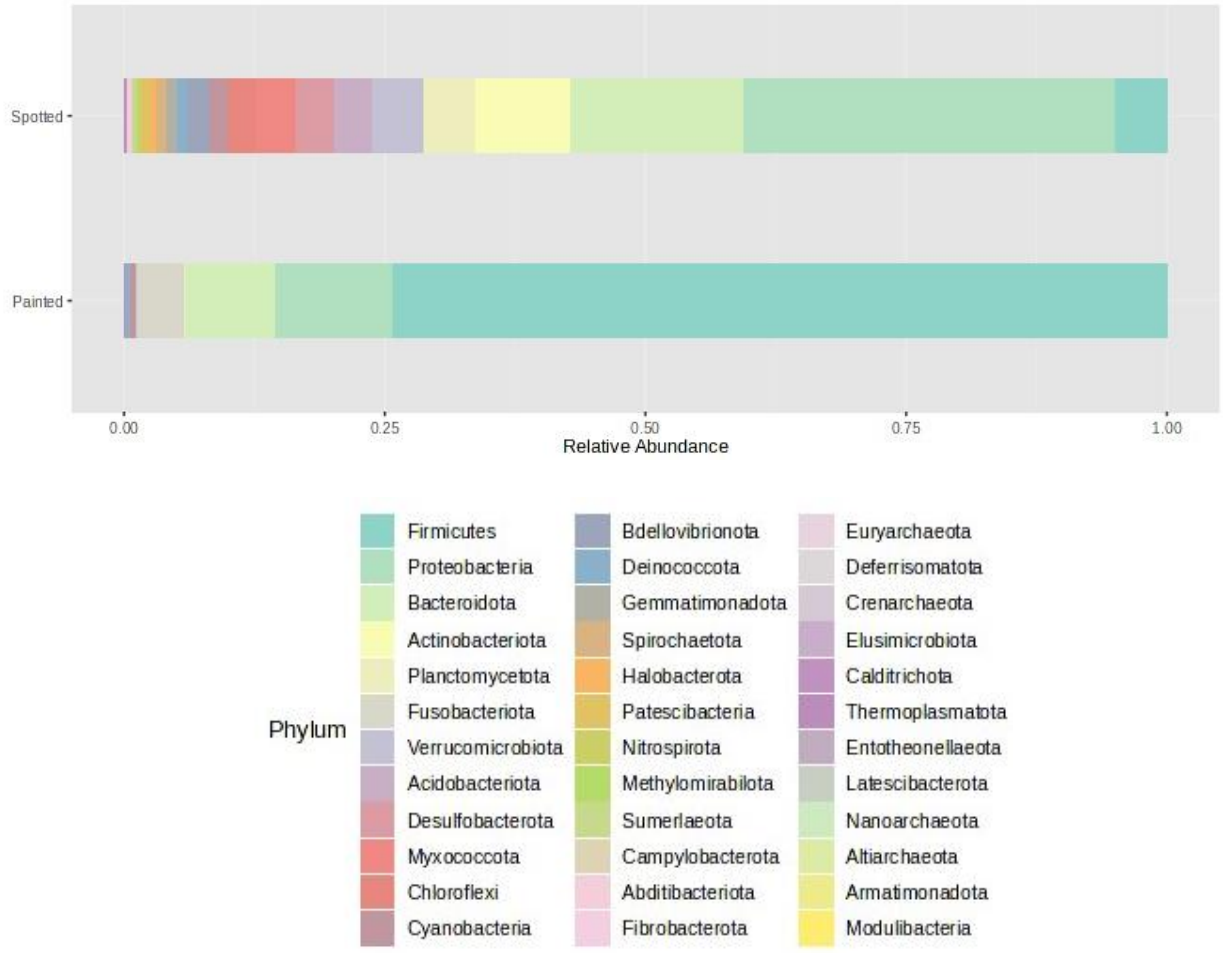


Figure 5. The relative abundances of the different bacterial phyla found in the gut microbiome of the spotted and painted turtles, merged on turtle species.

Although both species contain microbes from the same phyla, there are also many differences in present phyla and the abundance of those phyla. Both spotted and painted turtles have microbes from the *Firmicutes*, *Proteobacteria*, *Bacteriodiota*, *Actinobacteriota*, and some other less abundant phyla. However, the painted turtle gut microbiome contains a much higher abundance of Firmicutes making up almost 75% of the microbes present in the painted turtle gut microbiome. The spotted turtles overall seem to have a more even and rich microbiome at the phylum level.

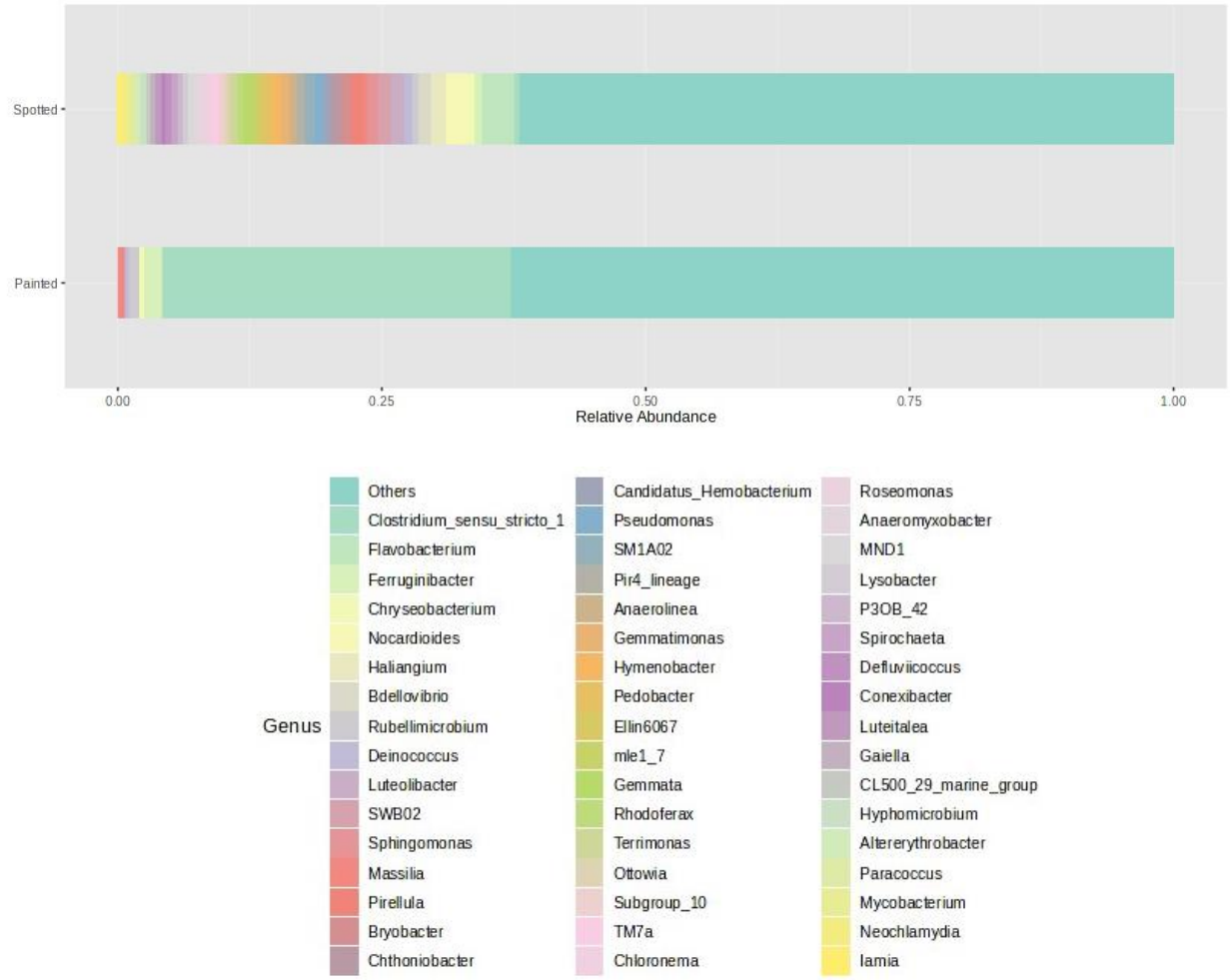


Figure 6. The relative abundances of the different bacterial genera found in the gut microbiome of the spotted and painted turtles, merged on turtle species. The top 50 abundant genera are included in the legend, while the remaining genera are grouped into "Others."

At the genus level, we see some similar genera between the two turtle species, however this visualization even more strongly highlights the higher genera richness present in the spotted turtle gut microbiome samples.

Characterization of the Spotted Turtle Gut Microbiome

The predominant bacterial phylum detected in across the spotted turtle samples was *Bacteriodota*, with the next most abundant phylum being *Proteobacteria* and *Actinobacterioda* third (Figure 7).

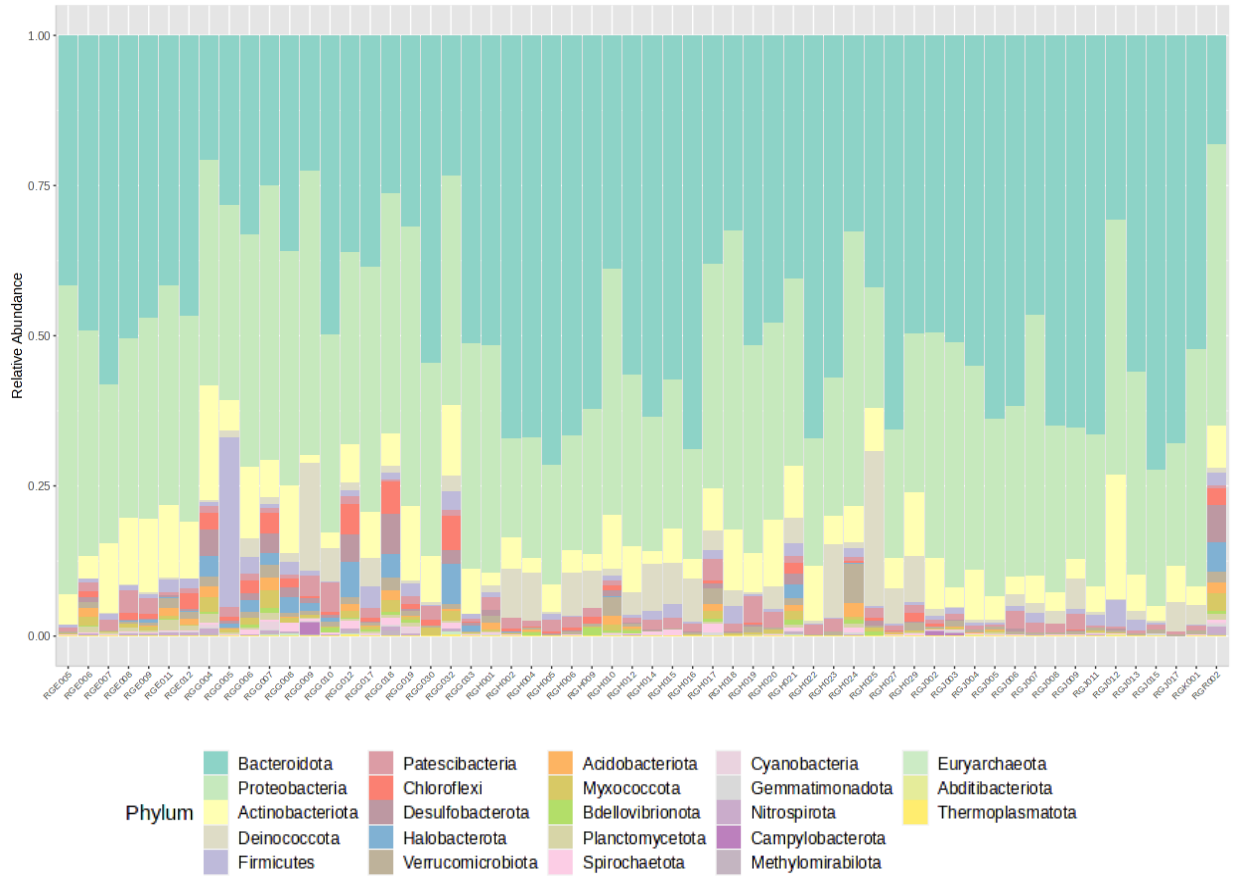


Figure 7. The relative abundances of the different bacterial phyla found in the gut microbiome of the spotted turtle with each bar representing a separate cloacal sample. 58 cloacal samples are included with a mix of turtle sex, collection site, and collection season.

The same visualizations were repeated at the genus level. This revealed the top 5 most abundant genera to be *Chryseobacterium*, *Ottowia*, *Thermomonas*, *Niabella*, and *Deinococcus* in the gut microbiome of the spotted turtle (Figure 8).

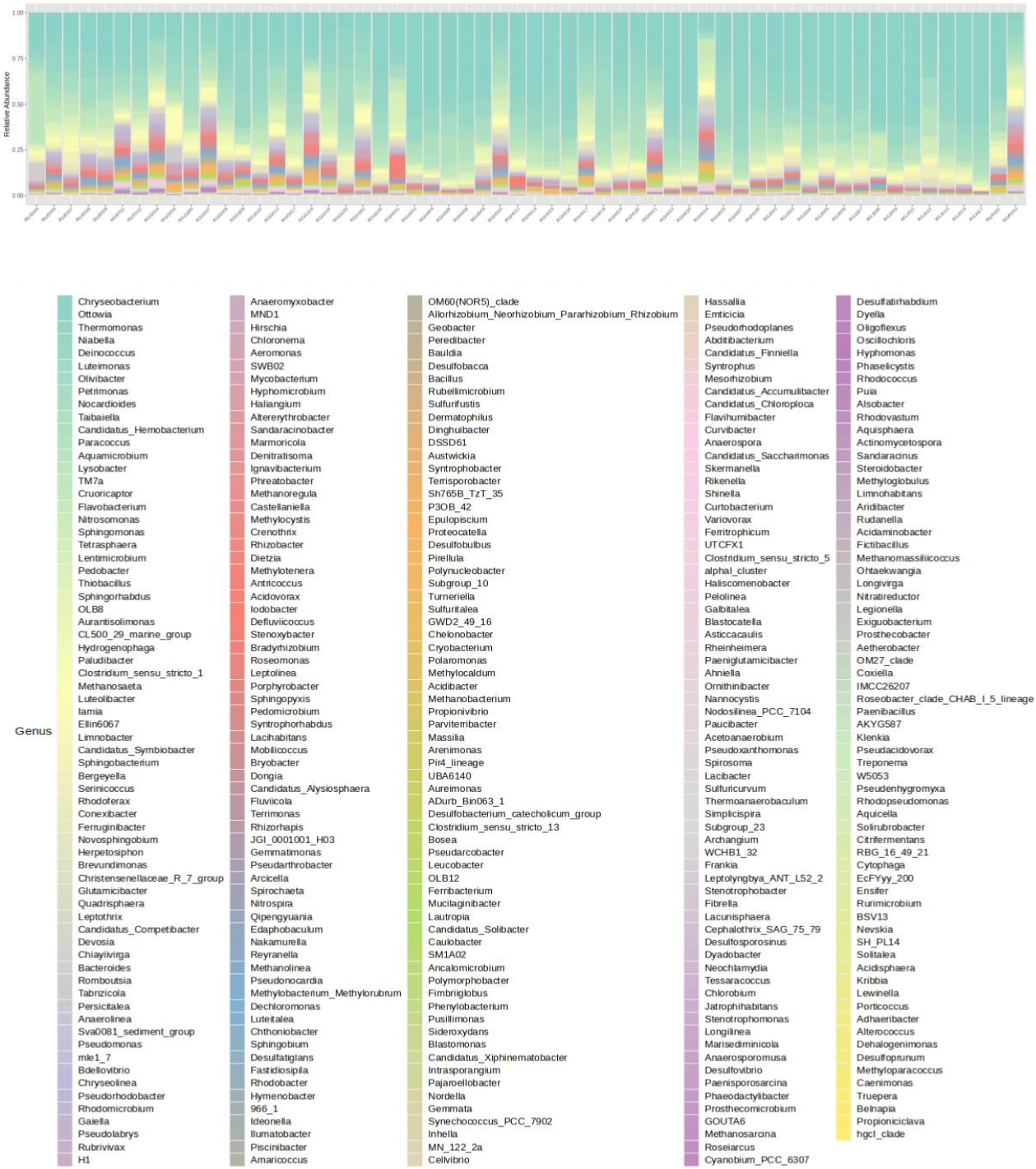


Figure 8. The relative abundances of the different bacterial genera found in the gut microbiome of the spotted turtle with each bar representing a separate cloacal sample. The same samples are shown as in Figure 7.

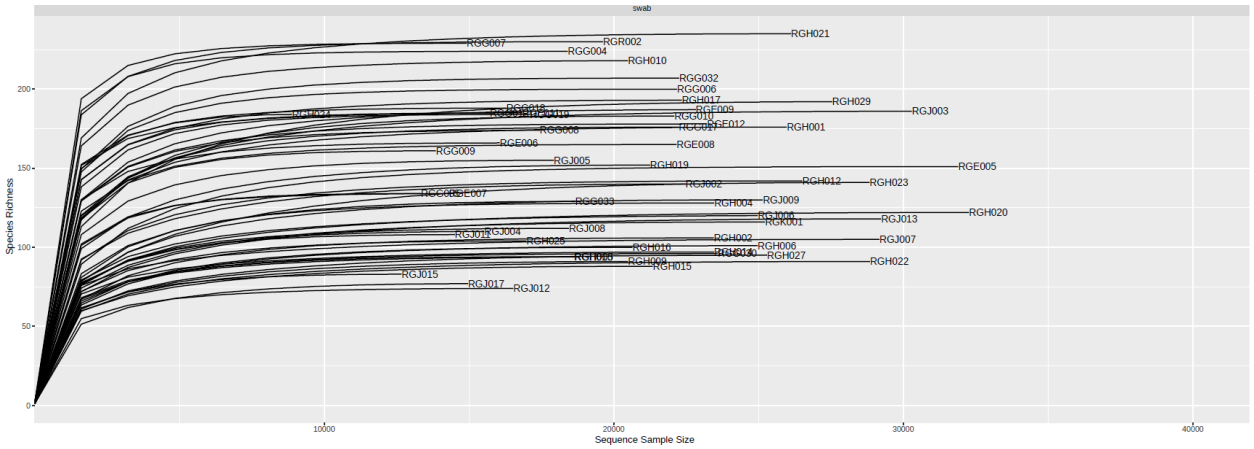


Figure 9. Rarefaction curve of all the spotted turtle cloacal samples. Based on this curve, samples were not rarified to the minimum library size, as it is not necessary.

Effects of Collection Site, Season, and Turtle Sex on Microbiome Composition

There are many factors which can influence the composition of and microbes present in the microbiome of an organism. Some of these factors can include seasonality (Maurice & Knowles, 2015), sex (Dominianni & Sinha, 2015), and body collection site, among others. For this reason, we analyzed if collection site, season, or turtle sex had any significant influence on the structure or composition of the gut microbiome.

First, comparing the relative abundances of the taxa found in each sampling location (site: Calumet or Pine Station), there does seem to be a visual difference between the taxa present and the amounts of taxa present between the two sites (Figure 10a). When comparing alpha diversity, there is not a significant difference between the two groups (p-value = 0.16). However, there is a significant difference between the groups in beta diversity (p-value = 0.002). Finally, when a multifactor analysis was performed using MaAsLin2 to compare the two sites, 83 significantly differently associated taxa were found (Table 2).

a.

b.

c.

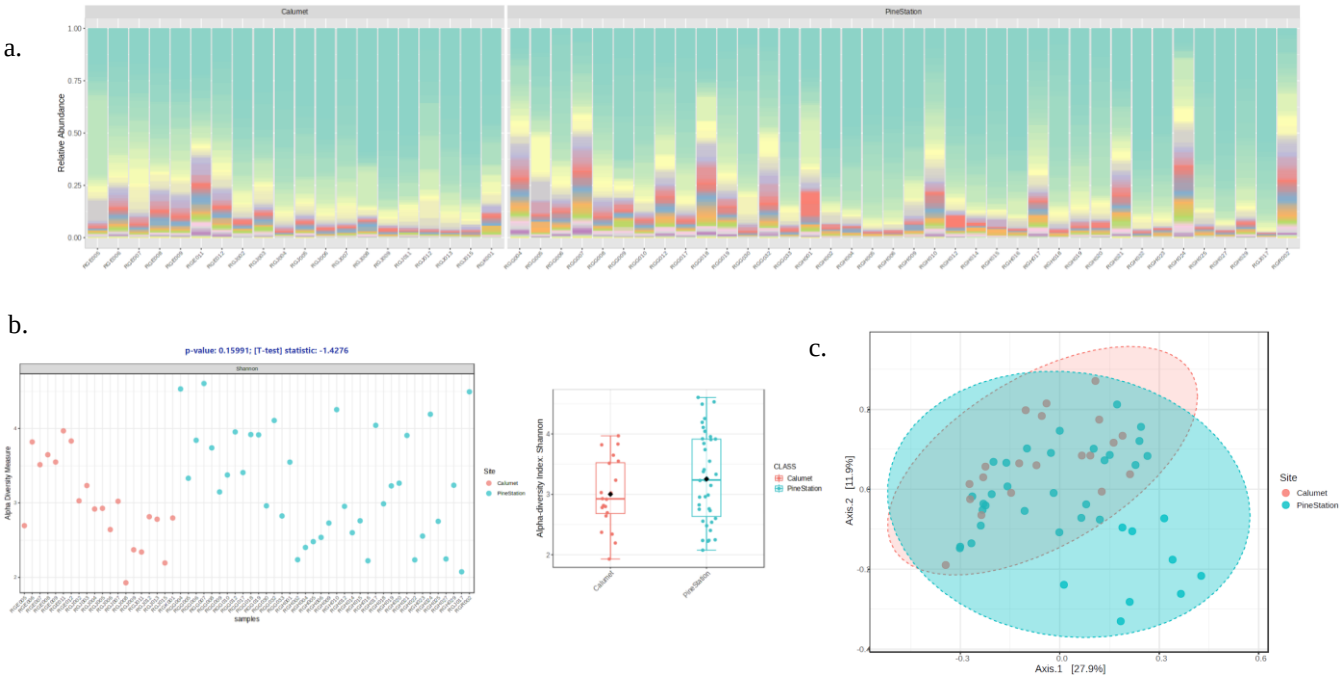


Figure 10. Visualizations of the microbiome comparisons between collection sites Calumet and Pine Station. (a) Relative abundance of cloacal samples grouped by turtle collection site. Refer to Figure 8 for the genera key. (b) Alpha diversity plot showing Shannon Diversity Index for each cloacal sample grouped by color (Calumet in red, Pine Station in blue) as well as a boxplot comparing those values. (c) PCoA plot of beta diversity comparing the cloacal samples from the two sites.

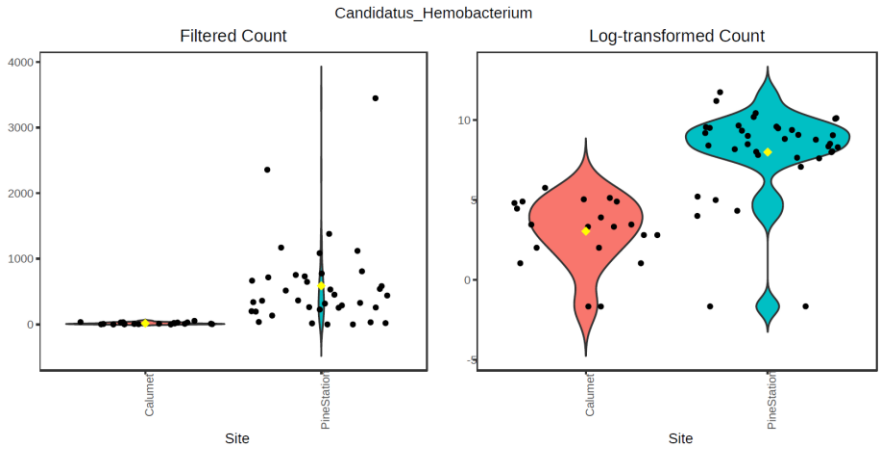


Figure 11. Violin plot of the top significantly differentially associated genus between sampling sites: *Candidatus hemobacterium*. This genus of microbes was present in a much higher abundance in the spotted turtle gut microbiome samples from Pine Station.

Genus	Log2FC	St.Error	P-value	FDR
Candidatus_Hemobacterium	4.84	0.651	6.53E-10	2.21E-07
Nakamurella	-2.63	0.425	7.20E-08	1.22E-05
Lentimicrobium	4.12	0.729	5.48E-07	5.16E-05
Rubellimicrobium	2.65	0.471	6.11E-07	5.16E-05
Pedobacter	-3.77	0.693	1.23E-06	8.34E-05
Crenothrix	3.39	0.637	1.86E-06	0.000105
Parviterribacter	2.3	0.435	2.22E-06	0.000107
Paracoccus	1.52	0.291	2.56E-06	0.000108
Sandaracinobacter	-2.35	0.478	7.89E-06	0.000296
Bdellovibrio	2.71	0.561	1.11E-05	0.000374
Deinococcus	2.68	0.567	1.60E-05	0.000491
Luteitalea	2.86	0.612	1.96E-05	0.000553
Chiayiivirga	3.07	0.701	5.32E-05	0.00138
Chloronema	2.96	0.695	8.07E-05	0.00182
Serinicoccus	-3.15	0.74	8.02E-05	0.00182
Phreatobacter	2.84	0.672	9.05E-05	0.00191
Methylobacterium_Methylorubrum	-2.42	0.585	0.000117	0.00234
Belnapia	-0.82	0.205	0.000183	0.00344
Adhaeribacter	-0.943	0.237	0.000203	0.0036
Pseudorhodobacter	-2.39	0.611	0.000256	0.00413
Synechococcus_PCC_7902	2.28	0.584	0.000249	0.00413
Candidatus_Udaeobacter	-1.62	0.418	0.000273	0.00419
Castellaniella	-2.42	0.634	0.000343	0.00481
OLB8	-2.61	0.687	0.000358	0.00481
Porphyrobacter	1.81	0.474	0.000339	0.00481
Streptomyces	-1.21	0.318	0.00037	0.00481
Altererythrobacter	-2.22	0.597	0.000469	0.00587
Aquamicrobium	-3.08	0.833	0.000504	0.00608
Parapusillimonas	-0.602	0.164	0.000526	0.00613
Haliscomenobacter	1.57	0.435	0.000651	0.00733
Sulfuritalea	2.21	0.623	0.000814	0.00888
Hyphomonas	1.24	0.354	0.000928	0.00923
Polaromonas	-1.77	0.506	0.000905	0.00923
Polynucleobacter	1.69	0.481	0.000886	0.00923
Proteiniclasticum	-1.34	0.388	0.00101	0.00976
Abditibacterium	-1.85	0.537	0.00106	0.00992
UBA6140	1.75	0.509	0.00112	0.0102
Mucilaginibacter	-1.49	0.447	0.00155	0.0138
Aurantisolimonas	1.56	0.475	0.00175	0.0151
Paenisporosarcina	1.17	0.358	0.00185	0.0157

Table 2. Top 40 out of 83 significantly differently associated taxa between sites (Pine Station vs Calumet)

Next, when comparing the relative abundances of the taxa found in each sampling time point (season: Spring or Fall), there also seems to be a visual difference between the taxa present and the amounts of taxa present between the two sites (Figure 12a). When comparing alpha diversity, there is a significant difference between the two groups (p-value = 5.58E-6). There is also a significant difference between the groups in beta diversity (p-value = 0.001). Finally, when a multifactor analysis was performed using MaAsLin2 to compare the two collection time points, 195 significantly differently associated taxa were found. Seeing this strong effect of season on the turtle gut microbiome, all consecutive multi factor analyses are performed controlling for season.

a.

b.

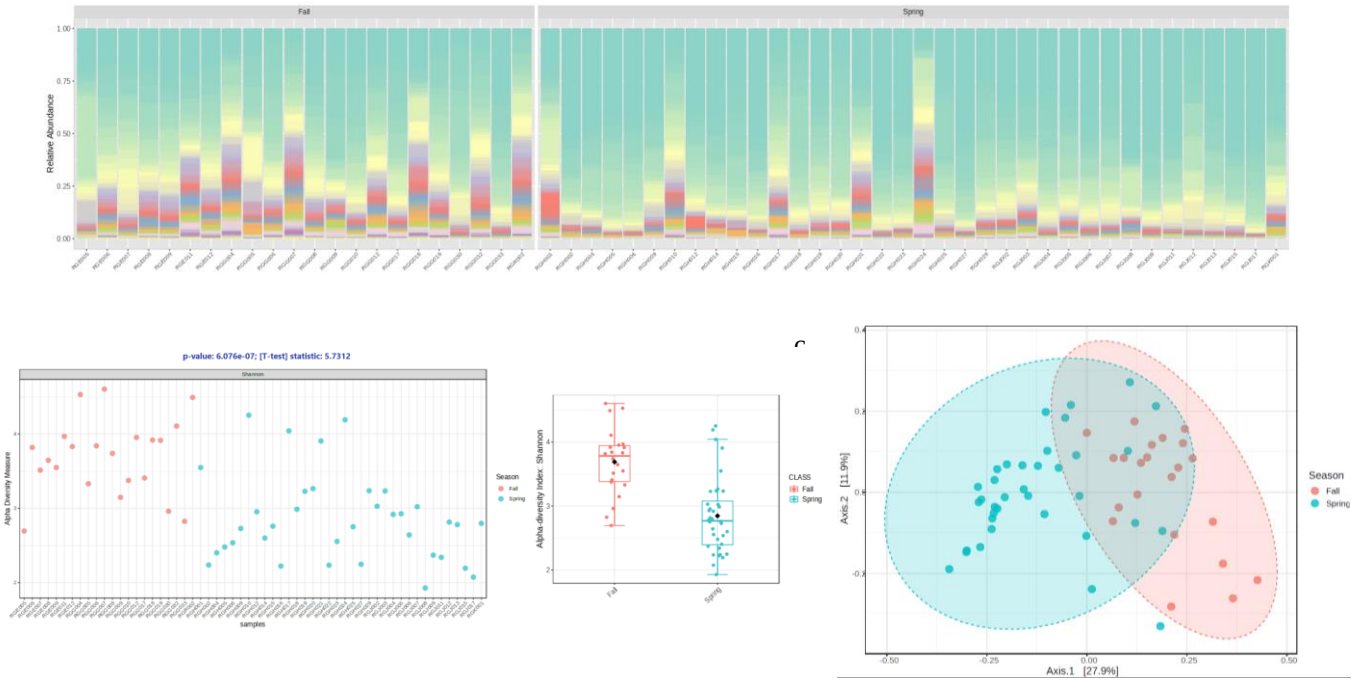


Figure 12. Visualizations of the microbiome comparisons between collection times Fall and Spring. (a) Relative abundance of cloacal samples grouped by turtle collection season. Refer to Figure 8 for the genera key. (b) Alpha diversity plot showing Shannon Diversity Index for each cloacal sample grouped by color (Fall in red, Spring in blue) as well as a boxplot comparing those values. (c) PCoA plot of beta diversity comparing the cloacal samples from the two times.

Genus	Log2FC	St.Error	P-value	FDR
M60.NOR5._clade	-4.05	0.374	2.24E-15	7.56E-13
Thiobacillus	-5.5	0.546	3.60E-14	6.09E-12
mle1_7	-4.39	0.487	1.72E-12	1.93E-10
Anaerolinea	-4.37	0.536	4.31E-11	2.52E-09
Glutamicibacter	-3.25	0.4	4.48E-11	2.52E-09
Sva0081_sediment_group	-4.64	0.569	4.26E-11	2.52E-09
Gaiella	-4.19	0.541	2.04E-10	9.83E-09
966_1	-3.61	0.474	3.33E-10	1.41E-08
Sh765B_TzT_35	-3.57	0.475	4.99E-10	1.87E-08
Desulfobacca	-3.88	0.521	6.27E-10	2.12E-08
Ellin6067	-4.87	0.667	1.14E-09	3.21E-08
Ignavibacterium	-3.41	0.467	1.08E-09	3.21E-08
Nitrospira	-3.6	0.5	1.61E-09	4.18E-08
Nordella	-2.79	0.395	2.74E-09	6.62E-08
Bacillus	-3.25	0.466	3.76E-09	8.47E-08
Sphingorhabdus	3.94	0.568	4.32E-09	9.13E-08
Syntrophorhabdus	-3.23	0.471	5.68E-09	1.13E-07
MND1	-4.2	0.614	6.30E-09	1.18E-07
Hirschia	-3.53	0.528	1.15E-08	2.05E-07
Leptolinea	-3.82	0.574	1.28E-08	2.17E-07
Sphingobacterium	-4.71	0.711	1.44E-08	2.32E-07
Pseudolabrys	-3.76	0.569	1.54E-08	2.36E-07
Methanobacterium	-3.07	0.473	2.27E-08	3.34E-07
Chryseolinea	-3.98	0.616	2.61E-08	3.67E-07
Bauldia	-3.25	0.513	4.19E-08	5.66E-07
Desulfatiglans	-3.23	0.511	4.54E-08	5.90E-07
Methylocaldum	-3.09	0.492	5.24E-08	6.33E-07
Sulfurifustis	-3.07	0.489	5.17E-08	6.33E-07
Methylotenera	3.28	0.528	7.04E-08	8.20E-07
Anaeromyxobacter	-3.36	0.548	9.35E-08	1.05E-06
Pedomicrobium	-3.49	0.575	1.20E-07	1.31E-06
Syntrophus	-2.5	0.416	1.44E-07	1.52E-06
Dinghuibacter	-3.14	0.528	1.84E-07	1.89E-06
DSSD61	-2.89	0.489	2.10E-07	2.09E-06
Methanosaeta	-4.36	0.74	2.26E-07	2.18E-06
Clostridium_sensu_stricto_1	-3.36	0.572	2.40E-07	2.25E-06
Mycobacterium	-3.13	0.534	2.48E-07	2.27E-06
SWB02	-3.5	0.602	3.04E-07	2.63E-06
Syntrophobacter	-2.94	0.506	2.98E-07	2.63E-06
OLB12	-2.89	0.498	3.24E-07	2.74E-06

Table 3. Top 40 out of 195 significantly differently associated taxa between seasons (Spring vs Fall)

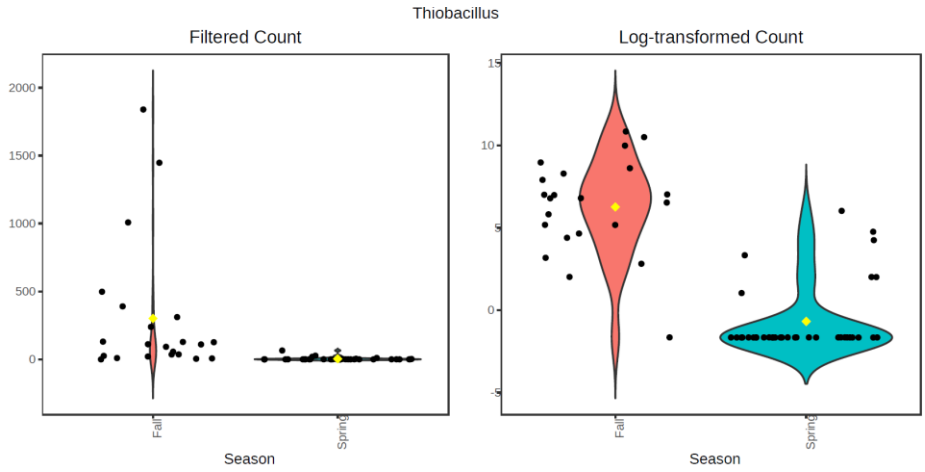


Figure 13. Violin plot of the top significantly differentially associated genus between sampling seasons: *Thiobacillus.* This genus of microbes was present in a much higher abundance in the spotted turtle gut microbiome samples from the fall.

Next, the microbiomes were compared based on the sex of the turtle from which the samples were collected. Of the 58 cloacal swabs, 17 were from female turtles, 35 were from male turtles, and the remaining 6 were unknown. The unknowns are present in the alpha diversity and beta diversity visualizations, but only the female and male turtles are included in the relative abundance and merged relative abundance bar charts. When comparing the relative abundances of the taxa found in each turtle sex (male or female), there does seem to be a visual difference between the taxa present and the amounts of taxa present between the two sites (Figure 14a). Upon merging the two groups based on turtle sex, however, the overall bars look very similar (Figure 14d). When comparing alpha diversity, there is not a significant difference between the two groups (p-value = 0.2). There is however a significant difference between the groups in beta diversity (p-value = 0.0028). Finally, when a multifactor analysis was performed using MaAsLin2 to compare the turtle sex, controlling for season, 12 significantly differently associated taxa were found.

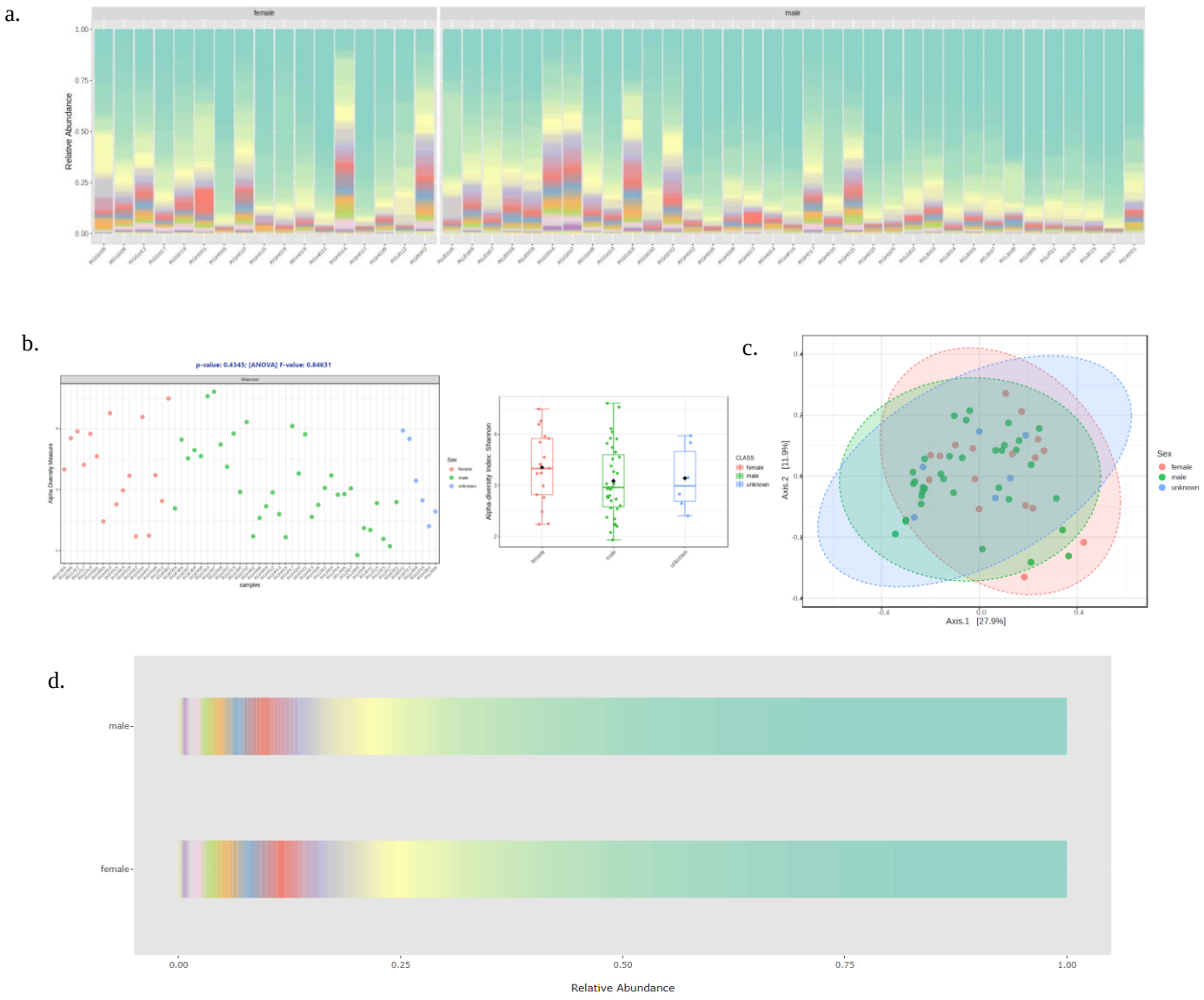


Figure 14. Visualizations of the microbiome comparisons between female and male turtles. (a) Relative abundance of cloacal samples grouped by turtle collection season. Refer to Figure 8 for the genera key. (b) Alpha diversity plot showing Shannon Diversity Index for each cloacal sample grouped by color (female in red, male in green, and unknown in blue) as well as a boxplot comparing those values. (c) PCoA plot of beta diversity comparing the cloacal samples from the different turtle sexes. (d) Relative abundance bar chart merged on turtle sex.

Genus	Log2FC	St.Error	P-value	FDR
Clostridium_sensu_stricto_1	-2.27	0.555	0.000143	0.00165
Terrisporobacter	-1.74	0.44	0.000226	0.00241
Chiayiivirga	-2.83	0.785	0.000694	0.00591
Chryseobacterium	0.794	0.224	0.000818	0.00669
Epulopiscium	-1.71	0.509	0.00146	0.0108
Taibaiella	1.64	0.537	0.0035	0.0227
Ferruginibacter	2.18	0.738	0.00467	0.0292
Fimbriiglobus	-1.54	0.531	0.00537	0.033
Anaerocella	-1.03	0.365	0.00675	0.0402
Hyphomonas	-1.09	0.397	0.00795	0.0458
Anaerosporomusa	-1.34	0.488	0.0081	0.0462
Thermomonas	-1.05	0.384	0.00826	0.0465
Pseudarthrobacter	-1.27	0.494	0.0132	0.0657
Romboutsia	-1.65	0.653	0.0146	0.0711
Petrimonas	-1.67	0.678	0.017	0.08

Table 4. Top 15 (12 of which are significantly differently associated) taxa between sex (Male vs Female)

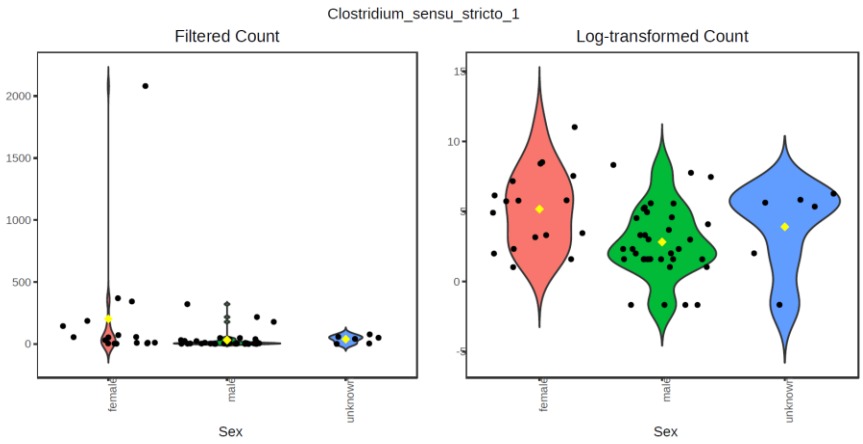


Figure 15**.** Violin plot of the top significantly differentially associated genus between turtle sex (male vs female): *Clostridium sensu stricto 1*. This genus of microbes was present in a higher abundance in the spotted turtle gut microbiome samples from the female turtles.

Effects of Heavy Metals on Microbiome Composition

In a subset of the spotted turtles included in this study, blood was sent out for heavy metal analysis. There are blood heavy metal concentrations available for 17 turtles from fall 2022. Those 17 samples are used in this section of the analysis. Each turtle's blood was tested for

six heavy metals: arsenic, cadmium, chromium, mercury, lead, and thallium. Those values were reported in parts per billion, with some metals reported below the limit of detection (<LOD or bLOD) for certain turtles.

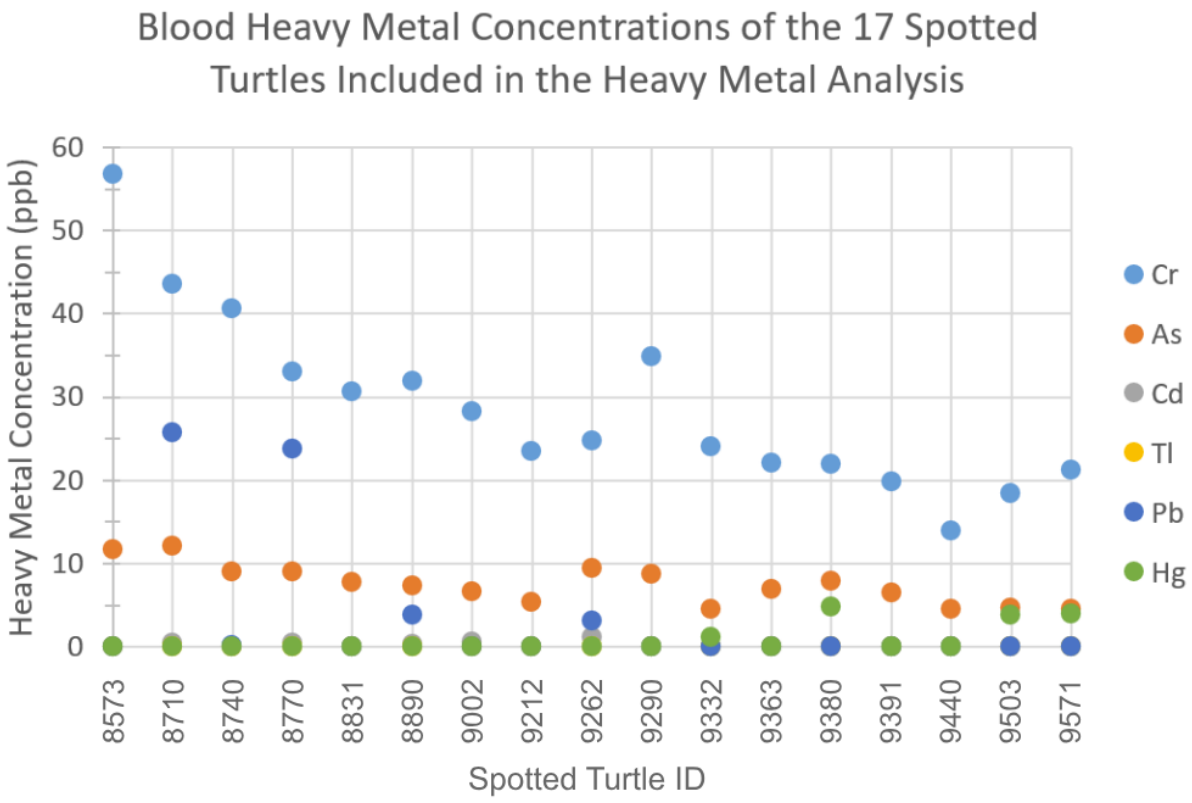


Figure 16. Plot visualizing the concentrations of the 6 different heavy metals in the 17 included spotted turtles.

	Arsenic	Cadmium	Chromium	Mercury	Lead	Thallium
Low	$\leq 15\ (ppb)$	$\leq 0.5\ (ppb)$	$\leq 15\ (ppb)$	$\leq 7.5\ (ppb)$	$\leq 15\ (ppb)$	$\leq 0.25\ (ppb)$
High	$> 15\ (ppb)$	$> 0.5\ (ppb)$	$> 15\ (ppb)$	$> 7.5\ (ppb)$	$> 15\ (ppb)$	$> 0.25\ (ppb)$

Table 5. Turtle heavy metal categories for the six heavy metals included based on blood metal concentrations (ppb)

For simpler comparisons turtles were grouped into three categories: <LOD, Low, and High for each of the six metals. Mercury also includes two turtles with No Data. All 17 of the turtles' blood concentrations fell below the limit of detection for thallium, so that visualization will be included in the relative abundance bar charts, but no further analysis will be done specific

to thallium. The cutoff between the Low and High categories was the mean or median reported value for each specific metal as there is not a baseline for heavy metal blood concentrations in spotted turtles and are no determined heavy metal level thresholds for acute or chronic exposure in reptiles. The EPA has water quality criteria for maintaining safe environments for aquatic organisms, however this is reported in water concentration.

Metal	**Arsenic**	**Cadmium**	**Chromium**	**Mercury**	**Lead**	**Thallium**
Limit (CCC-CCM ppb)	150-340	unknown-1.8	11-16 Cr (III) 74-570 Cr (IV)	0.77-1.4	2.5-65	unknown-13

Table 6. Heavy Metal Limits for Aquatic Life (Water Concentration (ppb)) listed with chronic-acute (Criterion Continuous Concentration (CCC) - Criterion Maximum Concentration (CMC)) exposure levels. (EPA, 2024b) (ATSDR, 1992)

Overall, samples were distributed mostly evenly among the different metal level categories in the arsenic and chromium comparisons, however some such as the cadmium, mercury, and lead groupings were much less even. First, looking at alpha diversity using the metric of the Shannon Index of Diversity to compare the gut microbiomes of turtles of different metal levels for each of the metals, only arsenic seemed to have a significant difference between those with Low and High arsenic concentrations (p-value = 0.006384). Following FDR correction using the Benjamini Hochberg correction for multiple testing, the adjusted p-value is still significant at 0.03192. Next, when comparing beta diversities of the gut microbiomes of the different metals levels for the heavy metals analyzed, one comparison was significant: the beta diversities of the gut microbiome for the arsenic Low vs High turtles are significantly different (p-value = 0.003, adjusted p-value = 0.015).

Finally, multifactor analysis was performed for each of the metals controlling for site and turtle sex. Not many significantly differently associated taxa were found for any of these analyses. For arsenic, 1 significantly differently associated taxon was found when comparing the

Low vs High groups. For cadmium, 1 significantly differently associated taxon was found when comparing the Low vs High groups and 1 when comparing the <LOD and Low groups.

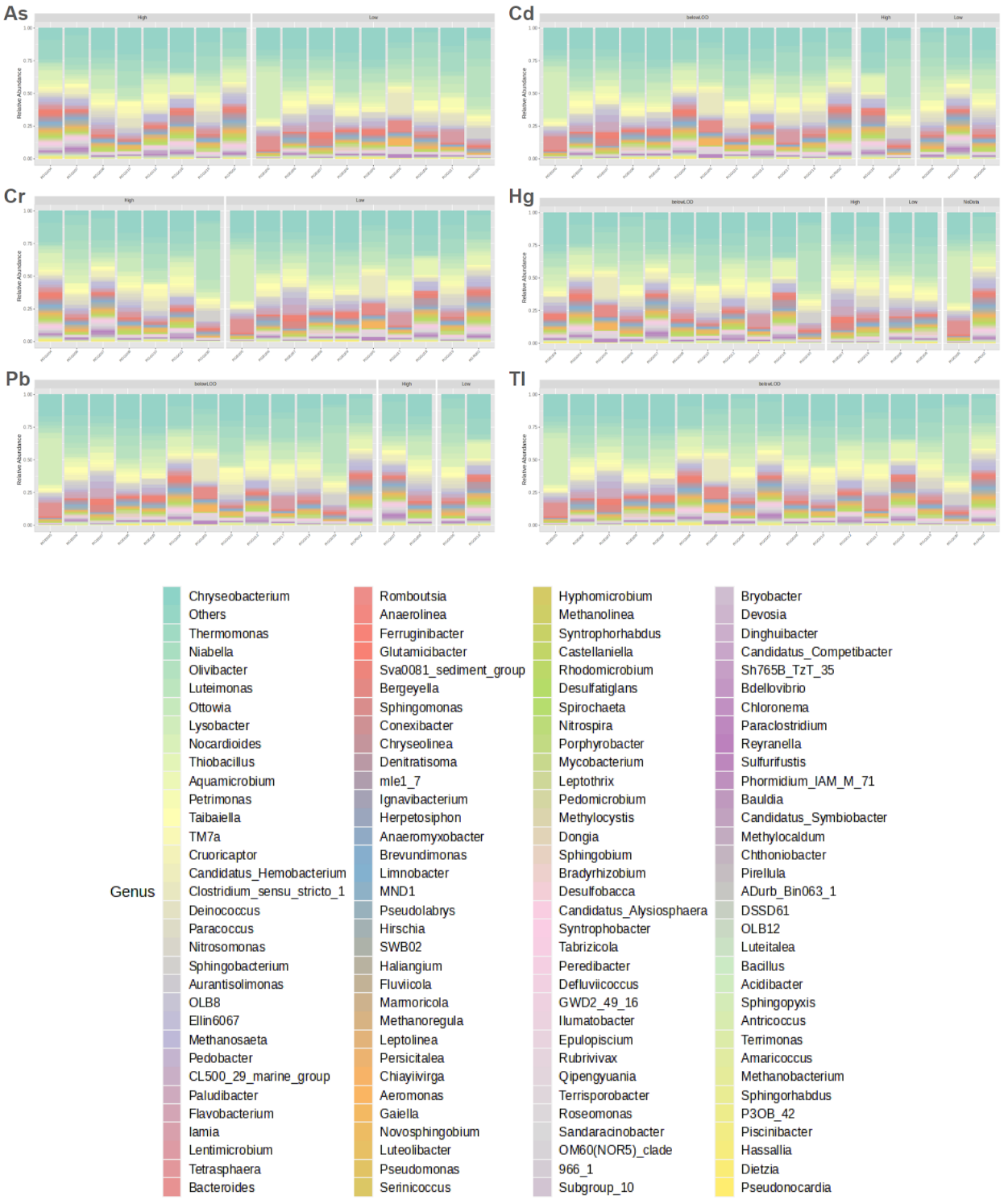


Figure 17. Relative Abundance Bar charts at the genus level, showing the top 131 genera, for the six heavy metals analyzed. Arsenic and chromium only have high vs low categories; cadmium and lead have high vs low vs below LOD categories; mercury has high vs low vs below LOD vs No Data; thallium only has below LOD values.

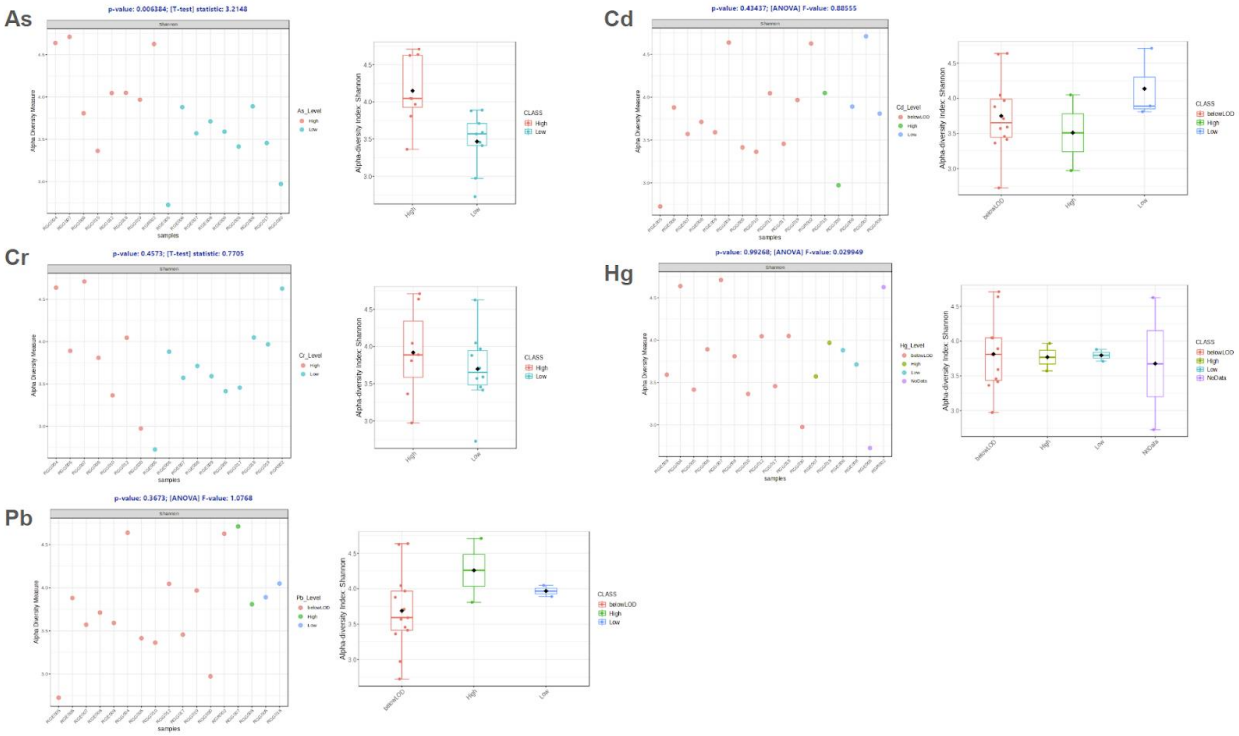


Figure 18. Alpha diversity plots showing the Shannon Diversity Index for each metal for each cloacal sample grouped by color (by metal level) as well as a boxplot comparing those values.

For chromium, no significantly differently associated taxa were found when comparing any of the groups. For mercury, 2 significantly differently associated taxa were found when comparing the Low vs High groups and 2 when comparing the <LOD and Low groups. any of the groups. Finally for lead, 1 significantly differently associated taxon was found when comparing the <LOD vs Low groups.

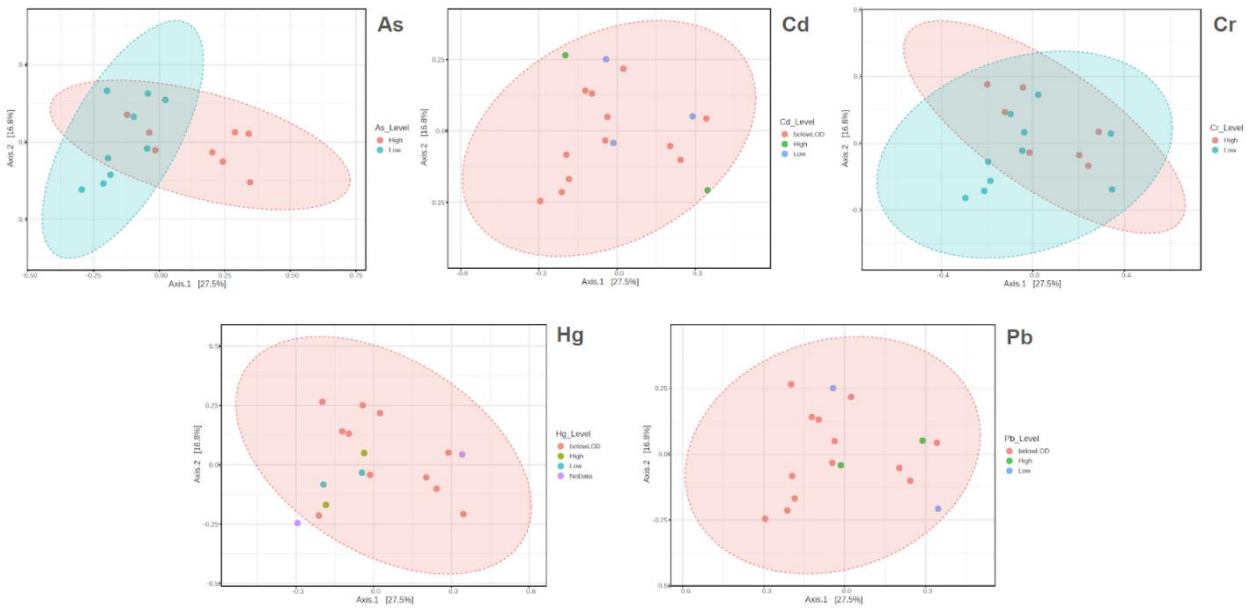


Figure 19. PCoA plots of beta diversity comparing the different cloacal samples associated with the different metal levels for each of the six metals analyzed.

Genus	**Log2FC**	**St.Error**	**P-value**	**FDR**
Tessaracoccus	1.81	0.19	3.19E-07	0.000388
Chthoniobacter	-2.88	0.685	0.00102	0.177
Blastopirellula	-2.73	0.84	0.00629	0.281
Denitratisoma	-4.79	1.33	0.00321	0.281
Dinghuibacter	-3.38	1.07	0.00745	0.281

Table 7. Top 5 (1 of which is significantly differently associated) taxa between Arsenic Level (Low vs High)

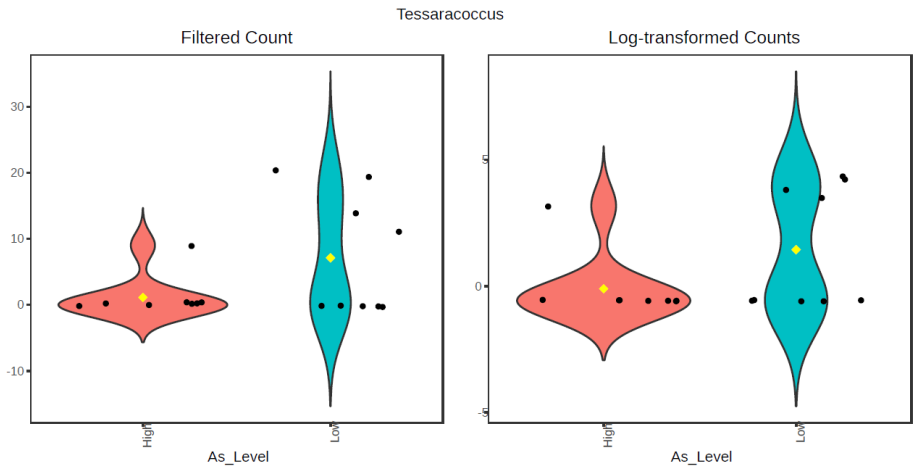


Figure 20. Violin plot of the top significantly differentially associated genus between Arsenic level (Low vs High): *Tessaracoccus.* This genus of microbes was present in a higher abundance in the spotted turtle gut microbiome samples from the low arsenic level turtles.

Genus	Log2FC	St.Error	P-value	FDR
Sterolibacterium	1.43	0.152	6.77E-07	0.0011
Candidatus_Altiarchaeum	0.712	0.251	0.0149	0.661
Candidatus_Chloroploca	4.59	1.43	0.00733	0.661
Candidatus_Methylospira	3.77	1.36	0.0169	0.661
Chlorobium	1.92	0.7	0.0178	0.661

Table 8. Top 5 (1 of which is significantly differently associated) taxa between Cadmium levels (Low vs High)

Genus	Log2FC	St.Error	P-value	FDR
Sterolibacterium	-1.54	0.119	2.05E-08	3.33E-05
Candidatus_Altiarchaeum	-0.797	0.197	0.0016	0.355
Prosthecobacter	-2.41	0.602	0.00175	0.355
Lacunisphaera	-3.96	1.07	0.00297	0.438
Acidovorax	2.35	0.688	0.00511	0.444

Table 9. Top 5 (1 of which is significantly differently associated) taxa between Cadmium levels (<LOD vs Low)

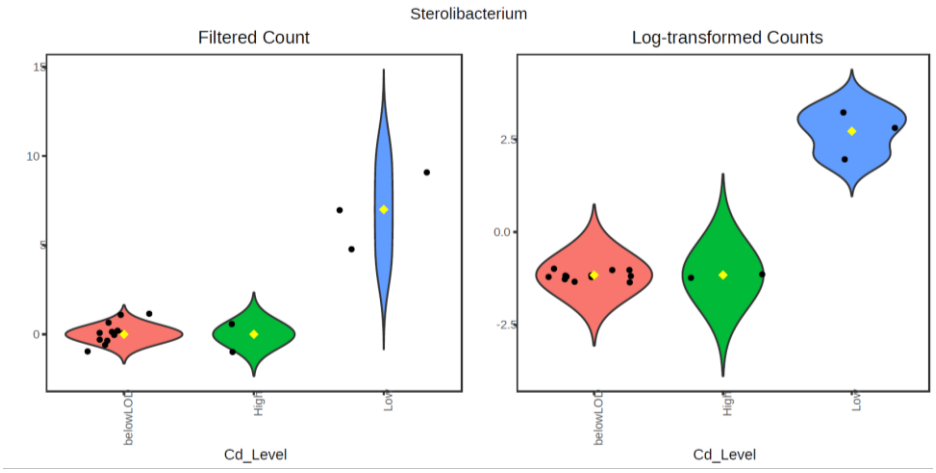


Figure 21. Violin plot of the top significantly differentially associated genus between Cadmium level (Low vs High and <LOD vs High): *Sterolibacterium*. This genus of microbes was present in a higher abundance in the spotted turtle gut microbiome samples from low cadmium level turtles.

Genus	Log2FC	St.Error	P-value	FDR
Candidimonas	4.87	0.796	7.49E-05	0.0394
Abditibacterium	-3.36	0.58	0.000122	0.0493
Stenoxybacter	2.45	0.522	0.00065	0.14
Adhaeribacter	-1.29	0.33	0.00248	0.268
Flavisolibacter	-0.979	0.253	0.00264	0.268

Table 10. Top 5 (2 of which are significantly differently associated) taxa between Mercury level (Low vs High)

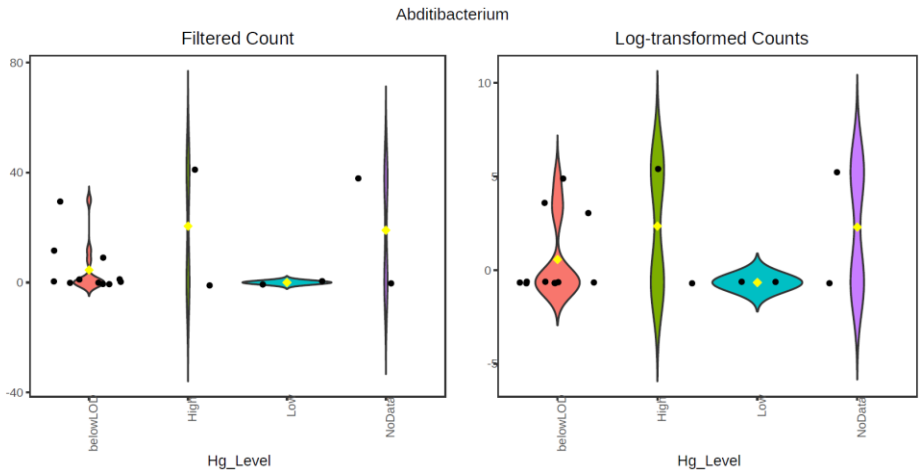


Figure 22. Violin plot of the second top significantly differentially associated genus between mercury level (Low vs High): *Abditibacterium*. This genus of microbes was present in a higher abundance in the spotted turtle gut microbiome samples from the high mercury level turtles.

Genus	Log2FC	St.Error	P-value	FDR
Candidimonas	-4.79	0.766	6.25E-05	0.0316
Abditibacterium	2.87	0.558	0.00032	0.0721
Belnapia	1.35	0.35	0.00275	0.222
Luteibacter	1.37	0.359	0.00285	0.222
Stenoxybacter	-1.94	0.502	0.00261	0.222

Table 11. Top 5 (1 of which is significantly differently associated) taxa between Mercury level (<LOD vs Low)

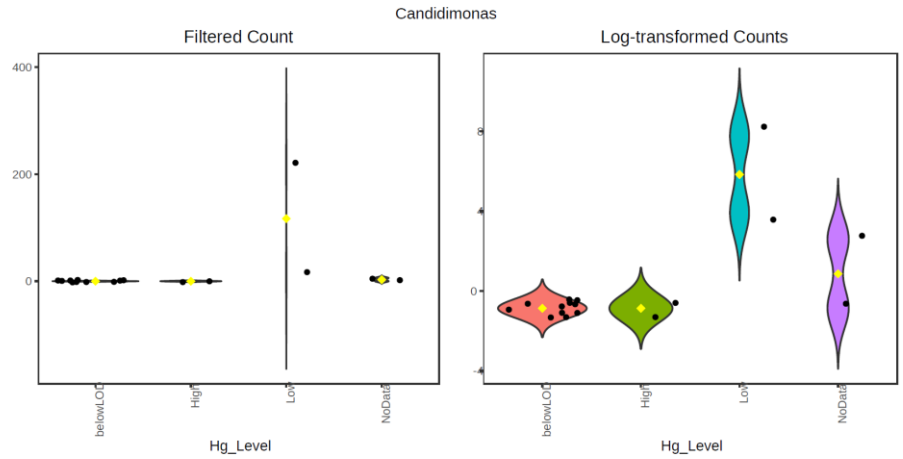


Figure 23. Violin plot of the top significantly differentially associated genus between Mercury level (<LOD vs Low): *Candidimonas*. This genus of microbes was present in a higher abundance in the spotted turtle gut microbiome samples from the low mercury level turtles.

Genus	**Log2FC**	**St.Error**	**P-value**	**FDR**
possible_genus_03	-1.53	0.213	1.11E-05	0.00598
Prosthecobacter	-2.72	0.606	0.000736	0.299
Crenothrix	-3.64	0.881	0.00138	0.339
Acidaminobacter	-1.35	0.423	0.00772	0.534
Methyloglobulus	-2.57	0.797	0.00739	0.534

Table 12. Top 5 (1 of which is significantly differently associated) taxa between Lead level (<LOD vs Low)

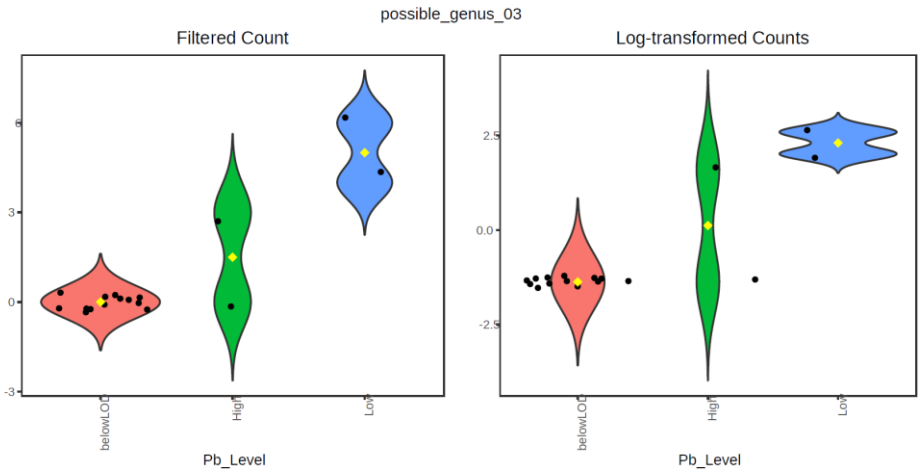
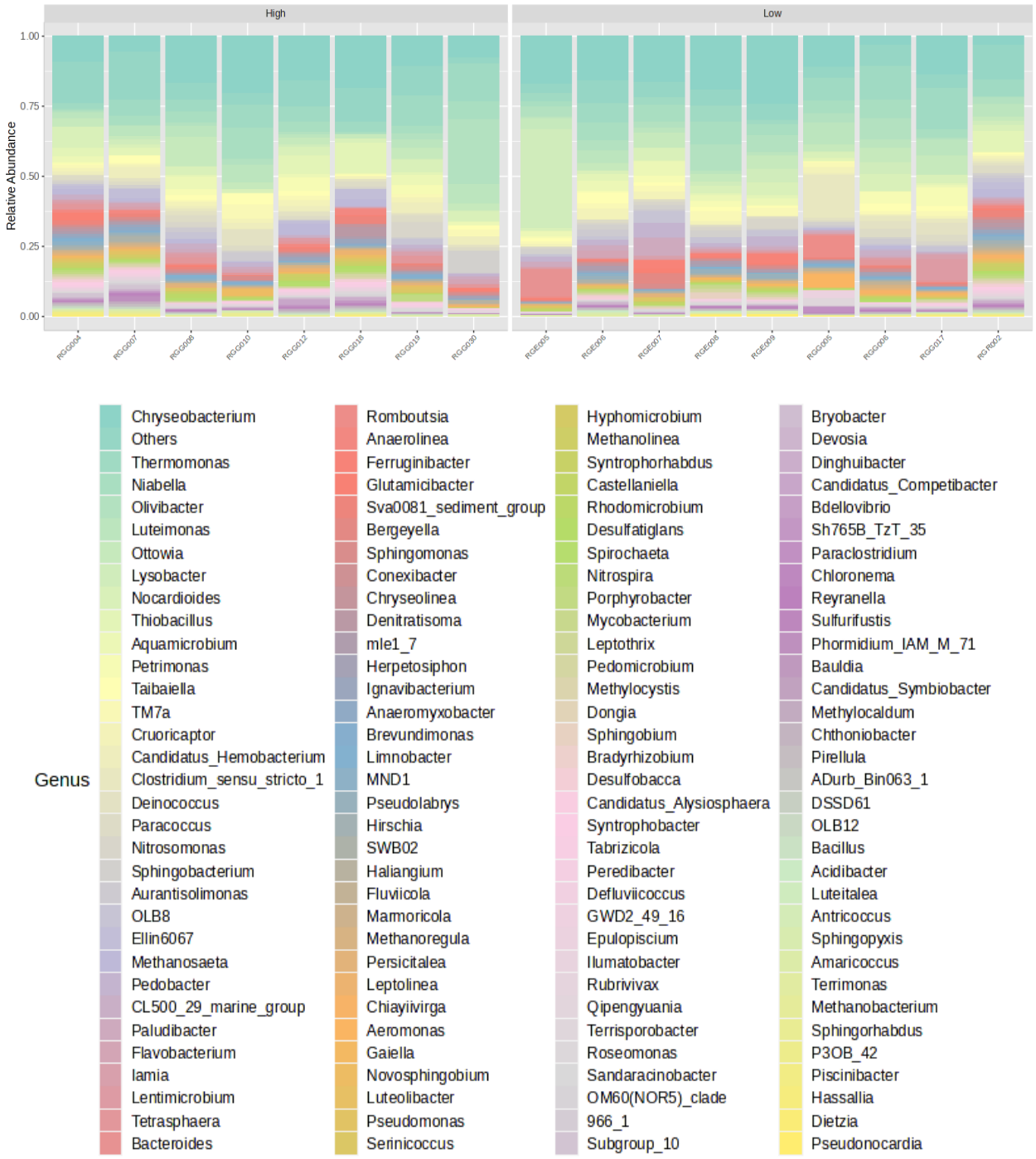


Figure 24. Violin plot of the top significantly differentially associated genus between Lead level (<LOD vs Low): *possible genus 03*. This genus of microbes was present in a higher abundance in the spotted turtle gut microbiome samples from the low lead level turtles.

Finally, to try to integrate the heavy metals into a more cohesive analysis, two different methods were used. The first method involved grouping turtle samples into either High or Low based on all six metal concentrations. If a turtle has two or more metals in the High category for that specific metal, the turtle overall was grouped into the High category. The relative abundance bar plots to visualize these groupings can be seen in Figure 25 below. When comparing alpha diversities and beta diversities, between the two groups, no significant differences were found. When a multifactor analysis was performed using MaAsLin2 to compare the Low vs High overall heavy metal concentration turtle samples, controlling for site and turtle sex, no significantly differently associated taxa were found.

The second method involved splitting turtle samples into groups depending on the number of metals in the below LOD category. In this method, the turtle samples were grouped into 2, 3, or 4 metals below the limit of detection. No turtle sample had the <LOD category for only one, five, or all six of the heavy metals analyzed. The relative abundance bar plots to visualize these groupings can be seen in Figure 26 below. When comparing alpha diversities and beta diversities, between the two groups, no significant differences were found. When a multifactor analysis was performed using MaAsLin2 to compare each of the different groups, controlling for season, no significant differences were found between any of the groups, either.

a.


High
Low
Relative Abundance
1.00
0.75
0.50
0.25
0.00
RGG004
RGG007
RGG008
RGG010
RGG012
RGG018
RGG019
RGG030
RGE005
RGE006
RGE007
RGE008
RGE009
RGG005
RGG006
RGG017
RGR002
Genus
Chryseobacterium
Others
Thermomonas
Niabella
Olivibacter
Luteimonas
Ottowia
Lysobacter
Nocardioides
Thiobacillus
Aquamicrobium
Petrimonas
Taibaiella
TM7a
Cruoricaptor
Candidatus_Hemobacterium
Clostridium_sensu_stricto_1
Deinococcus
Paracoccus
Nitrosomonas
Sphingobacterium
Aurantisolimonas
OLB8
Ellin6067
Methanosaeta
Pedobacter
CL500_29_marine_group
Paludibacter
Flavobacterium
Iamia
Lentimicrobium
Tetrasphaera
Bacteroides
Romboutsia
Anaerolinea
Ferruginibacter
Glutamicibacter
Sva0081_sediment_group
Bergeyella
Sphingomonas
Conexibacter
Chryseolinea
Denitratisoma
mle1_7
Herpetosiphon
Ignavibacterium
Anaeromyxobacter
Brevundimonas
Limnobacter
MND1
Pseudolabrys
Hirschia
SWB02
Haliangium
Fluviicola
Marmoricola
Methanoregula
Persicitalea
Leptolinea
Chiayiivirga
Aeromonas
Gaiella
Novosphingobium
Luteolibacter
Pseudomonas
Serinicoccus
Hyphomicrobium
Methanolinea
Syntrophorhabdus
Castellaniella
Rhodomicrobium
Desulfatiglans
Spirochaeta
Nitrospira
Porphyrobacter
Mycobacterium
Leptothrix
Pedomicrobium
Methylocystis
Dongia
Sphingobium
Bradyrhizobium
Desulfobacca
Candidatus_Alysiosphaera
Syntrophobacter
Tabrizicola
Peredibacter
Defluviicoccus
GWD2_49_16
Epulopiscium
Ilumatobacter
Rubrivivax
Qipengyuania
Terrisporobacter
Roseomonas
Sandaracinobacter
OM60(NOR5)_clade
966_1
Subgroup_10
Bryobacter
Devosia
Dinghuibacter
Candidatus_Competibacter
Bdellovibrio
Sh765B_TzT_35
Paraclostridium
Chloronema
Reyranella
Sulfurifustis
Phormidium_IAM_M_71
Bauldia
Candidatus_Symbiobacter
Methylocaldum
Chthoniobacter
Pirellula
ADurb_Bin063_1
DSSD61
OLB12
Bacillus
Acidibacter
Luteitalea
Antricoccus
Sphingopyxis
Amaricoccus
Terrimonas
Methanobacterium
Sphingorhabdus
P3OB_42
Piscinibacter
Hassallia
Dietzia
Pseudonocardia

b.

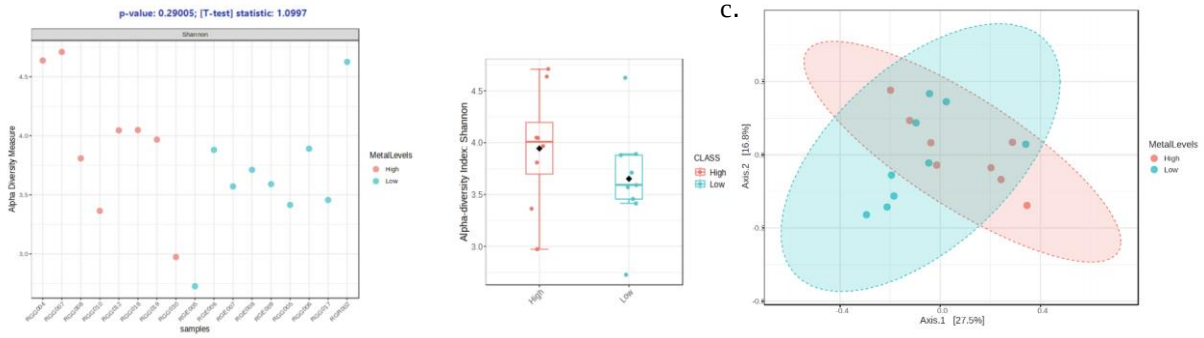
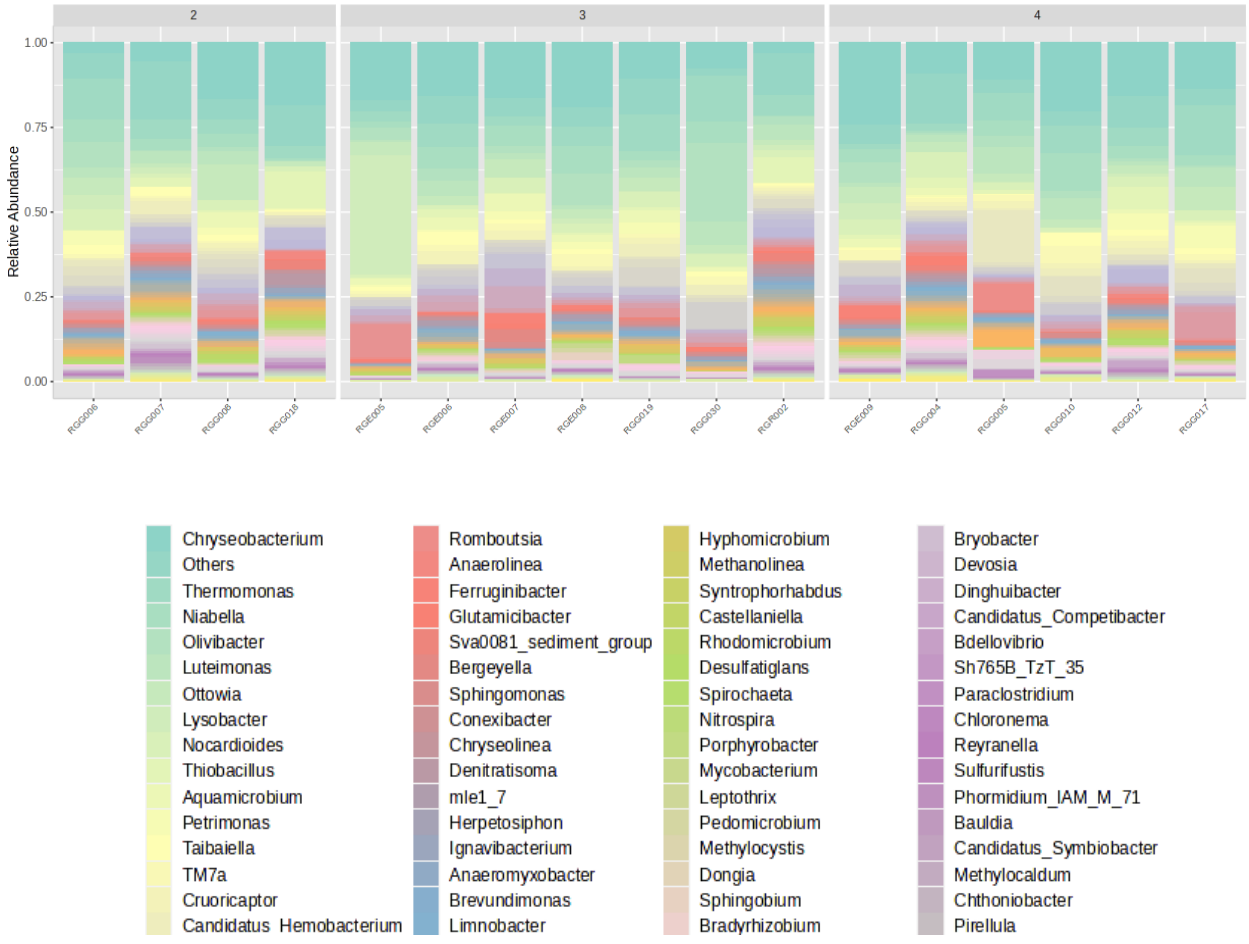


Figure 25. Visualizations of the microbiome comparisons between turtle samples grouped as High (2 or more heavy metals had High concentrations) or Low. (a) Relative abundance of cloacal samples grouped by overall heavy metal concentrations showing the top 131 genera. (b) Alpha diversity plot showing Shannon Diversity Index for each cloacal sample grouped by color (High in red, Low in blue) as well as a boxplot comparing those values. (c) PCoA plot of beta diversity comparing the cloacal samples by overall heavy metal concentrations.

a.



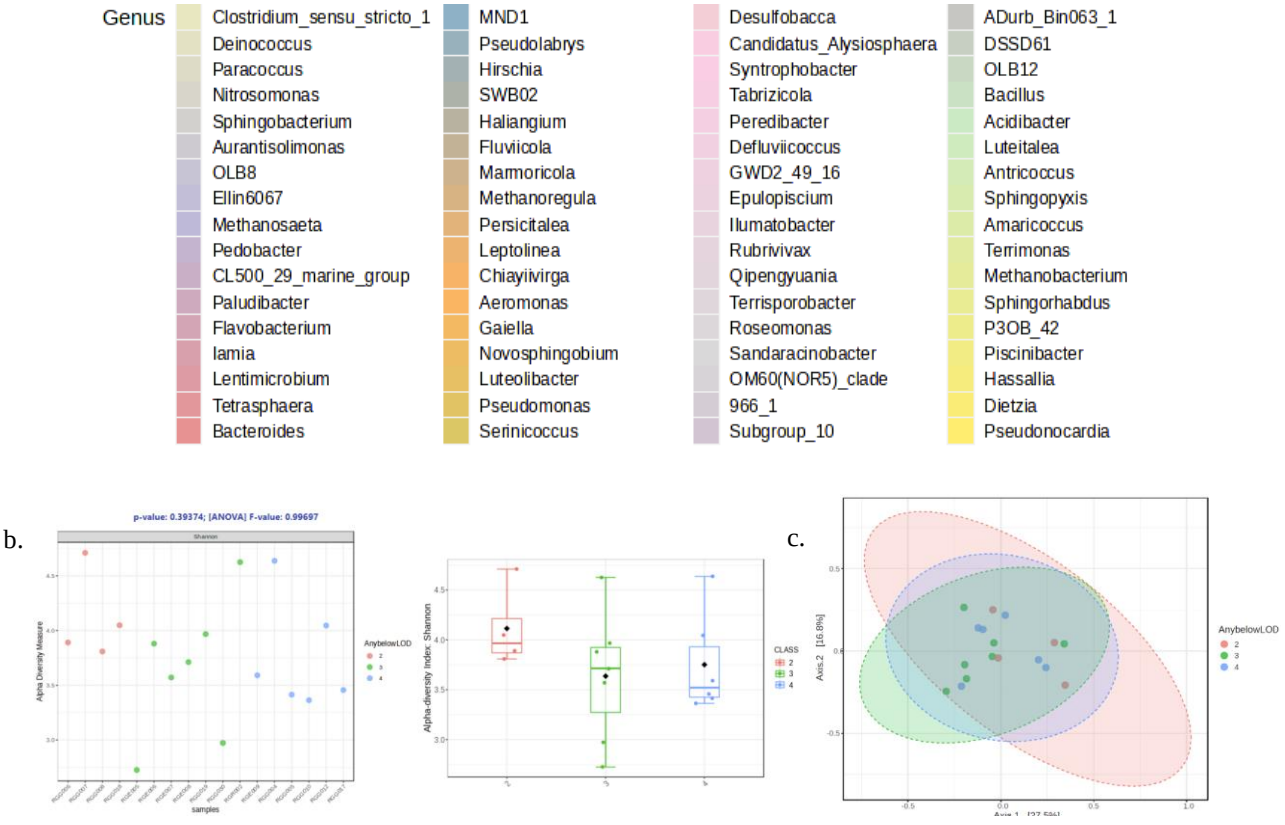


Figure 26. Visualizations of the microbiome comparisons between turtle samples grouped by number of heavy metal concentrations below the level of detection (2, 3, or 4). (a) Relative abundance of cloacal samples grouped by number of <LOD heavy metal concentrations. (b) Alpha diversity plot showing Shannon Diversity Index for each cloacal sample grouped by color (2 in red, 3 in green, and 4 in blue) as well as a boxplot comparing those values. (c) PCoA plot of beta diversity comparing the cloacal samples by number of heavy metal concentrations below LOD.

Discussion

In this study, we characterized the gut microbiome of the spotted turtle and explored the correlations of geographic location, season, turtle sex, and heavy metal contaminants with the composition of the spotted turtle gut microbiome. Season and geographic sampling location seemed to correlate with the largest changes in the spotted turtle gut microbiome, while not many changes were seen correlating with turtle sex and heavy metal contaminants. It is also important to note, that while the word “effect” is used, this study is based on observational data and a sample of convenience, so cause-and-effect may not be necessarily inferred from this data.

Characterization of the Spotted Turtle Gut Microbiome – Painted Turtle Comparison

Both spotted and painted turtles have microbes from the *Firmicutes, Proteobacteria, Bacteriodiota, Actinobacteriota*, and some other less abundant phyla present in their gut microbiomes. *Firmicutes* and *Bacteriodiota* are generally the most common and abundant phyla in vertebrate gut microbiomes although there is some variation by host species (Ley et al., 2008). The painted turtle gut microbiome contains a much higher abundance of Firmicutes that the spotted turtle, making up almost 75% of the microbes present in the painted turtle gut microbiome. This is mostly consistent with the results presented in Fugate et al., 2020. The spotted turtles overall seem to have a more even and rich microbiome at the phylum and genus levels. Although both turtles are North American freshwater species, they do have different ecosystem niches and diets which can help explain the differences seen here. Both diet and geographic location are known factors that correlate with differences in gut microbiota (Wang et al., 2022; Zhang, 2022). Also, with the painted turtle samples sequenced in 2017 verses 2024, the spotted turtle sequencing data contained both much higher read counts and read quality scores which can also contribute to the differences seen. Finally, all the painted turtle samples were from the fall, whereas the spotted turtle samples included collections in both the spring and fall. Season is also a know factor that correlates with differences in gut microbiota (You et al., 2022) so the spotted turtle samples likely show a greater variety of microbes due to this reason as well.

Characterization of the Spotted Turtle Gut Microbiome

Looking at the gut microbiome composition of the spotted turtles on their own, the most predominant bacterial phyla detected across the spotted turtle samples were *Bacteriodota, Proteobacteria,* and *Actinobacterioda*. The most abundant genera were *Chryseobacterium*, *Ottowia*, *Thermomonas*, *Niabella,* and *Deinococcus*. Proteobacteria are present in many

vertebrate gut microbiomes, however they are found in higher abundance in aquatic species than terrestrial ones (P. S. Kim et al., 2021). As the spotted turtle gut microbiome has not been previously characterized, there is not an available comparison for this species. As mentioned above, similar taxa were observed in a North American freshwater turtle species, however there are considerable differences between the two.

Effects of Collection Site, Season, and Turtle Sex on Gut Microbiome Composition

Based on the various comparisons performed, it is clear that geographic sampling location and sampling season correlated with large changes in the composition and structure of the spotted turtle gut microbiome. Although sampling location did not significantly affect the alpha diversity of the gut microbiome, it did significantly affect the beta diversity, which considers the actual taxa present and not just the number and evenness of the taxa present, such as alpha diversity measured do. Multifactor analysis also revealed 83 significantly differently associated taxa between sampling sites, showing that geographic location does correlate with the makeup of the gut microbiome. There were measurable differences in the two study sites including different environmental heavy metal concentrations and differences in overall habitat composition which could potentially affect the composition of the gut microbiome of the spotted turtles that live at each site. Previous research has also shown that geographic variations influence the gut microbiome even on small spatial scales and that animals which are more closely related tend to have more similar gut microbiomes (Goertz et al., 2019; Wang et al., 2022). We know that the turtles within each sampling location are closer to each other geographically than to those from the other site and it is likely that their ranges do not overlap. This could also indicate that the turtles within each site are likely more closely related than

between sites and may be why we see more similar gut microbiomes with the turtles from the same collection sites.

Sampling time or season also correlated with significant changes on both alpha and beta diversity of the spotted turtle gut microbiome. There was a significantly higher alpha diversity in the fall (Oct) than in the spring (March and April). A similar trend was found in white-lipped deer, where their gut microbiota had a higher alpha diversity in the grassy season (May-Oct) than the withering season (Nov-April) (You et al., 2022). Although spotted turtles are omnivores, this could partially be due to the variety and abundance of food sources available in the summer and fall as well as the fact that spotted turtles go through bromination in the late fall to early spring where they burrow, do not eat, and slow down their bodily functions to survive the winter. A large number (195) of differentially associated taxa between the two sampling times were also found. This is consistent with previous research that has found microbiome composition to change seasonally.

Some taxa of interest that were significantly differentially associated in the multifactor analysis between the seasons included *Bacilllus*, *Mycobacterium*, and *Pseudomonas*. These three genera were significantly more abundant in the spotted turtle gut microbiomes sampled in the fall than those in the spring. All three of these genera are capable of cellulose digestion and have been previously isolated from the earthworm gut (Yang et al., 2023). It is likely that these genera are more abundant in October as the turtles have been eating plant matter throughout the whole summer season. They are likely less abundant in the early spring as that is when the turtles once again become active and start eating following bromination in the colder months. The long period of inactivity and fasting can potentially decrease the presence of these microbes in the turtle gut microbiome, only increasing once the turtles begin to regularly consume food,

especially plant matter, after bromination. Although some other genera of interest with the potential for cellulose digestion were also found more abundant in the fall, they have not previously described in host gut microbiomes.

Finally, no significant difference was found between the male and female turtles in either alpha or beta diversities. Multifactor analysis then revealed 12 significantly differentially associated taxa between the male and female turtles. Although some sex differences have been described in the gut microbiome composition of eukaryotes, there is not a general consensus (Y. S. Kim et al., 2020). As the male and female turtles have the same general diets and niches, we would expect their gut microbiomes to overall be similar as we see from this analysis.

Effects of Heavy Metal Contaminants on Spotted Turtle Microbiome Composition

Although we hypothesized that turtles with high levels of heavy metal contamination will also have significantly different gut microbiome composition relative to animals that are uncontaminated or less contaminated as prior evidence indicates, our limited sample sizes and limited range of heavy metal contamination do not demonstrate this trend. Only the arsenic Low vs High turtles seemed to have a correlation between blood serum arsenic level and gut microbiome composition as both the alpha diversities and beta diversities were significantly different between the two groups. All the remaining heavy metal comparisons did not show any trends between contaminants and changes in the gut microbiome, with only a handful of significantly differently associated taxa highlighted through the multifactor analysis. The heavy metal analysis which grouped the turtles into low or high categories based on all of the metals together (2 or more individual metals in the high level categorized that sample to be high for the total metal comparison) as well as the analysis which grouped turtles based on the number of

metals below the limit of detection also did not reveal any significant differences between alpha or beta diversity between gut microbiome of the two groups.

Some potential problems with this study design that could have influenced these results include both limited sample sizes and limited ranges of heavy metal contamination. We do not have any data including truly "clean" or uncontaminated spotted turtles as a comparison. In addition, due to lack of baseline heavy metal information regarding aquatic life and reptiles, it is possible that the Low vs High cutoffs for the heavy metals used in this study are inaccurate. The EPA does have water heavy metal limits recommended for freshwater ecosystems, but there are no determined safe biological (blood) levels of heavy metals for turtles. Li et al. (2024) aimed to examine the correlation of heavy metal contamination on the sea turtle gut microbiome. Their data similarly suggests no statistically significant correlation between iron, zinc, copper, lead, and cadmium level and the gut microbiome composition of those sea turtles, however this study involved two species of captive sea turtles and used environmental heavy metal concentrations from the water that the turtles were in and not blood serum values and similarly also had a very small range of heavy metal levels (C. X. Li et al., 2024). Another study involving 20 wild Pine Snakes (*Pituophis melanoleucus*) from a relatively undisturbed habitat (although environmental heavy metal concentrations were not analyzed) found the average blood serum levels of arsenic, cadmium, chromium, mercury, and lead to be 7.0, 4.3, 42.5, 26.9, and 88.8 ppb (Burger et al., 2017). These values are on average all higher than our spotted turtle blood values except for arsenic, for which the spotted turtles on average had double the amount in their blood. Similar to this study, Burger et al, 2017 did not directly look at health effects for the animals sampled. Another study involving mugger crocodiles (*Crocodylus palustris*) in Iran from highly contaminated habitats (average sediment heavy metal concentrations for arsenic, cadmium,

chromium, mercury, and lead are as follows: 25490, 40, 27, 411, 4557 ppb) found the average blood serum levels of arsenic, cadmium, chromium, mercury, and lead to be 1.196, 15.22, 25.33, 507.2, and 333.6 ppb (Gholamhosseini et al., 2022). These values are once again on average all higher than our spotted turtle blood values except for arsenic, for which the spotted turtles on average had twelve times the amount in their blood. This is interesting as our heavy metal sediment concentrations for the Northern Indiana study sites were almost five times lower for arsenic than the Iran sediments (25490 ppb vs 5657 ppb) while being much higher for the remaining metals, except for mercury: cadmium 40 vs 549 ppb, chromium 27 vs 13869 ppb, mercury 411 ppb vs <LOD, lead 4557 vs 34436 ppb. This hints at differences in absorption and bioaccumulation of these metals between the spotted turtles and the mugger crocodiles. While Gholamhosseini et al. (2022) did not directly look at health effects for the animals sampled, they do hint at detrimental health effects for both the crocodiles living in these habitats as well as the humans nearby. Overall, it is important for more research to focus on specific blood heavy metal concentrations as previous work in humans has shown that blood serum levels of heavy metals do not clearly correlate with environmental samples. There is a weak correlation between age and diet and heavy metal blood contamination levels but less so between environmental sample levels and blood serum levels (Jose & Ray, 2018). This is especially important to remember as heavy metals bioaccumulate in turtle and other vertebrate tissue.

Conclusion and Future Directions

Previous research has brought to light the importance of the gut microbiome for overall organism health. In the case of some wild bird species, there was clear evidence that the gut microbiome composition correlates with fitness. Additionally, heavy metals in other vertebrates are known to influence gut microbial structure. The work presented here is an attempt to

synthesize these findings into a unified research program to simultaneously assess overall health, heavy metal exposure, and microbiome alterations in an at-risk wild turtle population that can serve as an indicator of overall ecosystem health. Although this work does not present many significant differences based on heavy metal contaminants, it characterizes the gut microbiome of the spotted turtle and explores the correlation of the gut microbiome with geographic site, season, and turtle sex. Hopefully this data can be used as starting points for further work and analysis of this topic. A future direction of this work could focus on improving the heavy metal analysis. Ideally, acquiring samples from a spotted turtle comparison group from an uncontaminated location to compare gut microbiome compositions would provide better insight on the true correlations between heavy metals and the spotted turtle gut microbiome composition.

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