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Microbiome diversity and composition varies across body areas in a freshwater turtle

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Abstract

There is increasing recognition that microbiomes are important for host health and ecology, and understanding host microbiomes is important for planning appropriate conservation strategies. However, microbiome data are lacking for many taxa, including turtles. To further our understanding of the interactions between aquatic microbiomes and their hosts, we used next generation sequencing technology to examine the microbiomes of the Krefft's river turtle (*Emydura macquarii krefftii*). We examined the microbiomes of the buccal (oral) cavity, skin on the head, parts of the shell with macroalgae, and parts of the shell without macroalgae. Bacteria in the phyla Proteobacteria and Bacteroidetes were the most common in most samples (particularly buccal samples), but Cyanobacteria, Deinococcus-Thermus, and Chloroflexi were also common (particularly in external microbiomes). We found significant differences in community composition among each body area, as well as significant differences among individuals. The buccal cavity had lower bacterial richness and evenness than any of the external microbiomes, and it had many amplicon sequence variants (ASVs) with a low relative abundance compared to other body areas. Nevertheless, the buccal cavity also had the most unique ASVs. Parts of the shell with and without algae also had different microbiomes, with particularly obvious differences in the relative abundances of the families Methylomonaceae, Saprospiraceae, and Nostocaceae. This study provides novel, baseline information about the external microbiomes of turtles and is a first step in understanding their ecological roles.

Introduction

Animals harbor diverse assemblages of microbial organisms that play key roles in host health and ecology [1–4] and may be important for conservation efforts [5, 6]. Thanks to

advances in high-throughput sequencing (HTS) technology, our knowledge of host microbiomes (particularly human microbiomes) and the diverse roles that they play has grown rapidly during the past two decades, with thousands of studies being published every year. Nevertheless, there are still many knowledge gaps to fill, and the microbiomes of many major taxonomic groups remain poorly studied. Indeed, one review found that over 90% of vertebrate microbiome studies focused on mammals, with comparatively few studies on each of the remaining vertebrate classes [7].

Turtles are among the groups that are in particular need of increased research. They are among the most imperiled vertebrates, with nearly two-thirds of their species listed as threatened or endangered [8, 9]. Further, although habitat loss, overconsumption, and poaching are the primary threats to most turtle species [10], emerging infectious diseases are also a serious concern and have decimated populations of several species [11–13]. Given the importance of microbiomes in both human health [14, 15] and emerging infectious diseases in groups like amphibians [16–18], it is likely that microbiomes are important for turtle health as well. Further, *Ranavirus* infections are of particular concern for turtles [19] and *Ranavirus* infections are negatively associated with higher microbial richness in amphibians [20], suggesting that microbiomes may be able to mitigate infections.

In addition to the potential role of microbiomes in turtle disease ecology, they may be important for conservation efforts that require turtles to be temporarily kept in captivity. Many conservation strategies for turtles rely heavily on captive assurance colonies, head-starting programs, and reintroduction programs, but in many taxa, such as gibbons [21], frogs [22], salamanders [23], and lizards [24], captivity alters microbiomes, and there is some evidence for this occurring in turtles [25–27]. Due to the link between microbial diversity and host health,

maintaining healthy microbiomes may be a key, but often overlooked, factor in the success of these efforts [5, 6]. However, monitoring and maintaining proper microbiomes in captivity requires baseline data on the composition and roles of microbiomes in wild populations, but those data are lacking for turtles.

Few studies have examined turtle microbiomes, and, as often is the case in turtle research, the literature is taxonomically biased, with most studies focusing on sea turtles [27–34] and their eggs [35–39], followed by tortoises [40–45]. These groups are certainly important and more studies should be conducted on them (particularly expanding the number of species covered), but these taxonomic groups are highly ecologically divergent from most turtle species, and they only represent three turtle families and 20% of extant species [9]. The remaining 11 families (80% of species) are only represented by a handful of studies using methods like culturing and fluorescent *in situ* hybridization (which only detect a limited portion of the microbiome)[25, 46–49] and, to the best of our knowledge just four studies (three species) using HTS methods [26, 50–52]. Further, one of these HTS studies sampled only two individuals [50], one was on turtles in a commercial turtle farm [52], and three were on captive individuals [50–52]. Given that captivity is known to affect the microbiomes of other taxa, a dearth of studies on wild populations is a serious knowledge gap. Further, with the exception of one culture-based study on *Phrynops geoffroanus* [49] and one culture-based study on *Podocnemis* eggs [53], to the best of our knowledge, all turtle microbiome work has focused on members of the suborder Cryptodira, while the other major branch of the turtle evolutionary tree (Pluerodria) remains unstudied. These suborders diverged roughly 200 million years ago [54] and may have important differences.

In addition to the taxonomic limitations of the current literature, most turtle microbiome studies have focused on gut/fecal microbiomes and cloacal microbiomes, with a few studies on oral microbiomes. No studies have looked at the external microbiomes of turtles (i.e., on the skin and shell). This knowledge gaps extends beyond turtles and applies to reptiles in general, with only a handful of studies published on their external microbiomes [7, 55–57]. Nevertheless, these external microbiomes may have important functions in host health and ecology and should be examined.

Studying turtle microbiomes, particularly external microbiomes, is also important not only for turtle ecology and conservation, but also for gaining a comprehensive understanding of the microbiomes of aquatic ecosystems. Turtles are ecologically significant, and often comprise a large portion of the vertebrate biomass in aquatic ecosystems [58, 59]. This potentially makes them an excellent and highly mobile reservoir for many bacterial species. Further, the keratin scutes on their shell are a fairly unique substrate in aquatic environments. Fish also have keratinized scales, but unlike turtles, they secrete an epidermal mucus that contains, among other things, many anti-microbial peptides, which no doubt affect the microbiome [60]. Indeed, the ability of turtles' shells to harbor specialized organisms has fascinated herpetologists for decades, and the macroalgae (hereafter, "algae") that covers many turtles' shells are actually members of a unique genus (*Basicladia*) that grows almost exclusively on turtles [61–63]. These algae have already been implicated in a number of ecological roles, including camouflage, seed dispersal, and harboring a community of crustaceans [64, 65]. They could also affect the microbiome by providing an additional substrate for bacteria, competing with benthic bacteria for access to turtles' shells, allowing bacterial colonization from other organisms living in the algae, trapping sediment particles, and retaining moisture when turtles bask.

115 The goal of the present study was to help fill these gaps in our knowledge by
116 documenting and characterizing the microbiomes of a wild population of the aquatic Krefft's
117 river turtle (*Emydura macquarii krefftii*) in the Chelidae family (suborder Pleurodira). We also
118 were specifically interested in external microbiomes and how they differed across parts of the
119 body. Therefore, we examined the microbiomes of the buccal cavity (which is an important
120 transition from the external environment to the internal environment), the skin on top of the
121 head, parts of the shell that were free of algae, and parts of the shell that supported algae. These
122 data will provide an important baseline on which future research can build.
123
124 **Methods**
125 *Sample collection*
126 We captured Krefft's river turtles (*Emydura maquarii krefftii*; suborder Pleurodira,
127 family Chelidae) in the Ross River (Townsville, Queensland, Australia) on 30 October 2016.
128 Ross River is ~30km long and 150m wide. It runs from the Ross River Dam to Cleveland Bay,
129 and usually has a low flow rate. Various authors have referred to our study species as *Emydura*
130 *krefftii* or *E. k. krefftii* [66], but we will follow the taxonomy proposed by the Turtle Taxonomy
131 Working Group [9] and refer to it as *E. m. krefftii*. Regardless of nomenclature preference, it is
132 the only *Emydura* that occupies Ross River, thus clarifying which organism we examined.
133 Turtles were captured using a single baited trap that was placed overnight. We captured
134 six adult turtles: one male and five females (mass = 0.6–2.0 kg; curved carapace length = 17.2–
135 26.1 cm). All turtles appeared healthy. We rinsed each individual with sterile water to remove
136 sediment and transient bacteria [67], then swabbed four body areas using a different swab for
137 each area. Sterile rayon-tipped swabs (Medical Wire, MW113) were used. We swabbed the

inside of the buccal cavity (mouth), the top of the head, part of the shell that did not have algae growing on it, and part of the shell that had algae growing on it (the algae were not characterized as part of this study, but they were assumed to be members of the genus *Basicladia* based on appearance and the extensive literature documenting the abundance of that genus on turtles). Swabs were rolled and moved around each area for 30 seconds, while attempting to cover a similar amount of surface area for each region (standardizing surface area was not possible for buccal swabs, so the swabs were moved around the inside of the mouth as much as possible). Additionally, two blank swabs were collected to control for background contamination. Both were removed from the sterile packaging, held in the air for 30 seconds, then placed into sterile vials. One swab had sterile water poured over it, the other swab did not. All swabs were immediately placed on dry ice and stored in a -80ºC freezer for four months.

Extraction, amplification, and sequencing

We extracted bacterial DNA using the cetyl trimethyl ammonium bromide (CTAB) protocol with a chloroform precipitation step [68]. To lyse gram positive bacteria, we added a lysozyme digestion step to the beginning of the protocol. Briefly, after allowing samples to thaw for ten minutes, 70 µL of a freshly mixed lysozyme solution (20 mM Tris-HCL, 2m M EDTA, 1.2% Tween, 20 mg/mL lysozyme powder) were added to each sample, and the samples were incubated at 37ºC for thirty minutes. Then, 650 µL CTAB buffer and 10 µL proteinase K (20 mg/ml BIOLINE) were added to each sample, and they were incubated at 56ºC for 14 hours. The standard CTAB protocol was used for the remaining steps. All samples (and the two blanks) were extracted simultaneously using a single batch of reagents.

We prepared samples for sequencing following the Illumina 16S Metagenomics Sequencing Library Preparation guide [69], including amplifying the V3V4 16S regions with the recommended S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primer pair [70]. We modified the Illumina protocol slightly to include 30 cycles for the amplification PCR (triplicate 10 µL reactions with KAPA HiFi DNA Polymerase) and 40 µL reactions for the indexing PCR (triplicates were pooled prior to the indexing PCR). Additionally, we used Sera-mag SpeedBeads (ThermoScientific, California, USA) for all cleanup steps [71]. We sequenced the samples on an Illumina MiSeq run that was shared with samples from other projects (Reagent kit V3 600 cycles PE, Illumina, USA; 10% PhiX spike-in). One algae sample and one head sample (different individuals) could not be sequenced, resulting in five samples for each of those areas.

Bioinformatics and quality control

The data were analyzed using DADA2 [72], within the QIIME2 environment [73], using the parameters outlined below. To remove the primer sequence, 20 bp and 21 bp of the 5'-end were trimmed from the forward read and reverse reads, respectively. To remove low quality base pairs at the 3'-end, forward and reverse reads were further truncated at position 270 and 230, respectively. Maximum ee value was set to 6 and chimeras were removed using the consensus method (detailed information on the scripts used can be found here: https://github.com/R-Huerlimann/MouseKD_analysis). Taxonomic assignment was done using the Silva taxonomic classifier (version 132) provided by QIIME2. Any ASV that comprised less than 0.01% of all reads was removed. Contaminant reads were removed from the samples using the R package microDecon on default settings [74]. In accordance with the package recommendations, both blanks were used, and each body area was set as a group.

183

184 *Analyses*

185 We used several methods to compare the taxonomic composition and community
186 structure of the different body areas. First, we used DESeq2 to compare the differential
187 abundance of ASVs between body areas [75, 76]. For this test, we first removed any ASVs that
188 were not present in at least three of our 22 samples, then we ran DESeq2 on default settings
189 comparing all body areas (the model was area + turtle ID). We then looked at each pairwise
190 comparison and extracted ASVs that were differentially abundant for a given comparison.
191 Because we were making many comparisons, we used a stringent false discovery rate (FDR) of
192 0.001 within the comparisons for each pair of body areas.

193 We ran PERMANOVAs via the adonis2 function in the R package vegan [77] to compare
194 the entire communities of each body area (5,000 iterations). We ran three tests: one based on
195 Bray-Curtis dissimilarities (which incorporate abundance), one based on the Jaccard index
196 (which is based only on presence/absence), and one based on weighted unifrac distances (which
197 incorporates both abundance and phylogenetic relationships among ASVs). For all tests, we
198 conducted post hoc tests between pairs of body areas by using PERMANOVAs to make pairwise
199 comparisons between areas (while accounting for turtle ID). For each set of comparisons, we
200 used a sequential Bonferroni correction to control the type 1 error rate. Additionally, we
201 constructed an ordination plot based on Bray-Curtis dissimilarities, and examined composite
202 dissimilarities by combining all samples per body area into a single sample (mean) and
203 comparing the Bray-Curtis dissimilarities of these mean samples. For all Bray-Curtis
204 dissimilarities and unifrac distances, we transformed the data to proportions (sometimes called
205 total sum normalization) prior to calculating the dissimilarities. This method is superior to

206 alternatives for many ecological questions [78]. For the composite dissimilarities, we
207 transformed samples to proportions, then calculated the mean proportion for each ASV within
208 each group.
209 Finally, we examined alpha-diversity by comparing body areas for both ASV richness
210 and evenness. For both metrics, we constructed linear models using the *aov* function in R [79]
211 with body area and turtle ID as the main effects. We could not fit an interaction because of the
212 two samples that could not be sequenced. Although rarefaction curves indicated that a sufficient
213 read depth had been achieved (Supporting Data), richness results were biased by differences in
214 read depth, particularly one sample from the head that had three times as many reads as the next
215 highest sample. Rarefying did not correct this problem; therefore, we include read depth as a
216 covariate in our model for richness. Significance was assessed with the *Anova* function in the car
217 package [80], and the *TukeyHSD* function was used to make post hoc comparisons. Model fit
218 was assessed with QQ plots and residual plots.
219
220 **Results**
221 *Sequencing output and dada2 processing*
222 The sequencing run produced 1,241,199 reads for the samples in this project (8.9% of
223 reads from the shared run), which were filtered with DADA2 denoising (998,438 reads retained),
224 merging (801,547 reads retained), and chimera filtering (772,249 reads retained), followed by the
225 removal of a sample with only 72 reads. microDecon was used to remove contaminant reads
226 (693,633 reads retained), after which the blank samples were removed from the data set. This
227 produced a final data set of 22 samples with a total of 640,328 reads (median = 23,132 reads per

sample). Rarefaction plots confirmed that sufficient read depth was achieved for all samples (see Supporting Data for plots and filtering details for each sample).

Taxa and differential abundance

Reads were segregated into 1,136 ASVs representing 19 phyla, 41 classes, 94 orders, and 130 families. Proteobacteria and Bacteroidetes were the most common phyla (especially in the buccal cavity), comprising an average of 30.6% (SD = 8.1) and 25.9% (SD = 11.0) of reads per sample, respectively (Fig. 1). Other common phyla varied among body areas (Fig. 1), particularly Cyanobacteria, which was highly abundant on parts of the shell without algae (mean = 19.0% of reads, SD = 9.4), but was rare in the buccal cavity (mean = 1.2 % of reads, SD = 1.1) and was moderately abundant on the head (mean = 5.1% of reads, SD = 4.2) and parts of the shell with algae (mean = 9.9% of reads, SD = 3.2). Differences among body areas became increasingly apparent at lower taxonomic levels, but there was a fairly high degree of consistency among samples within body areas (Fig. 2), with the exception of the families Weeksellaceae and Flavobacteriaceae in buccal samples. In four buccal samples, Weeksellaceae was common (mean = 28.5% of reads, SD = 1.9) and Flavobacteriaceae was fairly uncommon (mean = 4.1% of reads, SD = 2.3), but in the other two samples, Flavobacteriaceae was the most common family (74.5% and 36.3% of reads) while Weeksellaceae was low to moderately abundant (1.2% and 7.5% of reads).

Differential abundance tests also revealed interesting differences at the ASV level (Fig. 3). A total of 218 ASVs were significantly differentially abundant in at least one comparison. Most of these (209 ASVs) involved comparisons to the buccal cavity (some ASVs were also differentially abundant between other regions), and in most cases (151 ASVs), the buccal cavity

had a lower relative abundance. There were, however, exceptions. For example, the phyla Bacteroidetes and Patescibacteria contained both ASVs that had an increased relative abundance in the buccal cavity and ASVs that had a reduced relative abundance in the buccal cavity. Parts of the shell with algae generally had higher relative abundances than buccal samples, but often had lower relative abundances than either the head or parts of the shell without algae (e.g., several Bacteroidetes and Proteobacteria). There was a high degree of consistency within body areas in that only five ASVs were significantly more abundant for a particular area in one comparison and significantly less abundant for that area in a different comparison (Fig. 3). The consistency was particularly pronounced for the buccal cavity, where 56 ASVs showed the same pattern in all three comparisons, and 89 ASVs showed the same pattern in two comparisons and did not show a significant difference in the third. The other areas had lower consistency, but this was largely driven by the fact that most differences involved the buccal cavity (Fig. 3; Supporting Data).

Despite having a low relative abundance for many ASVs, the buccal cavity had more unique ASVs (120) than any of the other body areas (Fig. 4). For the buccal cavity, most of the unique ASVs were in the phyla Proteobacteria (63) or Bacteroidetes (26). Within Proteobacteria most were in the class Gammaproteobacteria (48), order Betaproteobacteriales (39) and family Burkholderiaceae (25). Additionally, several of the ASVs that were unique to the buccal cavity were fairly abundant. Six of them each comprised more than 1% of all buccal reads, and one (genus *Flavobacterium*) comprised 8.4% of all buccal reads. Eight other *Flavobacterium* were unique to the buccal cavity, and collectively, all unique buccal ASVs comprised 38.5% of all buccal reads. In contrast, for the other body areas, unique ASVs were fewer and present in lower abundances. Out of all three areas, only one unique ASV was present as more than 1% of the

reads for a given area (*Synechococcus* PCC-7902, a Cyanobacteria), which comprised 2.9% of all reads for the shell (without algae). Collectively, unique ASVs for body areas other than the buccal cavity only comprised 4.1% of all reads for the head, 6.9% for the shell (without algae), and 2.0% for the parts of the shell with algae (the head swab for turtle #5 was not included in the results in this paragraph because it had three times as many reads as other swabs, resulting in high levels of rare, unique ASVs in that sample, even after rarefying, see Supporting Data).

Communities

The PERMANOVA based on Bray-Curtis dissimilarities found significant differences in the communities among body areas ($F = 3.7$, $df = 3$, pseudo $P < 0.001$) and among individuals ($F = 1.5$, $df = 5$, pseudo $P = 0.009$). Post hoc tests found that each body area was significantly different from every other area (all pseudo P [after sequential Bonferroni correction] < 0.003). Bray-Curtis dissimilarities based on a composite of each body area showed that the strongest differences were for comparisons between the buccal cavity and external microbiomes (Bray-Curtis dissimilarities = 0.81–0.87). Also, although each area had a unique microbiome, the shell without algae was most similar to the shell with algae (Bray-Curtis dissimilarity = 0.50) followed by the head (Bray-Curtis dissimilarity = 0.59). The head was more different from parts of the shell that had algae (Bray-Curtis dissimilarity = 0.70) than parts of the shell with algae (Bray-Curtis dissimilarity = 0.59). These patterns are reflected in the PCoA (Fig. 5).

The PERMANOVA based on Jaccard indices showed the same patterns, with significant differences in the communities among body areas ($F = 2.4$, $df = 3$, pseudo $P < 0.001$) and among individuals ($F = 1.3$, $df = 5$, pseudo $P = 0.007$). Post hoc tests found that each body area was

296 significantly different from every other area (all pseudo P [after sequential Bonferroni
297 correction] ≤ 0.016).
298 The PERMANOVA based on weighted unifrac distances also found a significant
299 difference among body areas (F = 7.9, df = 3, pseudo P < 0.001), but the differences among
300 individuals did not quite achieve significance (F = 1.7, df = 3, pseudo P = 0.052). Post hoc tests
301 found that each body area was significantly different from every other area (all pseudo P [after
302 sequential Bonferroni correction] ≤ 0.002).
303 Buccal microbiomes had lower mean bacterial richness than any of the external body
304 areas (mean richness [SD]: buccal = 199 [44.7], head = 399 [109.5], shell without algae = 302
305 [92.8], shell with algae = 320 [66.4]). Within each individual, the buccal cavity had a lower
306 richness than any other body area. The ANOVA confirmed that richness differed significantly
307 among body areas (F = 7.6, df = 3, P = 0.004). It also showed that the number of reads per
308 sample was a significant covariate (F = 8.8, df = 1, P = 0.012). Differences among individual
309 turtles were nearly significant (F = 2.6, df = 5, P = 0.078). Post hoc Tukey's tests showed that the
310 buccal cavity had lower bacterial richness than all other body areas (all P ≤ 0.030). No other
311 comparisons were significant, but the difference between the shell (without algae) and head was
312 nearly significant (P = 0.055).
313 Buccal microbiomes also had lower bacterial evenness than any of the external body
314 areas (mean evenness [SD]: buccal = 0.71 [0.12], head = 0.87 [0.03], shell without algae = 0.83
315 [0.06], shell with algae 0.86 [0.04]). The ANOVA confirmed that evenness differed significantly
316 among body areas (F = 6.2, df = 3, P = 0.007), but not among individuals (F = 1.7, df = 5, P =
317 0.213). Post hoc tests found differences between the buccal cavity and the head (P = 0.015) and
318 the buccal cavity and parts of the shell with algae (P = 0.020). No other differences were

significant, but the difference between the buccal cavity and parts of the shell without algae was nearly significant (P = 0.056).

Contamination

Seventy-three ASVs amplified in the blanks, 41 of which also amplified in at least one sample. microDecon removes contamination by using information in blank samples to remove contaminant reads, rather than whole ASVs (though sometimes all reads for an ASV are removed). Thus, it can handle situations where a common environmental ASV is present on turtles, but also present as reagent contamination. It appeared to do a good job of removing the contaminant reads. Thirty-one ASVs were completely removed from all samples. For the ten ASVs that were not entirely removed, two were retained in samples from all four body areas. These bacteria (an Actinobacteria and a Gammaproteobacteria) were abundant in the samples, and rare in the blanks, suggesting that microDecon correctly identified them as being only partially from contamination and retained most of their reads. The next most common ASV (a Gammaproteobacteria) was retained in multiple samples from all groups except for parts of the shell with algae. Finally, one ASV was retained in multiple samples for both the head and shell without algae, and the remaining six ASVs were in multiple samples from the buccal cavity, but no samples from other groups. This type of separation between groups would be expected from accurately removing contaminant reads as opposed to whole ASVs (in contrast to a fairly random pattern that would be expected from residual contamination). Our results further support the use of microDecon as a technique to remove contamination from microbial samples. Full outputs from microDecon are available in the Supporting Data.

Discussion

This study provides several useful insights into turtle microbiomes. First, we found that microbiomes differed among all body areas tested, including all three external body areas. This result echoes research that found different external cutaneous microbiomes on different body areas in humans [81, 82], amphibians [83], and fish [84]. Perhaps unsurprisingly, in our data, the strongest difference was between the buccal cavity and the external microbiomes; this difference was partially due to an abundance of photosynthetic bacteria, such as Cyanobacteria and Chloroflexia [85], on the exterior surfaces, but many other bacteria were also differentially abundant between the buccal cavity and external microbiomes. Also, the buccal samples had lower ASV richness and evenness than the samples from external areas, as well as more unique ASVs. It is also worth noting that most buccal ASVs could be identified to the family level (mean = 92.8% of reads, SD = 4.8), but the proportion of ASVs that could be identified at the family level was lower for external areas (mean percent of reads [SD]: head = 68.9% [5.1], shell without algae = 79.7% [4.1], shell with algae = 76.3% [6.7]). It is difficult to interpret this result, but one obvious hypothesis is that this is a result of biases in the literature. Oral microbiomes have been more well-studied and characterized than the external microbiomes of aquatic species like turtles, which could result in a reduced ability to identify bacteria from extremal microbiomes. This emphasizes the need for greater research on this topic.

Another interesting result is that parts of the shell with algae had different microbiomes than parts of the shell without algae. This provides novel information about the ecological interactions between algae and their turtle hosts, and it could be an important consideration in captive husbandry and monitoring turtle health. For example, the family Methylomonaceae was more abundant on parts of the shell with algae (mean = 15.3% of reads, SD = 9.5) than on any

365 other body area (mean percent of reads [SD]: buccal = 0.7% [0.5], head = 3.3% [2.3], shell
366 without algae = 7.9% [11.8]). Methylomonaceae are methanotrophic bacteria that occur in a
367 variety of freshwater and marine environments, as well as in symbiotic relationships with deep-
368 sea invertebrates living around thermal vents, and it is interesting to learn that they also colonize
369 turtles' shells (particularly areas with algae) [86–89]. Parts of the shell with algae also had the
370 highest levels of bacteria in the family Saprospiraceae (mean percent of reads [SD]: buccal =
371 0.3% [0.4], head = 3.6% [2.3], shell without algae = 6.4% [2.7], shell with algae = 14.1% [2.5]),
372 a group that is noted for its important role in breaking down complex organic molecules [90].
373 Conversely, the family Nostocaceae was more abundant on parts of the shell without algae than
374 on other body areas (mean percent of reads [SD]: buccal = 0.2% [0.1], head = 2.4% [1.9], shell
375 without algae = 11.8% [10.8], shell with algae = 2.4% [1.3]). Nostocaceae are benthic
376 Cyanobacteria [91], and they may compete with macroalgae for access to the turtles' shells [92].
377 Both of the microbial communities on turtles' shells are likely affected by turtles molting their
378 scutes, followed by a re-growth of algae. None of our turtles were molting or appeared to have
379 molted recently, but this would be an interesting topic for future work.
380 Due to the general dearth of turtle microbiome studies, it is difficult to compare our
381 results to those of other microbiome studies, but a few comparisons are merited. First, Zancolli et
382 al. [50] used HTS methods to examine the oral microbiomes of several captive reptiles, and
383 found that, at the family level, Weeksellaceea was highly abundant in two turtles (*Trachemyes*
384 *scripta scripta*), a boa (*Acrantophis dumerili*), and a gecko (*Eublepharis macularius*), but was
385 rare in four pythons (*Python regius*). In contrast, Flavobacteriaceae was abundant in all four
386 pythons, but not in the other species. This is interesting, because we found that Weeksellaceae
387 and Flavobacteriaceae were abundant in the oral microbiomes of *E. m. krefftii*, but their

388 abundances alternated, with only one family being abundant in any individual. It is possible that
389 some form of competitive exclusion exists between these families in reptiles generally, but the
390 current data are too limited to draw that conclusion.

391 The high abundance of Flavobacteriaceae in some of our samples could also have
392 implications for disease ecology. Many members of Flavobacteriaceae are common in aquatic
393 environments, including living in high abundances on fishes' skin and gills [84], but several of
394 them are pathogens [93]. Indeed, one well-known fish pathogen (*Flavobacterium columnare*)
395 was present in one sample, where it comprised 1.9% of reads [94, 95], and 18 additional ASVs
396 were identified as the genus *Flavobacterium*, but the species could not be determined. To our
397 knowledge, *F. columnare* has not been documented to cause disease in turtles, nor did any of our
398 turtles show clinical signs of disease. It is possible that *F. columnare* (and the other
399 Flavobacteriaceae species) represent recent dietary acquisition, rather than being part of the
400 normal oral microbiome. Turtles are often opportunists and scavengers, serving as the vultures of
401 the aquatic world [58, 96]. This would provide an easy, although admittedly speculative, route
402 for a pathogen to pass from a dead or sick fish to a turtle.

403 Beyond Weeksellaceae and Flavobacteriaceae, there are several other interesting points
404 of comparison between our results and those of Zancolli et al. [50]. For example, Zancolli et al.
405 [50] found a high relative abundance of the family Chitinophagaceae in the buccal microbiomes
406 of all four pythons and the gecko, but only a low abundance in the two turtles and the boa. We
407 also found a low abundance of Chitinophagaceae in all body areas of our turtles (mean = 4.5% of
408 reads, SD = 3.3). Conversely, they reported high relative abundances of Cytopyhagaceae and
409 Moraxelleceae, whereas we did not find any Cytopyhagaceae in our buccal samples, and both
410 families were very rare in all body areas (mean = 0.03% and 0.19% of reads, respectively, SD =

411 0.04 and 0.25). Additionally, the family Deinococcaceae was abundant in our buccal samples
412 (mean = 8.9% of reads, SD = 6.4), but was rare or absent in Zancolli et al. [50].
413 Our results differed strongly from the results of a HTS study on the buccal cavities of
414 Bolson tortoises (*Gopherus flavomarginatus*)[42]. Both García-De la Peña et al. [42] and our
415 study found high levels of Proteobacteria, but levels were higher in García-De la Peña et al. [42]
416 (mean = 59%) than in our study (mean [for buccal samples] = 37.3% of reads, SD = 7.0).
417 Further, García-De la Peña et al. [42] reported moderate levels of Actinobacteria (15%) and
418 Firmicutes (10%), both of which were rare in our buccal samples (mean = 3.1% and 3.4% of
419 reads, respectively, SD = 2.4 and 3.1). Additionally, Bacteroidetes dominated our buccal samples
420 (mean = 40.2% of reads, SD = 11.0), but were not abundant in García-De la Peña et al. [42]
421 (mean = 7%). At the family level, García-De la Peña et al. [42] reported moderate to high levels
422 of Pasteurellaceae (30%), Moraxellaceae (11%), Micrococcaceae (9%), and Rhodobacteraceae
423 (8%). In contrast, we did not find any Pasteurellaceae or Micrococcaceae on any body area, and
424 Rhodobacteraceae (mean = 0.5% of reads, SD = 0.6) and Moraxellaceae (mean = 0.19% of
425 reads, SD = 0.25) were rare on all body areas. Additionally, our buccal samples contained high
426 levels of Burkholderiaceae and Weeksellaceae, which were absent or rare in García-De la Peña
427 et al. [42]. Nevertheless, both García-De la Peña et al. [42] and our study found that some
428 individuals had high levels of Flavobacteriaceae. Several factors likely contributed to the large
429 differences between these studies. First, *G. flavomarginatus* is a terrestrial, desert species,
430 whereas *E. m. krefftii* is an aquatic species. Further, *G. flavomarginatus* is an herbivore, whereas
431 *E. m. krefftii* is an omnivore [97, 98]. These differences highlight the importance of studying
432 turtles from multiple taxonomic and ecological guilds, rather than limiting research to a small
433 subset of species and niches.

It is also worth comparing our results to the results from Ferronato et al. [49]. They used culturing methods to identify bacteria from 17 genera in the buccal cavities of *Phrynops geoffroanus*, a South American turtle in the same family as *E. m. krefftii.* Culturing methods cannot detect the same range of bacteria that can be identified with sequencing methods, nor can they estimate relative abundance. Despite these limitations, there are some noteworthy comparisons between Ferronato et al. [49] and our study. For example, Ferronato et al. [49] identified 12 genera that were not present in our samples, including *Staphylococcus* (seven species), *Escherichia* (four species), and *Klebsiella* (four species; see Supporting Data). Several of these are potential pathogens. Only five genera were documented in both studies (*Acinetobacter*, *Aeromonas*, *Bacillus*, *Enterococcus*, and *Plesiomonas*). The reason that most of the genera documented in Ferronato et al. [49] were not documented in our study is unclear, especially given that our methods should have had increased detection power, and both studies looked at oral swabs from turtles in the same family. Two possibilities stem from the fact that Ferronato et al. [49] conducted research in disturbed habitats and the fact that many of their turtles were injured. Both habitat disturbance and injuries might allow opportunistic colonization, and this is a topic that should be studied.

Comparisons to additional turtle microbiome studies are constrained by the fact that most studies have examined gut or cloacal microbiomes, as opposed to oral and external microbiomes. Nevertheless, it is worth mentioning that Proteobacteria and Bacteriodetes were generally the most common phyla in our samples, and they have been reported among the most common phyla (often the most common phyla) in most turtle microbiome studies, despite differences in turtle taxonomic groups and body areas being studied [26, 27, 45, 50–52, 99, 28, 30–33, 42–44]. This is unsurprising, given that they are diverse and common phyla that occupy a wide range of

environments and have diverse ecological roles [100, 101]. The phylum Firmicutes was also among the most common phyla in most turtle studies [26–28, 30, 34, 42, 44, 45, 51] often achieving the highest relative abundance of any phylum [31–33, 43, 52], but it had a fairly low abundance in our study. Firmicutes is well-known for its important roles in digestion and is often the most common phylum in the guts of reptiles, birds, and mammals [102–104]. Its low abundance in our study is likely due to the fact that we did not examine gut or cloacal microbiomes, which are generally the areas where it dominates.

Conclusion

This study is among the first to document the microbiomes of wild freshwater turtles, and the first to use HTS methods to document either the external microbiomes of turtles or the microbiomes of turtles in the suborder Pleurodira. We found different microbiomes on each part of the turtles that we sampled, suggesting that different body areas are selecting for different microbiota, rather than simply representing the microbes in the environment. These differences may result from important ecological interactions that are key for understanding turtles' roles in their environments and designing appropriate captive husbandry plans. This is a largely neglected topic that is worth further study.

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Author Statements

All authors affirm that they have no conflicts of interest to declare.

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Ethical approval

This work was conducted with the approval of the James Cook University Ethics Committee (approval #A2344) and under a Queensland Department of Environment and Science research permit (#WISP13270413).

Supporting Material and Data Files

Supporting Material 1.pdf = Additional figures and tables of results

Supporting Material 2.xlsx = Original ASV table, decontaminated ASV table used in analyses, microDecon results, and full output of statistical tests.

The scripts used for bioinformatics and statistics are available at https://github.com/R-Huerlimann/MouseKD_analysis

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Figure Legends

Fig. 1. Bacterial phyla from each body area (mean percent of reads). All phyla are shown (the order in which they are listed is based on mean percent of reads from all samples). Algae = parts of the shell with algae, Shell = parts of the shell without algae.

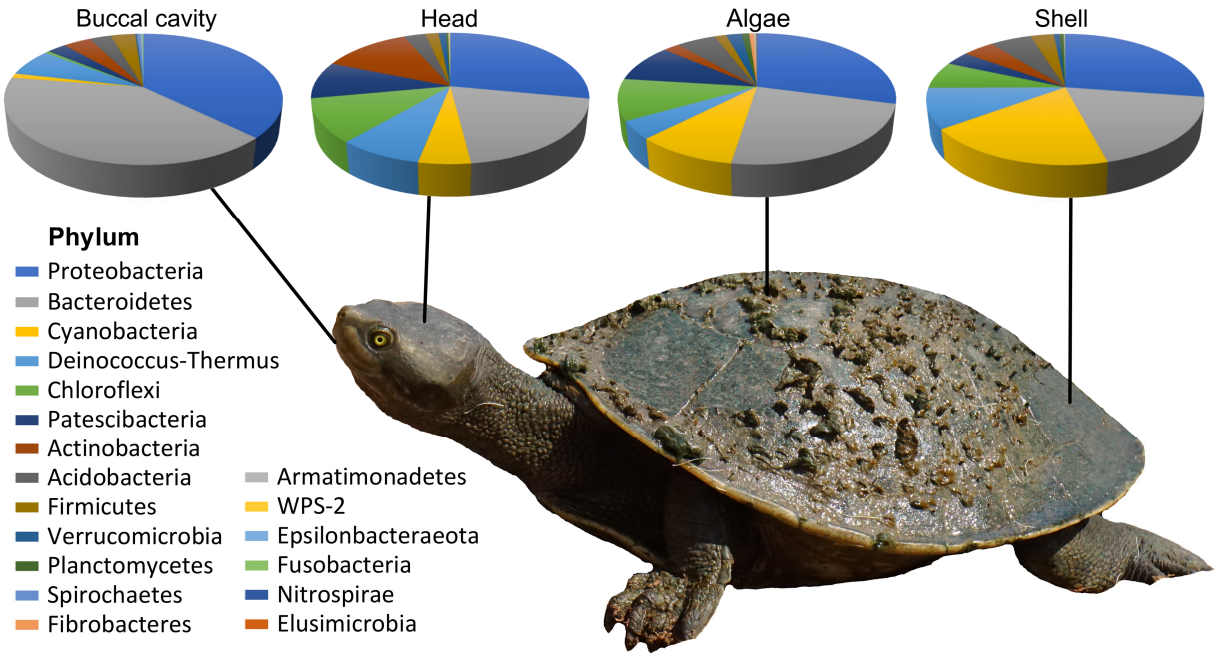


Fig. 2. Percent of reads (community) for each sample. Data are shown for the 20 most abundant classes, orders, and families (all other bacteria are lumped into the "Other" categories). Algae = parts of the shell with algae, Shell = parts of the shell without algae.

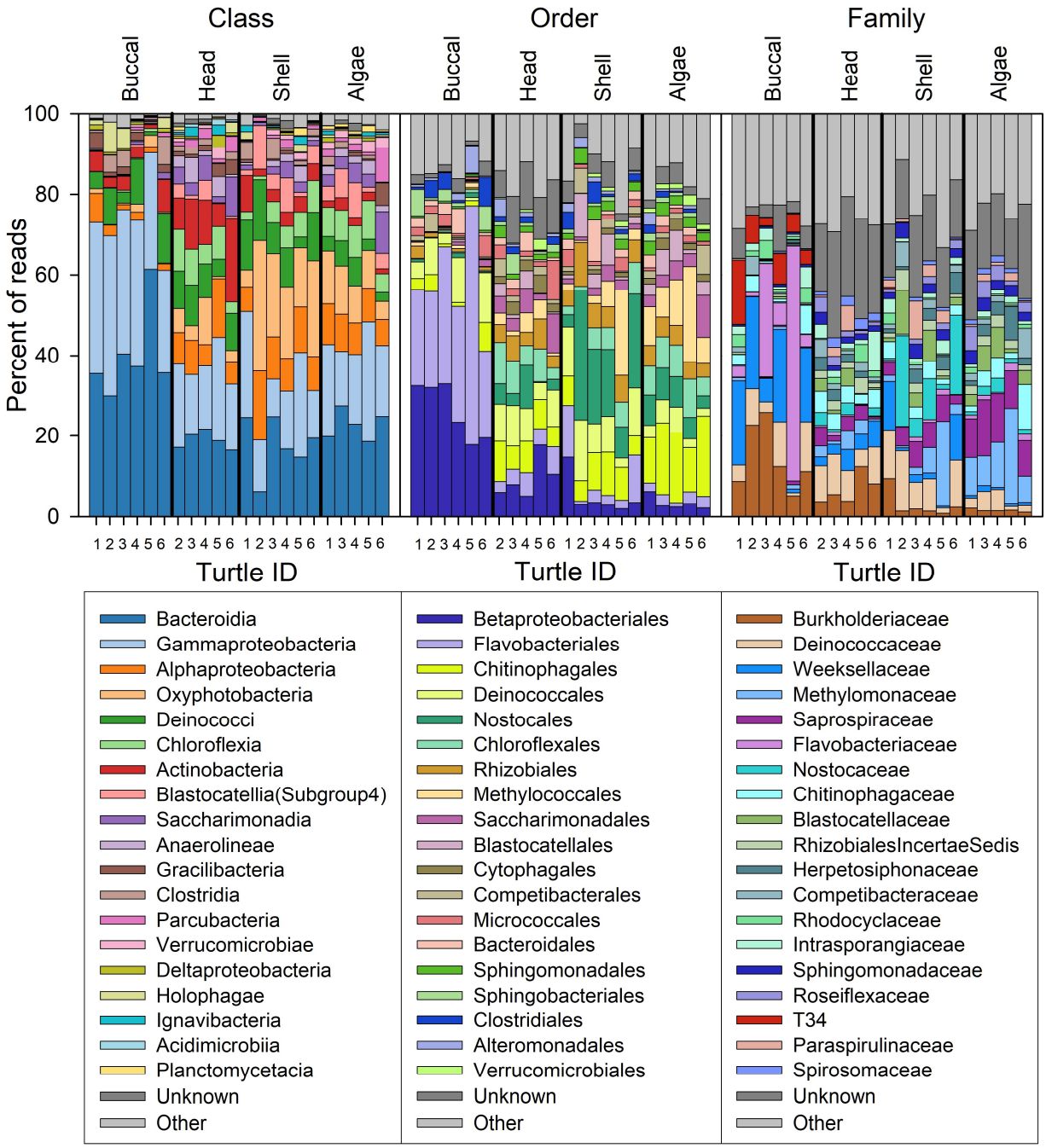
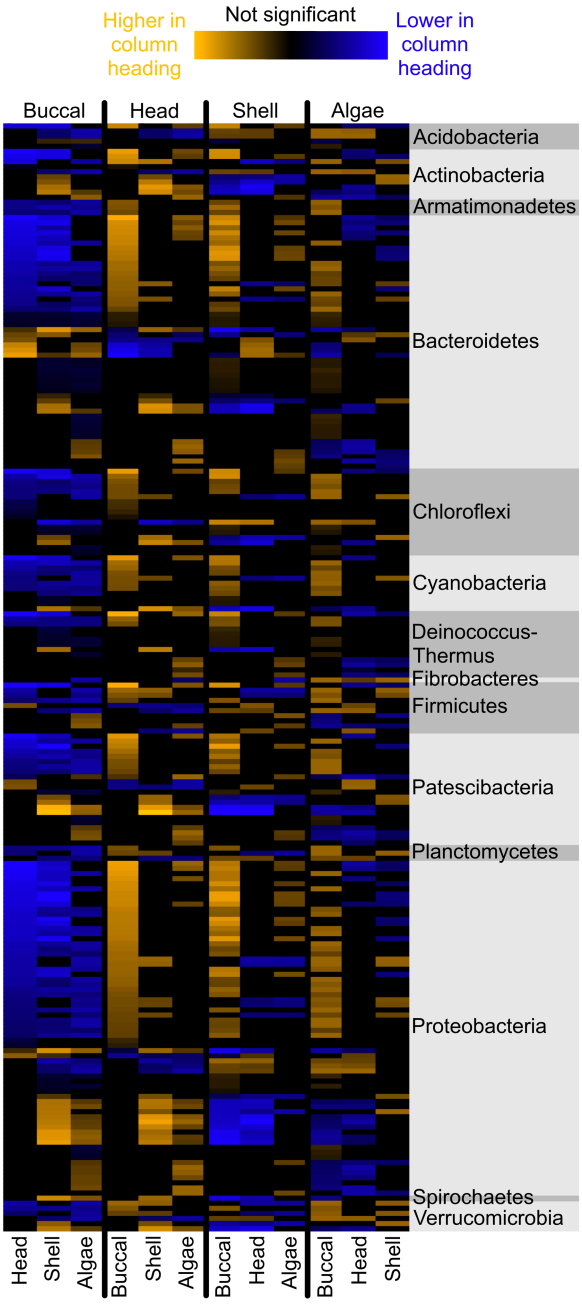


Fig. 3. DESeq2 results for ASVs that were differentially abundant between two groups. Yellow ASVs were significantly more abundant in the group in the column heading than the group in the column footer, whereas blue values were significantly lower in the column heading than in the column footer. Color intensity indicates strength of significance. Non-significant values are black. Data are grouped by phylum. Full taxonomic information is available in the Supporting Data. Algae = parts of the shell with algae, Shell = parts of the shell without algae.


Higher in column heading
Not significant
Lower in column heading
Buccal
Head
Shell
Algae
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Chloroflexi
Cyanobacteria
Deinococcus-Thermus
Fibrobacteres
Firmicutes
Patescibacteria
Planctomycetes
Proteobacteria
Spirochaetes
Verrucomicrobia
Head
Shell
Algae
Buccal
Shell
Algae
Buccal
Head
Algae
Buccal
Head
Shell

793

794

Fig. 4. Venn diagram of ASVs for each combination of body areas. One individual was removed from the head group due to an abnormally high number of reads, resulting in unique ASVs that were not corrected, even after rarefying. Data including that individual are presented in the Supporting Data. Data in this figure were not rarefied, but rarefied data are available in the Supporting Data. Algae = parts of the shell with algae, Shell = parts of the shell without algae.

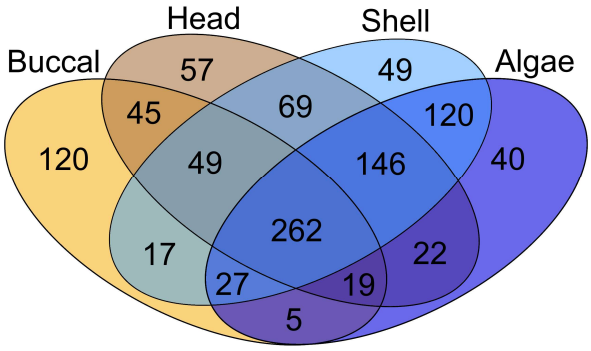


Fig. 5. PCoA comparing body areas (based on Bray-Curtis dissimilarities following normalization to proportions). All body areas were significantly different. Each shape represents a different individual (circles are the male, all other shapes are from females). Shaded polygons are simply a visual aid and do not represent confidence intervals or other statistical parameters. Algae = parts of the shell with algae, Shell = parts of the shell without algae.