

1 *Short title:* Bacterial diversity in feces of wild birds from the Pacific Northwest

2

3 RESEARCH ARTICLE

4 **Bacterial Diversity in Feces of Wild Bald Eagles, Turkey Vultures**
5 **and Common Ravens from the Pacific Northwest Coast, U.S.A.**

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31 **Abstract**

32 Birds harbor diverse microorganisms in their guts, which collectively fulfill important
33 roles in providing their hosts with nutrition and protection from pathogens. Although numerous
34 studies have investigated the presence of certain pathogenic bacteria in the feces of wild birds,
35 only a few have attempted to investigate the microbiota of the gut. This study analyzed the avian
36 bacteria present in the cloaca of avian scavengers captured on coastal beaches of Washington and
37 Oregon between 2013 and 2015: 10 turkey vultures (*Cathartes aura*), 9 bald eagles (*Haliaeetus*
38 *leucocephalus*), and 2 common ravens (*Corvus corax*). We used illumina sequencing based on
39 the V4 region of the 16s gene was to characterize the bacterial diversity. Our investigation
40 revealed phylum-level differences in the microbiome of turkey vultures, compared with bald
41 eagles and common ravens. Substantial microbiome differences were found between bald eagles
42 and ravens below the phylum level. Although little is known about the possible relations among
43 these microorganisms, our analyses provides the first integrated look at the composition of the
44 avian microbiota and serves as a foundation for future studies in this area.

45

46 *Keywords:* avian scavenger, turkey vulture, bald eagle, common raven, bacteria, coast,
47 microbiota, microbiome, raptor.

48 *Abbreviations:* OTU = operational taxonomic unit

49

50

51 **Introduction**

52 Birds harbor diverse microorganisms in their gut, which collectively fulfill important
53 roles in providing their hosts with nutrition and protection from pathogens [1]. Gut
54 microorganisms have been isolated and characterized by a variety of methods, such as culture-
55 based assays, culture-independent DNA sequencing, and fluorescence *in situ* hybridization
56 targeting the 16S rRNA gene [2]. Most of these studies are limited in scope and are focused on
57 pathogenic bacteria such as *Salmonella*, *Escherichia coli*, *Campylobacter*, and *Clostridium*
58 *perfringens* [3-5].

59 In recent years, metagenomics using next-generation sequencing has emerged as a way to
60 analyze complex microbial communities and their functions [6]. This approach is capable of
61 sequencing thousands or millions of amplified DNA molecules in a single run and, for bacteria,
62 does not require conventional cloning and amplification. Additionally, metagenomic studies
63 offer a powerful tool for the comprehensive and unbiased assessment of microbial diversity
64 within the complex gut ecosystem by allowing examination of organisms not easily cultured in
65 the laboratory [6, 7].

66 Few studies have used genetic sequencing to explore the intestinal microbiota of wild
67 birds [8]. Furthermore, these studies reported gene sequences to phylum or class levels only,
68 with no attempt to understand extant microorganism populations at the species level. In this
69 study, we analyze the avian microbiome present in the cloaca of bald eagles, turkey vultures, and
70 common ravens collected in coastal Washington and Oregon, U.S.A. Because research has
71 demonstrated that intestinal microorganisms can influence the lifecycle and reproduction of their
72 hosts [9], data presented in this paper can be important for a better understanding of the health

73 and nutrition of these apex scavengers that comprise key components of the food web of the
74 Pacific Northwest coast.

75

76 **Materials and methods**

77 We captured avian scavengers in coastal Washington and Oregon with a carcass-baited
78 net launcher [10]. We captured turkey vultures and common ravens from May through August
79 and bald eagles from February through June. Once in hand, we marked birds for identification
80 with visual identification leg bands (eagles and ravens) or patagial tags (vultures) [11].

81 *Aging.* We assigned captured birds to two age classes, adult and immature. We classified
82 bald eagles as adults (≥ 4 years old) with attainment of fully white head and tail plumage, yellow
83 beak and yellow iris [12]. We classified turkey vultures as adults (≥ 2 years old) with attainment
84 of a bright ivory bill [13]. Common ravens were ranked as adults (≥ 1 years old) by having
85 glossy black wing and tail feathers and as immatures by having dull black or brownish wing and
86 tail feathers [14].

87 *Sexing.* When possible, we determined the sex of individuals using measurements. We
88 sexed bald eagles using bill depth and hallux length measurements [15] and common ravens
89 using foot pad length and body mass [16]. Using this approach, the sex of one raven captured
90 was equivocal. In this case, we classified the individual as female because we observed it with
91 another raven that we assumed was male based on its demonstrably larger size [17]. We did not
92 sex captured turkey vultures because it is not possible to differentiate gender using plumage
93 characteristics or measurements [13].

94 *Fecal sampling.* We extracted up to 2 ml of fecal material directly from the cloaca of
95 each bird using a disposable 3 ml pipette. In the field, we transferred fecal samples to cryovials

96 and then placed them in a pre-frozen Golden Hour Thermal Container (www.credothermal.com).
97 With no more than 8 hours of elapsed time, samples were stored at -20°C for up to one month
98 and then at -70°C until processed in 2017.

99 Genetic sequencing of all 21 cloaca flora samples was conducted by MR DNA,
100 Molecular Research, LP (www.MrDNAlab.com; Shallowater, Texas). In summary, the 16S
101 rRNA gene V4 variable region PCR primers 515/806 were used in a single-step 30 cycle PCR
102 using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C
103 for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1
104 minute, after which a final, 5-minute elongation step at 72°C was performed on a MiSeq system
105 (Illumina Inc, San Diego, California). After amplification, PCR products were checked in 2%
106 agarose gel to determine the success of amplification and the relative intensity of bands. Samples
107 were pooled and purified using calibrated Ampure XP beads (Beckman Coulter, Life Sciences,
108 Indianapolis, Indiana). This purified PCR product was then used to prepare an Illumina DNA library
109 (<https://www.illumina.com/techniques/sequencing/ngs-library-prep.html>).

110 Sequence paired ends were processed using MR DNA taxonomic analysis pipeline
111 (www.MrDNAlab.com). In summary, the process included depletion of barcodes and primers
112 from genetic sequences, paired sequences were merged, followed by removal of sequences
113 <150bp, and then removal of sequences with ambiguous base calls and homopolymer runs
114 exceeding 6bp. Following these steps, operational taxonomic units (OTUs) were generated at
115 97% homology and chimeras were removed. Final OTUs were taxonomically classified using
116 BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against a database derived from Ribosomal
117 Database Project II (<http://rdp.cme.msu.edu>) and the National Center for Biotechnical
118 Information (www.ncbi.nlm.nih.gov). When a given sequence matched < 77% of a reference
119 sequence, no taxonomic identity was assigned. To designate a sequence in a phylum the

120 sequence was at least 77% identical to the reference sequence, for the class >80%, for the order >
121 85%, > 90% for the family, > 95% for the genus, and for the species >97%. We acknowledge
122 that use of the V4 region of the 16s does not give more than a tentative evaluation of the lower
123 phylogenetic levels (e.g. genus and species), yet we feel that some discussion of the findings in
124 terms of genus and species provided interesting insight as part of this unique survey. Normalized
125 to 20,000 sequences per sample, Alpha and Beta diversity analyses were completed using the
126 Qiime2 [18] microbiome analysis pipeline (<https://qiime2.org/>).

127

128 **Results**

129 We captured and sampled 10 turkey vultures, 9 bald eagles and 2 common ravens.
130 Except for one turkey vulture captured on an Oregon coastal beach, all individuals were captured
131 on southwest Washington coastal beaches or adjacent to Grays Harbor (Fig 1). Eight of the
132 turkey vultures were adults and two were immatures. Three of the bald eagles were adults and
133 six were immature; six were male and four were female. One common raven was an adult female
134 and the other was an immature male. We collected one fecal sample from each individual for a
135 total of 21 samples.

136

137 **Fig 1. Avian scavenger sampling sites.**

138 Locations where fecal samples were collected from turkey vultures, bald eagles and common
139 ravens in coastal Washington and Oregon. Numbers next to squares show the number of avian
140 scavengers trapped at that location.

141

142 Genetic sequencing resulted in 1,905,510 sequences from the 21 samples we collected.
143 Bacterial OTUs comprised 99.7% of the sequences found; 0.006% were Archaea. The other
144 0.25% constituted sparse cross-reactions of the primers with eukaryotes [19].

145 The total number of bacterial taxonomic OTUs detected among the 21 samples was 1065.
146 Turkey vultures presented the largest diversity of bacterial fecal microbiome (1022 OTUs; $n =$
147 10), followed by common ravens (454 OTUs; $n = 2$), and bald eagles (604 OTUs; $n = 9$). In
148 turkey vultures, 183 bacteria genera were identified, 141 in bald eagles, and 131 in common
149 ravens (Table 1). Rare faction curves of observed OTUs and Shannon diversity indices were
150 generated based on a rarefaction level of 20,000 sequences per sample (Fig 2). Non-parametric
151 pairwise comparisons made between turkey vultures and bald eagles (Fig 3) revealed significant
152 differences in the number of observed OTUs ($p < 0.001$) as well as Shannon diversity indices
153 between the two species ($p < 0.001$). The alpha diversity of turkey vultures was found to be
154 significantly greater than the alpha diversity of bald eagles with regards to both measured alpha
155 diversity metrics. Corresponding with alpha diversity analyses, based on ANOSIM pairwise
156 comparisons (S1 Table), beta diversity between the turkey vultures and both the bald eagles as
157 well as the common raven is shown. Beta diversity comparisons were based on the weighted
158 unifrac distance matrix values which can be better visualized via principal coordinate analysis
159 (PCoA) plot (Fig 4).

160

161 **Table 1. Number of bacteria taxonomic groups.**

Avian Species	Bacteria Taxonomy				
	Phylum	Class	Order	Family	Tentative Genus
Turkey vulture	13	23	49	94	183
Bald eagle	12	22	43	78	141
Common raven	12	19	39	76	131

162 Number of bacteria taxonomic groups identified in fecal samples collected from turkey vultures
163 (n = 10), bald eagles (n = 9) and common ravens (n = 2) captured on coastal beaches in
164 Washington and Oregon.

165

166 **Fig 2. OTU Rarefaction and Shannon Diversity Index Curve.**

167 16s rRNA sequence data was rarefied to 20,000 sequences per sample. (A) OTUs and (B)
168 Shannon diversity indices were plotted for three avian species; Bald Eagle (n=9), Common
169 Raven (n=2), and Turkey Vulture (n=10). OTUs were clustered at 97% identity.

170

171 **Fig 3. Kruskal-Wallis Pairwise Results (Observed OTUs).**

172 Significant differences present in alpha diversity metrics: (A) Observed OTUs and (B) Shannon
173 diversity indices; were identified using the non-parametric Kruskal-Wallis H Test and
174 differences between groups identified using multiple pairwise comparisons. Comparisons
175 involving the Common Raven were not reported due to limited sample size (n=2).

176

177 **Fig 4. Weighted UniFrac PCoA.**

178 Principal coordinate plot of weighted UniFrac [18] data with colors keyed on the three avian
179 species sampled. Bald Eagle (n=9; Red), Common Raven (n=2; blue), and Turkey Vulture
180 (n=10; orange). The phylogenetic assemblage of the Turkey Vultures is clearly distinct from the
181 remaining two sampled species.

182

183 The proportions of bacterial OTUs varied by phylum for each bird in the study (Fig 5).

184 Thirteen phyla were represented among bacteria detected in the fecal samples (Fig 5, S1 Figure,

185 and S2 Table). Overall, more than 80% of the bacterial sequences detected belonged to two
186 phyla, Firmicutes and Proteobacteria. In turkey vultures, the two most common phyla were
187 Firmicutes and Fusobacteria and in bald eagles and common ravens Firmicutes and
188 Proteobacteria were most common.

189

190 **Fig 5. Mean proportions of bacterial operational taxonomic units by phylum.**

191 Mean proportions of bacterial operational taxonomic units by phylum detected in fecal samples
192 collected from turkey vultures (n = 10) bald eagles (n = 9), and common ravens (n = 2) captured
193 on coastal beaches in Washington and Oregon. Proportions < 1% are not shown.

194

195 Twenty-three classes of bacteria were represented in the fecal samples. In turkey vultures,
196 class Clostridia contained the highest proportions of OTUs by individual, while in bald eagles
197 class Betaprotobacteria held this distinction (Fig 6). The two common raven fecal samples also
198 included OTUs in class Betaproteobacteria, with a larger proportion of OTUs in the sample from
199 the adult.

200

201 **Fig 6. Proportions of bacterial operational taxonomic units by class.**

202 Proportions of bacterial operational taxonomic units by class detected in fecal samples from
203 turkey vultures (n=10), bald eagles (n = 9) and common ravens (n = 2) captured on coastal
204 beaches in Washington and Oregon. Letters below columns are the visual identification codes on
205 bands (eagles and ravens) or wing-tags (vultures) applied to birds at capture. Proportions < 1%
206 are not shown.

207

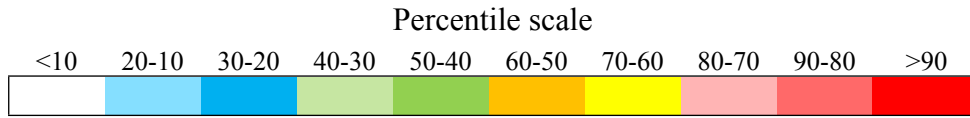
208 A total of 188 tentative bacterial genera were represented in the feces of the three avian
209 species. Of the 183 different genera detected in the 10 turkey vulture samples, 18 genera
210 accounted for 90% of the bacterial sequences, with two genera, *Clostridium* and *Fusobacterium*,
211 comprising 48% of the total on average (Table 2). A total of 141 genera were detected in the nine
212 bald eagle samples and 14 of these genera represented 90% of all sequences detected. The two
213 most common, *Burkholderia* and *Pseudomonas*, comprised 36% of the total on average (Table
214 2). While only two common ravens were sampled, 131 bacterial genera were detected with 9 of
215 these comprising 90% of the OTUs (Table 2). Based on hierarchal clustering of the top 35
216 genera detected amongst the three species, there is a clear distinction between the fecal
217 microbiome of turkey vultures and that of the bald eagle (Fig 4).

218

219

220 **Table 2. Proportions of bacterial operational taxonomic units by genus.**

221



222

223

A) Turkey Vulture

Genus	Individual Patagial Tag ID Codes										Average
	CA	AR	HE	AY	BV	AV	AL	BY	AK	AX	
Enterococcus	0.16	0.03	15.08	0.05	0.06	0.55	0.16	0.1	0.03	0.03	1.63
Vagococcus	0.04	0.03	9.72	0.04	0.03	0.2	0.14	0.58	0.02	0.07	1.09
Lactobacillus	1.01	0.13	1.41	2.36	0.12	7.28	18.01	1.34	0.08	12.4	4.42
Clostridium	22.69	4.25	34.99	18.06	9.86	49.1	50.35	28.14	66.49	33.31	31.66
Peptostreptococcus	7.31	5.94	0.48	8.47	16.18	18.03	2.45	4.86	1.55	1.3	6.66
Ruminococcus	5.34	34.66	0.06	0.44	0.08	0.06	0.1	12.09	0.07	2.68	5.56
Anaerovorax	0.42	8.4	0.02	0.41	1.37	0.04	0.27	0.45	0.14	1.33	1.28
Eubacterium	3.12	3.01	0.19	3.43	0.08	5.34	0.4	3.32	0.61	0.11	1.96
Cellulosilyticum	1.99	0.05	0.14	0.53	0.04	0.14	0.33	0.06	8.46	2.94	1.47
Peptoclostridium	0.42	0.74	1.5	1.58	0.53	0.93	14.42	1.04	0.42	0.18	2.14
Holdemania	0.06	7.72	0.02	1.92	0.01	0.02	0.02	0.01	0.32	0.01	1.01
Peptoniphilus	1	8.99	0.09	1.65	9.97	0.69	0.22	18.07	0.11	0.05	4.08
Atopobium	3.74	6.71	0.07	10.67	0.04	0.12	0.32	6.94	2.84	6.46	3.79
Fusobacterium	38.98	9.96	4.04	33.3	36.35	8.08	10.68	13.8	0.19	14.2	16.96
Bradyrhizobium	0.04	0.01	0.02	0.04	1.51	0.03	0.04	0.51	0.75	6.89	0.98
Salmonella	5.03	0.64	7.46	0.56	0.1	0.14	0.19	0.09	0.3	0.59	1.51
Pseudomonas	0.13	0.1	0.32	1.43	9.87	0.08	0.09	0.09	1.19	4.67	1.8
Chlamydia	0.53	0.01	13.43	0.01	0.01	0.01	0.02	0.01	< 0.01	0.01	1.4

224

226 B) Bald Eagle

Genus	Individual Leg Band ID Codes									
	R/O	M/C	P/O	N/D	M/E	N/D	K/2	N/K	D/O	Average
Streptococcus	0.62	0.49	0.04	0.01	0.04	15.49	19.42	0.02	28.22	7.15
Clostridium	23.29	0.41	0.37	0.33	0.42	0.33	1.29	0.48	0.32	3.03
Bifidobacterium	0.01	0.03	0.02	0.01	0.01	0.02	5.52	19.87	0.01	2.83
Sphaerotilus	0.34	0.05	17.17	0.07	0.11	0.16	0.03	0.07	0.17	2.02
Bacteroides	2.23	4.39	0.01	0.01	0.00	9.21	4.23	0.02	0.00	2.23
Paraprevotella	>0.01	0.07	>0.01	>0.01	nd*	0.01	0.10	12.71	nd	1.43
Prevotella	2.13	nd	nd	>0.01	nd	nd	>0.01	nd	nd	1.58
Bradyrhizobium	0.82	0.13	0.02	0.14	0.01	5.99	0.02	5.14	0.01	1.37
Burkholderia	2.14	22.11	27.06	12.86	32.28	19.69	8.71	12.59	39.10	19.62
Delftia	6.62	1.06	11.01	0.35	41.61	14.51	0.31	8.70	4.07	9.80
Methylophilus	0.01	14.64	0.05	0.02	0.05	0.02	0.03	0.02	0.03	1.65
Salmonella	12.10	0.18	0.33	2.20	0.09	0.62	0.05	0.09	0.28	1.77
Pseudomonas	17.17	12.19	29.52	48.94	15.95	2.21	1.50	14.29	6.61	16.49
Stenotrophomonas	21.07	0.30	0.17	20.30	0.08	18.71	0.25	0.07	15.36	8.48

227 *nd = not detected

228

229

230

C) Common Raven

Genus	Individual Leg Band ID Codes		
	RSYLOG	ROGLSG	Average
Psychrobacter	0.01	12.47	6.24
Atopostipes	7.11	0.04	3.58
Catelicoccus	18.12	0.05	9.09
Enterococcus	0.21	14.53	7.37
Vagococcus	0.15	11.81	5.98
Peptoclostridium	0.15	11.81	4.45
Erysipelothrix	0.02	12.64	6.33
Afipia	9.39	1.48	5.44
Daeguia	8.85	>0.01	4.43
Burkholderia	12.02	0.36	6.19
Delftia	14.27	1.23	7.75
Stenotrophomonas	11.14	5.11	8.12

231

232 Top 90th percentiles, expressed as a percentage of total, bacterial operational taxonomic units, by

233 genus in fecal samples collected from A) turkey vultures (n = 10), B) bald eagles (n = 9), and C)

234 common ravens (n = 2) captured on coastal beaches in Washington and Oregon. Letters above

235 columns are the visual identification codes on bands (eagles and ravens) or wing-tags (vultures)
236 applied to birds at capture. Color denotes percentile category within each column.

237

238 As with genera, most of the tentatively classified bacteria species detected across all three
239 avian species we studied comprised relatively small proportions of the overall fecal bacterial
240 diversity (Table 3). Moreover, bacteria that were relatively abundant in one avian species in our
241 study were less abundant or missing altogether in the other two. In turkey vulture samples, only
242 *Fusobacterium mortiferum*, *Clostridium perfringens*, and *Clostridium ruminantium* represented
243 over 10% each of the total bacteria species population. In bald eagles, *Burkholderia ubonensis*
244 comprised more than 10% of all bacterial species and *Delftia tsuruhatensis* comprised nearly
245 10%. In contrast, no bacteria species represented $\geq 10\%$ of the total bacteria in common ravens.

246 **Table 3. Top 10th percentiles of bacterial operational taxonomic units tentatively classified to the closest species match in fecal**
 247 **samples of turkey vultures, bald eagles and common ravens.**

Turkey Vulture (n = 10)	Number of OTUs (% of total)	Bald Eagle (n = 9)	Number of OTUs (% of total)	Common Raven (n = 2)	Number of OTUs (% of total)
<i>Fusobacterium mortiferum</i>	25,100 (17.0)	<i>Burkholderia ubonensis</i>	5,870 (17.8)	<i>Catelicoccus marimammalium</i>	5,632 (9.1)
<i>Clostridium perfringens</i>	21,764 (14.7)	<i>Delftia tsuruhatensis</i>	3,229 (9.8)	<i>Delftia tsuruhatensis</i>	4,801 (7.7)
<i>Clostridium ruminantium</i>	16,684 (11.3)	<i>Stenotrophomonas spp.</i>	2,598 (7.9)	<i>Stenotrophomonas spp.</i>	4,748 (7.7)
<i>Ruminococcus spp.</i>	7,943 (5.4)	<i>Streptococcus sanguinis</i>	2,229 (6.8)	<i>Enterococcus faecalis</i>	4,476 (7.2)
<i>Peptostreptococcus spp.</i>	7,526 (5.1)	<i>Pseudomonas spp.</i>	2,212 (6.7)	<i>Erysipelothrix tonsillarum</i>	3,924 (6.3)
<i>Peptoniphilus methionivorax</i>	6,046 (4.1)	<i>Pseudomonas veronii</i>	1,706 (5.2)	<i>Burkholderia ubonensis</i>	3,493 (5.6)
<i>Atopobium minutum</i>	5,560 (3.8)	<i>Pseudomonas plecoglossicida</i>	1,240 (3.8)	<i>Afipia spp.</i>	3,369 (5.4)
<i>Peptoclostridium clostridium sordellii</i>	2,771 (1.9)	<i>Bifidobacterium thermophilum</i>	933 (2.8)	<i>Peptoclostridium clostridium sordellii</i>	2,754 (4.4)
<i>Clostridium septicum</i>	2,374 (1.6)	<i>Clostridium spp.</i>	728 (2.2)	<i>Daeguia caeni</i>	2,745 (4.4)
<i>Lactobacillus hayakitensis</i>	2,336 (1.6)	<i>Sphaerotilus spp.</i>	665 (2.0)	<i>Psychrobacter proteolyticus</i>	2,394 (3.9)
<i>Peptostreptococcus anaerobius</i>	2,323 (1.6)	<i>Salmonella enterica</i>	584 (1.8)	<i>Atopostipes spp.</i>	2,202 (3.6)
<i>Enterococcus faecalis</i>	2,299 (1.6)	<i>Burkholderia spp.</i>	575 (1.7)	<i>Vagococcus carniphilus</i>	2,133 (3.4)
<i>Salmonella enterica</i>	2,235 (1.5)	<i>Bacteroides spp.</i>	575 (1.7)	<i>Vagococcus teuberi</i>	1,534 (2.5)
<i>Lactobacillus aviarius</i>	2,178 (1.5)	<i>Methylophilus spp.</i>	544 (1.7)	<i>Psychrobacter pulmonis</i>	1,431 (2.3)
<i>Cellulosilyticum spp.</i>	2,174 (1.5)	<i>Proteiniphilum acetatigenes</i>	472 (1.4)	<i>Ignatzschineria larvae</i>	1,226 (2.0)
<i>Chlamydia chlamydophila psittaci</i>	2,071 (1.4)	<i>Afipia spp.</i>	444 (1.3)	<i>Gardnerella vaginalis</i>	974 (1.6)
<i>Anaerovorax spp.</i>	1,901 (1.3)	<i>Bradyrhizobium spp.</i>	437 (1.3)	<i>Enhydrobacter aerosaccus</i>	965 (1.6)
<i>Holdemania spp.</i>	1,495 (1.0)	<i>Methylothera spp.</i>	429 (1.3)	<i>Pelomonas aquatica</i>	893 (1.4)
<i>Bradyrhizobium spp.</i>	1,407 (1.0)	<i>Prevotella spp.</i>	423 (1.3)	<i>Pseudomonas spp.</i>	869 (1.4)
<i>Pseudomonas spp.</i>	1,140 (0.8)	<i>Sphingobacterium spp.</i>	360 (1.1)	Other ^a	
<i>Campylobacter spp.</i>	1,100 (0.7)	<i>Ralstonia spp.</i>	319 (1.0)		
<i>Burkholderia ubonensis</i>	1,065 (0.7)	<i>Cytophaga hutchinsonii</i>	315 (1.0)		
<i>Stenotrophomonas spp.</i>	929 (0.6)	<i>Tepidimonas spp.</i>	314 (1.0)		
<i>Vagococcus carniphilus</i>	859 (0.6)	<i>Paenibacillus barengoltzii</i>	246 (0.7)		

*Thioalkalivibrio
denitrificans*

241 (0.7)

Sum of top 10 Percentile

121,280 (82.3)^b

27,447 (84.0)^b

54,276 (81.5)^b

248 Top 10th percentiles of bacterial operational taxonomic units sequenced from fecal samples of bald eagles, turkey vultures and

249 common ravens captured on coastal beaches in Washington and Oregon. Sequences detected in at least two avian species are

250 identified in bold (includes two in the footnote).

251 ^aBacteria not in top 10 percentile, nevertheless comprising 1.1 - 1.3% of the total: *Acinetobacter johnsonii*, *Cosenzaea proteus*

252 *myxofaciens*, ***Salmonella enterica***, ***Clostridium spp.***, *Staphylococcus epidermidis*. Bacteria shown in bold were also found in turkey

253 vulture and bald eagle samples.

254 ^bPercent of total bacterial operational taxonomic units included in the top 10th percentile.

255 Archaea were identified in one turkey vulture sample and in two bald eagle samples. We
256 tentatively classified these as uncultured *Candidatus nitrosocaldus*. No Archaea were identified
257 in common ravens-

258

259 **Discussion**

260 Our analyses showed substantial microbiome diversity among turkey vultures, bald
261 eagles, and common ravens. These differences probably represent their diverse anatomies,
262 physiologies, and also to an extent, differences in diet. Turkey vultures, bald eagles and common
263 ravens are among the many terrestrial vertebrates that scavenge opportunistically on the Pacific
264 Northwest coast [20] and also inland from the coast. It should be noted that ceca, which act as
265 sites of anaerobic activity in some species of birds, are vestigial in all three of our study species
266 [21].

267 The authors are aware that bacteria species classification based upon V4 is tentative. The
268 classification of ribosomal RNA genes has been the gold standard for molecular taxonomic
269 research for decades, but standard primers for bacterial species identification does not exist yet
270 [22]. On the other hand, sequences of the most commonly identified bacterial genera and species
271 (those that are the 10 top percentile) in this study exist in the databases. These bacteria were not
272 environmental or novel bacteria. For this reason, we discuss not only bacteria class, order, and
273 phylum, but also genus and species.

274 Two classes, Fusobacteria and Clostridia, which are comprised exclusively of anaerobes,
275 predominated the cloacal flora in turkey vultures. Roggnbuck et al. [23] also reported these two
276 classes as most prevalent in turkey vultures. *Fusobacterium mortiferum* , which on average

277 comprised 17% of all bacterial species detected in Turkey Vulture feces, is a normal inhabitant
278 of the alimentary tract in chickens where it protects against pathogenic bacteria [24].

279 Many clostridia species are normal gut flora and play a crucial role in gut homeostasis,
280 however under stressful gut conditions (i.e. inflammation, parasitism, etc) these bacteria may
281 release toxins that can cause serious diseases in humans and other animals, including birds [25,
282 26]. *Clostridium perfringens* and *C. ruminantium* accounted for 26% of the bacterial species we
283 detected in turkey vulture feces in our study. *C. perfringens* is known to be a normal component
284 of intestinal flora, but can cause necrotic enteritis in birds when intestinal dysbiosis occurs [27].
285 *C. ruminantium* is reported to contribute to rumen fermentation in cattle and it is also a food-
286 spoiling bacterium, but it has not been directly linked to animal diseases [28].

287 Bacterial toxins can also be present in carrion. Turkey vultures have a remarkable
288 tolerance for some of these toxins, such as botulinum [29, 30] and anthrax [23, 31]. Only 0.3% of
289 sequences assembled from turkey vultures feces were identified as *C. botulinum* and no *Bacillus*
290 *anthracis* was detected in these birds. Neither of the aforementioned species was present in bald
291 eagles or common raven samples.

292 Most *Enterococcus* (phylum Firmicutes) are commensal bacteria; however there are a
293 few species in this genus, including *E. faecalis*, that are associated with significant mortality in
294 humans and other animals [32, 33]. *Enterococcus faecalis* comprised 7.2 % of the bacterial
295 OTUs in the two common ravens we sampled and 1.6% in the turkey vultures. While this
296 bacterium was detected in all nine bald eagle fecal samples, they comprised < 0.1% of the
297 assembled sequences for that species. In Illinois, 25 raptors were tested for *Enterococcus* due to
298 their potential to develop resistance to antibiotics; 53 of 56 cloacal samples collected contained
299 *E. faecalis* [34]. *Streptococcus sanguinis* (phylum Firmicutes) comprised nearly 7% of all

300 bacteria in bald eagles but $\leq 0.3\%$ in turkey vultures and common ravens, Although these
301 bacteria are considered normal inhabitants of mammals and humans [35], there is no information
302 about the commonality of these bacteria or their significance in the digestive tracts of avian
303 species. *Erysipelothrix tonsillarum* comprised 6% of the OTUs in the two common raven fecal
304 samples and $< 0.1\%$ in turkey vultures and bald eagles. These non-pathogenic bacteria are
305 commonly isolated from healthy swine and poultry as well as poultry litter. *E. tonsillarum* is
306 closely related to the pathogenic species *E. rhusiopathae*, causative agent of erysipeloid in
307 mammals and erysipelas in poultry [36, 37].

308 Almost 70% of fecal bacteria in the bald eagles we sampled (n = 9) and 55% in the two
309 common ravens belonged to the phylum Proteobacteria. In contrast, Proteobacteria accounted for
310 less than 10% of the turkey vulture microbiome (n = 10). In bald eagles, the most common
311 species belonging to this phylum were tentatively classified as *Burkholderia ubonensis* and
312 *Delftia tsuruhatensis*, comprising 18% and 10% of all fecal bacteria detected respectively. Both
313 are common environmental bacteria that have been occasionally linked to infections in humans
314 [38, 39]. *B. ubonensis* was also detected in the two common ravens sampled, ranking seventh
315 most common and averaging 5.6%. *Catelicoccus marimammalium* was the most common
316 bacteria in the common ravens sampled, averaging 9% of the total. *C. marimammalium* was the
317 most common species in the feces of gulls in Wisconsin, U.S.A. [40].

318 Other notable bacteria detected from the Proteobacteria phylum included bacteria in
319 *Escherichia*, *Campylobacter*, *Pseudomonas*, and *Salmonella*. *E. coli* inhabits the large intestine
320 and distal ileum of most vertebrate species, including birds; the organism is shed in the feces.
321 Generally-speaking, the vast majority of the strains are components of normal intestinal flora and
322 are non-pathogenic. Some strains are of low virulence and may cause opportunistic infections. A

323 few are highly pathogenic and may cause septicemia. *E. coli* sequences were detected in the
324 cloacal samples from eight of the ten turkey vultures, eight of the nine bald eagles, and both
325 ravens. However, *E. coli* sequences were sparse and, on average, accounted for less than 0.01%
326 of all the bacterial OTUs per bird. No further analysis was done on these *E. coli* sequences to
327 determine if they had any pathogenic genes or not. *Campylobacter* sp. were detected in all the
328 fecal samples from turkey vultures and accounted for 0.7% of the bacterial OTUs (Table 3). In
329 bald eagles and common ravens, *Campylobacter* sp. were also detected in all samples, but the
330 OTU frequency was less than 0.01%. Members of *Pseudomonas* are ubiquitous and can be
331 opportunistic pathogens [41]. Bacteria from the *Pseudomonas* genus were detected in all fecal
332 samples. They constituted 17% of the fecal flora in bald eagles, but < 2% of all bacterial OTUs
333 in turkey vultures and common ravens.

334 Avian predators may become infected with *Salmonella* when they consume infected
335 animals [42]. *S. enterica* colonize the intestinal tract and have been isolated from wild birds with
336 or without clinical signs of disease [43]. In our study, *S. enterica* comprised 1-2% of bacterial
337 species detected in all three avian scavengers. In other research, *S. enterica* sampled from the
338 feces of scavenging birds ranged from 1% to 20% by isolation [44-46]. Differences in prevalence
339 between isolation and detection by meta-analysis may be due to the stability of the DNA
340 molecule that can be amplified even when the target organism is no longer viable. *S. enterica*
341 sequence was performed only to the species level, and subspecies or serotype were not
342 determined.

343 Archaea are single-celled prokaryotes that are difficult to culture [47]. They have been
344 found in the digestive tracts of some animals where they produce methane [48]; the role these
345 organisms play in the gut of birds is still unknown [1]. *Candidatus nitrosocaldus* is ubiquitous in

346 water environments being present in plankton, sediments, and surrounding soils of lakes and
347 oceans [49]. It has a particular affinity for hot spring environments. It is also common in waste
348 water treatment plants and fertilized soils. Its presence in the feces of two bald eagles and one
349 turkey vulture in our study may be a reflection of their contact with marine carrion.

350 In summary, this is the first study utilizing metagenomics to investigate the fecal
351 microbiome to the species level. This is also the first meta-analysis performed in bald eagles and
352 common ravens. We demonstrated that the fecal flora varies among the three scavenger species
353 even though they live in the same region and consume some of the same foods (D. Varland,
354 unpub. data). We discovered that over 50% of the microbiota in the cloaca belongs to a single
355 phylum in all three species, Proteobacteria for bald eagles and common ravens and Firmicutes
356 for turkey vultures. As expected numerous species of microorganisms were tentatively identified;
357 however, only about 10% of these tentative species contained > 1% of the OTUs. Finally, this
358 method for identification and characterization of microorganism populations can be used in
359 epidemiological, pathogen detection, and microbial diversity studies.

360

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367 and under state Scientific Collection Permits 13-014, 14-084 and 15-093 in Washington and
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369

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506

507 **Supporting information**

508 **S1 Figure. Proportions of bacterial operational taxonomic units by phylum.** Proportions of
509 bacterial operational taxonomic units by phylum detected in fecal samples from turkey vultures
510 (n = 10), bald eagles (n = 9), and common ravens (n = 2) captured in coastal Washington and
511 Oregon. Letters below columns are the visual identification codes on bands (eagles and ravens)
512 or wing-tags (vultures) applied to birds at capture. Proportions < 1% are not shown.

513

514 **S2 Figure. Hierarchal Clustering of Taxonomic Data.** Evaluation of the taxonomic
515 classification data using a dual hierarchal dendrogram. Each sample is clustered on the X-axis
516 labeled based on the sampled species. Samples with more similar microbial populations are
517 mathematically clustered closer together. The heatmap represents the relative percentages of
518 each genus. The predominant genera are represented along the right Y-axis. The legend for the
519 heatmap is provided in the upper left corner. The microbial population of Turkey Vultures
520 (n=10) is distinct from the population identified in the Bald Eagle (n=9).

521 **S1 Table. Pairwise ANOSIM Results (Weighted UniFrac)**

522 Significant differences present in the weighted UniFrac distance matrix values were identified
523 using the Analysis of Similarities (ANOSIM) Test and differences between groups identified
524 using multiple pairwise comparisons over 999 permutations.

Group 1	Group 2	Sample size	R ^a	p-value	q-value
BALDEAGLE	COMMONRAVEN	11	0.204204	0.239	0.239
BALDEAGLE	TURKEYVULTURE	19	0.928121	0.001	0.003
COMMONRAVEN	TURKEYVULTURE	12	0.81087	0.015	0.0225

525 *^aR scales from +1 to -1. A value of +1 indicating the most similar samples are in the same*
526 *group. A value equal to 0 indicating there is no relationship observed between similar and*
527 *dissimilar samples.*

528 **S2 Table. Mean number (and percent) of bacterial operational taxonomic units by phylum.**

529 Mean number (and percent) of bacterial operational taxonomic units by phylum detected in fecal
 530 samples from turkey vultures (n = 10), bald eagles (n = 9), and common ravens (n = 2) captured
 531 on coastal beaches in Washington and Oregon. For each species, phyla with the largest number
 532 of operational taxonomic units are identified in bold.

Phylum	Turkey Vulture		Bald Eagle		Common Raven	
	Mean	(Percent)	Mean	(Percent)	Mean	(Percent)
Acidobacteria	5.7	(< 0.1)	0.2	(< 0.1)	ND ^a	ND
Actinobacteria	5441	(4.3)	2,033.8	(5.4)	951	(2.1)
Bacteroidetes	261.4	(0.2)	2,912.2	(8.3)	336.5	(0.4)
Chlamydiae	2,000	(1.4)	6.9	(< 0.1)	5.5	(< 0.1)
Cyanobacteria	7.3	(< 0.1)	195.3	(0.6)	150.5	(0.2)
Deferribacteres	19.2	(< 0.1)	0.1	(< 0.1)	ND	ND
Firmicutes	10,6044.8	(67.1)	4,937.4	(13.6)	27,467	(40.9)
Fusobacteria	22,873.3	(17.3)	81.2	(0.2)	61.5	(0.1)
Gemmatimonadetes	4.1	(< 0.1)	111.4	(0.3)	1	(< 0.1)
Ignavibacteriae	7.9	(< 0.1)	208.3	(0.6)	1	(< 0.1)
Proteobacteria	11,345.5	(9.7)	22,445.3	(70.9)	32,631	(55.5)
Spirochaetes	2.4	(< 0.1)	0.3	(< 0.1)	372.5	(0.9)
Tenericutes	2.1	(< 0.1)	ND	ND	ND	ND

533 ^a ND = Not detected.

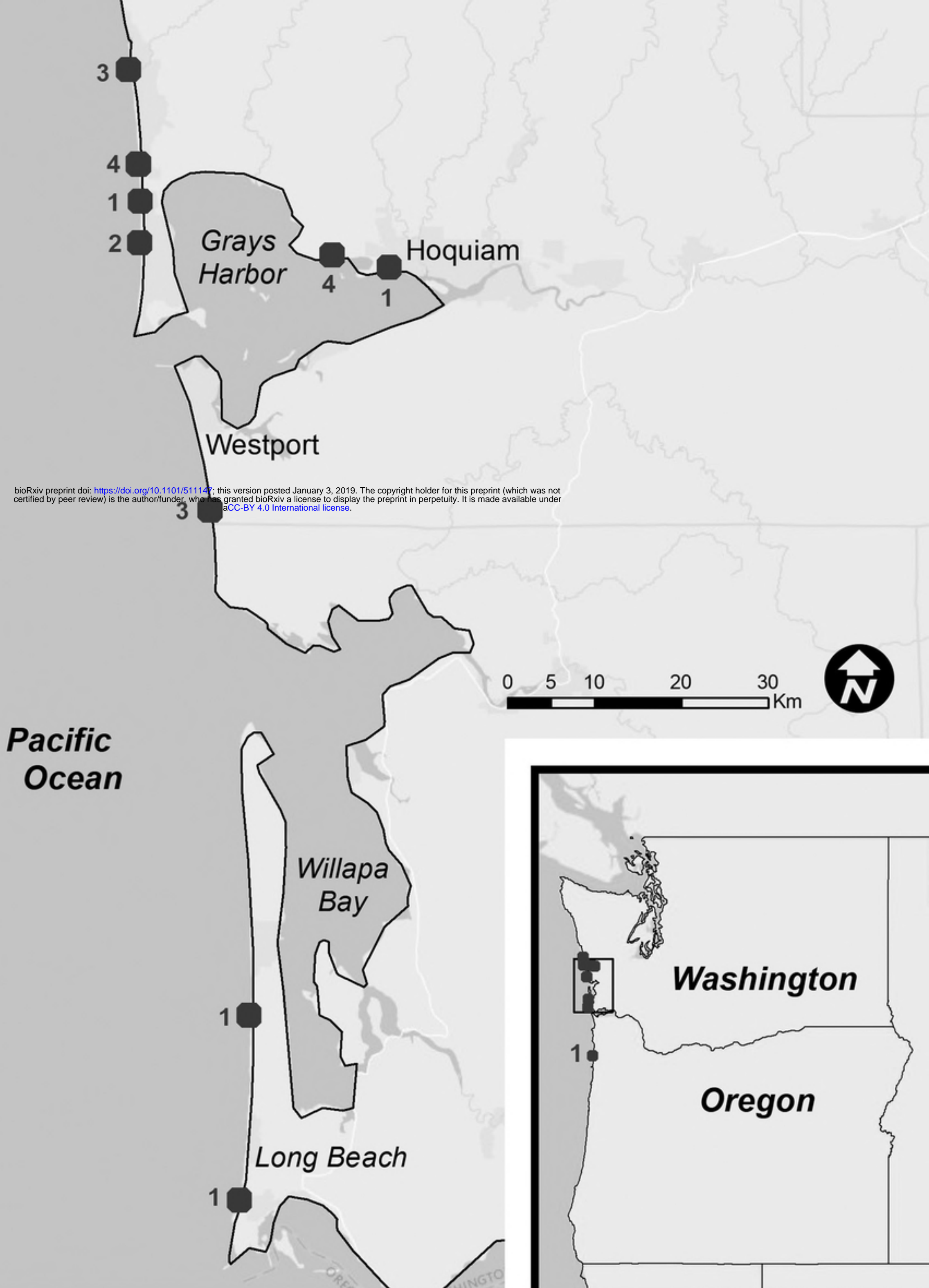


Figure 1

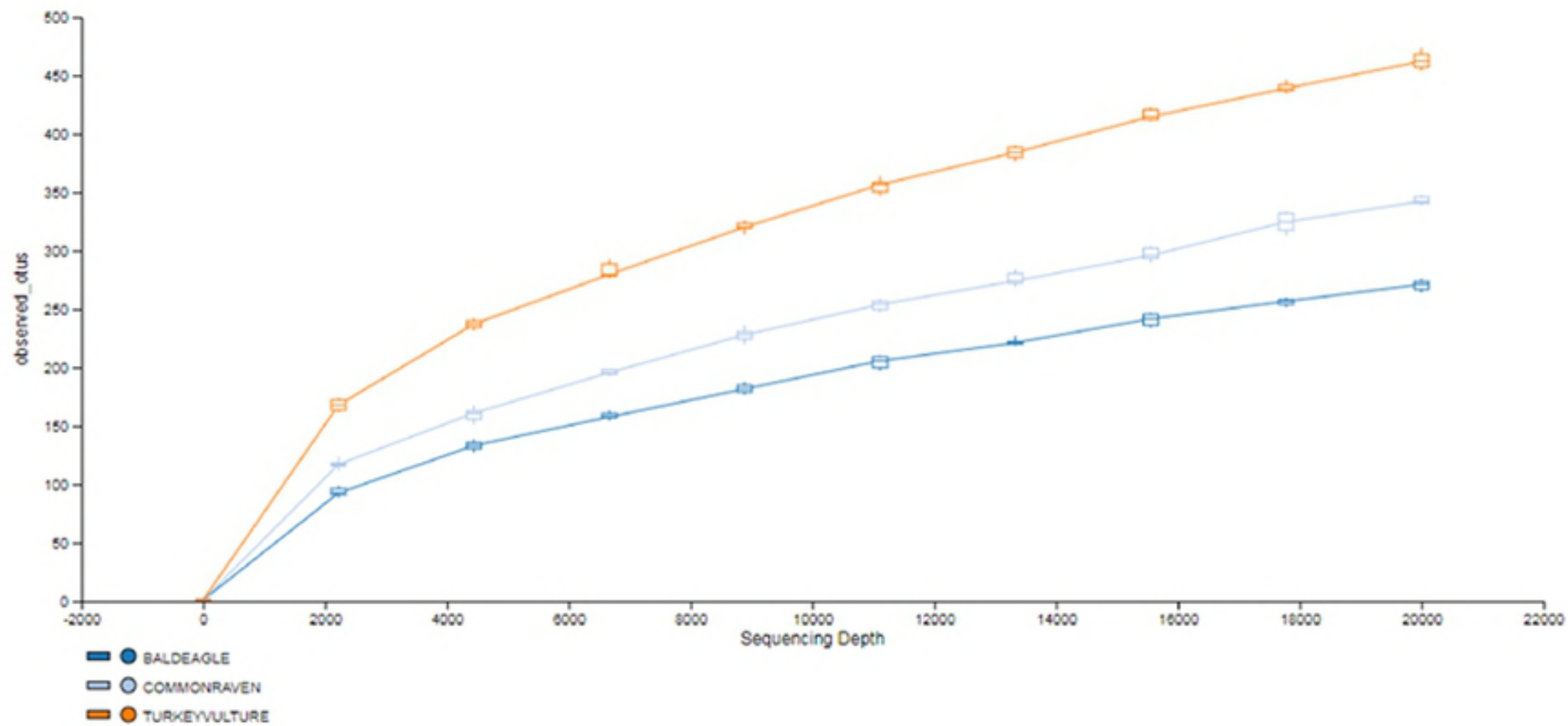


Figure 2a

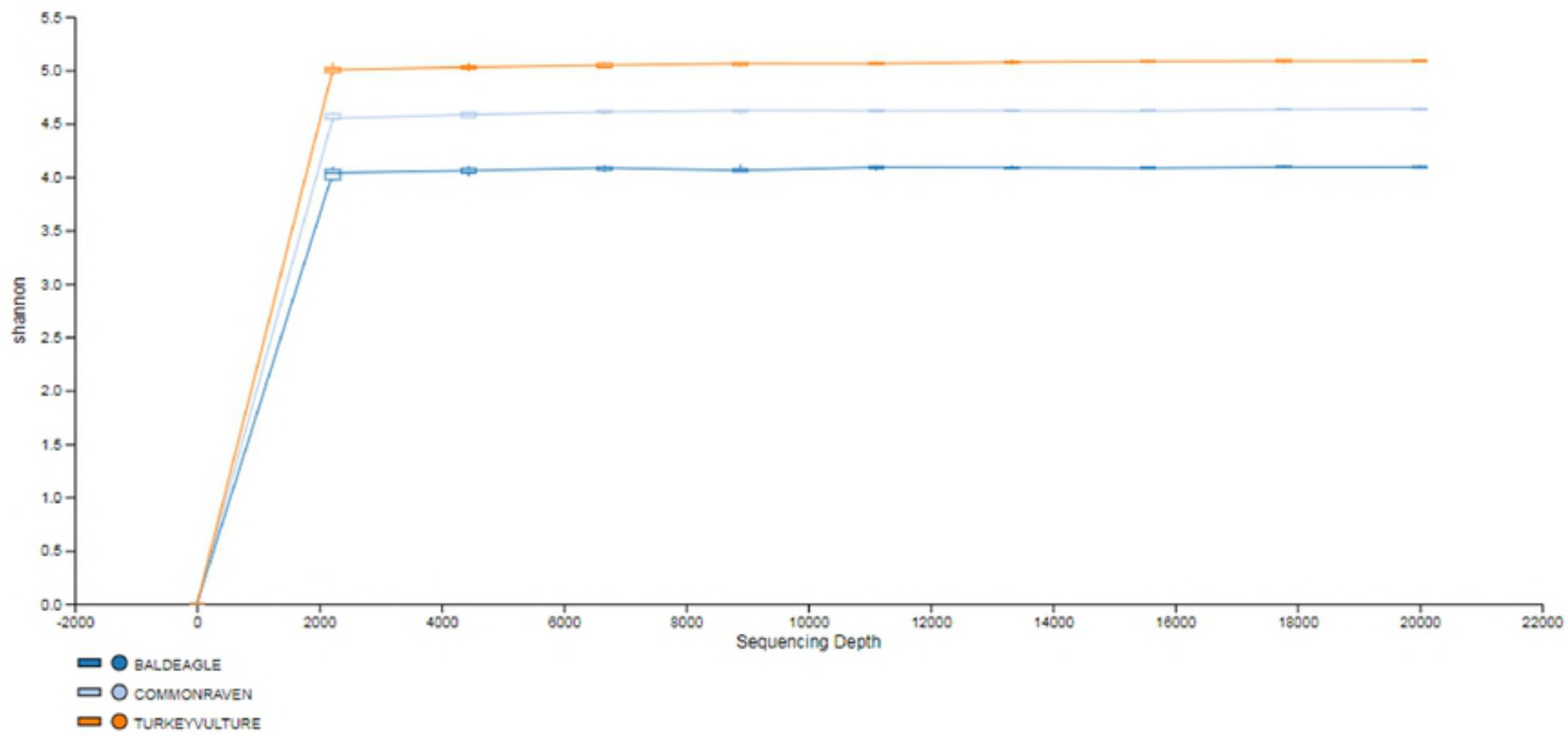
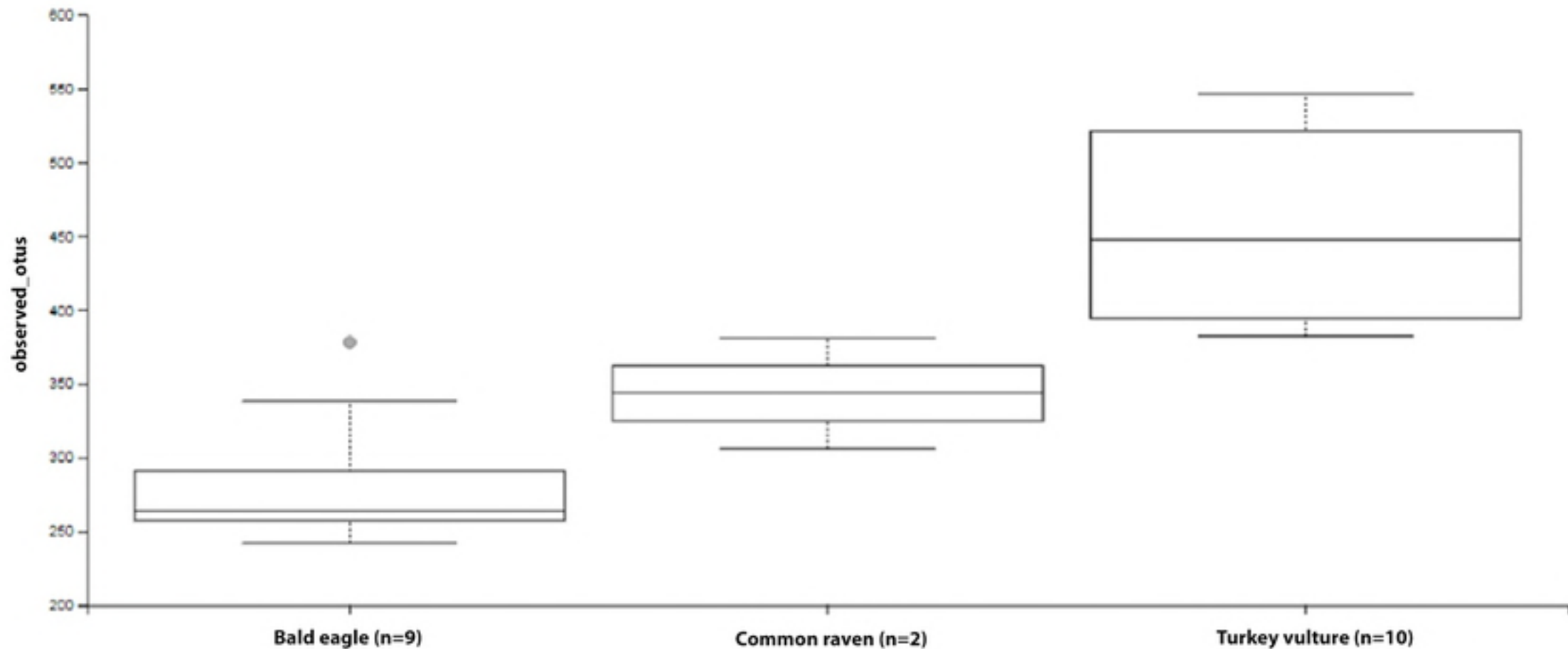
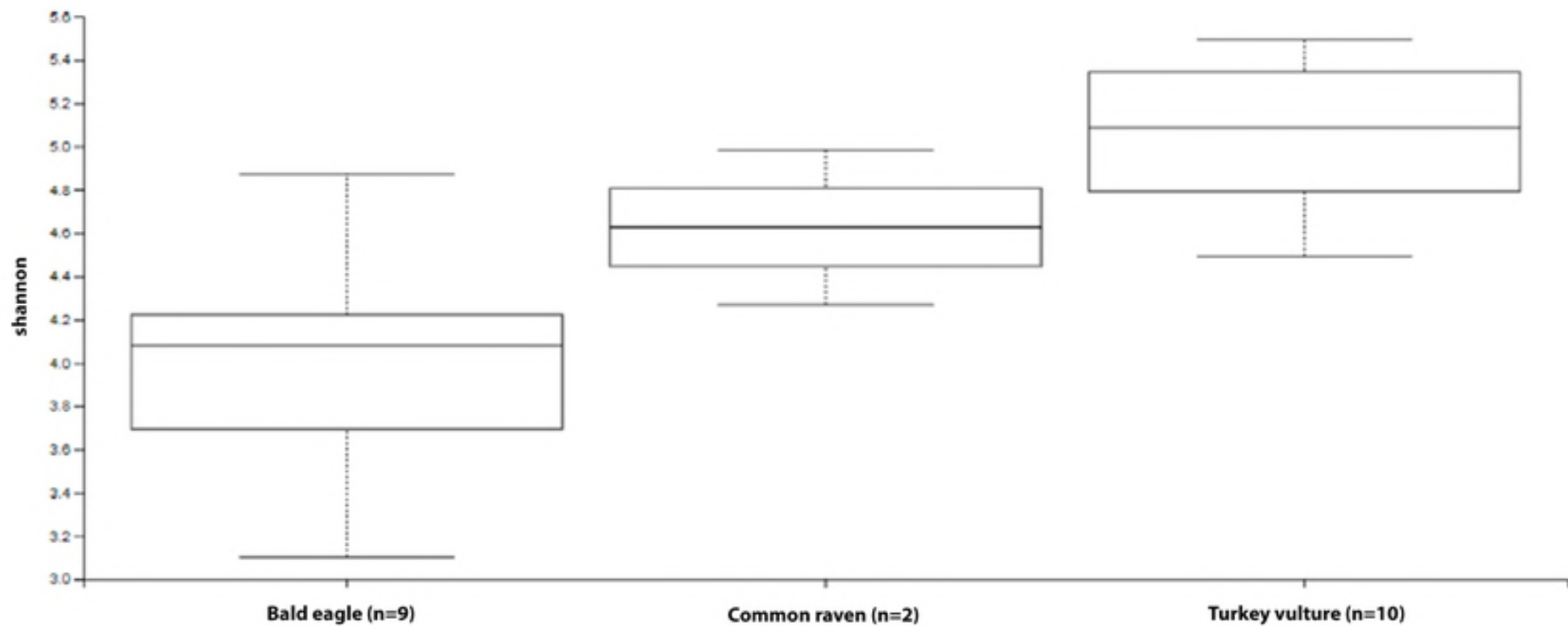


Figure 2b



Groups				
Group 1	Group 2	H	p-value	q-value
Bald eagle (n=9)	Turkey vulture (n=10)	13.5	0.000238563	0.00071569

Figure 3a



Groups				
Group 1	Group 2	H	p-value	q-value
Bald eagle (n=9)	Turkey vulture (n=10)	11.76	0.000605172	0.001815517

Figure 3b

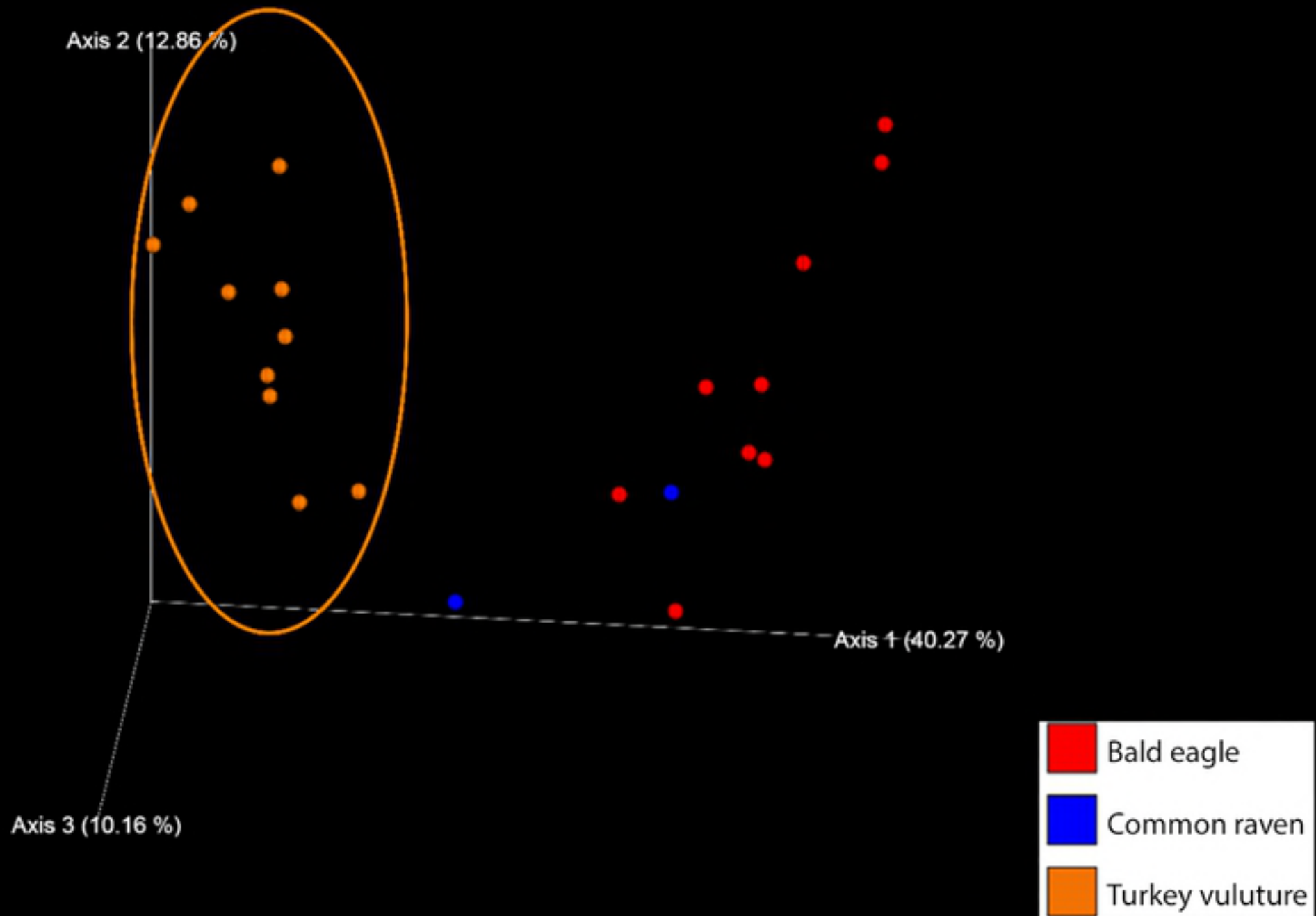


Figure 4

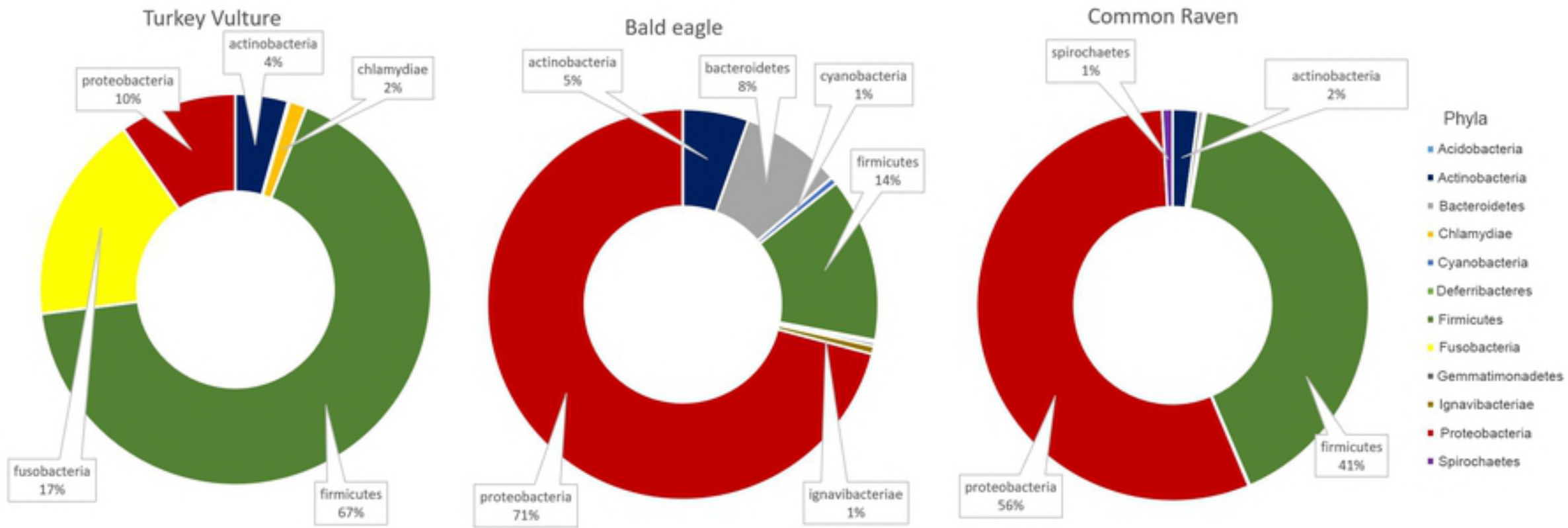


Figure 5

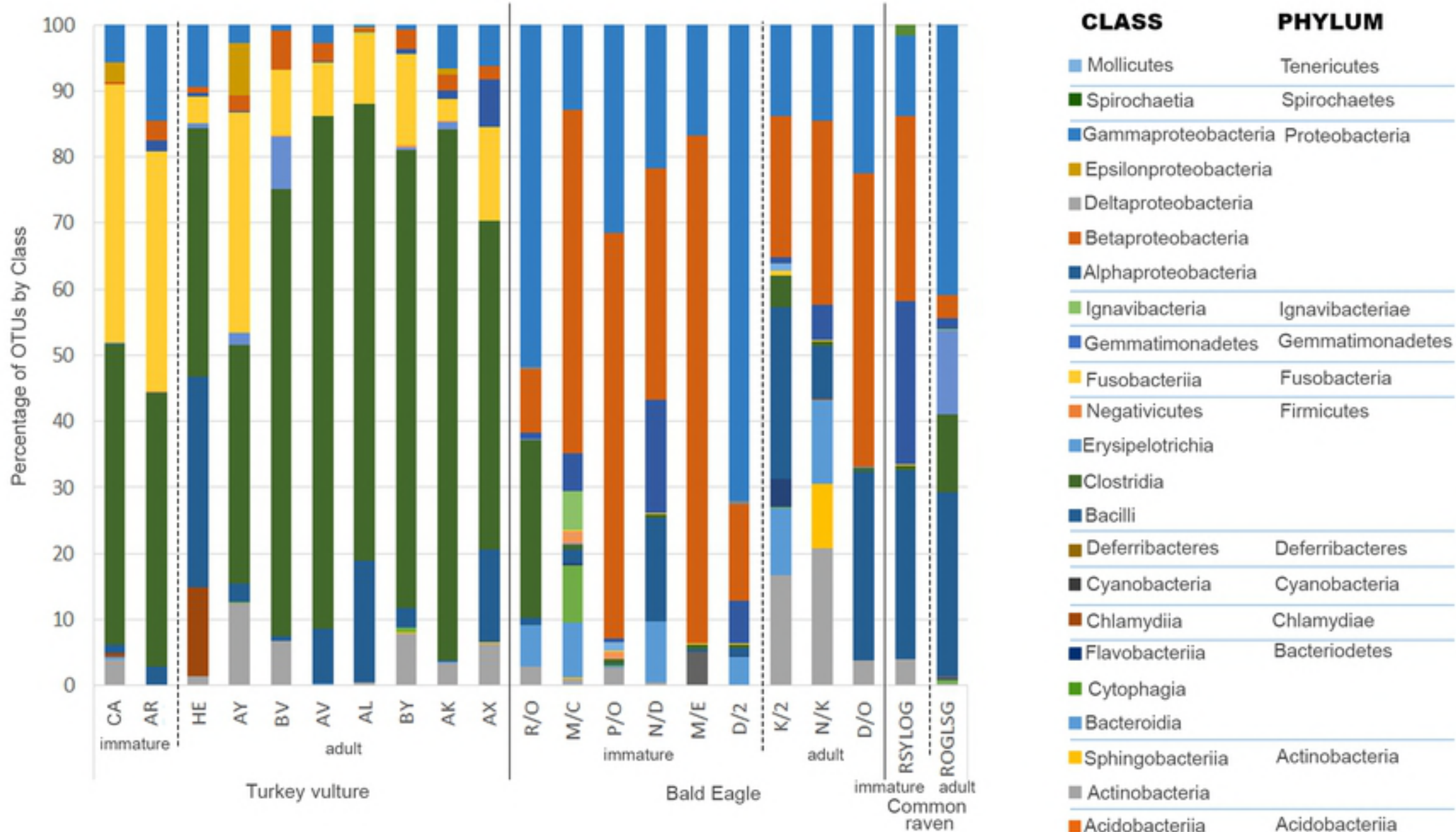


Figure 6