

1 **Running head:** Flower microbiome assembly in almond

2

3 **Title:** Ecological dynamics of the almond floral microbiome in relation to crop management and
4 pollination

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22 **Abstract**

23 1. Crop tissues harbor microbiomes that can affect host health and yield. However,
24 processes driving microbiome assembly, and resulting effects on ecosystem services,
25 remain poorly understood. This is particularly true of flowering crops that rely on
26 pollinators for yield.

27 2. We assessed effects of orchard management tactics and landscape context on the flower
28 microbiome in almond, *Prunus dulcis*. Fourteen orchards (5 conventional, 4 organic, 5
29 habitat augmentation) were sampled at two bloom stages to characterize bacterial and
30 fungal communities associated with floral tissues. The surveys were complemented by *in*
31 *vitro* experiments to assess effects of arrival order and fungicides on nectar microbial
32 communities, and effects of fungicides and microbes on honey bee foraging. Finally, a
33 field trial was conducted to test effects of fungicides and microbes on pollination.

34 3. As bloom progressed, bacterial and fungal abundance and diversity increased, across all
35 floral tissue types and management strategies. The magnitude by which microbial
36 abundance and diversity were affected varied, with host proximity to apiaries and orchard
37 management having notable effects on bacteria and fungi, respectively.

38 4. Experiments showed immigration history and fungicides affected the composition of
39 nectar microbial communities, but only fungicides affected pollinator foraging through
40 reduced nectar removal. Neither treatment affected pollination services.

41 5. *Synthesis and applications.* Our results shed light on routes through which management
42 practices can shape microbiota associated with flowers of a pollinator-dependent crop.
43 With growing appreciation for the role of floral-associated microbes in affecting biotic
44 interactions at the floral interface, understanding such drivers can potentially inform

45 microbial-derived ecosystem services in agricultural landscapes, including pollination
46 and biocontrol.

47 *Keywords:* agricultural landscapes, ecosystem services, flower microbiome, fungicide,
48 pollination, *Prunus dulcis*, sustainable agriculture

49

50 **Introduction**

51 Crop tissues harbor distinct microbiomes that affect host health and yield. For example,
52 microbiomes can affect host tolerance to stress and disease (Vurukonda *et al.*, 2016; Berg and
53 Koskella, 2018) and can stimulate growth through mobilization and transport of nutrients (Pii *et*
54 *al.*, 2015). However, despite the importance of the microbiome, few studies have investigated the
55 processes mediating microbiome assembly, especially in crop systems (but see Edwards *et al.*,
56 2015; Grady *et al.*, 2019). Assessing such processes is critical, as manipulation of the plant
57 microbiome can have wide-ranging effects, including improved crop performance, food safety,
58 associated ecosystem services, and sustainability (Mueller and Sachs, 2015; Busby *et al.*, 2017;
59 Toju *et al.*, 2018; Allard and Micallef, 2019).

60 Approximately 35% of crops produced globally benefit from pollination by arthropods
61 (Klein *et al.*, 2007). With an economic value exceeding \$300 billion globally (Lautenbach *et al.*,
62 2012), there is strong incentive to manage pollination of flowering crops during bloom. Bloom
63 management is also critical because some plant pathogens infect crops through flowers and can
64 be dispersed by pollinators (McArt *et al.*, 2014). To combat pathogens, growers often use
65 fungicides, bactericides, or other antibiotics during bloom. However, such chemicals can cause
66 non-target effects on the microbiome (Schaeffer *et al.*, 2017) and affect pollinators (Frazier *et al.*,
67 2015; Johnson, 2015).

68 In addition, agricultural intensification often decreases native pollinator abundance and
69 diversity, largely through habitat loss (Kremen *et al.*, 2002; Kovács-Hostyánszki *et al.*, 2017;
70 Grab *et al.*, 2019). Given that pollinators harbor distinct microbiomes and disperse microbes
71 between flowers, loss of pollinator biodiversity and reduced pollinator visitation may indirectly
72 affect microbiome assembly (Vannette and Fukami, 2017; Russell *et al.*, 2019). In orchard
73 systems, flowering strips, hedgerows, and nesting structures have been employed to support
74 pollinator populations (Scheper *et al.*, 2015; Williams *et al.*, 2015; Kremen *et al.*, 2019).
75 However, whether restored vegetation, which can act as a source of inocula, affects the assembly
76 of microbiomes in crop systems remains poorly understood (Lindow and Andersen, 1996;
77 Lymeropoulou *et al.*, 2016).

78 Here, we assessed how agricultural management affected flower microbiome assembly and
79 function in mass-flowering almond (*Prunus dulcis*). Almond depends on biotic pollination for
80 fruit set, and is heavily managed during bloom to ensure adequate pollination and prevention of
81 pathogens, namely *Monilinia laxa*, the causal agent of brown rot blossom blight. Synthetic
82 fungicides are applied during bloom to preempt *M. laxa* establishment in conventional orchards
83 (Adaskaveg *et al.*, 2017). However, increasing demand for sustainably produced almonds has
84 spurred adoption of organic management tactics in many orchards (Brodt *et al.*, 2009; Plattner *et*
85 *al.*, 2013), including use of copper and other materials instead of synthetics for disease control.
86 Given that pollinators of almond, including honey bees and bumble bees, are sensitive to the
87 chemical alterations that nectar microbes induce through metabolism of nectar resources (Rering
88 *et al.*, 2018; Schaeffer *et al.*, 2019), shifts in microbiome structure arising from different
89 management schemes may affect pollination services (Herrera *et al.*, 2013; Vannette *et al.*, 2013;
90 Schaeffer and Irwin, 2014).

91 To address these linkages, we conducted a field survey of microbial diversity on almond
92 flowers in orchards with different management schemes. We also conducted two experiments to
93 examine mechanisms that affect microbial assembly and function in almond. First, we examined
94 how fungicides interact with variation in microbial immigration history to affect microbial
95 community structure. Second, we examined effects of nectar microbes and fungicides on honey
96 bee foraging, and consequences for pollination. Overall, our results provide evidence that
97 variation in management of a blooming crop can shape microbiome assembly and the biotic
98 interactions that mediate ecosystem services in agricultural landscapes.

99

100 **Methods**

101 ***Study system***

102 California (CA) almonds flower in February through early March, and rely almost
103 exclusively on managed *Apis mellifera* colonies for pollination. Approximately 470,000 ha of
104 almond orchards in CA produced over 80% of the world's supply in 2018, generating \$5.8B in
105 farmer revenue (Sumner *et al.*, 2014; Almond Board of California, 2019). Despite these
106 numbers, fruit set in almond orchards typically ranges from 10-40% (Bosch and Blas, 1994),
107 partly due to limited pollinator availability and the quality of services they provide, in addition to
108 disease and inclement weather during bloom.

109

110 ***Orchard survey***

111 We surveyed 14 orchards (4 organic, 5 conventional, and 5 with supplemental forb
112 plantings) across the Sacramento Valley of CA (Figure 1). Supplemental forb plantings included
113 a mix of annual species native to CA, including *Calandrinia ciliata*, *Collinsia*

114 *heterophylla*, *Eschscholzia californica*, *Nemophila maculata*, *Nemophila menziesii*, *Phacelia*
115 *campanularia*, and *Phacelia ciliata*. Beyond the addition of forb plantings, forb-amended
116 orchards follow conventional management practices. Orchards were sampled twice between
117 February 15 and 24, 2017, once at early bloom (~10% of flowers open) and then at peak bloom
118 (>50% of flowers open). At each orchard and sampling event, six trees ('Nonpareil' variety)
119 were sampled; three near the edge of the orchard, and adjacent to the forb planting if available.
120 These trees were located in the second row in from the edge of the orchard ('edge'). The other
121 three trees were sampled from the orchard interior (row 10) ('interior'). We chose this sampling
122 scheme because semi-natural habitat in the surrounding landscape can increase visitation by
123 native pollinators such as bees and flies (Klein *et al.*, 2012). Pollinators can also be important
124 dispersal agents for microbes (Aizenberg-Gershtein *et al.*, 2013; Vannette and Fukami, 2017);
125 thus, we may see greater microbial abundance or diversity in flowers in close proximity to these
126 natural habitats. For each site (edge or interior) and sampling event, 30 open flowers ($N = 10$ per
127 tree) were collected using aseptic technique and then pooled at the site level. Flowers with flat,
128 fully-reflexed petals that had been open for approximately 3 days were chosen for collection (Yi
129 *et al.*, 2006). This choice increased the probability that flowers had been visited by pollinators
130 that disperse microbes. Once collected, flowers were placed in a cooler and transferred to the lab,
131 then stored at 4 °C until processing (within the following 24 h).

132 To assess effects of pollinator foraging activity on microbiome structure and diversity, we
133 used two complementary approaches. First, during flower collection, we measured the distance
134 between trees sampled and the nearest set of honeybee hives in the orchard. Honey bees often
135 forage near their hive (Gary *et al.*, 1978), and we hypothesized that tree proximity would be a
136 proxy for visitation frequency during bloom. Second, we measured the amount of semi-natural

137 habitat within 1 km buffer of each orchard, as prior work shows increased semi-natural habitat
138 can increase the diversity and visitation rates of native pollinators in orchards (Klein *et al.*,
139 2012). We classified land cover from the croplands data layer product (USDA, 2017) within a 1-
140 km radius of each orchard edge using ArcGIS (ESRI, Redlands, CA, USA). Natural habitat near
141 orchards in this study can primarily be classified as chaparral, oak woodland, or valley and
142 foothill riparian woodland (Barbour *et al.*, 2007). We predicted that more natural habitat would
143 promote more diverse floral microbiomes as pollinator species harbor distinct microbiomes
144 (Koch *et al.*, 2013; Graystock *et al.*, 2017) and disperse microbes (Vannette and Fukami, 2017;
145 Russell *et al.*, 2019).

146

147 ***Sample processing***

148 Whole flowers were dissected acropetally in sequence to minimize cross-contamination, as
149 previous work has shown that floral microbial communities can display taxonomic-structuring
150 across tissue types (Junker and Keller, 2015). Petals were removed first using sterile forceps.
151 Petals from all 30 flowers in a sample were then pooled in a 50 mL Falcon tube (Corning,
152 Corning, NY, USA), massed for fresh weight (g), then suspended in 20 mL of 1x-0.15% PBS-
153 Tween solution. The androecium and gynoecium (hereafter collectively referred to as ‘Anthers’)
154 were then removed from the base of each flower, pooled in a 15 mL Falcon tube, massed, then
155 suspended in 5 mL of 1x-0.15% PBS-Tween solution. To sample nectar, we ‘washed’ each
156 hypanthia with 2 μ L of 1x-0.15% PBS-Tween solution using a pipette, and pooled for each set of
157 30 flowers. Each wash was diluted with 1 mL of 1x-0.15% PBS-Tween solution. For petals,
158 androecium, and gynoecium, samples were sonicated (Branson CPX5800H, Danbury CT) for 10
159 min to dislodge epiphytic microbes. After sonication, debris was removed from sample tubes by

160 pouring samples through autoclaved cheesecloth into a sterile Falcon tube. Falcon tubes
161 containing debris-filtered samples were then centrifuged at 3000 rpm for 10 min at 4 °C to pellet
162 microbial cells. We then decanted the supernatant, re-suspended microbial cell pellets in 1 mL of
163 autoclaved PBS solution, vortexed tubes, then transferred the cell suspensions to new 1.7 mL
164 microcentrifuge tubes.

165

166 ***Microbial abundance***

167 To estimate microbial abundance across tissue types, we used dilution plating to estimate
168 the density of colony forming units (CFUs) for each sample. Selective media for growth of fungi
169 (yeast malt agar + chloramphenicol) and bacteria (R2A + cycloheximide) was used. Although
170 not all microbes are culturable, previous work suggests that most dominant species observed to
171 be associated with flowers are culturable on these media types (Morris *et al.*, 2020). Plates were
172 incubated for 5 days at 25 °C and colonies counted.

173

174 ***DNA extraction and sequencing***

175 Genomic DNA was extracted from samples using a ZymoBIOMICS® DNA Microprep kit
176 (Zymo Research, Irvine, CA, USA) at the University of California, Davis (Davis, CA, USA),
177 following the manufacturer's protocol. Extracted DNA was then sent to the Centre for
178 Comparative Genomics and Evolutionary Bioinformatics at Dalhousie University (Halifax, Nova
179 Scotia, Canada) for library preparation and 16S/ITS amplicon sequencing. Amplicon sequence
180 variants (ASVs) were assigned using DADA2 (Callahan *et al.*, 2016). See SI Materials and
181 Methods for information concerning amplification, sequencing, and bioinformatic processing of
182 data.

183

184 ***Microbial community assembly***

185 To assess effects of nectar composition, two different fungicides, and arrival order on
186 microbiome assembly, we conducted a sequential inoculation experiment. We used three
187 different synthetic nectar environments containing either copper, typically used in organic
188 orchards, or propiconazole, a synthetic, demethylation inhibitor (DMI) often used in
189 conventional orchards. We used microbes commonly observed in nectar, including *Asaia astilbis*
190 (bacterium), *Aureobasidium pullulans* (yeast), *Metschnikowia reukaufii* (yeast),
191 and *Neokomagataea thailandica* (bacterium). These species are frequently found on floral tissues
192 and nectar, including almond (Fridman *et al.*, 2012; Aizenberg-Gershtein *et al.*, 2013; Schaeffer
193 *et al.*, 2017). Colonies formed by these species are distinguishable on media. Strains used were
194 isolated from almond nectar or *Epilobium canum* (Onagraceae), a perennial herb native to the
195 foothills of California (Morris *et al.*, 2020). Yeast and bacteria strains were cultured on YMA
196 and R2A, respectively, and grown at 25 °C. After three days of growth, microbial cell
197 suspensions for inoculation were diluted to ca. 400 cells μL^{-1} using a hemocytometer just prior
198 to the beginning of the experiment described below.

199 To prepare the synthetic nectar environments, fungicides were added to filter-sterilized
200 15% (w/v) glucose:fructose solution supplemented with 0.32 mM amino acids from digested
201 casein (Vannette & Fukami 2014). Fungicide concentration (7500 ppb) was parameterized based
202 on residue analyses of almond flowers and those of other flowering crops (Frazier *et al.*, 2015).
203 In each nectar environment, we assessed the strength of priority effects following a two-way,
204 full-factorial design, with three different orders of species introductions (e.g., Tucker and Fukami
205 2014). Our introduction treatment groups included: (i) simultaneous introductions of the two

206 yeast and bacterial species to the artificial nectar on day 0; (ii) ‘yeast-first’ sequential
207 introductions, in which the two yeast species were introduced first (day 0) and then, 48 h later,
208 the two bacterial species; and (iii) ‘bacteria-first’ sequential introductions, in which the two
209 bacterial species were introduced first (day 0) and then, 48 h later, the two yeast species. The
210 experiment was performed in 200 μ L polymerase chain reaction (PCR) tubes (ThermoFisher
211 Scientific Corp.) and lasted for 4 d, which approximates the lifespan of an almond flower (Yi *et*
212 *al.*, 2006). To each tube, we added 9 μ L of synthetic nectar at the start, and 0.5 μ L suspensions of
213 each respective microbial species (ca. 200 cells) for the appropriate day and treatment
214 combination. We chose this timing as 48 h as a realistic time interval between microbial
215 immigration events in floral systems with displays open for such a duration (Peay *et al.*, 2012;
216 Vannette and Fukami 2014). Four days after the first inoculation the experiment was ended and
217 nectar from each tube was divided for chemical analysis and to determine microbial abundance.
218 All treatment combinations were performed in each of the three nectar environments ($N = 16$ per
219 treatment combination).

220

221 ***Pollination***

222 ***Pollination Experiment One*** - To test consequences, including non-additive effects, of
223 flower exposure to fungicides and microbes on pollinator foraging behavior, we performed a
224 field assay. Briefly, artificial flowers (Figure S2) designed to mimic those of almond were set out
225 in an array near an apiary at the Harry H. Laidlaw Jr. Honey Bee Research Facility (Davis, CA).
226 Flowers were treated with 200 μ L of artificial nectar (same as above) in a fully-crossed design,
227 with three levels for each treatment. For fungicides, treatment levels were: (1) no fungicide
228 (control), (2) organic (copper, 7500 ppb), or (3) conventional (propiconazole, 7500 ppb). With

229 respect to nectar-inhabiting microbes, treatments were: (1) no bacterium or yeast (control), (2) *N.*
230 *thailandica* (bacterium), or (3) *M. reukaufii* (yeast). Experimental arrays were set ~1–2 m from
231 the hives at the apiary in the morning each day the experiment was performed. Two hours after
232 the start of the experiment each day, the remaining nectar from each flower's tube was capped,
233 brought back to the laboratory, and weighed to estimate changes in volume. This assay was
234 performed four times ($N = 12$ flowers per treatment combination per assay). See SI Materials and
235 Methods for full experimental details.

236 *Pollination Experiment Two* - We performed an *in vivo* field assay at an orchard (Zamora,
237 CA, USA) to test for consequences, including non-additive effects, of flower exposure to
238 fungicides and microbes on the quality of pollination services. Briefly, fungicide/microbe
239 treatments mirrored those used in the first pollination assay, with treatment identity randomized
240 among 9 unvisited flowers within an individual tree ($N = 20$ 'Nonpareil' variety trees, spaced
241 across alternating rows, with five haphazardly selected in each row). Two microliters of treated
242 nectar was applied to each flower, and after two days of exposure to pollinators, flowers were
243 carefully removed along with the pedicel and placed in individual 1.5 mL microcentrifuge tubes
244 containing 0.5 mL of water. Flowers were positioned such that the stigma did not touch the
245 tube's surface and the pedicel was in water (Brittain *et al.*, 2013). Once returned to the lab,
246 flowers were stored in the dark at room temperature for 72 h to allow pollen tube growth. After
247 this period, pistils were fixed (Farmer's fixative) and then stored at 4 °C until further processing.
248 Pollen tube growth was assessed using a staining and microscopy procedure, following a
249 previously established protocol (Brittain *et al.*, 2013). See SI Materials and Methods for full
250 experimental details.

251

252 **Statistical analyses**

253 All analyses were performed in R v.4.0.2 (R Core Team, 2013). We fit linear mixed-effect
254 models with the *lme4* package (Bates *et al.*, 2014) to assess the impact of orchard management,
255 and other measured variables on microbial abundance (log-10 transformed CFU counts) and
256 diversity (ASV richness and Shannon diversity index). For each model and response variable
257 examined, management, bloom stage (early/peak), site (edge/interior) and a three-way
258 interaction among them, along with amount of semi-natural habitat surrounding orchards and
259 distance to the nearest apiary were included as predictors, with orchard identity as a random
260 effect to account for repeated sampling. Bacterial and fungal data were analyzed separately for
261 each floral tissue examined. Once fit, we used backward stepwise model selection in the
262 *lmerTest* package (Kuznetsova *et al.*, 2017) to identify the best-fit model for each response
263 variable examined. Fixed model terms were retained based on log-likelihood ratio tests, with
264 significance of each calculated using *F* tests, based on Satterthwaite approximation for
265 denominator degrees of freedom (Kuznetsova *et al.*, 2016). Finally, to determine if the relative
266 abundance of individual ASVs responded to orchard variables of interest, we used *DESeq2* with
267 Benjamini-Hochberg corrections for multiple testing (Love *et al.* 2014). Orchard management,
268 bloom stage, and apiary distance were treated as predictors in separate models.

269 Pairwise dissimilarities between fungal and bacteria communities were calculated using
270 the Bray–Curtis dissimilarity metric. We also calculated abundance-weighted UniFrac distances
271 for bacteria; the UniFrac metric uses phylogenetic information to calculate dissimilarities
272 between communities, and was weighted by the relative abundance of ASVs within a sample
273 (Lozupone and Knight, 2005). Finally, we used permutational multivariate analysis of variance
274 (PERMANOVA) to assess the contribution of management, bloom stage (early/peak), site

275 (edge/interior) and all (2- and 3-way) interactions among them, along with amount of semi-
276 natural habitat surrounding orchards and distance to the nearest apiary on community
277 composition. This analysis was performed using *vegan*, based on 1000 permutations (Oksanen *et*
278 *al.*, 2015).

279 To assess effects of fungicide identity and immigration order on microbiome assembly in
280 our *in-vitro* experiment, we fit linear mixed-effect models for each species with abundance
281 (CFUs) as the response variable and immigration order and fungicide treatment as fixed factors,
282 including their interaction.

283 For the behavior assay (*Pollination Experiment One*), we fit a linear mixed-effects model
284 with nectar remaining as the response, fungicide and microbe treatments as fixed factors, as well
285 as their interaction. Trial number was included as a random effect. For the in-orchard pollination
286 service assay (*Pollination Experiment Two*), we also fit linear mixed-effects models with pollen
287 germination and number of tubes as response variables, fungicide and microbe treatments as
288 fixed factors, as well as their interaction. Tree identity was included as a random effect.

289

290 **Results**

291 ***Microbial abundance and diversity***

292 Floral microbial abundance and diversity increased from early to peak bloom across all
293 tissue types, and management strategies, for both bacteria and fungi (Tables S1-S3). The
294 magnitude in which microbial abundance increased, however, varied considerably among these
295 factors. Culturable bacterial CFU abundance from petals ($F_{1,53} = 645.35, P < 0.0001$), anthers
296 ($F_{1,43.92} = 466.26, P < 0.0001$), and nectaries ($F_{1,54} = 929.91, P < 0.0001$) was eleven-, five-, and
297 eight-fold higher at peak bloom, respectively (Figure 2; Table S1) than at the initial sampling.

298 Like bacteria, fungal CFU abundance (Table S1) also increased from early to peak bloom,
299 increasing two-fold for petals ($F_{1,46.03} = 6.38, P = 0.02$), two-fold for anthers ($F_{1,52} = 70.97, P <$
300 0.0001), and three-fold for nectaries ($F_{1,44.04} = 41.11, P < 0.0001$).

301 Beyond bloom progression, additional orchard factors affected microbial abundance, but in
302 disparate ways for bacteria and fungi across tissue types. Fungi associated with anthers were
303 27% more abundant in organic orchards than those that were forb-amended or conventional in
304 management practice ($F_{2,52} = 4.61, P = 0.01$). Moreover, regardless of management scheme,
305 fungi associated with petals were 11% more abundant along the edges of orchards compared to
306 the interior ($F_{1,46.03} = 6.38, P = 0.02$). However, no effect of sampling location within orchards
307 was detected for bacteria, nor for the amount of natural habitat in the surrounding landscape on
308 the abundance of either bacteria or fungi.

309 Bacterial diversity increased as bloom progressed for communities associated with petals
310 (richness: $F_{1,54} = 8.85, P < 0.01$; Shannon index: $F_{1,54} = 6.05, P = 0.02$), anthers (richness:
311 $F_{1,35.60} = 25.99, P < 0.0001$; Shannon index: $F_{1,51} = 33.28, P < 0.0001$), and nectaries (richness:
312 $F_{1,40.32} = 102.96, P < 0.0001$; Shannon index: $F_{1,40.17} = 25.45, P < 0.001$) (Figure 3; Tables S2
313 and S3). All tissues were dominated by Proteobacteria (Figure 4), particularly members of the
314 Pseudomonadaceae, with two taxa being enriched from early to peak bloom overall (BactSeq2:
315 *Pseudomonas* sp., log2-fold change = 2.50, $P_{adj} < 0.001$; BactSeq10: *Pseudomonas* sp., log2-fold
316 change = 3.64, $P_{adj} < 0.01$). Tree proximity to apiaries within an orchard was found to be
317 associated with bacterial diversity, although the association was weak (Figure S2A): Shannon
318 diversity was higher on flowers closer to apiaries than those that were collected further away,
319 though a significant effect was only detected for anthers ($F_{1,51} = 7.39, P < 0.0001$) in our models.

320 With the exception of organic orchards, fungal ASV richness generally increased over
321 bloom (Figure 3). This trend was significant for nectaries (richness: $F_{1,42.37} = 6.81, P = 0.01$) and
322 petals (richness: $F_{1,41} = 5.32, P = 0.03$). Shannon diversity followed a similar pattern, with a
323 significant increase observed for nectaries ($F_{1,42.44} = 10.22, P < 0.01$) and petals; however, for the
324 latter this effect depended on orchard management scheme (Management \times Bloom stage: $F_{2,39} =$
325 3.50, $P = 0.04$). Specifically, fungal diversity (Shannon index) increased in both conventional
326 and forb-amended orchards by 59% and 12% respectively, while decreasing in organic orchards
327 by 26%. Other orchard-level variables examined, including apiary distance, natural habitat in the
328 surrounding landscape, and site sampled within orchards, had no effect on observed fungal ASV
329 richness or Shannon diversity (Tables S2 and S3). We also detected no correlation between
330 apiary distance and either diversity metric (Figure S2b); however, DESeq2 analyses revealed
331 *Vishniacozyma carnescens* (syn. *Cryptococcus carnescens*) ASVs that significantly declined in
332 abundance with tree distance from apiaries (FunSeq13: log2-fold change = -0.02, $P_{adj} < 0.001$;
333 FunSeq58: log2-fold change = -0.02, $P_{adj} < 0.01$).

334 Fungal communities associated with floral tissues were generally dominated by members
335 of the Aureobasidiaceae and Bulleribasidiaceae (Figure 4), including *Aureobasidium pullulans*,
336 *V. victoriae* (syn. *C. victoriae*), and *V. carnescens*. Over bloom, *A. pullulans* in particular was
337 found to significantly increase in relative abundance (FunSeq1: log2-fold change = 2.96, $P_{adj} <$
338 0.0001), along with *Gelidatrema spencermartinsiae* (syn. *C. spencermartinsiae*, FunSeq16: log2-
339 fold change = 2.27, $P_{adj} < 0.01$), *Filobasidium wieringae* (syn. *C. wieringae*, FunSeq34: log2-
340 fold change = 3.87, $P_{adj} < 0.0001$), and *Buckleyzyma aurantiaca* (syn. *Rhodotorula aurantiaca*,
341 FunSeq26: log2-fold change = 1.14, $P_{adj} < 0.01$). Taxa observed to significantly decline in
342 relative abundance included *Cladosporium delicatulum* (FunSeq3: log2-fold change = -1.52, P_{adj}

343 < 0.001), a widely distributed saprobe species, and *Naganishia friedmannii* (syn. *C. friedmannii*;
344 FunSeq27: log2-fold change = -3.28, $P_{adj} = 0.02$).

345 Bacterial and fungal species composition differed between sampling times (Table 1), with
346 bloom stage explaining 3-21% of variation in composition, depending on flower tissue. Orchard-
347 level predictors (e.g., natural habitat) generally explained less variation in composition (Table 1).
348 Orchard management was not associated with bacterial species composition, but did predict
349 variation in fungal composition in anthers ($R^2 = 0.073$, $F_{2,33} = 2.11$, $P = 0.001$) and petals ($R^2 =$
350 0.078, $F_{2,33} = 2.60$, $P = 0.005$). The amount of semi-natural habitat in the surrounding landscape,
351 as well as apiary distance, were both generally found to be associated with shifts in bacterial and
352 fungal community composition, with a particularly notable effect of apiary distance on bacteria
353 in nectar ($R^2 = 0.176$, $F_{2,20} = 5.65$, $P = 0.001$).

354

355 ***Microbial community assembly in floral nectar***

356 In the in-vitro sequential inoculation experiment, arrival order significantly affected
357 species' densities in nectar. However, this effect depended on the nectar environment and
358 presence of fungicide residues (Figure 5). The bacterium *Asaia* was the only species that could
359 persist across all nectar environments ($F_{2,134} = 2.64$, $P = 0.08$). Interestingly, the bacterium *Asaia*
360 reached higher densities when introduced after or simultaneously with yeast species ($F_{2,134} =$
361 22.89, $P < 0.0001$). Of the yeasts, persistence and growth were strongly dependent on the nectar
362 environment: *Metschnikowia* grew best in control nectar ($F_{2,134} = 31.63$, $P < 0.0001$), while
363 *Aureobasidium* performed better in the copper treatment ($F_{2,134} = 16.94$, $P < 0.0001$). Finally,
364 propiconazole had strong inhibitory effects on the growth of both species.

365

366 ***Microbial and fungicide effects on honey bee foraging and pollination***

367 Fungicides reduced the amount of nectar removed by honey bee foragers ($F_{2,311} = 130.76$,
368 $P < 0.0001$). In contrast, neither microbe affected forager nectar removal ($F_{2,311} = 1.90$, $P =$
369 0.15), nor did we detect a significant interaction between microbial inoculation and fungicide
370 treatment ($F_{4,311} = 2.19$, $P = 0.07$). In the orchard experiment, neither fungicide application nor
371 nectar-inhabiting microbes affected pollen germination or pollen tube number (Table S5).

372

373 **Discussion**

374 The results presented here demonstrate that orchard management practices can mediate
375 flower microbiome structure, though the magnitude of these effects can hinge on a variety of
376 factors. Of those examined, timing of bloom was the most consistent predictor: bacterial and
377 fungal abundance and diversity were higher at peak bloom than at the start across all floral tissue
378 types and management strategies. Sampling time with respect to bloom intensity has been
379 documented previously (e.g., Smessaert *et al.*, 2019), and may be driven by variation in a
380 combination of interrelated variables, including temperature, pollinator activity, and host
381 metabolism. However, other orchard-level variables affected microbial abundance and diversity,
382 with host proximity to apiaries and orchard management having significant effects on both
383 bacteria and fungi, respectively.

384 The results described here support a key role for pollinator foraging in the assembly of the
385 almond flower microbiome. For example, the orchard survey detected a strong signature of tree
386 proximity to apiaries on epiphytic bacterial diversity of almond flower nectaries and reproductive
387 structures, as both bacterial richness and diversity decreased the further trees were from apiaries.
388 Within orchards, honey bees forage more intensively near their apiary (Bates *et al.*, 2014),

389 dispersing bacteria among flowers (Vannette and Fukami, 2018; Russell *et al.*, 2019), and
390 generate spatial variation in microbial transmission and resulting flower microbial communities.
391 Moreover, while foraging on flowers, individual honey bees can display consistent behaviors,
392 including focus on either pollen and nectar collection only, affecting the degree of contact with
393 intrafloral tissues (Bosch and Blas, 1994; Thomson and Goodell, 2001). Finally, although contact
394 with petals can occur as foragers side-work for nectar (Thomson and Goodell, 2001), these
395 collective behaviors may explain the lack of a significant effect of apiary distance on bacterial
396 diversity observed on petals vs. nectar and reproductive structures of the flower. In sum, these
397 patterns point to increased consideration of both apiary spacing, and pollinator foraging
398 behavior, as interest grows in leveraging pollinators as vectors of microbial biocontrol agents to
399 combat disease (Kevan *et al.*, 2008; Menzler-Hokkanen and Hokkanen, 2017).

400 Pollinator foraging could also affect microbial arrival to flowers and community assembly,
401 generating priority effects between microbial species (Peay *et al.*, 2011; Vannette and Fukami,
402 2014). Indeed, in our laboratory experiment, immigration history had a significant effect on
403 species' persistence and densities in nectar. These effects, however, were dependent on both the
404 biotic environment and presence of fungicides. At the concentrations tested, both fungi were
405 highly susceptible to the synthetic fungicide propiconazole, while *Aureobasidium* was much less
406 so in comparison to *Metschnikowia* when challenged with copper. Surprisingly, the bacterium
407 *Asaia* persisted in all nectar environments, while *Neokomagataea* did not, in contrast to previous
408 findings (Tucker and Fukami, 2014). Additional work on the sensitivity of these taxa to
409 fungicides across different sugar environments may explain these patterns. Taken together, these
410 results suggest that arrival order and environmental filtering via the presence of fungicides can
411 potentially affect beta diversity among nectar microbial communities in managed flowering

412 systems. As nectar-inhabiting fungi and bacteria can differ in their effect on nectar chemistry
413 (Vannette and Fukami, 2018), it is possible that microbial effects stemming from differing
414 community compositions may manifest to affect the quality of pollination services provided
415 among managed flowering systems.

416 Honey bee foraging was affected by fungicides, but not by nectar-inhabiting microbes, in
417 contrast to previous work (Vannette *et al.*, 2013; Good *et al.*, 2014; Rering *et al.*, 2018).

418 Regardless of fungicide type, honey bees removed less nectar from artificial flowers with copper
419 and propiconazole residues, demonstrating that fungicide application and residual contamination
420 of floral rewards can affect forager decisions. Exposure to fungicide-contaminated rewards can
421 affect pollinator health and pollination in agroecosystems, including almond. Such consequences
422 range from negative effects on larval development, pollinator cognition, and even mortality
423 (Johnson, 2015), as has been observed with honey bee workers directly exposed to a range of
424 fungicides commonly employed for pathogen control in almonds. Although fungicide residues in
425 nectar deterred honey bees, these shifts in foraging behavior did not translate to noticeable
426 effects on pollination services in almonds measured through pollen germination and tube
427 number. Honey bees foraging on almond flowers however often alternate between foraging for
428 nectar or pollen (Bosch and Blas, 1994). These nectar and pollen foragers differ markedly in the
429 quality of services that they provide, with pollen foragers being on average five times more
430 effective in affecting fruit set than those foraging for nectar (Bosch and Blas, 1994). Given that
431 our flowers were exposed for a long enough duration to allow pollinators to forage for both
432 resources, those that foraged for pollen alone likely conferred adequate pollination observed in
433 our experiment.

434 Our results highlight multiple orchard management practices that can shape the assembly
435 of crop-associated microbiota during flowering and pollination. We documented temporal
436 changes in microbial abundance and composition, but also detected effects of managed
437 pollinators and natural areas, suggesting a key role of immigration in determining species
438 composition in many floral tissues. Combined with the potential for agrochemicals to
439 differentially affect microbial growth and species interactions, we outline a few factors that
440 likely contribute to flower microbiome assembly. Because flowers form the template for
441 potential reproductive output that is translated through interactions with pollinators,
442 understanding linkages between management, the assembly of the floral microbiome and its
443 impact on pollination in crops can reveal how microbial interactions affect both crop yield and
444 quality.

445

446 **Authors' contributions**

447 RS conceived the idea for the study and collaborated with DC, JB, TF, NW, and RV in designing
448 the survey and experiments performed. RS carried out the survey, experiments, analyses, and
449 wrote the first draft of the manuscript. DC and JI contributed ArcGIS data and analysis. All
450 authors contributed to revisions.

451

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459

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582

583 **Figure Legends**

584 **Figure 1.** Map of the study area, showing heterogeneity in land-cover classes (Almond: brown,
585 Forest: green; Shrubland: blue; Grass/Pasture: tan) across the Sacramento Valley of California, a

586 major production area for almond. Points represent sampled orchards and are color-coded by
587 management scheme (Conventional: black; Forb-amended: purple; Organic: blue).

588 **Figure 2.** Abundance of a) colony-forming units (CFUs) on R2A media (bacteria) and b) CFUs
589 on YMA (fungi) associated with floral tissues (Anther, Nectary, or Petal) of almond. Flowers
590 were sampled at two stages of bloom (Early or Peak) from orchards that employed different
591 management schemes (Conventional, Forb-amended, or Organic).

592 **Figure 3.** Observed sequence variant richness of a) bacteria and b) fungi associated with floral
593 tissues (Anther, Nectary, or Petal) of almond flowers. Flowers were sampled at two stages of
594 bloom (Early or Peak) from orchards that employed different management schemes
595 (Conventional, Forb-amended, or Organic).

596 **Figure 4.** Average relative abundance (Proportion of Sequences) of a) bacterial and b) fungal
597 families associated with floral tissues (Anther, Nectary, or Petal) of almond. Flowers were
598 collected from orchards that vary in management scheme (Conventional, Forb-amended, or
599 Organic).

600 **Figure 5.** Mean abundance of colony-forming units (CFUs) of bacteria (gold = *Asaia*, red =
601 *Neokamagataea*) and yeasts (tan = *Aureobasidium*, blue = *Metschnikowia*) when introduced in
602 different orders (Bacteria-first, Yeasts-first, or Simultaneously) to nectar environments treated
603 with fungicides (Copper or Propiconazole at 7500 ppb). Simultaneous introductions were carried
604 out on day 0, while sequential introductions on days 0 and 2.

605 **Figure 6.** Nectar remaining (g) in artificial almond flowers treated with fungicides (Copper or
606 Propiconazole) and nectar-inhabiting microbes (red = bacterium *Neokogamataea thailandica*,
607 blue = yeast *Metschnikowia reukaufii*).

608

609 **Table 1.** PERMANOVA results of Bray-Curtis dissimilarity between bacterial and fungal
610 communities associated with almond floral tissues.

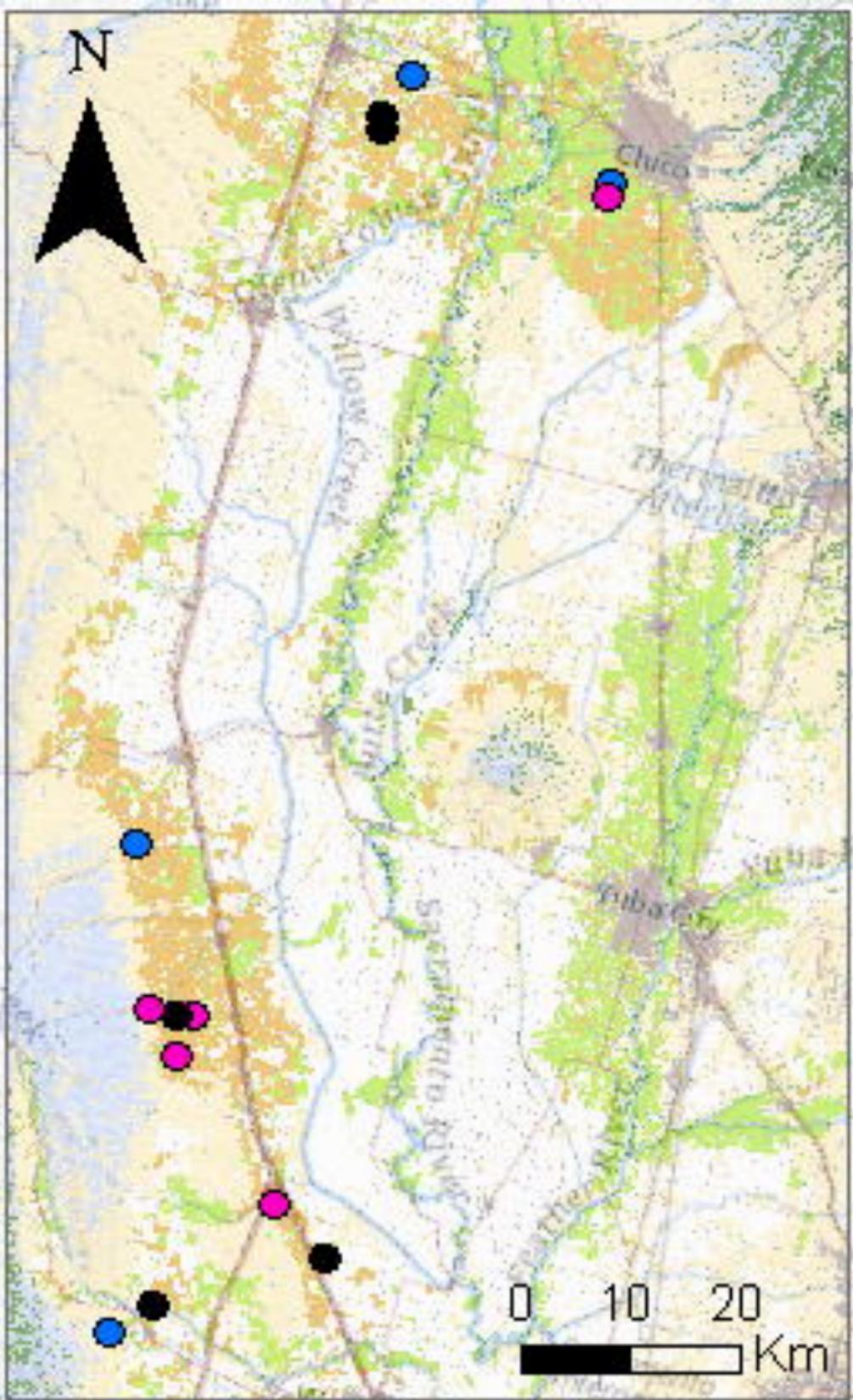
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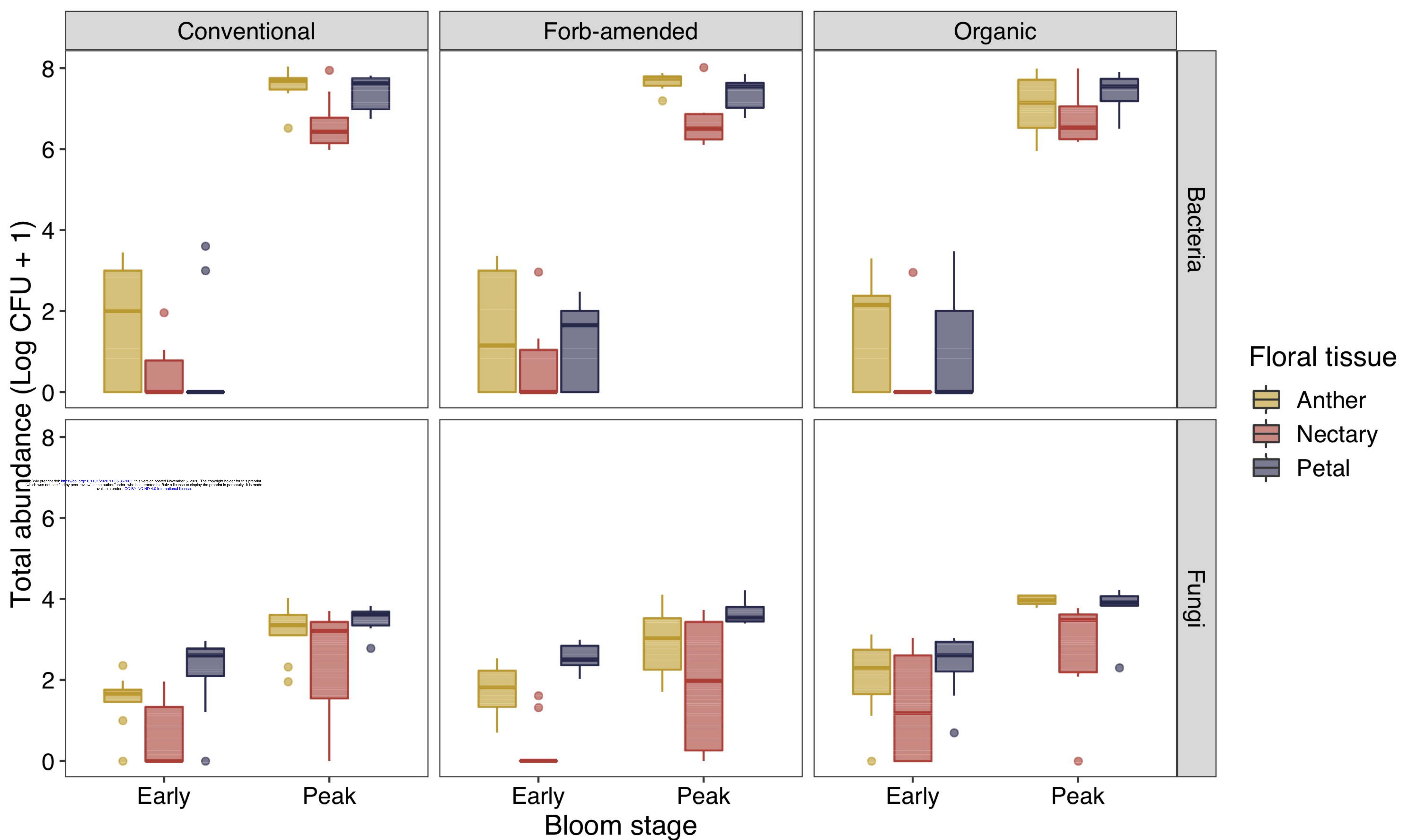
Table 1 - PERMANOVA results of Bray-Curtis dissimilarity between bacterial and fungal communities associated with almond floral tissues.

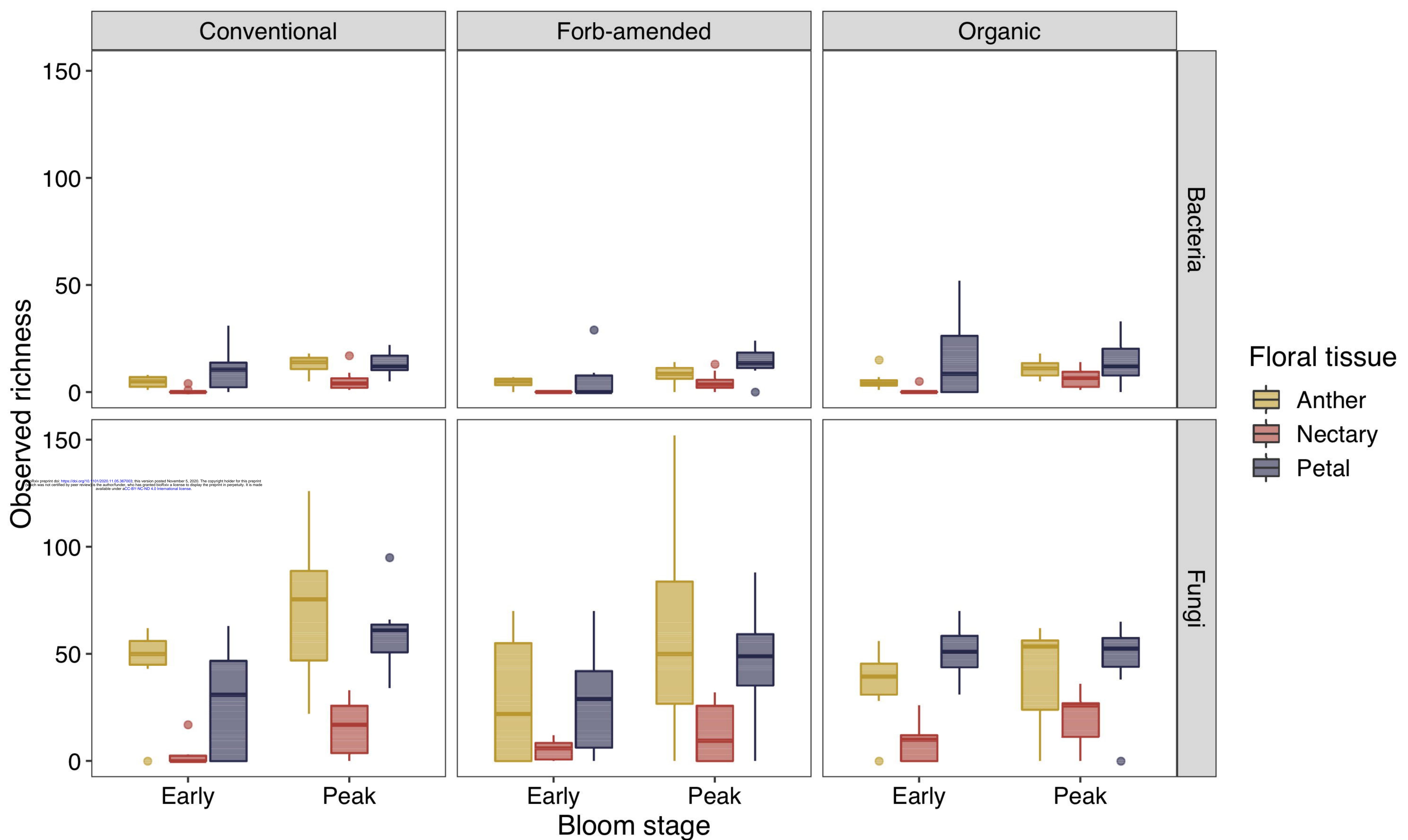
Bacteria	Anther			Nectary			Petal		
Variable	R ²	F	P	R ²	F	P	R ²	F	P
Management	0.043	1.10	0.299	0.039	0.63	0.740	0.061	1.26	0.157
Bloom stage	0.033	1.71	0.072	0.075	2.42	0.047	0.069	2.86	0.003
Site	0.024	1.23	0.234	0.030	0.96	0.374	0.018	0.75	0.744
Management x Bloom stage	0.022	0.56	0.963	0.027	0.87	0.471	0.062	1.30	0.147
Management x Site	0.029	0.76	0.791	0.019	0.30	0.983	0.036	0.74	0.848
Bloom stage x Site	0.013	0.67	0.797	-	-	-	0.026	1.10	0.360
Management x Bloom stage x Site	0.043	1.12	0.323	-	-	-	0.027	0.57	0.974
Natural habitat	0.041	2.10	0.017	0.010	0.33	0.880	0.022	0.90	0.564
Apiary distance	0.017	0.90	0.525	0.176	5.65	0.001	0.032	1.33	0.182
Fungi	Anther			Nectary			Petal		
Variable	R ²	F	P	R ²	F	P	R ²	F	P
Management	0.073	2.11	0.001	0.071	1.54	0.077	0.078	2.60	0.005
Bloom stage	0.137	7.89	0.001	0.146	6.33	0.001	0.210	14.01	0.001
Site	0.016	0.94	0.528	0.037	1.61	0.107	0.012	0.83	0.589
Management x Bloom stage	0.057	1.65	0.024	0.051	1.09	0.348	0.039	1.31	0.189
Management x Site	0.032	0.94	0.570	0.067	1.46	0.101	0.019	0.64	0.883
Bloom stage x Site	0.020	1.16	0.257	0.044	1.89	0.056	0.016	1.07	0.388
Management x Bloom stage x Site	0.033	0.97	0.511	0.044	0.96	0.515	0.022	0.74	0.774
Natural habitat	0.029	1.65	0.056	0.053	2.30	0.016	0.052	3.44	0.004
Apiary distance	0.032	1.83	0.021	0.027	1.15	0.304	0.056	3.73	0.002

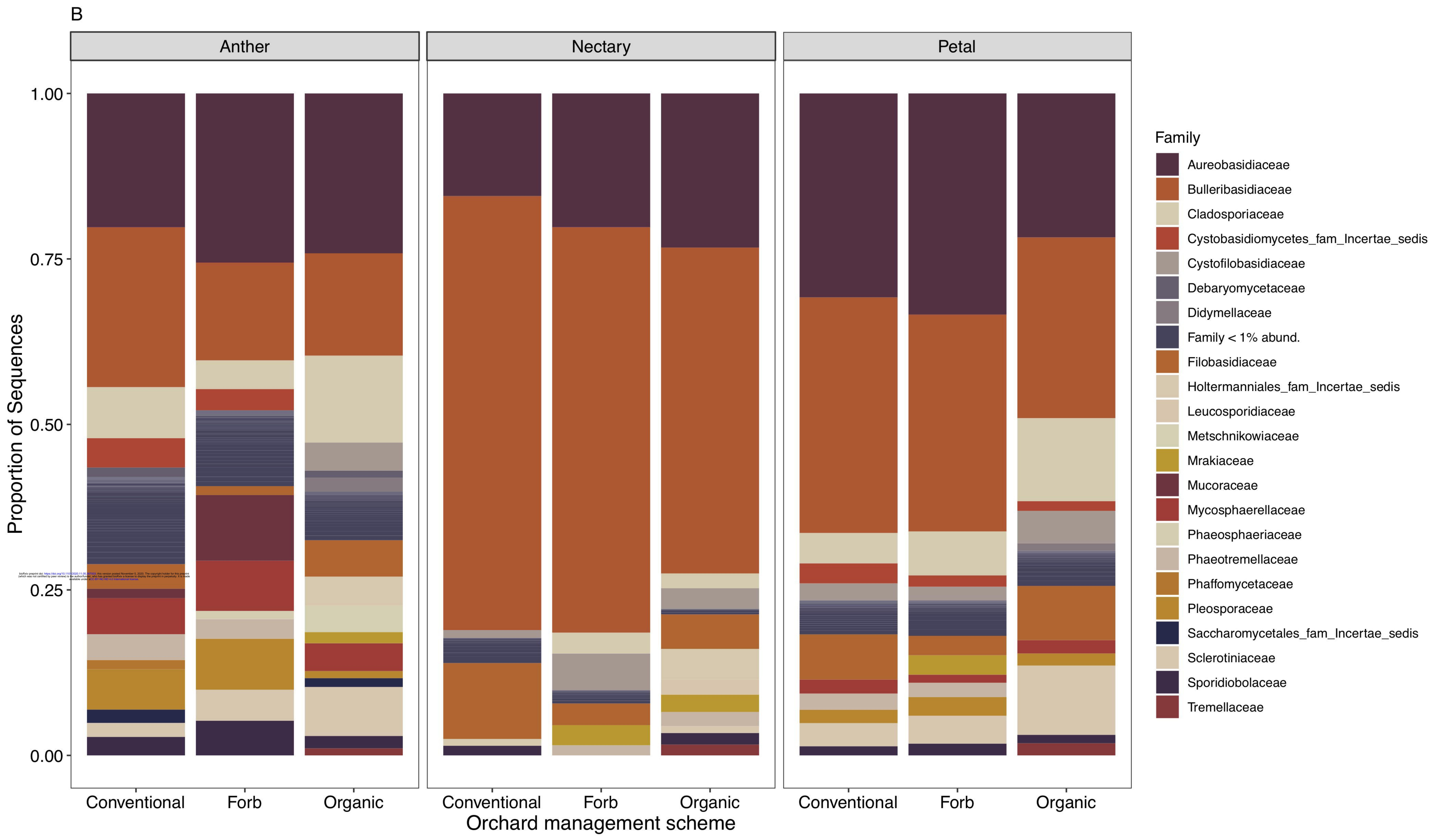
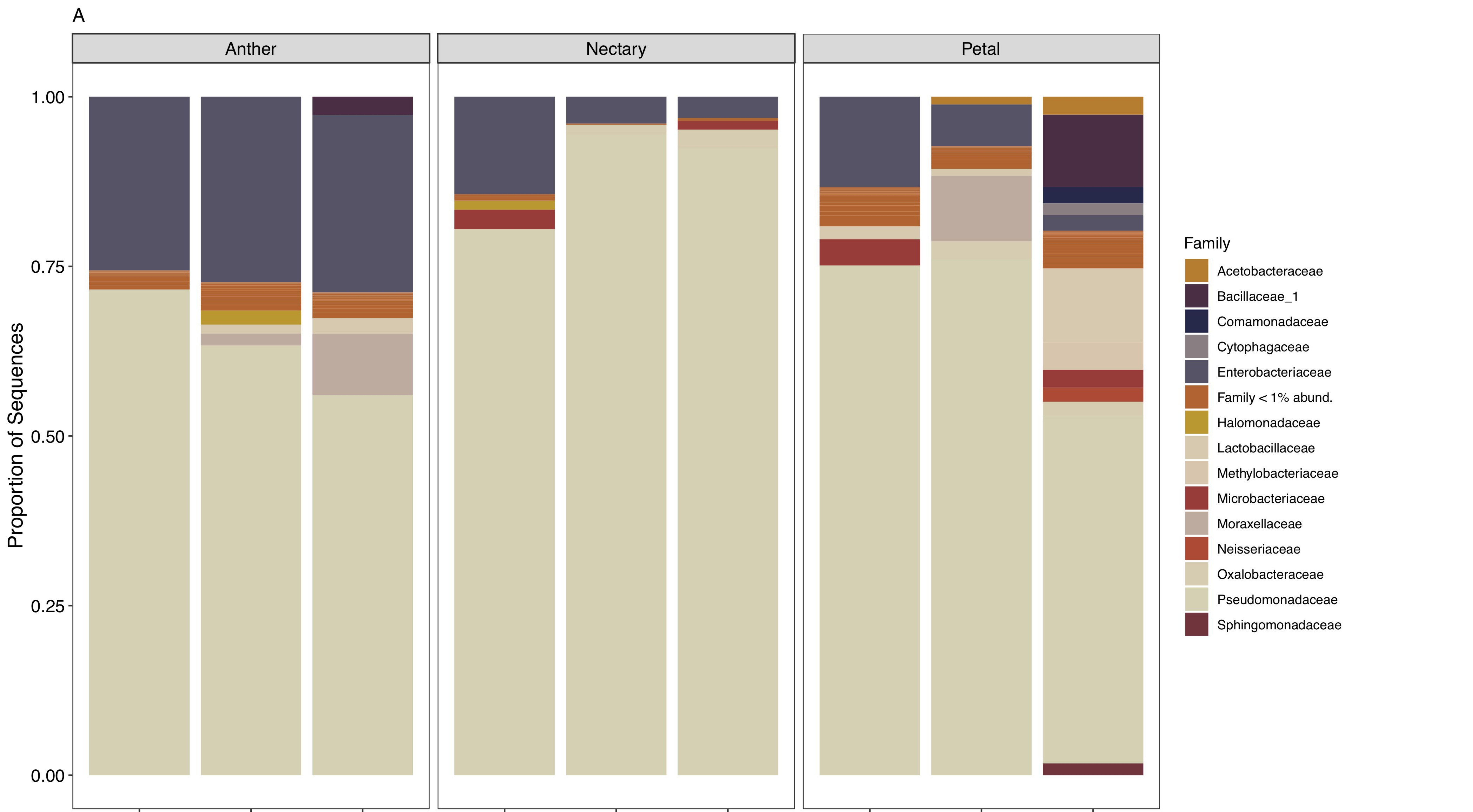
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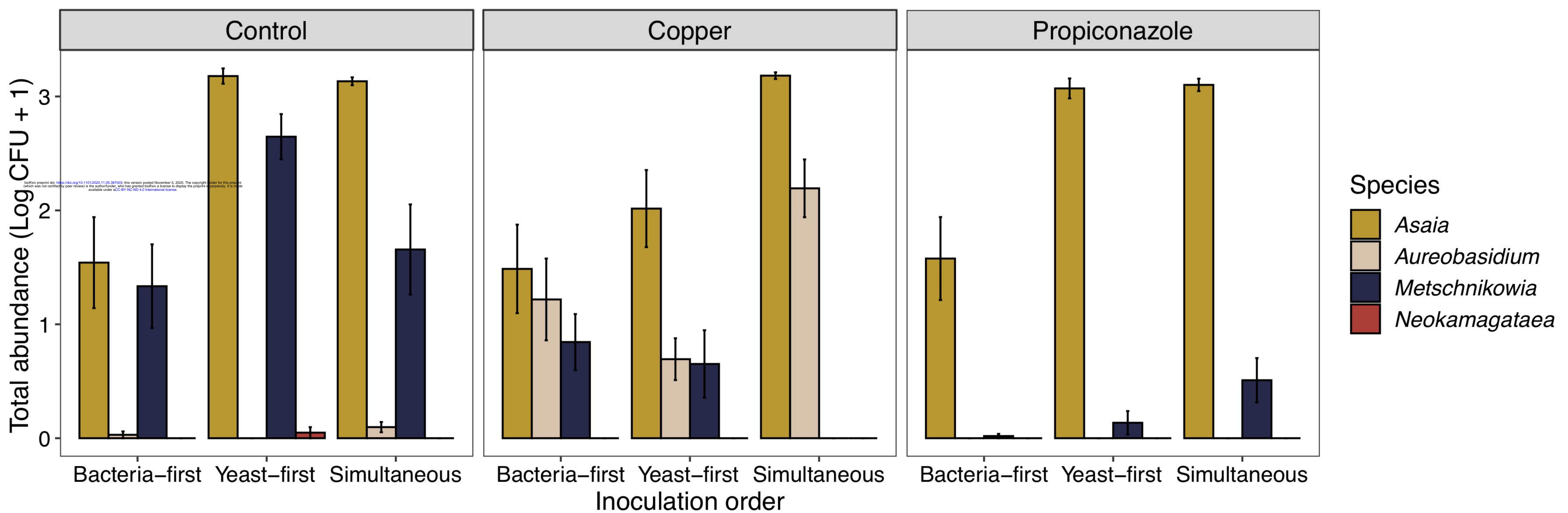
N











Nectar-inhabiting microbe

Control
Neokogamateaea
Metschnikowia

Nectar remaining (g)

1.4

1.2

1.0

Control

Copper
Fungicide

Propiconazole

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