

¹ **The effect of a commercial *Ascophyllum nodosum* extracts on tomato and pepper plant productivity and their associated fungal and bacterial communities.**

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⁸ Seaweeds have been used as a source of natural fertilizer and biostimulants for centuries. Here,
⁹ we used a commercially available *Ascophyllum nodosum* extract (ANE) in order to test its effect of
¹⁰ plant productivity in peppers and tomatoes. In addition, by using a metabarcoding high through-
¹¹ put sequencing approach, we characteristed the root and soil fungal and bacterial communities.
¹² We find that all productivity measures of root, shoot and fruit biomass differed significantly ac-
¹³ cording to species, and five of those were significantly different according to the fertilization treat-
¹⁴ ment. Local *a*-diversity was the highest in the bacteria-soil and fungi-soil samples, and the lowest
¹⁵ in the bacteria-root. In addition, *a*-diversity differed according to the fertilization treatment, but
¹⁶ this effect were small. Species composition among sites (*b*-diversity) differed according to the fer-
¹⁷ tilization treatment in all four communities measured (fungal-root, fungal-soil, bacterial-root and
¹⁸ bacterial-soil). Finally we identify a number of candidate taxa (Amplicon Sequence Variants) most
¹⁹ strongly associated with measures of productivity. Further studies for example using inoculum of
²⁰ microbial species linked to the presence of liquid seaweed extract may help to identify a causative
²¹ link between extracts, microbes and productivity.

²² *Keywords:* Stella Maris®, 16S, ITS, soil microbial diversity, Illumina MiSeq, ANE, Amplicon Se-
²³ quence Variants, OTU

24 INTRODUCTION

25 Seaweeds (also known as marine macroalgae) have been used as a source of organic matter and
26 nutrients for centuries, especially in coastal areas (Khan et al., 2009; Craigie, 2011). Liquid seaweed
27 extracts, developed in the 1950s in order to concentrate plant growth-stimulating compounds, fa-
28 cilitate their usage (Milton, 1952) and today, most commercially available extracts are made from
29 the brown algae *Ascophyllum nodosum*, *Ecklonia maxima* or *Laminaria spp.* One of the main ad-
30 vantages of seaweed extracts is that they are biodegradable, non-toxic and come from a renewable
31 resource, unlike modern chemical fertilizers (Dhargalkar & Pereira, 2005). Industry-funded bodies
32 such as the European Biostimulant Industry Coalition and the United States Biostimulant Coal-
33 ion have been working to accommodate biostimulants into mainstream legal architecture. These
34 organizations extoll benefits arising from modes-of-action research, agricultural applications and
35 positive effects on yield and quality of many commercial species (i.e. fruits, vegetables, turf and
36 ornamentals, and woody species). Legal recognition will further allow a fluid integration of vari-
37 ous biostimulants, including *Ascophyllum nodosum* Extracts (ANE) into sustainable long-term crop
38 management programs (Craigie, 2011; Jardin, 2015).

39

40 Several comprehensive reviews have described seaweed extracts and their effect on agricultural
41 plant productivity (Khan et al., 2009; Craigie, 2010, 2011; Battacharyya et al., 2015). The science
42 points to wide-ranging effects from biotic to abiotic resistance, effects on growth and develop-
43 ment, and ultimately, to their impact on plant establishment, crop yield and/or quality, and shelf
44 life. At the physiological level, these extracts have been found to influence hormonal changes that
45 in turn, influence physiological processes even at very low concentrations (Wally et al., 2013).

46

47 Starting in the 1990's, the development of high quality ANE has led to an increase in cause-effect
48 research, especially on plant diseases (Jayaraj & Ali, 2015). Noted increases in the activity of super-
49 oxide dismutase, glutathione peroxidase and ascorbate peroxidase helped support the argument
50 that ANE improve plant tolerance to oxidative stress (Ayad et al., 1997; Schmidt & Zhang, 1997;
51 Ayad, 1998; Allen et al., 2001). Positive effects were also found on phytoalexin production (Lizzi
52 et al., 1998; Jayaraj et al., 2008; Jayaraman, Norrie & Punja, 2010) suggesting that ANE may be

53 involved in suppressing disease infection through increased activity of these protective enzymes
54 that target oxidizing toxins naturally emitted by disease pathogens.

55

56 Improved plant stress resistance and tolerance to foliar and soil treatments is attributed to a cas-
57 cade of various physiological reactions. ANE can impact plant-signaling mechanisms through a
58 multitude of plant processes and cellular modifications including osmotic/oxidative stresses such
59 as salinity, freezing and drought stress (Jithesh et al., 2012). ANE can also impart drought-stress
60 tolerance to plants by reducing stomatal conductance and cellular electrolyte leakage (Shotton and
61 Martynenko, unpublished data; Spann & Little, 2011). These results suggest that ANE can influ-
62 ence cellular membrane maintenance leading to a higher tolerance for various osmotic stresses
63 and can mitigate oxidative damage.

64

65 Although there is an abundance of published evidence detailing systemic plant effects from ANE,
66 outstanding questions remain as to the effects of ANE on the soil rhizosphere. Various microbes,
67 small arthropods, nematodes, earthworms and insects thriving in the soil rhizosphere help con-
68 tribute to the aggregation of soil particles, enhance nutrient cycling and delivery to plants, degrade
69 toxic substances, allow better soil water and play a role in plant disease management. An exam-
70 ination of sustainable products that can positively influence microbial interactions between plant
71 roots and soil biota will in turn help to further understand soil borne plant-pathogens competition
72 dynamics. It has been suggested that the plant immune system is composed of inherent surveil-
73 lance systems that perceive several general microbial elicitors, which allow plants to switch from
74 growth and development into a defense mode (Newman et al., 2013). This may allow the plant
75 to avoid infection from potentially harmful microbes. The effect of ANE on the bacterial profile
76 suggests that ANE applications increased strawberry root and shoot growth, berry yield and rhi-
77 zosphere microbial diversity and physiological activity (Alam et al., 2013). Similar results were
78 found for sandy loam soils growing carrots as Alam et al. (2014) showed a strong relationship
79 between carrot growth, soil microbial populations and activity.

80

81 The recent development of culture-independent molecular techniques and high throughput se-
82 quencing will permit to circumvent the inherent biases of culture-based approaches by targeting

83 the ubiquitous component of life, its DNA. In turn, this should permit to identify a larger propor-
84 tion of the microbial diversity and lead to a better understanding of the soil microbial response
85 to seaweed extract. DNA barcoding targeting the internal transcribed spacer (ITS) region of the
86 nuclear ribosomal repeat and the bacterial V3-V4 region of the 16S ribosomal gene for fungi and
87 bacteria, respectively, are now regarded as a prerequisite procedure to comprehensively document
88 the diversity and ecology of microbial organisms (Toju et al., 2012; Klindworth et al., 2013).

89

90 Here the general objective was to quantify the impact of ANE on plant growth and test how the
91 bacterial and fungal communities responded to the addition of theses extracts. We also aimed to
92 identify specific taxon positively associated with increased in plant productivity following addi-
93 tion of ANE. We hypothesized that the inclusion of liquid seaweed extracts would improve pro-
94 ductivity and alter significantly the bacterial and fungal communities. We used a commercially
95 available ANE, Stella Maris®, developped by Acadian Plant Health (NS, Canada). Stella Maris®
96 is derived from the marine algae *A. nodosum*, and harvested from the nutrient-laden waters of the
97 North Atlantic off the Eastern Coast of Canada. We tested the effect of ANE on two commonly
98 used plants (tomato and pepper) using several measures of plant productivity and by measuring
99 soil and root bacterial and fungal diversity using High Throughput Illumina Miseq sequencing.

100

101

102 MATERIAL AND METHOD

103 *Experimental design*

104 Greenhouse experiments were set up in large trays (60x30x18 cm LxWxH) using two different
105 crops: tomato (*Solanum lycopersicum* L.) and pepper (*Capsicum annuum* L.). Tomato cultivar Totem
106 Hybrid#A371 was planted in November 16th 2015, while pepper cultivar Ace Hybrid#318 was
107 planted in December 9th 2015. Tomato and pepper seeds were purchased from William Dam
108 Seeds Ltd (ON, Canada). These cultivars were selected for greenhouse production. Soil was col-
109 lected from an agricultural field under organic regime at the IRDA research station in St-Bruno
110 (Qc, Canada, 45°32'59.6"N, 73°21'08.0"W) on October 7th 2015. The soil was a loamy sand and
111 was collected from the 15 cm top layer. Natural soil was mixed and put into trays, filled to 15 cm
112 in height. Soil analysis was done using a commercial service provided by AgriDirect (Longueuil,
113 QC) and soil characteristics are shown in Table 1. Eight seeds per tray were planted and after
114 germination, only four seedlings per tray were kept.

115

116 For each crop species, a randomized split block design (Table S1) was used with four trays set
117 up per block and eight blocks for each experiment. Half of the trays were fertilized (fertilization
118 treatment), as described below. Half of the trays were also planted (planting treatment) with four
119 plants per tray, while the other trays were not planted. This allowed a direct comparison of fungal
120 and bacteria soil communities with respect to fertilization and planting treatments.

121

122 Two different fertilization regimes were used according to the plant species. For tomatoes, plants
123 were fertilized using multipurpose organic fertilizer (pure hen manure, 18 g per tray repeated ev-
124 ery 4 weeks, 5-3-2) from Acti-sol (Notre-Dame-du-Bon-Conseil, QC) in addition to Stella Maris®
125 (3.5 ml per 1L, each tray received 250 ml, repeated every 2 weeks) for the duration of the ex-
126 periment. The other half was not fertilized. Stella Maris® is a commercial *Ascophyllum nodosum*
127 seaweed based product and its physical and chemical analyses are shown in Table S2 WHERE?.
128 For the pepper experiment, the fertilization regime consisted solely of Stella Maris® (3.5 ml per
129 1L, each tray received 250 ml, repeated every 2 weeks) for the duration of the experiment. The
130 other half was not fertilized. Both experiments were managed under organic farming practices.

131 Thrips were controlled using *Neoseiulus cucumeris* (syn. *Amblyseius cucumeris*) (1 bag per plant),
132 Fungus gnats were also controlled using predatory mite *Gaeolaelaps gillespiei* (1L; Natural Insect
133 Control, ON). Plants were treated once a week with Milstop, a Potassium Bicarbonate-based foliar
134 fungicide to control the powdery mildew on both crops.

135

136 *Plant productivity*

137 Tomato and pepper experiments were harvested on March 29th 2016. Measuring the following
138 traits assessed plant productivity: fruit number, fruit weight, shoots fresh weight and roots fresh
139 weight. Traits were measured on three plants chosen randomly per tray for each fertilization-
140 control treatment, crop (tomato/pepper) and block (eight blocks) for a total of 96 samples. In
141 addition, both shoots and roots were dried in a 70 degrees drying oven, and dry weights were
142 quantified after 48 hours. Together, these traits are expected to represent well the plant overall
143 productivity.

144

145

146 *Sample preparation, DNA extraction and High throughput sequencing*

147 Soil and root samples were taken for both experiments. Soil DNA was extracted using NucleoSpin®
148 Soil DNA extraction kit (Macherey-Nagel, BioLinx, ON) on 250 mg of soil, following the manu-
149 facturer's instructions. Roots were first washed with tap water and rinsed with sterile water.
150 Chopped roots sub-samples (100 mg) were subjected to DNA extraction using DNeasy Plant Mini
151 kit (Qiagen Inc - Canada, ON), following the manufacturer's recommendations. Amplicon se-
152 quencing targeting bacterial 16S rRNA gene and fungal ITS was performed on both root and soil
153 samples.

154

155 For fungal ITS, we used the following primers with the universal CS1 and CS2 adapters: CS1_ITS3_KYO2
156 (5'-ACA CTGA CGA CAT GGT TCT ACA GAT GAA GAA CGY AGY RAA-3') and CS2_ITS4_KYO3
157 (5'-TAC GGT AGC AGA GAC TTG GTC TCT BTT VCC KCT TCA CTC G-3') to produce a final
158 amplicon size of approximately 430bp including adapters (Toju et al., 2012).

159

160 For bacterial 16S, we used the following primers with CS1 and CS2 universal adapters: 341F (5'-

¹⁶¹ CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TACC AGG GTA TCT AAT C-3') to produce
¹⁶² a final amplicon size of approximately 460 bp and targeting specifically the bacterial V3-V4 region
¹⁶³ of the 16S ribosomal gene (Klindworth et al., 2013).

¹⁶⁴

¹⁶⁵ DNA samples were then barcoded, pooled and sequenced (2X300bp, paired-end) using an Il-
¹⁶⁶ lumina (San Diego, CA, USA) MiSeq sequencer through a commercial service provided by the
¹⁶⁷ Genome Quebec Innovation Centre (Montreal, QC). Sequences were demultiplexed by the se-
¹⁶⁸ quencing facility and further processed as described below.

¹⁶⁹

¹⁷⁰ *Bioinformatics*

¹⁷¹ All bioinformatics, statistical, and graphical analyses further described were performed in R 3.5.1
¹⁷² (Team, 2018) and detailed scripts are available here (https://github.com/seb951/Acadian_Seaplants).

¹⁷³

¹⁷⁴ We used the R package dada2 (Callahan et al., 2016) to infer *Amplicon Sequence Variants* (ASVs).
¹⁷⁵ Dada2 offers accurate sample inference from amplicon data with single-nucleotide resolution in an
¹⁷⁶ open source environment. Unlike the Operational Taxonomic Unit (OTU) approach (e.g. Schloss et
¹⁷⁷ al., 2009; Caporaso et al., 2010), ASV are not treated as cluster of sequences defined with an *ad hoc*
¹⁷⁸ sequence similarity threshold. Instead, after sequences are quality trimmed and error-corrected,
¹⁷⁹ dada2 reveals the unique members of the sequenced community, thus allowing sequences and
¹⁸⁰ abundance counts to be compared among studies (Callahan et al., 2016).

¹⁸¹

¹⁸² First, sequences were trimmed following strict quality thresholds (removing primers and low
¹⁸³ quality nucleotides, see parameter details in the accompanying R scripts). Following this, we
¹⁸⁴ applied the error model algorithm of dada2 which incorporates quality information after filter-
¹⁸⁵ ing, unlike other OTU based methods. Then dereplication, sample inference, merging of paired
¹⁸⁶ end reads and removal of chimera were performed in order to obtain a sequence (ASVs) table of
¹⁸⁷ abundance per sample. Taxonomy was also assigned using the Ribosomal Database Project (RDP)
¹⁸⁸ Naive Bayesian Classifier algorithm from Wang *et al.* (2007). Depending on support (minimum
¹⁸⁹ bootstrap support of 80), we assigned taxonomy from Kingdom to species. We used the silva
¹⁹⁰ database formatted for dada2 to infer bacterial taxa (Callahan, 2018). We used the Unite (Com-

191 munity, 2018) fasta release (including singletons) to infer fungal taxa after formatting it to the
192 dada2 format using a custom R script. The pipeline was run on a multithreaded (48 CPUs) com-
193 puter infrastructure provided by Westgrid (<https://www.westgrid.ca/support/systems/cedar>)
194 and Compute Canada (www.computecanada.ca). Note that the pipeline was run separately for
195 fungal-root, fungal-soil, bacteria-soil and bacteria-root samples given the markedly different nu-
196 cleotide compositions of the sequenced amplicons, unique taxa and specific error models of each
197 dataset.

198

199 *Statistical analyses - plant productivity*

200 We tested for the effect of species (tomato vs pepper), fertilization and their interaction on six plant
201 productivity measures (fruit number, average fruit weight, shoots fresh weight, roots fresh weight,
202 shoots dry weight, roots dry weight). We used linear mixed effect models (LMM) in the R package
203 nlme (Pinheiro et al., 2017), which are more appropriate than an Analysis of Variance (ANOVA)
204 given the current block design (blocks and replicates nested within a block were treated as random
205 variables). All six plant productivity measures were either square root or log transformed in or-
206 der to help satisfy the assumption of normality of the residuals in the LMM statistical framework.
207 For the variables *fruit number* and *average fruit weight*, we also used a permutation-based 2-way
208 ANOVA (Anderson & Legendre, 1999) given that the residuals of the LMM were not normally
209 distributed (results were similarly significant).

210

211 *Statistical analyses - microbial and fungal diversity*

212 Fungal-root, fungal-soil, bacterial-root and bacterial-soil ASV diversity was measured separately.
213 For each of these four datasets, we removed samples that showed poor sequencing output and
214 contained few ASVs. In order to do this, we summed the abundance of all ASVs for each sam-
215 ple ($\sum_{i=1}^n ASV$) and eliminated samples that had fewer than the mean sum minus four standard
216 deviations ($\overline{\sum_{i=1}^n ASV} - 4\sigma$). In addition, we removed ASVs from our dataset that were present
217 in fewer than 5% of the samples (less than ten individuals in the soil samples or less than five in
218 the root samples). This was done to remove very rare ASVs unique to a block or replicate, but not
219 found in the majority of samples.

220

221 We then conducted community-based analyses looking at the effect of the fertilization treatment
222 on the ASV taxa in the tomato and pepper experiments. To reduce the complexity of the datasets,
223 relative abundance of all taxa was calculated per family using the R package `dplyr` (Wickham et
224 al., 2015). Barplots were drawn using `ggplot2` (Wickham, 2016) to visualize communities. ASV
225 alpha (α)-diversity was calculated for each sample using the inverse Simpson diversity index in
226 `vegan` (Oksanen et al., 2013). The effect of the fertilization treatment, species (and planting for
227 soil communities) were assessed using a linear mixed-effect (LMM) model in the R package `nlme`
228 (Pinheiro et al., 2017), given the unbalanced, replicated block design. Alpha diversity was log
229 transformed in order to help satisfy the assumption of normality of the residuals of the LMM sta-
230 tistical framework.

231

232 Using the community matrix data of ASVs abundance, we performed PERmutational Multivari-
233 ate ANalysis Of VAriance tests (PERMANOVA; Anderson, 2001) to identify relationships between
234 the communities according to the experimental design. ASV abundance data was Hellinger-
235 transformed and significance was assessed using 10,000 permutations in `vegan` (Oksanen et al.,
236 2013). Blocks and replicates nested within blocks were factored as strata (blocks) in the model.

237

238 We also performed canonical correspondence analyses (CCAs) using Hellinger-transformed ASV
239 abundance data in `vegan` (Oksanen et al., 2013) to visually assess the grouping of samples, ASVs
240 and their association with productivity variables (*species* scaling based on ASV matrix). Data were
241 analyzed separately for fungal-root, fungal-soil, bacterial-root and bacterial-soil, but also accord-
242 ing to species (tomato/pepper), given that analyses of α -diversity showed that tomato and pep-
243 per were markedly different. This gave a total of eight CCAs. Data were constrained based on
244 four productivity measures (fruit number, average fruits weight, shoots fresh weight, roots fresh
245 weight). We excluded the shoot & root dry weights as constraints to simplify the model. In addi-
246 tion, these were highly correlated with the fresh weight already included as constraints ($r^2=0.98$
247 and 0.76 for shoot dry/fresh weights and root dry/fresh weights, respectively).

248

249 Finally, we attempted to identify candidate ASVs positively associated with productivity. As such,
250 we identified the ten ASVs most positively associated with the measures of fruit number, shoots

251 fresh weight and roots fresh weight from each canonical correspondence analysis for a total of 40
252 fungal and 40 bacterial candidate ASVs. We aligned candidate sequences from these candidates
253 ASVs using the Bioconductor R package `decipher` (Wright, 2016) and build pairwise distances
254 matrices using a JC69 substitution models of DNA sequence evolution (equal base frequencies,
255 (Jukes & Cantor, 1969) in `phangorn` (Schliep, 2010). Phylogenetic trees for bacteria and fungi were
256 plotted using `ape` (Paradis, Claude & Strimmer, 2004). This permitted to identify if similar candi-
257 date ASVs were found under different experimental conditions (soil/root, pepper/tomato), thus
258 reinforcing their role in productivity increase, and decreasing the false positive rate.

259

260 **RESULTS**

261 *Soil characteristics*

262 In Table 1, we present the characteristics of the soil which was collected at the IRDA research
263 station in St-Bruno (Qc, Canada) and used in the current experimental design.

264

Table 1: Soil characteristics (in ppm unless specified otherwise)

Soil Characteristics	Average value
pH	6.01
Conductivity (mmhos/cm)	0.68
Nitrate (N)	62.40
Ammonium	0.09
Phosphorus	0.41
Potassium	29.30
Calcium	64.40
Magnesium	13.80
Chloride	28.50
Sulfate	19.30
Sodium	17.80
Zinc	0.12
Manganese	0.06
Cooper	0.81
Iron	0.90
Aluminium	1.66

265

266

267 *Plant productivity*

268 The effects of the fertilization treatment were tested on overall plant growth and six measures of
269 productivity (i.e. fruit number, average fruit weight, shoots fresh weight, shoots dry weight, roots
270 fresh weight, roots dry weight) for both tomato and peppers. Visually, both above ground and
271 below ground plant structure grew larger in fertilized tomato (hen manure + ANE) and pepper
272 plants (ANE only), in addition to producing more fruits (see Figure 1 for some examples of the
273 differences between fertilized and unfertilized plants).

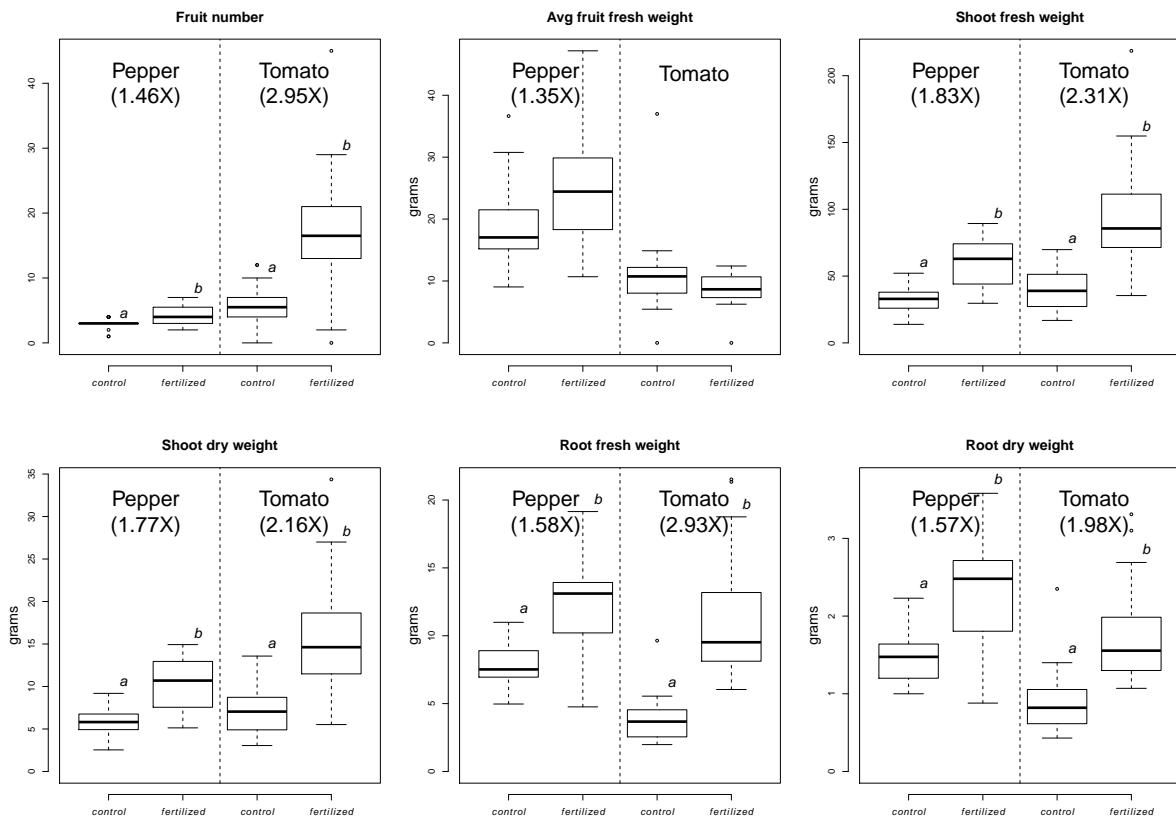


274

275 **Figure 1: Plant productivity. Photos were taken at the end of the experimental treatment. In**
276 **each photo, fertilized plants are on the left. A: pepper plants, B: pepper roots, C: pepper fruits**
277 **and D: tomato fruits.**

278

279 Statistically, all six productivity measures differed significantly according to species, and five of
280 those were significantly different according to the fertilization treatment (Figure 2). The fertil-
281 ization effect was stronger in the tomato plants (see fold changes in Figure 2), likely due to the
282 fact that these plants were fertilized with both hen manure and ANE. The only exception was
283 the average fruit weight that did not differ between fertilized and control plants (LMM, $F_{(1,69)} =$
284 1.27 , $p\text{-value}=0.26$). However, the model did reveal a significant interaction between treatment
285 and plant ($F_{(1,69)} = 9.6$, $p\text{-value}=0.0028$). In fact, when testing only the pepper plants, the effect of
286 fertilization on average fruit weight was significantly higher in the fertilized pepper plants ($F_{(1,23)}$
287 $= 10.84$, $p\text{-value}=0.0032$).



290 **Figure 2: Measures of plant productivity. a and b subscripts above boxplots denote significant
291 differences. Fold changes between the mean of the control and fertilized plants were also noted
292 for significant changes (for pepper and tomato separately).**

295 *Sequencing*

296 A total of 2.7 million paired-end raw reads were obtained for all samples combined (976,000 for
297 fungi-soil, 920,000 for fungi-root, 309,000 for bacteria-soil and 535,000 for bacteria-root, Table 2).
298 Note that sequencing samples were analyzed separately for fungal-soil, fungal-root, bacteria-soil
299 and bacteria-root conditions. On average, 47,664 paired-end reads were obtained per sample. Af-
300 ter quality filters were applied, including removing chimeras, and paired-end reads were merged,
301 an average of 19,690 sequences remained per sample. From 192 soil samples for fungi and bacte-
302 ria, and 96 root samples for fungi and bacteria sequenced, seven fungi-soil samples, 15 fungi-root
303 samples and one bacteria-root samples were removed because they had too few reads based on our

304 strict quality thresholds.

305

306 The dada2 pipeline inferred, on average, 170 Amplicon Sequence Variants per sample (average
307 of 176 fungal-soil ASV, 37 fungal-root ASVs, 269 bacterial-soil ASVs and 92 bacterial-root ASVs).
308 Many of those were unique to one of a few samples (total number of 6,112 fungal-soil, 845 fungal-
309 root, 9,352 bacterial-soil and 2,023 bacterial-roots ASVs). After quality filtering ASVs found in
310 fewer than 10% of the samples, we retained 413, 106, 811 and 325 ASVs. These retained ASVs
311 comprised 94%, 95%, 89% and 98% of all reads in the fungal-soil, fungal-root, bacterial-soil and
312 bacterial-root samples, respectively.

313

314

Table 2: Sequencing and ASV summary

	fungi-soil	fungi-root	bacteria-soil	bacteria-root
No sequences (sum)	976,000	309,000	920,000	535,000
No sequences (mean)	50,847	32,208	47,907	56,365
No seq. filtered (mean)	32,626	12,714	29,662	37,642
No seq. filt. merged (mean)	29,300	12,094	14,060	30,706
No seq. filt. merg. no chimeras (mean)	25,476	9,849	13,521	30,408
No samples	192	96	192	96
No samples trimmed	189	81	192	95
No ASVs (sum)	6,112	845	9,352	2,023
No ASVs trimmed (sum)	413	106	811	325
ASV per sample (mean)	176	37	269	92

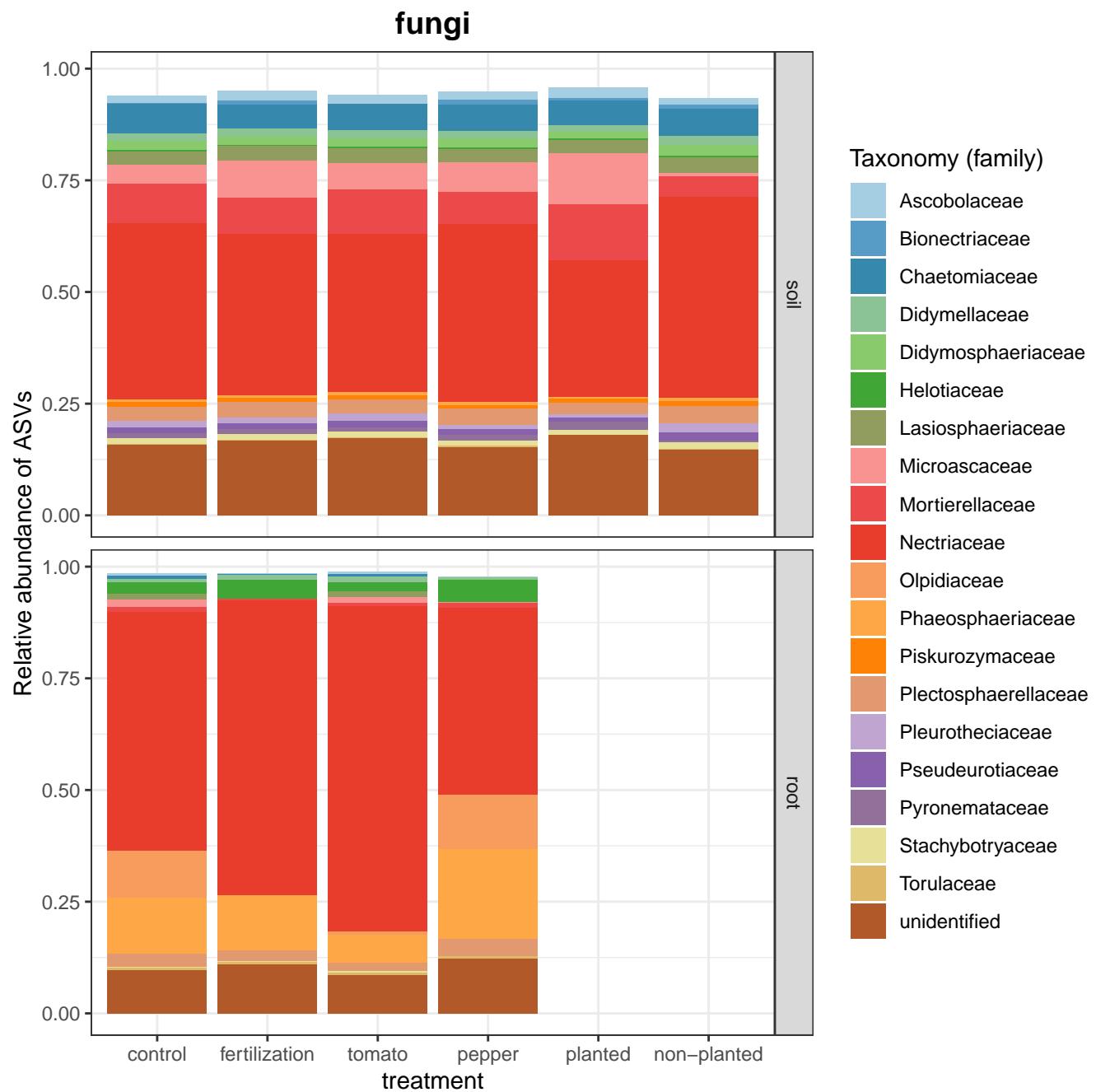
315

316 *Root, soil, microbial and bacterial diversity*

317 The entire community structure that was measurable in the soil was then analyzed and the relative
318 abundance of taxa (family) for the fungal-soil, fungal-root, bacteria-soil and bacteria-root condi-

319 tions was reported (Figure 3a & b). Fungal communities were dominated by Nectriaceae, both
 320 in the root and soil samples. The bacterial family Bacilaceae dominated to a lesser extent the soil
 321 communities. Bacterial root communities were largely dominated by the Cyanobacteria phylum
 322 (identified as *chloroplast* in the silva database according to the RDP Bayesian Classifier).

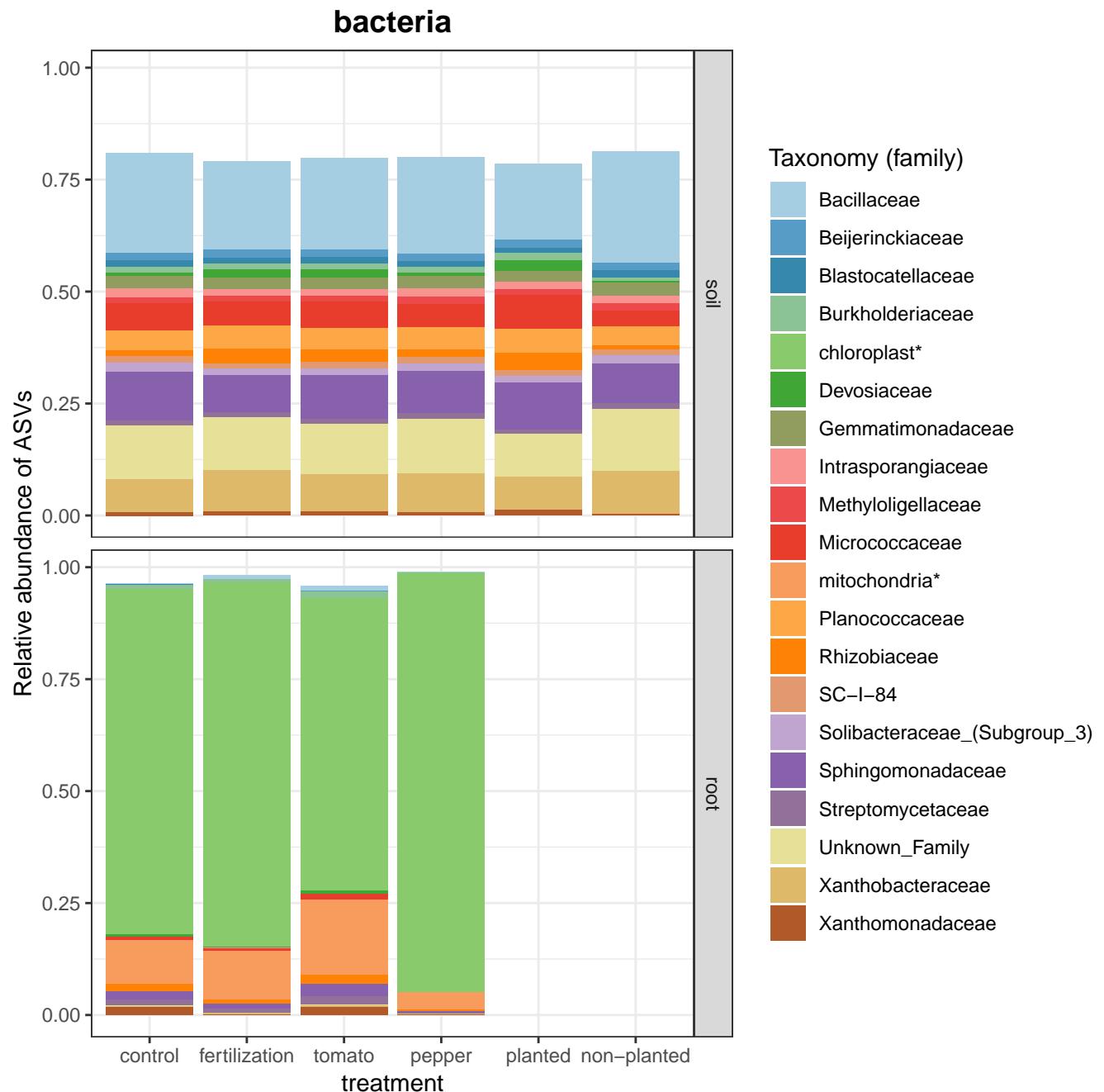
323



324

325 **Figure 3a: Barplots of the relative abundance of fungal ASVs for fungi**

326



327

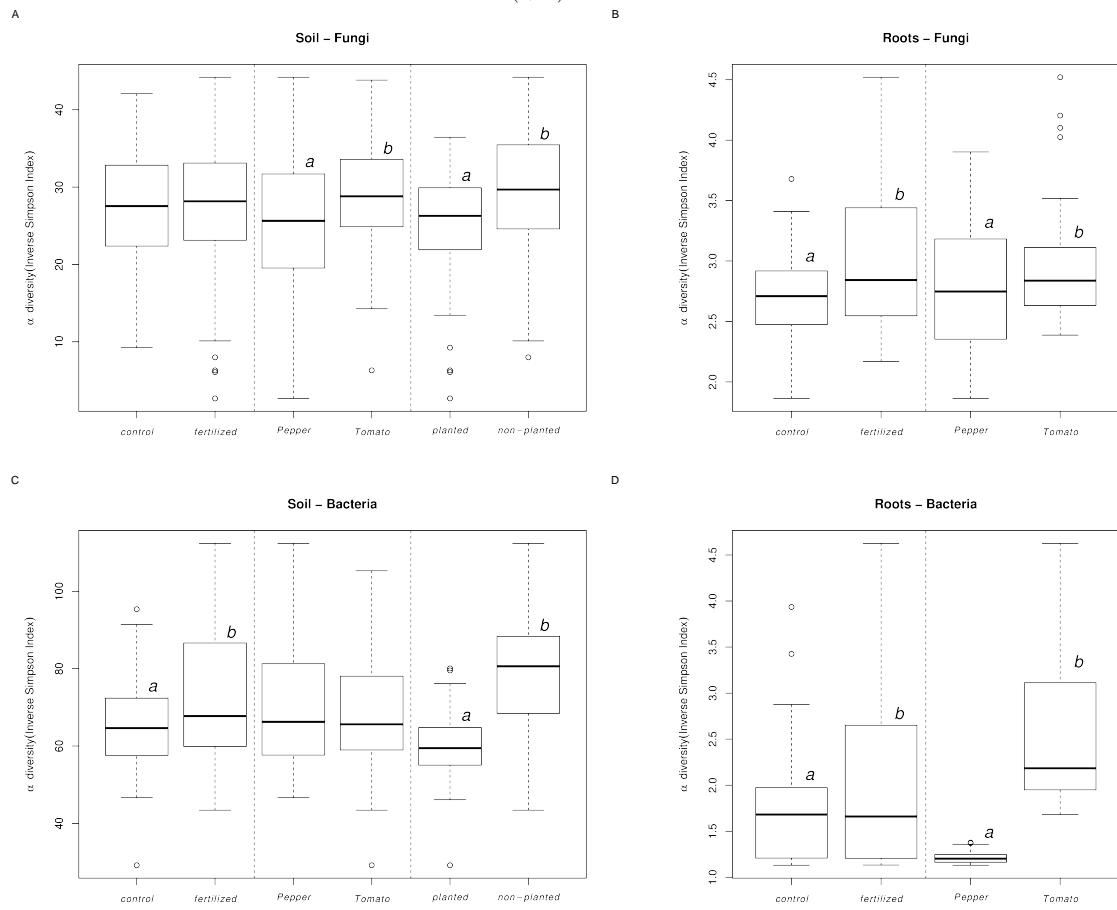
328 **Figure 3b: Barplots of the relative abundance of bacterial ASVs for bacteria**

329

330 *Local (a-diversity)*

331 The diversity of each site (*a*-diversity) was calculated separately for each sample and under each
 332 experimental condition (fungi-soil, fungi-root, bacteria-soil and bacteria-root, Figure 4). Total *a*-
 333 diversity was the highest in the bacteria-soil and fungi-soil samples, and the lowest in the bacteria-

root. Linear mixed effects models were used to assess significance. In soil samples, fungal diversity did not differ with respect to the fertilization ($F_{(1,161)}=0.17$, $p\text{-value}=0.6853$), but did so with respect to planting ($F_{(1,161)}=9.00$, $p\text{-value}<0.0032$) and species ($F_{(1,161)}=13.03$, $p\text{-value}=0.0003$) treatments. In root samples, fungal diversity differed with respect to the fertilization treatment ($F_{(1,56)}=10.1$, $p\text{-value}=0.003$), and the species tested ($F_{(1,56)}=4.5$, $p\text{-value}=0.04$). In soil samples, bacterial diversity differed with respect to the fertilization ($F_{(1,165)}=17.13$, $p\text{-value}<0.0001$), planting ($F_{(1,165)}=139.0$, $p\text{-value}<0.0001$) but not species ($F_{(1,165)}=1.89$, $p\text{-value}=0.17$) treatments. In root samples, bacterial diversity differed with respect to the fertilization treatment ($F_{(1,67)}=17.27$, $p\text{-value}=0.0001$), and the species tested ($F_{(1,67)}=359.69$, $p\text{-value}<0.0001$).



343

344 **Figure 4: Boxplot of alpha diversity according to the treatment, species and planting effect for**
 345 **fungal-root, fungal-soil, bacteria-soil and bacteria-root. *a* and *b* subscripts above boxplots de-**
 346 **note significant differences.**

347

348

349 *Differences in species composition among sites*

350 Using a PERMANOVA statistical framework, we identified that for all conditions, communities
 351 differed with respect to the fertilization treatment (Table 3). Soil fungal and bacterial communities
 352 differed the most according to whether the tray was planted (greatest % of variance explained by
 353 factor, Table 3), while root communities differed most with respect to the species (tomato/pepper)
 354 factor.

Table 3: summary of the factors tested in the PERMANOVAs
 $(r^2$ and p -values)*

	fungi-soil	fungi-root	bacteria-soil	bacteria-root
fertilization	0.02 (2e-04)	0.08 (1e-04)	0.04 (1e-04)	0.07 (1e-04)
planted	0.21 (1e-04)	NA	0.13 (1e-04)	NA
species	0.02 (1e-04)	0.26 (1e-04)	0.02 (3e-04)	0.52 (1e-04)
fertilization:planted	0.01 (0.003)	NA	0.02 (1e-04)	NA
fertilization:species	0.01 (0.006)	0.04 (0.002)	0.03 (1e-04)	0.05 (2e-04)
planted:species	0.01 (0.09)	NA	0.01 (0.004)	NA
fertilization:planted:species	0.01 (0.16)	NA	0.01 (0.04)	NA

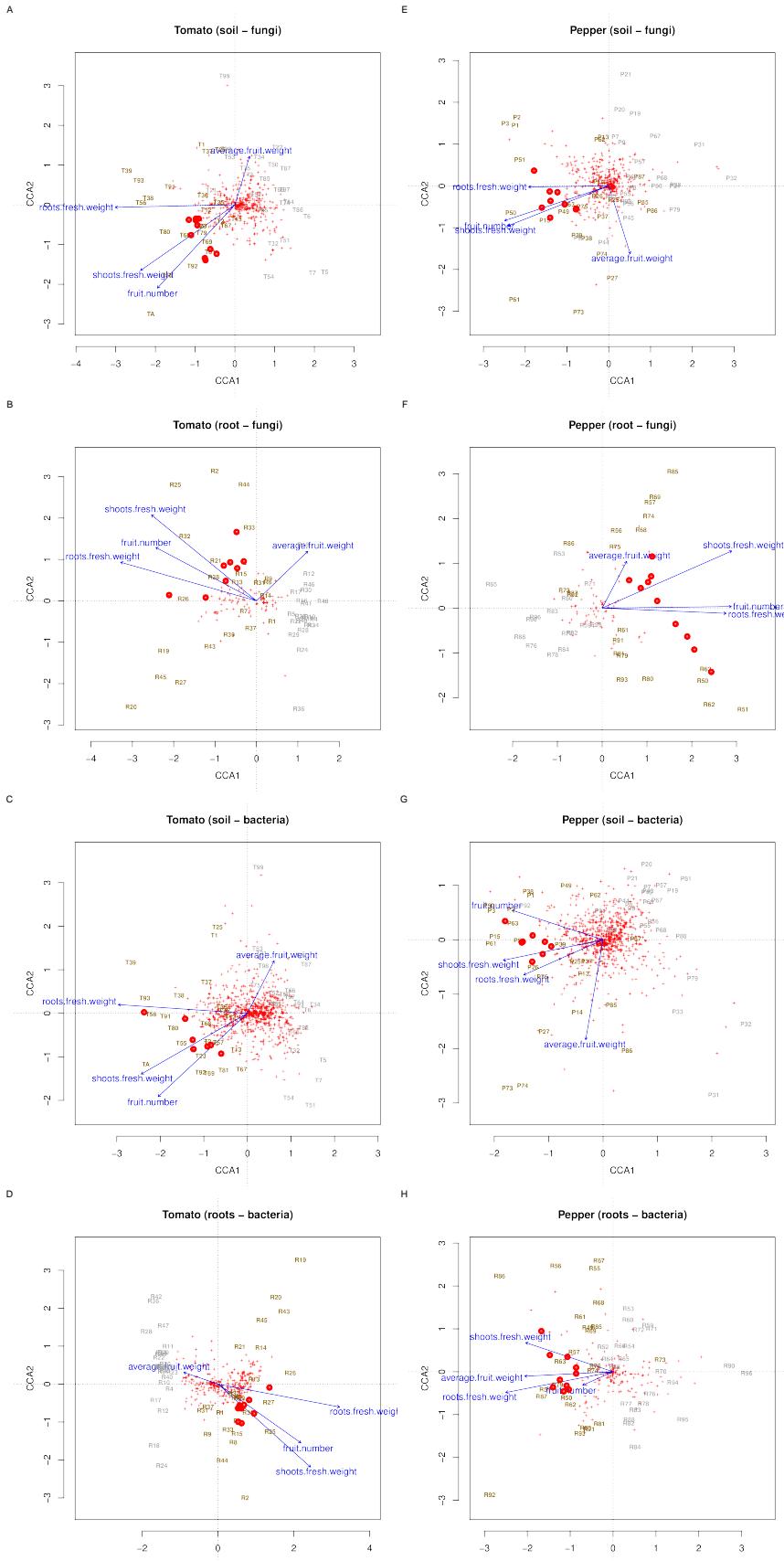
355 * r^2 (percentage of variance explained by the term in the model) and associated p -values in
 356 parentheses.

357

358 *Canonical correspondence analyses and candidate ASVs*

359 Canonical correspondence analyses (CCAs) indicated how fertilized samples clustered together
 360 according to their fungal or bacterial communities (Figure 6). It also shows a similar association of
 361 three of the constrain variables (productivity measures of root fresh weight, shoots fresh weight
 362 and fruit number), while average fruit weight behave differentially as noted previously in Figure
 363 2 (in fact nearly orthogonally to the other three constrains in most ordinations).

364

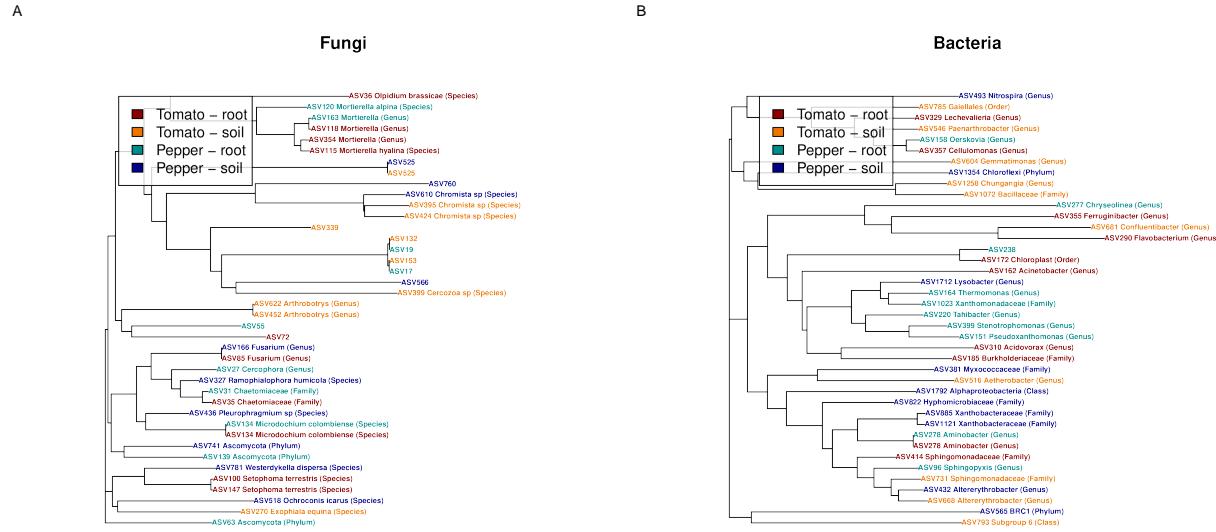


367 **Figure 6: Canonical correspondence analyses for tomato (A-D) and peppers (E-H) for soil-fungi,**
368 **root-fungi, soil-bacteria and root-bacteria.** Samples were labeled and colored in gray (unfertil-
369 **ized) or dark yellow (fertilized). Red crosses represent individual ASVs, while red points rep-**
370 **resent the ten ASVs most closely associated with the three productivity measures of root fresh**
371 **weight, shoots fresh weight and fruit number. Blue arrows are the four productivity measures**
372 **used as constrains in the ordinations.**

373

374 Next, we identified, for each ordination, the ten ASVs most closely related to the three constrains
375 of root fresh weight, shoots fresh weight and fruit number. These ASVs were considered as puta-
376 tive candidate sequences most positively impacted (increase presence of the ASV) by fertilization.
377 We further analyzed the corresponding sequences for these eighty candidate ASVs (ten candi-
378 dates * eight ordinations) in two separate alignments (one for fungi and one for bacterial ASVs)
379 and their accompanying phylogenetic trees. In fungi, we identified one cluster of ASVs taxonomi-
380 cally assigned to *Mortierella* (soil saprotrophs in the phylum Zygomycota) positively associated to
381 productivity in both tomato and pepper roots. In addition, we identified a cluster of four different
382 fungal ASVs in tomato soil (ASV132, ASV153) and pepper-root (ASV19 & AV17) closely related
383 phylogenetically. Given that no taxonomy was assigned to these sequences through the dada2
384 RDP bootstrap approach, we used a BLASTn (Altschul et al., 1997) approach (against NCBI nr)
385 to identify the most closely related sequences. We identified this cluster of ASVs as *Rhogostoma*
386 *schuessleri* (BLASTn, *e-value*=4e-76), a protist in the phylum Cercozoa, which is known to be present
387 in the rhizo and phyllo-sphere (Dumack et al., 2017). A number of putative plant pathogenic fungi
388 were also identified such as *Fusarium sp.*, *Microdochium colombiense* or *Setophoma terrestris*.
389 In bacteria-roots, we identified a large number of different ASVs most positively impacted (in-
390 crease presence of the ASV) by fertilization (Figure 6).

391



392

Figure 6: Neighbor-Joining trees of candidates ASVs (fungi & roots) associated with productivity measures. The most accurate taxonomy assigned according to the RDP bayesian classifier (from Phylum to species) was added as tip labels.

396

397 **DISCUSSION**

398 In the current study, we investigated the effects of *Ascophyllum nodosum* Extracts (ANE) on root
399 and shoot characteristics in addition to fruit yield and bacterial and fungal communities in tomato
400 and pepper. Overall measures of root, shoot and fruit productivity increased for both species fol-
401 lowing the addition of ANE. As such, our results corroborate previous studies documenting the
402 impact of ANE on productivity in strawberries (Alam et al., 2013) and carrots (Alam et al., 2014).

403

404 In the tomato experimental set up, the effect of fertilization was especially high, likely due to the
405 fact that plants were also fertilized with hen manure (5-3-2, 18g per tray / 2 weeks) in addition to
406 ANE (see Figure 2). This was not the case for the pepper plants and the increase in productivity
407 was solely due to the addition of ANE. The commercial extract (Stella Maris®, Acadian Seaplants
408 Ltd) used contained about 1% nitrogen, 0.5% phosphorus, 15% potassium, 0.4% calcium, 0.4%
409 magnesium, 155 ppm iron, 121 ppm manganese, 5 ppm copper, 91 ppm zinc, and 124 ppm boron.
410 In the current experimental setup, ANE was diluted to 3.5 g / L prior to application (250ml per
411 tray / 2 weeks). As such, these nutrients were given at very low concentrations relative to the crop
412 requirements and are not expected to significantly impact growth relative to a regular agricultural
413 fertility program (Alam et al., 2013). In fact, the amounts of Nitrogen and Phosphorus supplied
414 via the application of ANE were ~100 less than from the hen manure itself in the tomato plants.
415 Instead, bioactive compounds such as betaines, polyamines, cytokinins, auxins, oligosaccharides,
416 amino acids and vitamins have been found to have many overall beneficial productivity effects on
417 plant growth (Khan et al., 2009; Craigie, 2010, 2011; Bhattacharyya et al., 2015)

418

419 Here, one of primary goal of the study was to document how the bacterial and fungal commu-
420 nities responded to the addition of ANE. We used a metabarcoding high throughput sequencing
421 approach targeting a DNA region specific to all fungi (ITS) and bacteria (16S). Then, we identified
422 bacterial and fungal communities using a relatively novel bioinformatics approach developed by
423 Callahan et al. (2016). The approach, based on the widely used programming language R (Team,
424 2018), identifies unique, non-clustered, sequences (ASVs) that are then comparable among stud-
425 ies. In the current study, most ASVs identified were rare and unique to one or a few sample.

426 In fact, ~90% of all ASVs were discarded given that they were found in very few samples and
427 were thus not representative of a particular experimental treatment. Yet, these ASVs comprised a
428 small minority of all sequencing reads (~5% of all sequences). In addition, the current analytical
429 pipeline used a bayesian classifier approach to taxonomy rather than the widely used BLAST ap-
430 proach, thus providing more conservative, but more accurate taxonomic identifications (Wang et
431 al., 2007).

432

433 The total number of ASVs per site (*a*-diversity) for bacteria and fungi differed with respect to
434 the fertilization treatment in root samples and the soil (only for bacteria), but these effects were
435 small (Figure 5). Nectriaceae, a family of fungi in the order Hypocreales and often encountered as
436 saprophytes on decaying organic matter comprised most of the diversity both in the soil on root
437 of plants (between 25-70% of the total number of sequencing reads, Figure 4a). With respect to
438 soil-bacteria, communities were much more diverse and comprised many different family (Figure
439 4b). Surprisingly, most sequencing reads in the root-bacteria communities likely originate from
440 the plants themselves (identified as chloroplastic or mitochondrial in origin in Figure 4b), despite
441 the fact that the DNA primer pair used should have primarily targeted the bacterial V3-V4 region
442 of the 16S ribosomal gene.

443

444 Species composition among sites (*b*-diversity) differed according to the fertilization treatment in
445 all four communities measured (fungal-root, fungal-soil, bacterial-root and bacterial-soil). This
446 fertilization effect was small (2-7% of variance explained in the models, Table 3), but signifi-
447 cant. Most of the variance was explained by the planting treatment in the soil and the species
448 (tomato/pepper) in the roots (Table 3).

449

450 We also aimed to identify specific taxon positively associated with increased in plant productiv-
451 ity following addition of ANE. Here we discuss some of the candidates. In fungi, we identified
452 one cluster of ASVs taxonomically assigned to *Mortierella* (soil saprotrophs in the phylum Zy-
453 gomycota) positively associated to productivity in both tomato and pepper roots. In addition,
454 we identified several fungal ASVs in tomato soil and pepper-root linked to productivity. These
455 were assigned to *Rhogostoma schuessleri* (BLASTn, *e*-value=4e-76), a protist in the phylum Cercozoa,

⁴⁵⁶ which is known to be present in the rhizo and phyllo-sphere (Dumack et al., 2017). A number of
⁴⁵⁷ putative plant pathogenic fungi were also identified such as *Fusarium sp.*, *Microdochium colombi-*
⁴⁵⁸ *ense* or *Setophoma terrestris*. In bacteria-roots, we identified a large number of different ASVs most
⁴⁵⁹ positively impacted (increase presence of the ASV) by fertilization (Figure 6).

⁴⁶⁰

⁴⁶¹ DNA barcoding is now regarded as a prerequisite procedure to comprehensively document the
⁴⁶² diversity and ecology of microbial organisms (Toju et al., 2012; Klindworth et al., 2013). Further
⁴⁶³ studies for example using inoculum of microbial species linked to the presence of liquid seaweed
⁴⁶⁴ extract may help to identify a causative link between extracts, microbes and productivity.

⁴⁶⁵

⁴⁶⁶

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470 REFERENCES

471

- 472 Alam MZ., Braun G., Norrie J., Hodges DM. 2013. Effect of ascophyllum extract application on
473 plant growth, fruit yield and soil microbial communities of strawberry. *Canadian Journal of Plant
474 Science* 93:23–36.
- 475 Alam MZ., Braun G., Norrie J., Hodges DM. 2014. Ascophyllum extract application can pro-
476 mote plant growth and root yield in carrot associated with increased root-zone soil microbial ac-
477 tivity. *Canadian Journal of Plant Science* 94:337–348. DOI: [10.4141/cjps2013-135](https://doi.org/10.4141/cjps2013-135).
- 478 Allen V., Pond K., Saker K., Fontenot J., Bagley C., Ivy R., Evans R., Schmidt R., Fike J., Zhang
479 X., others. 2001. Tasco: Influence of a brown seaweed on antioxidants in forages and livestock—A
480 review 1. *Journal of Animal Science* 79:E21–E31.
- 481 Altschul SF., Madden TL., Schäffer AA., Zhang J., Zhang Z., Miller W., Lipman DJ. 1997.
482 Gapped blast and psi-blast: A new generation of protein database search programs. *Nucleic acids
483 research* 25:3389–3402.
- 484 Anderson MJ. 2001. A new method for non-parametric multivariate analysis of variance. *Aus-
485 tral ecology* 26:32–46.
- 486 Anderson MJ., Legendre P. 1999. An empirical comparison of permutation methods for tests
487 of partial regression coefficients in a linear model. *Journal of statistical computation and simulation*
488 62:271–303.
- 489 Ayad J. 1998. The effect of seaweed extract (*ascophyllum nodosum*) on antioxidant activities
490 and drought tolerance of tall fescue (*festuca arundinacea schreb*). *Ph D Thesis, Texas Tech University*.
- 491 Ayad J., Mahan J., Allen V., Brown C. 1997. Effect of seaweed extract and the endophyte in tall
492 fescue on superoxide dismutase, glutathione reductase and ascorbate peroxidase under varying
493 levels of moisture stress. In: *Proceedings*.
- 494 Battacharyya D., Babgohari MZ., Rathor P., Prithiviraj B. 2015. Seaweed extracts as biostimu-
495 lants in horticulture. *Scientia Horticulturae* 196:39–48. DOI: [10.1016/j.scienta.2015.09.012](https://doi.org/10.1016/j.scienta.2015.09.012).
- 496 Callahan B. 2018. Silva for dada2: Silva taxonomic training data formatted for dada2 (silva
497 version 132). *Zenodo*. DOI: [10.5281/zenodo.1172783](https://doi.org/10.5281/zenodo.1172783).
- 498 Callahan BJ., McMurdie PJ., Rosen MJ., Han AW., Johnson AJA., Holmes SP. 2016. DADA2:

- 499 High-resolution sample inference from illumina amplicon data. *Nature methods* 13:581.
- 500 Caporaso JG., Kuczynski J., Stombaugh J., Bittinger K., Bushman FD., Costello EK., Fierer N.,
- 501 Pena AG., Goodrich JK., Gordon JI., others. 2010. QIIME allows analysis of high-throughput
- 502 community sequencing data. *Nature methods* 7:335.
- 503 Community U. 2018.UNITE general fasta release. version 01.12.2017. Available at <https://files.plutof.ut.ee/doi/C8/E4/C8E4A8E6A7C4C00EACE3499C51E550744A259A98F8FE25993B1C7B9E7D2170B2.zip>
- 504 Craigie JS. 2010. Seaweed extract stimuli in plant science and agriculture. *Journal of Applied*
- 505 *Phycology* 23:371–393. DOI: [10.1007/s10811-010-9560-4](https://doi.org/10.1007/s10811-010-9560-4).
- 506 Craigie JS. 2011. Seaweed extract stimuli in plant science and agriculture. *Journal of Applied*
- 507 *Phycology* 23:371–393.
- 508 Dhargalkar V., Pereira N. 2005. Seaweed: Promising plant of the millennium.
- 509 Dumack K., Flues S., Hermanns K., Bonkowski M. 2017. Rhogostomidae (cercozoa) from soils,
- 510 roots and plant leaves (*arabidopsis thaliana*): Description of rhogostoma epiphylla sp. nov. and r.
- 511 *cylindrica* sp. nov. *European journal of protistology* 60:76–86.
- 512 Jardin P du. 2015. Plant biostimulants: Definition, concept, main categories and regulation.
- 513 *Scientia Horticulturae* 196:3–14. DOI: [10.1016/j.scienta.2015.09.021](https://doi.org/10.1016/j.scienta.2015.09.021).
- 514 Jayaraj J., Ali N. 2015. Use of seaweed extracts for disease management of vegetable crops. In:
- 515 Ganesan S, Vadivel K, Jayaraman J eds. *Sustainable crop disease management using natural products*.
- 516 CAB International, 160–183.
- 517 Jayaraj J., Wan A., Rahman M., Punja Z. 2008. Seaweed extract reduces foliar fungal diseases
- 518 on carrot. *Crop Protection* 27:1360–1366. DOI: [10.1016/j.cropro.2008.05.005](https://doi.org/10.1016/j.cropro.2008.05.005).
- 519 Jayaraman J., Norrie J., Punja ZK. 2010. Commercial extract from the brown seaweed asco-
- 520 phyllum nodosum reduces fungal diseases in greenhouse cucumber. *Journal of Applied Phycology*
- 521 23:353–361. DOI: [10.1007/s10811-010-9547-1](https://doi.org/10.1007/s10811-010-9547-1).
- 522 Jithesh MN., Wally OS., Manfield I., Critchley AT., Hiltz D., Prithiviraj B. 2012. Analysis of sea-
- 523 weed extract-induced transcriptome leads to identification of a negative regulator of salt tolerance
- 524 in *arabidopsis*. *HortScience* 47:704–709.
- 525 Jukes T., Cantor C. 1969. Evolution of protein molecules, pp. 21–132 in mammalian protein

- 528 metabolism, edited by munro hn.
- 529 Khan W., Rayirath UP., Subramanian S., Jithesh MN., Rayorath P., Hodges DM., Critchley AT.,
- 530 Craigie JS., Norrie J., Prithiviraj B. 2009. Seaweed extracts as biostimulants of plant growth and
- 531 development. *Journal of Plant Growth Regulation* 28:386–399.
- 532 Klindworth A., Pruesse E., Schweer T., Peplies J., Quast C., Horn M., Glöckner FO. 2013. Evalu-
- 533 ation of general 16S ribosomal rna gene pcr primers for classical and next-generation sequencing-
- 534 based diversity studies. *Nucleic acids research* 41:e1–e1.
- 535 Lizzi Y., Coulomb C., Polian C., Coulomb P., Coulomb P. 1998. Seaweed and mildew: What
- 536 does the future hold? *Phytoma La Defense des Vegetaux (France)*.
- 537 Milton R. 1952. Improvements in or relating to horticultural and agricultural fertilizers. *British*
- 538 *Patent* 664989.
- 539 Newman M-A., Sundelin T., Nielsen JT., Erbs G. 2013. MAMP (microbe-associated molecular
- 540 pattern) triggered immunity in plants. *Frontiers in Plant Science* 4. DOI: [10.3389/fpls.2013.00139](https://doi.org/10.3389/fpls.2013.00139).
- 541 Oksanen J., Blanchet FG., Kindt R., Legendre P., Minchin PR., O'hara R., Simpson GL., Solymos
- 542 P., Stevens MHH., Wagner H., others. 2013. Package “vegan”. *Community ecology package, version*
- 543 2.
- 544 Paradis E., Claude J., Strimmer K. 2004. APE: Analyses of phylogenetics and evolution in r
- 545 language. *Bioinformatics* 20:289–290.
- 546 Pinheiro J., Bates D., DebRoy S., Sarkar D., Team RC. 2017. Nlme: Linear and nonlinear mixed-
- 547 effects models. r package version 3.1-128. 2016. *R software*.
- 548 Schliep KP. 2010. Phangorn: Phylogenetic analysis in r. *Bioinformatics* 27:592–593.
- 549 Schloss PD., Westcott SL., Ryabin T., Hall JR., Hartmann M., Hollister EB., Lesniewski RA.,
- 550 Oakley BB., Parks DH., Robinson CJ., others. 2009. Introducing mothur: Open-source, platform-
- 551 independent, community-supported software for describing and comparing microbial communi-
- 552 ties. *Applied and environmental microbiology* 75:7537–7541.
- 553 Schmidt R., Zhang X. 1997. Influence of seaweed on growth and stress tolerance of grasses. In:
- 554 *Proceedings*.
- 555 Spann TM., Little HA. 2011. Applications of a commercial extract of the brown seaweed asco-
- 556 phyllum nodosum increases drought tolerance in container-grown ‘hamlin’ sweet orange nursery

- 557 trees. *HortScience* 46:577–582.
- 558 Team RC. 2018. R: A language and environment for statistical computing.
- 559 Toju H., Tanabe AS., Yamamoto S., Sato H. 2012. High-coverage its primers for the dna-based
- 560 identification of ascomycetes and basidiomycetes in environmental samples. *PloS one* 7:e40863.
- 561 Wally OS., Critchley AT., Hiltz D., Craigie JS., Han X., Zaharia LI., Abrams SR., Prithiviraj
- 562 B. 2013. Regulation of phytohormone biosynthesis and accumulation in arabidopsis following
- 563 treatment with commercial extract from the marine macroalga *ascophyllum nodosum*. *Journal of*
- 564 *plant growth regulation* 32:324–339.
- 565 Wang Q., Garrity GM., Tiedje JM., Cole JR. 2007. Naive bayesian classifier for rapid assign-
- 566 ment of rRNA sequences into the new bacterial taxonomy. *Applied and environmental microbiology*
- 567 73:5261–5267.
- 568 Wickham H. 2016. *Ggplot2: Elegant graphics for data analysis*. Springer.
- 569 Wickham H., Francois R., Henry L., Müller K. 2015. Dplyr: A grammar of data manipulation.
- 570 *R package version 0.4.3.*
- 571 Wright ES. 2016. Using decipher v2. 0 to analyze big biological sequence data in r. *R Journal* 8.